Catarina Maria Pinto Mora Pinto de Magalhães

Inorganic Nitrogen Dynamics in Intertidal Rocky Biofilms and Sediments of the Douro Estuary: Processes and Communities

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Dissertação de Candidatura ao grau de Doutor em Ciências do Meio Aquático submetida ao Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal.

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Co-orientador - Professor Doutor William J. Wiebe

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Ao Luís e ao Hugo
Aos meus Pais

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Abstract

Problems associated with increasing eutrophication have stimulated many investigators to examine primary productivity and nutrient dynamics and transformations in different coastal ecosystems. In this study we investigate the dynamics of inorganic nitrogen biogeochemistry and of the microorganisms involved in intertidal rocky biofilms and sediment of the Douro River Estuary, an euthrophic system with high nitrogen loading from freshwater and urban runoff. Questions related with the unrecognized role of rocky biofilms in N biogeochemistry, environmental controls on key nitrogen processes, and relationships between nitrifiers and denitrifiers community dynamics with environmental variables and inorganic nitrogen cycle biogeochemistry were addressed.

Firstly, the inorganic nutrient and oxygen fluxes in five intertidal rocky biofilm sites were evaluated in order to examine the importance of the inorganic nutrient and metabolic dynamics of intertidal rocky biofilms in the Douro River estuary. Results showed that while rocky biofilms occupy just 21% of the total linear (2-dimensional) area of the intertidal zone of Douro River estuary, because of rugosity and higher flux rates, they were responsible for approximate 43% of the NO_3^- uptake and 45% of the SiO_4^{4-} uptake, in terms of hourly removal capacity for the total intertidal area.

A more detailed study centralized on the several inorganic nitrogen processes and dynamics in intertidal rocky biofilms and sediments of the Douro River estuary were then performed. Results showed that during daylight nitrogen assimilation by benthic or epilithic primary producers was the major process of dissolved inorganic nitrogen removal; nitrogen fixation, nitrification and denitrification were non-important processes in the overall light dissolved inorganic nitrogen cycle. At night, denitrification together with dissimilatory nitrate reduction to ammonium appeared to be important pathways for water column nitrate removal. The conceptual model performed showed major differences between intertidal

sediment and rocky sites in terms of the absolute mean rates of dissolved inorganic nitrogen net fluxes and the processes involved (mainly nitrification and denitrification); with the biofilm substrata producing generally higher flux rates. Of particular significance, the N_2O produced in intertidal rocky sites, represents the highest N_2O release rates ever recorded for marine systems.

Then, a study focused on the regulatory effect of salinity and inorganic nitrogen (nitrate and ammonium) concentrations on nitrification, denitrification and nitrous oxide production was performed within intertidal sandy sediments and rocky biofilms of the Douro River estuary. This study revealed that inorganic nitrogen and salinity are important environmental variables in controlling the nitrogen processes evaluated. While salinity did not regulate denitrification rates in both environments, nitrifying communities at both sites showed maximum nitrification activity at intermediate salinities. Results also revealed that increasing nitrate concentration stimulated denitrification rates in sandy sediments and exerted no control on rocky biofilms denitrifying communities. In both environments, while nitrate availability influences N_2O production rates, other parameters, or the interactions between them were also important regulatory factors for N_2O production rates. Finally, ammonium concentration regulated nitrification rates at both sites, and it was demonstrated that rocky biofilms nitrifier community is more tolerant of high ammonium concentrations than sandy sediments nitrifiers.

Finally, the composition of the ammonia oxidizing and denitrifiers (*nosZ* genes) bacteria assemblages were related with *in situ* transformation rates and some environmental variables. Interestingly we report clear site-specific differences in the ammonium-oxidizing bacteria (AOB) communities through the growth of just *Nitrosomonas*-like communities in the rocky biofilm sites, and mainly *Nitrosospira*-like species in the intertidal sediment sites. This AOB community site-selection was related to site differences in some environmental variables and linked to nitrifying activity; higher nitrification rates were observed in the rocky biofilm samples than in sediment samples. Results also indicate low *nosZ* gene diversity in all sites but pronounced temporal shifts in the relative peak areas of some T-RFLPs, which were significantly related to some environment variables and denitrification rates, suggesting that the dynamics of denitrifier community observed could be due to responses to some environmental conditions mainly water column nitrate concentrations and salinity, which in turn reflected the seasonal variability in denitrifying communities function.

Resumo

Os problemas associados com a eutrofização levaram muitos investigadores a estudar a produtividade primária e a dinâmica dos nutrientes bem como as suas transformações em distintos ecossistemas costeiros. No presente estudo, investigou-se a dinâmica dos processos biogeoquímicos do azoto inorgânico bem como dos microrganismos envolvidos nas suas transformações, em sistemas quer de sedimento, quer de substrato rochoso intertidais do estuário do Rio Douro. Para tal, foram abordadas questões tais como a actividade biogeoquímica do ciclo do azoto nos sistemas rochosos intertidais, o efeito regulador das variáveis ambientais em processos chave do ciclo do azoto e as relações entre a dinâmica das comunidades nitrificantes e desnitrificantes com variáveis ambientais assim como com a magnitude dos processos mediados por estes microrganismos.

Inicialmente, os fluxos de oxigénio e nutrientes inorgânicos foram medidos em cinco locais distintos de biofilme rochoso intertidal com o objectivo de avaliar a importância destes sistemas nos fluxos de nutrientes e no metabolismo do estuário do Rio Douro. Os resultados obtidos sugeriram que alguns processos biogeoquímicos avaliados no substrato rochoso diferem em magnitude dos sedimentos intertidais. Apesar da área ocupada pelo substrato rochoso do estuário do Rio Douro representar apenas 21% da área total intertidal, estes sistemas são responsáveis pela remoção de 47% do NO_3^- e 58% da SiO_4^{4-}, em termos da capacidade de remoção da área total intertidal.

No seguimento da linha de investigação anterior, foi realizado um estudo mais detalhado e focado apenas nos processos do ciclo do azoto, quer em sedimentos quer em biofilms rochosos intertidais. Os resultados obtidos revelaram processos distintos de remoção de azoto consoante a presença ou ausência de luz. Durante o dia, a assimilação do azoto pelos produtores primários foi considerado o processo dominante de remoção de azoto. Pelo contrario, na ausência de luz, a desnitrificação e a redução dissimilatória dos nitratos

a amónia foram identificados como os processos mais importantes na remoção de azoto inorgânico da coluna de água. No modelo conceptual realizado, foi possível verificar diferenças nítidas nas magnitudes dos processos do ciclo do azoto, nomeadamente nas taxas de desnitrificação e nitrificação. Os biofilmes rochosos intertidais deram origem, de um modo geral, a taxas mais elevadas. Com particular significado, as taxas de produção de N_2O encontradas foram as mais altas obtidas em sistemas marinhos.

O efeito regulador da salinidade e do azoto inorgânico nas taxas de nitrificação, desnitrificação e na produção de N_2O foi também avaliado nos sedimentos e substratos rochosos do estuário do Rio Douro. Este trabalho revelou que o azoto inorgânico e a salinidade condicionaram de forma importante os processos estudados. Apesar da salinidade não ter apresentado nenhum efeito regulador nas taxas de desnitrificação em ambos os locais de amostragem, as comunidades nitrificantes apresentaram-se mais activas a salinidades intermédias. Por outro lado, experiências realizadas com diferentes concentrações de NO_3^- revelaram uma estimulação das taxas de desnitrificação a concentrações de NO_3^- mais elevadas, nos sedimentos arenosos. No entanto, não foi observado qualquer efeito regulador do NO_3^- sobre as taxas da desnitrificação em biofilmes rochosos. Em ambos os locais de amostragem verificou-se que, apesar da disponibilidade de NO_3^- influenciar as taxas de produção de N_2O, outros parâmetros ambientais poderão ser importantes na regulação da produção de N_2O observada. A concentração de amónia também teve um efeito regulador nas taxas de nitrificação e os resultados demonstraram que as populações nitrificantes do substrato rochoso foram mais tolerantes a concentrações mais elevadas de NH_4^+ do que as dos sedimentos intertidais.

Por último, a dinâmica espacial e sazonal da estrutura das comunidades nitrificantes e desnitrificantes foi relacionada com os processos do ciclo do azoto e com variáveis ambientais relevantes. Os resultados obtidos apontaram para diferenças espaciais claras nas comunidades bacterianas que oxidam a amónia. Assim, nos locais de biofilme rochoso observou-se apenas a presença de espécies pertencentes ao género *Nitrossomonas*, enquanto que nos sedimentos intertidais as espécies de nitrificantes pertenciam, na sua maioria, ao género *Nitrosospira*. Esta selectividade espacial foi relacionada com algumas diferenças espaciais no que diz respeito a variáveis ambientais e às taxas de nitrificação. No que diz respeito às populações de desnitrificantes, os resultados obtidos indicaram uma baixa diversidade do gene *nosZ* em todos os locais de amostragem. Os resultados também revelaram uma variação mensal pronunciada na área relativa dos picos de T-RFLP que, por

seu lado, está correlacionada com a variação de alguns parâmetros ambientais e das taxas de desnitrificação. Os resultados sugerem que a dinâmica das populações desnitrificantes observada poderá ser fruto das flutuações de alguns parâmetros ambientais, nomeadamente a concentração de NO_3^- na coluna de água e salinidade, condicionando, por sua vez, uma variabilidade sazonal nas taxas de desnitrificação.

Résumé

Des problèmes associés à l'eutrophisation ont stimulé de nombreux chercheurs à examiner la productivité primaire et la dynamique des nutriments ainsi que leurs transformations dans les différents écosystèmes côtiers. Dans cette étude, nos recherches ont porté sur la dynamique des processus biogéochimiques de l'azote inorganique ainsi que sur les microorganismes responsables de ces transformations au sein des systèmes de sédiments et des substrats rocheux intertidaux de l'estuaire du fleuve Douro. Dans ce travail, nous avons abordé des questions relatives à la méconnaissance de l'activité biogéochimique de l'azote dans les systèmes rocheux intertidaux, l'effet régulateur des variables environnementales dans des processus clefs du cycle de l'azote et les rapports entre la dynamique des communautés nitrifiantes et dénitrifiantes avec les variables de l'environnement, ainsi que la magnitude des processus effectués par l'intermédiaire de ces microorganismes.

D' abord, les flux d'oxygène et de nutriments inorganiques ont été mesurés en cinq locaux du biofilm rocheux intertidal à fin d'évaluer l'importance de ces systèmes de flux de nutriments et le métabolisme de l'estuaire du Douro. Les résultats obtenus ont suggéré que les processus biogéochimiques évalués dans le substrat rocheux diffèrent en magnitude des sédiments intertidaux. Même si les substrats rocheux de l'estuaire du Douro constituent seulement 21% de la surface totale intertidale, ces systèmes sont responsables de la disparition de 47% du NO_3^- et de 58% de la SiO_4^{4-}, par rapport à la capacité d'enlèvement sur la totalité de la surface intertidale.

En poursuivant les recherches précédentes, nous avons réalisé une étude plus détaillée et centralisée sur les processus du cycle de l'azote, à différents endroits des sédiments et des biofilms rocheux intertidaux. Les résultats obtenus ont démontré la prévalence des processus d'enlèvement de l'azote distincts selon la présence ou l'absence de lumière. Pendant la journée, l'assimilation de l'azote par les producteurs primaires a été considérée

comme le processus dominant de l'enlèvement d'azote en détriment de sa fixation et de la dénitrification. Dans l'obscurité, la dénitrification et la réduction de dissimilation des nitrates à ammonium ont été reconnues comme étant les processus les plus importants dans le retirement de l'azote inorganique de la colonne d'eau. Dans le modèle conceptuel réalisé, il est possible de vérifier de nettes différences dans les magnitudes des processus liés au cycle de l'azote, particulièrement en ce qui concerne les taux de dénitrification et de nitrification. Les biofilms rocheux intertidaux ont produit, d'une façon générale, des taux plus élevés. Particulièrement importants, les taux de production de N_2O ont été les plus élevés mesurés en systèmes marins.

L'effet régulateur de la salinité et de l'azote inorganique sur les taux de nitrification, de dénitrification et de la production de N_2O a aussi été évalué dans les sédiments et les substrats rocheux du fleuve Douro. Se travail a montré que l'azote inorganique et la salinité sont des variables environnementales importantes dans la régulation des processus évalués. Même si la salinité n'a présenté aucun effet régulateur vis-à-vis des taux de dénitrification dans les deux lieus d'échantillonnage, les communautés nitrifiantes ont montré une plus grande activité avec des salinités intermédiaires. Les essais réalisés avec différentes concentrations de NO_3^- ont prouvé la stimulation des taux de dénitrification dans les sédiments sableux, cependant, aucun effet régulateur des NO_3^- dans les taux de dénitrification des biofilms rocheux n'a été observé. Dans les deux locaux d'échantillonnage, nous avons observé que même si la disponibilité du NO_3^- influence les taux de production de N_2O, les autres paramètres de l'environnement n'ont pas plus d'importance régulatrice dans la variabilité de la production de N_2O observée. Les concentrations d'ammonium ont aussi eu un effet régulateur sur les taux de nitrification et les résultats ont démontré que les communautés nitrifiantes qui habitent le substrat rocheux sont plus tolérantes à des concentrations de NH_4^+ plus élevées que celles des sédiments intertidaux.

Enfin, la dynamique spatiale et saisonnière de la structure des communautés nitrifiantes et dénitrifiantes a été mise en rapport avec les processus du cycle de l'azote et avec des variables environnementales relevantes. Les résultats obtenus ont démontré des différences spatiales claires chez les communautés bactériennes qui oxydent l'ammonium, vu que dans les endroits de biofilm rocheux, nous avons uniquement observé la présence d'espèces appartenant au genre *Nitrossomas*, tandis que dans les sédiments intertidaux, les espèces nitrifiantes appartenaient, dans leur majorité, au genre *Nitrosospira*. Cette sélectivité spatiale a été mise en rapport avec quelques différences spatiales en ce qui concerne

certaines variables environnementales et avec les taux de nitrification. En ce qui concerne les populations de dénitrifiantes, les résultats obtenus indiquèrent une faible diversité parmi le gène *nosZ* dans tous les lieus d'échantillonnage. Les résultats ont aussi montré une variation mensuelle prononcée dans le domaine des pics de T-RFLP, qui à son tour est en rapport avec la variation de certains paramètres de l'environnement et avec les taux de dénitrification. Les résultats suggèrent que la dynamique des populations dénitrifiantes observée pourra être le fruit des fluctuations de quelques paramètres environnementaux, en particulier de la concentration NO_3^- au sein de la colonne d'eau et de la salinité, qui sont le reflet, à leur tour, de la variabilité saisonnière des taux de dénitrification.

List of Papers

This thesis is based on the following papers:

1. Magalhães C., A. A. Bordalo, and W. J. Wiebe. 2003. Intertidal biofilms on rocky substratum can play a major role in estuarine carbon and nutrient dynamics. Mar. Ecol. Progr. Ser. 258:275-281.

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

3. Magalhães C. M., S. B. Joye, R. M. Moreira, W. J. Wiebe, and A. A. Bordalo. 2005. Effect of salinity and inorganic nitrogen concentrations on nitrification and denitrification rates in intertidal sediments and rocky biofilms of the Douro River estuary, Portugal. Water Res. 39: 1783-1794.

4. Magalhães C. M., N. Bano, W. J. Wiebe, J. T. Hollibaugh and A. A. Bordalo. Comparison of ammonium oxidizing bacterial phylotypes and function between intertidal rocky biofilms and sediments of the Douro River estuary, Portugal. Submitted to Environ. Microbiol..

5. Magalhães C. M., N. Bano, W. J. Wiebe, A. A. Bordalo and J. T. Hollibaugh. Nitrous oxide reductase (*nosZ*) genes retrieved from intertidal rocky biofilm and sediment samples of the Douro River estuary (Portugal), and relation to denitrification rates. Submitted to App. and Environ. Microbiol..

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Chapter 1

General Introduction

This dissertation has been designed to improve the understanding on the dynamics of inorganic nitrogen biogeochemistry and microbiology in rocky and sediment boundaries of temperate intertidal environments. Fundamentally important questions related to the unrecognized role of rocky biofilms in N biogeochemistry, environmental controls on key nitrogen processes, and links between nitrifier and denitrifier community dynamics with inorganic nitrogen cycle biogeochemistry are addressed.

1.1 Motivation

In this study it was examine the unrecognized importance of intertidal hard substrata in estuaries for inorganic nitrogen cycling, and comparisons were established with intertidal sediments, which have been well studied. While not yet quantified, many estuaries and coastal areas have abundant natural and, importantly, man-made hard surfaces that have been virtually invisible to the microbiologists and biogeochemists concerned with the study of coastal and estuarine nitrogen N biogeochemistry. Rocky shores support a diverse mixture of plants and animals adapted to survive under unique conditions. Thus for several decades coastal rocky shores have received much attention by community ecologists, who have studied animal, macrophyte and microalgal distribution, controls of trophic structure, and food webs of these communities (Hillebrand and Sommer 2000, Worm et al. 2000, Knox 2000 and references herein). However, the extent of biogeochemical processes within the thin layer of organic matter that characterizes hard surfaces of many marine coastal

habitats has scarcely been considered. For example, Decho (2000) who reviewed microbial biofilms in intertidal systems and Knox (2000) who published a book on the ecology of hard and soft seashores do not mention this topic at all. Furthermore, while numerous studies on the nitrogen cycle have been conducted for decades on open ocean ecosystems and in coastal waters and sediment environments, including lagoons, bays, estuaries, coral reefs, seagrass meadows, mangroves, muddy and sandy flats, salt marshes, etc, virtually no studies have been conducted on the N biogeochemistry of the rocky intertidal zone in temperate regions. Thus, this study represents the first investigation of the N biogeochemistry of intertidal rocky biofilms environments, and we believe that the examination of this new habitat provides important information to be added to our knowledge of the global marine nitrogen cycle.

This thesis also deals with the increasing interest in establishing links between prokaryote species diversity, functional diversity and ecosystem processes, because it has been generally recognized that the current state of information on the ecology of the microorganisms that mediate biogeochemical transformations in natural environment is inadequate (Bothe et al. 2001, Webster et al. 2005). Since investigators start using molecular approaches to investigate the composition and diversity of natural bacterial communities involved in nitrogen biogeochemistry, they begun to recognize that the real world of the microorganisms that mediate nitrogen transformations is much more complex and diverse than had been considered. These studies also have demonstrated considerable diversity in nitrifiers, denitrifiers and nitrogen fixing bacteria and a prevalence of uncultured species in natural environments (Zehr and Ward 2002 and references herein). Thus, the bacteria which have been studied extensively in pure cultures over the last decades, may not be the major "players" of N cycling transformations in most natural habitats. In particular, in this study it was investigate potential links between environmental variations, nitrifier and denitrifier diversity dynamics and biogeochemical functions, by integrating major N biogeochemical processes and environmental characteristics with specific microbial assemblages variability. This study, together with recent investigations (McCaig et al. 1999, Caffrey et al. 2003, Cébron et al. 2003, Rich et al. 2003, Schramm 2003, O'Mullan and Ward 2005 and Webster et al. 2005) represents an important beginning to examine the influence of environmental factors on the diversity of nitrifier and denitrifier bacterial communities in nature and the consequent effects on the mediated N biogeochemical processes in ecosystems.

1.2 Background

1.2.1 Inorganic Nitrogen Cycle in Coastal Marine Environments

The growth of all organisms depends on the availability of different resources, especially mineral nutrients. Nitrogen is required in large amounts as an essential component of proteins and nucleic acids, two major macromolecular components of living organisms. Nitrogen is one of the most abundant element in living tissue and therefore plays a central role in biogeochemical cycles of marine and coastal ecosystems. Moreover, nitrogen is typically the nutrient that controls primary production in most temperate zone estuaries and other marine coastal ecosystems and as such, is commonly implicated in the eutrophication and consequently degradation of coastal marine systems due to anthropogenic nitrogen enrichment (Ryther and Dunstan 1971, Codispoti 1985, de Jonge 1995, Nixon et al. 1995).

The complexity of the nitrogen cycle is demonstrated in Fig.1.1, which shows that nitrogenous compounds undergo a series of oxidation/reduction reactions mediated by a metabolically diverse range of autotrophic and heterotrophic organisms. These transformations includes: (1) nitrate (NO_3^-) and nitrite (NO_2^-) reductions to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) (denitrification), (2) ammonium (NH_4^+) conversion to organic N by any one of several independent assimilatory processes, (3) NH_4^+ production from the decomposition of organic N (ammonification), (4) NH_4^+ oxidation to NO_2^- and NO_3^- (nitrification), (5) N_2 reduction to NH_4^+ and organic N (Nfixation), (6) NO_3^- reduction to NH_4^+ (dissimilatory nitrate reduction to ammonium), (7) NH_4^+ oxidation to N_2 with NO_2^- as an electron acceptor (anammox). Most of these N pool interconversions affect the oxidation state of N, and therefore the free energies of the various molecules and compounds. Consequently, these biogeochemical conversions are either energy-yielding (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).

All microbially mediated N transformations are strongly regulated by the prevailing environmental physico-chemical conditions. Thus, environmental specificities of each coastal ecosystem can affect the complex interactions of the several recycling nitrogen pathways, and the significance of the N processes can vary according to the idiosyncracies of each habitat.

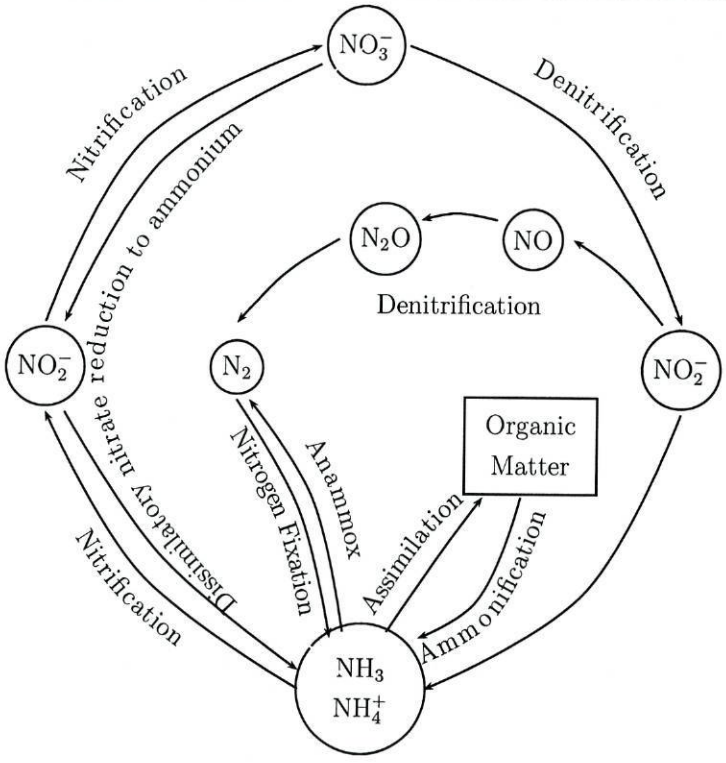

Figure 1.1: Schematic of the key processes involved in the nitrogen cycle.

1.2.1.1 Nitrogen Fixation

In temperate coastal systems it is generally considered that N fixation has a smaller contribution to N budgets than in the open ocean (Howarth et al. 1988, Seitzinger 1988). Although locally high rates of N fixation can be found in some temperate coastal environments, the overall contribution to the annual budget has been found to be modest (Hanson and Gunderson 1977, Nixon et al. 1981, Joye and Paerl 1993a). Also, several investigators have found that in eutrophic estuarine sediments N losses via denitrification were usually not balanced by N fixation, since N losses via denitrification greatly exceeded N inputs through N fixation (Capone and Kiene 1988, Seitzinger 1988, Welsh et al. 2000). Joye and Paerl (1993b) demonstrated that nitrogen fixation and denitrification in intertidal sediments quickly responded to inorganic N enriched runoff: Nfixation rates decreased, approaching zero, while denitrification rates increased by an order of magnitude. In agreement, in a coastal marine environment characterized by the presence of extensive meadows of rooted macrophytes, nitrogen fixation was unimportant due to the high nitrogen availability in water column and sediment (Welsh et al. 1996, 2000).

In some oligotrophic coastal lagoons nitrogen fixation has been found to significantly contribute to the annual nitrogen input into the system (Smith 1984, Hanson and Gundersen 1977). In tropical seagrass meadows, nitrogen fixation can supply up to 50% of the nitrogen requirements of the plant communities (Howarth et al. 1988). Nitrogen fixation is also known to be a key feature of the nitrogen cycle of many coral reefs (see D'Elia and Wiebe 1990); exceptions are locations with enhanced terrestrial nitrogen runoff (D'Elia and Wiebe, 1990).

1.2.1.2 Ammonification

Ammonification is the process by which primary amines are deaminated during decomposition of organic compounds. This process plays a central role in nitrogen recycling in marine environments.

In several studies of shallow water coastal ecosystems investigators have found that remineralization by the microbiota plays an important role in supporting primary production both in phytoplankton dominated systems and those where macrophytes are the dominant primary producers (Bebout et al. 1994, Trimmer et al. 2000). Deposition of organic matter in these environments can result from episodic events such as the rapid sedimentation of phytoplankton or benthic macroalgal blooms, or, in systems where rooted macrophytes are dominant, deposition of dead plant material can occur throughout the year. The quantity, quality and spatial distribution of the deposited organic matter in the sediment regulates the rates of benthic nutrient regeneration. For example, in macroalgal dominated sediment, mineralization can be accelerated in relation to systems dominated by vascular plants, because macroalgae have little structural material and decompose rapidly (Enoksson 1993, Duarte 1995). However, in these systems the high macroalgal requirements for dissolved inorganic nitrogen influences the flux of dissolved inorganic nitrogen between sediment and overlying water, and thus the dissolved inorganic nitrogen produced will supply the macroalgal N requirements (Trimmer 2000). 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 sediments is nitrified and subsequently denitrified (Seitzinger 1990).

1.2.1.3 Nitrification

Nitrification represents the oxidative part of the N cycle and refers to the two-step process whereby ammonium is oxidized to nitrite and subsequently to nitrate. This process completes the redox cycle of nitrogen from most reduced to most oxidized form. Usually, the two steps of nitrification are tightly coupled, and thus no accumulation of nitrite is observed in the environment.

It is recognized that oxidation of ammonium plays a pivotal role in the N cycle of shallow coastal sediments, since it generates a source of nitrate for denitrifying bacteria. The coupling of this obligate aerobic process (nitrification) with an anaerobic process (denitrification) leads to the loss of nitrogen to the atmosphere as nitrous oxide and/or dinitrogen (Seitzinger 1988). However, the degree of coupling between nitrification and denitrification vary according to inherent environmental characteristics of each system. For example, Jensen et al. (1996) found a generally low degree of coupling between nitrification and denitrification; in this study coupling dominated only during summer when the overlying water was almost nitrate depleted. Also Rysgard et al. (1994) demonstrated a great seasonal variability in nitrification and denitrification coupling; with lower coupling in winter NO_3^- rich waters and higher coupling in summer NO_3^- depleted waters. In agreement, Dong et al. (2000) found that uncoupled denitrification represented the larger proportion (40-100%) of the total denitrification, because of the high nitrate concentration in the water column and oxygen limitation on nitrification. Finally, Rysgard et al. (1994, 1995) suggested that nitrification and denitrification coupling might not be so important in shallow coastal systems that receive significant nutrient inputs.

A number of physico-chemical and biological factors are important in regulating nitrifying activity in coastal marine sediments. Thus, on a seasonal basis different patterns of nitrification rates are usually observed (Seitzinger et al 1984, Jensen et al. 1996). Nitrifying bacteria are obligate aerobes, and thus in sediments the depth distribution of nitrification is ultimately constrained by the limits of downward O_2 diffusion (Kemp et al. 1990, Caffrey et al. 2003), which varies according to the sediment characteristics, organic matter content, temperature, tidal energy and degree of mixing and bioturbation. Sulfide is also an important factor regulating the nitrification rates, since it has been shown to permanently inhibit the activity of nitrifying bacteria (Joye and Hollibaugh 1995). The interaction between oxygen and NH_4^+ availability is also central in controlling rates of nitrification (Caffrey et al. 2003). During the day, competition between nitrifiers and

benthic microalgae for inorganic N could be particularly intense due to the high rates at which primary producers assimilate NH_4^+ (Rysgaard et al. 1995). Thus the activity of primary produces may control the patterns of nitrification rates. Changes in salinity have also been shown to have a significant effect on nitrification activity (Rysgaard et al. 1999, Seitzinger et al. 1991, Gardner et al 1991); generally, nitrification rates tend to be smaller at higher salinities (Rysgaard et al. 1999, Gardner et al 1991). Seitzinger et al. (1991) proposed that the salinity-induced reduction in nitrification activity is due to the reduction of the total NH_4^+ concentration within the sediment, due to fact that salinity enhanced NH_4^+ adsorption to sediment and thus NH_4^+ would be not available to nitrifiers.

1.2.1.4 Denitrification

Denitrification comprises a series of reductions whose overall result is the reduction of nitrate to dinitrogen; a specific enzyme carries out each step. Nitrite reductase is usually closely coupled with subsequent enzymes in the reduction sequence to nitric oxide and nitrous oxide, since neither of these gases nor nitrite usually accumulates in the environment in large amounts.

Coastal sediments present an ideal environment for denitrification; they concentrate organic matter from the water column, which upon decomposition releases NH_4^+, available for nitrification, and subsequently NO_3^- would support denitrification (Seitzinger 1988). In addition, NO_3^- from overlying water can diffuse into the sediments and be directly denitrified. While this source of NO_3^- is especially important in systems where water column NO_3^- concentrations are high, in systems were external nitrogen inputs are small, nitrification is the principal source of nitrate to denitrification (Risgaard et al. 1994 and 1995, Dong et al. 2000). Nitrate advected through the sediments from groundwater, while less studied, may also provide an important source of NO_3^- for denitrification, especially where groundwaters are contaminated with nitrate from agriculture (Nowicki et al. 1998).

Denitrification is an ubiquitous process in aquatic sediments. Different sediment systems show a wide range of denitrifier activity (Seitzinger 1988, 2000), with a tendency for high rates in highly eutrophic estuarine sediments (Rysgaard et al. 1996, Ogilvie et al. 1997, Trimmer et al. 1998, Cabrita and Brotas 2000). Almost all studies performed describe distinct seasonal patterns, and it is evident that denitrification in marine sediments is subject to a complex array of regulatory mechanisms involving both physico-chemical and

biological factors. Numerous investigators have correlate higher denitrification rates with the progressive increasing of NO_3^- concentrations, showing the importance of NO_3^- as a regulatory factor of denitrification (Jorgensen and Sørensen 1988, Rysgaard et al. 1995, Jensen et al. 1996, Ogilvie et al 1997, Trimmer et al. 1998, Cabrita and Brotas 2000, Dong et al. 2000). The availability of organic carbon (Jorgensen and Sørensen 1988) and changes in oxygen regimes (Law et al. 1990) have been also recognized as major environmental controls of denitrification. The presence of infauna also affects the rates of denitrification, since infaunal activity typically results in complex patterns of oxic and anoxic microenvironments that significantly affect the mixing of O_2 and NO_3^- from the sediment surface to deeper layers (Rysgaard et al. 1995, Gran and Pitkanen 1999).

The presence of green algae, seagrass and microphytobenthos in shallow and intertidal soft bottom embayments and estuaries could compete with denitrification for the nitrate available in the pore water and also diminishing denitrification activity through the high rates of O_2 production during photosynthesis (McGlathery al. 2001, Sundbäck et al. 2003). Sundbäck et al. (2003) found that in Ragardsvik Bay, Sweden, which supports extensive green macroalgal mats in addition to microphytobenthos, denitrification accounted only for a small portion (20%) of NO_3^- removal, while 80% was due to assimilation by primary producers. Also estimates of N consuming processes by Sundbäck and Miles (2002) showed that denitrification played only a minor role as a N sink in autotrophic sediment systems dominated by microphytobenthic primary producers. Similarly, in intertidal sediments with high *Enteromorpha* spp. and *Ulva* spp. cover, denitrification rates were low, suggesting that 98% of the N mineralized remained within the system due to the high N assimilation by macroalgae (Trimmer et al. 2000). In agreement, Rysgaard et al. (1996) and Welsh et al. (2000) found that *Zostera noltii* assimilation was quantitatively more important than denitrification as a sink for nitrogen, due to the high assimilation rates of these plants.

Denitrification plays a particularly important role in nitrogen enriched coastal ecosystems, since this process permanently removes nitrogen from the system as dinitrogen gas, reducing the amount of N transported downstream and to the ocean, reducing consequently the nitrogen available for phytoplankton and macrophytes (Nixon et al. 1981). Thus, denitrification has been recognized as an important removal process for external N loading, since numerous studies indicate that denitrification rates were promoted as nutrient loading increased (Seitzinger 1988, Ogilvie et al. 1997, Seitzinger 2000). Seitzinger (2000), who ex-

amined denitrification rates in fourteen coastal marine systems, showed that denitrification removed an amount of nitrogen ranging from 3% to 100% of the total dissolved inorganic nitrogen loading into each system, with a mean of 48%.

1.2.1.5 Nitrous Oxide

Nitrous oxide is next to CO_2 and CH_4 in importance as a potent greenhouse gas, and together with NO, it is of much concern in terms in stratospheric ozone depletion (Dickinson and Cicerone 1986). Atmospheric concentrations of N_2O have been increasing during the last century by approximately 0.25% per year (Seitzinger and Kroeze 1998). There are numerous sources of N_2O including biogenic and abiogenic production. Abiogenic sources are primarily the result of fossil fuel combustion and nylon manufacture, while biogenic sources are produced by three microbially distinct processes: denitrification (Knowels 1982); nitrification (Yoshida and Alexander 1970) and dissimilatory reduction of nitrate to ammonia (Smith and Zimmerman 1981). Denitrification is involved in both the production and consumption of N_2O in marine systems. Nitrous oxide production due to dissimilatory nitrate reduction to ammonium has not yet been documented in marine sediments, however several investgators have measured N_2O production rates due to nitrification and/or denitrification in different coastal sediments. While Bauza et al. (2002) and Corredor et al. (1999) indicated that N_2O was produced mainly by nitrification in the sediments of a mangrove forest, N_2O production due to denitrification has been generally considered to be a more important process in most temperate coastal systems (Seitzinger 1988 and 1990). Robinson et al. (1998) demonstrated that benthic denitrification and not nitrification was the major source of N_2O in a high nitrate concentration estuary. This is in agreement with the experiments performed by Jorgensen et al. (1984), which concluded that denitrification was the major source of N_2O at low O_2 concentrations usually found in aquatic coastal and estuarine sediments. The higher production rates of N_2O in the more eutrophic sediments may be related to the lower O_2 concentrations in those sediments and/or the H_2S inhibition of N_2O reduction to N_2 during denitrification (Sørensen et al. 1980). The potential effect of anthropogenic activities on nitrous oxide production from aquatic sediments was demonstrated by Seitzinger and Nixon (1983), showing that nitrous oxide fluxes and the ratio of N_2O:N_2 increased markedly as the rate of N loading to the system increased.

1.2.1.6 Dissimilatory Nitrate Reduction to Ammonium

The second mechanism for reducing nitrate involves nitrate-reducing bacteria that mediate a process termed dissimilatory nitrate reduction to ammonium (DNRA). In contrast to denitrification where nitrogen is lost from the ecosystem, DNRA results in the conservation of fixed nitrogen within the system. Dissimilatory reduction of nitrate to ammonium could be important in sediments in which fermentative bacteria, with whose metabolism it is often associated, were numerically important (Herbert and Nedwell 1990, Cole and Brown 1980). In organically rich sediments, DNRA could account for more than half of the total nitrate reduced, whereas in sediments with low C:N ratios the rates of nitrate reduced by DNRA were lower (Koike and Hattori 1978, Macfarlane and Herbert 1984, Jorgensen 1989, Rysgaard et al. 1996). Enoksson and Samuelsson (1987) found that in coastal sediments with low organic carbon content (1.5-3.0%), DNRA accounted just for 10.3% of the nitrate uptake, while denitrification rates contributed for approximately 90% of the total nitrate consumption. These observations reflect the great variability in DNRA under different C:N conditions in the field. Data obtain by Ogilvie et al. (1997), demonstrated also that as the nitrate concentration increased in water column, DNRA became a less important process in nitrate reduction. In agreement, Smith et al. (1982) found that DNRA in salt marsh sediments decreased from 50% to 4% of the total nitrate reduced when nitrate concentrations increased. Kelly-Gerreyn (2001) suggested that temperature is an important controlling factor for partitioning nitrate reduction into DNRA and denitrification. An and Gardner (2002) proposed also that high sulphide concentrations enhance DNRA by providing an electron donor to the bacteria responsible for DNRA. However, high sulphide concentrations also inhibited nitrification rates, causing a consequent reduction in denitrification rate.

1.2.1.7 Anammox

Anammox constitutes a shortcut in the conventional N cycle, converting ammonium and nitrite anaerobically to dinitrogen gas. Until a few years ago, denitrification was recognized as the only significant process converting fixed nitrogen to gaseous N_2. However, it was recently discovered that the anaerobic oxidation of ammonium with nitrite -the anammox reaction- acts as an additional pathway for the removal of N from marine and coastal ecosystems (Dalsgaard et al. 2005 and references herein). The first direct evidence of

anaerobic oxidation of NH_4^+ came from a wastewater treatment plant facility (van de Graaf et al. 1995), and the first direct evidence for anaerobic oxidation of NH_4^+ in a natural environment was in marine sediments (Thamdrup and Dalsgaard 2002). Later, anammox was measured in anoxic water columns of a coastal bay in Costa Rica (Dalsgaard et al. 2003), and in the Black Sea (Kuypers et al. 2003), in other coastal sediments (Trimmer et al. 2003, Risgaard-Petersen et al. 2004, Risgaard et al. 2004) and in Arctic Sea ice (Risgaard et al. 2004).

Anammox activity measured in different estuarine sediment systems showed values of activity within the same range (Risgaard-Petersen et al. 2004, Trimmer et al. 2003, Dalsgaard and Thamdrup 2002). However, the relative importance of anammox and denitrification in nitrogen removal varies greatly between locations and in some cases anammox activity has even not been detected (Risgaard-Petersen et al. 2004). For example, anammox in estuarine sediments form Randers Fjord, Denmark (Risgaard-Petersen et al. 2004) was less important (5-25%) as a source of N_2 production than in marine sediments from the Baltic-North Sea, where the process accounted for 67-79% of N_2 production (Thamdrup and Dalsgaard 2002). These values represent the maximum reported relative importance of anammox in N_2 production (Thamdrup and Dalsgaard 2002). While what regulates the significance of anammox for N removal in aquatic systems is largely unknown (Dalsgaard and Thamdrup 2002), the relative importance of anammox to denitrification has been linked to the availability of NO_3^- (Risgaard-Petersen et al. 2004, Rysgaard et al. 2004). In agreement, Trimmer et al. (2005) found in slurry experiments that in the presence of environmental NO_2^- and NO_3^- concentrations, the production of N_2 via anammox increased progressively. The temperature optimum for marine anammox populations varies also according to the environment. For example, in the permanently cold sediment of Young Sound, Greenland, with a temperature of < -1 °C year round, the temperature optimum for anammox was 12°C (Rysgaard et al. 2004), and in the Baltic North Sea, with annual temperatures between 4 and 6 °C, the optimum temperature was 15 °C (Dalsgaard and Thamdrup 2002).

Most published studies on anammox in marine environment have been based on anoxic incubations of homogenized sediment or water (Dalsgaard and Thamdrup 2002, Risgaard-Petersen et al. 2004, Thamdrup and Dalsgaard 2002, Trimmer et al. 2003), thus there is an almost total lack of *in situ* anammox measurements in natural environments. Important

research remains to be carried out on the molecular biology, physiology and ecology of this recently discovered nitrogen process.

1.2.2 Linking N Biogeochemical Processes with Natural Nitrifier and Denitrifier Communities

Research on the biogeochemistry of nitrogen by direct measurement of the rates of processes themselves elucidates the distributions and dynamics of various nitrogen compounds in the environment (Herbert 1999, Galloway et al. 2004). However, critical processes in the N cycle are predominantly controlled by many different species of bacteria, and each of which can have very different reactions to many environmental variables. Thus, since their activities determine the distribution of the N compounds (Bock and Wagner 2003, Shapleigh 2003), environmental conditions can determine where and when each process occurs, the degree of exchange among various nitrogen pools, and the physical, chemical, and biological interactions that are possible. Thus, although detailed biochemical information about bacterially mediated reactions is required to understand the ecosystem-level controls on the nitrogen cycle, it is also necessary to assess the mechanisms that limit the distribution of organisms that have the genetic capability for the different processes. Thus, direct methods to measure *in situ* rates of nitrification and denitrification must be supplemented by others that focus on the organisms responsible for the reactions. This "microbiological" approach can address questions that the rate measurements do not, such as: (1) How do the observed rates depend on the species composition and diversity of different denitrifiers and nitrifiers present in the sample? (2) Are the biochemical models derived from cultured organisms, which we use to understand environmental rate processes, accurate? (3) Does species succession or competition among similar metabolic types influence the observed rates?, i.e., do nitrifier and denitrifier diversity and dynamics drive fluctuations in nitrification and denitrification rates? (4) How is chemical information from the environment reflected in regulation of bacterial community dynamics and therefore in net transformation rates? (5) Do nitrifier and denitrifier communities respond to micro- and/or macro-scale environmental changes?

The application of molecular techniques to microbial ecological studies has greatly increased in the last decade (Bothe et al. 2000). A variety of different polymerase chain reaction (PCR) techniques for specific amplification of 16S rRNA gene fragments are commonly

used methods for detection and characterization of both isolates and natural nitrifier and denitrifier communities (Bothe et al. 2000, Kawalchuk and Stephen 2001, Bock and Wagner 2003, Shapleigh 2003). However, one limitation of rRNA-based analysis is that it does not provide a direct link to physiology and metabolic capacities. The increasing interest in linking nitrogen biogeochemical processes with the mediating microorganisms, encourages the use of probes based on enzymes or genes directly involved in those transformations (Scala and Kerkhok 1999, Braker et al. 2000, Gieseke et al. 2001, Cébron et al. 2004, Horz et al. 2004, O'Mullan et al. 2005). Functional probes can yield information on the distribution and expression of genes involved in a certain biogeochemical pathway without regard to the taxonomic identity of the organisms that might possess those genes in the nature. Thus, these functional markers, encoding key enzymes of specific metabolic pathways, have been found to be useful in the detection of microorganisms belonging to different ecophysiological classes in complex microbial assemblages from diverse habitats. Also, the combination of those techniques with denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and with quantitative population structure analysis by applying fluorescence *in situ* hybridization were developed and recently applied for the study of natural nitrifier and denitrifier communities in a wide diverse environments (Stephen et al. 1998, Braker et al. 1998, Bano and Hollibaugh 2000, Braker et al. 2000, Scala and Kerkhok 2000, Avrahami et al. 2002, Cébron et al. 2004). In recent studies workers have also used reverse transcript (RT-PCR) approaches to investigate gene expression that encodes key nitrogen cycle enzymes in natural environments (Nogales et al. 2002, Sharma et al. 2005).

