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# Insights on the biogeochemistry of marine nitrogen and organic sulfur cycles and implications for nitrous oxide emissions

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# INSIGHTS ON THE BIOGEOCHEMISTRY OF MARINE NITROGEN AND ORGANIC SULFUR CYCLES AND IMPLICATIONS FOR NITROUS OXIDE EMISSIONS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Professor Doutor Adriano A. Bordalo e Sá

Categoria – Professor Associado com agregação

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*Like it or not, we humans are bound up with our fellows, and with the other plants and animals all over the world. Our lives are intertwined.*

Carl Sagan

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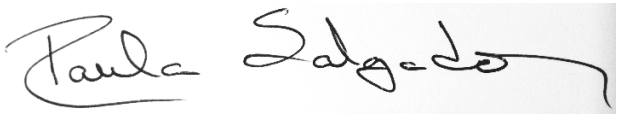

(PAULA SALGADO)

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The work contained in this thesis was based on the data from the following articles published or in preparation for publication:

- Salgado, P., Machado, A. and Bordalo, A. A. (2020). " Spatial-temporal dynamics of N-cycle functional genes in a temperate Atlantic estuary (Douro estuary, Portugal)" Aquatic Microbial Ecology 84:205-216. DOI: https://doi.org/10.3354/ame01935.

- Salgado, P., Ribeiro, H., Teixeira, C. and A. A. Bordalo. Molecular insights into the interaction between DMSP metabolism and the denitrification pathway in marine bacteria. *(In preparation for submission)*

- Salgado, P., Ribeiro, H., Teixeira, C. and A. A. Bordalo. Exploring the marine biogeochemical network interactions regulating nitrous oxide emissions. *(Submitted to iScience)*

The author of this thesis declares that had a main role on the conception, design, execution, interpretation and writing of the present study.

# ABSTRACT

Nitrogen (N) and sulfur (S) are essential for life and marine ecosystem function. Shifts in the fluxes of these elements alter the rates of basic processes, disturb biogeochemical cycles, and threaten the ecosystem sustainability. Today, with the worldwide concern of climate change urges the need to expand the knowledge about N cycle dynamics, the effects on the nitrous oxide ($N_2O$) emissions, a strong greenhouse gas, how microbial communities will respond to environmental change and in what conditions microorganisms couple elemental cycles.

An inhibitory interaction between dimethylsulphoniopropionate (DSMP) metabolism and the last step of denitrification, affecting the production of climate regulator dimethylsulfide (DMS) and, simultaneously, climate warming $N_2O$ gas was previously identified. Therefore, the purpose of this thesis was to assess the spatial-temporal dynamics of N cycle microbial communities in an estuarine system (Douro, Portugal), and explore the interaction between denitrification and DMSP metabolism in marine bacteria.

To address how N cycle microbial communities are regulated by the estuarine environmental features, water samples were collected, physical-chemical parameters measured, and specific functional genes (*nirS*, *nirK*, bacterial and archaeal *amoA* and the non-denitrifying *nosZ*II) abundance assayed. After, the inhibitory interaction between DMSP and denitrification was evaluated by the quantification of $N_2O$ and DMSP degradation compounds, and the expression of genes from denitrification (*nirS*, *nosZ*) and DMSP catabolism (*dmdA*, *dddW*), in Roseobacter strains.

Dissolved oxygen, temperature, and salinity were key factors in controlling the annual distribution of N cycle genes. The winter/spring seasons yielded a higher abundance of *nirS*, *nosZ*II, bacterial and archaeal *amoA*, which were also associated with increased turbidity. This feature was also linked with the strong correlation between *nirS* and bacterial and archaeal *amoA*, suggesting a potential coupling between nitrification and denitrification in particle-associated assemblages. When evaluating the DMSP effect on the denitrification in Roseobacter cell suspensions, it was observed that $N_2O$ accumulation followed the progressive increase of MeSH, predicting a repression on *nosZ* transcription. However, a different microbial regulation of *nosZ* expression was possibly the result of different sensitivities of marine bacteria towards DMSP, because of different sulfur requirements within microorganisms. Nevertheless, it was confirmed the direct effect of MeSH on the accumulation of $N_2O$ in *R. pomeroyi* cultures, but not on the functioning of the $N_2O$-reductase.

This work contributed to improve the understanding of the dynamics between N and organic S biogeochemical processes, and how the environmental conditions regulate the distribution and abundance of N-cycle marine microorganisms, that drive elemental cycles.

# RESUMO

O azoto (N) e o enxofre (S) são essenciais para a vida e funcionamento dos ecossistemas marinhos. As alterações nos fluxos destes elementos influenciam as taxas dos processos associados com reflexos nos ciclos biogeoquímicos, ameaçando a sustentabilidade de todo o ecossistema. Atualmente, com a evidente preocupação com as alterações climáticas é premente expandir o conhecimento sobre a dinâmica do ciclo do N, sobre os efeitos das emissões do óxido nitroso ($N_2O$), um forte gás do efeito de estufa, assim como respondem as comunidades microbianas às mudanças ambientais e em que condições os microrganismos associam ciclos da matéria.

Atualmente, é conhecida uma interação inibitória entre o metabolismo do dimetilsulfoniopropionato (DSMP) e a última etapa da desnitrificação, afetando a produção do dimetilsulfureto (DMS) – um regulador do clima e, simultaneamente, do gás $N_2O$, que aquece o clima, tendo um efeito contrário. Desta forma, o objetivo desta tese foi avaliar a dinâmica espácio-temporal das comunidades microbianas do ciclo do N num sistema estuarino (Douro, Portugal) e explorar a interação entre a desnitrificação e o metabolismo do DMSP em bactérias marinhas.

De forma a investigar como as comunidades microbianas da coluna de água associadas ao ciclo do N são reguladas pelas características ambientais estuarinas, foram determinados parâmetros físico-químicos chave e avaliada a abundância de genes funcionais específicos (*nirS*, *nirK*, *amoA* de Bacteria e Archaea e o *nosZ*II não-desnitrificante). Seguidamente, em estirpes do grupo Roseobacter, foi avaliada a interação inibitória entre o DMSP e a desnitrificação pela quantificação do $N_2O$ e dos compostos de degradação do DMSP, assim como pela expressão dos genes da desnitrificação (*nirS*, *nosZ*) e do catabolismo do DMSP (*dmdA*, *dddW*).

O oxigénio dissolvido, temperatura e salinidade foram determinantes no controlo da distribuição anual dos genes do ciclo do N. As estações inverno/primavera apresentaram maior abundância do *nirS*, *nosZ*II, *amoA* de Bacteria e Archaea, que também estavam associados ao aumento da turvação da água. Esta parâmetro mostrou, igualmente, uma forte correlação entre o *nirS* e o *amoA* de Bacteria e Archaea, sugerindo um potencial acoplamento entre nitrificação e desnitrificação em agregados associados a partículas da coluna de água. Ao avaliar o efeito do DMSP na desnitrificação em suspensões celulares de estirpes Roseobacter, observou-se que a acumulação de $N_2O$ acompanhou o aumento progressivo do MeSH, supondo existir uma repressão na transcrição do *nosZ*. No entanto, uma regulação

microbiana díspar da expressão do *nosZ* foi, provavelmente, consequência de diferentes sensibilidades das bactérias marinhas às concentrações do DMSP, derivado de requisitos de enxofre distintos nos microrganismos. Por outro lado, foi confirmado o efeito direto do MeSH na acumulação de $N_2O$, nas culturas da *R. pomeroyi*, e não no funcionamento da $N_2O$-redutase.

Este trabalho contribuiu, pois, para melhorar a compreensão sobre a dinâmica entre os processos biogeoquímicos do N e do S orgânico, assim como sobre o papel regulador das condições ambientais relativamente à distribuição e abundância dos microrganismos marinhos do ciclo N, essenciais para o funcionamento do ciclo da matéria, no geral.

# LIST OF ABBREVIATIONS

ANOVA – One-way analysis of variance

AMO – Ammonia monooxygenase

Anammox – Anaerobic ammonium oxidation

AO – Ammonia oxidizers

AOA – Ammonia-oxidizing Archaea

AOB – Ammonia-oxidizing bacteria

AOM – Anaerobic oxidation of methane

BLAST – Basic Alignment Search Tool

Chl*a* – Chlorophyll *a*

CND – Coupled nitrification-denitrification

DMS – Dimethyl sulfide

DMSP – Dimethylsulfoniopropionate

DNRA – Dissimilatory nitrate reduction to ammonium

DOC – Dissolved organic carbon

ECD – Electron-Capture Detector

ETSP – Eastern Tropical South Pacific

GC – Gas-chromatography

GHG – Greenhouse Gas

HAO – Hydroxylamine oxidoreductase

MBM – Marine basal medium

MeSH – Methanethiol

MMPA – methylmercaptopropionate

$N_2$OR – Nitrous oxide reductase

NAP – Periplasmic nitrate reductase

NAR – Respiratory nitrate reductase

N-damo – Nitrate/nitrite-dependent anaerobic methane oxidation

NIR – Nitrite reductase

NO – Nitric oxide

NOB – Nitrite-oxidizing bacteria

NOR – Nitric oxide reductase

OMZs – Oxygen minimum zones

qPCR – Quantitative polymerase chain reaction

P-FPD – Pulsed-Flame Photometric Detector

POM – Particulate organic matter

RDA – Redundancy analysis

RefSeq – Reference Sequence Database

RT – Reverse transcription

SPM – Suspended Particulate matter

TC – Total carbon

TN – Total nitrogen

VIF – Variation inflation factor

VOSC – Volatile Organic Sulfur Compounds

# CHAPTER 1

## General introduction

## 1.1. Environmental importance of nitrous oxide

The biogeochemical cycling of major elements such as nitrogen, sulfur, carbon, hydrogen, phosphorus, and oxygen, is largely mediated by microbial organisms, and their interactions are fundamental to maintain the health balance of the Planet (Madsen, 2011, Howarth et al., 2011). This complex network supports life through biological, geological, and chemical feedbacks that maintain climate adequate, supply the necessary food resources to the growth of organisms and, generally, sustain all ecosystem dynamics. Perturbations in biogeochemical cycles might result in an unexpected environmental impact.

Marine environments with a high supply in inorganic nutrients, such as estuarine and coastal waters, are very productive with dynamic metabolic pathways co-occurring and, consequently, the hotspot for biogeochemical cycling processes (Howarth et al., 2011, Rees, 2012b). The cycling of these elements is strongly dependent on the biological activity where microbial communities, a key player on nutrient cycling, have an important role on ecological processes and, ultimately, sustain life on Earth (Konopka, 2009, Prosser, 2012). The disturbance in these elements dynamics affects the physical, chemical, and biological characteristics, leading to environmental changes that can accelerate climate change to an overall decline of marine biological productivity, with commensurable impact on plankton growth, marine fisheries, loss of biodiversity, and even result in the destruction of ecosystems (Moore et al., 2018, Barbosa et al., 2010, Cheung et al., 2013). Fluctuating environmental conditions enable a co-occurrence of biochemical processes, regulated by microorganisms, where the interactions of reduction-oxidation (redox) reactions, essential for biogeochemical cycles, result in a permanence and/or switch of biochemical pathways in response to changing conditions and, consecutively, contribute for life maintenance (Lovelock, 1979, Falkowski et al., 2008, Auguères and Loreau, 2015). However, the rise of an Anthropocene Era represents serious consequences to the maintenance of ecosystems (Moore et al., 2018, Waters et al., 2016), interfering in chemical and biological interactions. One of the most alarming consequences is the potential exacerbation of the atmospheric GHGs (Greenhouse Gas), resulting from perturbations in ocean chemistry (Babbin et al., 2015), and the intensification of nitrous oxide has received increased attention due to its impact on climate (Wuebbles, 2009, Codispoti, 2010). The emissions of $N_2O$ are a huge contributor to the stratospheric ozone depletion through the reaction with the atomic oxygen, which consequently potentiates the increase of harmful UV-B radiation (Krupa and Kickert, 1989, Caldwell and Flint, 1994, Nevison and Holland, 1997, Ravishankara et al., 2009, Portmann et al., 2012). Additionally, $N_2O$ contributes to the radiative forcing of GHGs, representing an environmental problem with a global warming potential 298 times greater than Carbon dioxide ($CO_2$) for 100 years (Kroeze, 1994, Ciais et

al. 2013), and a long atmospheric lifetime of 114 years (Dickinson and Cicerone, 1986). However, until a few years ago, $N_2O$ impact was oblivious to the world and underestimations lead to an unawareness of the severe implications on the atmosphere by this trace gas. During decades, when climate alarm was buzzing throughout the world, the cause was indisputably linked to $CO_2$ emissions (Plass, 1956, Benton, 1970, Kimball and Idso, 1983, Lashof and Ahuja, 1990, Pearce, 1991, Cox et al., 2000), namely from burning fossil fuels (Broecker et al., 1979, Hall, 1989). Even so, it was unsatisfactory to explain the unequivocal acceleration of global warming. Indeed, $N_2O$ atmospheric concentration has increased about 20% over the past century mainly due to anthropogenic emission (Ciais et al., 2013). Consequently, it leads to a perturbation on the balance of nitrogen cycle, mainly due to the use of fossil fuels and intensification of agriculture, all related to a growing world population (Delwiche, 1970, Galloway et al., 2008, Galloway et al., 2013). Whilst anthropogenic sources represent a heavy weight on $N_2O$ emissions and a considerable and vast research about $N_2O$ terrestrial sources and sinks is available, little is known, though, about the underlying mechanisms that regulate marine biogenic $N_2O$ production. The oceanic emissions are estimated to be 4 TgN $yr^{-1}$, representing an important natural $N_2O$ source (~35%; Ciais et al., 2013). Still, it is very difficult to accurately measure the oceanic $N_2O$ emissions due to the complexity of the processes that are interlinked, which makes the estimations an ambiguous inference.

Marine ecosystems have a relevant role in the biogeochemical cycles of elements on Earth and is, therefore, necessary to (i) understand how microbial transformations contribute to $N_2O$ flux, (ii) how the involved microorganisms cope with environmental changes that may regulate it, (iii) how other biogeochemical cycles interact with $N_2O$-cycling and its environmental significance. However, the factors that regulate the microbial nitrogen-transforming networks are poorly understood.

## 1.2. **Nitrous oxide sources and networks within the N-cycle**

The characterization of marine $N_2O$ processes is rather difficult due to the complexity of the dynamic nitrogen cycle and its network of chemical and biological processes, and transformations associated with it. The classical N-biochemical pathways include four types of reduction (nitrogen fixation, assimilatory nitrate reduction, dissimilatory nitrate reduction to ammonia (DNRA), and denitrification). Two oxidation pathways (nitrification and anaerobic ammonium oxidation - anammox) are also associated, and both metabolic processes co-occur in several environments through metabolic handoffs (Zehr and Kudela, 2011, Thamdrup, 2012, Stein and Klotz, 2016, Kuypers et al., 2018, Albright et al., 2019). The main microbial pathways

of oceanic $N_2O$ production are denitrification and nitrification, where $N_2O$ is an intermediate product of both dissimilatory and detoxification (reduction of nitric oxide, NO) reactions or a by-product in nitrification (Bange et al., 2010, Stein, 2011a, Thomson et al., 2012). However, there is a more dynamic cluster of marine $N_2O$ production pathways which remains unclear and unaccounted. The coupling of processes in the water column and the production and consumption of nitrogenous compounds, were effectively highlighted in an updated Zafiriou (1990) "leaky pipe" model (**Figure 1.1**), where N flows through the metabolic pipes that represent nitrogen cycle reactions (Codispoti et al., 2005).

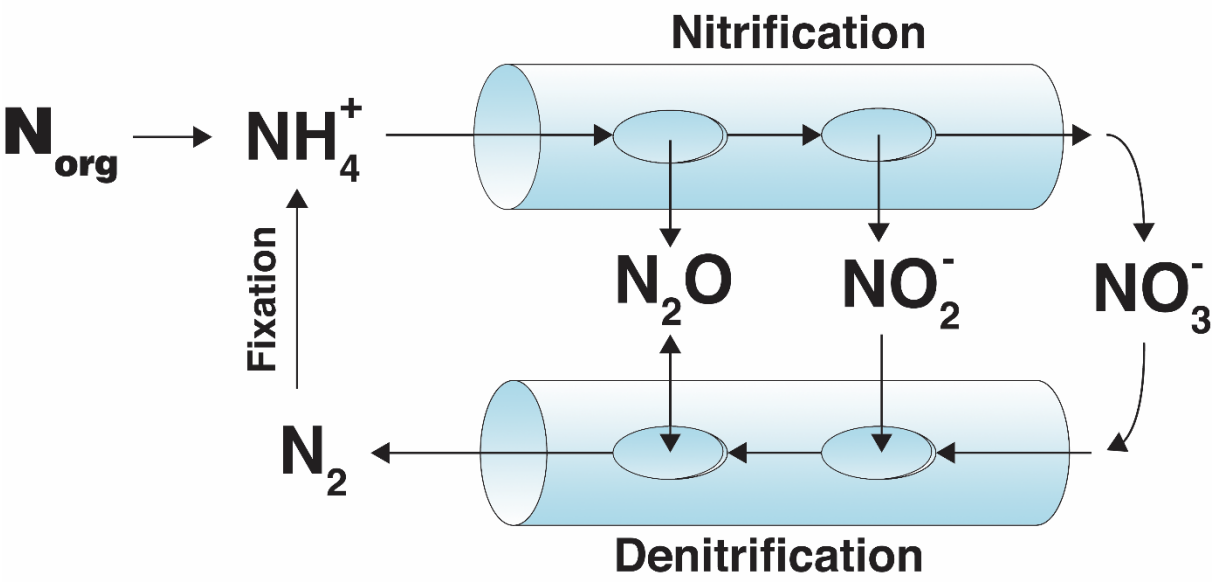


**Figure 1.1.** Conceptualization of the "leaky pipe" model of processes (adapted from Codispoti et al. (2005)).

### 1.2.1. Denitrification

Canonical denitrification is an anaerobic reduction process coupled to the oxidation of organic matter, where nitrogen oxides (nitrate, $NO_3^-$, or nitrite, $NO_2^-$) serve as alternate electron acceptors on low oxygen ($O_2$) waters (0 < O2 < 2-10 µmol L-1; Cline and Richards, 1972, Knowles, 1982, Naqvi, 1987, Tiedje, 1982, Codispoti et al., 2005, Bange et al., 2010). It constitutes one of the most important processes within the N-cycle, being a major player in the biological removal of inorganic nitrogen from the environment. This is achieved by catalyzing the reduction of N-oxides into the subsequent intermediates NO and the non-toxic $N_2O$, until the formation of dinitrogen ($N_2$), the lowest reduction state, completing the global nitrogen cycle (**Figure 1.2**).

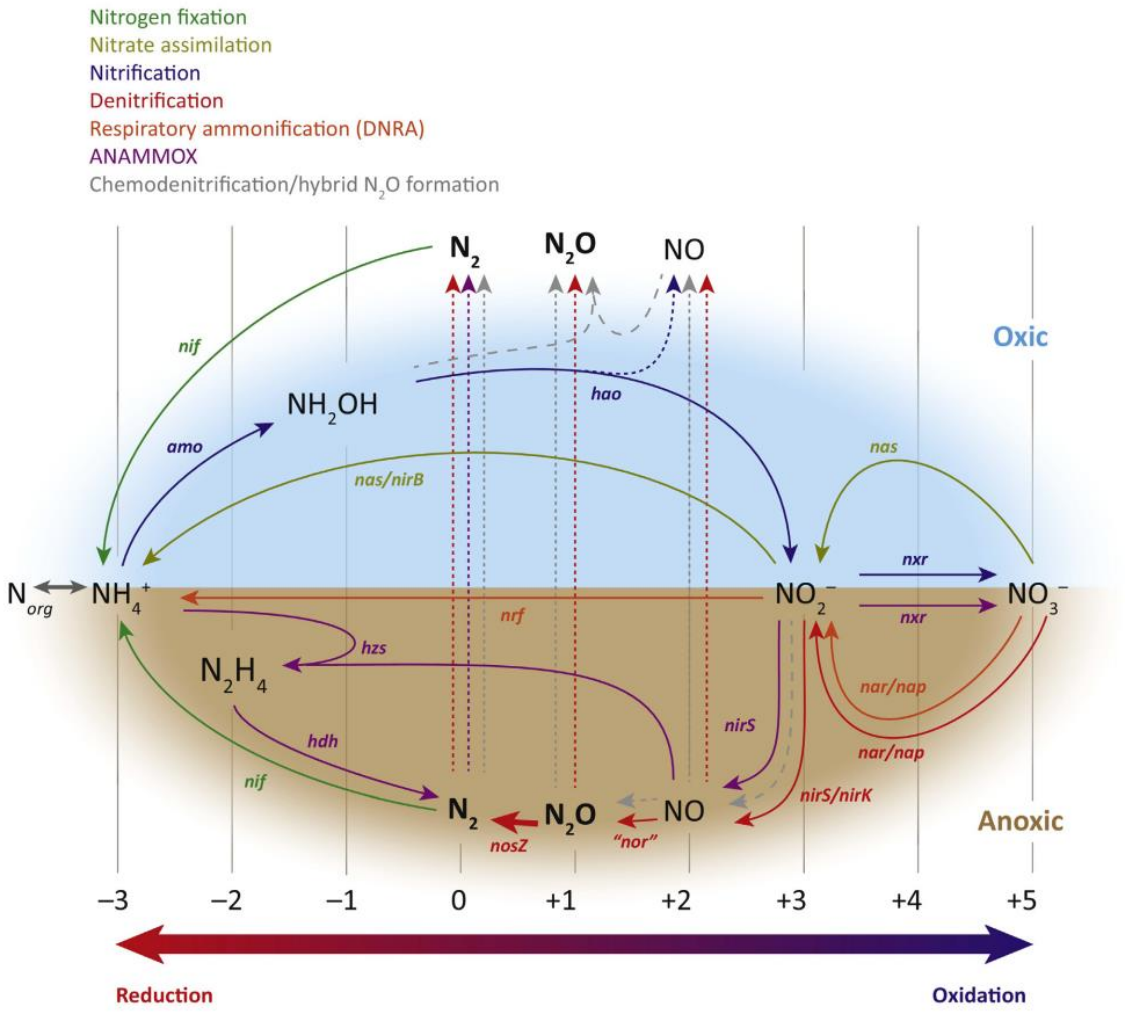


**Figure 1.2.** Conceptual diagram of N-cycle microbial processes and general relationships which regulate marine $N_2O$ emissions (source: Hallin et al. 2008).