The direct retrieval of 16S rRNA gene fragments or fragments of functional genes from environmental samples has confirmed the presence and enlarged the known distribution of several different physiological and phylogenetic groups including nitrifiers and denitrifiers. In addition these cultivation-independent molecular phylogenetic surveys have revealed a still increasing numbers of novel phylogenetic lineages unanticipated from culture-based techniques. Molecular techniques used in the analysis of natural nitrifier and denitrifier communities has been reviewed by several authors (Bothe et al. 2000, Kawalchuk and Stephen 2001, Koops and Pommerening-Röser 2001).

1.2.2.1 Nitrifiers

As mentioned above, nitrification is a two step process which involves two different groups of obligatory aerobic chemolithotrophic bacteria, that use the oxidation of inorganic nitrogen compounds as their major energy source (Bock and Wagner 2003). Ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite and nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate (Warrington 1978, Wodicka et al. 1997). Recently it has been discovered in wastewater treatment systems (Strous et al. 1997) and in several marine ecosystems that representatives of the genus Planctomyces (Schmid et al. 2003) are involved in the chemolithotrophic nitrification in anaerobic conditions. In addition to chemolithotrophic nitrification, heterotrophic nitrification can also be catalysed by several microorganisms including fungi and heterotrophic bacteria (Killham 1986, Crossman et al. 1997). However, the role of heterotrophic nitrification in natural environments is not well known and is usually considered to contribute only marginally to the global nitrogen cycle (Killham 1986, Crossman et al. 1997, Bock and Wagner 2003). Since this study was designed to examine the AOB assemblages, below we focus exclusively on the phylogeny and on the review of studies about the environmental diversity and dynamics of this lineage of nitrifying bacteria.

Unlike most functional groups, AOB-specific 16S rRNA amplification has being used to infer the phylogeny of the AOB and thus to identify unculturable organisms at a functional level, because they are restricted to monophyletic clusters (McCaig et al. 1999, Bano and Hollibaugh 2000, Burrell et al. 2001, Freitag and Prosser 2003). Also, during the past few years, the gene encoding the first subunit of ammonia monooxygenase (amoA) has been increasingly exploited as a molecular marker for AOB diversity research in natural systems (Gieseke et al. 2001, Cébron et al. 2004, Horz et al. 2004, O'Mullan et al. 2005). Parallel studies using both molecular markers found consistent affiliations between 16S rRNA gene and amoA based trees (Caffrey et al. 2003, Purkhold et al. 2003, O'Mullan et al. 2005).

Evolutionary relationship of ammonia oxidizing bacteria based on comparative sequence analysis of their genes encoding the 16S rRNA demonstrated that all recognized ammonia oxidizers are either members of β- or γ-subclass of the Proteobacteria (Stephen et al. 1996, Purkhold et al. 2000, Bock and Wagner 2003). 16S rRNA sequence analysis includes the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* in a closely related monophyletic assemblage within the β-subclass of Proteobacteria (Purkhold et al. 2000, Fig.1.2). The *Nitrosomonas* cluster can be further subdivide in to the

N.europaea/Nitrosococcus mobilis cluster, the *N. marina* cluster, the *N. oligotropha* cluster, and the *N. communis* cluster. *Nitrosomonas cryotolerans* forms a separate lineage within the β-subclass of Proteobacteria. The genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* are closely related and form the *Nitrosospira* cluster separate from the *Nitrosomonas* cluster. The other line of descent constitutes a separate branch within the γ-subclass of Proteobacteria including the genus *Nitrosococcus* (Fig.1.2). Purkhold et al. (2000, 2003) presented the phylogeny of all recognised species of ammonia oxidizers from cultures and environmental clones and obtained similar evolutionary relationships if comparative analysis of amoA sequences were performed.

The knowledge of the distribution, diversity and relative abundance of AOB in nature has proliferated rapidly in recent years. The power of the molecular approaches was demonstrated by finding considerable diversity of AOB in several sewage treatment plants (Schramm et al. 1998, Princic et al. 1998, Gieseke et al. 2001, Egli et al. 2003) and environmental samples from lakes (Bollmann and Laanbroek. 2001), rivers (Brümmer et al. 2000, Cébron et al. 2004), seawater from coastal and oceanic sites (Ward et al. 1997, Phillips et al. 1999, Bano and Hollibaugh 2000, Hollibaugh et al. 2002, Cébron et al. 2003, O'Mullan et al. 2005), coastal and estuarine sediments (Caffrey et al. 2003, Freitag and Prosser 2003) and from various terrestrial environments (Stephen et al. 1998, Bruns et al. 1999, Bartosch et al. 2002, Horz et al. 2004, Webster et al 2005). Most of the AOB ecology have been focused on the diversity of these communities in different habitats (Sinigalliano et al. 1995, Stephen et al. 1996 and 1998, Kowalchuck et al. 1997, Ward et al. 1997, Philips et al. 1999, Bano and Hollibaugh 2000, Bothe et al. 2000, Kowalchuk and Stephen 2001, Koops and Pommerening-Röser 2001, Hollibaugh et al. 2002, Freitag and Prosser 2003), and studies revealed that the most common sequences detected in environmental samples were not from the most easily cultured species. On the other hand, the detection of the same β-subclass nitrifiers sequences in different environments showed a wide distribution of those microorganisms (Hollibaugh et al. 2002). Because these studies showed that species composition of nitrifier assemblages can change according to the type of environment, nowadays there is an increase interest in collecting information about the ecological requirements and physiological capacities of AOB in relation to environmental factors. Thus, studies in natural habitats (Schramm et al. 1998, Stephen et al. 1998, Brunes et al. 1999, Avrahami et al. 2002, Caffrey et al. 2003, Cébron et al. 2004, Horz et al. 2004, O'Mullan and Ward 2005) together with enrichment culture experiments (Princic et al. 1998, Kowalchuk and Stephen 2001) and wastewater

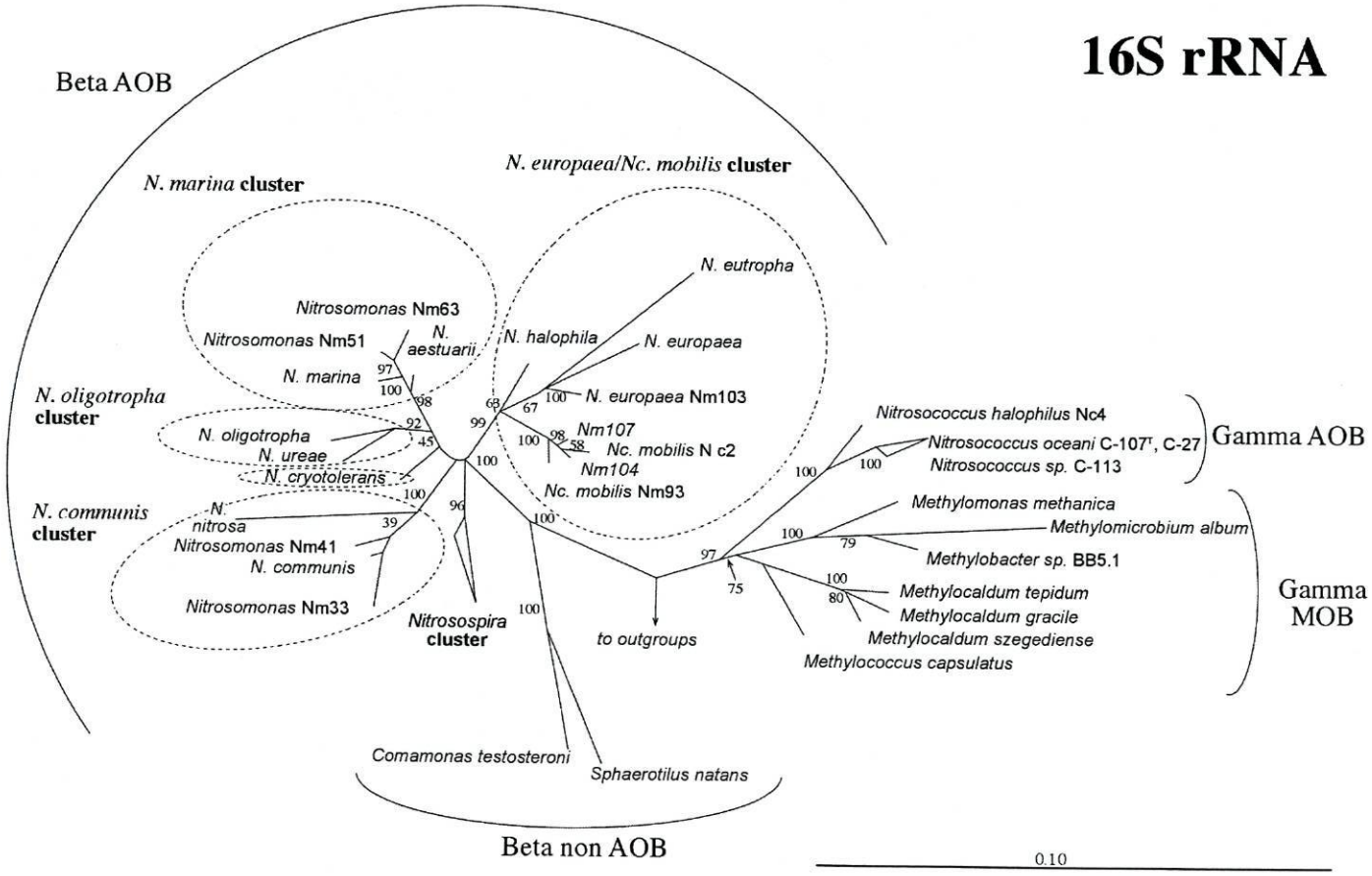

Figure 1.2: Phylogenetic neighbor-joing 16S rRNA tree showing the relationships of ammonia oxidizing bacteria and several reference organisms (adapted from Bock and Wagner 2003).

bioreactors investigations (Schramm et al. 1996 and 1998, Burrel et al. 2001, Gieseke et al. 2001, Egli et al. 2003) have revealed that there are physiological and ecological differences between the different AOB genera and lineages and that the different magnitudes of several environmental variables are of crucial importance in shaping the biogeography of AOB. Investigations on the potential links between environmental variables, AOB diversity, and biogeochemical function has also started in biofilm reactor systems of wastewater treatment plants (Schramm et al. 1996 and 1998, Gieseke et al. 2001, Egli et al. 2003). These kinds of studies have just recently been performed in a few natural aquatic (Caffrey et al. 2003, Cébron et al. 2004, O'Mullan and Ward 2005) and terrestrial ecosystems (Horz et al. 2004, Webster et al. 2005). Thus, insights about the diversity dynamics and function of marine AOB assemblages are just now beginning to emerge.

1.2.2.2 Denitrifiers

Nearly all of the prokaryotes defined as denitrifiers are identified because of their ability to reduce nitrate or nitrite to gaseous end products. Denitrifying bacteria are facultative anaerobes. While aerobic denitrification was described in several isolates (Robertson et al. 1995), its significance in the environment remains uncertain.

Bacteria constitute the vast majority of organisms capable of denitrification. However, denitrification also extends beyond the bacteria to the Archaea (Zumft 1997). Also, denitrification has been described for some fungi (Zumft 1997), however the ecological significance of denitrification in these organisms still needs to be characterized. The distribution of denitrification among the prokaryotes does not follow a distinct pattern, and thus the ability to denitrify is widespread among bacteria of unrelated systematic affiliations (Table 1.1). The majority of currently characterized denitrifiers fall in the group known as the Proteobacteria. The Proteobacteria was subdivided into five subdivisions (α, β, γ, δ, and ϵ) and denitrifiers have been found in four of these groups. The δ subdivision, which contains a number of strict anaerobes, has not been found to contain any strains that denitrify. The α subdivision contains a number of well characterized denitrifiers including *Paraccocus denitrificans*, various *Rhodobacter* and *Rhizobium* strains (Table 1.1). Denitrification in members of the γ subclass of proteobacteria has also been well studied in *Pseudomonas stutzeri* and *Ps. aeruginosa*. Although some of the β-proteobacteria denitrify, they are not as well studied as members of the α and γ subdivisions. Finally, a few denitrifiers have been found in the ϵ subdivision (Table 1.1).

Table 1.1: Listing of microbial genera that are suggested to include denitrifiers (adapted from Shapleigh 2003).

Archaea	*Oligella*
	Ralstonia (formely *Alcaligenes*)
Haloarcula	*Rubrivivax*
Halobacterium	*Thauera*
Haloferax	*Thermothrix*
Ferroglobus	*Thiobacillus*
Pyrobaculum	*Vogesella*
	Zoogloea
Bacteria	
Gram-negative	Gamma subdivision
Aquifex	*Acinetobacter*
Flexibacter (formerly *Cytophaga*)	*Alteromonas*
Empedobacter	*Azomonas*
Flavobacterium	*Beggiatoa*
Sphingobacterium	*Deleya*
Synechocystis sp. PCC 6803	*Halomonas*
	Marinobacter
Purple Bacteria	*Moraxella*
Alpha subdivision	*Pseudoalteromonas*
	Pseudomonas
Agrobacterium	*Rugamonas*
Aquaspirillum	*Shewanella*
Azosspirillum	*Thioploca*
Blastobacter	*Thiomargarita*
Bradyrhizobium	*Xanthomonas*
Gluconobacter	
Hyphomicrobium	Delta subdivision
Magnetospirillum	
Nitrobacter	None
Paracoccus	
Pseudomonas (G-179)	Epsilon subdivision
Rhizobium	
Rhodobacter	*Wolinella*
Rhodoplanes	*Campylobacter*
Rhodopseudomonas	*Thiomicrospira*
Roseobacter	
Sinorhizobium (formerly *Rhizobium*)	**Others**
Thiobacillus	Gram Positive
Beta subdivision	*Bacillus*
	Corynebacterium
Achromobacter	*Frankia*
Acidovorax	*Dactylosporangium*
Alcaligenes	*Dermatophilus*
Azoarcus	*Gemella*
Brachymonas	*Jonesia*(formerly *Listeria*)
Burkholderia	*Kineosporia*
Chromobacterium	*Micromonospora*
Comamonas	*Microtetraspora*
Eikenella	*Nocardia*
Hydrogenophaga	*Pilimelia*
Janthinobacterium	*Propionibacterium*
Kingella	*Saccharomonospora*
Microvirgula	*Saccharothrix*
Neisseria	*Spirrilospora*
Nitrosomonas	*Streptomyces*
Ochrobactrum	*Streptosporangium*

Because denitrifying bacteria belong to different phylogenetic groups (Zumft 1997), recent attempts to analyze denitrifying bacteria are based on the functional genes encoding nitrate-, nitrite-, nitric oxide- and nitrous oxide-reductase enzymes. The genes involved in denitrification contain highly conservative DNA regions, which can be successfully exploited for developing gene probes (Bothe et al. 2000).

Recent studies have involved molecular investigation on the ecology of denitrifier communities in distinct natural habitats, emphasizing mainly their distribution in time and space (Scala and Kerkhof 2000, Nogales et al. 2002, Priemé et al. 2002, Liu et al. 2003, Rich et al. 2003, Castro-González et al. 2005). Efforts have been directed toward amplification of functional genes from environmental samples involved in denitrification, mainly nitrite reductases genes (*nirS* and *nirK*, respectively), and a gene encoding nitrous oxide (*nosZ*). These PCR-based approaches revealed the presence of diverse denitrifying microbial communities in different natural ecosystems including continental shelf and estuarine sediments (Scala and Kerkhof 2000, Braker et al. 2001, Nogales et al. 2002, Liu et al. 2003), marine water column (Castro-González et al. 2005) and meadow and forest soils (Priemé et al. 2002, Rösch et al. 2002, Rich et al. 2003). Significant work in understanding the dynamics of denitrifier communities has also recently been accomplished in activated sludge (Song and Ward 2003, Yoshie et al. 2004), and cultivated soils systems (Stres et al. 2004, Throbäck et al. 2004, Wolsing and Priemé 2004, Sharma et al. 2005), but with contrasting interests of application. While in wastewater treatment plants, understanding the ecology of denitrifying communities is fundamental to determine optimal and stable operational conditions for denitrification processes (Yoshie et al. 2004), in agriculture related research the increasing knowledge of denitrifying bacterial communities would be of great importance to avoid land N removal and to develop research into denitrifiers gene repression (Sharma et al. 2005). All these studies together have contributed to the improvement of the complete or partial sequences of environmental nitrite and nitrous oxide reductase genes in the databases, and revealed a high diversity of denitrification genes in the environment, which often are divergent from the genes of cultured denitrifiers.

The effects of different environmental factors may underly temporal and spatial changes in denitrifying communities, and thus the study of the environmental controls on the dynamics of denitrifying communities is essential to develop an understanding of the factors that affect population dynamics and changes in rates of activity. However, just recently such studies are emerging. Liu et al. (2003) reported that biogeochemical conditions, especially

nitrate and oxygen, appear to be the key factors in controlling the structure of denitrifying communities in continental margin sediments. In agreement, Castro-González et al. (2005) demonstrated that NO_3^-, NO_2^-, O_2 and depth were important environmental factors governing shifts in denitrifiers communities of South Pacific water column O_2 minimum zone. Also, Avrahami et al. (2002) found that denitrifying community composition was related to ammonium (NH_4^+) addition in soils, and Yoshie et al. (2004) showed that salinity decreased nitrite reductase gene abundance in a wastewater treatment system. In addition, the impact of changes in microbial community structure and composition on the rate of denitrification has been recognized to be critical for the understanding of the global N dynamics and how N cycling might be altered with the global changes, but has been evaluated only in one study (Rich et al. 2003).

1.3 Douro River estuary: Description and Previous Research

The Douro River takes is source in Spain at 2070 m. Along its 930 Km the River drains 98000 km^2 (19% in Portuguese territory) before discharging into the Atlantic Ocean (41°08'30" N; 08°42'W). In the last 50 years thirteen large dams were built in the Portuguese section of the watershed, 10 of which in the main course of the River. Together with over 45 dams in the Spanish territory they retain potentially 8100 h m^3 of freshwater. The last dam was built in 1985 (Crestuma-Lever) and confined the Douro River estuary to its present 21.6 Km length. In the estuary, the river flows in a narrow granitic valley and just 3 km from the mouth occurs an enlargement of the margins. The mouth of the estuary is 1300 m wide and is partially obstructed by a 900-1100 m long unstable sand bar. The Douro River estuary is located in one of the most populated zones of Portugal. Three main cities, Porto, Vila-Nova-de-Gaia and Gondomar (750 000 inhabitants), are localized within the estuary exerting significant anthropogenic pressures in terms of urban runoff, sewage discharge, land reclamation and onshore constructions.

Douro River estuary has a mesotidal, semi-diurnal regime with an average depth of 8.2 m and a mean tidal amplitude of 2.8 m; freshwater discharge ranges from virtually 0 to 13,000 m^3 s^{-1}(average, 505 m^3 s^{-1}) with a residence time between 0.3-16.5 days (Vieira and Bordalo 2000). The estuary is vertically stratified even under low river flow conditions

(Vieira and Bordalo 2000). Water column temperatures range from 7 °C in winter to 22 °C in summer, and salinity in the lower estuary ranges from 0 to nearly 35 psu (practical salinity units).

Since 1985, a number of studies have been performed in the water column of the Douro estuary. This studies contributed for the understanding of the bacterioplankton and phytoplanktonic metabolism and nutrient dynamics (Bordalo 1991, Bordalo 1993, Bordalo and Lobo-da-Cunha 1994), and to the hydrodynamics (Bordalo 1997, Vieira and Bordalo 2000, Bordalo and Vieira 2005) of the estuary. Intertidal sediments of the Douro River estuary was also characterized in terms of macrobenthic community structure, and heavy metals concentration (Mucha et al. 2003, Mucha et al. 2004, Mucha et al. 2005). These studies revealed a clear signature of anthropogenic contamination of zinc (Zn), copper (Cu), lead (Pb) and chromium (Cr) and concentrations indicated possible toxic hazard for sediment biota. The importance of benthic primary production and inorganic nutrient fluxes between intertidal sediments and the water column was firstly evaluated by Magalhães et al. (2002). Results demonstrated that the productivity of the intertidal flats and their capacity for water column inorganic nutrient removal assumes particular importance within the estuary. More recently, the spacial and seasonal dynamics of the Bacteria and Archaea populations were characterized in the intertidal sandy sediments as well as in the water column of the lower Douro River estuary (Abreu et al. 2003, Abreu 2005). Results showed that while Bacteria community diversity showed no clear spatial and seasonal variability, the Archaea diversity presented clear seasonal trends.

1.4 Objectives

The main purpose of this study was to investigate the dynamics of the inorganic nitrogen cycle in intertidal rocky biofilm environments and establish a comparison with intertidal sediment environments of the Douro River estuary. Special attention was given to key N processes, the importance of some environmental controls and finally to the ecology of some bacterial communities that are responsible for important N transformations (Nitrifiers and Denitrifiers). In order to achieve this, five chapters are presented with the following detailed objectives:

1. Evaluate the role of the inorganic nutrient and metabolic dynamics of intertidal rocky biofilms in the Douro River estuary.

2. Characterize the inorganic nitrogen cycle dynamics in intertidal rocky biofilms and sediments of the Douro River estuary and provide a semi-quantitative comparison of the processes impacting inorganic nitrogen cycling in the different intertidal environments analysed.

3. Evaluate the effect of salinity and inorganic nitrogen (NO_3^- and NH_4^+) concentrations on nitrification, denitrification and N_2O production in sandy sediments and rocky biofilms of the Douro River estuary.

4. Characterize the ammonium oxidizing bacterial phylotypes in intertidal rocky biofilms and sediments of the Douro River estuary and establish relationships between community distribution and the rate of N processing and environment variables.

5. Evaluate *NosZ* gene variability in intertidal rocky biofilms and intertidal sediment sites of the Douro River estuary and establish a link between denitrifiers communities dynamics and key nitrogen processes and environmental variables.

Chapter 2

Intertidal Biofilms on Rocky Substratum can Play a Major Role in Estuarine Carbon and Nutrient Dynamics

2.1 Introduction

Worldwide, eutrophication of estuaries and coastal waters constitutes a serious, rapidly increasing problem (e.g. Jickells 1998). While intertidal and subtidal estuarine sediments are recognized as important sites for nutrient transformation and sequestration via biogeochemical cycling (Ogilvie et al. 1997, Cabrita and Brotas 2000, Schulz and Zabel 2000), many estuaries also possess extensive areas of hard surfaces (e.g. rocky coastlines, fjords, drowned sandstone and granitic valleys, carbonate platforms). Further, man-made structures (e.g. breakwaters and jetties, pilings and walls for marinas and port facilities) progressively replace sediments with hard surfaces. These rocky surfaces provide substrate for the proliferation of biofilms with a wide variety of metabolically diverse microorganisms.

The contents of this chapter were originally published in Magalhães C., A. A. Bordalo, and W. J. Wiebe (2003), Intertidal biofilms on rocky substratum can play a major role in estuarine carbon and nutrient dynamics, Mar. Ecol. Progr. Ser. 258:275-281.

Biogeochemical benthic-water coupling in rocky rivers and coral reef flats (carbonate platforms) are documented (e.g. Wetzel 1983, D'Elia and Wiebe 1990), and coastal rocky shores have received much attention by community ecologists, who have studied animal, macrophyte and microalgal distribution, controls of trophic structure, and food webs of these communities (Hillebrand and Sommer 2000, Knox 2000 and references herein, Worm et al. 2000). However, the extent of biogeochemical processes within the thin layer of organic matter that characterizes hard surfaces of many marine coastal habitats has scarcely been considered. For example Decho (2000) who reviewed microbial biofilms in intertidal systems and Knox (2000) who published a book on the ecology of seashores do not mention this topic.

In this study rates of primary production and respiration, and net fluxes of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-}, SiO_4^{4-} between hard surface biofilms and water column of intertidal zone of the Douro River estuary were examined and compared with comparable data from adjacent intertidal sandy and muddy sediments.

2.2 Material and Methods

2.2.1 Site Description

The Douro River estuary receives an average of 505 m^3/s^{-1} of freshwater, containing, on average, 8 µM NH_4^+, 80 µM NO_3^-, 2 µM NO_2^-, 2 µM PO_4^{3-} and 43 µM SiO_4^{4-} (Bordalo et al. unpublished data). In addition, largely untreated sewage from approximately 750 000 inhabitants is discharged within its lower reach. Throughout the estuary, much of the historic intertidal marsh and sediment flats has been dredged or filled to provide housing, parks, roads and port facilities. Today the total planar (2-dimensional) area of the estuary is 7.5 km^2, of which approximately 1.5 km^2 is intertidal sand and muddy sediment and 0.39 km^2 intertidal, natural and man-made hard surfaces. Because of its rugosity, the actual (3-dimensional) surface area of the intertidal hard surface rises to 0.63 km^2. The hard surfaces support extensive microbial biofilms intertwined with *Enteromorpha* spp. and *Fucus* spp. macroalgae, and with benthic microalgae (mostly diatoms).

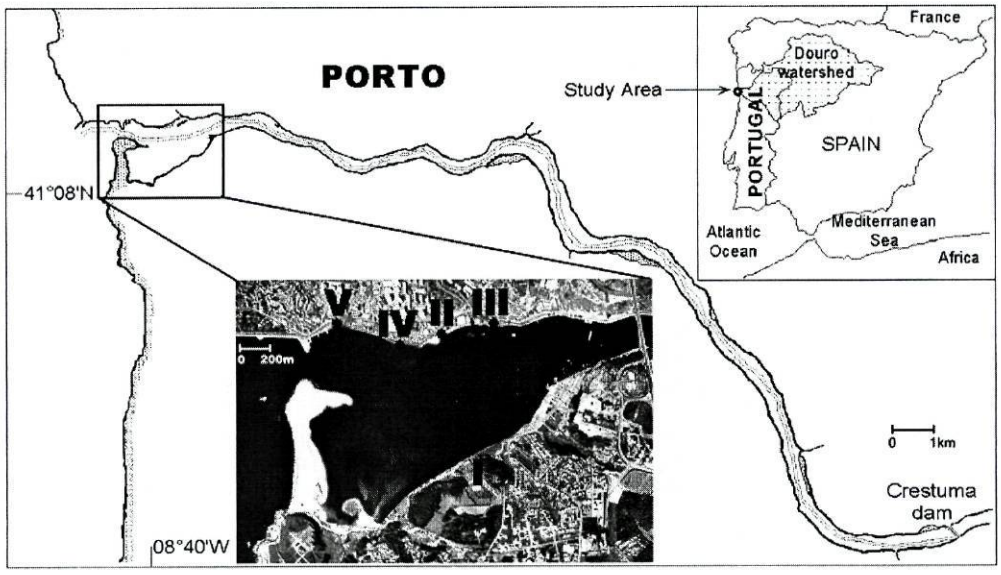

Figure 2.1: The Douro River estuary and location of sampling stations.

2.2.2 Flux Measurements

The exchange of oxygen and inorganic nutrients (ammonium, nitrate, nitrite, phosphate and silicate) between biofilm-covered rocks and water was measured on samples taken in the spring of 2000 from five mid-intertidal sites (between March and June) (Fig. 2.1 and Table 2.1). Rocks were collected at low tide, placed in plastic bags and returned immediately to the laboratory; 30 liters of estuarine water was also collected adjacent to the sampling sites. In the laboratory, the estuarine water was filtered through a 0.2 μM membrane filter; triplicate light and dark (wrapped with several layers of aluminum foil) Plexiglas chambers (11 cm diameter and 12 cm depth) were prepared for incubation. Individual rocks were placed at the bottom of each chamber, the chambers filled carefully with the filtered estuarine water, and all air bubbles removed. Light and dark chambers were incubated under natural light (> 800 μEinstein m^{-2} s^{-1}) at *in situ* temperature (Table 2.1). Triplicate light and dark control chambers consisted of only the filtered (0.2 μM) estuarine water. After incubation, each rock was molded with aluminum foil and the foil weighed after drying. The weight was converted to area (cm^2), using a linear regression between foil weight and the corresponding area. The coefficient of variation of this method was 1.8%.

Table 2.1: Characteristics of the estuarine water used for incubations (mean±SD).

Site	Date (dd/mm/yy)	Temperature (°C)	O_2 (mg l^{-1})	Salinity (psu)	NH_4^+ (µM)	NO_3^- (µM)	NO_2^- (µM)	PO_4^{3-} (µM)	SiO_4^{4-} (µM)
I	14-3-00	15.3±2.2	8.8±0.2	14.0	11.2±0.2	83.2±0.1	0.8±0.1	1.7±0.3	53.6±2.6
II/III	1-6-00	17.1±0.2	6.6±0.0	4.5	13.5±0.1	125.4±0.8	2.4±0.2	1.1±0.0	73.6±4.0
IV/V	8-6-00	19.5±0.6	6.5±0.4	9.5	8.6±0.6	91.6±2.1	1.1±0.1	1.4±0.2	70.3±4.9

Primary production and respiration were estimated from the difference between the initial (T_0) and T_1 dissolved oxygen concentrations in the triplicate light and dark incubation chambers. During this period of incubation (1 to 1.5 hours) chambers water volume did not change since T_0 overlying water sample (approximately 6% of the total volume) was replaced immediately with filtered estuarine water. Net oxygen fluxes were calculated according to Hargrave et al. (1983), following the equation;

$$F_O = \left(\frac{(T_1 - T_0)}{A \times t} \right) \times V \times 10^4 \tag{2.1}$$

where F_O is the flux of oxygen (mg $m^{-2}h^{-1}$), $(T_1 - T_0)$ the difference in oxygen concentration between time zero and time one of the incubation (mg O_2 l^{-1}); A is the rock surface area in cm^2; t is the incubation time (h); V is the estuarine water volume in each chamber (l) and 10^4 is the conversion factor from cm^2 to m^2.

Immediately after sampling, the oxygen samples were fixed and dissolved oxygen determinations performed in triplicate, using a modification of the Winkler method (Carpenter 1965). Oxygen values were corrected for the oxygen variations in the control chambers. Hourly respiration and primary production rates were calculated per square meter of biofilm as follows: Hourly net primary production rate (NPP) = mean oxygen production (mg m^{-2}) in transparent chambers / incubation time (h); Hourly respiration rate (R) = mean oxygen consumption (mg m^{-2}) in dark chambers / incubation time (h). Oxygen data were converted to carbon assuming that 1 mg of oxygen produced or respired is equivalent to 0.375 mg of organic carbon (Uthicke and Klumpp 1998).

For inorganic nutrients a T_0 sample was taken from each chamber, and the volume of water removed was replaced with estuarine water, as described below. Subsequently, four (T_1 to T_4), 50 µl subsamples of overlying water were taken approximately hourly from

each chamber during 4.5 to 5.5 hours of incubation period to assess linearity of the rates (Fig. 2.2). Water was gently mixed manually every 20 minutes. In controls (0.2 μM filtered estuarine water), subsamples were taken only at times T_0 and T_4. All subsamples were immediately syringe-filtered through 0.45 μM acid-washed membrane filters and stored at -21 °C in acid-cleaned polyethylene flasks until inorganic nutrient analysis. Nutrient analyses of each sample were performed in triplicate. Orthophosphate, nitrite, ammonium and reactive silicate were quantified using methods described in Grasshoff et al. (1983). Samples for dissolved silicate analysis did not have contact with glass and after thawing were kept one week at 5°C; this procedure completely depolymerizes the silicate (Grasshoff et al. 1983). Nitrate was assayed using an adaptation of the spongy cadmium reduction technique (Jones 1984), with the nitrite value subtracted from the total. The precision of all determinations was in the range 0.1 - 8%, depending on the particular nutrient concentration. For each incubation period, inorganic nutrient concentrations (μM) were corrected for the corresponding volume of chamber water (μmol), by multiplying the concentration value of each inorganic nutrient by the actual volume (l) of each chamber at each time.

Flux rates of inorganic nutrients were calculated using the slope of the linear relationship between the changes in the nutrient content (μM) in the water chamber with the time of incubation (h) (e.g. Barbanti et al. 1992), following the equation:

$$F_{\mathrm{N}} = \left(\frac{\alpha}{A}\right) \times 10^4 \tag{2.2}$$

where F_{N} is the flux of each inorganic nutrient (μmol $\mathrm{m^{-2}h^{-1}}$), α is the slope of the linear regression (μmol $\mathrm{h^{-1}}$), A is the rock surface area in ($\mathrm{cm^2}$) and 10^4 is the conversion factor from $\mathrm{cm^2}$ to $\mathrm{m^2}$.

When net nutrient flux rates were either positive or negative, the fluxes were linear over the entire incubation period (Fig. 2.2). Seven times (out of 50) null net fluxes were observed; in these cases each of the replicate chambers showed no change in nutrient concentration with incubation time. Significant differences in control chamber nutrient concentrations between T_0 and T_4 (ANOVA, $p > 0.05$) were never observed.

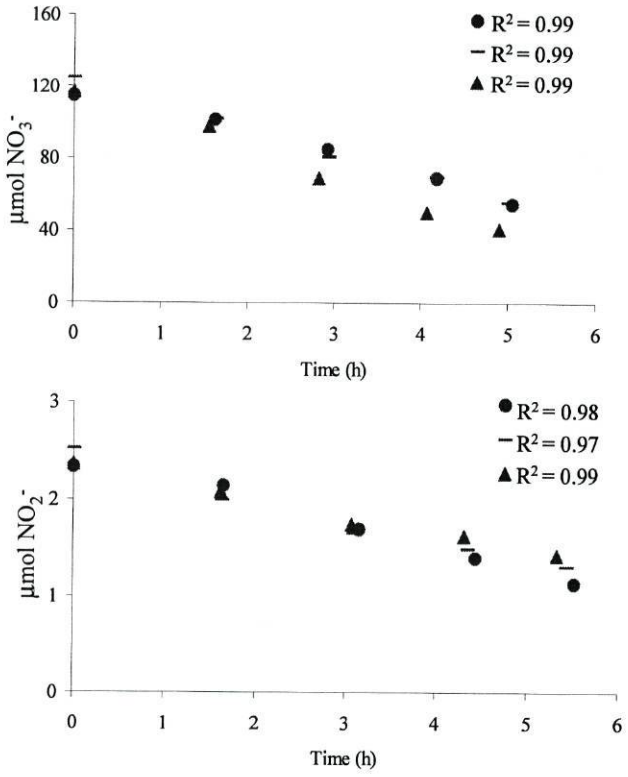

Figure 2.2: Examples of typical linearity between water nutrient concentrations and incubation period. Different symbols represent individual values for triplicate chambers.

2.2.3 Total Organic Matter and Chlorophyll *a*

Surface triplicate subsamples (1 cm diameter) were collected by scraping each rock at the end of the experiment and placing them into glass vials for chlorophyll *a* (chl *a*) and total organic matter (OM) analyses. Samples for chl *a* measurements were kept frozen (-21 °C) until analysis. Extraction was performed in a mixed solution of acetone, methanol and water (45:45:10, respectively), according to Joye et al. (1996). Also, Hill and Hawkins (1990) found that methanol extraction is a more accurate and precise method than acetone extraction to estimate chl *a* content in intact rocks. Chl *a* concentration was determined spectrophotometricaly, using standard equations (Parsons et al. 1984). Samples for organic matter content were processed immediately by drying to constant weight at 60 °C, followed by ignition in a muffle furnace at 500 °C for 4h and reweighing (data are presented either as % of total organic matter and g of total organic matter per m^2 of biofim).

Table 2.2: Spatial variation of chlorophyll *a* (chl *a*) and total organic matter content (mean±SD). na: not available

Site	g chl *a* m^{-2}	g OM m^{-2}	% OM
I	na	na	na
II	0.44±0.26	41.07±23.99	11.96±2.56
III	0.80±0.26	71.54±34.73	9.34±4.16
IV	1.24±0.31	124.53±19.76	16.23±9.67
V	0.84±0.22	101.45±33.47	16.86±13.90

2.2.4 Statistical Analysis

Data analysis was performed at the 95% confidence level ($p < 0.05$). Data were tested for normality, using the Kolmogorov-Smirnov test, and homoscedasticity, using the Leven's test (Zar 1996). In order to compare the means of each variable between the different stations, a one-way ANOVA (one degree of freedom) analysis was performed (Zar 1996).

2.3 Results and Discussion

Water column temperature, oxygen concentration, salinity and inorganic nutrient concentration for each sampling date are given in Table 2.1.

The average concentration of chl *a* on hard surfaces was 0.8±0.4 g m^{-2}. The average total organic matter per square meter of biofilm was 84.7± 41.8 g, and the mean value for percent total organic matter was 13.4±8 %. Showing that in spite of the high total organic matter per square meter of biofilm, there was also a high percentage of non-organic material since just 13.4±8 % of these biofilms are organic matter. Spatial data for these variables are shown in Table 2.2. The positive linear relationship found between chlorophyll *a* and total organic matter ($r = 0.84$, $p < 0.001$, $n = 24$) suggests that the majority of the organic matter in these biofilms was macroalgae.

Hourly net primary production (NPP; light incubation) exceeded hourly net respiration (R; dark incubation) by 5 to 11 times (Fig. 2.3, Table 2.3). NPP and R were similar between sites (Fig. 2.3); only Site III showed significantly higher NPP and R rates (ANOVA, $p < 0.05$). The mean hourly biofilm NPP averaged 146.0±58.1 mg C $m^{-2}h^{-1}$, similar to values

Table 2.3: Comparison of net inorganic nutrient fluxes (μmol m^{-2} h^{-1}), net primary production (NPP) and respiration rates (R) (mg O_2 m^{-2} h^{-1}) in hard surface-water and sediment-water interfaces, for the Douro River estuary (mean±SD). Sandy and muddy sediment data from Magalhães et al. (2002).

	NH_4^+ (light)	NH_4^+ (dark)	NO_3^-	NO_2^-	PO_4^{3-}	SiO_4^{4-}	NPP	R
Rocky biofilms	-123.2±22.5	144.5±151.9	-595.4±236.7	-0.57±9.1	1.4±10.5	-305.7±209.6	389.4±168.3	50.4±17.5
Sandy sediment	-64.1±25.1	-18.3±22.9	-338.6±170.1	-3.8±3.6	-4.8±3.1	-169.3±57.0	427.5±173.0	37.9±23.5
Muddy sediment	-55.8±33.0	33.5±67.1	-316.7±142.9	2.6±9.4	-3.9±3.5	-127.2±71.6	261.5±51	44.6±13.1

found in adjacent sediments (Magalhães et al. 2002) and in sediments elsewhere (e.g. Colijn and de Jonge 1984; Barranguet et al. 1996). NPP showed a positive correlation with respiration rates ($r = 0.64$, $p < 0.05$, $n = 30$), comparable to results from other intertidal sediment-water flux studies (e.g. Cammen 1991, Magalhães et al. 2002).

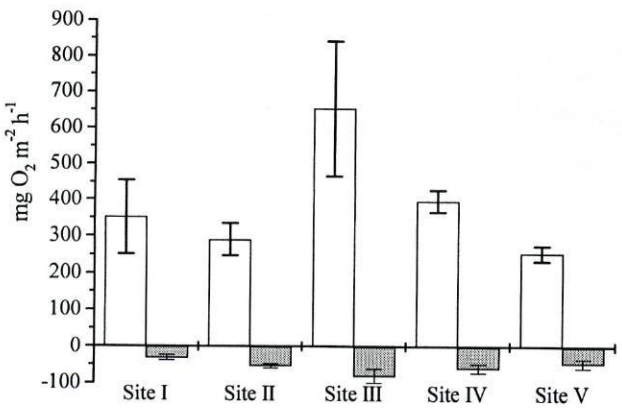

Figure 2.3: Hourly net production (white bars = light incubation) and respiration (grey bars = dark incubation) rates at each sampling site (mean±SD).

The direction and magnitude of light and dark incubated inorganic nutrient fluxes between hard substrates and water varied, depending on the nutrient examined (Fig. 2.4). Ammonium concentrations in incubation water decreased linearly over time in light incubations (negative fluxes) but showed variable responses in dark incubations (Fig. 2.4a). In contrast, nitrate, which in addition to providing fixed nitrogen for algae and bacteria is considered a serious contributor to coastal eutrophication, was consistently removed at similar rates in both light and dark incubations (Fig. 2.4b). These findings suggested an ammonium assimilation preference by the primary producers that inhabit the rocky biofilms. The

preferentially use of NH_4^+ instead of NO_3^- by macrophyte and microalgal has been documented (e.g. Korb and Gerard 2000, Tungaraza et al. 2003). Nitrite concentrations in incubation water ranged from 0.8 to 2.4 μM (Table 2.1); light vs dark incubation did not affect the flux direction or magnitude (Fig. 2.4c). Nitrite, produced via ammonium oxidation and during denitrification, is generally found in low concentrations, and benthic fluxes are reported to be minimal (e.g. Ogilvie et al. 1997, Cabrita and Brotas 2000).

No spatial variability was found in light net nitrate fluxes since the ANOVA analysis yielded no spatially significant differences, while Site III showed significantly higher dark uptake rates than sites I, II and V (ANOVA, $p < 0.05$). In all cases molar ratios of C:N (NH_4^+, NO_3^-, NO_2^-) net fluxes in light chambers (20.4±4.1; 10.4±1.8; 25.0±11.3; 18.9±0.6; 10.7±1.7, for sites I, II, III, IV and V, respectively) were not far from the optimal stoichiometric ratio of C:N = 7 given by Hillebrand and Sommer (1999) for periphyton. Further, Worm et al. (2000) found C:N ratios of *Enteromorpha* in spring and summer from 10 to about 40 depending in part on the ambient water nitrogen concentration. On the other hand the lack of statistical relationships between NPP and NO_3^- and NH_4^+ net uptake rates and the absence of significant differences between light and dark incubations in nitrate net fluxes, supports the idea that primary production alone is unlikely to be the major process controlling nitrogen uptake rates. Denitrification, as previously found for intertidal and subtidal sediments (c.f. Seitzinger 1990, Ogilvie et al. 1997), could also be largely responsible for the nitrate uptake rates reported here, and experiments are underway to assess this possibility.

Phosphate net fluxes showed no consistent trend in flux direction or magnitude (Fig. 2.4d). Phosphate, in addition to its biological importance, also rapidly adsorbs to clays and other particulate inorganic compounds (de Jonge and Villerius 1989, Sundby et al. 1992). As a result, clear, biologically-mediated, benthic-water fluxes are not commonly seen. In this study the absence of significant, positive relationships between net PO_4^{3-} uptake rates and NPP as well as the dominance of positive fluxes, suggest that net PO_4^{3-} uptake rates measured did not primarily reflect phosphate assimilation by epilitic primary producers.

Both light and dark dissolved silicate fluxes were negative (removal from water), with one exception when no net flux occurred in either light or dark incubations (Fig. 2.4e). Dissolved silicate, while required by diatoms for production of frustules (Tréguer et al. 1995), also can be adsorbed by hydrated oxides of aluminium, iron, manganese and magnesium (Edwards and Liss 1973), and thus the uptake can result from both biological and

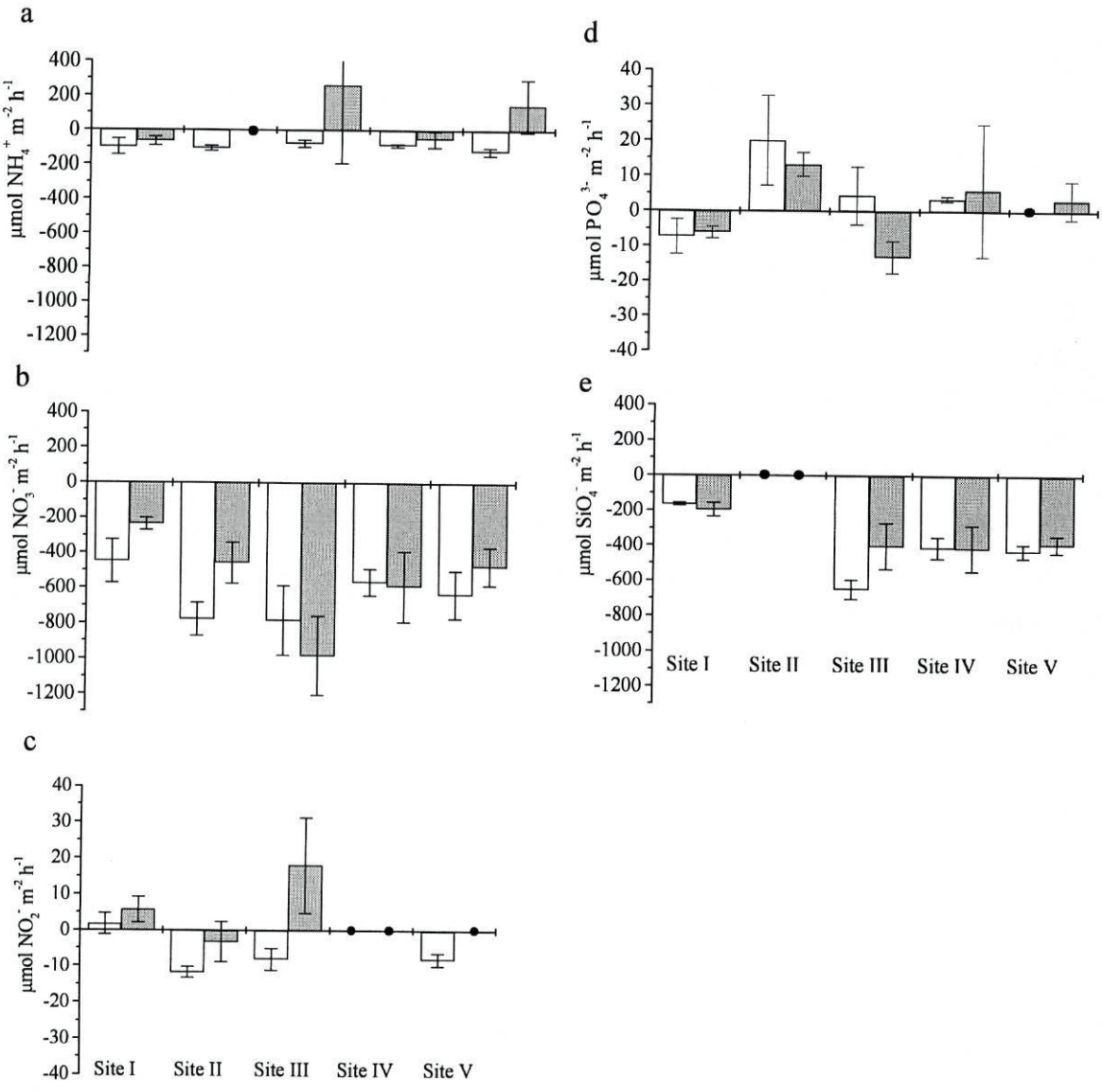

Figure 2.4: Averaged values (mean±SD) of Douro River estuary net fluxes of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and SiO_4^{4-} between the water column and rock biofilms (grey bars = dark incubation; white bars = light incubation; dark spots = null flux).

abiotic processes. However, since two samples gave no net uptake (Fig. 2.4e), biological processes probably were responsible for the kinetics seen. Sigmon and Cahoon (1997) demonstrated the potential importance of benthic diatoms as regulators of water column SiO_4^{4-} concentrations, but in this case dark and light flux rates differed. Correlation analysis between NPP rates and net SiO_4^{4-} fluxes in light chambers reveled a significantly positive relationship between NPP and net influx of dissolved silicate ($r = 0.69$, $p < 0.05$, $n = 15$), i.e. the higher the NPP, the higher uptake of SiO_4^{4-}. These results suggest that diatoms, which appear visually abundant in the biofilms, could account for a significant amount of the SiO_4^{4-} uptake. Also, net SiO_4^{4-} uptake rates found in this study were generally within the values reported by Tréguer et al. (1991) for rates of pelagic biogenic silicate production (90 to 950 $\mu M\ m^{-2}\ h^{-1}$) (Fig. 2.4e).

The comparisons between hard surface and sediment-water fluxes of oxygen and nutrients within the Douro River estuary reported in Table 2.3 show that NPP and respiration rates were virtually identical in the two systems (ANOVA, $p > 0.05$). This is in some ways remarkable given that the chl *a* in spring averaged 188±32 mg m^{-2} for muddy sediments, 631±91 mg m^{-2} for sandy sediments and 830±38 mg m^{-2} for rocky substrates. This emphasizes the importance of the microalgal contribution to the NPP, even when macroalgae dominate the plant biomass. The fact that only 34% of the variability found in NPP is explained by the chl *a* content strengthens this idea ($R^2 = 0.34$, p ¡ 0.05, n = 12). This is reminiscent of the finding by Pomeroy (1959), who found that the thin film of diatoms on the intertidal sediments of a *Spartina alterniflora* marsh contributed 20% of the marsh productivity.

In the case of inorganic nutrient net fluxes, while no statistical differences were found for NH_4^+, NO_2^- and PO_4^{3-} between these intertidal environments, hard surfaces removed significantly higher amounts of NO_3^- (ANOVA, $p < 0.002$) and SiO_4^{4-} (ANOVA, $p < 0.007$) from the water column than did adjacent muddy and sandy sediments (Table 2.3). In order to compare the biogeochemical significance of NO_3^- and SiO_4^{4-} fluxes between intertidal hard substrates and sediments, mean hourly values were calculated and integrated for the total exposed area of each component. While rocky biofilms occupy just 21% of the total linear (2-dimensional) area of the intertidal zone of Douro River estuary, because of rugosity and higher flux rates, they were responsible for approximate 43% of the NO_3^- uptake and 45% of the SiO_4^{4-} uptake, in terms of hourly removal capacity for the total intertidal area. These data provide a substantial case that hard surface zones of estuaries

can represent an important, albeit overlooked, component of estuarine and coastal carbon and nutrient cycling.

From a general biogeochemical perspective, it was unexpected that the metabolic rates and nutrient flux directions and magnitudes would produce such similar results for intertidal muddy sediment, sand and rock substrata, given the vastly different 3-dimensional physical structure of the biotic communities. The results presented here emphasize the importance of the boundary surface layer of the rock substrata for processing carbon and nutrients.

In summary inorganic nutrient and oxygen fluxes of rocky surfaces within the intertidal zone of the Douro River estuary function at comparable, and in some cases higher, rates than are found in adjacent sandy and muddy sediments. The results strongly suggest that these, overlooked components of the intertidal zones deserve inclusion in assessing biogeochemical fluxes of other estuarine systems and along coastlines with significant areas of hard surface.

Chapter 3

Inorganic Nitrogen Dynamics in Intertidal Rocky Biofilms and Sediments of the Douro River Estuary (Portugal)

3.1 Introduction

Soft and hard substrate intertidal habitats are highly dynamic. Both habitats are characterized by frequent fluctuations of a diverse suite of physical and chemical parameters creating physical and biochemical challenges and opportunities for the organisms that inhabit these boundary environments. In intertidal sediments, there is a growing interest in understanding the structure and function of a variety of metabolically diverse microorganisms, since microbial processes account for a majority of nutrient remineralization, carbon oxidation and often primary production (e.g. Asmus et al. 1998, Thomas and Christian 2001). A broad range of microbial-mediated N transformations occurs within intertidal sediment biofilms, and rates of N cycle processes have been quantified by a

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variety of techniques (e.g. Joye and Paerl 1994, Jensen et al. 1996, Cabrita and Brotas 2000). In systems containing high concentrations of fixed N, nitrogen fixation is often of minor consequence (e.g. Howarth et al. 1988). However, in such systems, denitrification can represent an important sink for natural and anthropogenic inputs of NO_3^- (Seitzinger 1988, Ogilvie et al. 1997, Seitzinger 2000). Remineralization of organic nitrogen and subsequent nitrification of NH_4^+ to NO_3^- can further stimulate the activity of denitrifying bacteria (Jensen et al. 1996). Also, denitrification in hyper-eutrified systems may result in increased fluxes of N_2O (Robinson et al. 1998), a potent greenhouse gas that also contributes to the destruction of atmospheric ozone (Dickinson and Cicerone 1986).

While intertidal rocky biofilms can represent important sites for biogeochemical activity (Magalhães et al. 2003*), nitrogen cycle dynamics within such biofilms have rarely been investigated (Decho 2000, Knox 2000). On the other hand, numerous studies have focused on the macro-ecology of coastal rocky shore (c.f. Knox 2000) and some investigations of the influence of inorganic nutrient supply on epilithic microalgae and macroalgae community structure have been conducted in marine and freshwater habitats (Guash et al. 1995, Hillebrand and Sommer 2000, Bokn et al. 2002).

The Douro River estuary (a drowned granitic valley) possesses extensive areas of both natural hard surfaces as well as man-made structures in addition to intertidal sand and mud flats. Throughout the estuary, much of the historic intertidal marsh and sediment flats have been dredged or filled to construct housing, parks, roads and port facilities. Over time, intertidal sediments have been progressively removed and replaced by rocks or other hard surfaces. The magnitude of NO_3^- inputs from freshwater has increased dramatically during the past decade, and no improvement is expected when, in future, several sewage treatment plants, without significant nutrient removal, begin operation.

The study described here had two distinct objectives. First, we quantified the different nitrogen cycle processes (mainly N fixation, nitrification, denitrification, uptake by primary production) contributing to the inorganic N fluxes between Douro River estuarine water and four, visually distinct, intertidal substrates: muddy and sandy sediments and *Enteromorpha* spp. and *Fucus* spp. colonized rocks, under both light and dark incubation conditions at four different times of the year. Second we developed a conceptual model of the nitrogen cycle dynamics for each intertidal environment to provide a semi-quantitative comparison of the processes impacting DIN cycling in each different environment.

*The contents of this article can be found in Chapter 2.

3.2 Methods

3.2.1 Study Area and Sampling Program

Physical and chemical characteristics of the Douro River estuary have been presented previously (Magalhães et al. 2002, Magalhães et al. 2003).

This study was conducted between 2001 and 2002 (Table 3.1) in the four dominant intertidal environments of the lower estuary (Fig. 3.1): two intertidal rocky areas (*Enteromorpha* spp. and *Fucus* spp. colonizing zones; EZ and FZ respectively), and two intertidal sediments (sandy and muddy areas; SZ and MZ respectively). Macrozoobenthic abundance and diversity, as well as particle size distribution, were previously reported for SZ and MZ sites (Magalhães et al. 2002, Mucha et al. 2003).

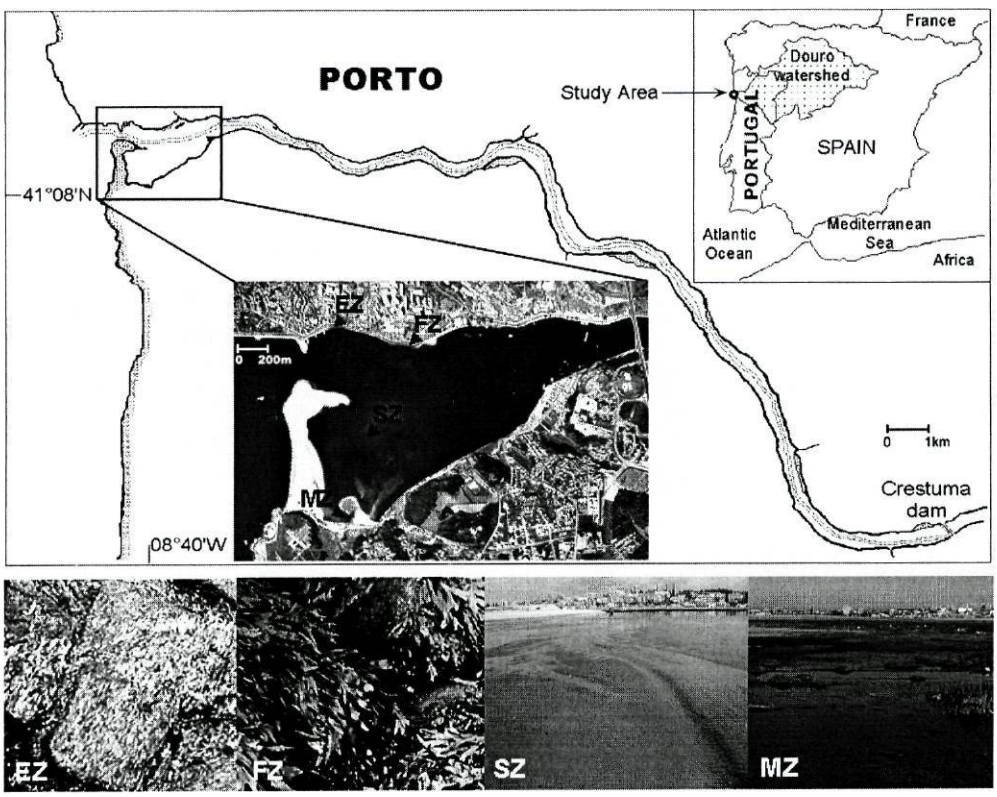

Figure 3.1: Douro River estuary and location of sampling sites (SZ, MZ, EZ and FZ).

Sampling was conducted at low tide between 2001 and 2002 (Table 3.1). MZ and SZ sediments were collected by inserting transparent Plexiglas tubes (12 cm inner diameter and 16 cm long, with a top fixed Plexiglas stopper with two 1 cm diameter holes) 10 cm

into the sediment; cores were then gently removed and sealed with rubber stoppers. Rocks at the EZ and FZ sites were collected either by hand or by drilling out a core. Within one hour of collection, six cores and/or rocks from each sampling site were kept cool and transported in the dark to the laboratory for processing. Estuarine water was collected in the vicinity of each sampling site on the following high tide for use in the experiments described below.

3.2.2 Incubation Set Up

In the laboratory, triplicate light and dark (wrapped with several layers of aluminum foil) chambers were prepared for incubation; sediment and rock samples were kept submerged overnight at the environmental temperature but the chambers were open to the atmosphere (Conley et al. 1997). The next day (8 h - 9 h), incubation chambers with sediment or rocks were carefully filled with filtered estuarine water (0.2 μm filtered, Schleicher & Schuell membrane filters) with care taken to exclude air bubbles. Controls consisted of 0.2 μm filtered estuarine water in the same Plexiglas tubes. Light and dark chambers were incubated at *in situ* temperature in a water bath under white fluorescent lamps at 400 μmol m^{-2} s^{-1}; this value as been reported to provide saturating irradiance for *Enteromorpha* spp. (Kirk 1983) and benthic microalgae (Pinckney and Zingmark 1993). However, light saturation may vary according with the different primary producer communities. Each chamber was gently stirred using a teflon magnet positioned 4 to 5 cm above the sediment or rock surface. Within each chamber two transparent vinyl gloves, positioned 2 cm below the top of the chamber were introduced in order to occupy, progressively, the water volume removed during each sub-sampling. This prevented introduction of headspace or air bubbles when sampling during the incubation period. The consistent absence of variation in the concentrations of dissolved inorganic nitrogen (DIN) compounds with time in control chambers (filtered estuarine water) (Fig. 3.2) confirms that leakage did not occur during the incubation period. After this initial incubation, overlying water was carefully removed, incubation chambers were stored at *in situ* temperature in the dark, and sediment cores and hard substrate samples were maintained submerged in chambers open to the air.

The following day, a second incubation was performed on the same samples. Chambers were refilled with freshly filtered estuarine water (as above), and 10% v/v of the overlying

Table 3.1: Characteristics of time zero filtered estuarine water used for the incubations (mean±SD), chlorophyll *a* (chl *a*), and total organic matter (OM) content in the substrate from the four sites (na: not available).

		Sampling Dates	Incub. Temp. °C	Salinity	pH	NO_3^- (μM)	NO_2^- (μM)	NH_4^+ (μM)	chl *a* (mg m^{-2})	OM (%)	OM (g m^{-2})
SZ	Summer	20 Jul 2001	20	15.4	7.8	45.9±13.9	1.8±0.1	17.1±1.7	113.4±19.9	0.4±0.0	38.9±5.8
	Fall	2 Oct 2001	19	20.0	na	33.7±4.5	1.0±0.1	17.6±2.2	na	1.3±0.1	161.8±16.1
	Winter	14 Mar 2002	11	5.0	7.7	88.5±11.1	0.7±0.1	17.6±0.8	296.6±22.5	0.6±0.1	65.1±11.2
	Spring	17 May 2002	18	11.8	7.6	40.0±8.2	0.9±0.2	21.1±1.5	510.1±120.9	0.9±0.1	91.5±9.7
MZ	Fall	20 Sep 2001	20	10.8	7.5	39.3±6.8	1.5±0.4	26.4±10.1	108.9±14.0	3.4±0.5	289.5±40.1
	Winter	27 Feb 2002	12	11.2	7.4	57.6±6.2	0.9±0.1	21.8±4.5	62.2±14.4	8.0±3.3	409.4±41.4
	Spring	14 May 2002	16	33.3	7.9	12.4±1.8	0.3±0.1	13.4±6.8	127.7±6.9	12.6±4.4	911.0±53.0
EZ	Summer	11 Jul 2001	19	16.1	7.7	62.3±19.7	3.8±1.9	50.5±14.2	1347.4±265.4	12.7±14.4	178.5±50.6
	Fall	10 Oct 2001	19	12.9	7.6	52.4±8.0	2.2±0.5	28.6±6.6	740.1±669.4	33.8±21.2	125.8±61.1
	Winter	19 Feb 2002	12	19.8	na	58.1±3.4	1.6±0.3	18.3±2.3	640.0±37.8	14.2±8.6	91.5±11.9
	Spring	10 May 2002	16	22.5	7.8	32.9±4.4	2.0±0.6	111.9±10.2	342.3±77.7	15.2±13.7	62.7±6.0
FZ	Summer	25 Jul 2001	18	20.2	7.6	54.6±9.9	1.5±0.3	17.0±4.7	941.7±348.4	7.0±2.1	459.0±149.9
	Fall	17 Oct 2001	18	21.0	7.3	50.4±2.5	1.7±0.1	13.9±2.3	1171.2±596.9	14.1±1.0	361.3±69.7
	Winter	4 Mar 2002	12	13.7	7.7	61.8±3.5	1.6±0.6	22.5±5.3	1087.8±116.5	12.0±15.0	385.2±243.7
	Spring	6 May 2002	17	7.2	7.8	83.8±11.8	2.2±0.4	91.6±26.7	513.3±406.7	25.3±15.4	75.9±73.6

water was replaced by acetylene-saturated estuarine water (C_2H_2 was produced from calcium carbide). The addition of acetylene allowed for the simultaneous determination of N fixation, denitrification and nitrification rates via the acetylene reduction and acetylene block techniques, respectively (Joye and Paerl 1994). To control for intrinsic changes (e.g. cell growth or death) that could affect the rates of reaction during the second day incubations, an additional set of samples without acetylene was run for each site. The absence of significant differences in rates between samples incubated without acetylene on day I and II (data not shown) confirmed that differences between net fluxes with and without acetylene for the same samples resulted from the addition of acetylene.

3.2.3 Oxygen Fluxes

For oxygen, 20 ml of overlying water were collected from each chamber, and samples were fixed immediately and processed within 24 h, according to Carpenter (1965). Almost no changes in O_2 concentration were found in control chambers, containing filtered overlying water, between T_0 and T_1 (data not shown). Oxygen net fluxes were estimated from the difference between initial (T_0) and time one (T_1) dissolved oxygen concentrations in triplicate light and dark incubation chambers (Hargrave et al. 1983), and concentrations (μM) were corrected for the corresponding volume of chamber water (l) at T_0 and T_1 to obtain the total mass (mmol in the chamber). Oxygen values were converted to carbon assuming that 1 mg of oxygen produced or respired is equivalent to 0.375 mg of organic carbon (Uthicke and Klumpp 1998).

3.2.4 Nitrogen Cycling Processes

Four sub-samples of overlying water were collected from each chamber at approximately 1 hour intervals for NH_4^+, NO_3^-, NO_2^-, N_2O and ethylene (C_2H_4) analysis. Sub-samples of 25 ml for DIN (NH_4^+, NO_3^-, NO_2^-) were collected with a syringe, immediately filtered (GF/F) and stored at -21 °C in acid-cleaned, polyethylene tubes until analysis. To quantify dissolved N_2O and C_2H_4 concentration, 12 ml of overlying water was collected into a gas-tight syringe where it was mixed with 12 ml of helium. The syringe was shaken vigorously for 1 min to allow the total transfer of N_2O and C_2H_4 to the helium phase. The gas phase was transferred to an 11 ml evacuated serum bottle for storage until analysis.

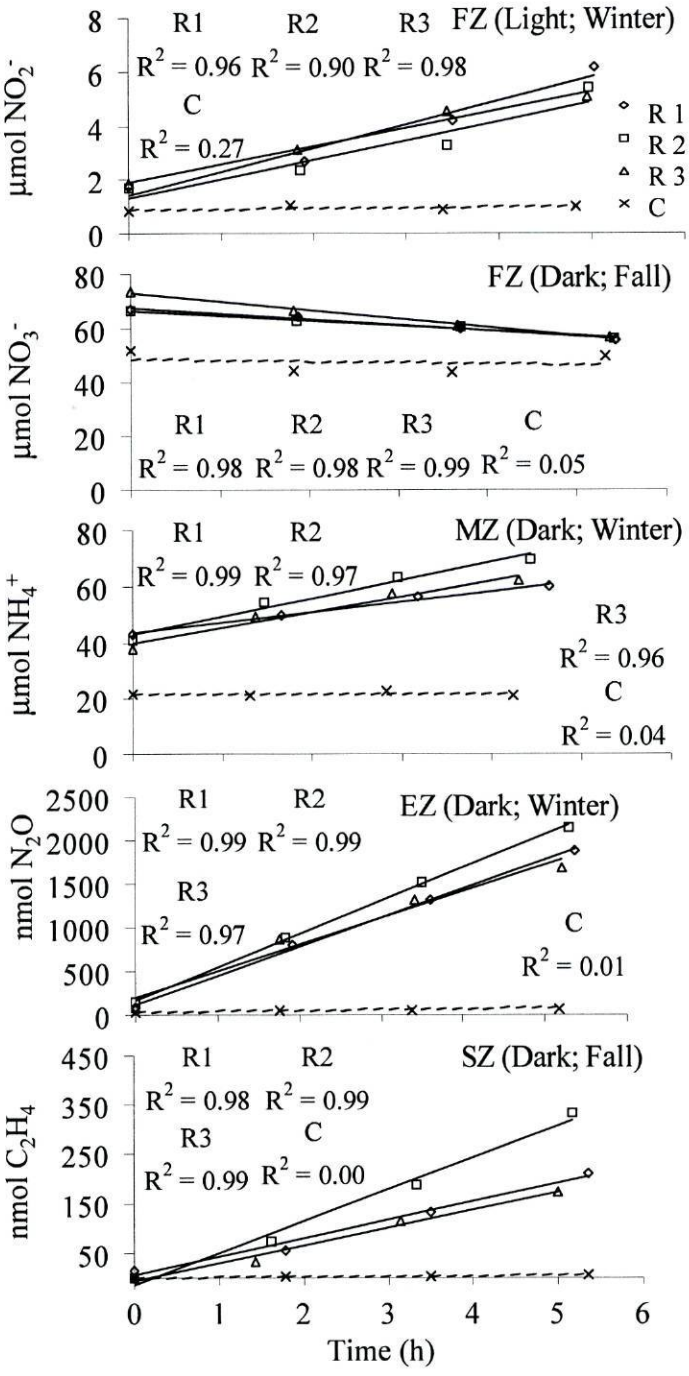

Figure 3.2: Examples of typical linear variation of NO_2^-, NO_3^-, NH_4^+, N_2O and C_2H_4 in triplicate chambers during the incubation period.

For each incubation period NO_2^-, NO_3^-, NH_4^+, N_2O and C_2H_4 concentrations (μM) were corrected for the corresponding volume of chamber water to obtain total mass (in μmol), which corrects for the volume changes during the incubation. Net fluxes were calculated using the slope of the linear relationship between the changes in the nutrient concentration in the water chamber versus the incubation time (Barbanti et al. 1992). Fluxes were linear over the entire incubation period (Fig. 3.2). When no net fluxes were observed, each of the three replicate chambers showed no net change in nutrient concentration during the incubation; this situation was observed in all control chambers (Fig. 3.2). After incubation, each rock was molded with aluminum foil and the foil weighed after drying. The weight was converted to area (cm^2), using a linear regression between foil weight and the corresponding area (Magalhães et al. 2003).

Nitrogen fixation rates were determined by the acetylene reduction method described in Joye and Paerl (1994) and calculated assuming a 3:1 ratio between ethylene production and N fixation (Fig. 3.3, Joye and Paerl 1994). In the same samples, denitrification rates were measured by the acetylene block method (Fig. 3.3), according to Joye and Paerl (1994); N_2 produced via denitrification was calculated as the difference between the N_2O produced with acetylene and the N_2O produced without acetylene (natural net N_2O flux). Because acetylene also inhibits ammonium monooxygenase, nitrification rates were calculated as the difference between NH_4^+ net flux measured in incubations with and without acetylene (Sloth et al. 1992, Caffrey and Miller 1995) (Fig. 3.3).

3.2.5 Analytical methods

Analysis of ethylene was performed using a Shimadzu gas chromatograph equipped with a flame-ionization detector at 200 °C. Samples (1 ml) were injected onto a 2-m Poropak T column (80 °C) with nitrogen as the carrier gas. N_2O was analyzed in a Shimadzu gas chromatograph equipped with an electron capture detector at 300 °C. Carrier gas (5% CH_4 in 95% Ar at 30 ml/min) was directed through a 0.5-m Hay Sep D pre-column at 20 °C, the 2-m main column Hay Sep D column at 80 °C, and the detector. C_2H_4 and N_2O concentrations were determined by comparison with purified standards (Scott Specialty Gases, Inc. Plumsteadville, PA). Inorganic nutrient analysis (NH_4^+ and NO_2^- and NO_3^-) were performed in triplicate as described in Magalhães et al. (2002).

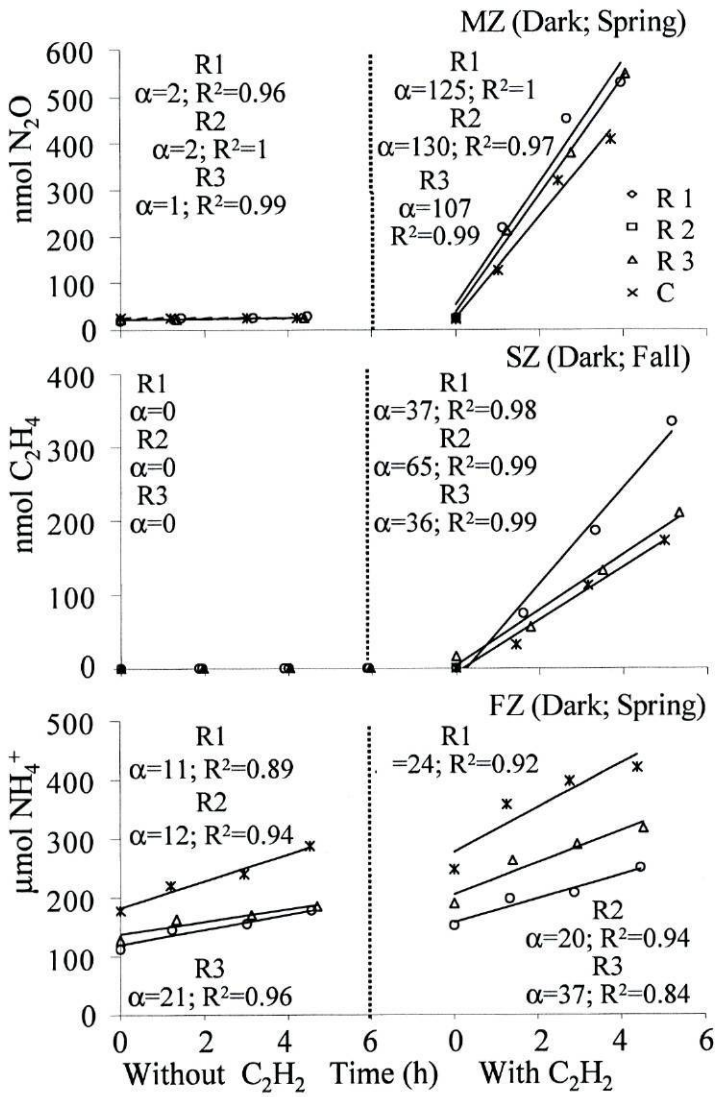

Figure 3.3: Examples of typical variation of N_2O, C_2H_4 and NH_4^+ in the triplicate chambers treated without and with acetylene.

Surface sub-samples (in triplicate) were collected by scraping each rock (1 cm^2) or by collecting sediment sub-cores (1 cm diameter and 0.5 cm depth) at the end of the experiment. Samples were placed into scintillation glass vials for chl *a* and total organic matter (OM) analysis. In the case of FZ rocks, scraping was performed after *Fucus* spp. was removed for dry weight analysis (dried at 60°C until constant weight); for the EZ rocks, *Enteromorpha* spp. was included in chl *a* and OM analyses as we found it impossible of separate this macroalgae from the rest of the surface biofilm. Samples for chl *a* measurements were kept frozen (-21 °C) until analysis. Extraction was performed in a mixed solution of acetone, methanol and water (45:45:10, respectively), according to Joye et al. (1996). Hill and Hawkins (1990) found that methanol extraction is a more accurate and precise method than acetone extraction to estimate the chl *a* content of intact rock biofilms. Chl *a* concentration was determined spectrophotometricaly, as described in Magalhães et al. (2003*). Samples for OM contents were processed immediately by ignition in a muffle furnace at 500 °C for 4h and reweighing (data are presented as percent OM and grams of total OM per m^2).

3.2.6 Statistical analysis

Statistical analysis were performed at the 95% confidence level ($p < 0.05$). Data were tested for normality, using the Kolmogorov-Smirnov test and for homoscedasticity using the Leven's test (Zar 1996). A one-way ANOVA analysis was used to compare the means of each parameter between the different stations or seasons (Zar 1996). Student's *t*-test (Zar 1996) was used to examine differences in mean hourly flux rates (triplicate chambers) between dark and light incubations. Linear regression analysis (Zar 1996) was conducted to establish relationships between the N processes and/or environmental parameters measured.

3.3 Results

3.3.1 General Features

Variation in NO_3^-, NO_2^- and NH_4^+ water column concentrations for each sampling site are given in Table 3.1. NO_3^- concentrations varied between 12.4±1.8 and 88.5±11.1 μM

*The contents of this article can be found in Chapter 2.

and were generally lowest at the muddy sediment sites. NO_2^- mean values did not differ significantly between the sites (1.1±0.5; 0.9±0.6; 2.4±1.0; 1.75±0.3 μM, respectively for SZ, MZ, EZ and FZ). NH_4^+ concentrations ranged between 13.4±6.8 and 111.9±10.2 μM and showed strong temporal variation at the rocky biofilm sites (EZ, FZ).

OM and %OM varied according to the type of substrate, the lowest values were observed in intertidal sandy sediments and the highest values at MZ and EZ (Table 3.1). The lowest chl *a* concentrations occurred in intertidal muddy sediments (MZ), intermediate values in intertidal sandy sediments (SZ) and the highest values at the rocky sites (EZ and FZ) (Table 3.1). Mean values for *Fucus* spp. dry weight in the triplicate samples were 700.5±172.7, 453.9±192.9, 919.1±450.9 and 1125.0±592.8 g dw m^{-2} for summer, fall, winter and spring samples, respectively.

3.3.2 Primary Production and Respiration

Rates of respiration and net primary production (NPP) are shown in Table 3.2 (net oxygen fluxes measured in dark and light chambers, respectively). In all samples, significantly higher mean values of NPP were observed at the FZ site (ANOVA, $p < 0.001$), and lowest values in the MZ site, where oxygen consumption (negative net O_2 values) in light incubations were observed during winter incubations (Table 3.2). Highest community respiration rates were also observed at the FZ site (ANOVA, $p < 0.001$). During fall, no significant differences in community respiration rates were observed between the other three sites. The MZ site showed significantly higher respiration rates than the SZ and EZ in winter (ANOVA, $p < 0.001$). EZ had significantly higher respiration rates compared to the MZ and SZ sites during the summer (ANOVA, $p < 0.001$).

At the SZ and MZ sites, the concentration of chl *a* in the sediment surface was related linearly to the hourly GPP (gross primary production) rates, i.e., the higher the chl *a* content the higher the oxygen production rate ($R^2 = 0.65$, $p < 0.001$, $n = 9$ and $R^2 = 0.72$, $p < 0.001$, $n = 9$; respectively for SZ and MZ). At the FZ site, *Fucus* spp. biomass but not the chl *a* content of the biofilm without *Fucus* spp., was related linearly to hourly GPP rates ($R^2 = 0.70$, $p < 0.001$, $n = 12$). This suggests that *Fucus* spp. was a major contributor for the oxygen production rates measured at the FZ site. In EZ, there was no clear relationship between GPP rate and chl *a* content.

Table 3.2: Oxygen net fluxes (mmol m^{-2} h^{-1}), net NH_4^+, NO_3^- and NO_2^- net fluxes (µmol m^{-2} h^{-1}) performed without C_2H_2 and N_2O:N_2 ratio in light and dark incubations (mean±SD). Negative values indicate uptake and positive values indicate release).

		O_2		NH_4^+		NO_3^-		NO_2^-		N_2O:N_2	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
SZ	Summer	1.6±0.2	Null flux	-66.8±11.8	-105.0±41.5	-283.9±20.3	-263.6±170.0	-4.5±1.1	-3.5±1.8	0.0±0.0	17.1±4.5
	Fall	2.3±0.5	-0.6±0.1	-40.7±14.3	163.2±65.4	-196.6±10.0	-145.2±12.7	-3.8±0.7	Null flux	0.0±0.0	0.0±0.0
	Winter	2.9±0.8	-0.3±0.1	-32.4±28.0	-51.0±19.5	-111.8±78.0	-209.8±152.6	Null flux	Null flux	0.0±0.0	0.0±0.0
	Spring	4.5±0.7	-0.4±0.7	-85.9±15.6	-30.3±52.4	-178.0±66.9	-237.0±106.7	-1.9±1.2	1.4±2.9	0.0±0.0	1.8±1.2
MZ	Fall	1.3±0.3	-0.5±0.3	-50.4±25.6	54.3±52.4	-38.1±21.3	-25.5±44.1	-0.3±1.3	9.5±5.8	0.0±0.0	4.2±7.3
	Winter	-3.0±0.3	-4.4±0.6	222.0±133.3	398.8±114.8	-152.1±48.0	-303.8±94.4	2.2±3.8	15.2±7.5	0.7±0.2	1.6±0.9
	Spring	0.9±1.1	-1.2±0.7	227.9±114.8	505.6±83.2	-25.0±12.8	-11.6±33.9	1.6±1.2	5.3±1.6	0.0±0.0	1.6±0.9
EZ	Summer	6.8±1.1	-2.4±0.9	-372.0±274.0	603.1±285.3	275.5±195.4	-317.9±75.4	14.2±8.6	69.2±46.3	7.1±4.5	40.5±15.1
	Fall	5.1±1.0	-0.4±0.9	-223.6±213.2	371.7±433.8	-155.0±88.6	-239.2±118.0	5.7±18.6	32.1±16.6	6.5±11.2	13.2±5.5
	Winter	7.4±1.7	-1.1±1.0	-199.7±118.4	-244.9±224.7	-169.3±113.7	-237.1±99.4	3.1±3.0	19.6±12.8	41.8±36.7	5.4±3.2
	Spring	8.9±2.9	-3.3±0.5	-696.3±328.1	325.9±117.3	-67.8±80.0	-170.2±108.2	-0.9±4.8	43.6±26.6	0.0±0.0	3.8±0.7
FZ	Summer	18.3±2.3	-4.5±1.8	Null flux	620.2±251.4	-516.0±199.3	-641.1±10.1	-9.7±3.2	213.9±105.8	34.0±29.7	15.0±6.1
	Fall	17.3±2.8	-3.8±0.6	-104.8±92.5	70.7±78.3	114.6±189.8	-201.5±86.8	7.7±4.3	119.3±71.8	0.0±0.0	11.1±11.3
	Winter	34.6±4.4	-7.3±2.2	-362.0±110.8	684.1±464.8	Null flux	-252.1±161.4	64.2±30.0	94.2±23.3	20.3±4.7	7.1±2.5
	Spring	48.3±2.5	-6.7±2.0	-1654.0±394.7	1970.4±805.3	1014.6±577.1	-1528.6±434.3	113.4±122.8	335.0±181.1	14.5±13.2	4.9±1.3

3.3.3 Inorganic Nitrogen Net Fluxes

Light and dark mean values of net NH_4^+, NO_3^- and NO_2^- fluxes (μmol $m^{-2}h^{-1}$) measured in incubations without C_2H_2 are given in Table 3.2. At the MZ, EZ and FZ sites, NH_4^+ net fluxes differed between light and dark incubations (student t-test, $p < 0.05$), with lower uptake or higher release rates observed in dark treatments (Table 3.2). There was one exception to this generalization; during winter, no significant differences in light and dark fluxes were observed at the EZ site. At the SZ site, no significant differences in dark and light NH_4^+ net fluxes were found (ANOVA, $p < 0.05$), with the exception of fall (Table 3.2).

Mean NO_3^- net fluxes were almost always negative for all sampling times and sites under both light and dark incubations; exceptions occurred in light incubations of FZ samples during spring and fall, and EZ samples during summer, where effluxes of NO_3^- were observed (Table 3.2).

NO_2^- concentrations in the Douro River estuarine water were, as expected, low compared to NO_3^- and NH_4^+ (Table 3.1). In the SZ and MZ sites, net NO_2^- fluxes were low and unpredictable in both light and dark incubations; however high rates of NO_2^- release were observed in rocky biofilm sites (EZ, FZ), mainly during dark incubations (Table 3.2).

N_2O net fluxes ranged between 0 and 23.2 μmol m^{-2} h^{-1}, with consistently higher rates observed in rocky biofilms (Fig. 3.4). To evaluate the magnitude of the N_2O accumulation during denitrification, N_2 to N_2O ratios were determined (Table 3.2). Generally, N_2O: N_2 ratios in sediments were low compared to N_2O:N_2 ratios obtained for rocky biofilms (Table 3.2).

3.3.4 Rates of Nitrogen Cycle Processes

3.3.4.1 N Fixation

At all four intertidal habitats, nitrogen fixation rates ranged between 0 and 4.07±1.42 μmol C_2H_4 m^{-2} h^{-1} (Fig. 3.4). No N fixation was ever observed at the EZ site, but the other sites showed zero N fixation rates frequently (Fig. 3.4).

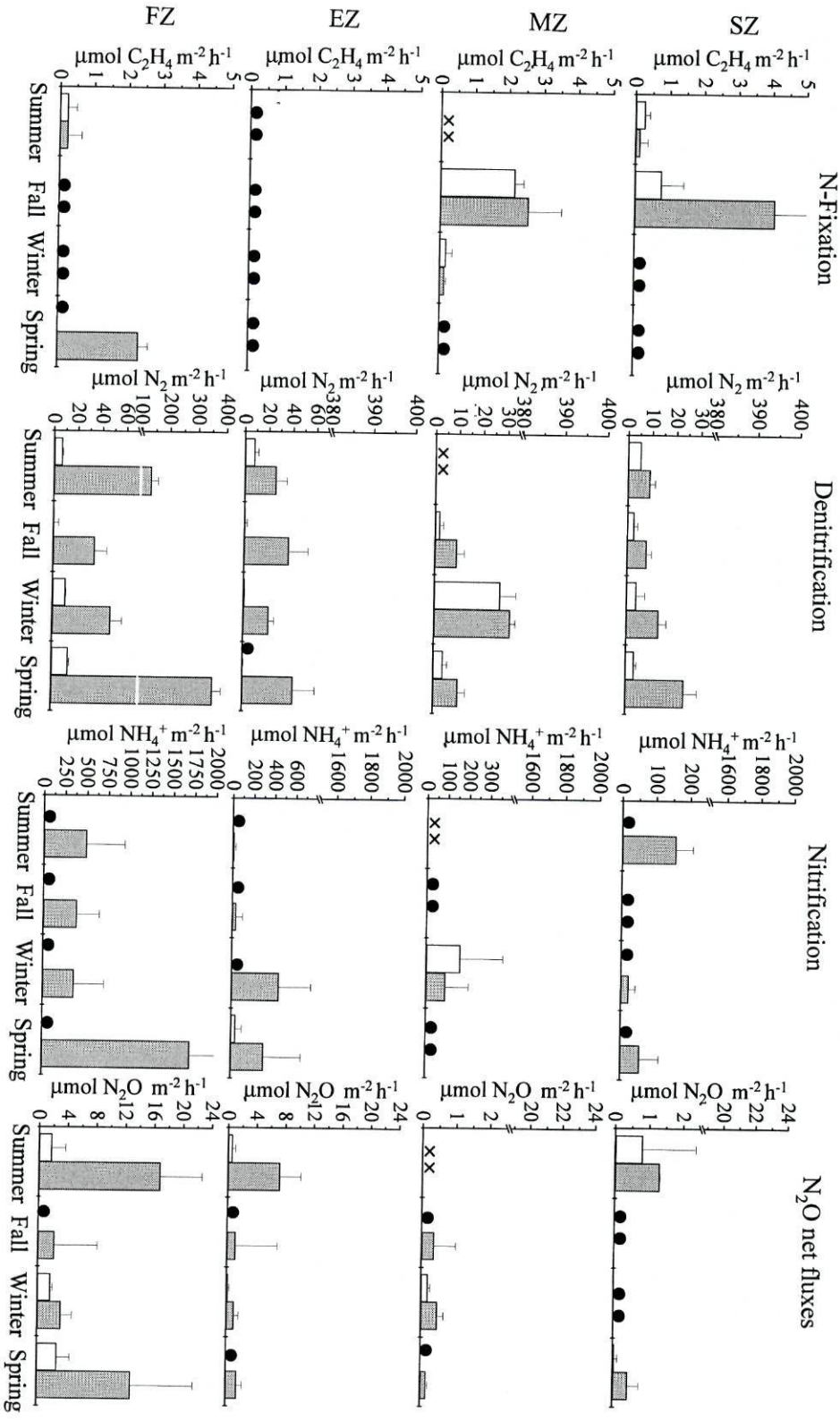

Figure 3.4: Seasonal and spatial mean±SD nitrogen fixation, denitrification and nitrification rates and N_2O net fluxes in the Douro River estuary (white bars = light incubation; grey bars = dark incubation; black spots = zero values; × = not measured).