In the N reduction process, nitrate ($NO_3^-$) is metabolized by a respiratory (encoded by *nar* gene), or periplasmic (encoded by *nap* gene) nitrate reductase (NAR or NAP, respectively). Nitrite ($NO_2^-$) is reduced by either a cytochrome $cd_1$, encoded by *nirS*, or a Cu-containing enzyme encoded by *nirK* (nitrite reductase – NIR; Zumft, 1997). The nitrogen oxides are further potentially reduced to the gaseous intermediates nitric oxide (NO), $N_2O$, and dinitrogen ($N_2$). This respiratory process, concomitant with energy conservation by electron transport phosphorylation in each of the reduction steps, is prevalent mostly in heterotrophic bacteria and archaea (Tiedje, 1982, Philippot, 2002). Hitherto, it is the only known biological sink of $N_2O$ via dissimilative reduction to $N_2$ catalyzed by the nitrous oxide reductase ($N_2OR$), a metalloenzyme encoded by the *nosZ* gene (Arai et al., 2003, Pauleta et al., 2013). Concomitantly, a potential $N_2O$ fixation by diazotrophic organisms, under extreme environmental conditions, may also occur (Farías et al. (2013). On the other hand, $N_2O$

emissions can be enhanced by microorganisms that have a truncated denitrification, where they lack the *nosZ* gene and terminate the pathway with $N_2O$ (Zumft, 1997). Additionally, it was already described that the metabolic feature of microbial $N_2O$ reduction has evolved into two lineages. In the clade I, $N_2O$ reductase gene *nosZ* is related to the so-called "true denitrifiers" that own other denitrification genes, occurring among the Alpha-, Beta- and Gammaproteobacteria. In the phylogenetically distinct clade II, that possess an atypical *nosZ* within both Bacteria and Archaea groups of marine and terrestrial environments, other denitrification genes are usually absent (Jones et al., 2008, Sanford et al., 2012, Jones et al., 2013, Graf et al., 2014, Sun et al., 2017, Angell et al., 2018). Accordingly, Graf et al. (2014) analyzed public available microbial genomes from organisms belonging to different environments, and found non-random distribution of *nosZ* concomitant with *nirS* genes than with *nirK*, which implies that *nirS* organisms can potentially carry out **complete denitrification** and, thus, reduce $N_2O$ emissions. This evidence suggests that denitrification is a highly modular pathway, with different variants of the genes involved in each reduction step posing an advantage in terms of energy and growth rate of the organisms, as in a cross-feeding interaction (Zumft, 1997, Pfeiffer and Bonhoeffer, 2004, Hallin et al., 2018).

### 1.2.2. **Nitrification**

Nitrification occurs in zones where dissolved $O_2$ concentrations are usually above 10 µmol $L^{-1}$ (Ryabenko et al., 2012), and involves the autotrophic oxidation of ammonia ($NH_3$) to nitrite, via hydroxylamine ($NH_2OH$), and nitrate. Therefore, the process is associated with the remineralization of organic matter (Ward, 2013). The first phase that mediates the oxidation of $NH_3$ to $NO_2^-$ is catalyzed by ammonia monooxygenase, encoded by the gene *amoA*, which hydroxylates $NH_3$ to $NH_2OH$. Then, $NH_2OH$ is oxidized to $NO_2^-$ by a multiheme enzyme, hydroxylamine oxidoreductase (HAO). The last oxidation step results in the transformation of nitrite to nitrate (nitratation), by nitrite-oxidizing bacteria (NOB), supplying denitrification with $NO_3^-$. It was previously considered a modular process, in which the last step was carried out by different types of organisms (Costa et al., 2006), until the recently discovered complete nitrification by a chemolithoautotrophic microorganism belonging to the genus *Nitrosospira* (Daims et al., 2015, van Kessel et al., 2015).

Nitrification was originally thought to be exclusive of ammonia-oxidizing bacteria (AOB) belonging to some Proteobacteria groups, namely the β-subclass (Alzerreca et al., 1999, Bano and Hollibaugh, 2000, Kowalchuk and Stephen, 2001, Norton et al., 2002). But the discovery of ammonia monooxygenase genes, associated with archaeal scaffolds within marine metagenomic datasets (Venter et al., 2004), subsequently confirmed the existence of marine

ammonia-oxidizing archaea – AOA (Könneke et al., 2005), which seem to dominate ocean nitrification and play an important role in the nitrogen cycle as well as in $N_2O$ production in the near-surface ocean (Francis et al., 2005, Wuchter et al., 2006, Francis et al., 2007, Santoro et al., 2011, Löscher et al., 2012, Tolar et al., 2016). However, it has remained unclear about the biological pathways in AOA that produce $N_2O$ (Vajrala et al., 2013, Kozlowski et al., 2016, Hink et al., 2017).

#### 1.2.2.1. $N_2O$ production from Nitrification

$N_2O$ may be formed as a by-product during the oxidation of hydroxylamine ($NH_2OH \rightarrow NOH \rightarrow N_2O$), or as an intermediate species from nitrite reduction by nitrifiers that denitrify, under microaerophilic conditions (Hooper et al., 1997). Von Breymann (1982) observed empirically the $N_2O$ production in seawater samples, via immediate oxidation of hydroxylamine using Fe (III) ions. This reaction was later demonstrated in bacterial cultures of the heterotrophic *Alcaligenes faecalis* revealing an increased in $N_2O$ as a by-product of $NH_2OH$ oxidation (Otte et al., 1999). Recently, it was measured for the first time $NH_2OH$ in the open ocean, which was associated with $N_2O$ concentrations, enabling to consider $NH_2OH$ as a potential precursor of $N_2O$ carried out by nitrifying organisms in the ocean (Ma et al., 2019, Korth et al., 2019). In addition, it has been proposed a direct enzymatic pathway where an oxidized mono-heme enzyme called cytochrome (cyt) P460 is used by AOB, for detoxifying $NH_2OH$ through $N_2O$ release under anoxic conditions (Caranto et al., 2016).

As abovementioned, $N_2O$ can also be formed as an intermediate product from heterotrophic nitrification under oxic to suboxic conditions, where nitrification links to aerobic denitrification via $NO_2^- \rightarrow NO \rightarrow N_2O$ (Fig. 2), through a process called nitrifier-denitrification, carried out by AOB (Goreau et al., 1980, Jørgensen et al., 1984, Ryabenko et al., 2012). It has been suggested that, when the levels of oxygen are minimum, $NO_2^-$ acts as an alternative terminal electron acceptor. Also, high concentrations of $NO_2^-$ promote nitrifier-denitrification and, consequently, enhance $N_2O$ emissions (Chandran et al., 2011). The sequential aerobic reduction of $NO_2^-$ to NO, and then $N_2O$ is catalyzed by the combined action of the enzymes NIR and nitric oxide reductase (NOR), encoded by the genes *nirS* and *norB*, respectively, whose orthologs have also been found in nitrifying bacteria (Casciotti and Ward, 2001, Casciotti and Ward, 2005, Cantera and Stein, 2007, Arp et al., 2007). This $N_2O$ formation mechanism enables the maintenance of intracellular redox balance and depends on the environmental conditions that potentiate the switch or interaction between pathways (Stein, 2011a, Frame and Casciotti, 2010). Nitrifier denitrification, which has been known to yield higher $N_2O$ than autotrophic nitrification, has been the subject of a growing research interest due to its importance for the global $N_2O$ budget (Bange et al., 2010, Stein, 2011b, Wrage-

Mönnig et al., 2018). Although the nitrifier-denitrification microbial metabolism seems relevant for $N_2O$ dynamics in the lower euphotic zone of the open ocean (Wilson et al., 2014), the importance and contribution of this process for oceanic $N_2O$ production is still poorly studied.

#### 1.2.2.2. $N_2O$ production from coupled Nitrification and Denitrification

Alternatively, interactions between nitrifiers and denitrifiers may also occur, supplying the denitrifiers with the oxidized nitrifier products (Codispoti and Christensen, 1985, Santoro et al., 2011), contributing therefore, to the mitigation of eutrophication through N removal and release it to the atmosphere as $N_2O$ or $N_2$. These coupled nitrification-denitrification (CND) processes, which are different from the nitrifier-denitrification pathway, can take place in environments with conditions that favor the occurrence of both processes. The association between AO and denitriers is particularly important in oxic-anoxic interfaces of the water column, for example in estuarine and coastal sediments, where nitrification occurs in the upper, oxidized phases, and denitrification on the lower, reduced layer (Jenkins and Kemp, 1984, Codispoti and Christensen, 1985, Ward et al., 1989, Rysgaard et al., 1994, Ward, 1996, An and Joye, 2001, Francis et al., 2005, Rao et al., 2008, Gao et al., 2010, Fernandes et al., 2016). As a result, the expected $N_2O$ production is high. This coupling of processes, which can occur in close proximity to each other, is constrained by the diffusive or advective transport of compounds (Hulth et al., 2005 and references therein). Additionally, the co-occurrence of nitrification and denitrification activity has been associated to suspended particles in the water column of estuaries with high turbidity. Denitrification can occur under oxic conditions inside of organic particles, which provide an anoxic microenvironment for denitrifiers to thrive and connect with outer nitrifiers (Zhang et al., 2014, Zhu et al., 2018).

Another important contribution to $N_2O$ release from marine ecosystems results from the bioturbation by benthic macrofauna. Tube-dwelling animals play a key role on the regulation of nitrification and denitrification, through the continuous renewal of $O_2$ in their burrows in the sediment, which may enhance $N_2O$ emission rates (Stief et al., 2009, Stief et al., 2010, Carpintero Moraes et al., 2018).

Several environmental conditions and regulatory factors, such as nutrient availability, temperature, and salinity can influence nitrogen transformation processes in the ocean and potentiate the combination of pathways (Tiedje, 1988, Zumft, 1997, Huesemann et al., 2002, Thamdrup and Dalsgaard, 2002, Beman et al., 2011, Hayashik et al., 2013, Fernandes et al., 2016, Rees et al., 2016). Likewise, the biological sources and sinks of $N_2O$ are influenced by environmental features (e.g. availability of oxygen, N compounds such as ammonium and

nitrate), that drive microbial communities to respond through co-occuring redox processes (Codispoti et al., 2001). Among other environmental conditions, oxygen is a key factor regulating the switch or coupling between nitrification and denitrification in the water column and sediments in marine environments. This interference by oxygen will be addressed below.

### 1.2.3. **Anammox**

Besides denitrification, anaerobic ammonium oxidation (anammox) is an additional anaerobic process responsible for nitrogen removal from marine systems, coupling $NH_4^+$ oxidation with $NO_2^-$ reduction (Dalsgaard et al., 2003, Kuypers et al., 2003, Thamdrup and Dalsgaard, 2002). However, the debate about the dominance of each process (denitrification vs. anammox) over the other, mainly in oxygen-deficient zones that are potential hotspots for $N_2O$ production, is still ongoing (Bange et al., 1996 and references therein, Naqvi et al., 1998, Naqvi et al., 2000, Arévalo-Martínez et al., 2015, Arévalo-Martínez et al., 2019).

Nowadays, a major environmental concern deals with the overall decline in oceanic oxygen concentrations, or 'deoxygenation', and a subsequent expansion of the mid-depth oxygen minimum zones (OMZs) due to increasing temperature and anthropogenic N input, which potentially increases $N_2O$ production (Stramma et al., 2008, Keeling et al., 2010). These oxygen-deficient waters tend to propitiate the denitrification and, eventually, a linked anammox, with denitrification providing substrates (nitrite) for anaerobic ammonium oxidation (annamox). Furthermore, the input of electron acceptors (oxidized N), required by denitrification, is arranged by anammox (Zehr and Kudela, 2011).

Anammox was originally discovered in a waste-water sludge in 1995, and is generally performed by bacteria belonging to the order Plantomycetales. They are able to generate $N_2$ via oxidation of ammonia in anoxic conditions, using nitrite as an electron acceptor instead of oxygen (Kuenen, 2008). But, this process was later detected in natural marine environments, such as the OMZs in the upwelling areas off the coasts of Namibia, Chile, and Peru (Kuypers et al., 2005, Thamdrup et al., 2006, Hamersley et al., 2007, Galán et al., 2009, Lam et al., 2009, Dalsgaard et al., 2012), in anoxic basins (Dalsgaard et al., 2003, Kuypers et al., 2003, Hannig et al., 2007), and in marine sediments (Thamdrup and Dalsgaard, 2002, Rysgaard et al., 2004, Engström et al., 2005, Bale et al., 2014, Devol, 2015, Fernandes et al., 2016). Because of toxic NO release during anammox, bacteria need to detoxify since NO can bind to metal centers and damage bacterial proteins, lipids and DNA, being $N_2O$ formed as a result (Kartal et al., 2007). Under ammonium limitation, anammox bacteria will depend on other biogeochemical pathway, and could be mediating dissimilatory nitrate reduction to ammonium

(Kartal et al., 2007). Consequently, $N_2O$ is produced from the anammox intermediate NO, released as a result of detoxification (Strous et al., 2006).

Oxygen-deficient waters are expanding due to the warming of the water column, and is a must to improve the understanding of the sources of $N_2O$ in these environments (Wright et al., 2012).

### 1.2.4. Dissimilatory nitrate reduction to ammonium (Respiratory ammonification)

Like denitrification, dissimilatory nitrate reduction to ammonium (DNRA) is an anaerobic process that removes nitrate from the environment. But, while denitrification (the stepwise reduction of $NO_3^-$ to $N_2$) removes fixed nitrogen, DNRA retains nitrogen as $NH_4^+$. In the initial step of DNRA, the nitrate-ammonifiers reduce nitrate to nitrite through the same enzymatic reaction as in denitrification (**Figure 1.2**). After, nitrite is reduced to ammonium, a reaction which is catalyzed by a pentaheme cytochrome *c* nitrite reductase (Nrf), yielding a total of eight electrons transfer, when compared to five electrons in denitrification (Kraft et al., 2011). It is considered that this pathway serves as an important electron sink for anaerobes in the oxidation reactions and, therefore, a favorable step in anaerobic environments where there is a limitation in electron acceptors (Tiedje, 1988, Bonin, 1996, Kraft et al., 2011). In fact, the occurrence of DNRA was documented in the water of the Benguela upwelling system, where anammox bacteria are active (Kuypers et al., 2005, Kartal et al., 2007). This coupling has been evidenced in the OMZs of the Eastern Tropical South Pacific (ETSP) and Arabian Sea, where the requirement of $NH_4^+$ for anammox was substantially provided by DNRA, and probably occurs intracellularly by anammox bacteria themselves (Kartal et al., 2007, Lam et al., 2009, Jensen et al., 2011). Both processes seem to be very important in oxygen-deficient waters and a tight DNRA-anammox coupling, a mechanism that could be confused as denitrification (Kartal et al., 2007), ultimately results in $N_2$ loss and could also mediate $N_2O$ formation.

Although the exact mechanisms of $N_2O$ production from DNRA are still unclear, it has been proposed that, in nitrate ammonifiers, $N_2O$ derives as a by-product of NO reduction (reviewed in Torres et al., 2016). NO is not an obligatory intermediate in DNRA and is generated through a chemical or enzymatic reaction as a signal and defense molecule which, in order to avoid nitrosative stress conditions, stimulates the cell to detoxify through the release of $N_2O$ (Poole, 2005).

## 1.3. Nitrous oxide production coupled to other biogeochemical cycles

Nitrogen is an essential element for the construction of biomolecules and can only be biologically assimilated in certain oxidized (e.g., $NO_3^-$), or reduced (e.g., $NH_4^+$) forms influencing primary productivity, thereby linking global biogeochemical cycles and affecting the climate of the Earth (Gruber and Galloway, 2008, Canfield et al., 2010a, Fowler et al., 2013, Voss et al., 2013). The understanding of chemical cycles in the ocean and its impact on marine ecosystem services reflect the need to incorporate the interactions between elements when studying the processes, fluxes, and modelling of biogeochemical cycles (Wollast, 1981, Williams, 1987). Indeed, global biogeochemical cycles can be intimately coupled, generally, through electrons transfer and establishment of redox gradients, where one element is oxidized while other is simultaneously reduced (reviewed in Falkowski et al., 2008). Also, in terrestrial subsurface, distinct coexisting microorganisms can perform single or isolated steps of redox transformations within each element cycling (Anantharaman et al., 2016), This can be achieved by microbes working as a co-op community through metabolic handoffs and where the final product of one organism is the substrate of the other (Pfeiffer et al., 2001, Angell et al., 2018, Hug and Co, 2018).

### 1.3.1. Oxygen regulation on $N_2O$ emissions

Oxygen is a key element in the N-cycle, defining which pathway prevails or can also influence the co-occurrence of pathways (Kalvelage et al., 2011). The most relevant implications that $O_2$ might have on the nitrogen cycle are the changes in ocean ecosystems associated with the OMZs expansion and potential impact in ocean-climate feedback through the production of $N_2O$ (Schmittner and Galbraith, 2008). Ocean deoxygenation is a predictable environmental consequence, derived from the anthropogenic influence, altering water chemistry, and leading to biogeochemical adjustments through the expansion of anaerobic metabolisms which are oxygen-sensitive in anoxic regions (Stramma et al., 2008, Keeling et al., 2010, Deutsch et al., 2011, Lam and Kuypers, 2011, Ulloa et al., 2012). Moreover, it was recently demonstrated from model simulations that anaerobic metabolisms, such as denitrification and sulfate reduction, can also occur in oxygenated waters but in anoxic microenvironments developed inside marine snow particles implying, therefore, a strong impact on nitrogen cycling (Bianchi et al., 2018).

The microbial $N_2O$ reduction step, which is catalyzed by the nitrous oxide reductase, is the most sensitive to $O_2$ and is, therefore, a strong regulator of denitrification influencing the

emissions of $N_2O$. Dissolved oxygen affects the rates of aerobic and anaerobic processes (Henriksen et al., 1981, Rysgaard et al., 1994), and mediates the switch or coupling of nitrification and denitrification processes in the oxic-anoxic interface in marine environments, particularly in OMZs where AOA can have an important influence on the $N_2O$ production (Ji et al., 2015, Trimmer et al., 2016, Qin et al., 2017). Generally, when $O_2$ decreases (< 20 µmol $L^{-1}$), aerobic ammonia oxidizers are the main responsible for $N_2O$ production, but when $O_2$ is consumed the remineralization/oxidation of organic matter is driven by an alternate electron acceptor (e.g. $NO_3^-$) stimulating complete denitrification where $N_2O$ is reduced to $N_2$ (Codispoti and Christensen, 1985, Lam and Kuypers, 2011). Additionally, studies on ETSP oxygen minimum zone waters verified higher $N_2O$ yields at the oxic-anoxic interface above the oxygen-deficient zone where the slightly oxygenated waters support both nitrification and denitrification (Ji et al., 2015). This interaction can occur as a nitrifier-denitrification or coupled nitrification-denitrification (**Figure 1.2**), where microorganisms use the $NO_2^-$ from partial nitrification as terminal electron or the regenerated $NO_3^-$ from complete nitrification, respectively. This coupling contributes to the removal of remineralized N from the environment and the subsequent release to the atmosphere as $N_2O$ or $N_2$. The gradients of dissolved oxygen in several marine environments (e.g. intertidal sediments, OMZs) strongly regulate $N_2O$ production through the balance between N-cycle pathways and adaptation of the microbial community composition to changing conditions (Kalvelage et al., 2011, Kock et al., 2016, Wittorf et al., 2016, Barnes and Upstill-Goddard, 2018). Therefore, oxygen plays an important role in inducing the shift or the coexistence of aerobic and anaerobic N-cycle processes, with repercussions on $N_2O$ production rates.

### 1.3.2. Sulfur cycle and $N_2O$ implications

In anaerobic conditions, coupled abiotic-biotic reactions can occur where some microorganisms are able to use inorganic compounds as electron donors, instead of organic carbon, to generate energy in a process called chemolithotrophy (Peck, 1968, Gottschalk, 1986, Kelly, 1999).

Denitrifying microorganisms are metabolically diverse and versatile, with the ability to switch between aerobic and anaerobic metabolisms (Zumft, 1997, Philippot, 2002). Denitrifiers are mostly heterotrophs, that obtain energy through the oxidation of dissolved organic matter, whereas others can grow autotrophically gaining energy from the oxidation of inorganic compounds (e.g. reduced sulfur compounds, hydrogen). In some environments with the absence of oxygen, organisms perform chemolithoautotrophic denitrification wherein can use oxidized nitrogen compounds, that act as electron acceptors, via reduced sulfur (e.g. hydrogen

sulfide, $H_2S$) oxidation (Lavik et al., 2009, Lam and Kuypers, 2011, Stewart, 2011, Grote et al., 2012, Ulloa et al., 2013). The anthropogenic nutrient load to marine ecosystems promotes the export of organic matter from the surface to deeper waters, leading to the oxygen depletion, enhancing the denitrification levels which potentially expands and intensifies OMZs throughout the oceans (Codispoti, 2010, Ji et al., 2015, Martinez-Rey et al., 2015). This coupling of denitrification and sulfide oxidation has been observed in suboxic zones, at the boundary between the oxic layer and sulfidic deep waters (Brettar and Rheinheimer, 1991, Stewart, 2011, Dalsgaard et al., 2013). Additionally, $N_2O$ production is redundant in suboxic/anoxic environments and is usually associated with sulfide oxidation, namely in OMZs, representing a serious environmental threat to global climate due to the massive $N_2O$ release to the atmosphere (Canfield et al., 2010b). This happens due to an imbalance in the suboxic zones between bacterial processes that produce $N_2O$ and those who consume it generating an excess of $N_2O$ liberated to the atmosphere (Babbin et al., 2015). In these suboxic zones, the presence of sulfide as an electron donor for microbial nitrate/nitrite respiration via denitrification (Kraft et al., 2014), is associated with the inhibition of $N_2O$ reduction in the denitrification pathway (Canfield et al., 2010b).

The N-S interaction was previously observed in the redox transition zone of a coastal marine sediment near the sulfide maxima concentration (Sørensen, 1978). The sulfide inhibition was later confirmed on the nitrous oxide reduction pathway of denitrification resulting in $N_2O$ accumulation (Sørensen et al., 1980, Seitzinger et al., 1984). This apparent regulation of the N cycle opened a precedent for researching new and possible sulfur compounds which could influence the nitrogen biogeochemical pathways. A new interaction between marine organic sulfur and nitrogen has been previously explored (Magalhães et al., 2012a). Indeed, dimethylsulfoniopropionate (DMSP) degradation product(s) were found to interfere with the $N_2O$ reduction step of denitrification in different estuarine sediments and rocky biofilms. DMSP is an important component of global sulfur cycle constituting ca. 24% of the total organic sulfur in surface seawater (Bates et al., 1994), and biosynthesized mainly by marine phytoplankton and macroalgae (Gage et al., 1997, Summers et al., 1998). It was identified a DMSP breakdown product – methanethiol (MeSH) – which directly affects the accumulation of $N_2O$ in benthic coastal environmental samples and in *R. Pomeroyi* DSS-3 cultures, which could possibly have an inhibitory effect on the last/terminal reduction step of denitrification (Magalhães et al., 2011). These studies confirm the dynamic interaction between marine N and S cycling, and the need of understanding the complexity of these intricate processes to make better estimations about gas emissions to the atmosphere and their consequent impact on the climate. Furthermore, it is essential to determine if MeSH is influencing the microbial denitrification activity at a transcriptional and/or enzymatical level and elucidate about what

type of regulation on $N_2O$ reduction occurs in natural environments. The mechanism(s) responsible for the interaction between organic S and N cycles and the potential regulation of $N_2O$ accumulation are further explored in the following chapters.

## 1.4. Relevance and objectives of the thesis

Nitrogen and sulfur are essential elements to maintain life on Earth. The anthropogenic pressure in the marine environment can alter these biogeochemical cycles, influencing gas emissions (e.g. dimethyl sulfide – DMS, $N_2O$) into the atmosphere and potentially affect global climate. There is an increasing need to understand the formation processes of N compounds and the factors that influence these processes, the complex network of pathways within the N-cycle and the connection to other biologically important elements, such as sulfur.