3.3.4.2 Nitrification

In light incubations, nitrification rates were zero in all but two cases (EZ samples in spring and MZ samples in winter; Fig. 3.4). In dark incubations, higher nitrification rates were generally observed in rocky biofilm samples (EZ and FZ) (Fig. 3.4). At the SZ site, net NH_4^+ uptake rates were related positively to the nitrification rates ($R^2 = 0.5$, p ¡ 0.05, $n = 12$). At the EZ and FZ sites, nitrification rates were related positively to the NH_4^+ efflux ($R^2 = 0.61$, p ¡ 0.01, $n = 12$ and $R^2 = 0.92$, $p < 0.001$, $n = 12$; for EZ and FZ, respectively). At the MZ site nitrification was only observed during winter incubations (Fig. 3.4).

3.3.4.3 Denitrification

Differences between light and dark incubations suggest that oxygen inhibited denitrification; rates were higher in dark treatments (student t-test, $p < 0.005$), except at the MZ site during winter (Fig. 3.4). In dark incubations, a clear positive and linear relationship was observed between community respiration and denitrification rates ($R^2 = 0.78$, $p < 0.001$, $n = 12$, $R^2 = 0.81$, $p < 0.001$, $n = 9$, $R^2 = 0.54$, p ¡ 0.05, $n = 12$ and $R^2 = 0.59$, p ¡ 0.01, $n = 12$; for SZ, MZ, EZ and FZ, respectively,). Seasonal patterns of denitrification rates differed between the study sites (Fig. 3.4). However, the highest rates occurred at the FZ site (Fig. 3.4). For the SZ, MZ and FZ, dark denitrification rates were positive and related linearly to NO_3^- concentration in the incubation water and with net NO_3^- uptake rates (Fig. 3.5), while EZ samples showed no relationships between those processes (Fig. 3.5).

3.4 Discussion

3.4.1 Primary Production and Community Respiration

During this study (2001-2002), net primary production rates in the muddy and sandy sediment samples (Table 3.2) were much lower than the net primary production rates measured at the same sites during a 1998 study, when NPP rates ranged between 5.5±0.4 to 19.3±2.5 mmol O_2 m^{-2} h^{-1} for sandy sediments and between 6.2±0.5 and 9.4±0.3 mmol O_2 m^{-2} h^{-1} for muddy sediments (Magalhães et al. 2002). These results show a large inter-

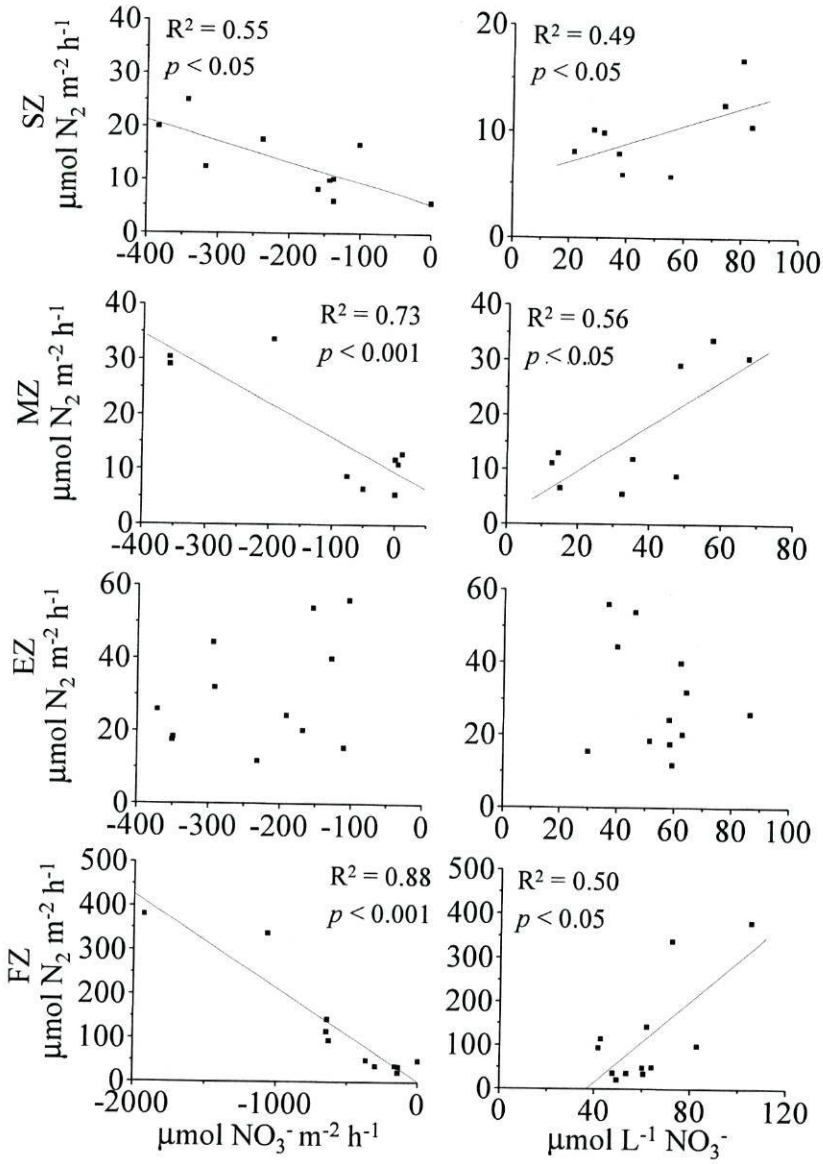

Figure 3.5: Relationships between dark denitrification rates and dark net NO_3^- fluxes, and between dark denitrification rates and T_0 incubation water NO_3^- concentration.

annual variability in primary producer dynamics of the MZ and SZ sites of the Douro River estuary. At the EZ site, comparison of NPP rates measured at different years showed a somewhat different pattern; NPP values during this present study (Table 3.2) fell within the lower range of the mean net primary production rates measured during 2000 (between 7.9±0.6 and 20.5±5.9 mmol O_2 m^{-2} h^{-1}; Magalhães et al. 2003*). In intertidal sediments (SZ and MZ) unpredictable large-scale (winter 2000/2001 floods) or small scale changes in estuarine hydrological dynamics, could explain the differences in NPP observed between the different years. Epilithic communities may be more resistant to these inter annual changes in estuarine hydrological dynamics, due to the inherent physical characteristics of the rocks.

The rate of oxygen uptake can be used to indicate the magnitude of organic mineralization (Trimmer et al. 2000). In this study, the generally significant, linear relationship between net oxygen consumption and dark net NH_4^+ effluxes ($R^2 = 0.46$, $p < 0.001$, $n = 12$; $R^2 = 0.59$, $p < 0.001$, $n = 9$, $R^2 = 0.34$, p ¡ 0.05, $n = 12$; $R^2 = 0.64$, $p < 0.001$, $n = 12$; for SZ, MZ, EZ and FZ, respectively) link NH_4^+ mineralization rates with respiration. The FZ site had the highest rates of oxygen uptake, with the exception of MZ site in winter, where similar rates of oxygen consumption were registered (Table 3.2). While the SZ and MZ community respiration rates were clearly within the range reported for other intertidal sediments (c.f. Magalhães et al. 2002), oxygen uptake rates for both macroalgal-dominated sites (FZ and EZ) were in the lower range compared to those measured by Trimmer et al. (2000) in intertidal sediments heavily covered by the macroalgae *Enteromorpha* spp. and *Ulva* spp. Differences in wave exposure between intertidal rocky and intertidal sediment environments may determine the differences in biomass accumulation and export rates for these environments. Biomass burial and remineralization is expected for macroalgae on intertidal sediments with higher mineralization rates and consequently higher oxygen consumption than in intertidal rocky environments colonized by macroalgae and exposed to wave action, where biomass export should dominate.

3.4.2 O_2 Production/Consumption and DIN Cycling

Clear differences in net NH_4^+ fluxes were observed between light and dark incubations of MZ, EZ and FZ samples (Table 3.2). At these sites, NH_4^+ uptake in light incubations

*The contents of this article can be found in Chapter 2.

almost always switched to net NH_4^+ efflux during dark incubations (Table 3.2), suggesting that the primary producers assimilated NH_4^+ in the light and that uptake diminished or ceased during dark incubations.

For SZ, EZ and FZ samples, NO_3^- uptake rates in light incubations were negatively and linearly related to T_0 NH_4^+ concentrations in the incubation water, ($R^2 = 0.54$, $p < 0.01$, $n = 12$, $R^2 = 0.61$, $p < 0.01$, $n = 12$; $R^2 = 0.71$, $p < 0.001$, $n = 12$; respectively for SZ, EZ and FZ), i.e. the higher the NH_4^+ concentration in water column the lower the NO_3^- uptake rate. This suggests that NO_3^- uptake rates were a function of the NH_4^+ concentration in the water column, and this pattern illustrates a discrimination against NO_3^- when NH_4^+ is available, which is expected in primary producer communities (Korb and Gerard 2000).

It is difficult to quantify the contribution of the various DIN fluxes to specific primary producers at the FZ and EZ sites, due to the uncertainty concerning the proportion of the primary production attributable to macroalgae or the epilithic and/or epiphytic microalgae. For periphyton, Hillebrand and Sommer (1999) found an optimal stoichiometric uptake C:N ratio of 7, which is close to the Redfield ratio (C:N = 6.6). Phytoplankton tend to be enriched in N relative to C compared to macroalgae, which often have N concentrations below the critical amount required for maximum growth (Duarte 1992). Higher macroalgal C:N ratios are commonly reported (C:N mean of 21; C:N range 10 to 40; C:N range of 10 to 20 for Atkinson and Smith 1983, Bokn et al. 2002, Worm et al. 2000, respectively).

In samples where a net uptake of NH_4^+ occurred, molar uptake ratios of C:NH_4^+ in light treatments consistently exceeded the Redfield ratio (range between 22 to 128) for all sites. However C:NH_4^++ NO_3^- ratios for the SZ site (5±0.2, 13±2, 16±9 and 21±6, respectively for summer, fall, winter and spring) and EZ (13±5, 18±9 and 12±6, respectively for fall, winter and spring) were mostly often close to the Redfield ratio. Similar C:N uptake ratios have been reported for *Enteromorpha* spp. in other studies (Trimmer et al. 2000, Worm et al. 2000). These results suggest that light assimilation rates of both NH_4^+ and NO_3^- supplied the SZ and EZ primary producers with most of their N requirements, with the exception of SZ during spring where C:NH_4^++ NO_3^- was 21±6, suggesting that NH_4^++ NO_3^- from water column was not the only source of nitrogen. NH_4^+ uptake rates were sufficient to support the rates of carbon fixation measured at the EZ site during summer (Table 3.2), with C:NH_4^+ ratios of 16±8. In the FZ, NH_4^+ uptake rates, and even NH_4^++NO_3^- combined in the case of summer (Table 3.2), did not fulfill the likely N

requirements for *Fucus* spp., since C:N uptake ratios (39.4±17.0, 105.0±33.9, 102.2±33.5 and 47.5±31.1, respectively for summer, fall, winter and spring) were higher than the expected C:N uptake ratios reported by Worm et al. (2000) for *Fucus vesiculosus*. In this case, *in situ* mineralization must have also fueled a portion of the primary producer N demand. The direct assimilation of dissolved organic nitrogen (DON), for example urea, bypassing organic nitrogen mineralization, may also fulfill the nitrogen requirements of some macroalgal species (as suggested by Tyler et al. 2001). The NH_4^+ uptake in the MZ light incubations only occurred during fall (Table 3.2), and in this case the C:$NH_4^+$$NO_3^-$ ratio was 16±4. While winter NO_3^- uptake rates in the MZ could fulfill the benthic microalgal demand for N (C:NO_3^- = 9±1), in spring the C:NO_3^- for light-incubated samples greatly exceeded the Redfield ratio (149±40). Thus within the MZ, mineralization appeared to be an important process for the primary producers to meet their N requirements.

3.4.3 Dynamics of Nitrogen Processes

3.4.3.1 Denitrification

Significant correlations between dark denitrification rates, dark net NO_3^- fluxes, and water column NO_3^- concentrations observed at SZ, MZ and FZ sites (Fig. 3.5), suggest that denitrification influenced net NO_3^- uptake rates; similar results have been described elsewhere (e.g. Ogilvie et al. 1997). The absence of a significant relationship between these variables at the EZ site (Fig. 3.5) suggests that this denitrifier community was not NO_3^- limited, and that NO_3^- concentrations in these biofilms were above the saturation values for denitrifiers, in agreement with recent data (Magalhães et al. 2005b*). The light-induced reduction in denitification rates agrees with earlier results (Rysgaard et al. 1994) and appears to be closely linked to oxygen production during the photosynthesis (Nielsen et al. 1990, Sundbäck et al. 2000). The significant relationship between denitrification rates and net oxygen uptake rates in dark chambers ($R^2 = 0.78$, $p < 0.001$, $n = 12$; $R^2 = 0.81$, $p < 0.001$, $n = 9$, R^2 =0.54, $p < 0.001$, $n = 12$; $R^2 = 0.59$, $p < 0.001$, $n = 12$; respectively for SZ, MZ, EZ and FZ) corroborates the idea that oxygen inhibits denitrification . The only case where no significant difference was observed between denitrification rates in light and dark incubations (MZ in winter; Fig. 3.4), coincided with an absence of net oxygen

*The contents of this article can be found in Chapter 4.

production in light chambers (Table 3.2). Thus, denitrification is of minor importance when photosynthetic rates, and as a result, O_2 concentrations, are high.

In dark incubations, assuming that 2 mol of NO_3^- produce 1 mol of N_2 during denitrification, the percentage of NO_3^- uptake accounted for by denitrification was 10 - 48%, 25 - 50%; 10 - 47% and 31 - 41% for the SZ, MZ, EZ and FZ, respectively. While the percentage of net NO_3^- uptake by denitrification varied between sites and times, denitrification never accounted for even 50% of the total net NO_3^- uptake. Several authors have reported similar results, and the percentages calculated here are within the same range of those estimated elsewhere (Cabrita and Brotas 2000, Trimmer et al. 2000, Kelly-Gerreyn et al. 2001). For example, Kelly-Gerreyn et al. (2001) found that the proportion of NO_3^- uptake due to denitrification varied between 28%±16% and 49%±14% and was strongly temperature dependent. In contrast, we observed no temperature dependence for denitrification in this study (Table 3.1, Fig. 3.4)

The percentage of NO_3^- uptake via denitrification suggests that an alternative sink for NO_3^- during dark incubations must exist. Dissimilatory NO_3^- reduction to NH_4^+ (DNRA) could also account for a high proportion of NO_3^- reduction in different estuarine sediments (Gilbert et al. 1997, Kelly-Gerreyn et al. 2001, Tobias et al. 2001, An and Gardner 2002). Though we did not measure DNRA directly, we estimated the proportion of NO_3^- reduction accounted for by DNRA by assuming that dark influxes of NO_3^- were equivalent to the NO_3^- reduction fuelled by NO_3^- from the overlying water. This assumption is valid when net NO_3^- influxes are significantly correlated to denitrification rates and to net NH_4^+ effluxes, since this suggests a strong link between the NO_3^- fluxes and dissimilatory NO_3^- reduction process (Trimmer et al. 1998, Kelly-Gerreyn et al. 2001). For MZ and FZ samples, we observed strong correlations between net dark incubation NO_3^- influxes and denitrification (Fig. 3.5) and between net dark NO_3^- influxes and net dark NH_4^+ effluxes ($R^2 = 0.60$, $p < 0.001$, $n = 18$; $R^2 = 0.70$, $p < 0.001$, $n = 24$; respectively for MZ and FZ), suggesting that denitrification and DNRA both contributed to the observed dark net NO_3^- uptake. Linking the denitrification rates with the measured net NO_3^- fluxes enabled us to partition the NO_3^- uptake from the water column that was reduced to NH_4^+, by subtracting the proportion of NO_3^- uptake due to denitrification (calculated above) from the contemporaneous net NO_3^- uptake rates. The resulting percentage of the total NO_3^- uptake that was subsequently reduced to NH_4^+ was estimated for the MZ (50%, 75% and 60%, respectively for fall, winter and spring), and the FZ (69%, 64%, 59% and

67%, respectively for summer, fall, winter and spring). According to these calculations, while DNRA was quantitatively the more significant process, denitrification co-occurred, suggesting considerable overlap of these processes at these 2 sites. The DNRA rates reported here fell within the range reported for other coastal environments (Rysgaard et al. 1996, c.f. Kelly-Gerreyn et al. 2001). Although interstitial water sulfide concentrations were not measured, high porewater sulfide concentrations have been suggested to favor DNRA over denitrification (An and Gardner 2002).

Dark nitrate assimilation by primary producers could also account for nitrate removal from the overlying water, but the switched of NH_4^+ uptake in the light to NH_4^+ efflux during dark at MZ, EZ and FZ sites (Table 3.2), suggested that N assimilation by primary producers diminished or ceased during dark incubations. Moreover assimilation of NO_3^- by primary producers will be converted in organic nitrogen, which will not explain the NH_4^+ effluxes observed at MZ, EZ and FZ sites, since estimated mineralization cannot account for the all of the NH_4^+ effluxes observed (Fig. 3.6).

3.4.3.2 Nitrous Oxide Net Fluxes

While the N_2O net effluxes observed in the SZ and MZ samples (Fig. 3.4) were similar to those reported for other coastal marine sediments (Seitzinger 1988), N_2O rates at the EZ and FZ sites were much higher (Fig. 3.4). The summertime and springtime net N_2O effluxes from FZ samples reached values of 23.2 μmol N_2O m^{-2} h^{-1}, exceeding the highest previously reported values for marine coastal systems by 7.8 μmol N_2O m^{-2} h^{-1} (Corredor et al. 1999). These high N_2O effluxes are significant because N_2O is a potent greenhouse gas and contributes to the destruction of atmospheric ozone (Dickinson and Cicerone 1986).

Higher N_2O net effluxes are coupled to high NO_3^- availability and consequently to higher denitrification rates (Kieskamp et al. 1991, Robinson et al. 1998, Seitzinger 2000, Trimmer et al. 2000). N_2O can also be produced via nitrification (Goreau 1980). While our data do not allow discrimination of the process(es) involved in N_2O production, using linear correlation analysis between N_2O net fluxes and net inorganic nitrogen fluxes, denitrification and nitrification rates, we were able to estimate the likely source of N_2O for each site. At the MZ, EZ and FZ sites dark net N_2O fluxes were significantly correlated to the overlying water-column NO_3^- concentrations (Fig. 3.7), and with net NO_3^- uptake rates (Fig. 3.7). For the MZ and FZ samples, a significant and positive relationship was

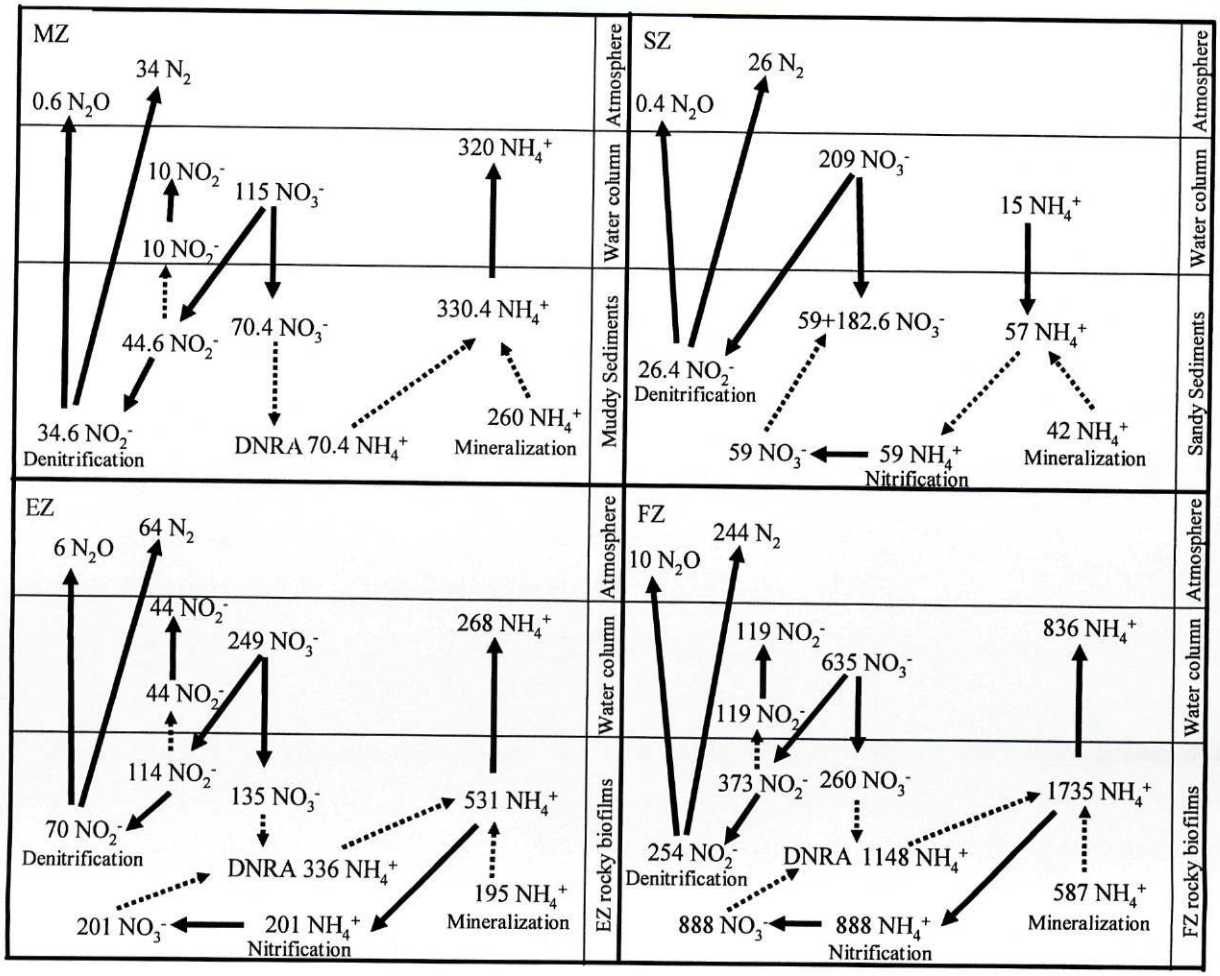

Figure 3.6: General conceptual model for nitrogen cycle dynamics (dark incubation) of each different intertidal environment studied (SZ, MZ, EZ, FZ) in Douro River estuary (solid arrows indicate measured processes; dotted arrows indicate estimated processes). All values are in μmol m^{-2} h^{-1}.

observed between denitrification rate and net N_2O efflux (Fig. 3.7). These correlations suggest that changes in NO_3^- availability regulate net N_2O effluxes at MZ, EZ and FZ, and that denitrification was the major source of N_2O in the MZ and FZ sites as it was observed by Kester et al. (1996). For the SZ samples, the highly significant and positive relationship between N_2O effluxes and nitrification rates ($R^2 = 0.86$, $p < 0.001$, $n = 12$) and the absence of correlation between N_2O net effluxes and water NO_3^- concentrations, NO_3^- net fluxes or denitrification rates (Fig. 3.7), suggests that nitrification was the major N_2O source at this site. These results are similar to those of Svensson (1998), who reported that the source of N_2O in an eutrophic lake sediments was via nitrification.

Ratios of N_2O:N_2 in the environment tend to be very low (< 5%) (Seitzinger 1988), but the EZ and FZ samples produced relatively high values (Table 3.2). Increases in N_2O efflux associated with anthropogenic nitrogen loading may drive denitrification rates and N_2O:N_2 flux ratios in some systems (Robinson et al. 1998, Corredor et al. 1999). In this study, similar water column NO_3^- concentrations at different sites produced very distinct effluxes of N_2O and N_2O:N_2 ratio patterns (Table 3.1 and Table 3.2, Fig. 3.4).

3.4.3.3 Nitrite Net Fluxes

Benthic NO_2^- net fluxes are generally small compared to other inorganic N species fluxes (e.g. Sundbäck et al. 1991, Cowan et al. 1996, Cabrita and Brotas 2000). In SZ samples, NO_2^- net fluxes in both light and dark incubations and the NO_2^- net fluxes in light incubations for MZ, EZ and FZ samples approached zero (Table 3.2). However, dark NO_2^- net fluxes in MZ, EZ and FZ samples were consistently positive, and particularly high NO_2^- fluxes were observed within EZ and FZ samples (Table 3.2). Significant positive correlations were also found between net NO_2^- release rates and dark net NO_3^- uptake rates and with denitrification rates in MZ, EZ and FZ samples (Fig. 3.7), suggesting that NO_2^- effluxes resulted from incomplete denitrification. The linear and positive relationship found between dark NO_2^- net effluxes and dark NH_4^+ net effluxes for EZ and FZ ($R^2 = 0.47$, p ¡ 0.05, $n = 12$; $R^2 = 0.62$, p ¡ 0.05, $n = 12$; respectively) suggest that DNRA may contribute to some of the observed NO_2^- efflux. Also, high concentrations of NO_2^- found in rivers, under slow-flowing conditions, have been previously attributed to the inhibition of the DNRA NO_2^- reductase system (Kelso et al. 1997). While NH_4^+ oxidation by nitrifiers can produce elevated water column NO_2^- concentration (Smith et al. 1997), the lack of significant relationships between nitrification rates and net NO_2^- fluxes for all four

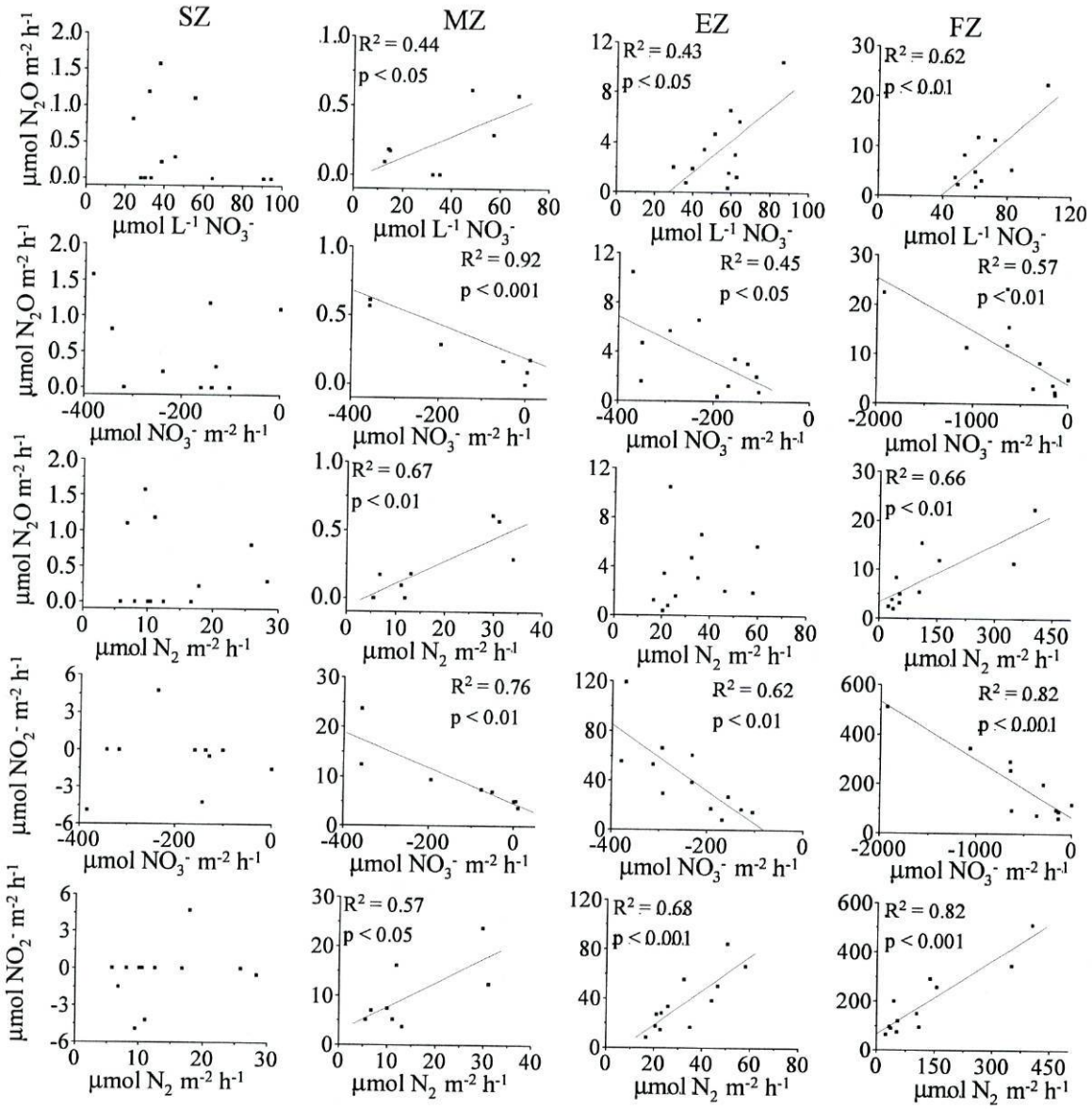

Figure 3.7: Relationships of dark net N_2O fluxes with T_0 incubation water NO_3^- concentrations, dark net NO_2^- fluxes, and dark denitrification rates, and relationships of dark net NO_2^- fluxes with T_0 incubation water NO_3^- concentrations and dark denitrification rates.

sites suggests that the high NO_2^- effluxes reported cannot be attributed to the nitrification process.

3.4.3.4 Nitrification

Results from studies of other intertidal sediments suggest that nitrification is stimulated during light incubations, due to the molecular oxygen required for the oxidation of NH_4^+ (Rysgaard et al. 1994, An and Joye 2001). In this study, with only 2 exceptions, nitrification occurred exclusively in dark incubations (Fig. 3.4). Competitive uptake of NH_4^+ by primary producers could explain the absence of nitrification in light treatments; NH_4^+ limitation of nitrification by benthic microphytes has been reported by several authors (Henriksen and Kemp 1988, Rysgaard et al. 1996, An and Joye 2001).

In MZ samples, light and dark nitrification played only a minor role in the N cycle, since rates were near zero. Even during winter, when nitrification was measurable, the high variability between replicates did not permit a clear interpretation of the results (Fig. 3.4). The high concentrations of sulfide (Joye and Hollibaugh 1995) and the low oxygen concentrations (Rysgaard et al. 1994) that often characterize muddy sediments could inhibit the activity of nitrifying bacteria in these environments. Dark nitrification rates of SZ samples were positively correlated with NH_4^+ net fluxes, i.e., the higher the nitrification rate the higher the NH_4^+ uptake rate (see Section 3.3.4.2). In addition, the only time dark release of NH_4^+ to the overlying water was detected (Table 3.2) coincided with a zero nitrification rate (Fig. 3.4). These findings suggest that a strong coupling exists between nitrification rates and NH_4^+ net fluxes within the SZ. This argument is strengthened by the fact that rates of NH_4^+ uptake were in the same range as the nitrification rates that were measured simultaneously (Table 3.2; Fig. 3.4). FZ samples showed the highest rates of dark nitrification. For both FZ and EZ samples, nitrification rates were positively correlated to the net NH_4^+ effluxes (see Section 3.3.4.2), suggesting a link between nitrification rates and DNRA in the rocky biofilms sites.

As stated earlier, the methods used do not allow accurate measurements of nitrification and denitrification coupling. However, the absence of significant differences between NO_3^- net fluxes measured without (Table 3.2) and with C_2H_2 (data not shown) together with the significant correlations found between dark denitrification rates, net NO_3^- fluxes, and water column NO_3^- concentration at SZ, MZ and FZ (Fig. 3.5), suggested that nitrification

does not regulate denitrification rates in these environments. In addition the zero dark nitrification rates found at MZ, SZ and EZ during some seasons (Fig. 3.4) confirms that nitrification and denitrification are not coupled in these particular situations. The degree of nitrification/denitrification coupling is highly variable according to the type of environment studied and the NO_3^- concentrations in overlying water as well as microenvironment oxygen conditions (Rysgaard et al. 1994, Dong et al. 2000). In environments containing high NO_3^- concentrations in overlying water several evidences for low coupling between both processes were documented (Jensen et al. 1996, Welsh et al. 2000). Further studies are necessary to improve our knowledge of the factors influencing nitrification and denitrification coupling in these intertidal environments.

3.4.3.5 N Fixation

N fixation was of minor importance in all four intertidal environments. Assuming a molar ratio of 106C:16N, and that 1 mol of N_2 fixed is converted into 2 mol of NH_4^+, N fixation rates of light-incubated samples would have supplied between 0% to 0.5% of the calculated N requirements for community GPP. The N fixation rates observed here were much lower than those reported for other intertidal environments, where N fixation supplies between 6 to 12% of the N demand (Welsh et al. 2000). In the Douro River estuary, N losses via denitrification were not balanced by N fixation. Also, in other temperate eutrophic estuarine sediments, N losses via denitrification greatly exceeded N inputs through N fixation (Capone and Kiene 1988, Seitzinger 1988). Bebout et al. (1993) showed that N fixation rates in dark incubations of microbial mats were stimulated three to four times when they occurred following a full day of photosynthesis, due to the utilization of storage products of oxygen photosynthesis. In this study, dark N fixation rates were measured in samples subjected to a 12h of dark period; thus our nighttime N fixation rates could be underestimated. However, even with four times higher N fixation rates still would not be important in terms of the overall DIN cycle at these sites, as expected for systems containing high concentrations of fixed N (Howarth et al. 1988).

3.4.4 General N Cycle Dynamics

In the present study all N-cycling processes were measured on inundated samples, but *in situ* intertidal environments are subject to normal tidal cycles undergoing to both exposed

and submerged situations. In the following discussion, the N budget presented represent only the submerged situation of the intertidal sites studied and all data are presented in hourly bases (μmol m^{-2} h^{-1}) in order to avoid any extrapolations errors.

3.4.4.1 N Budget: Light Incubation

During light incubations, N assimilation by primary producers accounted for virtually all of the uptake of both NH_4^+ and NO_3^- from the water column. Differences in inorganic N uptake rates between the four sites were resolved by the different N requirements for primary production, which varied according to the differences in C:N ratios of the primary producer communities. N fixation rates supplied only between 0.0% to 0.5% of the N requirements for GPP, nitrification rates were almost always zero (Fig. 3.4), and the NO_3^- uptake that could be accounted for by denitrification was negligible (0.6 to 7.7%, with the exception of MZ during winter when NPP was lower than respiration). Calculated mean values of NH_4^+ and NO_3^- uptake rates (μmol $m^{-2}h^{-1}$) for all light incubations at each site, together with the estimated rates of NH_4^+ remineralization (O_2:N= 7; Redfield 1934) based on the dark oxygen net fluxes measured (Trimmer et al. 2000), gave a mean ratio of carbon fixed to nitrogen assimilated (C:N) of 9.6±4.1, 7.8±5.5, 10.1±3.7 and 21.7±9.9 for SZ, MZ, EZ and FZ, respectively. Because these ratios are within the range reported for micro and macroalgae communities (c.f. Hillebrand and Sommer 1999, Worm et al. 2000, Bokn et al. 2002), measured NH_4^+ and NO_3^- uptake rates together with the estimated ammonification rates could supply the different N requirements of the primary producer communities. Together, these data lead us to the conclusion that SZ, MZ, EZ and FZ intertidal environments have an N budget in the light where the majority of the inorganic N is cycled from the overlying water to the benthic, epiphytic or epilithic primary producers.

3.4.4.2 N Budget: Dark Incubation

In dark incubations, measurements of nitrification, denitrification and net N nitrogen fluxes (N_2O, NO_3^-, NO_2^- and NH_4^+) allowed construction of N budget for each site, based on the calculated mean values (μmol $m^{-2}h^{-1}$) of each process (Fig. 3.6). N fixation is not included since negligible rates were observed. Though the coupling between denitrification and NO_2^- effluxes and between nitrification and DNRA were based on statistical

relationships observed between the different processes, the flux values for all other N transformations resulted from actual measurements.

The highest rates for all measured or estimated processes occurred in the FZ samples; denitrification, NO_3^- reduction to NO_2^- and DNRA appear to be the main sources for uptake of NO_3^- from the water column in the dark. On average at the FZ site, 41% of the NO_3^- removed from the overlying water was reduced to NH_4^+ through DNRA, 19% reduced to NO_2^- and subsequently diffused into the water column and 40% denitrified, of which 37% was converted to N_2 and 3% into N_2O(Fig. 3.6). Mineralization and dissimilatory NO_3^- reduction to NH_4^+ could have supported the measured nitrification rates and also contribute to the NH_4^+ efflux to the water column (in unknown proportions).

At the EZ site, denitrification could account for 28% of the NO_3^- removed from the overlying water, of which 26% was converted to N_2 and 2% to N_2O. NO_3^- reduction to NO_2^- and subsequent diffusion of NO_2^- into the water column could account for 18% of the NO_3^- uptake. NO_3^- produced through nitrification together with NO_3^- from the overlying water, could have been reduced to NH_4^+, which together with mineralization would supported the NH_4^+ effluxes to the water column as well as nitrification rates (Fig. 3.6).

At the MZ site, nitrification was excluded from the diagram, since the rates were almost always zero. On average, denitrification accounted for 39% of water column NO_3^- uptake, of which 9% could be released as NO_2^- to the water, 30% denitrified to N_2 and 0.4% denitrified to N_2O (Fig. 3.6). The other 61% of NO_3^- removed from the water column was probably reduced to NH_4^+ and supplied 22% of the NH_4^+ released. A large fraction (78%) of the NH_4^+ net effluxes measured appear to be supplied by mineralization (Fig. 3.6).

SZ samples showed generally the lowest rates of N cycle processes (Fig. 3.6). Denitrification accounted for 20% of the net NO_3^- uptake from the overlying water and just 0.2% was released as N_2O (Fig. 3.6). The low NH_4^+ uptake rates and mineralization could have supported the measured nitrification rates. There was an average of about 166.6 μmol m^{-2} h^{-1} of N for which the processes we measured cannot account for. It as been demonstrated that advective transport could constitute an important process in controlling biogeochemical fluxes in permeable sandy beads (Huettel et al. 1998), and thus physical diffusion or adsorption processes could be responsible for the N removal. The

possibility of dark N assimilation by primary producers, which was not measured in this study, may have led to the imbalance in the N cycle process that we observed at SZ site.

Mineralization rates were estimated from the dark oxygen net fluxes (Trimmer et al. 2000) and the Redfield equation for decomposition of organic matter (O_2:N = 7; Redfield 1934). For the SZ, EZ and FZ sites, the oxygen required to produce NO_3^- during nitrification (O_2:N-NH_4^+ = 2; Metcalf and Eddy 1991) was accounted for in the calculations. Using these calculations, the overall mean value of NH_4^+ production through mineralization was 260, 42, 195 and 587 μmol N-NH_4^+ m^{-2} h^{-1}, respectively for the MZ, SZ, EZ and FZ sites. Interestingly, for the MZ, EZ and FZ sites, the demand of NH_4^+ through mineralization according to the conceptual model developed (250, 133 and 576 μmol N-NH_4^+ m^{-2} h^{-1}, respectively; see Fig. 3.6) agreed well with the estimated NH_4^+ mineralization values given above. In the SZ site, the estimated NH_4^+ mineralization rates were very low but together with the NH_4^+ uptake rates measured; they could support the NH_4^+ requirements for the nitrifiers (Fig. 3.6).

3.4.4.3 Overall N Balance: Light and Dark Incubation

In order to classify the four intertidal environments as DIN sources or DIN sinks during daily immersion periods, an overall DIN balance for each site was calculated, taking into account the combined mean immersion daily values of each light and dark N process calculated above (μmol N m^{-2} h^{-1} times 6h; assuming that on average these intertidal sites were flooded half of the day). Net fluxes of NH_4^+, NO_3^- and NO_2^- were considered a N uptake or N release process, according to the measured flux direction. Through denitrification, N is exported to the atmosphere, as N_2 or/and N_2O; nitrification and DNRA were not included since they are internal N cycling processes.

Different total DIN balances were obtained for the four sites. While the MZ site functioned as a DIN-release environment, the SZ, EZ and FZ sites functioned as DIN removal environments. The MZ site showed a total daily net release of inorganic N to the water column of +998 μmol N d^{-1} m^{-2}, largely via NH_4^+ effluxes. The high organic matter content and the low NPP rates observed contributed to the observed net N loss. In contrast, SZ and EZ samples removed DIN from the system, since DIN sink processes clearly exceeded DIN release processes. At these sites, the magnitudes of net daily DIN uptake were similar (-2825 μmol N $d^{-1}m^{-2}$ and -3324 μmol N $d^{-1}m^{-2}$, respectively for SZ and EZ). The FZ

site also operated as a net DIN removal intertidal subsystem in the Douro River estuary; here the N uptake rates were highest, since under flooded conditions daily DIN uptake and DIN release processes combined gave a total of -6288 μmol N d^{-1} m^{-2} removed.

While DON fluxes were not included in this study, they may represent an important component of the overall N flux in benthic macroalgal dominated environments (Tyler et al. 2001). Macroalgae can release large amounts of DON compounds to the water column during active growth and during decomposition of algal tissues (Tyler et al. 2001). Nevertheless, the wave exposure of intertidal hard surfaces environments tends to export dead macroalgae and consequently we expected that DON effluxes due to macroalgal senescence should be much lower in rocky environments.

Chapter 4

Effect of Salinity and Inorganic Nitrogen Concentrations on Nitrification and Denitrification Rates in Intertidal Sediments and Rocky Biofilms of the Douro River Estuary, Portugal

4.1 Introduction

The progressive increases of nitrogen loading and reduction of freshwater discharge into estuaries have led to worldwide eutrophication problems in coastal systems (Jickells 1998, de Jonge et al. 2002, Montagna et al. 2002). In the Douro River estuary, the concentration of NO_3^- in freshwater flowing into the estuary increased dramatically during the 1990's (INAG 2003), and no amelioration is expected in the near future. In addition, water

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diversion for agriculture within the watershed and climate changes has led to a progressive decrease in freshwater flow into the estuary, altering the estuarine salinity regime (Vieira and Bordalo, 2000).