The biogeochemical interaction of N and organic S cycles is of global ecological significance in marine ecosystems. However, research on these complex interactions is still scarce, and little is known about the molecular and biogeochemical mechanisms behind the respective pathways.

Therefore, this thesis aims (i) to characterize the organic S and N cycles at the biogeochemical and molecular level, (ii) understand how organic S compounds interact with key steps of denitrification, and (iii) how it affects $N_2O$ production. Because DMS, one of DMSP degradation compounds, and $N_2O$ have contrasting effects on global climate, this study represents an advance in the understanding of the global dynamics of both marine S and N cycles, by providing clues to the global quantification and balance of each of these climate-relevant compounds.

Therefore, the overarching goal of this thesis is to analyze and characterize the marine biogeochemistry of organic S and N cycles, and explore the coupling of DMSP catabolism with the denitrification pathway, leading to a detailed understanding of the biogeochemical network linking these two elements.

The specific objectives are:

- Analyze the spatial-temporal dynamics and the influence of environmental factors on N-cycle microbial communities, through the distribution and abundance of nitrification and denitrification key genes, as well as the non-denitrifying nitrous oxide reducers, using as proxy the temperate Douro estuary.

- Characterize the inhibitory interaction between DMSP metabolism and the last step of denitrification, by examining the molecular mechanism(s) involved in this interaction at the biogeochemical and transcriptomic levels in marine bacteria.
- Evaluate the influence of methanethiol, a DMSP degradation compound, on the $N_2O$ reduction step of denitrification by analyzing the expression of denitrifying genes and $N_2O$ production on a model organism.

This thesis is structured in five chapters. The current **CHAPTER** 1 begins with a literature review that explores the marine biogeochemical networks of nitrogen cycle mediating nitrous oxide flux, followed by a brief description of the objectives of this study. **CHAPTER 2** analyzes the influence of the spatial-temporal variability of environmental factors, in the temperate Atlantic Douro estuary, on the annual distribution of N-cycle functional genes (bacterial and archaeal ammonia oxidizers, denitrifiers and the newly recognized non-denitrifying organisms). After assessing the microbial N-cycle dynamics in an estuarine system, **CHAPTER 3** focus on the biogeochemical and molecular characterization of the interaction between organic S and N cycles, by monitoring gas (DMS, MeSH and $N_2O$) accumulation and analyzing the gene expression changes related to DMSP catabolism and denitrification in bacterial cultures. **CHAPTER 4** evaluates the direct influence of MeSH, the final product of the preponderant DMSP catabolism pathway in the ocean - demethylation, on the $N_2O$ accumulation by identifying the molecular and enzymatic mechanism(s) that underlie that interaction. Then, **CHAPTER 5** delivers the general conclusions of this study and recommends the future research directions.

# CHAPTER 2

## Spatial-temporal dynamics of N-cycle functional genes in a temperate Atlantic estuary (Douro, Portugal)

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# Abstract

Understanding the spatial and seasonal dynamics of nitrogen (N) – cycle microbial communities is pivotal for the knowledge of the N biogeochemistry. This research dealt with the spatial-temporal variability of nitrification (bacterial and archaeal *amoA*) and denitrification (*nirS*, *nirK* and *nosZ*I) key genes, as well as of non-denitrifying nitrous oxide ($N_2O$) reducers (*nosZ*II), coupled with key environmental variables, in an estuarine ecosystem (Douro, NW Portugal). Samples were collected on a monthly basis over one year, key physical-chemical parameters measured, and specific functional genes abundance assayed. The results revealed a clear seasonality for *nirS*, *nosZ*II, bacterial and archaeal *amoA* abundance, with an increase during the winter/spring seasons. This period was especially characterized by high levels of dissolved oxygen, low temperature, low salinity, and increased turbidity. Indeed, turbidity emerged as the key factor controlling the distribution of *nirS*, *nosZ*II bacterial, and archaeal *amoA* abundance. In contrast, the abundance of *nosZ*I increased during the summer, while the *nirK* abundance was enhanced from the fall to late spring. Additionally, the availability of nitrogen dissolved nutrients had no commensurable effect on N-cycle functional genes. This study covers the annual variation of N-cycle functional genes in a temperate Atlantic estuary and provides a major contribution for the understanding on how environmental factors potentially influence the distribution and abundance of N-cycle microbial communities.

# 2.1. Introduction

Understanding how environmental factors control the abundance and distribution of microorganisms is fundamental to microbial ecology studies and to evaluate the impact on global biogeochemical cycles (Fuhrman et al., 2006, Steele et al., 2011). Estuaries are dynamic ecosystems with distinct spatial and temporal environmental variations (McLusky and Elliott, 2004), that may shape the structure of microbial communities. These transition systems receive increased loads of nutrients, particularly nitrogen which can be transformed by microbes and promote critical biogeochemical functions, leading eventually to its removal (Damashek and Francis, 2018). Denitrification and ammonia oxidation are major microbially mediated processes that play a key role in the global nitrogen cycle (Voss et al., 2013). Canonical denitrification occurs under low oxygen conditions, and is a metabolic respiratory process which consists in the dissimilatory stepwise reduction of nitrate ($NO_3^-$) through nitrite ($NO_2^-$), nitric oxide (NO), $N_2O$ to dinitrogen gas (N2; Knowles, 1982, Zumft, 1997). The reduction of $N_2O$ to $N_2$ is catalyzed by a metalloenzyme encoded by the *nosZ* (henceforth addressed of *nosZ*I) gene (Pauleta et al., 2013). Denitrification represents an ecological important process. In the one hand, promotes the biological removal of nitrogen from natural or anthropogenic systems (Seitzinger et al., 2006), reducing the eutrophication risk. On the other hand, can affect global climate due to the potential emission of the greenhouse gas (GHG) $N_2O$ (Philippot et al., 2011). Moreover, a newly recognized and abundant phylogenetic clade of $N_2O$-reducers, which generally have an atypical NosZ protein (encoded by the, henceforth addressed of, *nosZ*II), act as effective $N_2O$ consumers (Sanford et al., 2012, Jones et al., 2013, Hallin et al., 2018).

Nitrification is an autotrophic, aerobic process in which microorganisms obtain energy by converting ammonia ($NH_3$) to $NO_3^-$ (Bock and Wagner, 2013). The first oxidation step of nitrification is the transformation of $NH_3$ into $NO_2^-$ (nitritation), catalyzed by the ammonia monooxygenase (AMO), which most environmental studies have originally focused on the ammonia-oxidizing bacteria (AOB), belonging to the *β*-subclass (Kowalchuk and Stephen, 2001). The discovery of ammonia monooxygenase genes associated with archaeal scaffolds within marine metagenomic datasets (Venter et al., 2004), subsequently confirmed the existence of marine ammonia-oxidizing archaea (AOA; Könneke et al., 2005), usually dominating ocean nitrification (Francis et al., 2005, Wuchter et al., 2006, Santoro et al., 2011). The last oxidation step results in the transformation of $NO_2^-$ to $NO_3^-$ (nitratation), by nitrite-oxidizing bacteria (NOB), eventually supplying denitrification with the required $NO_3^-$. Microbial ecology molecular studies use *nirS*, *nirK*, and *nosZ* as marker genes of denitrifying bacteria in order to quantify the functional community in several environments (Henry et al., 2006,

Kandeler et al., 2006). The bacterial and archaeal *amoA*, that encodes the ammonia monooxygenase-α subunit, serve as proxy for the quantification of ammonia oxidizers (AO; Rotthauwe et al., 1997, Mincer et al., 2007).

With increasing awareness about the vital role of microbial communities to ecosystem functions, and how they may be impacted by climate change (Cavicchioli et al., 2019), the need to comprehend the feedback response of microorganisms to environmental change constitutes a fundamental quest for microbial research. In estuaries, some studies advocate that the AO and the denitrifying communities are strongly influenced by salinity fluctuations (Bernhard et al., 2010, Francis et al., 2013). Additionally, ammonium concentration seems to be a relevant factor for the niche separation between AOB and AOA (Urakawa et al., 2014). Nitrate can influence the selection between denitrification and dissimilatory reduction to ammonia (DNRA), coupled with the nitrate affinity or the availability of other elements (e.g. sulfide, $Fe^{2+}$), that may favor nitrate ammonifiers (Brunet and Garcia-Gil, 1996, Kessler et al., 2018).

Since habitat specific features of the ecosystems determine the diversity and distribution of microbial communities, we hypothesized that the seasonal variability of key environmental parameters may influence the distribution of N-cycle microbial communities in a temperate estuary. Using real-time PCR, we assessed and characterized the spatial-temporal variations of key functional genes of AOB and AOA (*amoA*), denitrifying (*nirS*, *nirK*, *nosZ*I), and the novel non-denitrifying $N_2O$ reducers (*nosZ*II). In addition, the relationship between key environmental parameters and the functional gene abundance were also assessed to determine the potential environmental drivers on the distribution of N-cycle microbial communities.

# 2.2. Material and Methods

## 2.2.1. Study area and water sampling

Water samples were retrieved from the Douro river estuary (NW Portugal, **Figure 2.1**). The Douro river drains an international watershed of 97,682 $Km^2$, and the estuary is limited by a hydroelectric power dam located 21.6 Km from the mouth (Bordalo and Vieira, 2005). The mean annual flow reaches 505 $m^3 s^{-1}$, ranging from 0 to >13,000 $m^3 s^{-1}$ during the summer and winter, respectively (Vieira and Bordalo, 2000, Azevedo et al., 2010). The water is contaminated by treated and untreated sewage disposal (Ribeiro et al., 2018). The lower, middle and upper estuarine sectors were based on the salinity gradient, according to Vieira and Bordalo (2000).

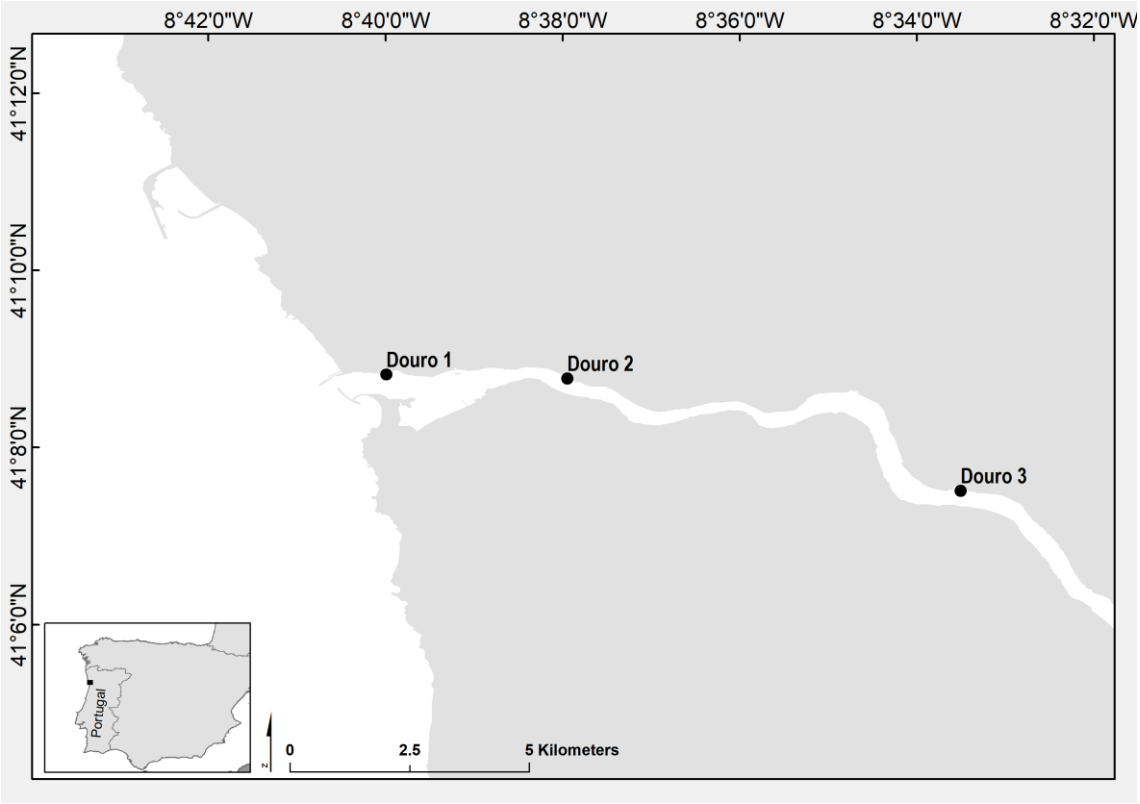


**Figure 2.1.** Location of the sampling sites in the Douro estuary. The three sampling areas correspond to the lower, middle and upper stretches, according to Vieira and Bordalo (2000).

Monthly sampling surveys were performed for one year (from July 2009 to June 2010), at low tide, at three sampling sites (lower, middle, and upper stretches), covering the

present full length of the estuary (**Figure 2.1**). The exact position of each sampling site was obtained by means of GPS (Magellan 600, San Dimas, CA, USA). Key physical and chemical parameters, namely temperature, salinity, pH, oxygen saturation, and turbidity were measured *in situ,* using a YSI6920 CTD multiparameter probe (YSI Inc., Yellow Springs, OH, USA). Surface water samples were collected using 500 mL acid-cleaned polyethylene bottles for chemical parameters analysis, and sterile bottles for microbiology analysis. All samples were kept in the dark, refrigerated in ice chests until further analysis.

Nutrient concentration of phosphate ($PO_4^{3-}$), nitrite ($NO_2^-$), nitrate ($NO_3^-$), and ammonium ($NH_4^+$), were determined colorimetrically using methods described in Grasshoff et al. (Grasshoff et al., 1983). Chlorophyll *a* (Chl*a*) concentration was assayed according to Parsons et al. (1984). Particulate organic matter (POM) was measured as percentage of weight loss on ignition (500 ºC, 2 h) (APHA 1992). Determination of dissolved total carbon (TC), organic carbon (DOC), and total nitrogen (TN) was performed by high temperature catalytic oxidation with a TOC-VCSN analyzer coupled to a total nitrogen-measuring unit (Shimadzu Instruments, Kyoto, Japan), according to the manufacturer instructions.

### 2.2.2. Nucleic acids isolation

Water samples were concentrated onto cellulose nitrate membranes (0.22 µm pore size, 0.47 mm diameter, Whatman, GE Healthcare, UK), and total DNA of each sample was extracted using the DNeasy PowerSoil Kit (QIAGEN, Germany) following the manufacturer instructions. The DNA quality was checked on 2% (w/v) agarose gel, and quantified on a Qubit fluorometer (Life Technologies, Carlsband, CA, USA), using the Quant-iT dsDNA assay.

### 2.2.3. Quantification of N-cycle functional genes

All qPCR reactions were carried out in duplicates containing 30 – 180 ng (for *nirS*, *nirK* and *nosZ*I amplification; Table S1), or 5 – 60 ng (for *nosZ*II and *amoA* from AOB and AOA amplification; Table S1) of DNA template, and *Power* SYBR® Green PCR Master Mix (2X), that included ROX as a passive reference, to a final 20 µl reaction volume. All reactions were performed in MicroAmp Fast Optical 96-Well Reaction Plates (Bio-Rad), using optical adhesive cover, and performed on a CFX96™ Real-Time System/C1000™ Thermal cycler (Bio-Rad). A no-template control was included for each run. Gene-

specific primers sequences and thermal cycling conditions are detailed in Table S1. A composite sample was prepared by adding equal volumes of all the extracted DNA samples from the Douro. This composite sample was used as template for amplification of each gene, the resulting PCR amplicon analyzed by agarose gel electrophoresis, and subsequently quantified by fluorometry. Quantification of functional gene abundance was achieved through calibration curves for each target gene by serially diluting (10-fold) the PCR amplicon. Gene copy numbers were determined based on the calibration curves, assuming one gene copy *per* genome (except the AOB, carrying an average of 2.5 copies), and normalized against mass (ng) of the extracted DNA. To evaluate qPCR reactions specificity, a melting curve analysis was performed at the end of each reaction, and all PCR products were analyzed on 2% (w/v) agarose gel to ensure that only expected target genes were amplified without artifacts. qPCR results were analyzed with the software Bio-Rad CFX Manager 1.6 (Bio-Rad Laboratories).

### 2.2.4. **Data analysis**

Statistical analysis was performed with the open source-software R version 3.3.3 (R Development Core Team 2013). The proportion of N-cycle functional genes and total microbial community was estimated by normalizations against RNA polymerase subunit gene (rpoB; Dahllöf et al., 2000, Vos et al., 2012). Significant changes in the ratios and abundance of each functional gene were analyzed by means of the Mann-Whitney U test ($p < 0.001$), after examining the distribution and homoscedasticity of data using the Shapiro-Wilk and Levene tests, respectively (Levene, 1960, Shapiro and Wilk, 1965). For the ordination analysis, non-normal distributed data were log transformed. The temporal associations between environmental parameters and nitrogen cycling functional genes were analyzed with the standardized data (to zero mean and unit variance), through a constrained redundancy analysis (RDA) performed with the vegan package (Oksanen et al., 2019). Collinearity between environmental variables was evaluated using the variance inflation factor (VIF). Collinear variables with a VIF > 10 were excluded from the RDA model. General associations between environmental parameters and the functional genes abundances were further explored with the Spearman's rank correlation coefficient, being the correlation *p*-values calculated by means of the corrplot and Hmisc packages (Wei and Simko, 2017, Harrell Jr, 2019).

## 2.3. **Results**

### 2.3.1. Environmental characterization

Temperature ranged from lower values in the winter months (8.0 – 10.9 ºC), to higher values in the summer (21 – 24.7 ºC; **Figure 2.2a**). Salinity decreased from November to May (0.1 – 4.8), due to higher river runoff (**Figure S2-1**), while September displayed the highest salinity in the range 13.9 – 29.5 (**Figure 2.2b**). The lowest dissolved oxygen concentrations were observed in July (2.0 mg $L^{-1}$ – lower estuary), while higher oxygenation was found during the winter season (10.8 – 15.0 mg $L^{-1}$, **Figure 2.2c**). Phytoplankton Chl *a* peaked in July (9.8 µg $L^{-1}$ – upper estuary), and August (10.1 µg $L^{-1}$ – lower estuary), corresponding to the summer phytoplankton bloom (**Figure 2.2d**). During the winter months, turbidity increased, namely in January at the lower estuary (33.5 NTU, **Figure 2.2e**), and in March at the upper stretch (33.2 NTU, **Figure 2.2e**). Total nitrogen (TN) ranged 0.017 – 3.21 mg $L^{-1}$ (**Figure 2.2f**), with overall higher concentrations in the winter, while POM levels fluctuated during the sampling surveys (**Figure 2.2g**). The annual distribution of ammonium and nitrite concentrations ranged 0.8 – 134.8 µM (**Figure 2.2h**), and 0.2 – 2.8 µM (**Figure 2.2i**), respectively, with a peak in September in the upper estuary. Nitrate was the most abundant inorganic nitrogen form (24.6 – 373.1 µM; **Figure 2.2j**), with the highest concentrations observed in August in the lower stretch.

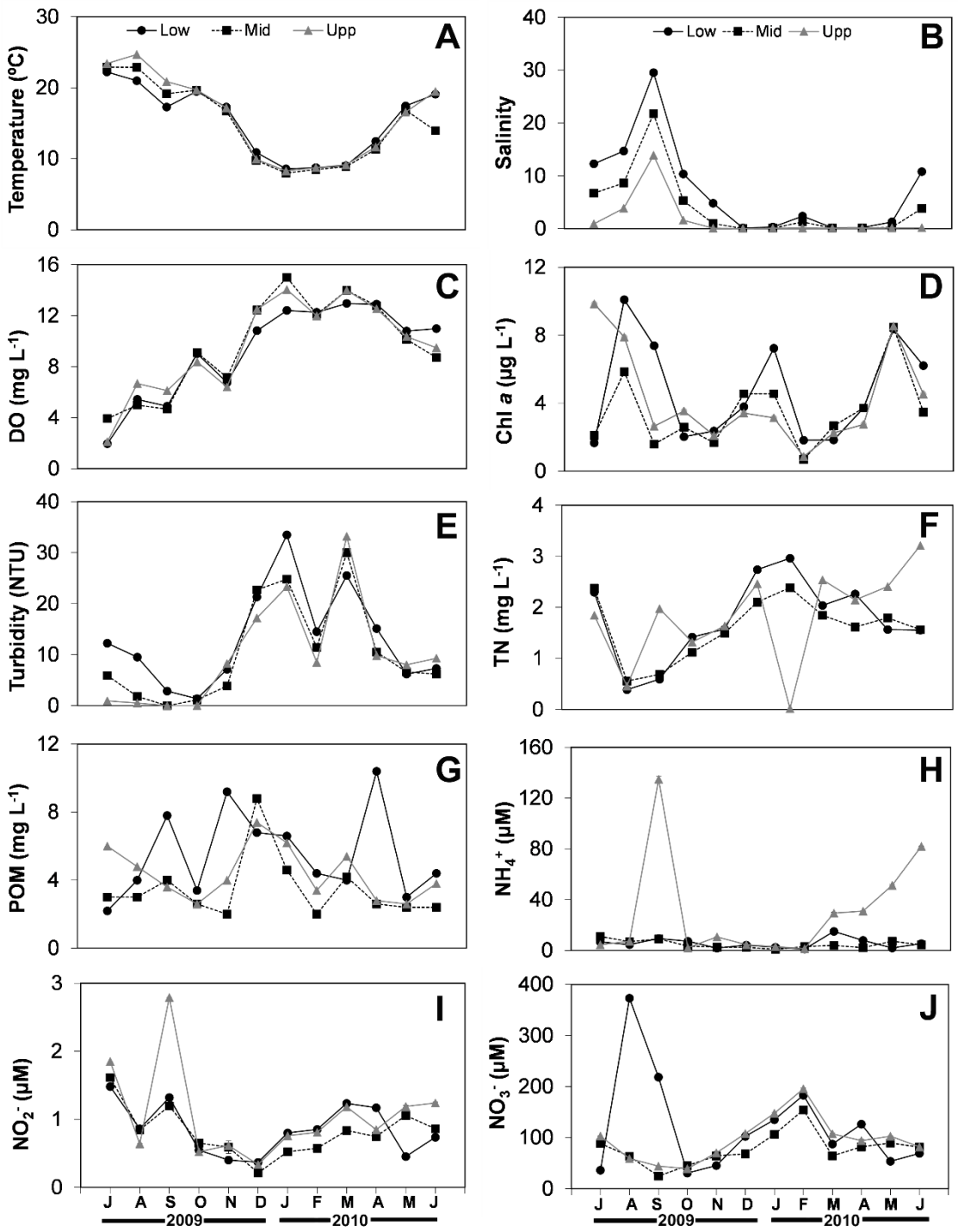


**Figure 2.2.** Seasonal variability of Temperature (ºC), Salinity, Dissolved Oxygen (mg $L^{-1}$), Chlorophyll *a* (mg $L^{-1}$), Turbidity (NTU), Total Nitrogen (TN; mg $L^{-1}$), Particulate Organic Matter (POM; mg $L^{-1}$), and dissolved nutrient concentrations (mean ± SD, 3 replicates), in the three sampling stations of the Douro river estuary, between July 2009 and June 2010.

### 2.3.2. Relationships between environmental variables and functional genes

The Douro estuary presented environmental gradients associated with seasonality, a characteristic of temperate ecosystems. After selecting the studied environmental variables, we used a subset of the matrix for the constrained redundancy analysis, in order to determine which environmental variables best correlated with the variance of N-cycle functional genes. As a result, the entire microbial community variation could be explained by the environmental variables (90.1 %, **Figure 2.3**), which significantly ($R^2$ = 0.507; $p < 0.05$, 999 permutations) influenced the N-cycle functional genes. Overall, all N-cycle functional genes in the Douro estuary thrived in well oxygenated waters, being negatively influenced by temperature and salinity. Particularly *nirS*, *nirK*, bacterial, and archaeal *amoA* which presented higher abundance in the fall and winter months when the temperature and salinity were the lowest (Fig. 2), and displayed significant correlations with DO ($p < 0.01$, **Figure 2.4**, **Table S2-2**). TN was significantly linked to the abundance of the *nosZ*II and *nirK* ($p < 0.05$), *nirS* and *nosZ*I ($p < 0.01$, **Figure 2.4**, **Table S2-2**). Curiously, both AO were not associated with the availability of ammonium, typically the substrate used by ammonia oxidizers. Instead, AO were more related to turbidity, which was the explanatory variable most strongly related with the first RDA axis (**Figure 2.3**). Turbidity in the estuary was strongly linked to DO ($p < 0.001$, **Figure 2.3** and **Figure 2.4**). As expected, the seasonal profiles of turbidity and river flow were similar, with an increase during winter and spring (**Figure 2.2e** and **Figure S2-1**). In addition, linear regression analysis suggested that 37 – 47% of the variability in *nirS*, *nirK*, and AOA abundance (**Figure S2-2**) was significantly explained by the river flow ($p < 0.05$).

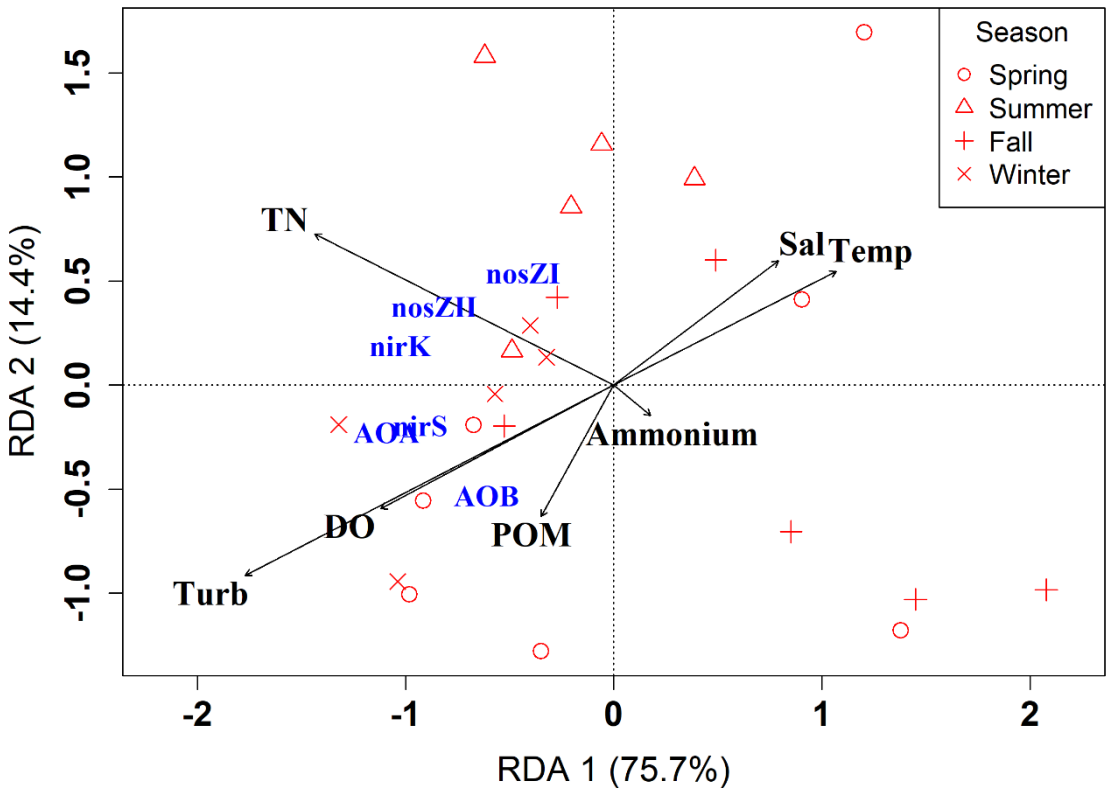


**Figure 2.3.** Ordination triplot of redundancy analysis between physical-chemical parameters and the seasonal distributions of ammonia oxidizers (AOB, AOA), denitrifying (*nirS*, *nirK*, *nosZ*I), and non-denitrifying (*nosZ*II) functional gene abundance, in the Douro estuary. Each ordination axis represents the proportion of variance explained by environmental variables. AOB – bacterial *amoA* gene; AOA – archaeal *amoA* gene; Temp – Temperature; Sal – Salinity; DO – Dissolved Oxygen; Turb – Turbidity; POM – Particulate Organic Matter; TN – Total Nitrogen.

The redundancy analysis showed that archaeal and bacterial *amoA* and *nirS* were mostly associated with the winter and spring seasons, also evidenced by the temporal profiles of the functional genes abundance which was clearly enhanced in those seasons (**Figure 2.5**), when the water was well oxygenated and murky. Additionally, *nirS* was significantly correlated with all functional genes ($p < 0.001$), namely with the bacterial *amoA*, which was also demonstrated by the similar spatial-temporal gene abundance patterns (**Figure 2.3**).

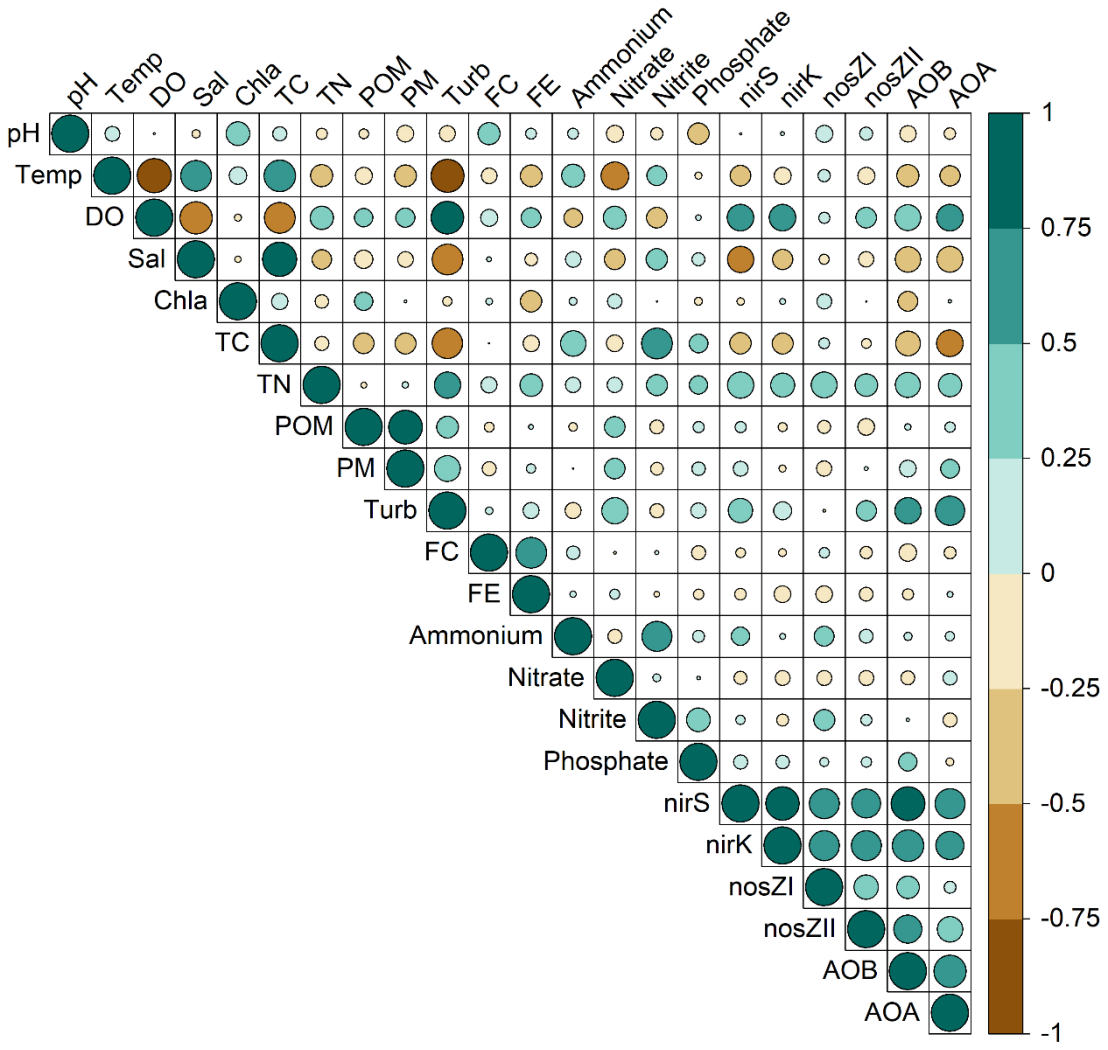


**Figure 2.4.** Correlogram of Spearman's rank correlation coefficients between the environmental features and the gene abundances of nitrogen cycle functional genes. Temp – Temperature; DO – Dissolved Oxygen; Sal – Salinity; Chl *a* – Chlorophyll *a*; TC – Total Carbon; TN – Total Nitrogen; POM – Particulate Organic Matter; PM – Particulate Matter; Turb – Turbidity; FC – Fecal coliform; FE – Fecal enterococci; AOB – bacterial *amoA* gene; AOA – archaeal *amoA* gene.

The bacterial community (based on *rpoB* gene abundance) revealed a different temporal profile, with higher values occurring mainly in the Fall (**Figure S2-3**). On the other hand, the proportion of the bacterial N-cycle functional genes to the *rpoB* gene abundance, yielded a significant seasonal variability ($p < 0.01$; **Figure S2-4**), in the three sections of the estuary. The highest ratio was observed in August, occurring at the same time with the highest Chl *a* concentration (**Figure 2.2d**).

### 2.3.3. Distribution of N-cycle functional genes

Overall, results displayed distinct patterns of N-cycle functional gene abundance (**Figure 2.5**). The archaeal *amoA* peaked in mid-summer (July) at the middle estuary, to recover again during the winter months (**Figure 2.5a**). The bacterial *amoA* abundance also increased during late winter (February-March), at the upper and lower stretches, while the middle estuary showed lower abundance (2.6 x $10^6$ gene copies $ng^{-1}$, **Figure 2.5b**).

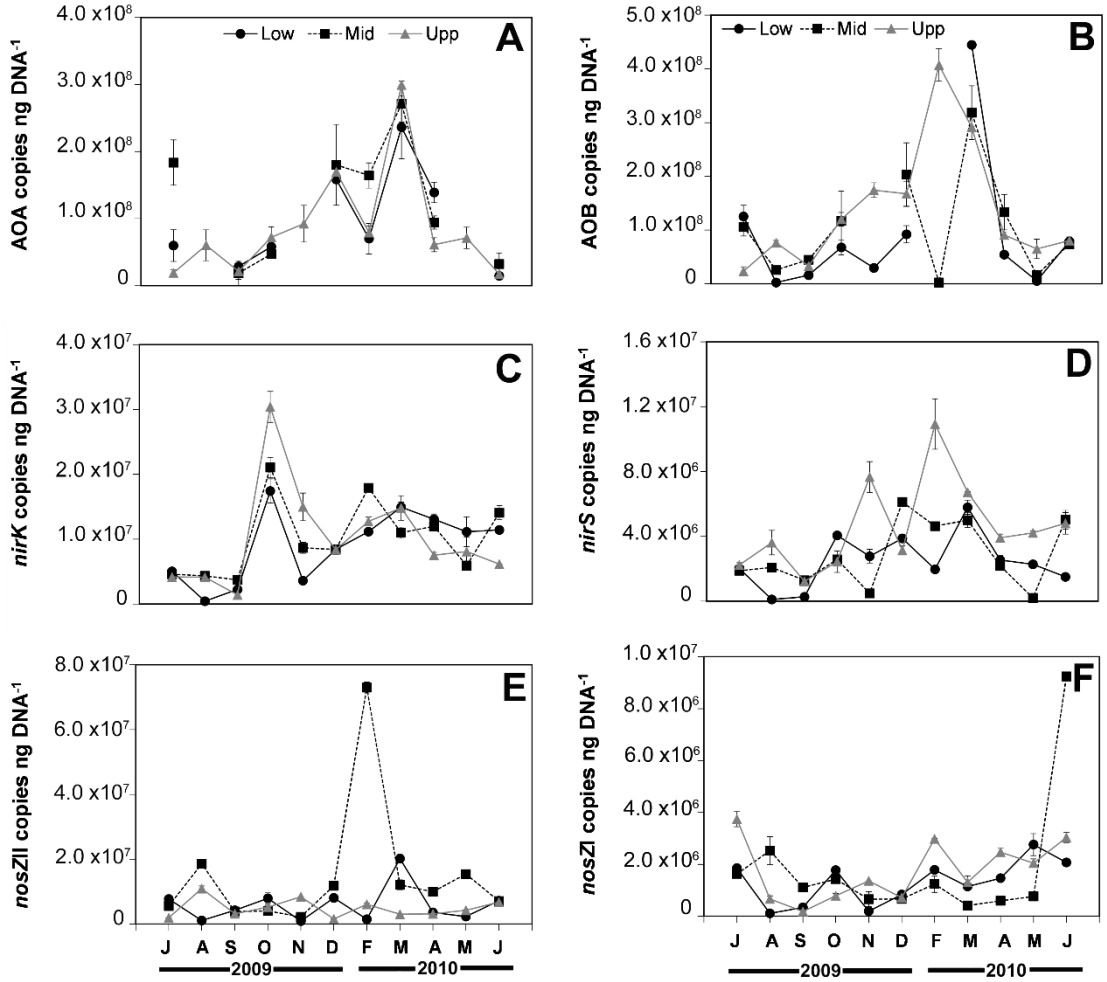


**Figure 2.5.** Spatial-temporal distribution of (a) archaeal *amoA*, (b) bacterial *amoA*, (c) *nirS*, (d) *nirK*, (e) *nosZ*I and (f) *nosZ*II, in water samples from the Douro estuary, from July 2009 to June 2010. The relative abundance of each functional gene is expressed as gene copies normalized to ng of extracted DNA. Error bars represent the standard deviations of the means. Samples from January (2010) were missing, and the month was not included in the analysis.

The denitrifying communities had different temporal distributions throughout the year. For instance, *nirK*-type denitrifiers abundance varied 4.4 x $10^5$ – 3.0 x $10^7$ gene copies $ng^{-1}$ (**Figure 2.5c**), with higher concentrations in early fall (October) within the estuary, decreasing towards late winter (February). The abundance of *nirS* was higher upstream in mid fall (November), expanding throughout the estuary during winter (**Figure 2.5d**).

The *nosZ*II abundance, related to the non-denitrifying $N_2O$ reducers, increased during the winter period (December – March), particularly in the middle estuary (**Figure 2.5e**). The *nosZ*I denitrifying communities remained low throughout the year ($1.1 \times 10^5 – 3.7 \times 10^6$ gene copies $ng^{-1}$), with a seasonal profile different from the *nirS* and *nirK*. Increased abundance was observed at the onset of the summer (June), within the middle estuary (**Figure 2.5f**).

## 2.4. **Discussion**

In the Douro estuary, the microbial nitrogen-cycle genes abundance had distinct seasonal patterns, constrained by environmental factors. All studied N-cycle functional genes were generally more abundant in well oxygenated, lower temperature and salinity waters, typical of the winter season. Therefore, the distribution of denitrifying *nirS*, *nirK*, *nosZ*II, and bacterial and archaeal *amoA* was influenced by the seasonality of environmental factors. Previous studies (Santoro et al., 2006, Jones and Hallin, 2010, Francis et al., 2013), have demonstrated that the diversity and abundance of denitrifying communities can be governed by environmental variables, responding differently to estuarine salinity and temperature gradients. Likewise, Lee and Francis (2017) observed that *nirK* had a higher relative abundance with low temperatures, while *nirS* was enhanced with high nitrate concentrations. But, in the Douro estuary, *nirS* had higher gene abundance namely during the winter season, with low temperature and salinity, highlighting the specificities of different estuaries. The denitrifying enzymes NirK or NirS (encoded by the *nirK* and *nirS* genes, respectively) are structurally different and believed to be non-homologous, responding differently to environmental gradients (Jones and Hallin, 2010). Additionally, the distribution of denitrifying *nosZ*I and non-denitrifying *nosZ*II genes, was predominantly related to dissolved oxygen, an important environmental factor that leads to niche partitioning between *nosZ* clade I and II, as it was demonstrated by Wittorf et al. (2016). Nevertheless, all studied N-cycle genes, including denitrifying, were abundant in high oxygenated estuarine waters. Although denitrification and $N_2O$ reduction are anaerobic processes, the occurrence of these functional genes in well oxygenated waters can be derived from the presence of microorganisms that also possess denitrifying genes.

The present study evaluated the microbial community by DNA analysis, and the presence of functional genes does not necessarily indicate the expected activities. However, certain organisms can possess and express denitrification genes under

aerobic growth, being the activity stimulated when organisms face a switch between anoxic to oxic conditions (Ka et al., 1997, Morley et al., 2008, Marchant et al., 2017). On the other hand, denitrification can occur in the presence of low levels of oxygen when microorganisms are metabolizing in anoxic microenvironments, such as within suspended particles (reviewed in Brezonik, 2013). Estuaries are dynamic bodies of water that receive freshwater inputs resulting in high inorganic nutrient and phytoplankton fluxes, stimulating primary production (Joint and Pomroy, 1981). Additionally, the freshwater runoff delivers dissolved and particulate materials that foster turbidity in the estuary. Indeed, in the Douro estuary, turbidity played a major role on the distribution of denitrifying *nirS*, the non-denitrifying $N_2O$-reducers (*nosZ*II), and the AO, which may be an indication of particle-attached microorganisms also capable of ammonia oxidation (Jing et al., 2018, Cai et al., 2019). Indeed, the occurrence of denitrification activity has been associated to small and large particles in coastal, river, and estuarine waters (Michotey and Bonin, 1997, Liu et al., 2013). The existence of anoxic microenvironments inside suspended or sinking particles, associated with high turbidity, has been suggested as hotspots for POM remineralization and a niche for the occurrence of heterotrophic denitrification process in the water column (Simon et al., 2014, Bianchi et al., 2018, Zhu et al., 2018). Still, since denitrifiers are also capable of aerobic respiration, the presence of denitrifying genes is not necessarily an indication of denitrification activity.

The Douro estuary is highly dynamic, influenced by the river flow (Vieira and Bordalo, 2000). The increased abundance of archaeal and bacterial *amoA* in the winter season (December – March) was strongly associated with turbidity when the river flow peaks. Indeed, high nitrification rates have been observed in turbid estuarine waters, with strong correlations with suspended particulate matter (SPM), possibly influenced by resuspension of benthic nitrifiers or ab ammonium release from sediment (Damashek et al., 2016, Sanders and Laanbroek, 2018). On the other hand, the higher turbidity in the lower estuary may also be related to sewage discharge, associated with increased levels of ammonium and fecal indicators (Azevedo et al., 2006, Azevedo et al., 2008). However, the abundance of fecal indicators and the levels of ammonium were not correlated with any of the studied N-cycle functional genes, suggesting that the sewage does not seem to be a relevant source for those genes in the Douro estuary. Additionally, river flow can act as a source of biogenic matter and shape the estuarine community, as well as influence the physical-chemical characteristics of estuaries, changing the environmental conditions (de Oliveira and Margis, 2015, Fortunato and Crump, 2015, Smith et al., 2019). Although the river runoff, in the Douro estuary, statistically explains the variability of *nirS*, *nirK*, and AOA abundance, river flow fails to show significant ($p > 0.05$)

relationships with the abundance of N-cycle genes. Therefore, its importance is not evident to explain the spatial-temporal dynamics of the abovementioned functional genes. During the freshet, winter season, the residence time in the water column decreases (Vieira and Bordalo, 2000). Consequently, during that period the river runoff continuously exports microbial populations towards nearshore and/or until they reach a 'hotspot' with extended water retention which gives enough time to increase the relative growth rate (Crump et al., 2004).

Ammonia oxidation kinetics can influence the niche separation between AOB and AOA (Martens-Habbena et al., 2009). Surprisingly, in the Douro estuary, the AOA and AOB were not correlated with ammonium. Although AO are known to use $NH_3$, rather than $NH_4^+$ as a substrate for their chemotrophic growth (Suzuki, 1974), the presence of ammonium in the water column, independently of its origin, is indicative of a nitrification potential (Sanders and Laanbroek, 2018). The high inorganic nutrient loads may not be limiting the nitrifying and denitrifying activity, which may explain the absence of correlation to N-cycle functional genes. Besides ammonium, other environmental parameters influenced AO abundance. Both AOB and AOA may exhibit a wide adaptive range of tolerance to environmental gradients and adapt to different estuarine characteristics (Bernhard et al., 2007, Gao et al., 2018). Salinity gradients have been associated with changes in the community structure and nitrification activity, but without consistent results on AO community shifts and distribution patterns (de Bie et al., 2001, Bernhard et al., 2005, Bernhard et al., 2007, Santoro et al., 2008). Although Magalhães et al. (2009) observed that the abundance of AOB in sandy sediments of the Douro estuary dominated over AOA, and positively correlated with salinity, the present study found a different pattern in the water column. Indeed, the AO abundance was more associated with low temperature and low salinity waters, as well as with high concentrations of DO and high levels of turbidity, usually occurring during the winter months.

Although the normalization against total DNA is a quantification approach used in molecular studies to measure the potential for N-cycle processes in the ecosystem (e.g. Christman et al., 2011, Wakelin et al., 2011, Soper et al., 2018), it is possible that on a temporal and spatial scale, changes in non-bacterial DNA contents could have biased the results. Additionally, because ecological data, especially from estuarine studies, are complex and explanatory variables are often correlated among each other (multicollinearity), caution must be made when interpreting results and making inferences. The co-occurrence between the functional genes can potentially represent an interaction between microorganisms, which reflect the influence of the environment

on their distribution (Faust and Raes, 2012, Graf et al., 2014). In this study, the strong relationship between *nirS* and bacterial and archaeal *amoA* indicated that these genes can be correlated with the same environmental variables. Nevertheless, the similar spatial-temporal distribution pattern between these genes, and the association with turbidity may also suggest the potential coupling between nitrification and denitrification processes, namely for particle-associated assemblages (Zhang et al., 2014, Zhu et al., 2018, Zhang et al., 2019). AO can remineralize the available organic matter while fueling the oxidation of the reduced inorganic nitrogenous compounds to nitrite/nitrate, making it available for denitrifiers to reduce (Zhu et al., 2013). Surprisingly, a noticeable association between *nirK* abundance and the *nosZ*II group was observed. Denitrifying organisms that harbor *nirK*, instead of *nirS*, are likely to have a truncated denitrification lacking the last step of $N_2O$ reduction (Graf et al., 2014). This link can hypothetically represent the interaction of microorganisms that perform single or isolated steps of each denitrification process, working as a co-op community through metabolic handoffs, and the final product of one organism is the substrate of the other (Hug and Co, 2018). This last occurrence can represent an interesting topic to be further explored, since there is a growing awareness of the interactions between organisms and how these networks shape and link biogeochemical cycles (Anantharaman et al., 2016).