Benthic microbes play an important role in estuarine nitrogen transformations. Under oxic conditions, nitrifying bacteria convert NH_4^+ NO_2^- and subsequently to NO_3^-, while in anoxic zones, denitrifying bacteria convert NO_3^- or NO_2^- into gaseous forms, either N_2 N_2O. Together, these microbially mediated processes may reduce land-derived N loading to coastal waters (Jensen et al. 1996, Seitzinger 2000). In hyper-nutrified coastal systems denitrification increase, and consequently the amount of N_2O produced by denitrification also increases (Seitzinger et al. 1983, Robinson et al. 1998, Corredor et al. 1999). N_2O is a much stronger greenhouse gas than carbon dioxide (CO_2) or methane (CH_4) and is believed to account for a significant fraction of greenhouse warming; its degradation in the atmosphere contributes to ozone depletion (Dickinson and Cicerone 1986).

Because benthic nitrification and denitrification influence the inorganic nitrogen budget in estuaries, understanding how environmental factor(s) control(s) these processes is of both fundamental and practical importance. Different relationships have been described between the influence of salinity and inorganic nitrogen concentration of key sediment nitrogen cycle processes (Ogilvie et al. 1997; Rysgaard et al. 1999). Often, it has been difficult to explain the results to a specific parameter, since throughout an estuary many environmental and chemical parameters are correlated or interact. In the present study, a monthly sampling program and laboratory enrichment experiments were conducted to evaluate the effect of salinity and inorganic nitrogen (NO_3^- and NH_4^+) concentrations on nitrification, denitrification and N_2O production within the two most representative intertidal environments of the Douro River estuary; sandy sediments and rocky biofilms.

4.2 Study Area Description

Physical and chemical characteristics of the Douro River estuary have been presented previously (Magalhães et al. 2002, Magalhães et al. 2003*, Chapter 1 of this thesis). Freshwater flow into the estuary since activation of the dam in 1985 has been reduced from 660±6 m^3s^{-1} (average between 1960-1984) to 505±56 m^3s^{-1} (average between 1985-2002).

*The contents of this article can be found in Chapter 2.

The NO_3^- concentration in the Crestuma-Lever reservoir, which supplies virtually all of the freshwater to the estuary, has increased dramatically during the past decade (Fig. 4.1).

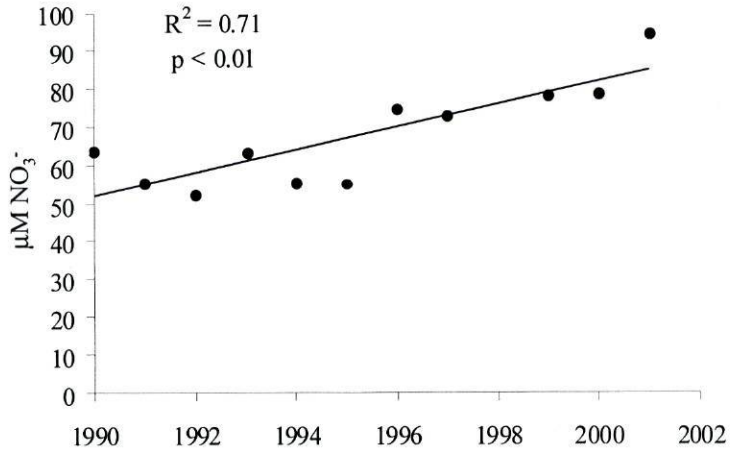

Figure 4.1: Linear regression model between yearly averaged NO_3^- concentration and time (1990-2001) in the Crestuma reservoir. Data from the Portuguese Ministry of Environment (INAG, 2003).

This study was conducted in the two dominant intertidal environments within the lower estuary (Fig. 4.2); sandy sediments (Site I) and rocky biofilms overlain by the macroalgal, *Enteromorpha* spp (Site II). Both sites have been characterized in previous studies in terms of total organic matter and chlorophyll *a* concentrations and, in the case of sandy sediments, the macrofauna, microphythobenthos and particle size distribution (Magalhães et al. 2002, Magalhães et al. 2003, Mucha et al. 2003).

4.3 Methods

4.3.1 Monthly Surveys

At the intertidal rocky and sandy sites (Fig. 4.2), monthly surveys were conducted at low-tide from February 2002 to January 2003. Each month, a total of 10 sediment cores (3 cm diameter and 10 cm depth) were collected from the sandy site, homogenized and stored in a sterilized plastic bag, and rocky biofilms were removed by scraping the rocks in the field, homogenizing approximately 80 g of material and stored in sterilized plastic

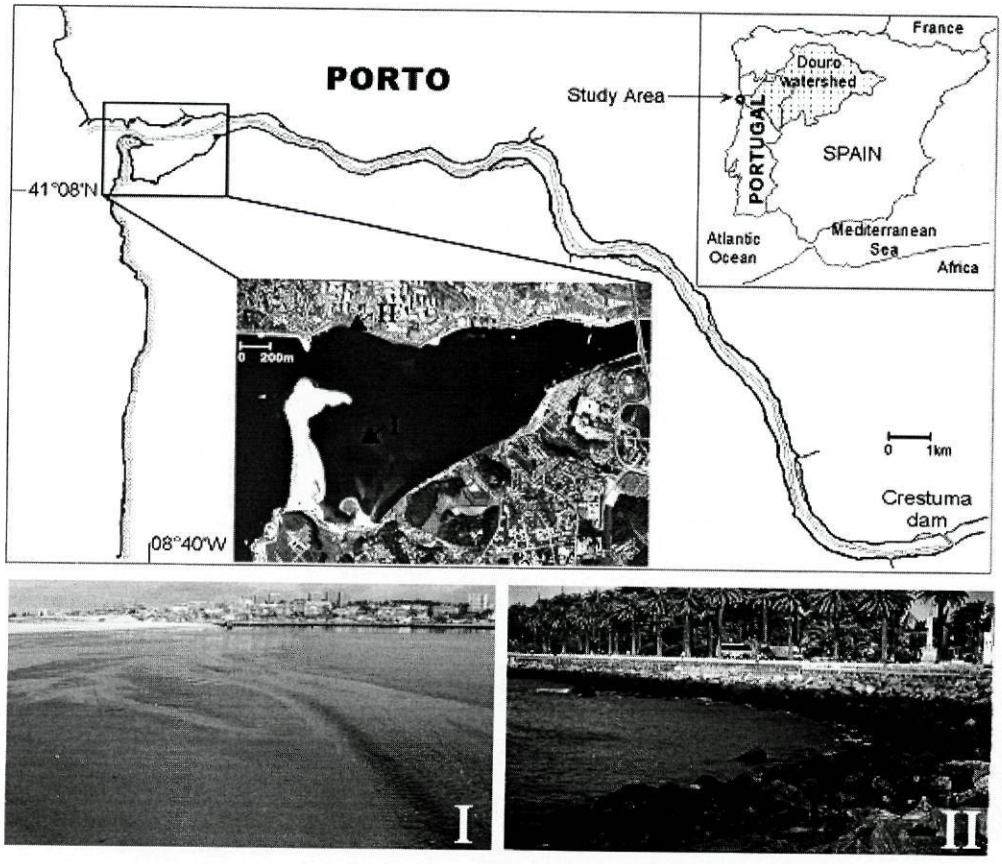

Figure 4.2: Douro River estuary and location of sampling sites.

bags. In addition, 1 l of water from each site was collected and stored in an acid-cleaned polyethylene bottle. Samples were refrigerated in ice chests and transported in the dark to the laboratory for processing, no later than 1 h after collection. In the laboratory, the estuarine water from each site was immediately filtered (0.2 μm, Schleicher & Schuell membrane filters), and samples were processed to measure denitrification and nitrification, according with the techniques described below.

4.3.2 Laboratory Enrichment Experiments

Rocky biofilms and sandy sediment samples were collected in August 2002, according to the techniques described above. Additionally, estuarine brackish water from the Douro estuary with 14 psu salinity, 63 μM NO_3^- and 2 μM NH_4^+, as well as freshwater (0 psu) from the Crestuma-Lever reservoir with 60 μM NO_3^- and 5 μM NH_4^+ were collected in acid-cleaned polyethylene bottles.

Table 4.1: Concentration of $NO_3^- + NO_2^-$ and NH_4^+ as well as salinity of the incubation water used for the different salinity and nitrogen treatments performed in intertidal rocky biofilms and sandy sediment sites.

		Salinity (psu)	NH_4^+ (μM)	NO_3^- (μM)
Nitrogen treatments				
1		14	2	63 (0)
2	Denitrification	14	2	163 (100)
3		14	2	363 (300)
1		14	2 (0)	63
2	Nitrification	14	22 (20)	63
3		14	202 (200)	63
Salinity treatments				
1		0	5	60
2	Denitrification	15	5	60
3		30	5	60
1		0	5	60
2	Nitrification	15	5	60
3		30	5	60

In the laboratory, water samples were immediately filtered (0.2 μM), and additions of NO_3^- (100 and 300 μM, using a standard solution of KNO_3) and NH_4^+ (20, 200 μM using a standard solution of $(NH_4)_2SO_4$) were added to aliquots of the collected brackish water in order to obtain the final concentrations of NO_3^- and NH_4^+ reported in Table 4.1. The impact of salinity was evaluated by amending the Douro River freshwater with salts, using the artificial seawater formula of Cavanaugh (1975) to achieve different salinities (15 and 30 psu; Table 4.1). Denitrification and nitrification rates were measured in triplicate slurries at each salinity treatment and at the three concentrations of NO_3^- and NH_4^+, respectively, according with the methods described below.

4.3.3 Denitrification and Nitrification Rate Measurements

Denitrification rates were measured using the acetylene inhibition technique according to Sørensen (1978). Slurries were prepared by adding 10 ml of incubation water (Table 4.1

and 4.2) to 50 ml serum bottles containing homogenized and weighed sandy sediment (around 3 g) or scraped rocky biofilm (around 1 g). Serum bottles were hermetically sealed with butyl stoppers and aluminum crimp seals. Each serum bottle, with the sample and incubation water, was purged with helium to remove the O_2; triplicate samples with and without acetylene (20% vol:vol) were run and a separate set of time zero samples was sacrificed immediately after acetylene addition. All samples were incubated in the dark for 4 h at constant temperature (20 °C) and stirring (70 rpm). At time zero and 4 h, 12 ml of gas were collected (after headspace equilibration via vigorous shaking), from each serum bottle, and stored in a 12 ml evacuated serum vial for later analysis of N_2O. The 4 h gas samples were collected from each serum bottle by simultaneously adding 12 ml of a 3M NaCl solution and recovering the gas displaced by salt addition (Joye et al. 1996). N_2 produced via denitrification was calculated as the difference between the N_2O produced with and without acetylene. N_2O was quantified using a Shimadzu gas chromatograph equipped with an electron-capture detector and concentration was calculated using certified gas standards (Joye et al. 1996).

Nitrification rates were measured in separate slurries by adding 25 ml of oxygenated (air-saturated) water (Table 4.1, 4.2) to 50 ml serum bottles with homogenized and weighed sandy sediment (around 3 g) or rocky biofilm (around 1 g). Samples were run in triplicate with and without difluoromethane (DFM) (10% vol:vol) according to Miller et al. (1998). All samples were incubated in the dark for 4h at constant temperature (20 °C) and stirring (70 rpm). At time zero and 4 h, 11 ml of overlying water were collected. These samples were centrifuged, 0.2 μm filtered and frozen (-20 °C) for later quantification of NH_4^+ and NO_3^-+NO_2^-. Nitrification rates were calculated by the difference between NH_4^+ production measured in treatments with and without DFM. The NH_4^+ concentration was quantified as described in Grasshoff et al. (1983), and NO_3^-+NO_2^- was determined using an adaptation of the spongy cadmium reduction technique (Jones 1984).

Incubation temperature was maintained at a constant 20°C in order to focus on the effect of seasonal variation of inorganic NO_3^- and NH_4^+ concentrations as well as salinity in denitrification and nitrification rates. Thus in this study any possible temperature regulatory effect on denitrification and nitrification rates was eliminated.

Table 4.2: Monthly incubation water salinity and time zero NO_3^-+NO_2^- and NH_4^+ concentration (mean±standard error of the mean of the replicates) for sandy sediment (Site I) and rocky biofilms (Site II).

	Salinity (psu)		NO_3^-+NO_2^- (μM)		NH_4^+ (μM)	
	Site I	Site II	Site I	Site II	Site I	Site II
Feb-02	22.7	14.9	34.5±1.1	51.7±0.1	21.1±0.7	37.0±1.1
Mar-02	15.1	4.5	31.9±0.9	99.8±0.1	21.9±0.5	25.6±1.2
Apr-02	16.0	16.0	39.59±0.3	49.6±0.5	38.6±1.9	67.0±5.2
May-02	24.0	20.1	21.5±0.1	38.3±1.8	36.9±0.8	51.65±4.3
Jun-02	26.8	16.5	5.2±0.3	41.4±1.1	100.0±3.7	97.6±1.1
Jul-02	25.0	24.8	12.8±0.2	31.5±1.0	22.0±1.9	158.0±5.6
Aug-02	13.9	13.9	65.0±0.6	59.1±1.3	40.8±1.1	14.9±2.2
Sep-02	13.2	16.2	36.0±0.5	37.4±0.7	168.6±1.7	101.5±4.2
Oct-02	22.7	14.9	20.6±1.1	32.9±0.1	49.9±0.7	37.0±1.1
Nov-02	9.0	0.3	59.1±1.8	80.8±3.3	121.7±0.9	145.3±0.9
Dec-02	3.4	0.5	59.9±1.0	103.2±2.2	46.7±1.6	67.64±3.9
Jan-03	1.5	0.1	91.89±2.8	90.2±2.4	65.4±1.5	44.3±2.8
Overall mean	16.1±2.4	11.9±2.4	39.8±7.2	59.7±7.7	61.1±13.3	70.6±13.3

4.4 Results and Discussion

The salinity and NO_3^-+NO_2^- and NH_4^+ concentrations at time zero incubation water are shown in Table 4.2. Salinity varied greatly over time at both sites (Table 4.2). Mean estuarine water column NO_3^-+NO_2^- concentrations at Site I were 39.9±7.2 μM; concentrations were generally higher at Site II (59.7±7.7 μM). Time zero incubation water mean values of NH_4^+ concentrations did not vary greatly between sites (61.2±13.3 and 72.5±13.1 μM NH_4^+, respectively for Site I and II); however, strong temporal variations were observed (Table 4.2). At both sites, time zero NO_3^-+NO_2^- concentrations were inversely related to salinity (Fig. 4.3a, b); thus dilution, rather then *in situ* processes, is the major factor affecting the concentration of NO_3^- of the Douro River estuary.

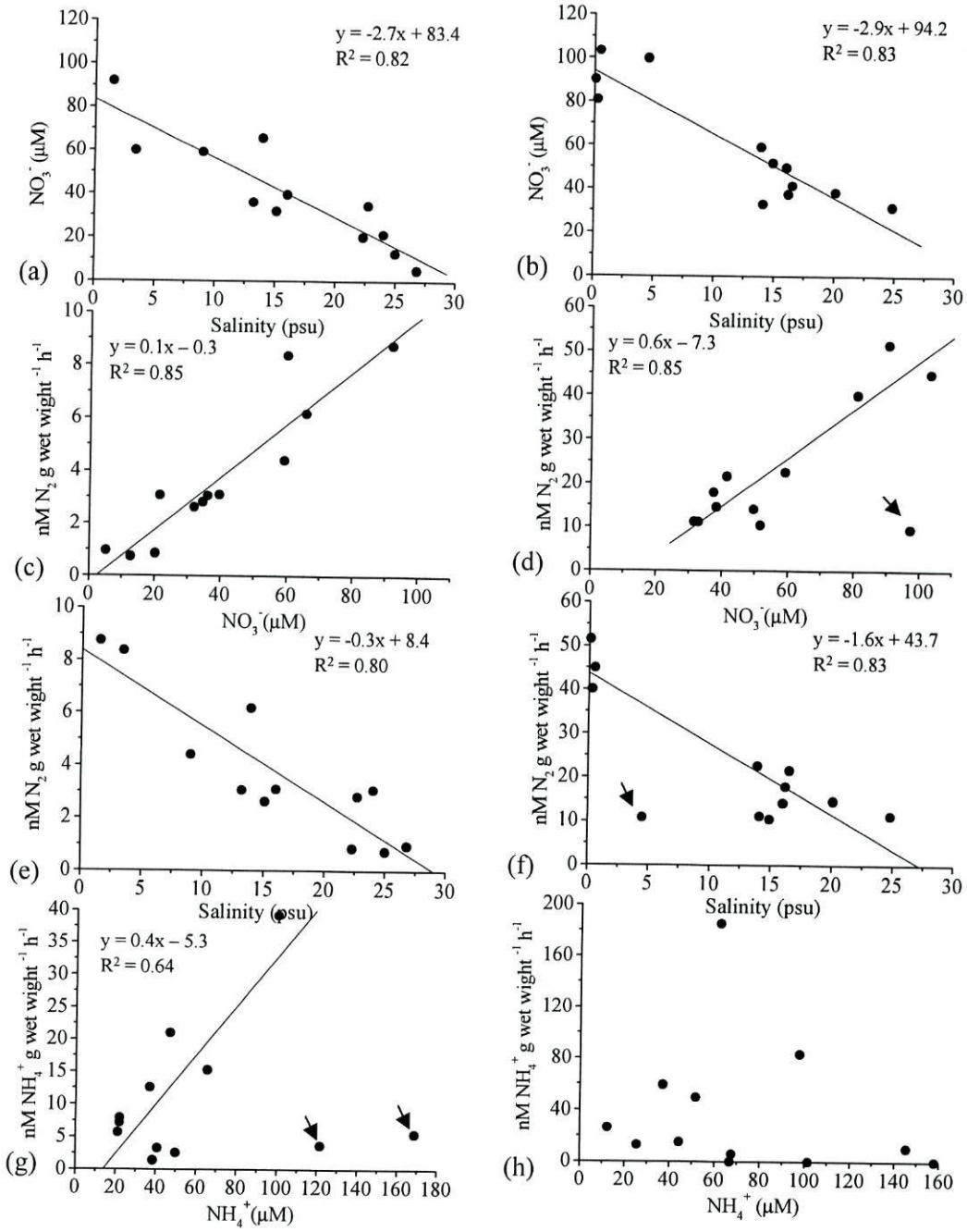

Figure 4.3: Linear relationships established for monthly surveys between NO_3^- concentration and salinity (Site I- a, Site II- b), denitrification rates and NO_3^- concentration (Site I- c, Site II- d), denitrification rates and salinity (Site I- e, Site II- f) and between nitrification rates and NH_4^+ concentration (Site I- g, Site II- h). Arrows indicate outliers removed from the regression analysis.

4.4.1 Denitrification Rates

Denitrification rates varied during monthly sampling in sandy sediments (Fig. 4.4a), with highest rates in December and January (ANOVA, $p < 0.001$) and significantly lower rates in June, July and October (ANOVA, $p < 0.05$). Also, sandy sediment denitrification rates were positively correlated to NO_3^- concentration ($r = 0.92$, $p < 0.001$, $n = 12$; Fig. 4.3c) and inversely related to salinity ($r = 0.89$, $p < 0.001$, $n = 12$; Fig. 4.3e). A different picture emerges, however, when the results of NO_3^- and salt addition experiments are examined (Fig. 4.5a, b). NO_3^- addition experiments confirmed the monthly sampling results, since a progressive increase in denitrification rates (ANOVA $p < 0.001$) with increasing NO_3^- availability was found (Fig. 4.5a). This agrees well with Ogilvie et al. (1997), who found maximum denitrification rates when water column NO_3^- was greatest. However, no significant differences were observed in denitrification rates between the different salinity treatments (ANOVA, $p = 0.21$) (Fig. 4.5b). Thus, the reason that there appeared to be a salinity effect in the monthly survey is that the source of NO_3^- was mostly from the fresh water and thus NO_3^- concentration was a function of the salinity (Fig. 4.3a).

Results from intertidal rocky biofilm (Site II) monthly surveys showed a positive and significant relationship between denitrification rates and the concentration of NO_3^- ($r = 0.67$, $p < 0.05$, $n = 12$), which varies between 31.5 and 103.2 μM NO_3^-. If the data from March sampling were removed as an outlier, a much stronger relationship emerges ($r = 0.92$, $p < 0.001$, $n = 11$; Fig. 4.3d). This suggests a progressive and linear increase of denitrification with increased of NO_3^- availability, up to at least NO_3^- concentration of 103μM; thus denitrifing activity in rocky biofilm sites is regulated by NO_3^- availability. In contrast, NO_3^- addition experiments produced just a weak stimulation of denitrification within the 63 to 163 μM NO_3^- range (Fig. 4.5c), and the lack of statistical differences between treatments (ANOVA, $p = 0.40$) indicating no NO_3^- concentration control on denitrification within the range of concentrations tested. However when denitrification rates are compared between monthly surveys (Fig. 4.4b) and lab experiments (Fig. 4.5c), the mean values for the 3 months where denitrification rates were higher varied between 40.0±7.5 and 51.5±9.5 nmol N_2 g wet weight^{-1} h^{-1} and similarly for the NO_3^- addition experiments denitrification rates varied between 39.5±8.5 and 50.4±17.3 nmol N_2 g wet weight^{-1} h^{-1}. The rates observed in the field and laboratory experiments were not significantly different (ANOVA, $p = 0.98$). taken together these results suggest that there is a maximum denitrification rate for rock biofilms samples, whether it is related to bacterial

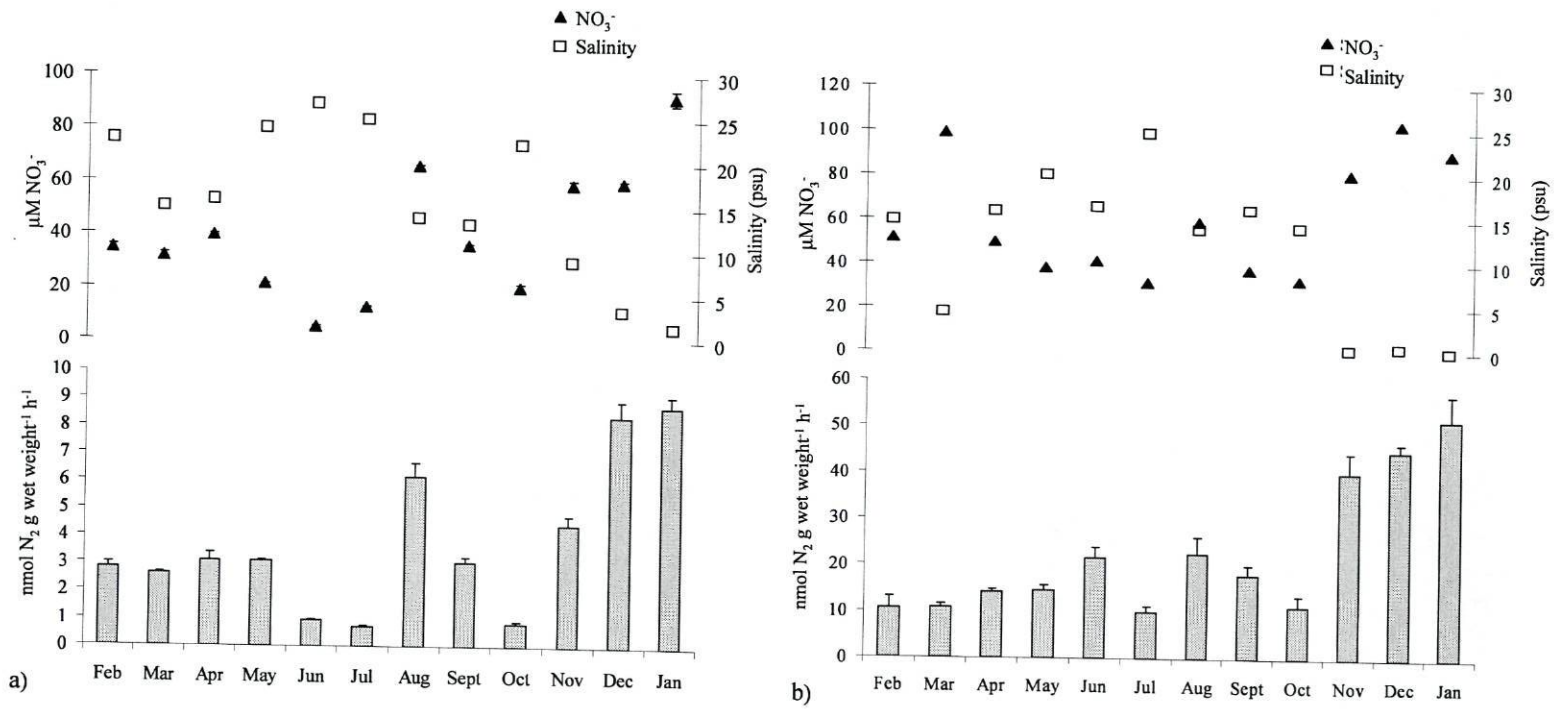

Figure 4.4: Seasonal variation of denitrification rates and estuarine water salinity and time zero NO_3^- concentration (error bars = standard error of the mean of three replicates). a) Site I; b) Site II.

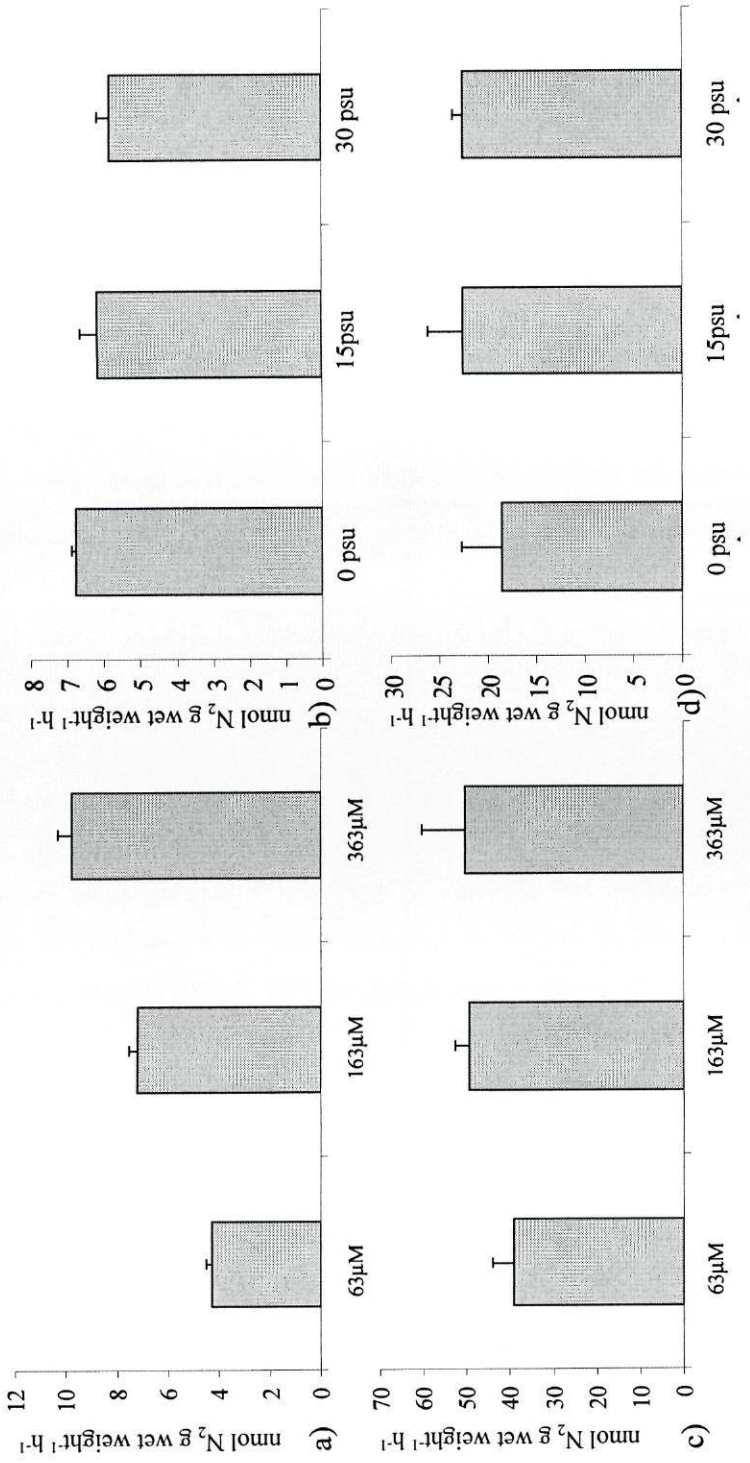

Figure 4.5: Denitrification rates in Site I (a, b) and Site II (c, d) at different salinities and NO_3^- treatments (error bars = standard error of the mean of three replicates). For NO_3^- treatments (a, c), 63 µM NO_3^- estuarine brackish water was amended with 100 µM of NO_3^- and 300 µM of NO_3^-. For the salinity treatments (b, d), Douro River freshwater (0 psu) was amended with different concentrations of salts to reach 15 psu and 30 psu.

population size or their metabolism is unknown. While in the NO_3^- addition experiments the maximum denitrification rates were reached at a concentration of 63 μM NO_3^-, in the monthly survey such rates were observed only at concentrations between 80.8 and 103.2 μM NO_3^-. This may be because in seasonal surveys other parameters acted in concert with NO_3^- availability to influence denitrification rates; however, at this stage we are unable to identify the cause of the discrepancy observed between lab and field experiments.

For salinity in the rocky biofilms site samples, a positive and significant relationship between salinity variation and denitrification rates in monthly surveys was observed ($r = 0.78$, $p < 0.002$, $n = 11$). If the data from March were removed as an outlier, a much stronger relationship would be found ($r = 0.91$, $p < 0.001$, $n = 11$; Fig. 4.3f). In contrast, no clear salinity effect was observed in the salinity manipulation experiments (Fig. 4.5d), since no statistical differences between denitrification rates were observed at the three salinities tested (ANOVA, $p = 0.61$). All these data agree with the data obtained for sandy sediments (Site I); the apparent salinity effect on denitrification rates in field samples was due to co-variation of salinity and NO_3^- in incubation water (Fig. 4.3b). However, salinity and NO_3^- addition experiments reveal that only NO_3^- concentration regulated effectively denitrification rates.

The absence of a clear salinity effect on denitrification rates at both sites indicates that halotolerant denitrifying bacteria inhabit both intertidal environments. In contrast, results from Danish estuarine sediments (Rysgaard et al. 1999), showed that denitrification decreased with increasing salinities, with the most pronounced reduction, of approximately 50%, observed when the salinity was raised from 0 psu to 10 psu in sediments where *in situ* salinities ranged from 3 psu to 13 psu.

4.4.2 N_2O Production and N_2O:N_2 Ratios

During the monthly surveys, a wide range of N_2O production rates were observed (Fig. 4.6a, b). Rates of N_2O production from sandy sediments varied between 0 to 1.1±0.5 nmol N_2O g wet weight^{-1} h^{-1} (Fig. 4.6a); in rocky biofilms values ranged between 0 to 7.5±2.0 nmol N_2O g wet weight^{-1} h^{-1} (Fig. 4.6b). To evaluate the magnitude of the N_2O accumulation during denitrification, the relative production of N_2 and N_2O (N_2O:N_2 ratio) was calculated (Fig. 4.6a, b). Ratios of N_2O:N_2 tend to be very low (< 5%), and seasonal N_2O:N_2 ratios in sandy sediments were consistent with literature values

(Fig. 4.6a) (Seitzinger 1988). However, the rocky biofilm N_2O:N_2 ratios were much higher than those in sandy sediments, and interestingly, the highest ratios were not found where the highest rates of denitrification were observed (Fig. 4.4b, 4.6b). During monthly surveys no significant relationship between N_2O production rates and NO_3^- concentration was observed, suggesting that NO_3^- concentration, *per se*, did not control the observed N_2O production rates. However, for both sites, NO_3^- addition experiments showed increased rates of N_2O production with higher concentrations of NO_3^- (Fig. 4.7a, b), which is in agreement with studies performed in other coastal systems, where higher N_2O net effluxes were coupled to higher NO_3^- availability, and consequently to higher denitrification rates (Kieskamp et al. 1991, Robinson et al. 1998, Seitzinger 2000, Trimmer et al. 2000). In addition, in sandy sediments N_2O:N_2 ratios rose from 3.5±0.2% at ambient NO_3^- concentration to 17.8±0.8% at 163 μM NO_3^-, maintaining this value for the 363 μM NO_3^- samples (Fig. 4.7a). On the other hand, rocky biofilms N_2O:N_2 ratios increased progressively with the same NO_3^- concentrations shown above (11.8±1.7% to 21.9±1.3% to 34.5±5.8%, Fig. 4.7b). These results demonstrate that increasing NO_3^- concentration enhanced both the N_2O production rates and the ratio of N_2O:N_2 (Fig. 4.7a, b).

Von Schulthess et al. (1994) found that high NO_2^- concentrations (higher than 143 μM NO_2^-) as well as aerobic conditions (higher than 8 μM O_2 m^{-3}) favor the production of N_2O. However, while NO_2^- concentrations were not monitored in this study, the low NO_2^- concentrations of the Douro River water column (Magalhães et al. 2002, Magalhães et al. 2003*) and the fact that the incubations were performed under anaerobic conditions suggest that neither of these variables would affect N_2O production rates measured. Finally, the discrepancy found between the results of the monthly surveys and the enrichment experiments shows that while NO_3^- availability influences N_2Oproduction rates, other parameters, or the interactions between them, also regulate N_2O production rates in these environments. Several other factors that were not included in this study are known to increase N_2O:N_2 ratio in the denitrification process, e.g. lowered pH and increased H_2S (Betlach and Tiedje 1981, Firestone and Davidson 1989).

*The contents of this article can be found in Chapter 2.

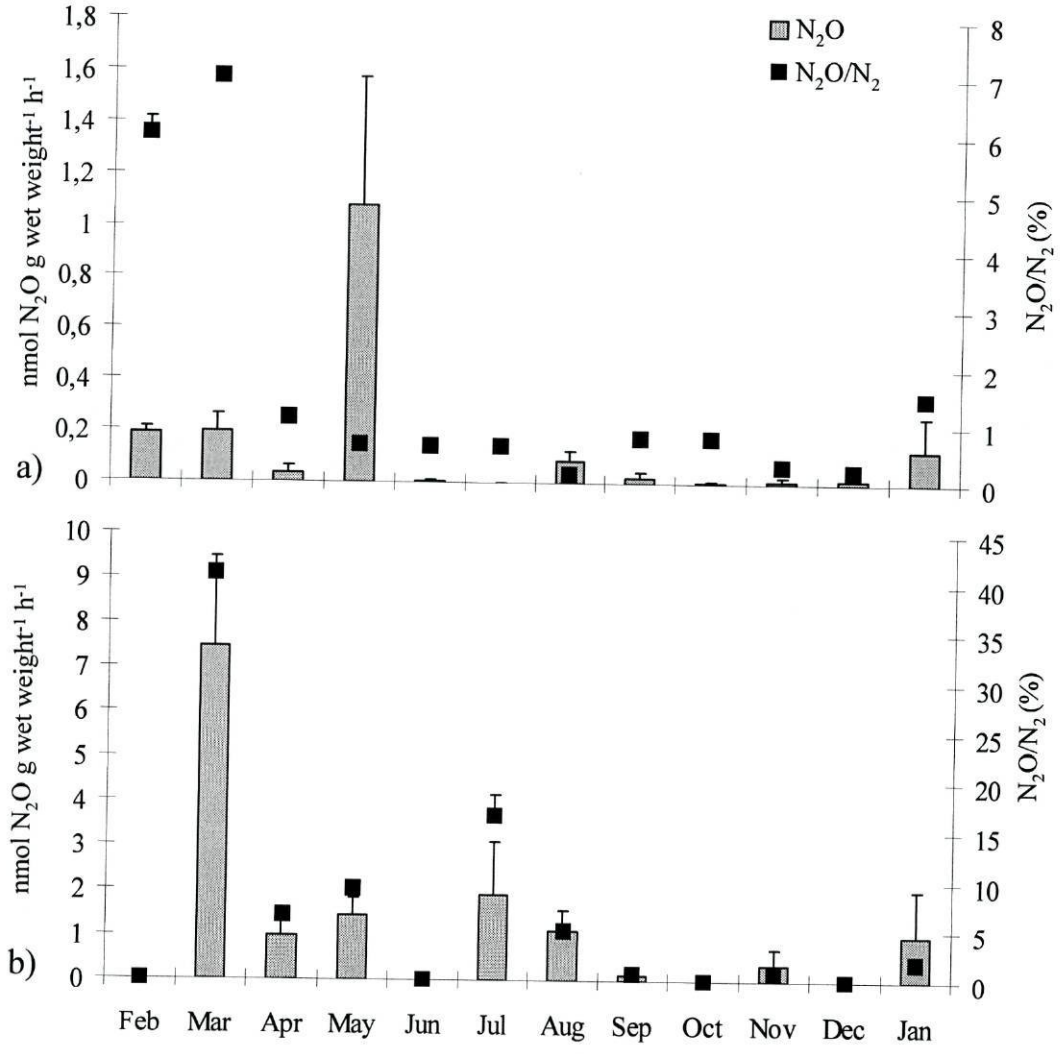

Figure 4.6: Monthly variation of N_2O production rates and N_2O:N_2 ratios (error bars = standard error of the mean of three replicates). a) Site I; b) Site II.

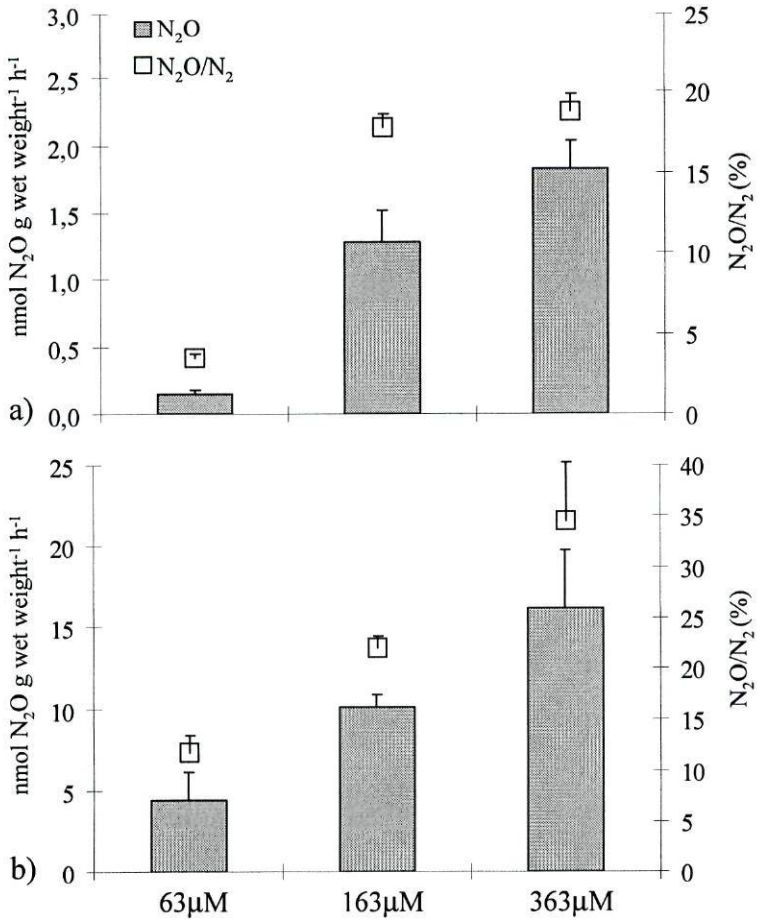

Figure 4.7: N_2O production rates and N_2O:N_2 ratios in Site I (a) and Site II (b) at different NO_3^- treatments (error bars = standard error of the mean of three replicates).

4.4.3 Nitrification Rates

The analysis of monthly data from sandy sediments (Site I) showed a significant, positive and linear relationship between nitrification rate and NH_4^+ concentration ($r = 0.80$, $p < 0.01$, $n = 10$; Fig. 4.3g) within the 21.1 to 100.0 µM range, when September and November data were removed from the analysis. These latter data were identified as outliers of the linear regression model according to the normal Q-Q plot of errors. For September and November, when higher ammonium concentrations occurred at time zero incubation water (168.6 and 121.7 µM NH_4^+, respectively), drastic decreases in nitrification rates were observed (Fig. 4.8a). Interestingly, in the ammonium enrichment experiment, nitrification

rates were stimulated by 35% for the 22 μM NH_4^+ but inhibited when 200 μM NH_4^+ was added to the natural concentration of 2 μM NH_4^+ (Fig. 4.9a), suggesting that ammonium availability stimulates nitrifying activity only within a restricted range. The combination of monthly survey data with lab experiment results suggested that sandy sediment nitrifying bacteria were stimulated by NH_4^+ availability up to ≈100 μM of NH_4^+, but that further increase resulted in inhibition of the nitrifying community. Monthly surveys at different "natural" salinities (Table 4.2) did not show a clear relationship between nitrification rates and salinity variation in intertidal sediments (Site I). In contrast, enrichment experiments showed that nitrification was stimulated by 40% and 17% in incubations with 15 psu and 30 psu, respectively, compared to 0 psu (Fig. 4.9b).

Monthly nitrification rates for rocky biofilms were variable (Fig. 4.8b). Correlation analysis did not show any relationship between monthly nitrification rates and seasonal water column NH_4^+ concentration (Fig. 4.3h) or salinity variations. However, in the enrichment experiments, the regulatory effect of each of these two parameters was demonstrated (Fig. 4.9c, d). A clear stimulation of nitrification (65%) was found at the highest ammonium concentration tested (Fig. 4.9c) and in the salinity experiment, nitrifier activity was stimulated by 50% at the intermediated salinity (15 psu), but almost totally inhibited at 30 psu (Fig. 4.9d). The lack of evidence for ammonium concentration and/or salinity as regulatory parameters for rocky biofilms during the monthly surveys, suggests that other parameter(s) could have (an) important role(s) in regulating nitrifier activity in these biofilms. For example Joye and Hollibaugh (1995) demonstrated that sulfide could be an important regulatory factor for nitrification.