## 2.5. **Conclusions**

Few studies have addressed how N-cycle processes and microbial communities respond to environmental change in the water column. Our research contributes to an improved understanding of how the microbial communities involved in the N-cycle are influenced by environmental factors in the waters of an estuarine system, and the potential implications on the microbial structure and N-biogeochemical cycle. The Douro river estuary had a specific environmental fingerprint that influenced the distribution and abundance of N-functional genes, with potential biogeochemical feedbacks. Overall, temperature, salinity, turbidity, and dissolved oxygen exerted a stronger influence on the variability of the genes encoding the nitrite-reductase (*nirS* and *nirK*), the non-denitrifying *nosZ*II, and bacterial and archaeal ammonia monooxygenase-α subunit (*amoA*). To some extent, the environmental patterns benefited the relationships between the N-cycle functional genes. Therefore, the obtained results supported the assumption that the combination of habitat-specific environmental variables, and not an isolated factor, may shape the structure of microbial communities, and control their abundance and

distribution. Overall, the present study provides an insight on the potential dynamics of N-cycle microbial communities responding to environmental change in a temperate estuary, and on the regulation of the newly recognized marine non-denitrifying nitrous oxide ($N_2O$) reducers.

## 2.6. **Supplementary Information**

**Table S2-1** Primers and qPCR cycling conditions used in this study.

| Target gene | Primer name | Primer sequence (5'-3') | Cycling conditions | Template (ng) | Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| *nirS* | nirScd3aF<br>nirSR3cd | AACGYSAAGGARACSGG<br>GASTTCGGRTGSGTCTTSAYGAA | (95°C, 7 min) x 1<br>(95°C, 15 s;(65°C – 60°C, -1°/cycle), 30 s; 72°C, 30 s) x 6<br>(95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 80°C, 30 s) x 35<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 40 – 60 | 96.2 | Kandeler et al., 2006 |
| *nirK* | NirK876<br>NirK1040 | ATYGGCGGVCAYGGCGA<br>GCCTCGATCAGRTTRTGGTT | (95°C, 7 min) x 1<br>(95°C, 15 s; 58°C, 30 s; 72°C, 30 s; 80°C, 30 s) x 35<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 30 – 50 | 89.6 | Henry et al., 2004 |
| *nosZ* (clade I) | nosZ2F<br>nosZ2R | CGCRACGGCAASAAGGTSMSSGT<br>CAKRTGCAKSGCRTGGCAGAA | (95°C, 7 min) x 1<br>(95°C, 15 s; 65°C, 30 s; 72°C, 30 s; 80°C, 30 s) x 35<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 130 – 180 | 90.1 | Henry et al., 2006 |
| *nosZ* (clade II) | nosZII-F<br>nosZII-R | CTIGGICCIYTKCAYAC<br>GCIGARCARAAITCBGTRC | (95°C, 7 min) x 1<br>(95°C, 30 s; 54°C, 1 min; 72°C, 1.5 min) x 40<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 5 – 10 | 93.9 | Jones et al., 2013 |
| Bacterial *amoA* | amoA-1F<br>amoA-2R | GGGGTTTCTACTGGTGGT<br>CCCCTCKGSAAAGCCTTCTTC | (94°C, 15 min) x 1<br>(94°C, 15 s; 56.2°C, 1 min; 72°C, 1.5 min; 78°C, 30 s) x 45<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 10 – 20 | 92.3 | Rotthauwe et al., 1997 |
| Archaeal *amoA* | CrenAmoAQ-F<br>CrenAmoAModR | GCARGTMGGWAARTTCTAYAA<br>AAGCGGCCATCCATCTGTA | (94°C, 15 min) x 1<br>(94°C, 15 s; 52°C, 30 s; 72°C, 1.5 min; 80°C, 30 s) x 40<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 5 – 10 | 103.3 | Mincer et al., 2007 |
| *rpoB* | rpoB1698F<br>rpoB2041R | AACATCGGTTTGATCAAC<br>CGTTGCATGTTGGTACCCAT | (95°C, 5 min) x 1<br>(95°C, 30 s; 54°C, 30 s; 72°C, 30 s; 80°C, 30 s) x 35<br>(95°C, 15 s;(60 to 95° C, 10 s, increment 0.5°)), x 1 | 5 – 10 | 90.5 | Dahllöf et al., 2000 |

**Table S2-2** Matrix of Spearman's coefficients with the correlation p-values between the environmental variables and the gene abundances of nitrogen cycle functional genes. Temp – Temperature; DO – Dissolved Oxygen; Sal – Salinity; Chl *a* – Chlorophyll a; TC – Total Carbon; TN – Total Nitrogen; POM – Particulate Organic Matter; PM – Particulate Matter; Turb – Turbidity; FC – Fecal Coliform; FE – Fecal Enterococci; Amm – Ammonium; Nitra – Nitrate; Nitri – Nitrite; Phos – Phosphate; AOB – bacterial *amoA* gene; AOA – archaeal *amoA* gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

| | pH | Tem | DO | Sal | Chl *a* | TC | TN | POM | PM | Turb | FC | FE | Amm | Nitra | Nitri | Phos | *nirS* | *nirK* | *nosZ*I | *nosZ*II | AOB | AOA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **pH** | 1.00 | 0.17 | 0.00 | -0.05 | 0.40* | 0.15 | -0.10 | -0.07 | -0.21 | -0.19 | 0.36* | 0.09 | 0.09 | -0.22 | -0.11 | -0.33* | 0.01 | 0.01 | 0.21 | 0.13 | -0.20 | -0.10 |
| **Temp** | | 1.00 | -0.85*** | 0.69*** | 0.23 | 0.72*** | -0.36* | -0.22 | -0.34* | -0.79*** | -0.18 | -0.35* | 0.38* | -0.55*** | 0.29 | -0.04 | -0.31 | -0.22 | 0.11 | -0.22 | -0.35 | -0.30 |
| **DO** | | | 1.00 | -0.75*** | -0.04 | -0.68*** | 0.40* | 0.25 | 0.27 | 0.77*** | 0.22 | 0.29 | -0.26 | 0.38* | -0.31 | 0.03 | 0.51** | 0.51** | 0.10 | 0.31 | 0.49** | 0.51** |
| **Sal** | | | | 1.00 | -0.03 | 0.83*** | -0.28 | -0.25 | -0.18 | -0.68*** | 0.02 | -0.12 | 0.18 | -0.31 | 0.34* | 0.13 | -0.50** | -0.30 | -0.08 | -0.18 | -0.48** | -0.49** |
| **Chl *a*** | | | | | 1.00 | 0.20 | -0.14 | 0.25 | 0.01 | -0.07 | 0.04 | -0.34* | 0.05 | 0.16 | 0.00 | -0.05 | -0.04 | 0.03 | 0.16 | 0.00 | -0.29 | 0.01 |
| **TC** | | | | | | 1.00 | -0.15 | -0.31 | -0.32 | -0.67*** | 0.00 | -0.20 | 0.48** | -0.21 | 0.68*** | 0.26 | -0.33 | -0.32 | 0.09 | -0.07 | -0.45* | -0.51* |
| **TN** | | | | | | | 1.00 | -0.03 | 0.03 | 0.51** | 0.20 | 0.36* | 0.18 | 0.18 | 0.32 | 0.25 | 0.50** | 0.42* | 0.49** | 0.37* | 0.46* | 0.39 |
| **POM** | | | | | | | | 1.00 | 0.82*** | 0.34* | -0.07 | 0.02 | -0.06 | 0.32 | -0.14 | 0.09 | 0.10 | -0.06 | -0.13 | -0.21 | 0.03 | 0.09 |
| **PM** | | | | | | | | | 1.00 | 0.49** | -0.15 | 0.07 | 0.00 | 0.32 | -0.12 | 0.13 | 0.16 | -0.04 | -0.18 | 0.01 | 0.21 | 0.25 |
| **Turb** | | | | | | | | | | 1.00 | 0.04 | 0.19 | -0.20 | 0.49** | -0.15 | 0.18 | 0.43* | 0.24 | -0.01 | 0.30 | 0.51** | 0.62*** |
| **FC** | | | | | | | | | | | 1.00 | 0.68*** | 0.13 | -0.01 | 0.01 | -0.15 | -0.07 | -0.05 | 0.08 | -0.12 | -0.23 | -0.11 |
| **FE** | | | | | | | | | | | | 1.00 | 0.03 | 0.08 | -0.02 | -0.08 | -0.10 | -0.21 | -0.21 | -0.14 | 0.10 | 0.03 |
| **Amm** | | | | | | | | | | | | | 1.00 | -0.15 | 0.65*** | 0.11 | 0.25 | 0.03 | 0.29 | 0.14 | 0.05 | 0.07 |
| **Nitra** | | | | | | | | | | | | | | 1.00 | 0.05 | 0.01 | -0.13 | -0.17 | -0.17 | -0.17 | -0.14 | 0.15 |
| **Nitri** | | | | | | | | | | | | | | | 1.00 | 0.41* | 0.07 | -0.11 | 0.33 | 0.09 | 0.01 | -0.15 |
| **Phos** | | | | | | | | | | | | | | | | 1.00 | 0.15 | 0.14 | 0.06 | 0.08* | 0.25* | -0.05 |
| ***nirS*** | | | | | | | | | | | | | | | | | 1.00 | 0.80*** | 0.66*** | 0.60*** | 0.82*** | 0.65*** |
| ***nirK*** | | | | | | | | | | | | | | | | | | 1.00 | 0.65*** | 0.65*** | 0.71*** | 0.57** |
| ***nosZ*I** | | | | | | | | | | | | | | | | | | | 1.00 | 0.43* | 0.37* | 0.11 |
| ***nosZ*II** | | | | | | | | | | | | | | | | | | | | 1.00 | 0.57*** | 0.47* |
| **AOB** | | | | | | | | | | | | | | | | | | | | | 1.00 | 0.74*** |
| **AOA** | | | | | | | | | | | | | | | | | | | | | | 1.00 |

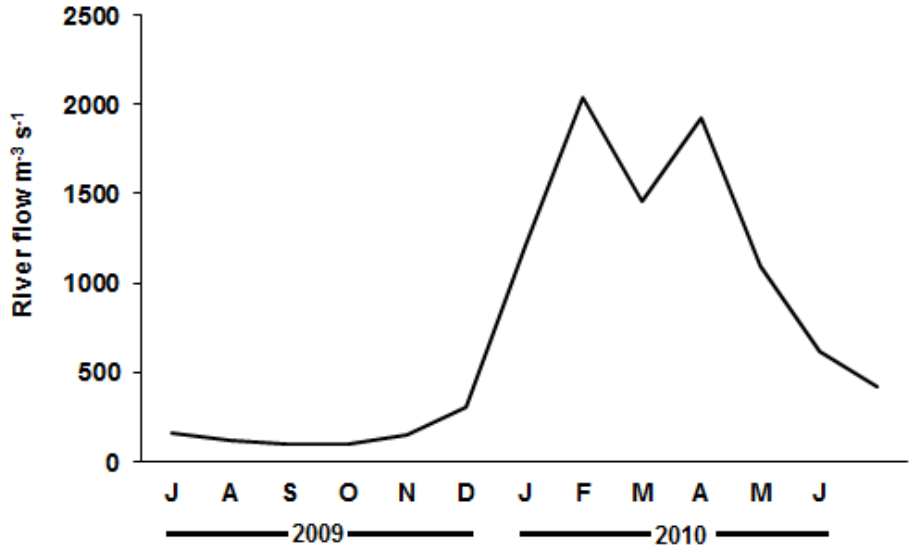


**Figure S2-1** Monthly river flow in the Douro river estuary, from July 2009 to June 2010.

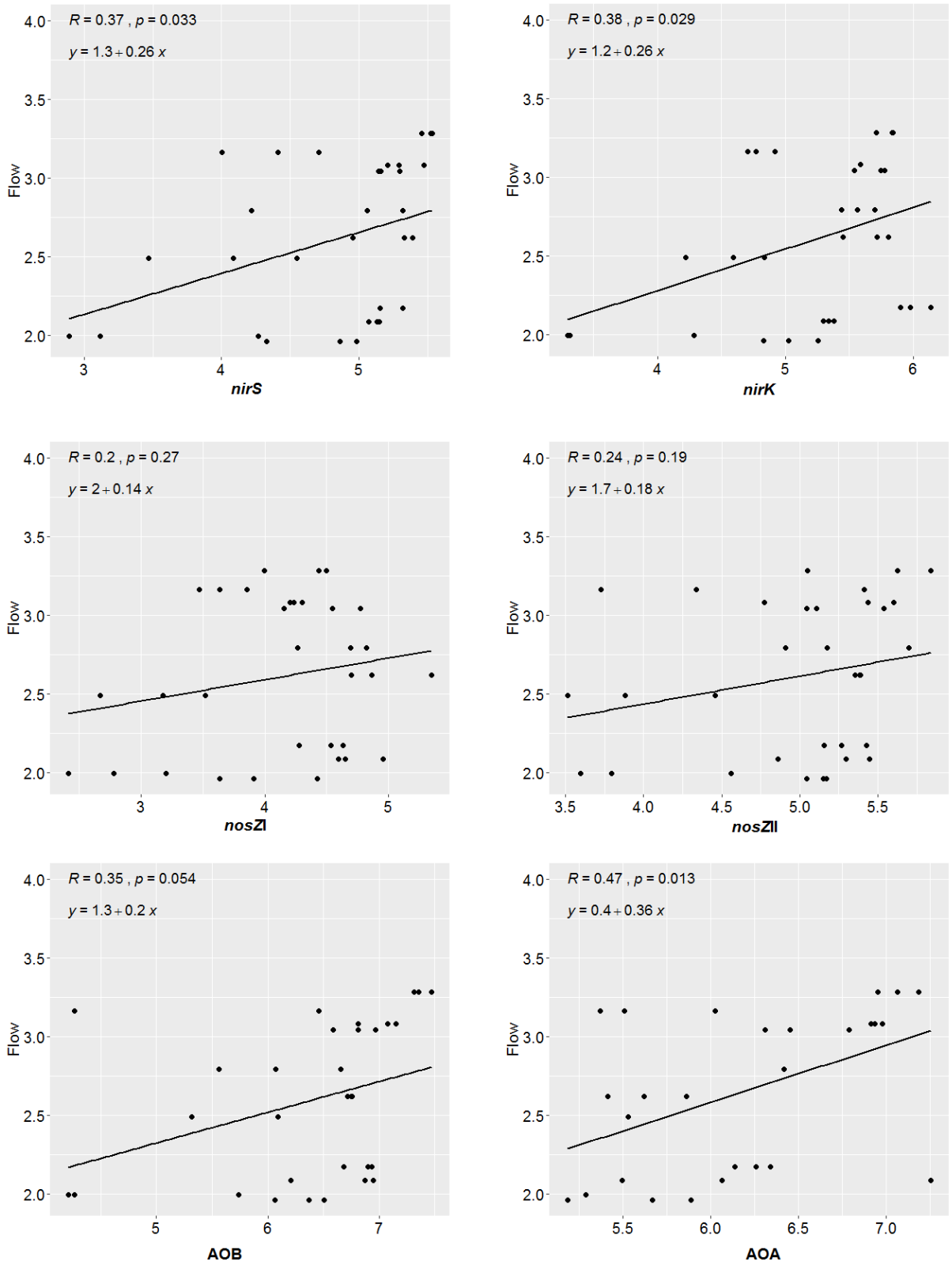


**Figure S2-2** Linear regressions to test the relationship between abundance of N-cycle genes and river flow (data were log transformed).

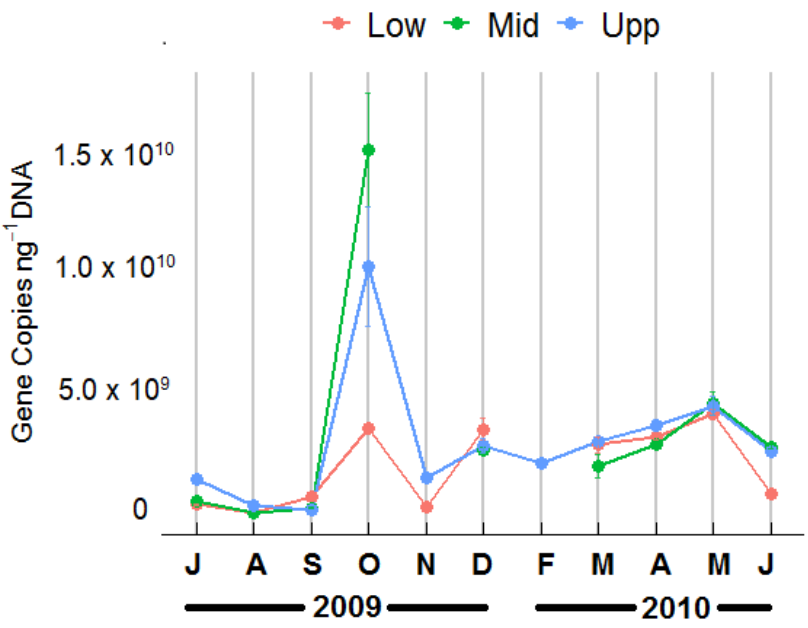


**Figure S2-3** Seasonal variability of bacterial *rpoB* abundance in the three sampling stations of the Douro river estuary, from July 2009 to June 2010.

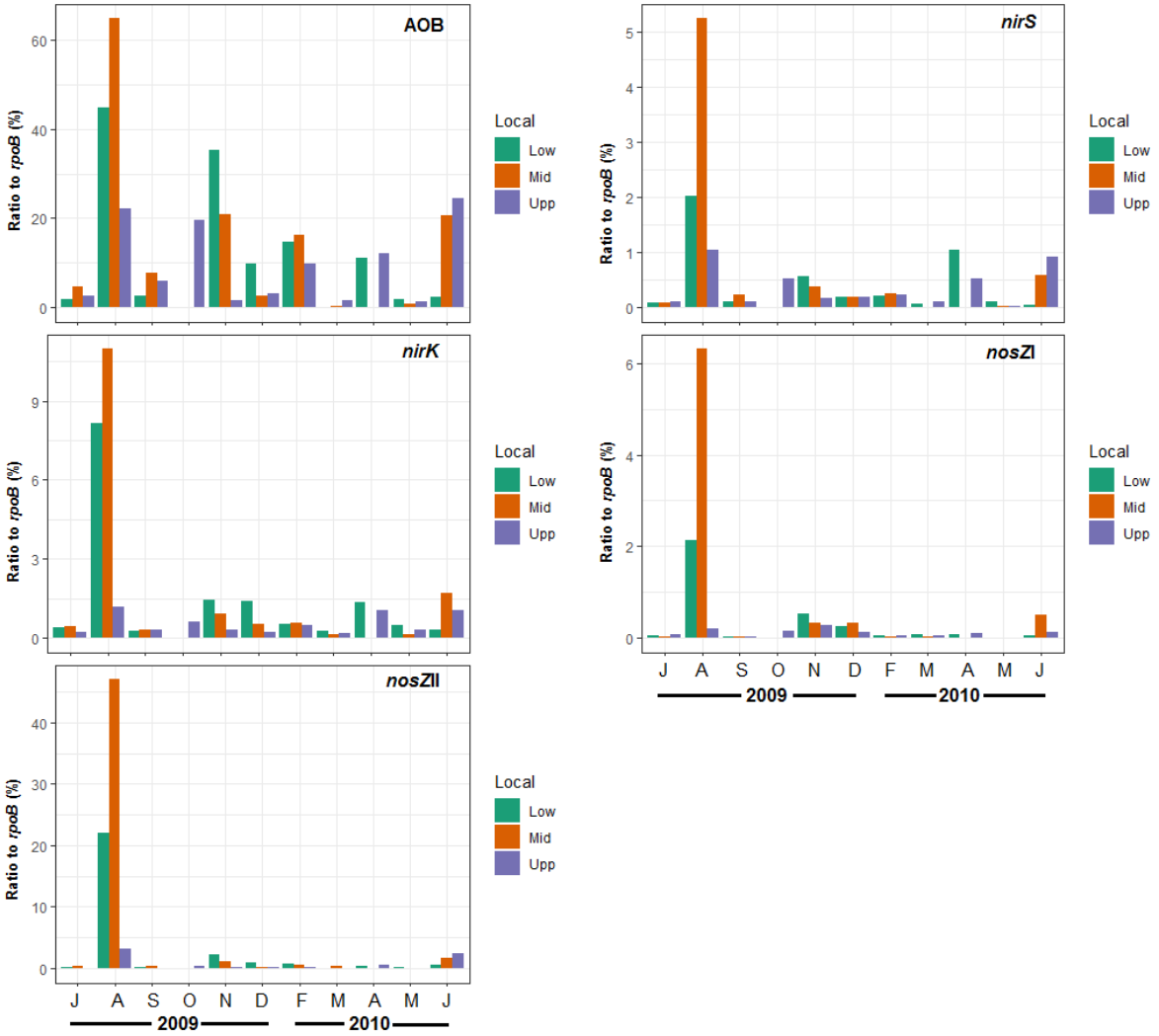


**Figure S2-4** Proportions of bacterial N cycling genes in the three sampling stations of the Douro river estuary (located at the lower, middle and upper stretches), from July 2009 to June 2010. Proportions of functional gene abundances were calculated as ratios of the *rpoB* community.

# CHAPTER 3

## Molecular insights into the interaction between DMSP metabolism and the denitrification pathway in marine bacteria

# Abstract

The occurrence of an inhibitory interaction between dimethylsulphoniopropionate (DMSP) degradation pathways and denitrification, influencing the production of climate cooling dimethyl sulfide (DMS) and, simultaneously, climate warming nitrous oxide ($N_2O$) gas, has been already acknowledged. However, little is known on the regulatory pathways at the cellular level. The present study intended to characterize and identify the molecular mechanism(s) involved in this interaction, by quantifying the $N_2O$ and DMSP degradation compounds (DMS and methanethiol – MeSH) and evaluate the expression of genes involved in the catabolism of DMSP (e.g.: *dmdA*, *dddW*), and denitrification (*nirS*, *nosZ*), in three representative Roseobacter strains (*Ruegeria pomeroyi* DSS-3, *Roseobacter denitrificans* OCh114 and *Ruegeria lacuscaerulensis* ITI-1157) cultured with different DMSP concentrations. The $N_2O$ and MeSH accumulation exhibited a similar profile, increasing with progressive DMSP amendments ($p < 0.001$) in Roseobacter cell suspensions, except for *R. lacuscaerulensis* ITI-1157 ($p > 0.05$). However, the *nosZ* transcription had different responses by the bacterial strains. Furthermore, in DSS-3 and ITI-1157 cell suspensions, *nosZ* expression was downregulated when $N_2O$ accumulation increases. On the other hand, in OCh114 the high $N_2O$ accumulation induced the expression of *nosZ*, but the amendment with 500 µM of DMSP resulted in the repression of *dmdA*, *nirS* and *nosZ* expression. Overall, this study shows that the reduction of $N_2O$ is differentially regulated by microbes, even under the same conditions. These novel findings contribute to a better understanding on how bacteria may regulate the underlaying molecular mechanisms responsible for metabolic processes, with potential implications in climate-relevant gas emissions.

# 3.1. Introduction

Biogeochemical cycles of nitrogen and sulfur play a vital role on the release and consumption of climate-regulating gases, such as $N_2O$ and DMS (Charlson et al., 1987, Ravishankara et al., 2009). DMS, which is the predominant form of volatile sulfur compound in the oceans, is believed to play a critical role in the global sulfur cycle, and in the radiation balance of the Earth (Andreae, 1990). On the other hand, denitrification can contribute to the natural marine emissions of the greenhouse gas $N_2O$ (Seitzinger et al., 2000, Frame and Casciotti, 2010). $N_2O$ is an obligate intermediate of denitrification, a respiratory process that reduces N oxides ($NO_3^-$ or $NO_2^-$) to the gaseous compounds nitric oxide (NO), $N_2O$, or $N_2$ (Zumft, 1997). Consequently, the anthropogenic impact on both metabolisms can influence the Earth natural balance, with implications on climate change.

The metabolism of organic sulfur represents an important process of the global sulfur cycle. In the case of DMSP, an organic sulfur compound largely produced by phytoplankton and macroalgae constitutes ca. 24% of the total organic sulfur in surface seawater (Bates et al., 1994, Groene, 1995). Due to the role as a biological precursor of DMS and methanethiol, has been thoroughly studied (MeSH; Kiene, 1996, Kiene et al., 2000, Reisch et al., 2011a). Bacteria are able to metabolize DMSP through an enzymatic cleavage, mediated by specific DMSP lyases (encoded by dddD, dddL, dddP, dddQ, dddW, dddY and dddK genes; Dey, 2017), resulting in the release of DMS, the most important fraction of biogenic sulfur for the maintenance of the Earth climate (Charlson et al., 1987). Alternatively, the “demethylation pathway” also produces methanethiol (MeSH), a compound that is consequently incorporated into S-containing biomolecules (Kiene et al., 2000), as a final product of a four-step pathway (Johnston et al., 2016). The first step of the demethylation/demethiolation pathway generates 3-methiolpropionate (MMPA). The process is mediated by DMSP demethylase DmdA, which is encoded by the gene *dmdA*, the most widespread and abundant DMSP degradation gene in marine metagenomes (Howard et al., 2008, Howard et al., 2011, Johnston et al., 2016). Subsequently, the downstream steps are catalyzed by DmdB, -C and -D, also widespread in bacteria (Reisch et al., 2011a). Research on DMSP catabolic pathways has been recently enhanced by the development of molecular biology tools with several identified bacterioplankton genes encoding DMSP degradation pathways, and with whole genomes of microbial DMSP-degraders now available (e.g. Ruegeria pomeroyi; Moran et al., 2004, Newton et al., 2010, Moran et al., 2012). Roseobacters, an α-Proteobacteria clade, are the most abundant bacterial group developing during phytoplankton blooms, and are often associated with major DMSP-producers, namely dinoflagellates (Zubkov et al., 2001, Onda et al., 2015). However, the complexity of the dynamic physical, chemical, and biological characteristics of

the marine environment has limited our understanding of the multiple processes and factors regulating and controlling DMSP cycling.

A novel inhibitory interaction between marine organic sulfur compound(s) derived from DMSP and the nitrogen biogeochemical cycle has been previously described (Magalhães et al., 2011, Magalhães et al., 2012a, Magalhães et al., 2012b). These studies demonstrated that DMSP degradation product(s) may interfere with the final step of denitrification, limiting nitrogen loss through $N_2$, and enhance greenhouse related nitrogen release via $N_2O$. However, research on these complex interactions between N and organic S cycles is in the preliminary stages. Therefore, little is known about the regulation of these pathways at the cellular level.

The present study aims to understand the inhibitory interaction between DMSP metabolism and the denitrification process, through a biogeochemical and molecular characterization of the interaction. Experiments were performed with different concentrations of DMSP on cell suspensions of representative Roseobacter strains, in order to evaluate the effect on $N_2O$ production, and to analyze the transcription signatures of the genes involved in the DMSP catabolism (e.g.: *dmdA*, *dddW*), and denitrification key functional genes (*nirS*, *nosZ*). Since the interference of DMSP degradation compounds on the denitrification process affecting $N_2O$ release is known, in this study we hypothesized that the abovementioned biogeochemical interaction induces distinctive responses on gene expression of different organisms. Therefore, the identification of the molecular mechanism(s) responsible for the inhibitory interaction between DMSP metabolism and the last step of denitrification in different Roseobacter strains is a must, together with the examination of the expression of *nosZ*, *dmdA*, *dddP* and *dddQ* in other environmental studies, retrieved from marine metatranscriptomic data sets.

## 3.2. Material and methods

### 3.2.1. Growth conditions of bacterial strains

*Ruegeria pomeroyi* DSS-3, *R. lacuscaerulensis* ITI-1157, and *Roseobacter denitrificans* OCh114 can grow heterotrophically, metabolize DMSP, and possess denitrifying genes. All strains were grown in Marine Basal Medium (MBM), containing 50 mM Tris-HCl; 19 mM $NH_4Cl$ (pH 7.5), 0.33 mM $K_2HPO_4$ x $3H_2O$, 10 mM glucose, and 0.1 mM $FeSO_4$. Cell suspensions were inoculated under aerobic conditions with constant rotary shaking (80 rpm), until near the end of the exponential phase ($OD_{600}$ 0.9). Cultures of ITI-1157 and OCh114 were amended

with $NO_3^-$, as nitrogen source, while $NO_2^-$ was added to cultures of DSS-3, since this strain lacked the gene for $NO_3^-$ reduction (Moran et al., 2004). All cultures were obtained from the CECT (Valencia, Spain).

### 3.2.2. DMSP assays and quantification by GC

After inoculation, 12 ml crimp-topped serum vials containing 3 ml of cell culture, were sealed with a Teflon stopper, purged with $N_2$ for 15 min (to simulate anoxic conditions), and amended with DMSP (Supelco). Each DMSP treatment was incubated in triplicate for 4 h at 28º C (for DSS-3 and OCh114 cell suspensions) and 45º C (for ITI-1157 cell suspensions), with constant rotary shaking (80 rpm), in the dark, and with three different concentrations of DMSP (5, 50, 500 µM), to maximize the DMSP metabolism, along with a nonamended control. Volatile sulfur compounds (DMS and MeSH) were measured in the headspace after 4 h of incubation, and analyzed by pulsed flame-photometric detection (GC/P-FPD), as described previously (Salgado et al., 2014). $N_2O$ was quantified with an electron-capture detector (ECD), with two Hay Sep D columns. Negative controls contained only sterile medium.

### 3.2.3. RNA extraction and cDNA synthesis

Bacterial cells were pelleted by centrifugation at 5,000 rpm for 5 min at 4 ºC, and total RNA of each sample was extracted using the RNeasy Mini kit (Qiagen, Germany), following the instructions from the manufacturer. DNA was removed from all RNA samples treated with TURBO DNA-*free*™ Kit (Invitrogen). RNA quality was evaluated by electrophoresis on 2% (w/v) agarose gel, and the absence of contamination with genomic DNA was confirmed by PCR using RNA polymerase subunit gene (*rpoB*) primers. Reverse transcription (RT) of RNA was carried out by using the NZY First Strand cDNA synthesis kit (NZYTech, Lisbon, Portugal), according to the instructions from the manufacturer. Synthesized cDNA was quantified on a Qubit fluorometer (Life Technologies, Carlsband, CA, USA) using the Qubit™ ssDNA Assay Kit, and further used for amplification according to the protocol described below. All working materials were previously treated with RNase Away® Reagent (Life Technologies, USA).

### 3.2.4. Primer pair design and quantification of functional genes

Novel qPCR primers used in this study (**Table S3-1** Primers used in this study for qPCR amplification.

**Table S3-2**) were designed in Primer-BLAST (Ye et al., 2012), using the nucleotide sequence of the functional gene from the respective organism model as query sequence obtained from the Roseobase (http://www.roseobase.org/). Then, an *in silico* test was performed to validate and confirm the primer specificity and non-amplification of unintended targets, by using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) on the generated primer pairs.

mRNA expression of the functional genes (**Table S3-3**) was quantified by qPCR using cDNA as template. All qPCR reactions were carried out in quadruplicates containing 10 – 80 ng of cDNA template, 400 nM of forward and reverse primers, and Power SYBR® Green PCR Master Mix (2X), that included ROX as a passive reference, to a final 25 µl reaction volume. Relative quantification of functional genes was performed on a CFX96TM Real-Time System/C1000TM Thermal cycler (Bio-Rad). A no-template control, which consisted of all the reagents except the template, was included at each run. The thermal cycling conditions consisted of an initial denaturation of a 5 min pre-cycling at 95 ºC, then 35 cycles of denaturation at 95°C for 30 s, annealing at the respective primer temperature (**Table S3-1** Primers used in this study for qPCR amplification.

**Table S3-2**) for 30 s, and extension at 72 °C for 30 s, followed by a melting curve with an increasing temperature from 55 to 95°C, at a rate of 0.5°C per second. To evaluate qPCR reactions specificity, a melting curve analysis was performed at the end of each reaction, and all PCR products were analyzed on 2% (w/v) agarose gel to ensure that only expected target genes were amplified without artifacts. The RNA polymerase subunit gene (*rpoB*) was used as reference gene for normalization, and fold changes of genes in response to different conditions were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). qPCR results were analyzed with the software Bio-Rad CFX Manager 1.6 (Bio-Rad Laboratories).

### 3.2.5. **Metatranscriptomic analysis**

For an initial analysis, metatranscriptomic data sets were obtained from ENA repository (Table S3-4). The quality of fastq files was assessed with FastQC (v. 0.11.7) (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), and raw reads were filtered with Trimommatic (v. 0.32) (Bolger et al., 2014). Reads with an average quality score ≤ 15 along a sliding window of 4 bp were truncated as well as the start and end of reads with a quality ≤ 3. Reads shorter than 50 bp were discarded. Additionally, the vsearch program (v. 2.13.6)

(Rognes et al., 2016), was used to improve the previous filtering step by removing reads with an expected error > 2, and dereplicate 100% identical fasta sequences. Reference enzyme sequences of NosZ and DmdA were retrieved from the NCBI Reference Sequence Database (RefSeq), from an organism that has an experimentally verified version of the respective gene, and analyzed by using the BLASTP program to find orthologs of that gene in other organisms to confirm sequence similarity. These reference sequences were indexed to be subsequently used as subject sequences for analysis of expression of genes on the selected marine metatranscriptomic data sets. The transcripts were annotated against the reference protein sequences with a cutoff e-value of $1e^{-20}$ by using the BLASTX program (v. 2.5.0) (Camacho et al., 2009).

An alternative *in silico* analysis was performed with selected metatrascriptomes datasets from the IMG/JGI (https://img.jgi.doe.gov) database, which consisted of several marine environmental studies (**Table S3-5**). To evaluate the distribution of Roseobacter genes involved in DMSP (*dmdA*, *dddP*, *dddL*) and denitrification (*nirS*, *nosZ*) metabolism, the corresponding amino acid sequences of *R. pomeroyi* DSS-3 were used as query for a BLASTP analysis to find orthologs, with a cutoff e-value of $1e^{-20}$, and a stringency level of >40% similarity. The count of retrieved sequences was size-normalized by the ratio of the query and RecA length, which was subsequently used to obtain the percentage of bacteria expressing the genes at each study.

### 3.2.6. Statistical analysis

Statistical analysis was performed with the open source-software R version 3.3.3 (R Development Core Team 2013). Volatile compounds accumulation data was examined for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively (Levene, 1960, Shapiro and Wilk, 1965), and a Wilcoxon Signed-Rank Test ($p < 0.05$) was used to evaluate the statistical differences between the different DMSP amendments. Significant changes ($p < 0.01$) in the relative expression of each gene, between DMSP amendments, were analyzed using a One-way analyses of variance (ANOVA) on the log transformation of fold-change values, followed by a Tukey's HSD post hoc test. Correlations between the different genes were performed using the log-transformed fold-changes, and results visualized with heatmaps using the ComplexHeatmap package (Gu et al., 2016).

## 3.3. Results and discussion

### 3.3.1. DMSP influence on $N_2O$ production

The experiment was designed with DMSP as the only sulfur source present in the cell suspension of the different strains. The progressive amendments resulted in significantly ($p < 0.001$) different changes in $N_2O$ accumulation patterns (**Figure 3.1**), except for the *R. lacuscaerulensis* ITI-1157 ($p > 0.05$). As expected, the production of DMS by *R. pomeroyi* DSS-3 (henceforth addressed as DSS-3), increased with progressive amendments of DMSP, while MeSH had a higher production with 50 µM of DMSP, and a lower MeSH accumulation at 500 µM of DMSP (**Figure 3.1a**). On the other hand, no accumulation of any of the DMSP degradation compounds at the 5 µM DMSP amendment occurred, probably due to metabolization or oxidation of volatile sulfur compounds. In fact, Eyice et al. (2018) observed that the transcription of *mtoX*, the gene encoding the MeSH oxidase, was enhanced in *R. pomeroyi* cultured with DMSP in aerobic conditions. Additionally, $N_2O$ production also increased with progressive concentrations up to 50 µM of DMSP, to decrease towards the highest DMSP concentration, following the same response as MeSH. Indeed, an inhibitory interaction between DMSP, a MeSH precursor, and the denitrification pathway was observed in both sediment slurries and *R. pomeroyi* cell suspensions explained by an effect of DMSP on $N_2O$ fluxes (Magalhães et al., 2012a). Another study identified MeSH as the direct cause for the inhibitory effect, resulting in a progressive $N_2O$ accumulation (Magalhães et al., 2011). The obtained results showed that higher MeSH accumulation caused higher $N_2O$ release, confirming the same influence of MeSH on the reduction of nitrous oxide. Although, at the highest DMSP concentration (500 µM), MeSH decreased, eventually and consequently to a more active $N_2O$ reduction to $N_2$, leading to less nitrous oxide accumulation by bacteria. Additionally, the high DMSP availability may exceed the bacterial sulfur demand resulting in a larger fraction of DMSP being cleaved into DMS instead of MeSH (Kiene et al., 2000, Moran et al., 2012). This may explain the lower MeSH production at 500 µM of DMSP. The *R. denitrificans* OCh114 and *R. lacuscaerulensis* ITI-1157 (henceforth addressed as OCh114 and ITI-1157, respectively) do not possess the *dmdD* gene (**Table S3-3**), which encodes the enzyme DmdD responsible for the release of the final demethylation compound, MeSH. However, MeSH was detected in cell suspensions of both strains (**Figure 3.1b and c**), possibly due to a non-orthologous isofunctional enzyme that may have replaced *dmdD* in some bacteria, and capable of catalyze the reaction (Reisch et al., 2011b). On the other hand, no DMS production was detected (**Figure 3.1b and c**, respectively).

Despite being previously assumed the absence of the cleavage phenotype (reviewed in Moran et al., 2012), both strains possessed the *dddP* gene (**Table S3-3**) that encodes the DMSP lyase DddP, which cleaves DMSP into DMS and acrylate (Todd et al., 2009, Curson et al.,

2011). Later, it was proposed that the cleavage of DMSP by DddP enzymes proceeds by a different catalytic mechanism in both OCh114 and ITI-1157 (Hehemann et al., 2014, Wang et al., 2015, Dey, 2017). Nevertheless, it was not observed any DMS production in OCh114 and ITI-1157 cell suspensions, which may be an indication of bacterial preference (>50%) for the demethylation/demethiolation pathway (Kiene et al., 1999, Kiene and Linn, 2000b). Moreover, marine metagenomic surveys have found that the most abundant genes belong to the bacterial DMSP demethylation pathway (Howard et al., 2006, Howard et al., 2008). Consequently, MeSH emerges as the leading volatile sulfur product (~75%) of initial DMSP metabolism (Kiene and Linn, 2000b), probably due to the metabolic importance of direct incorporation of MeSH into proteins by bacterioplankton (Kiene et al., 1999, Simó et al., 2000). However, the MeSH accumulation was only quantified at the highest DMSP amendments, but without significant ($p > 0.05$) differences between treatments. The low production rates of MeSH can be the result of rapid turnover or degradation (Kiene et al., 1999, Dickschat et al., 2010, Reisch et al., 2011b). On the other hand, it was observed that $N_2O$ production in OCh114 cell suspensions changed significantly ($p < 0.001$), and increased when MeSH accumulation was higher, resembling to the DSS-3 performance. In the ITI-1157 cell suspensions, however, no significant ($p > 0.05$) differences were observed in $N_2O$ and MeSH production along the DMSP amendments. It is worth of notice that $N_2O$ production decreased at the 5 µM amendment of DMSP.

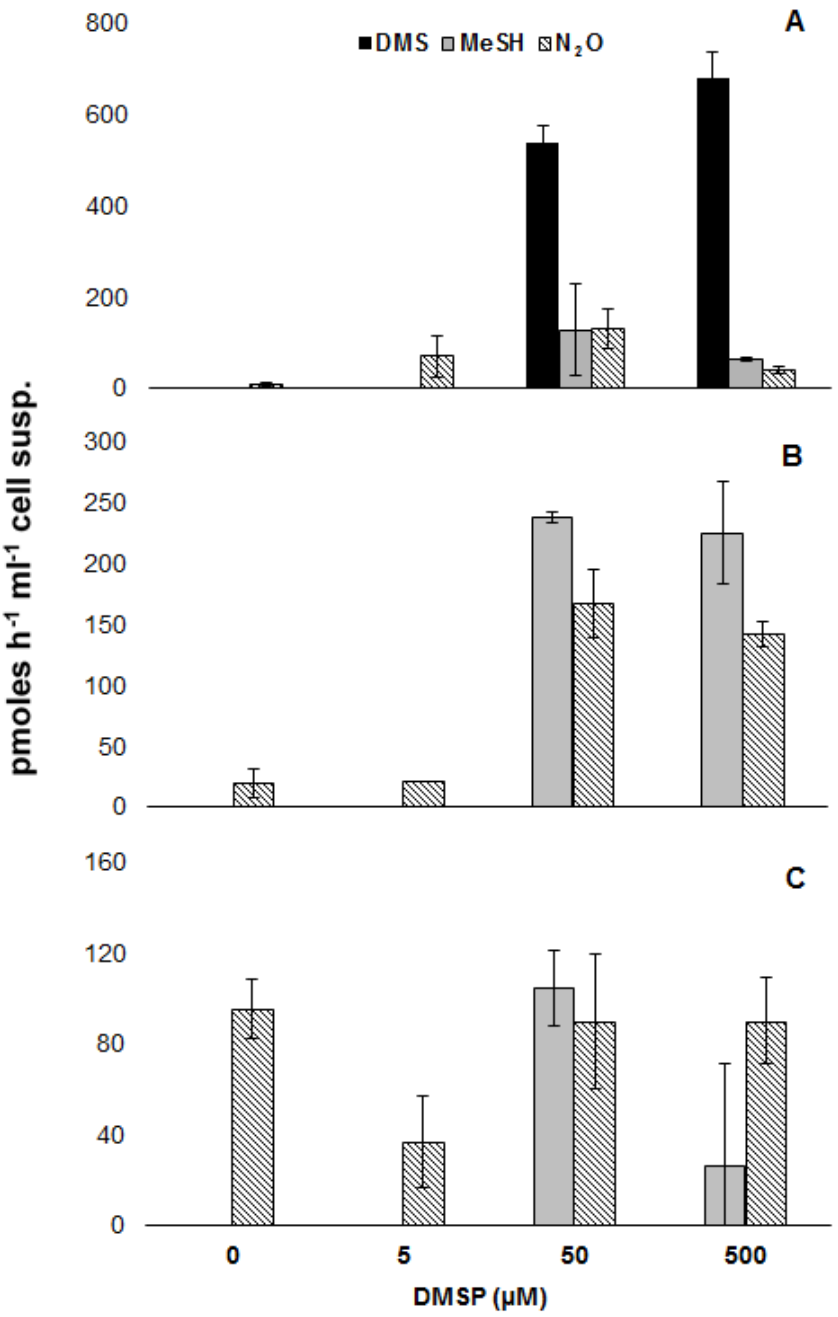


**Figure 3.1.** Net accumulation of DMSP degradation compounds (DMS and MeSH), and $N_2O$ in cell suspensions of (a) *Ruegeria pomeroyi* DSS-3, (b) *Roseobacter denitrificans* OCh114 and (c) *Ruegeria lacuscaerulensis* ITI-1157, under different DMSP concentrations. Values represent the mean of replicates and errors bars the standard deviation (n=3).

### 3.3.2. **DMSP and denitrification genes expression**

The DMSP amendments in cell suspensions of the studied Roseobacter strains resulted in different expressions of the transcripts of DMSP degradation and denitrification genes. After evaluating the effect of DMSP concentrations in $N_2O$ production, we expected to observe a

downregulation of *nosZ* transcription, which may explain the increasing production of $N_2O$ along the progressive DMSP amendments as a result of the possible gene inhibition by DMSP or its degradation compounds. However, when analyzing the *nosZ* expression in DSS-3 cell suspensions (**Figure 3.2**), it was found that, despite non-significant ($p > 0.05$) changes, only at the intermediate 50 µM DMSP concentration the *nosZ* transcription was downregulated (> 2-fold), whereas in the other treatments the *nosZ* expression was slightly induced (0.5 and 0.08-fold in 5 and 500 µM DMSP amendments, respectively). Indeed, $N_2O$ production was higher at the 50 µM DMSP concentration, which agrees with these results suggesting that *nosZ*, which encodes the catalytic domain of $N_2O$ reductase (N2OR; Zumft, 1997), may be inhibited and, consequently, the reduction of $N_2O$ was incomplete due to insufficient enzyme that catalyzes the reduction. In the present study, the rapid turnover of MeSH, the potential inhibitor of *nosZ* transcription (Magalhães et al., 2011), may explain the consequent lower $N_2O$ production and the upregulation of *nosZ* (**Figure 3.1a** and **Figure 3.2**, respectively), in the presence of 5 µM DMSP, in the case of DSS-3.