In this study, communities at both sites showed maximum nitrification activity at intermediate salinities, which is in agreement with some culture experiments showing that estuarine isolates of ammonia oxidizers exhibit optimum nitrification rates at intermediate salinities (5-10 psu) (Jones and Hood, 1980) or even between 0-20 psu (MacFarlane and Herbert 1984) with a subsequent reduction of the activity or inactivation at higher salinities. In contrast, recent investigators have shown that progressive salinity increases can reduce nitrification activity (Rysgaard et al. 1999, Campos et al. 2002). In addition, differences between the responses of each environment to the higher NH_4^+ concentrations occurred; rocky biofilm nitrification was greatly stimulated at the higher concentration of NH_4^+ (202 μM); however sandy sediments showed a clear inhibitory effect for such treatment (Fig. 4.9a). These results suggest the occurrence of differences in nitrifiers communities

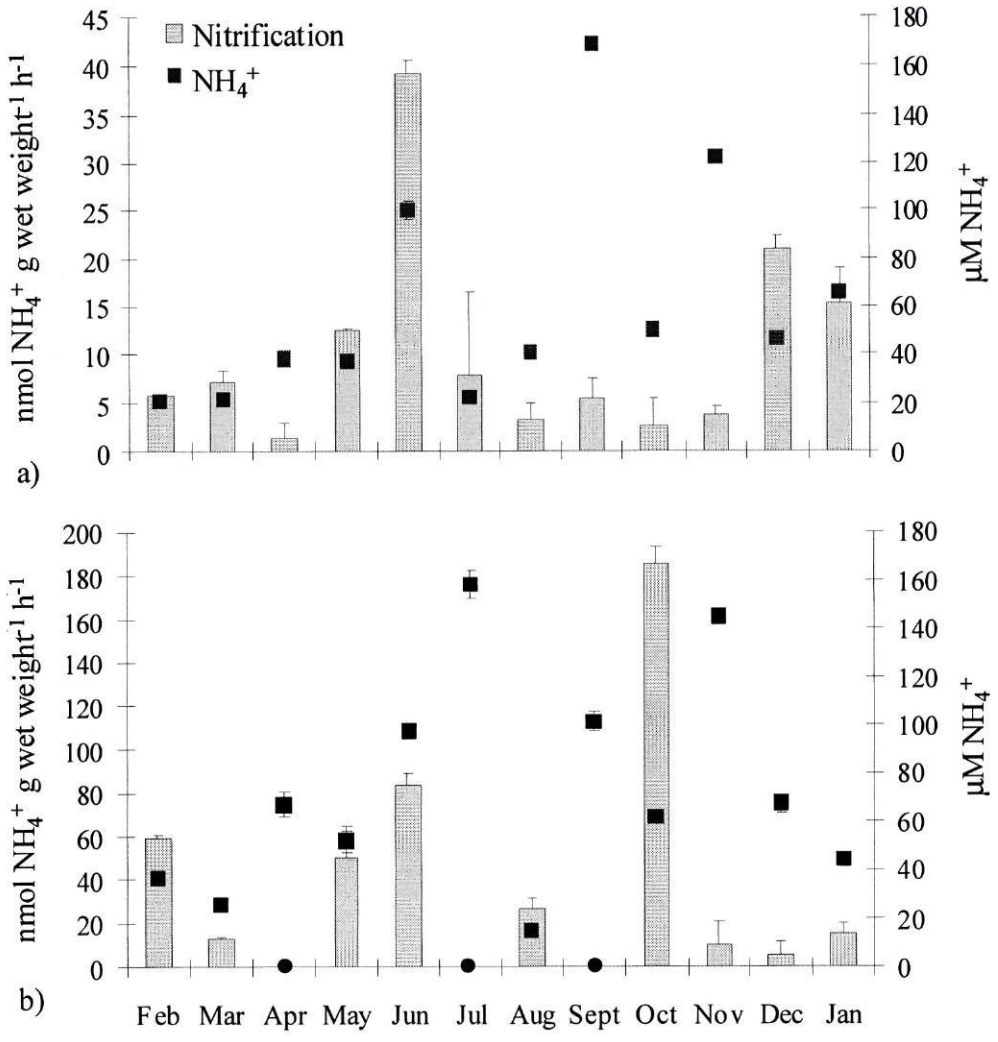

Figure 4.8: Monthly variation of nitrification and time zero NH_4^+ concentration (dark spots = null rates; error bars = standard error of the mean of three replicates). a) Site I; b) Site II.

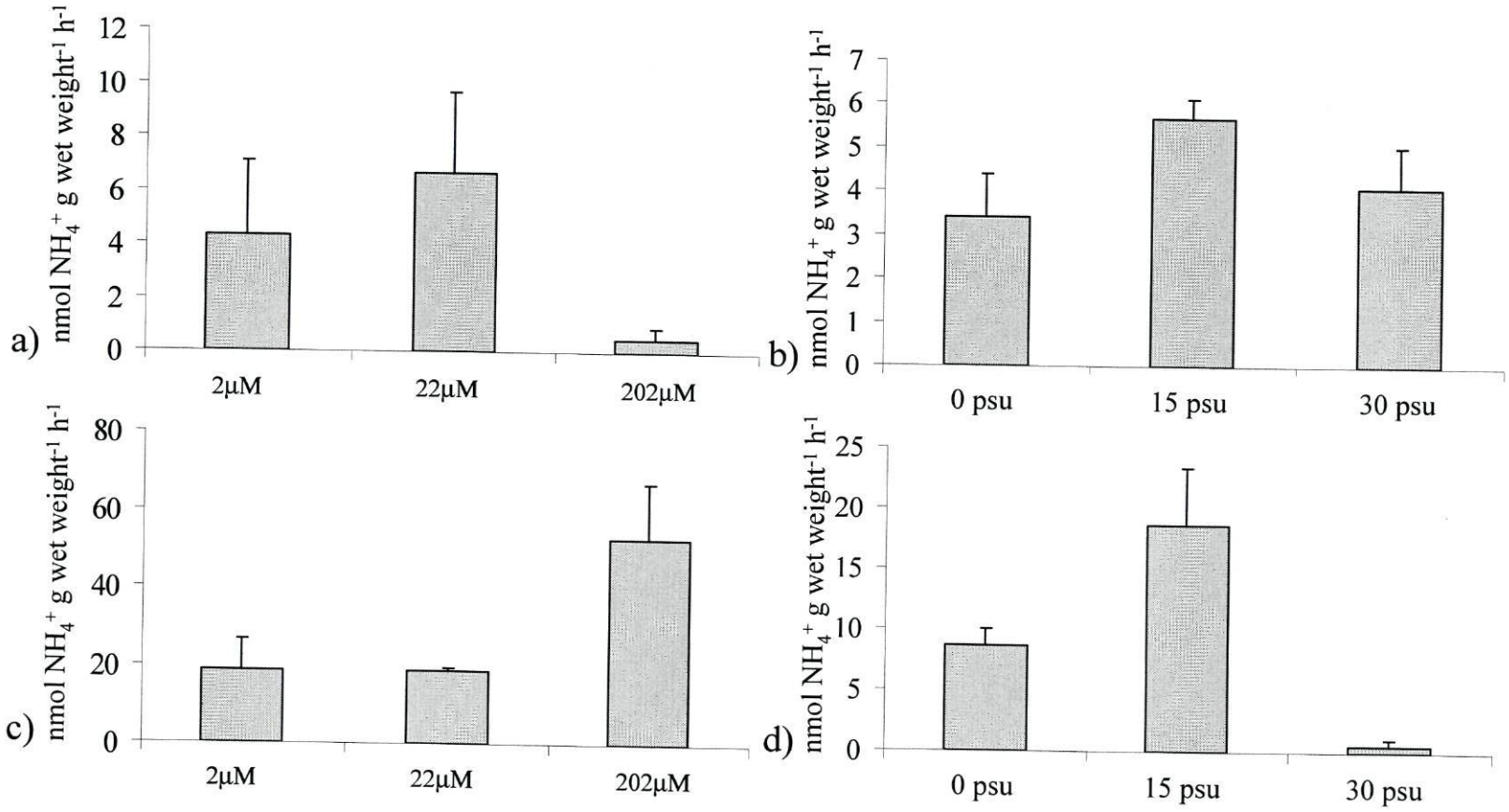

Figure 4.9: Nitrification rates in Site I (a, b) and Site II (c, d) at different salinities and NH_4^+ treatments (error bars = standard error of the mean of three replicates). For NH_4^+ treatments (a, c), estuarine brackish water was amended with 0 μM of NH_4^+, 20 μM of NH_4^+ and 200 μM of NH_4^+. For the salinity treatments (b, d), Douro River freshwater was amended with different concentrations of salts to reach 0, 15 and 30 psu.

of each environment study, while nitrifying bacteria of rocky biofilms are tolerant to high NH_4^+ concentrations, the nitrifier community of sandy sediments have lower tolerance for the increase of NH_4^+. Similarly, Butturini et al. (2000) showed a strong ammonium regulatory effect on the nitrification activity in stream sediment biofilm.

Finally, the results from the monthly survey program and the enrichment experiments suggest that salinity and NH_4^+ concentration have different, but important, regulatory effects on the nitrifier communities in sandy sediments and rocky biofilms.

Chapter 5

Comparison of Ammonium Oxidizing Bacterial Phylotypes and Function Between Intertidal Rocky Biofilms and Sediments of the Douro River Estuary, Portugal

5.1 Introduction

Nitrogen biogeochemistry is predominantly controlled by prokaryotes, thus their activities determine the species of nitrogen compounds present in an environment and their fate (Ward et al. 1997). To understand the ecosystem-level controls of the nitrogen cycle, it is necessary to know how environmental constraints influence the distribution of organisms that have the genetic capability for different N cycle processes. Nitrification is central to nitrogen biogeochemical cycling, consisting of the sequential oxidation of NH_4^+ to NO_2^- and subsequently to NO_3^-. These reactions represent a critical link between mineralization

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The contents of this chapter can be found in Magalhães C. M., N. Bano, W. J. Wiebe, J. T. Hollibaugh and A. A. Bordalo, Comparison of ammonium oxidizing bacterial phylotypes and function between intertidal rocky biofilms and sediments of the Douro River estuary, Portugal, Submitted to Environ. Microbiol..

of organic matter and the loss of fixed nitrogen through denitrification. The first oxidation step in nitrification is carried out by chemolithoautrophic ammonium oxidizing bacteria (AOB) of the β- and γ-subdivisions of the class Proteobacteria that gain energy via conversion of NH_4^+ to NO_2^-.

Culture techniques coupled with phylogenetic analysis of 16S rRNA genes of samples from various environments have revealed a total of 16 species of AOB (Bock and Wagner 2003). Two of these isolates (*Nitrosococcus halophilus* and *Nitrosococcus oceani*) were placed into the class of γ-Proteobacteria, while the remaining 14 species form a monophyletic group within the class β-Proteobacteia, comprising the genera *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*).

Due to the difficulty of culturing AOB, in recent years many investigators have resorted to cultivation-independent molecular ecological approaches to analyze the composition and distribution of the AOB in various environments, including soils (Bartoch et al. 2002, Bruns et al. 1999, Stephen et al. 1998), rivers (Brümmer et al. 2000), lakes (Bollmann and Laanbroek 2001), Arctic and Antarctic oceans (Bano and Hollibaugh 2000, Hollibaugh et al. 2002), temperate estuarine and ocean waters (Phillips et al. 199, Cébron et al. 2003) and coastal sediments (Stephen et al. 1998, Caffrey et al. 2003, Freitag and Prosser 2003). These investigations discovered that AOB were much more diverse than previously found using the classical isolation plus cultivation approach.

Ammonium is the essential energy source of AOB and there is some evidence that distribution of distinct species of nitrifiers depends on the ammonium concentrations in the systems (Phillips et al. 1999, Bollmann and Laanbroek 2001, Koops and Pommerening-Röser 2001). The effect of salinity on the growth and physiology of AOB has also been demonstrated; some AOB require salt for growth, while salt is inhibitory for others (Bock and Wagner 2003). The different magnitudes of these and other environmental variables are of crucial importance in shaping the biogeography of AOB. For example, polluted marine fish farm sediments (McCaig et al. 1999) and marine organic aggregates (Phillips et al. 1999) have been shown to be dominated by populations of *Nitrosomonas*-like AOB, whereas Arctic and Antarctic Ocean waters (Bano and Hollibaugh 2000, Hollibaugh et al. 2002) and Monterey Bay waters (O'Mullan and Ward 2005) appear to be dominated by *Nitrosospira*-like organisms.

A large number of *in situ* measurements demonstrate that different aquatic environments are characterized by different magnitudes of nitrification rates (Jensen et al. 1996, Kristensen et al. 1999). In previous studies performed in the Douro River estuary, it was found that intertidal sediments had lower nitrification rates and were less able to respond to elevated NH_4^+ concentrations than intertidal rocky biofilms (Magalhães et al. 2005a*, Magalhães et al. 2005b†). At present, with few exceptions (McCaig et al. 1999, Caffrey et al. 2003, Cébron et al. 2003, O'Mullan and Ward 2005), little has been done with respect to relating the composition of the nitrifier assemblages with *in situ* transformation rates and environmental variables.

In this study, molecular approaches (DGGE, cloning and sequencing of 16S rRNA) were used to compare the composition of the AOB assemblage at four different estuarine habitats of the Douro River estuary; two intertidal rocky biofilm sites and two intertidal sediment sites to investigate species selection or adaptation in the different environments. In addition, nitrification rates and net NO_3^-, NH_4^+ and NO_2^- fluxes determined previously (Magalhães et al. 2005a) were related to AOB assemblage composition. It was hypothesized that different AOB assemblages would be associated with different intertidal environments of the Douro River estuary, and that differences in AOB assemblages would be related to the rate of N processing and other environment variables.

5.2 Material and Methods

5.2.1 Description of Study Area

Physical and chemical characteristics of the Douro River estuary have been described previously (Magalhães et al. 2002, Magalhães et al. 2003‡). The present study was conducted between 2001 and 2002 in the four dominant intertidal environments of the lower estuary (Fig. 3.1): two intertidal rocky areas (*Enteromorpha* spp. and *Fucus* spp. colonized zones; EZ and FZ respectively), and two intertidal sediments (sandy and muddy areas; SZ and MZ respectively).

*The contents of this article can be found in Chapter 3.
†The contents of this article can be found in Chapter 4.
‡The contents of this article can be found in Chapter 2.

5.2.2 Sample Collection

For AOB assemblage analysis, duplicate sediment cores (3 cm diameter and 10 cm long) or samples of rocky biofilms (approximately 80 g, scraped from rocks), were collected at each season and sampling site. The samples were homogenized, stored in sterile plastic bags and transported to the laboratory in the dark in refrigerated ice chests and immediately frozen at -70 °C until DNA extraction.

5.2.3 N Processes and Environmental Variables

Net fluxes of NO_2^-, NO_3^- and NH_4^+ and nitrification rates were determined in intact cores and rocks in incubation chambers at *in situ* conditions. These data were presented in a previous paper where the details of the methods involved are described (Magalhães et al. 2005a*). In this study AOB assemblages dynamics were related to the N biogeochemistry data from dark incubations because nitrification was found to be an irrelevant process in light incubations (Magalhães et al. 2005a). Samples for inorganic N fluxes, nitrification rates, chl *a*, total organic matter (OM) and water column NO_3^-, NO_2^- and NH_4^+ concentrations were collected at the same sites and times as the samples collected for phylogenetic analysis of the AOB assemblage, details of the sampling methods and processing of these environmental variables are also described in Magalhães et al. (2005a).

5.2.4 DNA extraction and PCR amplification of 16S rRNA

DNA was extracted from 0.5 to 1.5 g of wet weight of sediment or biofilm samples using an Ultra clean Soil DNA Isolation Kit (MoBio laboratories Inc, Solana Beach, Calif.) following the manufacturer's instructions. Primers used in PCR amplifications were synthesized by Operon Technologies (Oakland, Calif.), and all PCR reactions were performed on an MJ Research PTC-200 Peltier thermal cycle. PCR products were visualized by standard agarose gel electrophoresis, with a 1-kb ladder (Promega). Negative control reaction mixtures containing no template were included in all amplifications performed. AOB 16S rRNA genes were amplified using a nested PCR approach (Ward et al. 1997). The initial amplification was carried out using the general bacterial primers 27F (5'-

*The contents of this article can be found in Chapter 3.

AGAGTTTGATCMTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT), which amplify nearly complete Bacteria 16S rRNA genes (1.4 kb). PCR was run with 20-60 ng of template DNA in 50 µl reaction volume using the following conditions: initial denaturation of the template DNA at 95 °C for 5 min, pause at 82 °C to add Taq DNA polymerase (5 U, Promega) then 30 cycles consisting of 94 °C for 45 s, 48 °C for 45 s, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. A portion of the PCR product (4 µl) obtained was used in a secondary amplification using primers nitA (5'-CTTAAGTGGGGAATAACGCATCG) and nitB (5'-TTACGTGTGAAGCCCTACCCA), specific for ammonium-oxidizing bacteria of the β-subclass of the class Proteobacteria. PCR conditions were the same as described in Bano and Hollibaugh (2000). These primers amplify a 1.1-kb fragment between positions 137 and 1234 of the *Escherichia coli* 16S rRNA gene (Voytek and Ward 1995). The nitA/nitB PCR products were precipitated with 2 volumes of ice-cold ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) overnight at -20 °C and then were centrifuged at 12000× g for 30 min at 4 °C. The DNA pellet was dried in a DNA SpeedVac (Savant) for 10 min and resuspended in 15 µl of nuclease-free water (Promega). A portion (4 µl) of this DNA was used as template for reamplification with primers 356f (5'-CCTACGGGAGGCAGCAG) and 517r (5'-ATTACCGCGGCTGCTGG). These primers amplify a fragment of the 16S rRNA gene between positions 340 to 533 of the *Escherichia coli* gene (Murray et al. 1996). A 40-bp GC clamp (5'-CGCCCGCCGCGCCC CGCGCCCGTCCCGCCGCCCCCGCCCC) was added to the 5'-end of the 356f primer (Myers et al. 1985) and fluorescein was attached to the 5' end of primer 517r. PCR reaction conditions were similar to those used by Bano and Hollibaugh (2000, 2002). The concentration of the resulting PCR product was estimated by the Hoechst dye assay (Paul and Myers 1982) and then the mixed-template product was analyzed by denaturing gradient gel electrophoresis (DGGE).

5.2.5 DGGE

DGGE was performed by using a CBS Scientific DGGE system (Del Mar, Calif.) essentially following Bano and Hollibaugh (2000, 2002). For each sample, 500 ng of PCR product was loaded on a 6.5% polyacrylamide gel containing a gradient of denaturant (urea and formamide) from 45 to 65%. PCR reactions containing genomic DNA from *Clostridium perfringens* and *Bacillus thuringiensis* (Sigma) were used as DGGE standards. Gels were run for 15 h at a constant voltage of 75 V in 1× TAE buffer at 60 °C. Gels were scanned by

using an FMBIO II (Hitachi) gel scanner set to measure fluorescein fluorescence. Bands of interest were excised from the gel, and DNA was eluted into 50 μl of water by incubation at 50 °C for 1 h. DNA extracted from the excised bands was reamplified with primers 356f and 517r as described above. The resulting PCR product was purified using the Qia-quick PCR purification kit (Qiagen, Valencia, Calif.) and sequenced on an automated sequencer at the Molecular Genetics Instrumentation Facility (MGIF) of UGA.

5.2.6 Cloning

Spring samples from each site were selected to generate clone libraries. AOB 16S rRNA genes were amplified with nitA and nitB primers as described above, but using only 25 cycles. PCR product was electrophoresed on an agarose gel (1.5%), bands of the appropriate size (1.1kb) were excised, DNA was extracted from bands using Qia-quick gel extraction kit (Qiagen, Valencia, Calif.), and cloned into the pCR 2.1 vector (Invitrogen Corp., Carlsbad, Calif.) following the manufacturer's protocol. Approximately 20 colonies were selected randomly from the library for each sample, then plasmids were isolated from *Escherichia coli* host cells with a Qiaprep Spin Miniprep kit (Qiagen, Valencia, Calif). Insert size was verified by digestion with EcoRI and clones with the correct insert size were sequenced. Sequencing reactions were carried out by dideoxy termination using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif) with nitA or plasmid-specific primers (M13 forward and reverse) and read on an ABI automatic sequencer at MGIF, yielding 800 bp of informative sequence per read.

5.2.7 Phylogenetic Analysis

All sequences 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 Ribosomal Database Project's CHECK CHIMERA program, and also by generating phylogenetic trees with different regions of the sequences. Sequences were aligned using the Genetics Computer Group Inc. package (Wisconsin package version 10.0, 1999). Phylogenetic trees were constructed using Jukes-Cantor distances and the neighbor-joining method (PHYLIP package; 7). Tree robustness was tested by bootstrap analysis (100

replicates). The clone sequences have been deposited in GenBank under the accession numbers DQ002435 to DQ002466 and DQ059323.

5.2.8 Statistical Analysis of AOB Distribution and Environmental Data

The DGGE gel image was converted to a densitometry scan, and band patterns (presence/absence) of samples were compared by cluster analysis (Jaccard index, UPGMA method) using image analysis software (Molecular Analyst version 1.12, Bio-Rad) as described by Ferrari and Hollibaugh (1999) and Bano et al. (2004). The presence and absence of DGGE bands in each sample were used as input variables to evaluate differences in AOB assemblage composition by the ordination method of non-metric multi-dimensional scaling (MDS) based on Bray-Curtis similarities. Samples in MDS plots that were close together were most similar and the extent to which the two-dimensional plots displayed the relationships between samples (i.e. goodness of fit) was determined by a stress coefficient. A stress coefficient < 0.1 indicate a good portrayal of data with no real prospect of misleading interpretation (Clarke and Warwick 1994).

In order to evaluate relationships between environmental function and AOB assemblage composition, the magnitudes of individual N cycle processes (nitrification rates, net NO_2^- fluxes, net NH_4^+ fluxes and net NO_3^- uptake rates) for each sample were shown on the MDS ordination plot as circles whose size varied with rate. In the case of net NO_2^- and NH_4^+ fluxes where both positive (release rates) and negative (uptake rates) values were recorded, absolute value of the most negative rate was added to all the other values in order to eliminate negative values (essentially, the data set was re-zeroed with the most negative rate set to zero). ANOSIM analysis (Clarke and Warwick 1994) tested whether differences in assemblage grouping in the MDS were significant; the values of the R statistic are an absolute measure of how well the groups are separated, and range between 0 (indistinguishable) and 1 (well separated). Hierarchical clustering with group-average linking was applied to the log-transformed environmental variables and normalized Euclidean distances were calculated. PRIMER version 5 Software (Clarke and Warwick 1994) was used for all multivariate statistical analysis of the environmental and biological data.

5.3 Results and Discussion

5.3.1 Environmental Variables

Concentrations of NO_3^-, NO_2^- and NH_4^+ in the water column of each sampling site are given in Table 5.1. NO_3^- concentrations varied between 12.4±1.8 and 88.5±11.1 μM and were generally lower at the muddy sediment site. NO_2^- and NH_4^+ concentrations ranged between 0.3±0.1 to 3.8±1.9 and 13.4±6.8 to 111.9±10.2 μM, respectively, with the higher values observed at the rocky biofilm sites (EZ, FZ). Chl *a*, and %OM varied according to the type of substrate. Samples from intertidal sediments (MZ, SZ) had generally lower values when compared with samples from rocky biofilm sites (Table 5.1).

Hierarchical cluster analysis was performed based on seasonal and spatial distribution of the variables presented in Table 5.1. The resulting dendogram yielded two clusters separated at the normalized Euclidean distance level of 2.8 (Fig. 5.1). Cluster *a* was composed of all samples from the rocky biofilm sites, which were characterized by higher water column NO_2^- and NH_4^+ concentrations as well as higher %OM and chl *a* contents (EZ and FZ); whereas cluster *b* contained samples from intertidal sediment sites (MZ and SZ), which were characterized by lower concentrations of NO_2^- and NH_4^+ and lower %OM and chl *a* contents. There were no significant differences in the variables measured among samples collected from the same site at different seasons or within sites at the rocky areas (EZ and FZ) and sediment areas (SZ and MZ).

5.3.2 Inorganic Nitrogen Net Fluxes and Nitrification Rates

Inorganic NO_3^-, NO_2^- and NH_4^+ net fluxes are reported for all sampling times and sites in Table 5.2. Similar rates of NO_3^- uptake were observed between sites, except one extremely high value at the FZ site during spring (Table 5.2). The net NO_2^- fluxes were very low at intertidal sediment sites but high rates of nitrite release were observed at rocky biofilm sites (Table 5.2). Net effluxes of NH_4^+ were observed at all sites except at the SZ site, where NH_4^+ net fluxes were negative with one exception (Table 5.2). Variability in nitrification rates between the four sites studied was high (Table 5.2). Generally higher nitrification rates were observed in rocky biofilm samples (EZ and FZ), where higher NH_4^+ effluxes

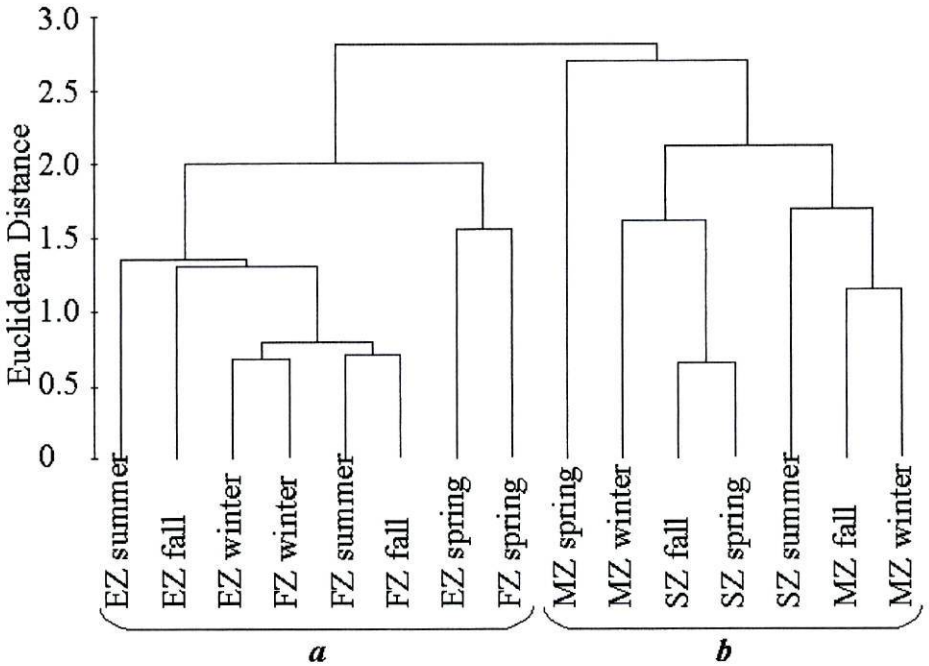

Figure 5.1: Dendogram for hierarchical clustering of the 15 samples based on group-average linking of Euclidean distance calculated for log-transformed environmental data presented in Table 5.1.

(Table 5.2) and higher amplitudes of water column NH_4^+ concentrations (Table 5.1) were generally observed. In MZ samples, nitrification was only found during winter (Table 5.2).

5.3.3 AOB Composition and Distribution

Our attempts to amplify AOB 16S rRNA genes directly from the extracted DNA with the nitA/nitB primer set were not successful for any of the samples; however, PCR product was obtained with the nitA/nitB primer set in all samples when amplified using a nested PCR approach. All of the sequences obtained from DGGE profiles of the nitA/nitB product (Fig. 5.2) were closely related to sequences from β-subdivision ammonium oxidizers.

DGGE fingerprints revealed that some bands were common to all samples, while others showed spatial and temporal variation (Fig. 5.2). Cluster analysis (Jaccard index, UPGMA) of DGGE banding patterns showed that the rocky biofilm and intertidal sediment samples clustered separately (Fig. 5.3). All of the AOB sequences retrieved from DGGE profiles of samples from rocky biofilm sites (EZ and FZ), were closely related to *Nitrosomonas*-like sequences; whereas the majority of sequences retrieved from samples

Table 5.1: Characteristics of the estuarine water used for the incubations and chlorophyll *a* (chl *a*) and total organic matter (%OM) content of the substrate from the four sites. Data are from Magalhães et al. (2005a*) and are given as mean±SD.

		Incub. Temp.	Salinity	NO_3^-	NO_2^-	NH_4^+	chl *a*	OM
		°C	psu	(μmol L^{-1})	(μmol L^{-1})	(μmol L^{-1})	(mg m^{-2})	(%)
SZ	Summer	20	15.4	45.9±13.9	1.8±0.1	17.1±1.7	113.4±19.9	0.4±0.0
	Fall	19	20.0	33.7±4.5	1.0±0.1	17.6±2.2	na	1.3±0.1
	Winter	11	5.0	88.5±11.1	0.7±0.1	17.6±0.8	296.6±22.5	0.6±0.1
	Spring	18	11.8	40.0±8.2	0.9±0.2	21.1±1.5	510.1±120.9	0.9±0.1
MZ	Fall	20	10.8	39.3±6.8	1.5±0.4	26.4±10.1	108.9±14.0	3.4±0.5
	Winter	12	11.2	57.6±6.2	0.9±0.1	21.8±4.5	62.2±14.4	8.0±3.3
	Spring	16	33.3	12.4±1.8	0.3±0.1	13.4±6.8	127.7±6.9	12.6±4.4
EZ	Summer	19	16.1	62.3±19.7	3.8±1.9	50.5±14.2	1347.4±265.4	12.7±14.4
	Fall	19	12.9	52.4±8.0	2.2±0.5	28.6±6.6	740.1±669.4	33.8±21.2
	Winter	12	19.8	58.1±3.4	1.6±0.3	18.3±2.3	640.0±37.8	14.2±8.6
	Spring	16	22.5	32.9±4.4	2.0±0.6	111.9±10.2	342.3±77.7	15.2±13.7
FZ	Summer	18	20.2	54.6±9.9	1.5±0.3	17.0±4.7	941.7±348.4	7.0±2.1
	Fall	18	21.0	50.4±2.5	1.7±0.1	13.9±2.3	1171.2±596.9	14.1±1.0
	Winter	12	13.7	61.8±3.5	1.6±0.6	22.5±5.3	1087.8±116.5	12.0±15.0
	Spring	17	7.2	83.8±11.8	2.2±0.4	91.6±26.7	513.3±406.7	25.3±15.4

taken at intertidal sediment sites (MZ and SZ) were related to *Nitrosospira*-like sequences (Table 5.3 and data not shown). Phylogenetic analysis of clone libraries constructed from DNA extracted from spring samples (Fig. 5.4) also showed that the majority of the sequences (14 out of 17) retrieved from intertidal sediment sites (MZ and SZ) clustered in the marine *Nitrosospira*-like cluster 1 (Stephen et al. 1996), while all of the sequences (total of 16) retrieved from rocky biofilm sites (EZ and FZ) clustered with the *Nitrosomonas*-like group. These results confirmed that the composition of the AOB assemblages described by sequences retrieved from DGGE bands agreed with the composition obtained from analysis of nitA/nitB fragments cloned from the spring samples.

Nitrosospira: Five of the seven clones from muddy sediments (MZ) and nine of the ten clones from sandy sediments (SZ) felt into marine *Nitrosospira*-like group 1 (Stephen et al. 1996). Sequences in cluster 1 were further divided into three sub-clusters (A, B, and C, Fig. 5.4). Sequences of these clones were >99% similar to the sequences obtained from other marine environments (Fig. 5.4): anoxic marine sediment at a water depth of 120 m from Loch Duich, Scotland (Freitag and Prosser 2003); intertidal sediment samples collected at

Table 5.2: Net fluxes of NH_4^+, NO_3^- and NO_2^- in μmol m^{-2} h^{-1} and nitrification rates μmol NH_4^+ m^{-2} h^{-1} (mean±SD). Negative values indicate uptake and positive values indicate release. Data from Magalhães et al. (2005a*) and are given as mean±SD.

		NH_4^+ fluxes	NO_3^- fluxes	NO_2^- fluxes	Nitrification rates
SZ	Summer	-105.0±41.5	-263.6±170.0	-3.5±1.8	157.3±61.8
	Fall	163.2±65.4	-145.2±12.7	Null flux	Null rates
	Winter	-51.0±19.5	-209.8±152.6	Null flux	33.7±4.9
	Spring	-30.3±52.4	-237.0±106.7	1.4±2.9	35.2±9.9
MZ	Fall	54.3±52.4	-25.5±44.1	9.5±5.8	Null rates
	Winter	398.8±114.8	-303.8±94.4	15.2±7.5	91.98±110.68
	Spring	505.6±83.2	-11.6±33.9	5.3±1.6	Null rates
EZ	Summer	603.1±285.3	-317.9±75.4	69.2±46.3	10.2±8.7
	Fall	371.7±433.8	-239.2±118.0	32.1±16.6	35.2±21.1
	Winter	-244.9±224.7	-237.1±99.4	19.6±12.8	449.3±206.6
	Spring	325.9±117.3	-170.2±108.2	43.6±26.6	310.1±225.4
FZ	Summer	620.2±251.4	-641.1±10.1	213.9±105.8	498.4±135.2
	Fall	70.7±78.3	-201.5±86.8	119.3±71.8	388.1±261.4
	Winter	684.1±464.8	-252.1±161.4	94.2±23.3	360.58±209.9
	Spring	1970.4±805.3	-1528.6±434.3	335.0±181.1	1712.4±666.6

the Elkhorn Slough estuary in California (Caffrey et al. 2003); water samples collected in the central Arctic Ocean (Bano and Hollibaugh 2000); polluted sediment samples from a fish farm located in United Kingdom (McCaig et al. 1999). Comparison between cloned sequences and sequences of DGGE bands show that sequences in sub-cluster A were 99.3 -100% similar to the sequence from DGGE Band MZ3, sequences in sub-cluster B were 99.3 -100% similar to the sequence from DGGE Band MZ4, and most of the sequences in sub-cluster C were 100% similar to the sequence from DGGE Band SZ4 (Table 5.3 and Fig. 5.2). Sequences from four other DGGE Bands (MZ6, MZ7, MZ5, and SZ2, Fig. 5.2) did not cluster with cloned sequences, but they also fell into *Nitrosospira*-like cluster 1 and were 93-95% similar to *Nitrosospira* sp. clone LD1-A1 obtained from anoxic marine sediment from Loch Duich, Scotland (Freitag and Prosser 2003).

Nitrosomonas: All sequences obtained from intertidal rocky biofilm samples fell into cluster Nm143, a newly described cluster of the *Nitrosomonas*-like group (Purkhold et al. 2003), and into clusters 5 and 6 described by Stephen et al. (1996) (Fig. 5.4). Three cloned

Table 5.3: Similarity of 16S rRNA gene sequences retrieved from DGGE bands to sequences retrieved from clone libraries constructed from spring samples.

Band	Length	Closest match with clone sequence (%)	Cluster[a]
MZ3	144	SZS4B (99.3); MZS-4 (100); MZS-17B (100)	Nitrosospira Cluster 1A
MZ4	144	MZS-1 (99.3); MZS-16B (100); SZS10B (100)	Nitrosospira Cluster 1B
SZ4	144	SZS3 (100) SZS5B (100) SZS9B (100) SZS3B (100) SZS11B (100) SZS2B (100) SZS6B (100) MZS12B (98.6) ZS4B[b] (99.3)	Nitrosospira Cluster 1C
EZ1	141	MZS-7 (99.3); EZS-13B (99.3)[c]; SZS1B (99.3) EZS-5B (98.6); EZS-3B (99.3)	Nitrosomonas Cluster Nm143
MZ8	141	EZS-11B (97.9)	Nitrosomonas Cluster Nm143
FZ4	141	FZS15B (99.3)[d]	Nitrosomonas Cluster Nm143
SZ1, EZ2	144	EZS-12B (97.9); FZS8 (100); EZS-2B (100) EZS-5 (100); EZS-9B (100); EZS-3 (100); FZS4 (100)[d]	Nitrosomonas Cluster 6A
No band		FZS3; FZS11B, EZS-4B; EZS2[d]	Nitrosomonas Cluster 6B
FZ1	144	FZS2 (100); FZS12 (100)[b]; FZS7 (100); FZS1 (100)	Nitrosomonas Cluster 6C
MZ1	144	MZS-2 (95.1)	Nitrosomonas Cluster 6D
MZ2	144	EZS-10B (100)[e]; EZS-1 (99.3)	Nitrosomonas Cluster 5

[a]Based on the phylogenetic tree derived from ≈800 bp sequences (Fig. 5.4).
[b]Short sequence not shown in phylogenetic tree (Fig. 5.4).
[c]Short sequence (774 bp) not shown in phylogenetic tree but 99.6% similar to MZS-7.
[d]Chimeric sequence not shown in phylogenetic tree but 100% similar to FZS8 after 120bp deleted.
[e]Chimeric sequence not shown in phylogenetic tree.

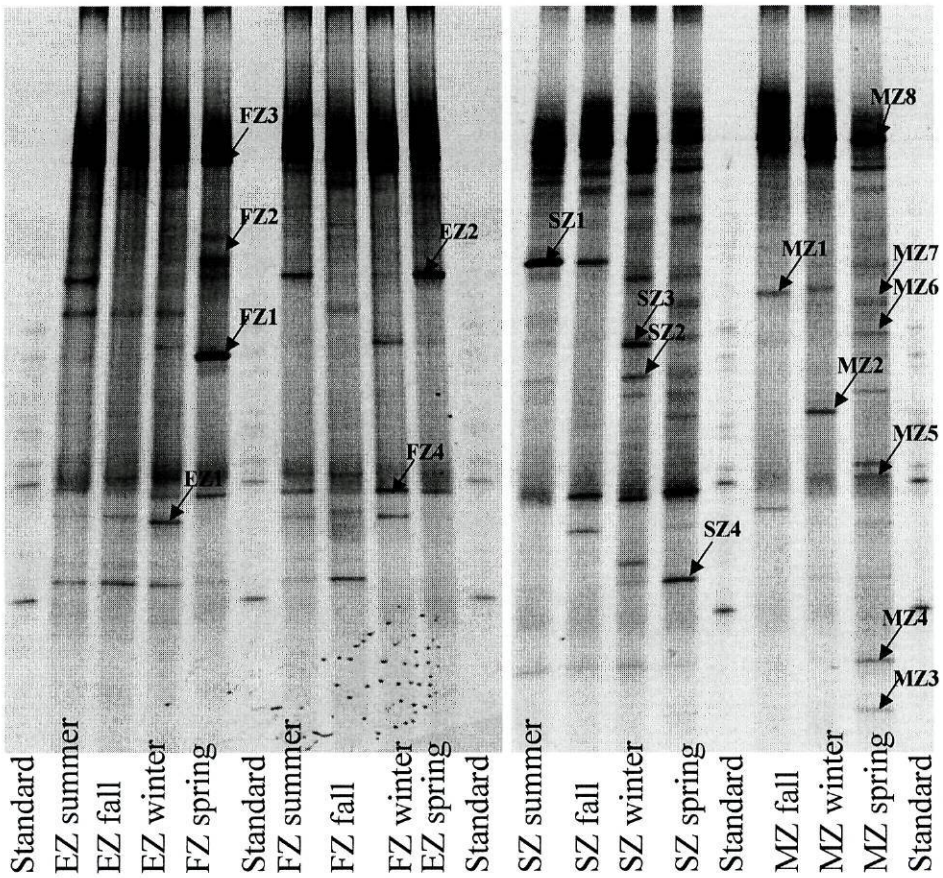

Figure 5.2: Image of the DGGE gels of AOB 16S rRNA gene fragments amplified from SZ, MZ, EZ and FZ samples collected at four different times. DNA sequences were retrieved from the bands indicated.

sequences from the EZ and one each from the MZ and SZ grouped within the Nm143 cluster, these sequences have between 97.9 and 99.3% identities to EZ1, MZ8 and FZ4 DGGE Bands, (Table 5.3 and Fig. 5.2). Sequences included in cluster Nm143 were 97% similar to *Nitrosomonas* sp. Nm143, isolated from an estuary in the Dominican Republic (Purkhold et al. 2003), and to the sequence of clone LD1-B6, isolated from anoxic marine sediment of Loch Duich, Scotland (Freitag and Prosser 2003). Representatives of cluster Nm143 were previously documented in a wide variety of estuarine and marine habitats, confirming a ubiquitous distribution of this lineage (Purkhold et al. 2003).

Thirteen sequences, all from FZ and EZ sites, with the exception of MZS-2, fell into cluster 6 of the *Nitrosomonas* group (Fig. 5.4). Cébron et al. (2004) also found that the majority

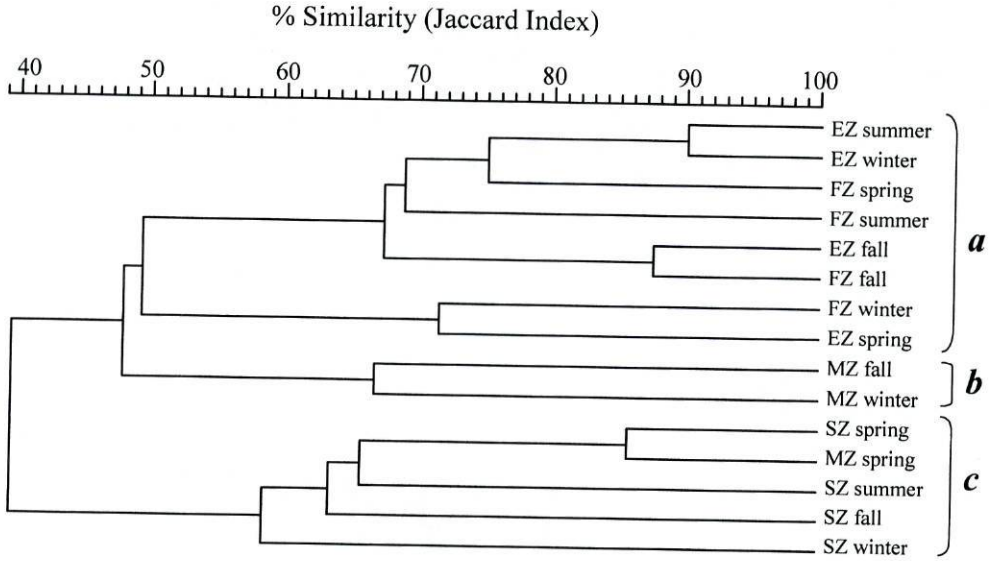

Figure 5.3: Dendrogram showing similarity of DGGE banding patterns (Jaccard index, UPGMA) for all samples shown in Fig. 5.2.

of AOB present samples from the lower Seine River belonged to this cluster. Cluster 6 was further divided into four sub-clusters; A, B, C, and D. Sequences in sub-clusters A, B and C were 98-99% similar to the sequence from clone AZP2-2, obtained from an eutrophic estuarine system at Azevedo Pond, California, characterized by an average water column DIN concentration of 47 μM (Caffrey et al. 2003) and to the sequence of *Nitrosomonas ureae*, isolated from oligotrophic freshwaters (Bock and Wagner 2003). However, several studies have demonstrated the occurrence of many AOB affiliated with cluster 6 in a wide range of environments including in eutrophic systems (de Bie et al. 2001, Cébron et al. 2004). Sequences in sub-cluster A were 100% similar to sequences from DGGE Bands EZ2 and SZ1 (Table 5.3). These intense DGGE bands were present in lanes containing amplicons from samples of SZ and EZ sites collected during summer and spring respectively (Fig. 5.2). Their occurrence coincided with high rates of nitrification relative to rates observed at other seasons (Table 5.2). Sequences included in sub-cluster C (Fig. 5.4) were 100% identical to sequences from DGGE Band FZ1 (Table 5.3 and Fig. 5.2). This DGGE band was only found in FZ during spring, where the highest rates of nitrification were

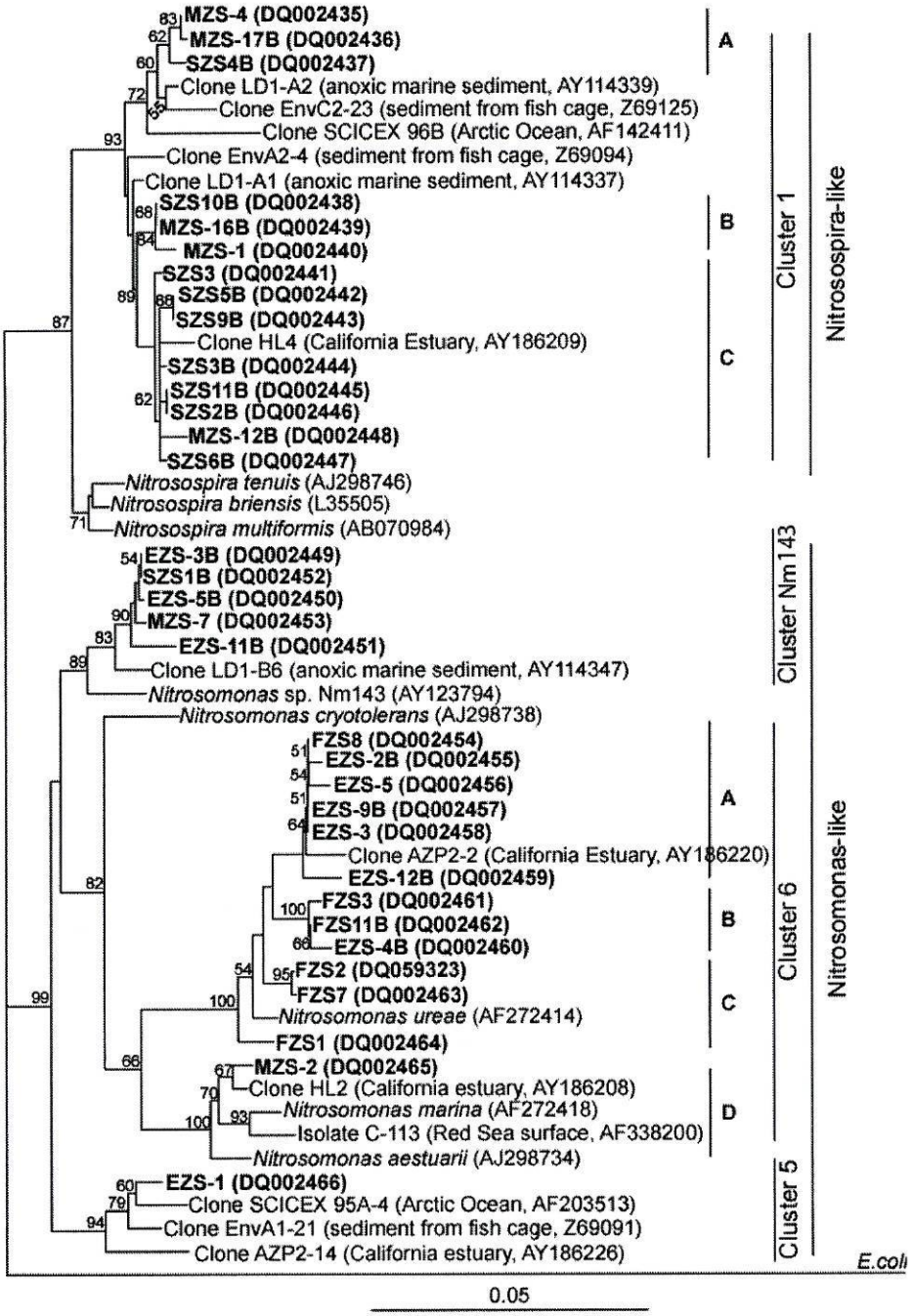

Figure 5.4: Neighbor-joining tree showing phylogenetic relationships between 16S rRNA gene sequences (≈800 bp) retrieved from SZ, MZ, EZ and FZ spring samples and AOB sequences from the database. Bootstrap values (100 iterations) greater than 50% are shown.

observed (Table 5.2). Only one sequence (MZS-2) fell into sub-cluster D and was 99.3% similar to the sequence from clone HL2 obtained from Elkhorn Slough estuary in California (Caffrey et al. 2003), 98.1% similar to C-113 clone from Red Sea surface (Voytek 1996) and 98.5% and 98.7% similar to the sequence *Nitrosomonas marina* and *Nitrosomonas aestuarii*, respectively. The sequence from DGGE Band MZ1 was 95.1% similar to the sequence from clone MZS-2 (Table 5.3 and Fig. 5.2).

Only one clone (EZS-1) contained a sequence that felt into cluster 5 (Fig. 5.4), composed of novel *Nitrosomonas*-like partial sequences originating from environments usually characterized by high nutrient concentrations. The sequence of clone EZS-1 was 98- 99% similar to sequences retrieved from highly polluted sediment beneath a salmon cage (Stephen et al. 1996), from nutrient rich Arctic Ocean water samples (Bano and Hollibaugh 2000), and from estuarine sediment (Caffrey et al. 2003) receiving nutrient runoff from agricultural fields. This clone is 99.3% similar to the DGGE Band MZ2. This DGGE band was observed in winter at the MZ site, the only time that nitrification was detectable at this site (Table 5.2 and Fig. 5.2). Finally the phylogenetic relationships established here showed that while a clear differentiation of AOB assemblages were observed in two different intertidal environments (intertidal sediments and rocks) of the same estuary, all sequences of AOB were grouped in already described *Nitrosospira* and *Nitrosomonas* clusters frequently observed in a wide variability of environments, confirming a broad biogeographic dispersion of the Douro River Estuary ammonium oxidizing bacteria.

5.3.4 Controls on AOB Assemblage Composition

The primary purpose of this study was to investigate relationships between environmental variables; N cycle processes, namely nitrification; and the distribution of AOB populations. Dendograms of samples based on the similarity of environmental characteristics (Fig. 5.1) and similarity of AOB assemblage composition (Fig. 5.2) had similar topologies. Samples included in cluster *a* of the dendrogram based on environmental characteristics (Fig. 5.1) correspond to samples included in cluster *a* of the AOB assemblage (Fig. 5.2). These clusters contained all of the samples from rocky biofilm sites, and are characterized by higher NH_4^+ and NO_2^- concentrations, %OM and chl *a* contents, and by an ammonium oxidizer assemblage containing only *Nitrosomonas*-like sequences. While, all of the sediment samples felt into cluster *b* of the environmental characteristics (characterized by lower

values for the environmental variables, Table 5.1 and Fig. 5.1), they were found in clusters *b* and *c* of the dendrogram based on DGGE banding patterns (Fig. 5.2), and the majority of sequences retrieved from these samples were *Nitrosospira*-like (Fig. 5.4). These results suggest that the differences in environmental conditions between intertidal rocky (EZ and FZ) and sediment (MZ and SZ) sites may affect the selection of particular populations of ammonium-oxidizing bacteria.

Magalhães et al. (2005b*), found that nitrification rates in EZ samples increased progressively with increasing NH_4^+ concentration; however, nitrification rates were inhibited at high NH_4^+ concentration (maximum tested 202 μM) in SZ samples. No information on the composition of AOB assemblages at these sites was available when these experiments were performed; however, the results presented here reveal that the AOB assemblage at site EZ was composed primarily of *Nitrosomonas*-like species, which were shown to be more tolerant of high NH_4^+ concentrations (Magalhães et al. 2005b). The AOB assemblage at the SZ site was composed mainly of *Nitrosospira*-like species and samples from this site displayed a lower tolerance to high NH_4^+ concentrations (Magalhães et al. 2005b). These results suggests a connection between the phylogenetic composition of the AOB assemblage and the physiological ability to tolerate high ammonium concentrations. Also, Stephen et al. (1996, 1998) and McCaig et al. (1999) found that most AOB sequences retrieved from marine sediment enriched with NH_4^+ fell into *Nitrosomonas* spp. clades, while *Nitrosospira* spp. sequences dominated samples from natural environments, suggesting that NH_4^+ enrichment favored the growth of *Nitrosomonas* spp. while low concentrations of NH_4^+ favored the growth of *Nitrosospira* spp. The occurrence of *Nitrosomonas*-like sequences in environments with higher substrate concentrations was also suggested in other previous investigations (Philips et al. 1999, Bano and Hollibaugh 2000).

In agreement with UPGMA cluster analysis (Fig. 5.2), non-metric multidimensional scaling analysis (MDS) of AOB assemblages identified differences in the distribution of AOB assemblages among different environments (MDS stress coefficient 0.09, Fig. 5.5a). Rocky biofilm AOB assemblages were clearly separated from sediment assemblages (Fig. 5.5a, group *a*). The sediment samples fell into two groups: one that included mainly sandy sediment (Fig. 5.5a, group *b*) and a second containing muddy sediments collected during the fall and winter (Fig. 5.5a, group *c*). ANOSIM analysis showed that ordination of the three groups in MDS plot differs significantly ($R = 0.95$, p ¡ 0.01).

*The contents of this article can be found in Chapter 4.

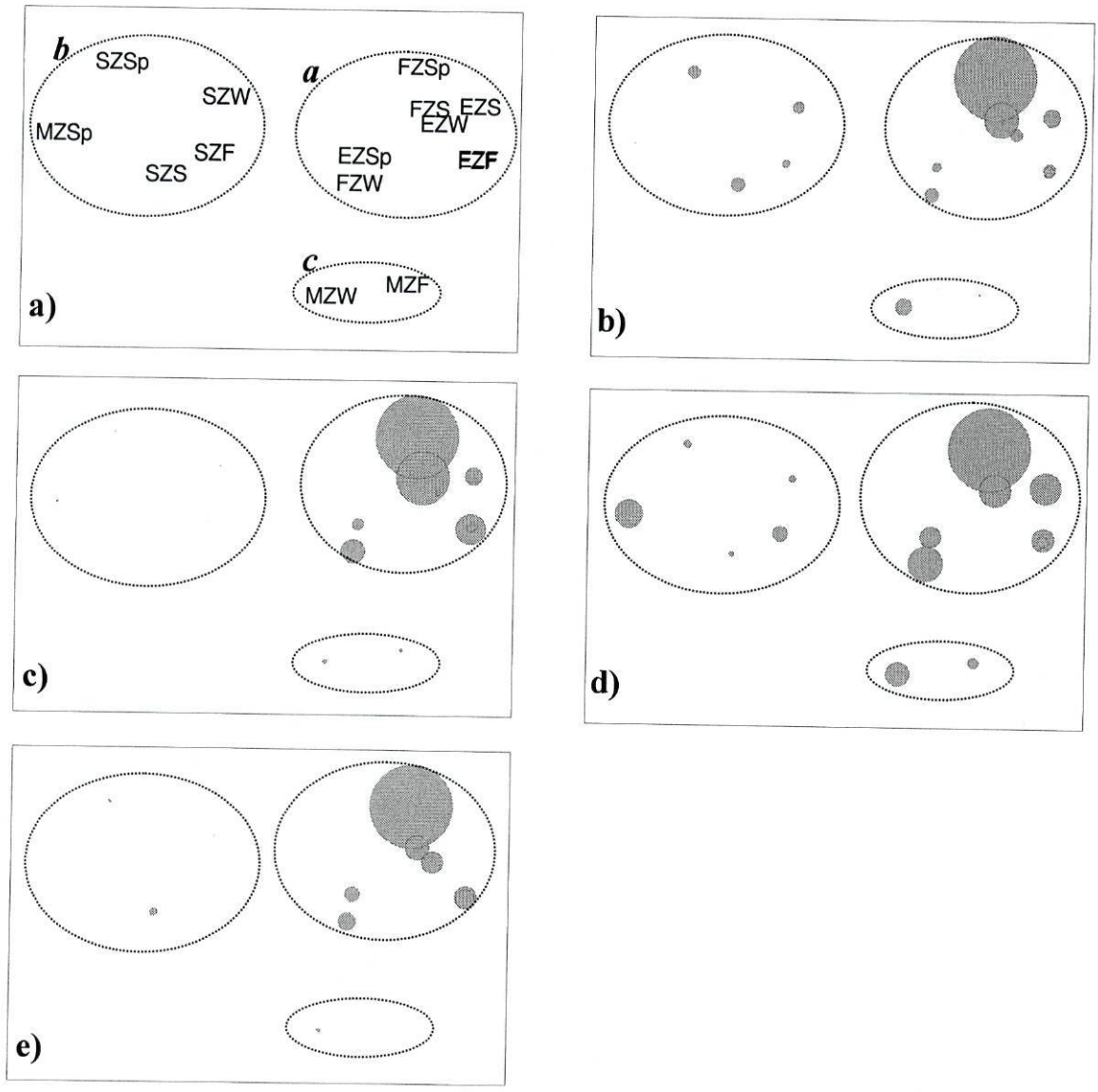

Figure 5.5: a) Non-metric multidimensional scaling (MDS) ordination of the sites calculated from the presence/absence of the DGGE Bands. Stress value = 0.09 for all panels. Large ovals indicate discrete groups of samples discussed in the text. b through e): values of environmental variables for each sample represented as circles of diameter scaled linearly to the magnitude of the value. b) NO_3^- uptake rates; c) NO_2^- fluxes, the absolute value of SZ summer was added to all of the NO_2^- flux values; d) NH_4^+ fluxes, the absolute value of EZ winter was added to all of the NH_4^+ flux values; e) nitrification rates.

Relationships between AOB assemblage composition and N cycling processes were established by plotting net process rates on MDS ordination diagrams (Fig. 5.5b-e). This analysis showed that higher net fluxes of NO_2^- were observed in samples that fell into MDS group *a* (ANOVA, p ¡ 0.05), containing all of the rocky biofilm samples (Fig. 5.5a, c). In contrast, much lower flux rates of these processes were observed for the samples that fell into MDS groups *b* and *c*, that included all intertidal sediment samples (Fig. 5.5a, c). While there were no significant differences between NO_3^- and NH_4^+ fluxes between rocky biofilm and sandy sediment samples (ANOVA, $p > 0.05$), the highest rates of these processes were always observed in rocky biofilm samples (Fig. 5.5b, d). Finally, our analysis revealed that, with few exceptions, high nitrification rates were always observed in the samples that fell into MDS group *a* (ANOVA, p ¡ 0.05) (Fig. 5.5e), with an AOB assemblage composed of *Nitrosomonas*-like species. Lower nitrification rates were measured in samples that fell into MDS groups *b* and *c* that were dominated by *Nitrosospira*-like sequences (Fig. 5.5e).

Taken together, these results indicate that different environmental characteristics of intertidal rocks and sediments might define the composition of different AOB assemblages and resulted in differences in the biogeochemical processes evaluated. In agreement with this study, Cébron et al. (2003) found replacement of *Nitrosospira*-like by *Nitrosomonas oligotropha* and AOB related to *N. ureae* in a site with high NH_4^+ concentrations, corresponded to the highest nitrifying bacterial biomass and higher nitrification rates. However, in a recent investigation (O'Mullan and Ward 2005), while spatial variation in the distributions of different AOB were observed, nitrification rates and/or environmental variables were not related to AOB abundance or assemblage composition. Finally, Caffrey et al. (2003) did not detect any spatial variation in the distribution of sediment AOB among different environments of the Elkhorn Slough estuary.

Chapter 6

Nitrous Oxide Reductase Genes (*nosZ*) Retrieved from Intertidal Rocky Biofilm and Sediment Samples of the Douro River Estuary (Portugal), and Relation to Denitrification Rates

6.1 Introduction

Denitrification involves a stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) and the production of the gaseous products; nitric oxide (NO), nitrous oxide (N_2O) and mainly dinitrogen (N_2), under suboxic conditions. This process directly impacts nitrogen availability and ultimately net primary production in many coastal ecosystems, because it contributes to the loss of fixed nitrogen (Seitzinger 2000). Denitrifying bacteria produce

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The contents of this chapter can also be found in Magalhães C. M., N. Bano, W. J. Wiebe, A. A. Bordalo and J. T. Hollibaugh, Nitrous oxide reductase (*nosZ*) genes retrieved from intertidal rocky biofilm and sediment samples of the Douro River estuary (Portugal), and relation to denitrification rates, Submitted to App. and Environ. Microbiol..

nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase that allow them to use NO_3^-, NO_2^-, NO, and N_2O, respectively, as terminal electron acceptors. Nitrous oxide reduction, the final step of denitrification, is catalyzed by nitrous oxide reductase, and in the environment this enzyme most often does not reduce all of the available N_2O to N_2, resulting in the release of N_2O to the atmosphere in different proportions according to environmental conditions (Seitzinger 1988, Corredor et al. 1999, Magalhães et al. 2005a*). Incomplete reduction of N_2O to N_2 assumes particular environmental importance, since the atmospheric N_2O accumulation contributes to global warming and to the destruction of stratospheric ozone (Devol 1991).

Given the importance of denitrification, there have been an enormous number of studies that characterizes denitrification rates in a wide variety of environments. Investigations have mainly focused on spatial and seasonal variation of denitrification rates (e.g. Seitzinger 1988, Cabrita and Brotas 2000, Magalhães et al. 2005a), regulating factors (e.g. Cornwell et al. 1999, Magalhães et al. 2005b†) and the large-scale environmental importance of this process (e.g. Seitzinger 2000). Recent investigations have begun to characterize the composition and diversity of natural denitrifier communities (Braker et al. 2000, Scala and Kerkhof 2000, Primé et al. 2002, Rösch et al. 2002, Liu et al. 2003, Stres et al. 2004). However, only a few attempts have been made to combine studies of community diversity with denitrifier activity (Rich et al. 2003).

Most of the characterized denitrifiers belong to the Proteobacteria (Shapleigh 2003). However, capacity for denitrification occurs in nearly 130 species of Bacteria and Archaea in more than 50 genera (Table 1.1). Denitrifiers are found in all major physiological groups except the Enterobacteriaceae (Shapleigh 2003). Functional marker genes encoding key enzymes of characteristic metabolic pathways (e.g. denitrification) have been found to be useful in the detection of microorganisms belonging to different ecophysiological groups in complex microbial assemblages. The advantage of this approach is that it gives information about the function of unknown microorganisms. Recent investigations showing that functional genes encoding nitrous oxide reductase (Scala and Kerkhof 2000, Rich et al. 2003, Stres et al. 2004) and nitrite reductase (Braker et al. 2000, Priemé et al. 2002) are useful molecular markers for investigating the diversity of denitrifying bacteria in different natural environments. However, while the gene encoding nitrous oxide reductase (*nosZ*)

*The contents of this article can be found in Chapter 3.

†The contents of this article can be found in Chapter 4.

is largely unique to denitrifying bacteria, the gene that encodes nitrite reductase (*nirK*) has been found in several isolates of β-subdivision ammonium-oxidizing bacteria (Casciotti and Ward 2001).

In the Douro River estuary, Magalhães et al. (2005a) revealed significant spatial heterogeneity in denitrification rates. Intertidal rocky biofilms generally had higher N_2 efflux rates than intertidal sediment sites, and the N_2O release rates in rocky biofilms sites were 10 times higher than in intertidal sediments (Magalhães et al. 2005a). In this study *nosZ* gene variability was evaluated in two intertidal rocky biofilms and two intertidal sediment sites of the Douro River estuary. In addition, intra-site variation of *nosZ* gene occurrence was investigated monthly in intertidal sandy sediments during a period of one year in order to document variability of the denitrifier assemblage in a single location. These results were combined with N biogeochemistry processes, and environmental variables to examine possible links between denitrifier community dynamics with some key N processes and environmental variables.

Inter- and intra-site genetic heterogeneity of the *nosZ* gene was evaluated by sequencing cloned *nosZ* gene fragments and using terminal restriction fragment length polymorphism analysis (T-RFLP) to survey samples. This molecular approach has been shown to be useful for assessing the complexity of bacterial community structure and for community comparison between different samples (e.g. Scala et al. 2000, Rich et al. 2003).

6.2 Methods

6.2.1 Site Description

Physical and chemical characteristics of the Douro River estuary have been presented previously (Magalhães et al. 2002, Magalhães et al. 2003,*). This study was conducted in the four dominant intertidal environments of the lower estuary (Fig. 3.1): two intertidal rocky areas colonized predominantly by *Enteromorpha* spp. and *Fucus* spp. (EZ and FZ respectively), and two intertidal sediments (sandy and muddy areas; SZ and MZ respectively).

*The contents of this article can be found in Chapter 2.

6.2.2 Sampling

Between 2001 and 2002, a sampling program for denitrification rates and inorganic nitrogen flux measurements was performed quarterly at EZ, FZ, SZ and MZ sites (for details see Magalhães et al. 2005a*). Monthly surveys were conducted just at the sandy sediment site (SZ) between February 2002 to January 2003, where potential denitrification rates and N_2O production rates were evaluated as described in Magalhães et al. (2005b†). During all sampling programs, sediments and/or rocky biofilms were collected for *nosZ* gene analysis. Two sediment cores (3 cm diameter and 10 cm depth) were collected at mudflat and sandflat stations; at the rocky stations, biofilms were sampled by scraping the rocks (approximately 80 g). All sediment and rocky biofilms samples were homogenized, stored in sterilized plastic bags, transported in the dark to the laboratory in refrigerated ice chests and immediately stored at -70 °C for DNA extraction.

6.2.3 DNA Extraction and PCR Amplification

Community DNA was extracted from 1 to 1.5 g wet weight sediment and rocky biofilm using the Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories Inc., Calif.). Fragments of *nosZ* genes were amplified with the primer set Nos661F (5'-CGGCTGGGGGCTGACCAA), fluorescently labeled at the 5' end with 6-carboxyfluorescein [6-FAM]), and Nos1773R (5'-ATRTCGATCARCTGBTCGTT) which amplify ≈1100bp of the *nosZ* gene (Scala and Kerkhof 1998). Primers used in PCR were synthesized by Operon Technologies (Oakland, Calif.). Amplification was performed using Ready-to-Go PCR beads (Amersham, Biosciences) in reactions containing 20 ng of template DNA and 2.4 μM of each primer in a final volume of 25 μl. All PCRs were performed in a DNA Engine thermocycler (MJ Research) with 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 with a final extension at 72 °C for 10 min. DNA from *Pseudomonas stutzeri* was use as a positive control. PCR products were separated by agarose gel electrophoresis; the gels were stained with ethidium bromide and a band of the correct size (≈1100bp) was extracted and purified with the QIAquick gel extraction kit (Qiagen). The concentration of the resulting PCR purified product was estimated by the Hoechst dye assay (Paul and Myers 1982).

*The contents of this article can be found in Chapter 3.

†The contents of this article can be found in Chapter 4.

6.2.4 T-RFLP Analysis

Approximately 300 ng of purified PCR product from environmental samples and 50 ng of plasmid DNA from the clone libraries were digested for 6 h at 37 °C in a final volume of 10 µl with 10 U of HinP1 restriction enzyme (5′...G˘CGC...3′) (New England Biolabs). The digested DNA was precipitated in 0.1 volume of 3M sodium acetate and 2 volumes of ice-cold ethanol followed by centrifugation at 12000× g for 15 min. The DNA pellet was dried in a DNA SpeedVac (Savant). Immediately prior to analysis, the pellet was resuspended in a mixture of 12 µl of deinonized formamide and 1 µl of GeneScan 2500 TAMRA size internal standard (Applied Biosystems) then denatured for 5 min at 95 °C and immediately placed on ice for at least 3 min.

Restriction fragments were separated and detected using an ABI Prism 310 genetic analyzer in Gene-Scan mode, which displays for each sample the various T-RFLP's as a series of peaks. Each fragment was expressed as a percentage of the total peak area for all peaks in each T-RFLP profile, giving the relative abundance of each terminal restriction fragment in the PCR product of each sample. Fragments with a signal below 1.5% of the sum of all peaks were removed from the analysis (Rich et al. 2003).

6.2.5 Clone Libraries and Phylogenetic Analysis

In order to identify the major T-RFLP peaks, *nosZ* gene fragments generated from the PCR of a total of 9 environmental samples were cloned; EZ summer, EZ fall, EZ winter, EZ spring, FZ spring, MZ spring, SZ winter and SZ spring from 2000/2001 sampling and SZ March from 2002/2003 monthly sampling. The PCR product obtained was gel purified as described above. The amplicons were cloned using the TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions, with the exception of slight modifications (vector DNA and salt solution was decreased to 0.5 µl and chemically competent cells decreased to 23 µl). A total of 54 randomly selected clones were sequenced on an automated sequencer in the Molecular Genetics Instrumentation Facility (MGIF) of UGA, screened using HinP1 restriction enzyme, and the fragment length detected as described above. Nucleotide sequences were aligned and converted to inferred amino acid sequences using Genetic Computer Group (GCG) package (Madison, WI). Sequences (nucleotide and amino acid) were compared to known sequences using BLAST (Altschul et al. 1990). The inferred

amino acid tree was constructed with PHYLIP, using Kimura distances and the neighbor-joining method with a bootstrap analysis of 100 replicates (MEGA package version 2). One chimera was detected with the Ribosomal Database Project CHECK-CHIMERA program. The sequences are going to be submitted to the GenBank.

6.2.6 Statistical Analysis

Non-metric multi-dimensional scaling analysis (MDS) was used to detect inter-site differences in denitrifiers assemblage sampled during 2000/2001. Data on relative T-RFLP peaks area of the *nosZ* genes (from T-RFLP) was 4th root transformed and Bray-Curtis similarities calculated. The ANOSIM test (Clarke and Warwick 1994) was used to examine the statistical differences between the MDS groups. Hierarchical clustering and principal components analysis (PCA) was also applied to the environmental variables measured during the 2002/2003 sampling program. The software package PRIMER version 5 (Clarke and Warwick 1994) was used to perform the latter multivariate statistic analysis.

For the monthly sampling (2002/2003), relationships between *nosZ* gene assemblages, environmental variables and N processes were analyzed with canonical correspondence analysis (CCA, ter Braak 1986), using the software package CANOCO (version 4.5, Microcomputer Power, Ithaca, NY) (ter Braak and Smilaeur 2002). For CCA the relative T-RFLP peaks area for each sample was log transformed, and the environmental variables were entered into the CCA in their standardized form (i.e. adjusted for a mean of 0 and SD of 1). Monte Carlo permutation test was used to assess the statistical significance of relationships. The variables used were salinity, water column $NO_3^- + NO_2^-$ concentration, denitrification rate, N_2O production rate and N_2O:N_2 ratios. In the CCA ordination diagram, the angle and length of the arrow relative to a given axis reveals the extent of correlation between the variable and the canonical axis (environmental gradient). The position of different T-RFLP peaks on an axis reveals their associations with the environmental gradient (i.e. increasing relative abundance of the T-RFLP peak with high or low values for certain environmental variables).

6.3 Results

6.3.1 Inorganic N Fluxes and Denitrification Rates

Results of dark net NO_3^-, NO_2^-, N_2O fluxes, dark denitrification rates and N_2O:N_2 ratios for the 2000/2001 sampling program were presented and discussed in Magalhães et al. (2005a*); these data are compiled in Table 6.1. In the present study *nosZ* assemblages dynamics were related to the N biogeochemistry data from dark incubations because denitrification was found to be an irrelevant process during light incubations (Magalhães et al. 2005a).

Hierarchical cluster analysis (Fig. 6.1a), performed on data from the monthly sandy sediment survey (2002/2003 sampling program), formed three separate clusters based on the environmental and N process variables previously evaluated (Magalhães et al. 2005, see data summarized in Table 6.1). In the 2-dimensional principal components analysis (PCA), PCA1 and PCA2 together explained 96% of the total variability. While PCA 1 was closely related to NO_3^-+NO_2^- concentrations and denitrification rates (this axis explains 59% of the total variability), PCA 2 was more related to the N_2O:N_2 ratio and N_2O production rates. Superimposition of denitrification rates (Fig. 6.1b), NO_3^-+NO_2^- availability (Fig. 6.1c) and net N_2O fluxes (Fig. 6.1d), as circles of different sizes on the PCA plot, show that samples from August, November, December and January were characterized by higher denitrification rates and NO_3^-+NO_2^- concentrations. However samples from February and March were characterized by higher N_2O flux rates and N_2O:N_2 ratios, and May, June, July and October samples by low denitrification rates and NO_3^-+NO_2^- concentrations (Fig. 6.1b-d).

6.3.2 Cloning of *nosZ*

The *nosZ* primer set amplified the expected 1100 bp fragment from SZ, MZ and FZ 2000/2001 samples and from SZ 2002/2003 samples. However the 25 clones sequenced from the clone library of EZ were not identified as a *nosZ* gene (i.e. not identifiable using the GenBank's BLAST search). Sequence analysis, based on comparisons with *nosZ* sequences available in the NCBI database, revealed that 29 of the cloned sequences from MZ, SZ and

*The contents of this article can be found in Chapter 3.

Table 6.1: Salinity and nitrogen geochemistry variables measured during 2000/2001 and 2002/2003 sampling programs (mean±SD of three replicates). Negative values indicate uptake and positive values indicate release. Data from Magalhães et al. (2005a*) and Magalhães et al. (2005b†).

		Samples from 2000 to 2001 sampling program				
		N_2 fluxes	N_2O fluxes	N_2O:N_2	NO_3^- fluxes	NO_2^- fluxes
		μmol m^{-2} h^{-1}	μmol m^{-2} h^{-1}		μmol m^{-2} h^{-1}	μmol m^{-2} h^{-1}
SZ	Summer	9.1±2.1	1.28±0.25	17.1±4.5	-263.6±170.0	-3.5±1.8
	Fall	8.0±2.2	Null flux	0.0±0.0	-145.2±12.7	Null flux
	Winter	13.2±3.2	Null flux	0.0±0.0	-209.8±152.6	Null flux
	Spring	24.0±5.4	0.44±0.32	1.8±1.2	-237.0±106.7	1.4±2.9
MZ	Fall	9.1±3.3	0.37±0.64	4.2±7.3	-25.5±44.1	9.5±5.8
	Winter	31.6±2.2	0.49±0.18	1.6±0.9	-303.8±94.4	15.2±7.5
	Spring	10.3±3.3	0.15±0.05	1.6±0.9	-11.6±33.9	5.3±1.6
EZ	Summer	26.0±9.4	7.25±2.29	40.5±15.1	-317.9±75.4	69.2±46.3
	Fall	37.1±16.2	4.05±1.43	13.2±5.5	-239.2±118.0	32.1±16.6
	Winter	21.1±4.6	1.06±0.62	5.4±3.2	-237.1±99.4	19.6±12.8
	Spring	42.3±18.0	1.56±0.71	3.8±0.7	-170.2±108.2	43.6±26.6
FZ	Summer	134.3±23.0	16.87±5.81	15.0±6.1	-641.1±10.1	213.9±105.8
	Fall	34.0±10.3	5.28±4.18	11.1±11.3	-201.5±86.8	119.3±71.8
	Winter	47.9±9.5	3.27±1.55	7.1±2.5	-252.1±161.4	94.2±23.3
	Spring	359.6±29.8	13.01±8.65	4.9±1.3	-1528.6±434.3	335.0±181.1

		Samples from 2002 to 2003 sampling program				
		N_2 fluxes	N_2O fluxes	N_2O:N_2	NO_3^-+NO_2^-	Salinity
		(nmol $weight^{-1}h^{-1}$)	(nmol $weight^{-1}h^{-1}$)		(μM)	psu
SZ	Feb 2002	2.81±0.21	0.19±0.02	6.03±0.27	34.5±1.1	22.7
	Mar 2002	2.62±0.05	0.20±0.06	7.02±0.12	31.9±0.9	15.1
	Apr 2002	3.09±0.31	0.03±0.03	1.11±0.10	39.6±0.3	16.0
	May 2002	3.07±0.05	1.08±0.50	0.66±0.03	21.5±0.1	24.0
	Jun 2002	0.96±0.02	0.01±0.00	0.65±0.03	5.2±0.3	26.8
	July 2002	0.74±0.04	0.00±0.00	0.65±0.03	12.8±0.2	25.0
	Aug 2002	6.17±0.47	0.08±0.04	0.14±0.03	65.0±0.6	13.9
	Sep 2002	3.05±0.18	0.02±0.02	0.78±0.06	36.0±0.5	13.2
	Oct 2002	0.85±0.04	0.01±0.01	0.78±0.06	20.6±1.1	22.7
	Nov 2002	4.41±0.33	0.01±0.01	0.30±0.00	59.1±1.8	9.0
	Dec 2002	8.36±0.57	0.02±0.02	0.24±0.03	59.9±1.0	3.4
	Jan 2003	8.75±0.39	0.13±0.13	1.48±0.07	92.0±2.8	1.5

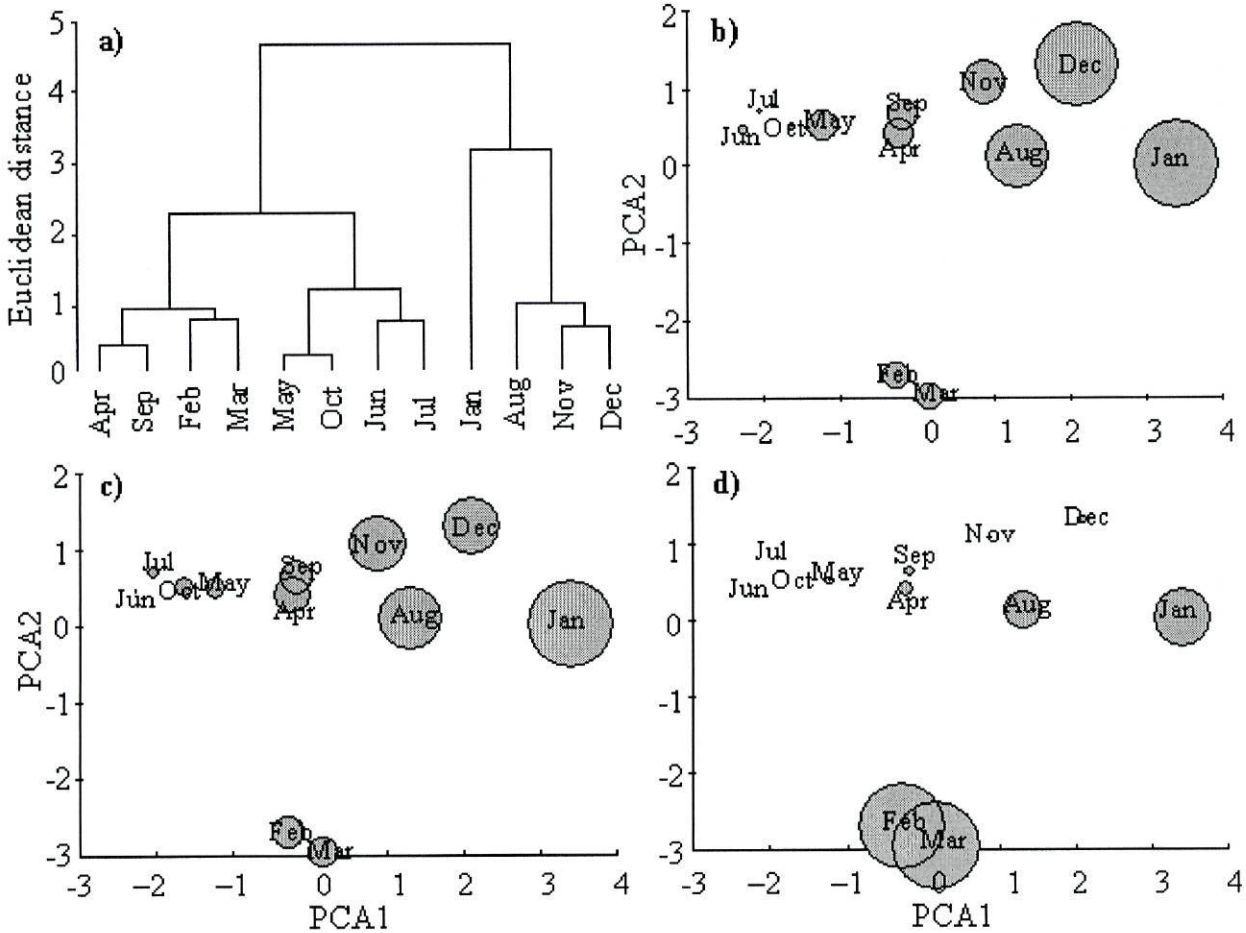

Figure 6.1: Hierarchical cluster analysis (a) and principal components analysis (PCA) from N biogeochemistry and environmental variables measured monthly (Table 6.1). Values of denitrification rates (b) $NO_3^- + NO_2^-$ (c) and net N_2O fluxes (d) for each sample are represented as circles of diameter proportional to the magnitude of the value

FZ sites were homologous to known *nosZ* sequences and that these sequences fell into 7 distinct clusters of *nosZ* genes (cluster A to cluster G, Table 6.2). Accordingly, among the 29 clones, just 7 *nosZ* genotypes were identified on the basis of the HinPI T-RFLP profiles of each clone; differences in T-RFLP peak sizes of the different *nosZ* sequences cloned are shown in Table 6.2. Each cluster includes remarkably similar sequences, between 99.5 to 100% identity. In this study amino acid sequence divergences less than 0.5% were considered errors and thus do not indicating a novel *nosZ* gene. Thus we considered that each sequence cluster (Table 6.2) represents a different bacterial phylotype.

Table 6.2: Correspondence between sequences from *nosZ* clone libraries and terminal restriction fragment lengh (T-RFLP).

Cluster identifier	Clone identifier	T-RFL (bp)
A	SP1, SP4	43
B	A22 , A24, A27	28
C	1FP8, FP3, FP6	200
D	AP9	245
E	AI1, AP1, AP2, AI10 , AI7, AP4, SP3	148
F	FP5, FP2, FP7, FP9	125
G	SP7, SP8, SP5, SP2, SP10, AP8, FP8, 1FP3, SP6	190

6.3.3 Phylogenetic Analysis

The phylogenetic tree was constructed with 341 deduced amino acids from one representative sequence of each cluster (99.5-100% similar, Fig. 6.2). The similarities of different *nosZ* gene clusters (A to G) ranged between 55% to 84%. The phylogram contains sequences available in the data banks and the data obtained from 12 cultured bacteria. None of the Douro River estuary sequences was completely identical with any known sequence. However, phylogenetic analysis revealed that almost all of our *nosZ* gene sequences were affiliated with α-Proteobacteria species. The exceptions were two 99.9% similar DNA sequence (100% similar protein sequence) from the MZ site (cluster A) that fell into γ-Protebacteria group (Fig. 6.2).

6.3.4 Inter and Intra-Site Variation of *nosZ* T-RFLP Profiles

T-RFLP is PCR based, and because of potential PCR bias and largely unknown copy numbers in microbial genomes the quantitative interpretation of fingerprinting data requires caution. However, Lueders and Friedrich (2003) evaluated PCR amplification bias by T-RFLP in pure cultures and soil samples and demonstrated that the T-RFLP analysis evaluated could give a quantitative view of the template pool. The relative peak area of the restriction fragments in T-RFLP profiles from each sampling is graphically represented in Fig. 6.3. All T-RFLP profiles from all sites contained one to three dominant fragments

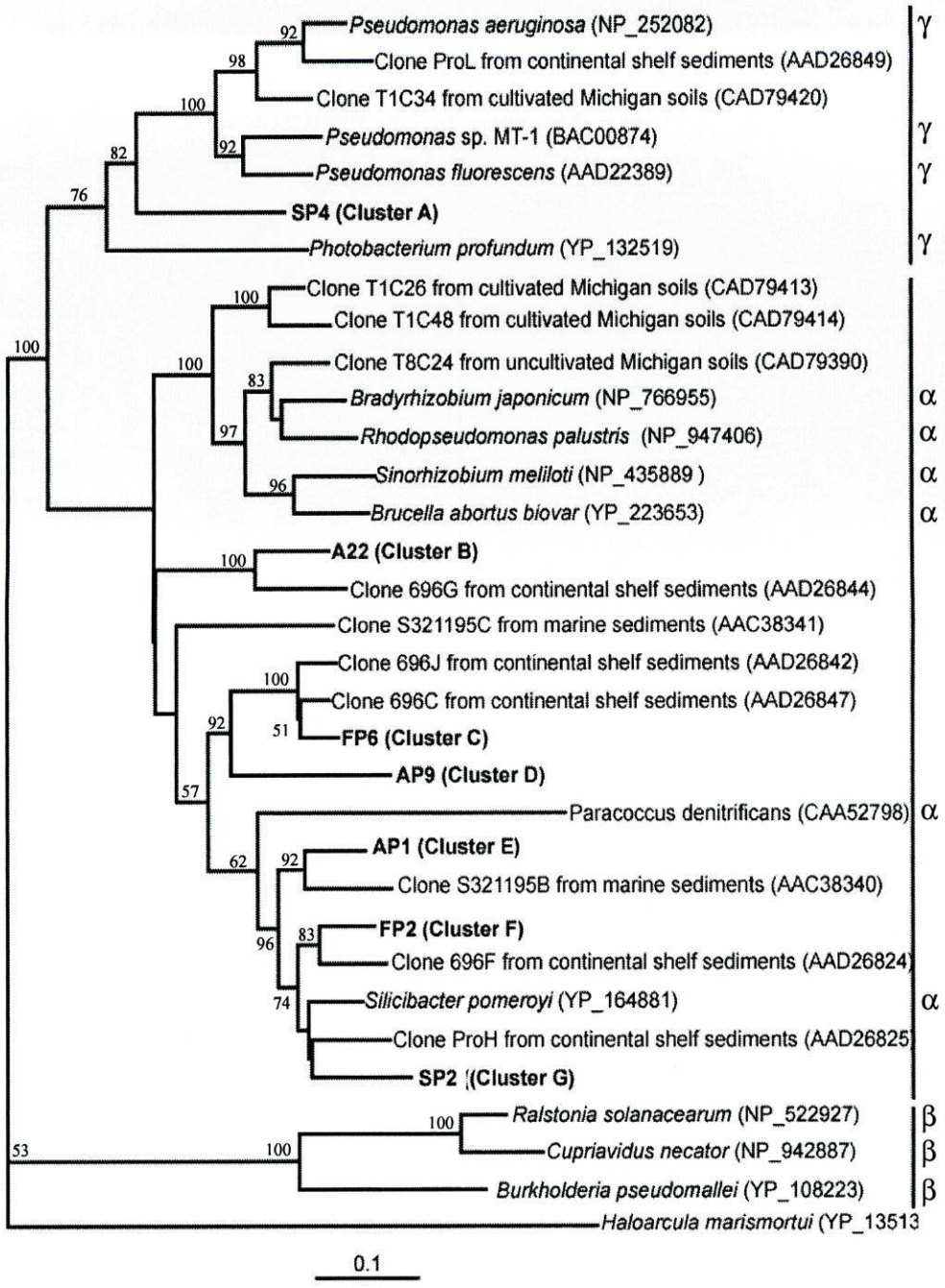

Figure 6.2: Dendogram showing similarities between *nosZ* gene fragments retrieved from Douro estuary and sequences from the database. The tree is based on sequences of 341 deduced amino acids and was constructed based on Kimura distances and the neighbor-joining method. Bootstrap values (100 iterations) greater than 50% are shown

and generally had low richness (Fig. 6.3). Samples from the SZ site had always more fragments in the T-RFLP profiles, (between 6 to 8, Fig. 6.3) than the MZ and FZ sites, which contained 3 and 1-5 restriction fragments in each sample, respectively. The 29 clone sequences allowed us to assign sequences to the 7 T-RFLP peaks of the environmental samples. These sequences represented 54% of the total variability of the T-RFLP peaks found. T-RFLP analysis of the *nosZ* clones gave terminal restriction fragments lengths between 28 and 245 bp long (Table 6.2). Profiles of SZ samples, where greater variation in *nosZ* gene sequence was noted, contained 4 peaks (18bp, 60bp, 75bp and 97bp) that did not match any of the restriction fragments from cloned sequences. Similarly, FZ samples contained two fragments (54 bp and 180 bp) not represented in the cloned sequences. Nevertheless, all of the T-RFLP fragments from the MZ site were identified by the cloning analysis performed. Relative abundances of different phylotypes from clone libraries agreed with relative occurrence of TRFL-P fragments in profiles from environmental sample; the most common *nosZ* sequences in the clone library (clusters G and E with 31% and 24% frequency, respectively) corresponded to TRFL-P peaks that were detected in the greatest number of samples (190 bp and 148 bp, Table 6.2). The restriction fragment 190 bp, corresponding to *nosZ* sequences included in cluster G, were found at all sites and seasons, with the exception of the FZ site during winter (Fig. 6.3a). Clusters F and C and the un-identified restriction fragments 54bp and 180bp were unique to the FZ site and were present in all FZ samples except those collected in winter where only T-RFLP fragment 54bp was observed (Fig. 6.3a). The 148 bp fragment, corresponding to cluster E sequences in clone libraries, was found in all MZ and SZ samples (Fig. 6.3a and b). Sequences included in clusters B and D and the un-identified restriction fragments 18bp, 60bp, 75bp and 97bp were only found in the SZ site, while the restriction fragment corresponding to cloned sequences included in cluster A were observed only in MZ samples (Fig. 6.3a).