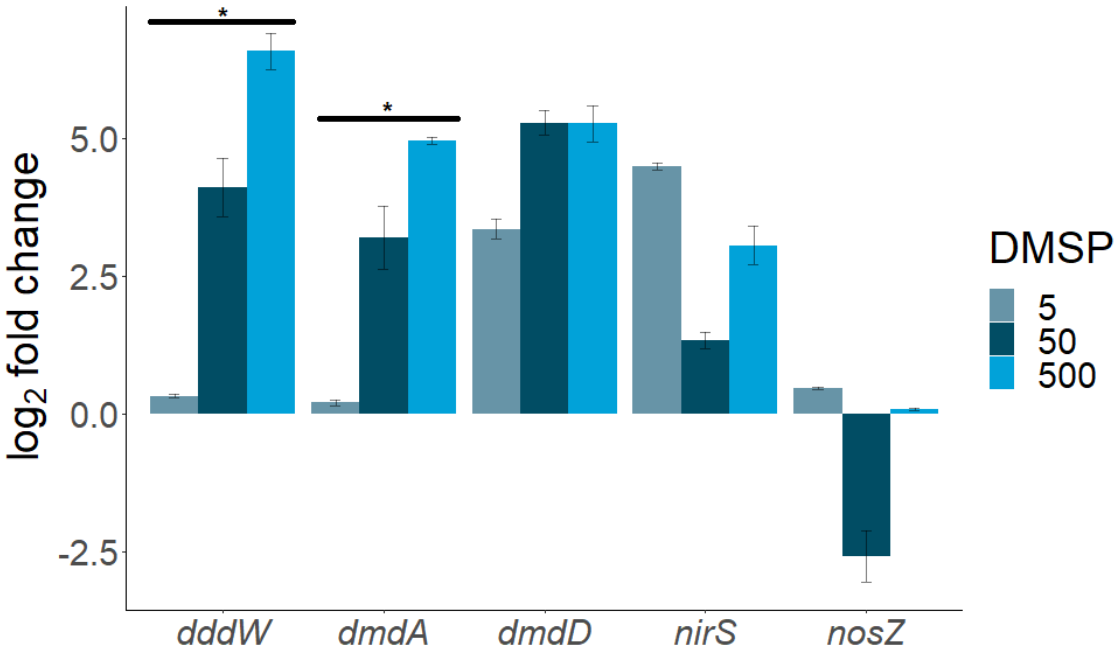


**Figure 3.2.** Expression of DMSP catabolism (*dmdA*, *dmdD* and *dddW*) and denitrification (*nirS*, *nosZ*) genes of *R. pomeroyi* DSS-3. Log-transformed fold change ($log_2$ fold change) values were generated by comparing the expression of genes at each DMSP treatment vs. control (without DMSP). Data are presented as means ± standard error (SE) from four independent replicates. Significant changes between DMSP (µM) treatments are noted by an asterisk.

Besides the recently acknowledged of $N_2O$ reduction performed by non-denitrifying organisms (Jones et al., 2013), the only known pathway of $N_2O$ consumption was the final step of denitrification catalyzed by the $N_2OR$. This enzyme is a copper-containing protein with two copper centers, the $Cu_A$ and "$Cu_Z$ center", that catalyze the reduction of $N_2O$ to dinitrogen (Pauleta et al., 2013). The structural gene for the $N_2OR$ is *nosZ*, included in the *nosRZDFYL* gene cluster which encode several proteins needed for the assembly of the enzyme (Viebrock and Zumft, 1988, Honisch and Zumft, 2003). Despite the extensive revision at the biochemical and molecular mechanisms of $N_2OR$ (Zumft and Kroneck, 2006, Pauleta et al., 2013), the catalytic reaction in $N_2OR$ is still unclear. Several environmental factors (e.g. oxygen) and transcriptional regulators that differ between organisms, such as *nosR* and *nosC*, control the genes and/or operon encoding the $N_2OR$ (reviewed in Spiro, 2012). On the other hand, the transcription of *nirS* was always induced in all DMSP amendments of DSS-3 cell suspensions, under anoxic conditions, but without significant ($p > 0.05$) changes. However, at the 50 µM DMSP concentration, the *nirS* expression was lower.

Although *R. pomeroyi* DSS-3 possesses several cleavage genes (**Table S3-3**), it was verified that *dddW* was the most differentially expressed gene of the DMSP cleavage pathway in *R. pomeroyi* culture (Landa et al., 2017). As expected, the transcription of *dmdA*, *dmdD* and *dddW* in DSS-3 cell suspensions increased following the progressive amendment of DMSP, and was significantly ($p < 0.01$) differentially expressed (**Figure 3.2**) in response to DMSP, except for the *dmdD*. However, the expression at the latter was comparatively higher (3.4-fold change) than the expression of *dmdA* and *dddW* at the 5 µM DMSP amendment (0.2 and 0.3-fold change, respectively). The high values of *dmdD* transcription meets the hypothesis of previous studies, in which DmdD appears to have evolved to become more adapted to DMSP metabolism and, consequently, highly efficient (Reisch et al., 2011b, Bullock et al., 2017). Additionally, *R. pomeroyi* can accumulate DMSP as an osmolyte and metabolize only the sulfur that exceeds the requirements of the cells for osmoprotection (Reisch et al., 2008, Salgado et al., 2014). DMSP concentrations can reach higher values, from 10 nM in the euphotic zone to 4,240 nM in dinoflagellates blooms (Galí et al., 2015, Kiene et al., 2019). The present results demonstrate that up to a 5 µM amendment of DMSP, DSS-3 cells may accumulate DMSP without metabolizing it, which explains the low expression of the genes mediating the initial steps of the two competing DMSP pathways, *dmdA* and *dddW*, and possibly the missing influence on the *nosZ* transcription.

Most DMSP research has been carried out with the universal selected model organism, *R. pomeroyi* DSS-3, but the present study aimed also to evaluate the transcriptomic response in other members of the Roseobacter clade, already acknowledged as DMSP-degraders (Moran et al., 2012). According to the initial hypothesis, the biochemical and molecular signatures of

OCh114 and ITI-1157, under the same conditions as DSS-3, were supposedly similar. Therefore, we sought to verify if different DMSP amendments generated the same response for the $N_2O$ production and expression of *nosZ* and *nirS*. The latter gene encodes the enzyme responsible for the reaction (reduction of nitrite) which distinguishes denitrifiers from nitrate respirers in the environment (Hallin and Lindgren, 1999).

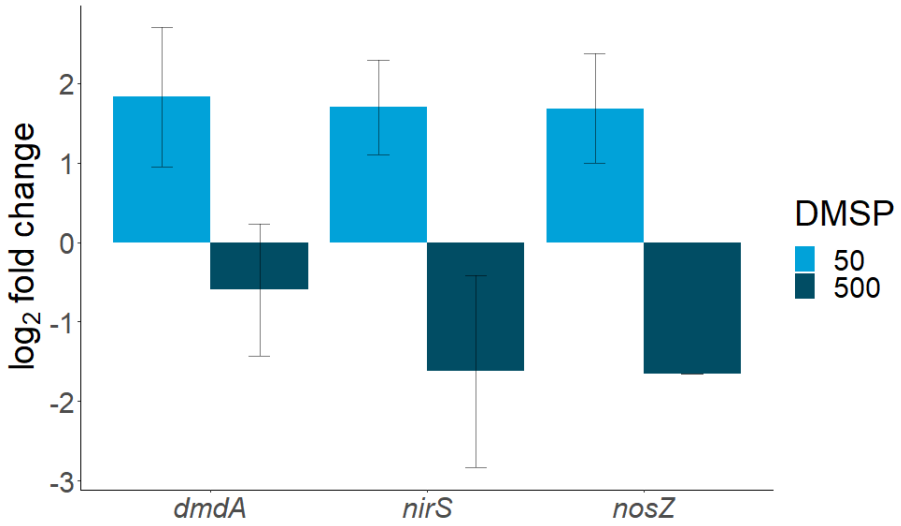


**Figure 3.3.** Expression of genes encoding DMSP demethylase (dmdA) and denitrification reductases (*nirS*, *nosZ*) of *Roseobacter denitrificans* OCh114. Log-transformed fold change ($log_2$ fold change) values were generated by comparing the expression of genes at each DMSP (µM) treatment vs. control (without DMSP). Data are presented as means ± standard error (SE) from four independent replicates.

OCh114 cell suspensions lead to different transcriptional performance as in DSS-3 cell suspensions, with *nosZ* transcript being upregulated at the 50 µM of DMSP (1.7-fold change), when $N_2O$ production was higher (**Figure 3.1b**), and downregulated (-1.6-fold change) with 500 µM of DMSP (**Figure 3.3**). The transcriptional signal showed an opposite response to the presence of metabolites, in the sense that the functional gene [*nosZ*] was induced, probably as a response to the increased $N_2O$ accumulation, but the enzyme [$N_2OR$] was not reducing efficiently or sufficiently the $N_2O$, since concomitant accumulation was observed. As abovementioned, the number of transcriptional units of *nos* gene cluster can differ between organisms belonging to the $\alpha$-Proteobacteria division (Pauleta et al., 2013), suggesting that the transcription of *nosZ* may be different between DSS-3 and OCh114, a compete denitrifier. Despite the regulation of *nos* genes still lacks extensive characterization, the gaseous intermediate of denitrification, NO, seems to be a stronger inducer than $N_2O$ for the transcription of the *nos* genes (Arai et al., 2003, Velasco et al., 2004).

Both *dmdA* and *nirS* showed the same response as *nosZ*, with a downregulation at the 500 µM DMSP concentration (-0.6 and -1.6-fold change, respectively; **Figure 3.3**), which indicates that the OCh114 strain had a lower tolerance towards the DMSP accumulation. The gene repression suggests that DMSP metabolism decreased at the 500 µM amendment, probable due to osmolyte saturation, which results in lower MeSH production. Moreover, the expression of these genes in OCh114 is regulated differently than that of DSS-3 (**Figure 3.4**). While in DSS-3, the denitrifying genes (*nirS* and *nosZ*) are negatively related to the DMSP degradation genes, in OCh114 only *nirS* showed a negative association with the demethylation gene (*dmdA*). This may indicate that the regulatory effect may alternatively occur on the gene transcription before the $N_2O$ reduction.

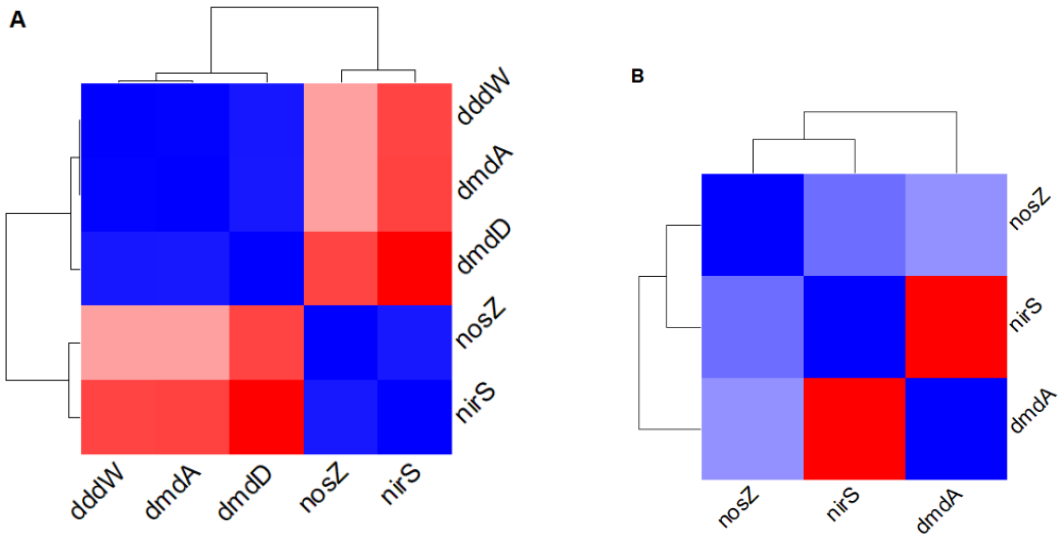


**Figure 3.4.** Heatmaps representing positive (blue) and negative (red) correlations between the DMSP catabolism (*dmdA*, *dmdD* and *dddW*), and denitrification (*nirS* and *nosZ*) genes in (a) *Ruegeria pomeroyi* DSS-3, and (b) *Roseobacter denitrificans* OCh114 cell suspensions. The dendrogram represents the distance between clusters calculated from Pearson's correlation coefficient.

The expression of *nosZ* in ITI-1157 cell suspensions revealed a significantly ($p < 0.01$) different gene regulation along the DMSP amendments (**Figure 3.5**). On the 5 µM DMSP treatment, when $N_2O$ production was lower compared to the control, the transcription of *nosZ* was upregulated (5.5-fold change), and the enzyme [$N_2OR$] was effectively reducing $N_2O$. At the higher DMSP amendment (500 µM), a downregulation (-3.3-fold change) of *nosZ* expression occurred, which resulted in higher $N_2O$ accumulation (**Figure 3.1c**). However, the distinction

between the ITI-1157 and the other strains genotypes relied on the fact that in its essence is not a complete denitrifier nor does possess the *nor* gene, which encodes the reductase that converts NO to $N_2O$ (**Table S3-3**). As previously mentioned, NO is the inducer molecule for the transcription of *nos* genes (Arai et al., 2003), and since ITI-1157 lacks the *norB* gene the $N_2OR$ activity was not affected by the absence of NO. Therefore, the DMSP addition or MeSH may directly influence the transcription of *nosZ* and not the $N_2OR$.

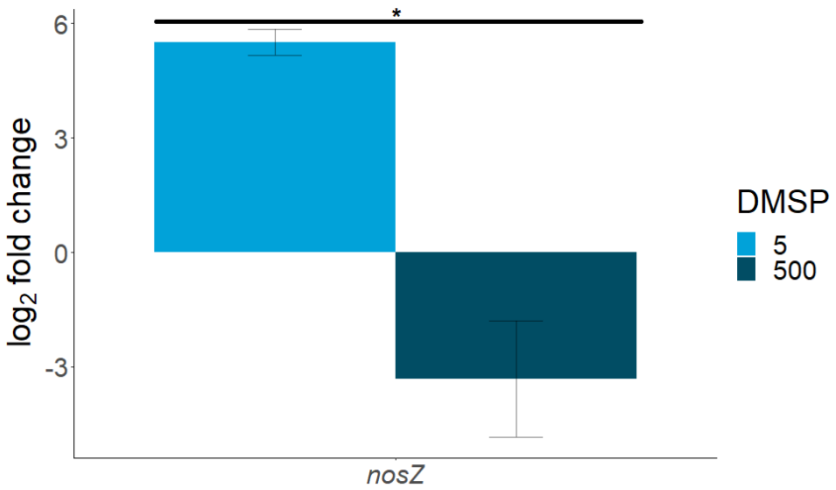


**Figure 3.5.** Expression of the gene *nosZ*, encoding the nitrous oxide reductase, in *Ruegeria lacuscaerulensis* ITI-1157 cell suspensions. Log-transformed fold change ($log_2$ fold change) values were generated by comparing the expression of genes at each DMSP (µM) treatment vs. control (without DMSP). Data are presented as means ± standard error (SE) from four independent replicates. Significant ($p < 0.01$) changes between DMSP treatments are noted by an asterisk.

Overall, these results suggest that $N_2O$ production was indeed influenced by the addition of DMSP or it(s) degradation compounds. However, the discrepancies of the *nosZ* transcription suggests that the regulation of the denitrifying genes, and consequent influence on $N_2O$ accumulation, is different among the Roseobacter strains. While in DSS-3 and ITI-1157 the *nosZ* transcription responds to the surrounding environment with a negative feedback (e.g. when the levels of the $N_2O$ increases, the signaling molecule represses the transcription of *nosZ*), in OCh114 the expression of *nosZ* is induced when $N_2O$ accumulation was higher, likely as a molecular response to $N_2O$ that is not being sufficiently reduced.

### 3.3.3. Gene expression in marine metatranscriptomic datasets

After the empirical approach to analyze the interaction between both pathways – DMSP catabolism and denitrification, the next step was to evaluate the extension of this association in marine metatranscriptomic studies. Initially, the distribution of *dmdA* and *nosZ* in ENA datasets was analyzed, and the proportion of both genes evaluated. The applied pipeline did not detect the expression of the target genes in all the selected studies. Overall, the metatranscriptomes revealed the prevalent distribution of *dmdA* in marine environments, but without *nosZ* transcripts (**Table 3.1**). As expected, *dmdA* was absent, and *nosZ* was expressed in anaerobic samples from a microbial mat (freshwater biome; study PRJEB5474).

**Table 3.1.** ENA metatranscriptomic data sets used for the analysis of *dmdA* and *nosZ* transcript abundance.

| ENA accession | Study project | Biome | No. transcripts | |
|---|---|---|---|---|
| | | | *dmdA* | *nosZ* |
| PRJEB5474 | Unraveling the stratification of an iron-oxidizing microbial mat by metatranscriptomics | Freshwater | 0 | 9 |
| PRJEB2064 | Western English Channel diurnal study | Oceanic | 6 | 0 |
| PRJEB4843 | Illumina and 454-based metatranscriptomic analyses of a diatom-induced bacterioplankton bloom in the North Sea | Marine | 3 | 0 |
| PRJEB5205 | Metatranscriptome of a marine bacterioplankton in the North Sea assessed by total RNA sequencing | Marine | 1 | 0 |

On the IMG/JGI metatranscriptomic datasets (**Table 3.2**), the general dominance of *dmdA* (encoding DmdA enzyme) in different marine environments was confirmed. On the other hand, the transcripts related to denitrifying enzymes were only present in favorable environmental conditions (e.g. oxygen minimum zones), where the DMSP catabolism enzymes were less abundant.

According to Dickschat et al. (2015), all four *dmd* genes from demethylation pathway are only present in few organisms belonging to the Roseobacter clade. Previously, an analysis performed on the unassembled Global Ocean Sampling (GOS) metagenomic dataset, the bacterial DMSP demethylation genes (*dmdA*, *dmdB* and *dmdC*) were more abundant than the cleavage ones (reviewed in Moran et al., 2012). Even though the “demethylation” pathway is widespread among marine bacteria, analyses performed on metagenomic data, and in representative bacterial genomes revealed low occurrence of DmdD, a Methylthioacryloyl-CoA hydratase leading to MeSH production (Reisch et al., 2011a, Reisch et al., 2011b, Bullock et al., 2017), also confirmed by our results. Overall, the proportion of expressed genes between DMSP catabolism and denitrification pathways was opposite.

**Table 3.2.** Distribution of genes (normalized to *rpoB*) encoding DMSP degradation and Denitrification enzymes in marine metatranscriptomes from the IMG/JGI database.

| Genome Name / Sample Name | DmdA | DmdD | DddP | DddQ | DddD | NirS | NosZ |
|---|---|---|---|---|---|---|---|
| *Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient (Fall)* | 3.75 | 0 | 1.24 | 0 | 0 | 0.85 | 1.25 |
| *Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient (Spr)* | 0 | 0 | 0 | 0 | 1.15 | 7.30 | 1.33 |
| *Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient (Summ)* | 7.77 | 0 | 1.54 | 0 | 0 | 0 | 0 |
| *Estuarine microbial communities from the Columbia River estuary - R.1175 metaT (Metagenome Metatranscriptome)* | 11.01 | 1.93 | 2.73 | 0 | 1.58 | 0 | 0 |
| *Estuarine microbial communities from the Columbia River estuary - R.871 metaT (Metagenome Metatranscriptome)* | 0 | 0 | 0 | 0 | 2.08 | 0 | 0 |
| *Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_10m* | 25.22 | 7.23 | 2.27 | 0 | 7.92 | 0 | 0 |
| *Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_100m* | 32.10 | 0 | 3.54 | 0 | 0 | 2.43 | 0 |
| *Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_200m* | 0 | 0 | 0 | 0 | 0 | 78.82 | 28.76 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 250_A metaT* | 12.72 | 0 | 3.60 | 0 | 3.14 | 0 | 0 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 250_B metaT* | 21.14 | 0 | 2.33 | 0 | 0 | 0 | 0 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 AAIW_A metaT* | 12.32 | 0 | 2.22 | 0 | 3.87 | 0 | 2.23 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 Bottom_A metaT* | 6.08 | 0 | 2.01 | 0 | 7.00 | 0 | 0 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 DCM_A metaT* | 10.92 | 0 | 2.46 | 0 | 0.43 | 0 | 0 |
| *Marine microbial communities from the Southern Atlantic ocean - KN S19 DCM_B metaT* | 8.94 | 0 | 1.97 | 0 | 0 | 0 | 0 |
| *Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 40m* | 15.99 | 0.37 | 1.06 | 0 | 3.99 | 0 | 0 |
| *Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 100m* | 3.55 | 0.29 | 1.35 | 0 | 0.47 | 0 | 0 |
| *Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 200m* | 21.57 | 0.41 | 6.50 | 0 | 4.66 | 0 | 0 |
| *Metatranscriptome of coastal salt marsh microbial communities from the Groves Creek Marsh, Georgia, USA* | 625.96 | 0 | 114.63 | 36.92 | 58.21 | 0 | 0 |
| **Average** | **45.50** | **0.57** | **8.30** | **2.05** | **5.25** | **4.97** | **1.87** |

## 3.4. Conclusions

The obtained results confirmed that MeSH production is positively correlated with $N_2O$ accumulation in bacterial cultures, mimicking the trend with estuarine sediment samples. However, the gene-expression profile and the gaseous compounds accumulation indicated that the individual organisms responded differently to the exposed conditions, manifested by a varied regulation of *nosZ* expression and/or possibly influence of $N_2O$R activity. In addition, the metatranscriptomic analysis revealed an opposite relation between the distribution of the DMSP degradation and denitrification genes in several marine biomes.

Overall, the present study delivers an insight into the regulation of genes belonging to DMSP catabolism and $N_2O$ reduction performed by one of the most cosmopolitan and abundant marine bacterial groups. Furthermore, this research contributes to the understanding of how the organic S and N biogeochemical cycles may interact in marine environments. Also, because DMS and $N_2O$ have contrasting effects on global climate, this study represents a major contribution for the knowledge of the global dynamics of both marine S and N cycles, by providing indication on the balance of each of these climate-relevant compounds. Future research needs to address how the physiological and metabolic state of the organism may regulate the transcription of gene regulatory proteins, and how this on-off switch of genes impacts the adaptation of bacterial cells.