The monthly analysis of *nosZ* gene variability conducted in the SZ site, indicated a conservative denitrifier assemblage over the entire year of sampling (2002/2003). The same T-RFLP peaks were observed in all samples, although with different relative peak areas (Fig. 6.3b). As an example of these mothly shifts, January and July T-RFLP profiles are shown in Fig. 6.4.

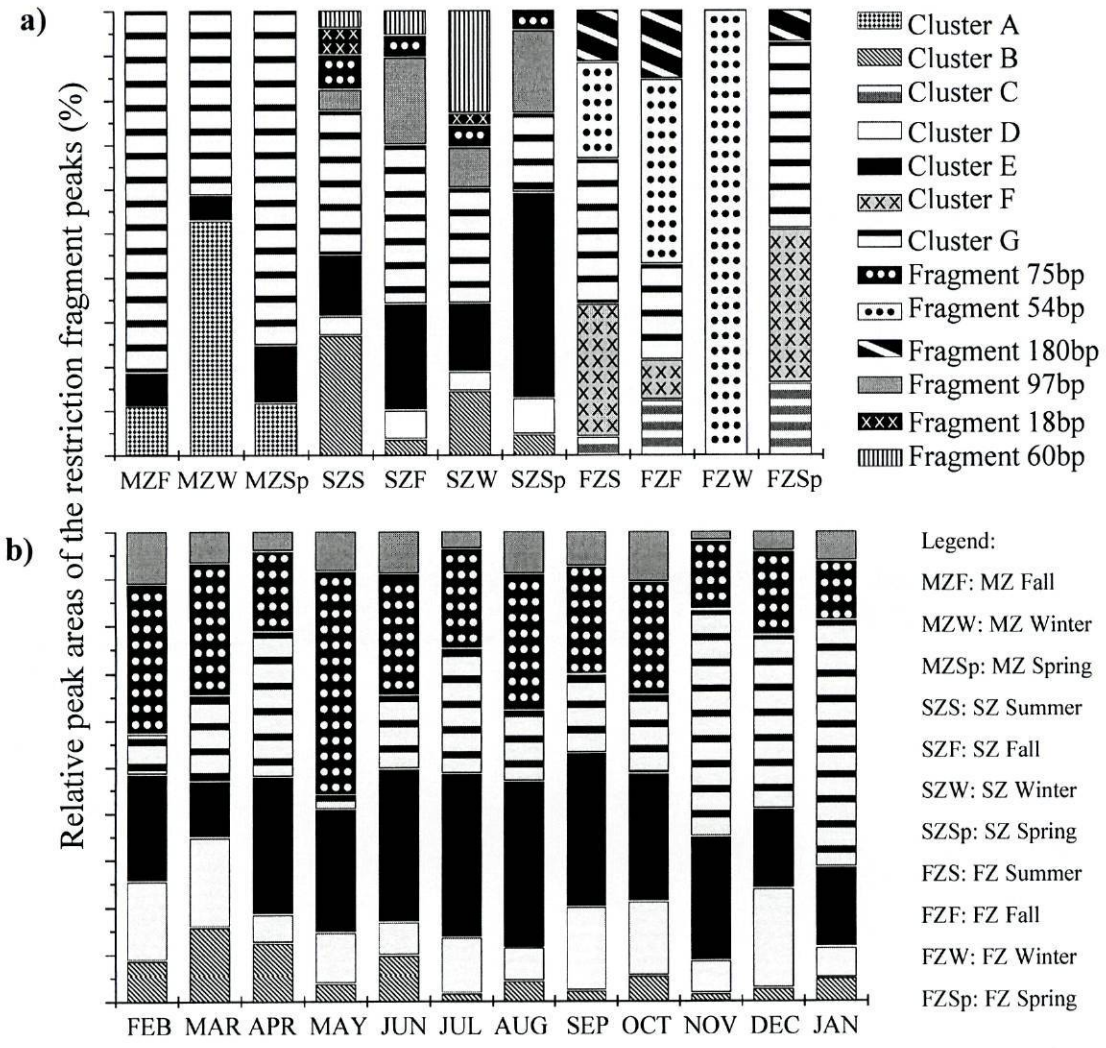

Figure 6.3: Spatial and seasonal variation of the relative peak height of the *nosZ* T-RFLPs. a) - 2000/2001 sampling program, b) - 2002/2003 sampling program.

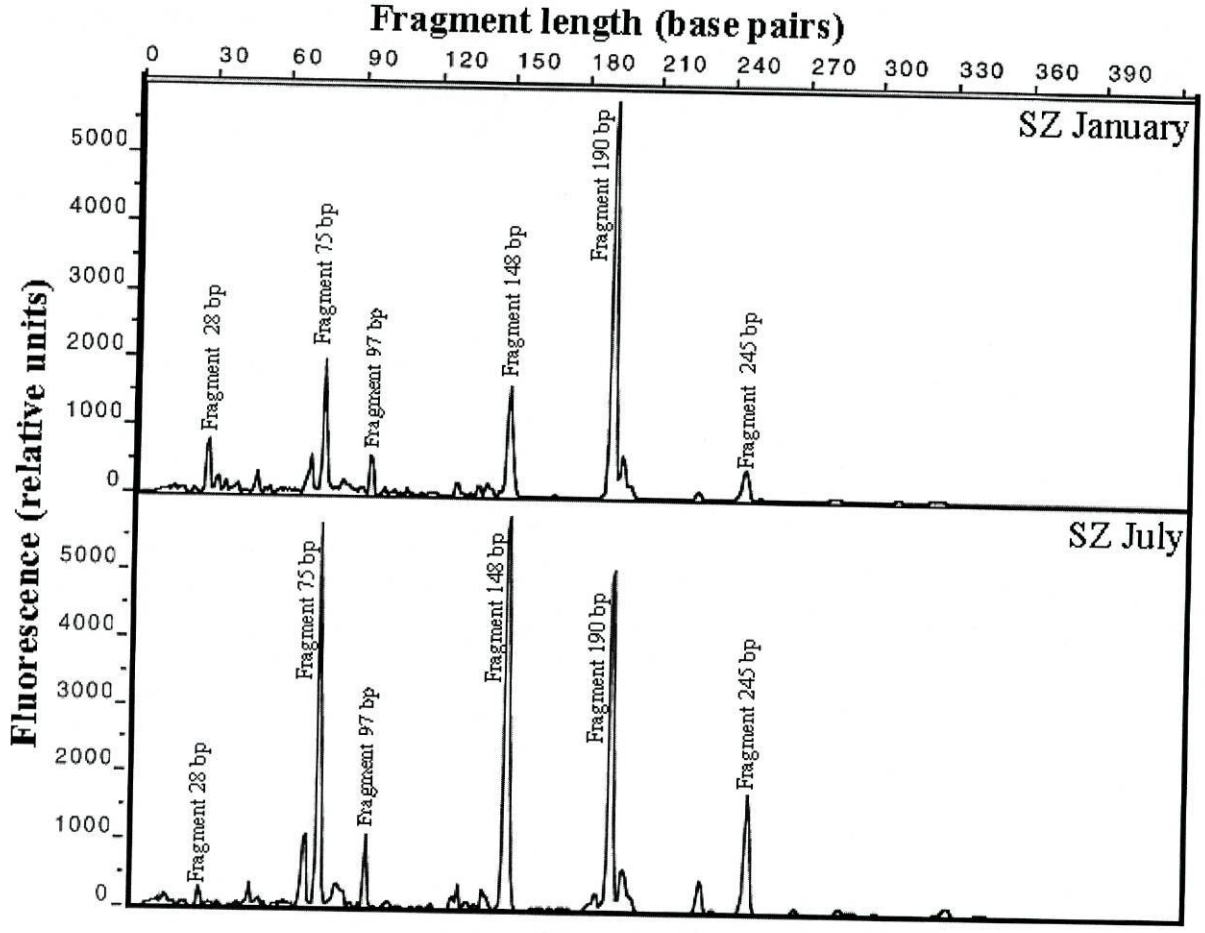

Figure 6.4: Examples of environmental T-RFLP profiles from sandy sediments obtained during monthly sampling.

6.3.5 Inter-Site Variability in *nosZ* Assemblages

The observed differences between the T-RFLP profiles of MZ, SZ and FZ sites from 2000/2001 sampling are a first indication of differences in the denitrifying microbial communities between the three environments. MDS analysis and hierarchical cluster analysis, performed with the relative *nosZ* T-RFLP peaks area in all samples from 2000/2001, showed that T-RFLPs profiles for all samples were grouped by sampling stations (Fig. 6.5a and b). Assemblages from different sampling locations were clearly distinct, and samples from the same sampling location clustered tightly together, with the exception of FZ winter, which contained just one T-RFLP peak (Fig. 6.3a). The ANOSIM test revealed significant statistical differences between these three MDS groups ($n = 11$, $R = 0.76$, p ¡ 0.01). In addition hierarchical cluster analysis revealed that denitrifiers assemblages from intertidal

sediment sites (MZ and SZ) were more similar to each other than to the rocky biofilm samples (FZ). The FZ samples clustered separately at a similarity level 41%, while the MZ and SZ samples were 50% similar (Fig. 6.5b).

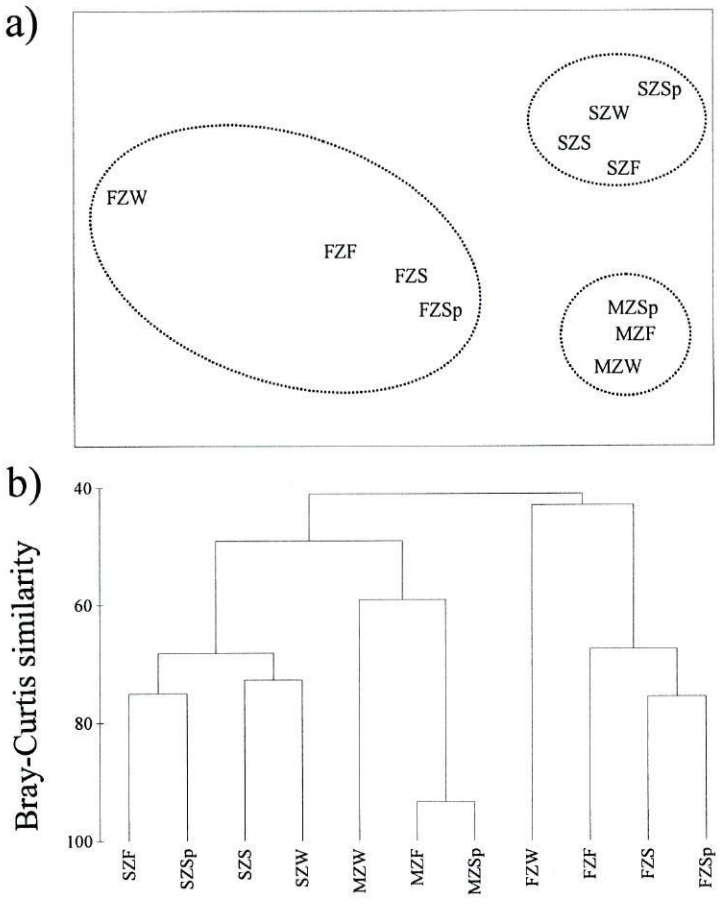

Figure 6.5: Non-metric multidimensional scaling (Stress value = 0.03) ordination of the sites (a), and dendrogram for hierarchical clustering of the three sites using Bray-Curtis similarities calculated on fourth root transformed *nosZ* gene T-RFLP peak height (b).

6.3.6 Intra-Site *nosZ* Variability and Environmental Variables

Correlations between environmental variables, N biogeochemistry and *nosZ* assemblages were identified using canonical correspondence analysis (CCA, Fig. 6.6). All T-RFLP data from the monthly sampling (Fig. 6.3b) were included in the CCA. The first two CCA

axes explained 90.5% of the total cumulative percentage of *nosZ* genotype variance and accounted for 90.2% of the cumulative percent variance of *nosZ* genotype-environment relation. In this analysis, all environmental variables contributed significantly to the explanation of *nosZ* genotype distribution according to Monte Carlo test of F-ratios ($p < 0.05$), and the effect of the combined variables on explaining distribution of the canonical axes was significant as well ($F = 2.628$ and $p < 0.05$, Monte Carlo permutation test). The variables that correlated most strongly with CCA 1 were denitrification rates and N_2O:N_2 ratios, whereas salinity, NO_3^- concentrations and N_2O production rates correlated best with CCA axis 2 (Fig. 6.6). Thus, restriction fragments that appeared closer to high values of CCA 1 occurred with higher relative peak area in samples where denitrification rates were higher and N_2O:N_2 ratios were lower. On the other hand, restriction fragments located closer to high values of CCA 2 occurred with high peak area when high NO_3^-+NO_2^- water column concentration, low salinity and high N_2O efflux rates were observed. Fig. 6.4 clearly shows the relative dominance in January of the restriction fragment corresponding to cluster G (fragment 190 bp), where higher rates of denitrification were observed (Table 6.1), compared to the July sample, where the lowest denitrification rates were registered (Table 6.1). In this sample, several restriction fragments with relatively high peak area were observed and thus a non-dominant restriction fragment was found.

6.4 Discussion

6.4.1 Occurence of *nosZ*

The endonuclease HinPI used in this study produces a large number of terminal restriction fragments of environmental samples allowing a high level of resolution (Scala and Kerkhof 2000). However the T-RFLP analysis indicated low complexity of *nosZ* gene in the denitrifying communities that inhabit MZ, SZ and FZ sites (Fig. 6.3). These results were confirmed by analysis of clone libraries, where all 29 *nosZ* clones could be subdivided into just 7 groups with very similar sequences in each (Table 6.2). The *nosZ* primer set used in this study has yielded useful sequence information in previous studies; Scala and Kerkhof (1999) found a total of 71 distinct T-RFLPs in continental shelf sediment samples, and Rösch et al. (2002) found many *nosZ* phylotypes in their samples, in contrast

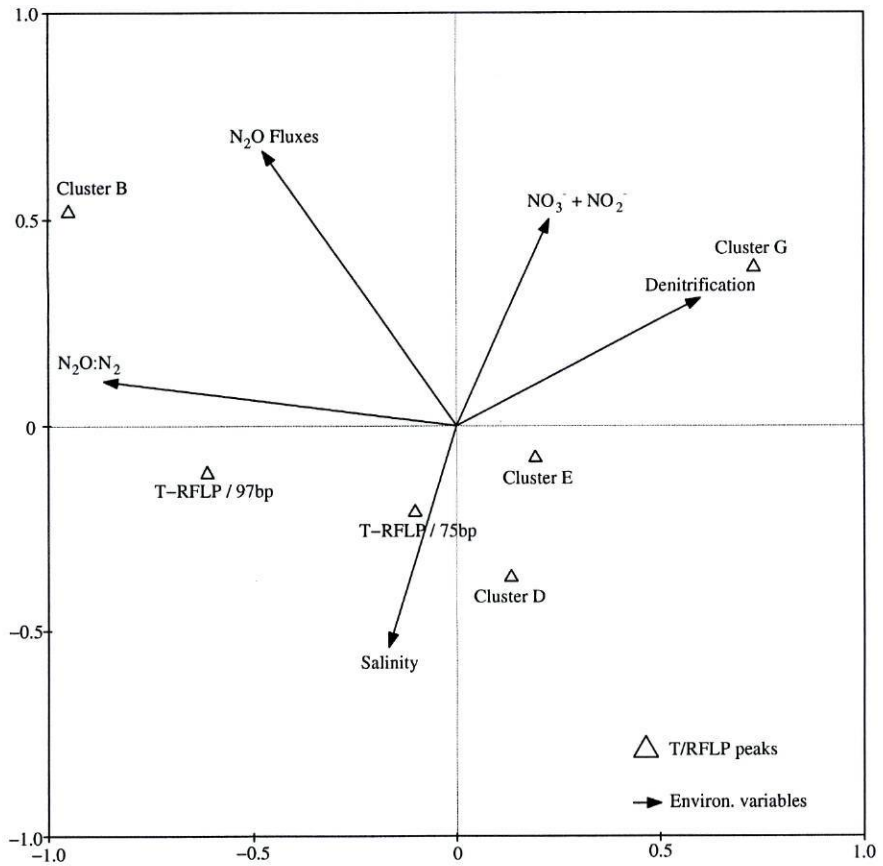

Figure 6.6: CCA ordination plot for the first two canonical correspondence analysis dimensions with the relationships between distribution of the T-RFLP peaks of the monthly sampling and denitrification rates, net N_2O fluxes, $NO_3^- + NO_2^-$ and salinity. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows.

with *nirS* and *nirK* genes, where just a few phylotypes were obtained. However, the discrepancies found between the number of distinct restriction fragments in this study and previous investigations (Scala and Kerkhof 1999, Rösch et al. 2002) suggests that the intertidal area of the Douro estuary has less *nosZ* gene variability than ocean sediments and acid forest soils. Surprisingly, all cloned amplicons from the rocky biofilm site colonized by *Enteromorpha* spp. turned out not to be recognizable *nosZ* genes. The quality of the DNA harvested from this site was suitable for PCR amplification, because we successfully amplified ammonium-oxidizing bacterial 16S rRNA genes from these same samples (Magalhães et al. submitted*). On the other hand, complete denitrification (N_2 production) was measured at the EZ site (Magalhães et al. 2005a† and Magalhães et al. 2005b‡, Table 6.1); thus denitrifiers able to reduce nitrous oxide were present at this site. However, these communities must be divergent from *nosZ* genes of the known denitrifiers, and thus *nosZ* primers used in this study, developed based on cultured denitrifiers and denitrifiers assemblages from Mid-Atlantic marine sediments (Scala and Kerkhof 1998), did not successfully characterize the unknown denitrifier community that inhabited the EZ site. Also these results stressed the idea that nitrous oxide reductase genes from SZ, MZ and FZ may not be fully evaluated, because it is unknown whether the non characterized denitrifiers that inhabit the EZ zone are important at the other sites studied. Schramm et al. (1998) reported a similar dilemma with nitrifying bacteria; they could not detect nitrifying populations in aggregates where intense nitrification was measured. However, when new probes were designed, they found that new *Nitrosospira* and *Nitrospira* strains inhabited these environments. The EZ uncharacterized denitrifiers may be adapted to the microenvironments within the *Enteromorpha* spp. dominated biofilms and thus these denitrifiers could be specific of that site. This specific microenvironmental conditions might also be the reason for the total absence of nitrous oxide reductase gene sequences at the EZ site. Of particular interest Magalhães et al. (2005b) found that, at the EZ site, contrary to what was observed for SZ, MZ and FZ sites, denitrification rates did correlate positively with water column NO_3^- concentration or with net NO_3^- uptake rates. Also in NO_3^- addition experiments, NO_3^- concentration did not control denitrification rates in slurries with scraped biofilms from the EZ site (Magalhães et al. 2005b). Taken together, these results suggest that the metabolism of the denitrifier community that inhabits the EZ site is independent of the NO_3^- availability in the system, which differ from the denitrifiers

*The contents of this article can be found in Chapter 5.

†The contents of this article can be found in Chapter 3.

‡The contents of this article can be found in Chapter 4.

communities that inhabit SZ, MZ and FZ sites. This is in agreement with the great differences in *nosZ* gene that may occur between EZ site and all the other sites (SZ, MZ and FZ). At this stage it is not clear whether EZ denitrifiers are adapted to the specificities of the *Enteromorpha* spp. colonized sites or if they are cosmopolitan microorganisms adapted to a wide range of environmental conditions. However, the unsuccessful amplification of *nosZ* genes at the EZ site clearly shows surprisely differences in denitrifiers assemblages that inhabit two physically similar and adjacent sites (FZ and EZ).

6.4.2 Phylogenetic Analysis

Phylogenetic analysis revealed that *nosZ* sequences of cluster A (all retrieved from the MZ site) were the most divergent (Fig. 6.2) from the other Douro estuary *nosZ* gene sequences, with just between 55 to 61% similarities. However, cluster A sequences were 71-73% related to *nosZ* genotypes from γ-Protebacteria denitrifiers that include *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, and to environmental sequences PROL and T1C34 retrieved from samples of continental shelf sediments and cultivated Michigan soils, respectively (Scala and Kerkhof 1999, Stres et al. 2004) (Fig. 6.2). The *nosZ* clade with α-Proteobacteria-like sequences contains two main sub-clusters; one that includes sequences from *Brucella abortus* and *Bradyrhizobium japonicum* and environmental sequences retrieved from cultivated Michigan soil (Stres et al. 2004) and a second containing sequences from *Silibacter pomeroyi* and *Paracoccus denitrificans* as well as environmental sequences retrieved predominantly from Atlantic and Pacific oceans sediment samples (Scala and Kerkhof 1999). All of the *nosZ* gene sequences of the Douro River estuary were included in the second sub-cluster (Fig. 6.2). Sequences from acid soil from Choobusch Forest (Rösch et al. 2002) were not included in the analysis presented here, because they did not fully overlap the region of the *nosZ* gene covered by the sequences of present study. However, a separate tree was constructed (data not shown), and revealed that they were not closely related to the sequences from the Douro Estuary. *NosZ* genes from this study do not form a separate group from Pacific and Atlantic oceans sediment sequences (Scala and Kerkhof 1999), showing generally greater similarity with some *nosZ* sequences from those regions (Scala and Kerkhof 1999) than with each other. These results suggest that while there is a clear divergence between terrestrial and marine N_2O reductase genes, the marine denitrifiers communities do not have restricted geographical distribution, as was suggested by Scala and Kerkhof (1999).

6.4.3 Inter-Site *nosZ* Variability and Relation to Environmental Variables

Results from this study showed that denitrifying assemblage composition, based on the relative T-RFLP peak areas, differed significantly according to the site (Fig. 6.5a and b). Only a few T-RFLP fragments were found to be common between the different sites, and a number of genotypes were found to be unique to each location, suggesting that environmental and/or geomorphological conditions at each site selects unique assemblages of *nosZ* gene denitrifying bacteria. Several authors also found that different denitrifier populations developed under the different environmental conditions present at different locations (Braker et al. 2001, Rich et al. 2003). Sequences of cluster G (Table 6.2) were the most common *nosZ* sequences in clone library and were found as a T-RFLP peak in all environmental samples except FZ during winter. This *nosZ* genotype clustered with sequences from the Pacific Ocean (Scala and Kerkhof 1999), and must be from microorganisms able to tolerate a wide range of environmental conditions, since they were found in different physical environments (rocks and sediments). The geomorphic differences between sandy sediments, muddy sediments (Magalhães et al. 2002) and rocky sites and consequently differences in pH, total organic matter and oxygen availability likely influenced differences in *nosZ* gene variability and richness in the denitrifying assemblages. The lower pH inherent to muddy sediments may have contributed to the lower *nosZ* richness in these sediments, since low pH has been suggested to inhibit denitrifiers (Cavigelli and Robertson 2001, Avrahami et al. 2002). Oxygen penetration is another environmental variable that is likely to differ between MZ, SZ and FZ sites. Cavigelli and Robertson (2001) found substantial differences in the sensitivity of nos enzymes from 156 denitrifying soil isolates to oxygen. As discussed by Magalhães et al. (2005a*), the sediment (MS, SZ) and rocky sites (FZ) functioned quite differently during the 2000/2001 sampling program (Table 6.1). The main differences were that the rocky biofilm samples (FZ) always had the highest denitrification rates, net NO_2^- effluxes and N_2O production rates. Differences in denitrifier assemblages together with differences in other environmental factors, like inorganic nitrogen availability, that have been previously found to be important in regulating denitrification rates and N_2O production rates at these sites (Magalhães et al. 2005b†), appear to have contributed to the differences in rates observed between the rocky

*The contents of this article can be found in Chapter 3.

†The contents of this article can be found in Chapter 4.

site (FZ) and sediments sites (SZ, MZ). It is difficult to relate intra-site shifts in denitrifier communities to N biogeochemistry with just four (FZ and SZ) or three (MZ) samples. However, for the MZ site, while the same three *nosZ* sequences were always present, the relative peak area of each restriction fragment was clearly different in winter, where higher rates of denitrification were found (Table 6.1 and Fig. 6.3a). In agreement hierarchical cluster analysis clearly showed distinct differences in *nosZ* gene distributions in winter compared with fall and spring samples (Fig. 6.5b). At the FZ site, higher denitrification rates were observed in spring and summer (Table 6.1), where similar proportions of the different *nosZ* T-RFLP peaks were observed. Also, hierarchical cluster analysis shows that these two samples have more similar denitrifier assemblages than fall and winter samples, where lower denitrification rates were observed (Fig. 6.5b). In sandy sediments, denitrification rates did not differ between summer, fall and winter and only slightly higher rates were observed in spring (Table 6.1); the pattern of relative abundance of *nosZ* gene fragments differed according to the sampling and no clear relationship was established between the relative peaks area of *nosZ* gene T-RFLPs and the pattern of denitrification rates (Fig. 6.3a).

6.4.4 Intra-Site *nosZ* Variability and Environmental controls

At the SZ site, where the highest *nosZ* gene diversity was observed (Fig. 6.3a), an additional sampling program of 12 months was performed during 2002/2003 to evaluate intra-site variability of the denitrifier assemblage in more detail and to try to establish clear relationships between *nosZ* gene variability and N biogeochemistry. The T-RFLP analysis suggested that while intra-site composition of the denitrifying assemblage was stable, shifts in the relative proportions of different denitrifying genotypes might occur (Fig. 6.3b). Hierarchical clustering of denitrification rates, N_2O effluxes, water column $NO_3^- + NO_2^-$ concentrations and N_2O:N_2 ratios (Fig. 6.1a) was compared with the dendogram that resulted from analysis of the distribution of the different *nosZ* T-RFLP peaks (Fig. 6.7). Both analyses placed November, December and January samples in a distinct cluster (Fig. 6.1a and Fig. 6.7). In terms of environmental variables, August clustered together with November, December and January (characterized by higher denitrification rates and $NO_3^- + NO_2^-$ concentrations; Fig. 6.1a-c, respectively). However, in terms of the composition of the denitrifying assemblage, the August sample was more closely related to the June sample than to November, December and January samples (Fig. 6.7). CCA analysis revealed

that, while there was no variation in *nosZ* during the 12 months of sampling, the relative abundance of the different *nosZ* gene fragments included in cluster G were highly correlated with denitrification rates (i.e. T-RFLP fragments corresponding to cluster G had relatively high peak areas in samples where high denitrification rates were reported, Fig. 6.6). On the other hand, monthly sandy sediment denitrification rates were found to be significantly and positively related to NO_3^-+NO_2^- concentrations (Magalhães et al. 2005b*), suggesting that these denitrifying bacteria have higher Kms for nitrate and thus have an advantage under NO_3^- enriched conditions. Interestingly, this group of denitrifiers was also present in SZ, MZ and FZ sites (Fig. 6.3), suggesting that organisms with this *nosZ* genotype are well adapted to a variety of environmental conditions. CCA analysis showed also that the high relative peak height of fragment 75bp (unidentified in the clone libraries) was close related to high salinities and low NO_3^-+NO_2^- water column concentrations (Fig. 6.6). All of these results taken together suggest that the apparent monthly variation in the relative proportions of the different *nosZ* genes in the SZ denitrifying communities was forced by environmental variables (NO_3^-+NO_2^- availability and salinity), which reflects differences in denitrifier community functioning (denitrification rates, N_2O production rates). Thus, certain denitrifier strains appear to have competitive advantage over the others when NO_3^- and/or salinity fluctuate(s) in the system. Avrahami et al. (2002), also detected similar shifts in *nirK* gene frequencies in a denitrifying population in response to ammonium enrichments.

In summary, this study showed low *nosZ* gene diversity and clear spatial differences in *nosZ* assemblages in the distinct intertidal environments of the Douro River estuary. The absence of *nosZ* amplification in *Enteromorpha* spp. colonized rocks, where complete denitrification rates were measured, reveals that the *nosZ* primers used in this study as well as in previous investigations (Scala and Kerkhof 2000, Rich et al. 2003) probably missed a relevant percentage of the denitrifiers community that could have an unknown but importance relevance in worldwide denitrifiers communities and consequently in systems function. Even with the inherent limitations of the T-RFLP fingerprint analysis for the quantitative interpretation of the data, results from the monthly survey indicate that the apparent stability of the denitrifiers community based on the presence or absence of distinct genotypes may mask the dynamics of the denitrifier community. Finally, this investigation lead to a better understanding of the function of denitrifiers populations in intertidal estuarine environments and to the understanding of the possible environmental

*The contents of this article can be found in Chapter 4.

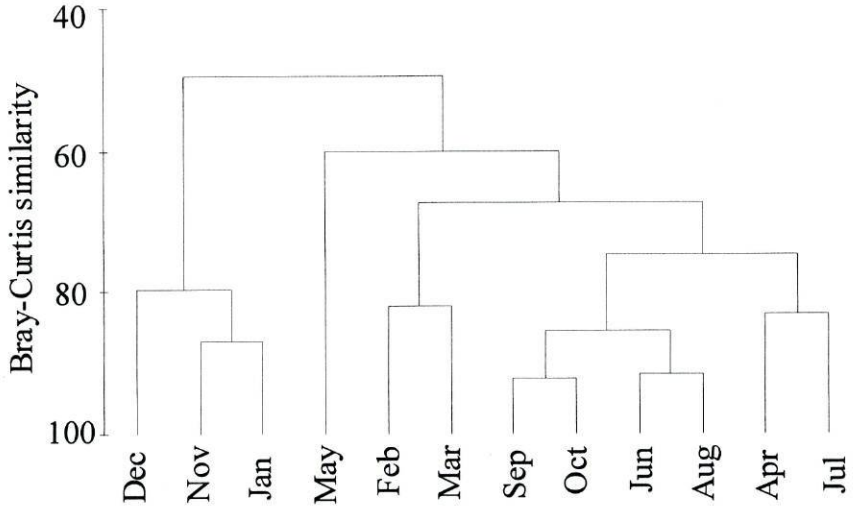

Figure 6.7: Dendogram for hierarchical cluster analysis of the 12 monthly samples in sandy sediments site. Analysis was based on group-average linking of Bray-Curtis similarities calculated from 4th root-transformed T-RFLP peak height data.

forces governing their dynamics. However, future research should be oriented in order to design specific new probes to characterized the unknown denitrifiers communities that inhabited EZ site, which are responsible for the denitrification rates measured.

Chapter 7

General Conclusions and Future Directions

The biogeochemical cycling of inorganic nitrogenous compounds in marine environments has received a great deal of attention over the last several decades, with a worldwide literature available for most of marine nitrogen biogeochemical transformations. The main contributions of this thesis leads with the fact that it represents the first comprehensive investigation of the N processes and environmental controls in coastal intertidal rocky biofilms environments. In addition a comparison between these biogeochemicaly unknown environments and intertidal sediment sites, for which there is an abundant global literature, was established. This study represents also a beginning attempt in terms of the knowledge of links between environmental variations, nitrifiers and denitrifiers diversity, and N biogeochemical function. In addition to the important results obtained, this study also has opened new windows of investigation that must be explored in future research. This chapter describes the general conclusions of this thesis and some possible directions for future investigations.

The present study began with a first evaluation of the metabolism and inorganic nutrient net fluxes between hard surface biofilms and the water column in order to examined the possible contribution of this environment to the inorganic nutrient fluxes within the intertidal zone of the Douro River estuary. Results from this study showed surprisingly similarities in metabolic rates of some nutrient flux directions and magnitudes (NH_4^+, NO_2^- and PO_4^{3-}) with intertidal sediments (Magalhães et al. 2002) and rock substrata,

given that the physical and biotic communities differ between these habitats. However, hard surfaces removed significantly higher amounts of nitrate and silicate from the water column than did adjacent muddy and sandy sediments. A general estimation for each intertidal environment showed that while biofilms occupy just 21% of the total linear (2-dimensional) area of the intertidal zone of Douro River estuary, they were responsible for approximate 43% of the nitrate uptake and 45% of the dissolved silicate uptake, in terms of hourly removal capacity for the total intertidal area.

Because nitrogen is the most serious cause of eutrophication in estuaries and coastal marine waters, and in the Douro River estuary the concentrations of nitrate inputs from freshwater have increased dramatically since the early 1990's, more detailed studies were then performed to focus on the dynamics and environmental controls of N biogeochemistry in intertidal rocky and sediment environments. During these studies, rates of O_2, NH_4^+, NO_3^-, NO_2^- and N_2O fluxes, and the processes responsible (nitrification, denitrification and N fixation), were compared between two intertidal sites for which there is an abundant global literature, i.e. muddy and sandy sediments, and two sites representing the rocky intertidal zone, where N biogeochemical processes have scarcely been investigated. These two sites, covered mainly with *Enteromorpha* spp. and *Fucus* spp. were representative of the macroalgal biofilms of the Douro estuary hard surfaces. Results revealed that during daylight, NH_4^+ and NO_3^- uptake rates together with ammonification could supply the different N requirements of the primary producer communities of all four sites. N assimilation by benthic or epilithic primary producers was the major process of daytime DIN removal, and N fixation, nitrification and denitrification were minor processes. At night, distinctly DIN cycling processes took place in the four environments, and denitrification together with DNRA appeared to be important pathways for NO_3^- processing. A conceptual model, developed in order to compare processes between the different sites, showed major differences between intertidal sediment and rocky sites in terms of the absolute mean rates of DIN net fluxes and processes involved. Rocky biofilm substrata produced generally higher flux rates. Of particular significance, the intertidal rocky biofilms released 10 times the amount of N_2O produced in intertidal sediments, representing the highest N_2O release rates ever recorded for marine systems. Finally, based on the overall DIN balance described, it was found that while the muddy sediment site functioned as a net DIN source subsystem, sandy and rocky biofilm sites functioned as net DIN sink subsystems of the entire Douro River estuary. This study, together with the previous research (Chapter 2), demonstrates

that rocky surface zones of estuaries can be highly biogeochemicaly active and thus can represent an important component of estuarine and coastal carbon and nutrient processing.

Besides the relationships established between specific inorganic nitrogen processes and the control of some environmental variables obtained in the above study (Chapter 3), more detailed experiments were performed in order to investigate the regulatory effects of salinity and inorganic nitrogen compounds (nitrate and ammonium) on nitrification and denitrification in intertidal sandy sediments and rocky biofilms covered by *Enteromorpha* spp. Results showed that salinity did not regulate denitrification rates in either environment, suggesting that halotolerant bacteria dominated these denitrifying communities. However, nitrifying communities at both sites showed maximum nitrification activity at intermediate salinities, confirming a salinity regulatory effect on the activity of those communities. Nitrate addition experiments confirmed the results obtained in the previous study (Chapter 3), revealing that increasing nitrate concentration clearly stimulated denitrification rates in sandy sediments but exerted no control in *Enteromorpha* spp. covered rocky substrata denitrifying communities, suggesting that the latter denitrifying communities were saturated at lower concentrations of nitrate. Nitrate enrichment experiments also revealed that N_2O:N_2 ratios increased rapidly when nitrate increased; however the combination of monthly survey data with laboratory experimental results suggest that while nitrate availability influenced N_2O production rates, other parameters, or the interactions between them were also important regulatory factors for N_2O production rates in both environments. Finally, the ammonium concentration regulated nitrification rates at both sites, and the different responses observed between sites suggest that the rocky biofilm nitrifier community was more tolerant of higher ammonium concentrations.

In order to link key N processes with the bacterial communities that mediate those transformations, the phylogenetic composition of ammonia oxidizing bacteria (AOB) of the class β-Proteobacteria was analyzed quarterly in samples from the same intertial sites evaluated in the study described in Chapter 3. The possible relationships between AOB assemblage compositions with nitrification rates, and net nitrate, ammonium and nitrite fluxes were investigated. Results showed that all sequences obtained from intertidal rocky biofilm sites exhibited phylogenetic affinity to *Nitrosomonas* lineages, whereas a majority of the sequences from the intertidal sediment sites were most similar to marine *Nitrosospira* lineages. Also, hierarchical cluster analysis based on environmental variables identified two main groups of samples. The first contained samples from intertidal rocky biofilm sites

that were characterized by high concentrations of nitrite and ammonium, and high organic matter and chl *a* content. The second group contained all of the intertidal sediment samples and was characterized by lower values of those variables. Also, significantly higher nitrification rates in the rocky biofilm samples compared with sediment samples were found. All these results together suggest that differences in environmental and/or physical characteristics of rocky biofilms and sediments led to selection for particular ammonia oxidizing bacterial assemblages that, in turn, function differently with respect to the N biogeochemical processes evaluated. In contrast, no clear seasonal variation in AOB assemblage composition was observed for any site, and thus no clear correlations were found between seasonal AOB diversity with environmental variables or nitrification rates. Thus the observed intra-site variability in AOB activity must be controlled by changes in AOB abundance and/or cell-specific activity not detected by nested PCR plus DGGE techniques. Finally, this study elucidated the findings obtained in the previous work (Chapter 4), since the different responses observed for nitrifying activity to ammonium additions in sandy sediments and *Enteromorpha* spp. could be explained by the differences found in AOB assemblages between the two sites. The *Enteromorpha* spp. site was composed primarily of *Nitrosomonas*-like species, which have been shown to be more tolerant of high ammonium concentrations (see Chapter 4), while the AOB assemblage at the sandy site was composed mainly of *Nitrosospira*-like species and samples from this site displayed a lower tolerance to high ammonium concentrations (see Chapter 5). These results demonstrated a connection between the phylogenetic composition of the AOB assemblage and the physiological ability to tolerate high ammonium concentrations.

In a similar analysis to the previous study, denitrifier assemblages were also evaluated by the distribution of nitrous oxide reductase (*nosZ*) genotypes in the same intertidal rocky biofilm and sediment samples where AOB were characterized. Concomitantly, other variables, such as denitrification rates, net DIN fluxes and nitrate availability were examined to test whether the distribution of *nosZ* genotypes could be related to some environmental property(ies) or nitrogen biogeochemistry. *NosZ* gene diversity was generaly low; the greatest species richness was observed at the sandy sediment site. Results showed that only a few T-RFLP OTUs were shared between the different sites, and indicated that *nosZ* populations from each location were significantly different. Surprisingly, all cloned amplicons from the rocky biofilm site colonized by *Enteromorpha* spp. were not *nosZ* genes; however complete denitrification (N_2 production) was measured at this site. Thus denitrifiers able to reduce nitrous oxide must have been present at the *Enteromorpha* spp. covered site,

but the *nosZ* primers used in this study did not successfully characterize the unknown denitrifier community. Intra-site temporal dynamics of *nosZ* genotypes were evaluated at the sandy sediment site during a more exhaustive survey (12 months of sampling), and results indicated considerable denitrifiers assemblage stability over time, since the same genotypes were present in the sandy sediment site all year round. However, pronounced temporal shifts in the relative peak areas of each T-RFLP profile were registered, suggesting considerable quantitative dynamics of this denitrifier community. In addition, canonical correspondence analysis revealed that the monthly peak area variability of some *nosZ* restriction fragments was significantly related to denitrification rates, nitrate availability and salinity. Thus, shifts in relative abundance of some *nosZ* restriction fragments could be explained by fluctuations of water column nitrate concentrations and salinity, since certain denitrifier strains appear to have competitive advantage over the others when nitrate and/or salinity fluctuate(s) in the system. These results together revealed that monthly variation in the relative proportions of the different *nosZ* phylotypes in sandy sediments was influenced by some environmental variables, which in turn reflected differences in denitrifier community functioning (denitrification rates and N_2O production rates).

Because the role of intertidal rocky habitats for processing nutrients typically has been overlooked, more detailed studies along the line of the research presented in this thesis are needed to be performed in different estuarine and other coastal systems with important areas of hard surfaces, in order to corroborate the findings presented here. In this study, most processes related to the nitrogen cycle were measured in intertidal rocky biofilm environments and compared to the more commonly measured sediment-water nutrient fluxes in the same estuary. However DNRA, which appears to have an important role in the N dynamics of these environments, deserves future direct measurements in order to confirm the results presented here. Also further research is necessary to improve our knowledge of the factors influencing nitrification and denitrification coupling in these intertidal environments to clarify the hypothesis presented. One of the interesting findings from this study is the observation that extremely high nitrous oxide fluxes occurred in rocky habitats; because N_2O release into the atmosphere is of much concern, N_2O production dynamics deserve future detailed investigation in order to find the cause(s) and control(s) of such high N_2O production rates in the rocky hard surfaces.

Factors controlling natural AOB and denitrifier community structure and the links between them with nitrifying and denitrifying activity are currently poorly understood, in part because of the present inability to convert phylogenetic data into a truly quantitative assessment of each species and its *in situ* activity. Thus, while this study contributes to a better understanding of the ecology of nitrifying and denitrifying bacteria, further investigations are necessary to fully understanding how nitrifier and denitrifier community diversity and dynamics affect ecosystem function. Primer design is one of the most critical aspect of microbial environmental diversity surveys and thus future research should be oriented to design specific new probes to characterize the unknown denitrifier and nitrifier communities that inhabit natural ecosystems in order to obtain a more accurate assessment of the distribution of nitrification and denitrification functional genes in diverse habitats. Future applications of new technologies that are continually emerging and being made available to microbial ecologists, including mRNA analysis, functional genes microarrays and microscale *in situ* analysis (combined microsensors and FISH methods), offer the promises to surmount a fundamental obstacle to progress in microbial ecology; attributing a microbially catalysed process, measured in a complex environmental system, with (a) specific microorganism(s).

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