## 3.5. Supplementary Information

**Table S3-1** Primers used in this study for qPCR amplification.

| Target Gene | Primer | Strain | Amplicon size (bp) | Sequence (5'-3') | Primer temp. (° C) | Reference |
|---|---|---|---|---|---|---|
| *dmdA* | RPdmdAF<br>RPdmdAR | DSS-3 | 155 | ATTGCGCCCATCTGAAGGAA<br>CGTGGGCACGTAGTAACACT | 54 | This study |
| | RddmdAF<br>RddmdAR | OCh114 | 212 | ACGGTCTACAACCGCATGTT<br>TCCACGATGGGCACGTAAAA | 56 | |
| *dmdD* | dmdD_F508<br>dmdD_R652 | DSS-3 | 145 | CTGACCGGTCGTGTCTATCA<br>AACAGATGGCGAAGTTGGTC | 61 | Reisch et al. 2011 |
| *dddW* | dddW_RpF1<br>dddW_RpR1 | DSS-3 | 259 | ATGCCATTGACGCCGAAAAC<br>ATATAGAGCGCGACACCTGC | 59 | This study |
| *nirS* | RPnirSF1<br>RPnirSR1 | DSS-3 | 253 | GCTCTTCGTGAAATCGCACC<br>TTCCAGATCCTTGGCGTTCC | 61 | |
| | RdnirSF<br>RdnirSR | OCh114 | 174 | GTCGTGGCGCCAATATCAAC<br>GCGATTTCACGAAGAGCGAC | 56 | |
| *nosZI* | nosZ2F<br>nosZ2R | Universal | 267 | CGCRACGGCAASAAGGTSMSSGT<br>CAKRTGCAKSGCRTGGCAGAA | 60 | Henry et al., 2006 |

**Table S3-3** Presence of genes of DMSP degradation and denitrification pathways in the genomes of Roseobacter model marine bacteria. The cross indicates a positive presence. DMSP demethylation genes – *dmdA*, *dmdB*, *dmdC*, *dmdD*; DMSP cleavage genes – *dddD*, *dddL*, *dddP*, *dddQ*, *dddW*, *dddY*; Methanethiol oxidase – *mtoX*; Denitrification – *napA* (gene encoding dissimilatory perisplamic nitrate reductase), *narG* (gene encoding dissimilatory respiratory nitrate reductase), *nirS*/*nirK* (gene encoding dissimilatory nitrite reductase), *norB* (gene encoding dissimilatory nitric oxide reductase), *nosZ* (gene encoding dissimilatory nitrous oxide reductase). Adapted from Newton et al. (2010), Moran et al. (2012), Luo and Moran (2014), and Bullock et al. (2017).

| | | *Sulfur cycle* | | | | | | | | | | | *Nitrogen cycle* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | *dmdA* | *dmdB* | *dmdC* | *dmdD* | *dddD* | *dddL* | *dddP* | *dddQ* | *dddW* | *dddY* | *mtoX* | *napA* | *narG* | *nirS* | *nirK* | *norB* | *nosZ* |
| *Ruegeria pomeroyi* | DSS-3 | × | × | × | × | × | | × | × | × | | × | | | × | | × | × |
| *Ruegeria lacuscaerulensis* | ITI-1157 | × | × | × | | | | × | × | | | | × | | | | | × |
| *Roseobacter denitrificans* | OCh 114 | × | | | | | | × | | | | | × | × | × | | × | × |

**Table S3-4** ENA metatranscriptomes information.

| *ENA accession* | *Biome* | *Study Name* | *Samples* | *Analyses* | *Centre name* |
|---|---|---|---|---|---|
| *PRJEB5474* | Freshwater | Unraveling the stratification of an iron-oxidizing microbial mat by metatranscriptomics | 14 | 14 | CNRS and University of Rennes |
| *PRJEB2064* | Oceanic | Western English Channel diurnal study | 10 | 20 | Plymouth Marine Laboratory |
| *PRJEB4843* | Marine | Illumina and 454-based metatranscriptomic analyses of a diatom-induced bacterioplankton bloom in the North Sea | 5 | 5 | MPI BREMEN |
| *PRJEB5205* | Marine | Metatranscriptome of a marine bacterioplankton in the North Sea assessed by total RNA sequencing | 1 | 2 | JACOBS |

**Table S3-5** IMG/JGI metatranscriptomes information.

| Study Name | Genome Name / Sample Name | IMG Genome ID | Genome Size assembled | Gene Count assembled |
|---|---|---|---|---|
| Aqueous microbial communities from the Delaware River/Bay and Chesapeake Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series | Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series - DEBay_Fall_15_>0.8_RNA2 (Metagenome Metatranscriptome) | 3300006602 | 169099453 | 577125 |
| Aqueous microbial communities from the Delaware River/Bay and Chesapeake Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series | Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series - DEBay_Spr_0.19_>0.8_RNA2 (Metagenome Metatranscriptome) | 3300006375 | 108356578 | 375334 |
| Aqueous microbial communities from the Delaware River/Bay and Chesapeake Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series | Aqueous microbial communities from the Delaware River and Bay under freshwater to marine salinity gradient to study organic matter cycling in a time-series - DEBay_Sum_22_D_>0.8_RNA1 (Metagenome Metatranscriptome) | 3300006383 | 163247289 | 472995 |
| Estuarine microbial communities from the Columbia River estuary, to analyze effect of nutrient fluxes, a time series | Estuarine microbial communities from the Columbia River estuary - R.1175 metaT (Metagenome Metatranscriptome) | 3300019200 | 65032598 | 140512 |
| Estuarine microbial communities from the Columbia River estuary, to analyze effect of nutrient fluxes, a time series | Estuarine microbial communities from the Columbia River estuary - R.871 metaT (Metagenome Metatranscriptome) | 3300019207 | 99105613 | 214159 |
| Marine microbial communities from expanding oxygen minimum zones in the northeastern subarctic Pacific Ocean | Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_10m (Metagenome Metatranscriptome) | 3300002692 | 41844446 | 101032 |
| Marine microbial communities from expanding oxygen minimum zones in the northeastern subarctic Pacific Ocean | Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_100m_A (Metagenome Metatranscriptome) | 3300002688 | 28948939 | 76966 |
| Marine microbial communities from expanding oxygen minimum zones in the northeastern subarctic Pacific Ocean | Marine microbial communities from expanding oxygen minimum zones in the Saanich Inlet - MetaT SI072_200m_A (Metagenome Metatranscriptome) | 3300002685 | 28981149 | 66797 |

| Study Name | Genome Name / Sample Name | IMG Genome ID | Genome Size assembled | Gene Count assembled |
|---|---|---|---|---|
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 250_A metaT (Metagenome Metatranscriptome) (version 2) | 3300011294 | 45766441 | 110617 |
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 250_B metaT (Metagenome Metatranscriptome) (version 2) | 3300011291 | 43115002 | 102948 |
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 AAIW_A metaT (Metagenome Metatranscriptome) (version 2) | 3300011318 | 100572331 | 226061 |
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 Bottom_A metaT (Metagenome Metatranscriptome) (version 2) | 3300011316 | 95664870 | 219308 |
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 DCM_A metaT (Metagenome Metatranscriptome) | 3300007612 | 135174406 | 441603 |
| Marine microbial communities from the Southern Atlantic ocean transect to study dissolved organic matter and carbon cycling | Marine microbial communities from the Southern Atlantic ocean - KN S19 DCM_B metaT (Metagenome Metatranscriptome) | 3300007596 | 64515886 | 216098 |
| Marine archaeal communities from Monterey Bay, CA, that are ammonia-oxidizing | Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 40m 12015 (Metagenome Metatranscriptome) | 3300021350 | 540012713 | 1079125 |
| Marine archaeal communities from Monterey Bay, CA, that are ammonia-oxidizing | Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 100m 12015 (Metagenome Metatranscriptome) | 3300021291 | 65529902 | 151261 |
| Marine archaeal communities from Monterey Bay, CA, that are ammonia-oxidizing | Metatranscriptome of ammonia-oxidizing marine archaeal communities from Monterey Bay, California, United States - M2 200m 12015 (Metagenome Metatranscriptome) | 3300021348 | 404827694 | 908865 |

| Study Name | Genome Name / Sample Name | IMG Genome ID | Genome Size assembled | Gene Count assembled |
|---|---|---|---|---|
| Coastal salt marsh microbial communities from the Groves Creek Marsh, Skidaway Island, Georgia | Metatranscriptome of coastal salt marsh microbial communities from the Groves Creek Marsh, Georgia, USA - 011501AT metaT (Metagenome Metatranscriptome) | 3300016733 | 235002426 | 510441 |

# CHAPTER 4

## Methanethiol influence on the expression of denitrifying genes and nitrous oxide production in the marine bacterium *Ruegeria pomeroyi* DSS-3

# Abstract

It was shown previously that methanethiol (MeSH) produced during degradation of dimethylsulfoniopropionate (DMSP) interferes with the final reduction step of denitrification, enhancing nitrogen release via nitrous oxide ($N_2O$), a greenhouse gas with a stronger potential than $CO_2$ for global warming. To examine how methanethiol, affects the $N_2O$ reduction step of denitrification, cell suspensions of the model organism *Ruegeria pomeroyi* DSS-3 were titrated with MeSH, and the $N_2O$ potential rates and the expression level of denitrification genes assessed. Indeed, $N_2O$ accumulation was significantly exacerbated ($p < 0.001$) with increased MeSH, a pattern expected from previous studies. Transcripts from *nirS* and *norB* (the genes encoding the nitrite and nitric reductases, respectively) did not reveal significant differences ($p > 0.05$) in magnitude in the different MeSH treatments. However, *nosZ* (the nitrous oxide reductase gene) transcription was significantly overexpressed ($p < 0.001$) in accordance with progressively higher MeSH additions, rather than an expected inhibition. Preliminary analysis of NosZ activity revealed that MeSH does not interfere directly with the functioning of the reductase. Therefore, this study suggests that $N_2O$ accumulation, due to additions of MeSH, is not caused by an inhibition at the transcriptional, but most probably due to an alternate regulatory point in the denitrification process of *R. pomeroyi* DSS-3. Overall, this interference could result in higher $N_2O$ emissions from marine microbial communities in the presence of organic sulfur, with possible implications for global climate.

# 4.1. Introduction

Life on Earth depends on the dynamic network of biogeochemical cycles and their impact on ecosystem processes (Falkowski et al., 2008). It is extremely relevant to understand how major elements cycles interact regarding the biological, chemical and geological processes. Likewise, the co-occurrence of marine sulfur and nitrogen cycling processes has been previously reported. Indeed, Sørensen (1978) noticed a major accumulation of denitrification intermediates near the sulfide-rich zone of coastal marine sediment, being later confirmed an inhibition on $N_2O$ reduction step by sulfide in denitrifying bacterial cells (Sørensen et al., 1980). Besides the effect on denitrification, sulfide ($HS^-$) additions on estuarine sediment slurries resulted also in an inhibition on nitrification rates (Joye and Hollibaugh, 1995). On the other hand, the coupling of nitrogen and sulfur biogeochemical processes was also observed in freshwater sediments where sulfide, generated from dissimilatory sulfate reduction in anaerobic conditions, acts as an electron donor stimulating, thus, dissimilatory nitrate reduction to ammonia (DNRA; Brunet and Garcia-Gil, 1996). Later, it was discovered a novel inhibitory interaction between organic sulfur and nitrogen cycles, specifically addressing an influence by MeSH, a DMSP degradation compound, on denitrification in benthic coastal environmental samples and in *R. Pomeroyi* DSS-3 cultures (Magalhães et al., 2011, Magalhães et al., 2012a), by inhibiting the reduction of $N_2O$ (**Figure 4.1**), a powerful greenhouse gas with an extended atmospheric lifetime of 116 ± 9 years (Prather et al., 2015). However, the mechanism of the MeSH effect on microbial denitrification activity is not known, nor is the level (transcriptional and/or enzymatic) at which it operates. The potential regulation of $N_2O$ accumulation by MeSH could influence $N_2O$ emissions and ultimately its influence on global climate.

DMSP, a precursor of MeSH, is synthesized mainly by micro- and macroalgae (Gage et al., 1997, Summers et al., 1998, Stefels, 2000) but also by higher plants (Greene, 1962, Hanson et al., 1994, Trossat et al., 1996), corals (Raina et al., 2013), and even by certain marine Alphaproteobacteria (Curson et al., 2017). DMSP provides several physiological roles for algae, acting as an osmolyte, cryoprotect, antioxidant (Dickson and Kirst, 1987, Karsten et al., 1990, Kirst et al., 1991, Stefels, 2000, Sunda et al., 2002, Husband et al., 2012, Talarski et al., 2016), herbivore deterrent (Van Alstyne et al., 2001, Strom et al., 2003, Fredrickson and Strom, 2009), and a chemical signal for foraging (Geng and Belas, 2010, Garren et al., 2014, Lee et al., 2016). Also, DMSP represents an important sulfur and carbon source for bacterioplankton, and is one of the most ubiquitous and abundant organosulfur compounds in the marine environment (Kiene et al., 2000, Kiene and Linn, 2000a, Yoch, 2002). Once it is released by marine algae, DMSP is easily assimilated by S-requiring marine bacteria, and degraded via a demethylation/demethiolation pathway that ends with the formation of MeSH. Alternatively, a

cleavage pathway leads to DMS, an important biogenic sulfur source emitted to the atmosphere (Charlson et al., 1987, Andreae, 1990, Vallina and Simó, 2007). About 90% of dissolved DMSP in seawater is channeled via demethylation (Kiene, 1996, González et al., 1999, Kiene and Linn, 2000b, Kiene et al., 2000), making it an extremely important process in global sulfur cycle. DMSP demethylation also dominates in anoxic sediments (Kiene and Taylor, 1988).

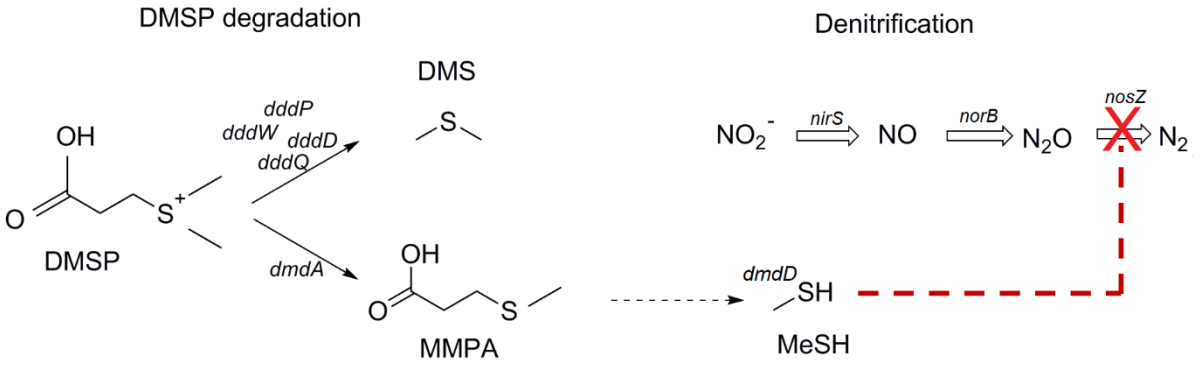


**Figure 4.1.** Simplified diagram of the interaction between DMSP degradation and denitrification reduction steps, in *Ruegeria pomeroyi* DSS-3. Genes involved in the bacterial DMSP cleavage pathway are *dddP*, *dddW*, *dddD* and *dddQ*, which encode the lyases that convert DMSP in DMS. In the demethylation pathway, the DMSP demethylase, encoded by *dmdA*, generates MMPA which, after the downstream steps (not shown in the diagram), releases MeSH, the final product of the demethylation/demethiolation pathway. DMS – dimethyl sulfide; MMPA – methylmercaptopropionate; MeSH - methanethiol; *nirS* – nitrite reduction gene, *norB* – nitric oxide reduction gene; *nosZ* – nitrous oxide reduction gene.

There is still a limited understanding of the key factors regulating DMSP degradation pathways (Vila-Costa et al., 2006, Salgado et al., 2014). However, recent advances in molecular biology and genomics are providing tools to unveil the complexity of bacterial DMSP transformations. We now know that DMSP catabolism genes are widely distributed across marine environments (Varaljay et al., 2012, Cui et al., 2015, Zeng et al., 2016), with about half the bacteria in ocean surface waters harboring the diagnostic *dmdA* gene for the demethylation pathway (Howard et al., 2006, Howard et al., 2008). The demethylation pathway has also been found to be nearly exclusive to marine Alphaproteobacteria, including SAR11 and Roseobacter members (Bullock et al., 2017).

A growing concern of climate scientists is the increase in anthropogenic emissions of greenhouse gases. Atmospheric $N_2O$, with a global warming potential 265-298 times greater than $CO_2$ for a 100-year timescale (Ciais et al., 2013), has increased in concentration about

20% over the past century, mainly due to human activities and strongly impacting the depletion of the stratospheric ozone layer (Ravishankara et al., 2009, Wuebbles, 2009). Microbial-mediated processes are a major source of $N_2O$ (reviewed in Thomson et al., 2012), and oceans represent about 34% of natural sources (Ciais et al., 2013), either by denitrification or nitrification, where $N_2O$ appears as an intermediate or side product, respectively (Freing et al., 2012).

In this study, the influence of MeSH, the end product of the dominant bacterial DMSP degradation pathway in the ocean (Kiene and Linn, 2000b, Kiene et al., 2000, Howard et al., 2008), was examined on the accumulation of the biogenic $N_2O$ from denitrification process. The goal was to characterize the MeSH-$N_2O$ interaction and identify the molecular or enzymatic mechanism(s) responsible, using cultures of the model marine bacterium *Ruegeria pomeroyi* DSS-3, a Roseobacter representative within the Alphaproteobacteria that has the capacity to denitrify as well (Moran et al., 2004). We hypothesized that the increase in $N_2O$ production might be the result of incomplete denitrification due to a lack of a functional *nosZ*, the gene encoding the catalytic subunit of the nitrous oxide reductase (N2OR; Philippot et al., 2011), or inhibition by MeSH of the enzyme $N_2OR$, that catalyzes the reduction of $N_2O$ to $N_2$ (Knowles, 1982, Brunet and Garcia-Gil, 1996, Senga et al., 2006, Bergaust et al., 2012). Therefore, a specific inhibitor of denitrification rates could be important in setting oceanic $N_2O$ emissions and potentially affect global warming.

## 4.2. Material and Methods

### 4.2.1. *Ruegeria pomeroyi* DSS-3 culture conditions

*R. pomeroyi* DSS-3 cells were grown in 30 ml of liquid Marine Basal Medium (MBM; González et al., 1999) containing 50 mM Tris-HCl pH 7.5, 19 mM $NH_4Cl$, 0.33 mM $K_2HPO_4.3H_2O$ (Baumann and Baumann, 1981), with the addition of 2 mM glucose, 0.1 mM $FeSO_4$ (González et al., 1999), and 100 µM of $NaNO_2$ as an electron acceptor. Cell suspensions were incubated for 48 h at 28°C on a rotary shaker (80 rpm) until near the end of the exponential phase ($OD_{600}$ 0.9). Three milliliters of cell culture were placed in a 12 ml crimp-toped serum vial, sealed with a Teflon stopper and purged with $N_2$ for 15 min. Cell suspensions were spiked with different amounts (150 – 1500 µl) of MeSH gas from a Dynacal® permeation tube (VICI Metronics), in a sealed 100 ml serum vial flushed with $N_2$. Controls without MeSH were also run in triplicate. Samples were incubated for 4h at 28 ºC in triplicate vials and the accumulation of $N_2O$ and denitrification gene transcripts were measured at the end of the incubation as described below.

Negative controls with only sterile MBM media (without bacterial cells), and with MeSH amendments were also run in triplicate.

### 4.2.2. Analytical techniques

Nitrous oxide was quantified by collecting 8 ml of headspace from the serum vials with a simultaneous addition of 8 ml of 3 M sodium chloride solution (NaCl; Joye et al., 1996). Samples were injected in a Bruker SCION gas chromatograph (436-GC) equipped with an electron-capture detector (ECD), two HayeSep D columns held at 80 ºC. The carrier gas was a mixture of 5 % methane in argon and the detector temperature was 250 ºC. $N_2O$ had a retention time of 1.5 min. The detection limit for $N_2O$ was 15 nM and $N_2O$ concentration was calculated using a standard curve generated from certified gas standards ($N_2O$ in He, Scott Specialty Gas).

### 4.2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the cell suspensions of each MeSH treatment using an NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal), according to the instructions from the manufacturer. DNA was removed from RNA by treatment with 1 U $\mu L^{-1}$ of DNase I (Sigma) using DNA- and RNA-free reagents. Reverse transcription (RT) of RNA was performed using the NZY First Strand cDNA synthesis kit (NZYTech, Lisbon, Portugal) by adding 5 µL of total RNA to a 15 µL RT mixture containing 10 µL of NZYRT 2x Master Mix and 2 µL NZYRT Enzyme Mix. Samples were incubated at 25 ºC for 10 min and after at 50 ºC for 30 min, and the reaction was inactivated by heating at 85 ºC for 5 min, and then chilled on ice. Synthesized cDNA was further used for amplification according to the protocol described below. All the working material was previously treated with RNase Away® Reagent (Life Technologies, USA).

### 4.2.4. Primer pair design

PCR primers for amplification of the denitrification *nirS* and *norB* genes (**Table 4.1**), specific to the nitrite and nitric oxide reduction, respectively, were designed in Primer-BLAST (Ye et al., 2012) using the respective nucleotide sequence of the abovementioned genes from *R. pomeroyi* DSS-3 as query sequence obtained from Roseobase (http://www.roseobase.org/). It was performed an *in silico* test to validate and confirm the primer specificity and non-amplification of unintended targets by using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) on the generated primer pairs.

**Table 4.1.** Primer pairs used in this study.

| Target gene | Primer | Sequence 5'- 3' | Amplicon size (bp) | Annealing temp (ºC) | Source |
|---|---|---|---|---|---|
| *nirS* | RPnirSF1 | GCTCTTCGTGAAATCGCACC | 253 | 61 | This study |
| | RPnirSR1 | TTCCAGATCCTTGGCGTTCC | | | |
| *norB* | RPnorBF1 | CTCCTCGCTCGAGGTCATTC | 287 | 61 | This study |
| | RPnorBR1 | ACAGGATCGGCATCGCATAG | | | |
| *nosZ* | nosZF2 | CGCRACGGCAASAAGGTSMSSGT | 267 | 58 | Henry et al. (2006) |
| | nosZR2 | CAKRTGCAKSGCRTGGCAGAA | | | |
| 16S rRNA | 27F | AGAGTTTGATCMTGGCTCAG | 394 | 56 | Heuer et al. (1997) |
| | 1492R | TACGGYTACCTTGTTACGACTT | | | |

### 4.2.5. Amplification of *nirS*, *norB* and *nosZ* genes

Quantification of *nirS*, *norB* and *nosZ* genes was performed in a Real-Time PCR System (StepOnePlus™, Applied Biosystems, USA) using the primer sets described in Table 1. Real-time PCR reactions were run in quadruplicate with a 20 µL reaction volume containing 10 µL of *Power* SYBR® Green PCR Master Mix (2X) that included ROX as a passive reference, 0.5 µL of each primer (10 mM), 8.5 µL of nuclease-free water (Promega) and 0.5 µL (~2.5 ng) of the template. The negative control consisted of all the reagents except the template. The thermal cycling conditions consisted of an initial denaturation for 5 min at 95 ºC, then 35 cycles of denaturation at 95°C for 30 s, annealing at the respective primer temperature (see **Table 4.1**) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min, followed by a melting curve with an increasing temperature from 55 to 95°C at a rate of 0.5°C per second. To confirm the absence of genomic DNA, a PCR using the universal primers gene sequences of prokaryotic 16S rRNA was run in all cDNA samples (**Table 4.1**). Standard curves were generated in duplicate for each primer set and consisted of five serial dilutions (10-fold) of DNA extracted from *R. pomeroyi* cultures, with $R^2$ values ranging between 0.94 and 0.99. Ct values were then converted into relative gene copy numbers based on each respective standard curve.

### 4.2.6. Whole-cell enzyme assays

Nitrous oxide reductase ($N_2OR$) activity was measured spectrophotometrically using the methyl viologen-linked assay described by Jones et al. (1992). Briefly, cells were grown

aerobically at 28 ºC, under constant gently shaking, in a batch culture of 2 L liquid MBM with addition of 2 mM glucose and 100 µM sodium nitrite. After two days of incubation, cells were harvested by centrifugation at 10,000x *g* and 4 ºC for 30 min. The enzyme activity assay was determined in anoxic 1 ml stoppered cuvettes in which the reaction mixture consisted of 200 µM methyl viologen, 12.5 mM dithionite dissolved in 10 mM potassium phosphate buffer (pH 7.1), and $N_2O$-saturated water. After the background oxidation rate stabilized, the reaction was initiated by the injection of 25 µL of a protein-containing sample (300 µg $ml^{-1}$), from whole cells lysate, to initiate the reaction. On the treatment sample, MeSH was added before the reaction being initiated. The absorbance was regularly monitored at 600 nm over 20 min until the methyl viologen was completely oxidized. Control assays which consisted of the complete reaction mixture without $N_2O$ (negative control), displayed no bleaching of the reduced methyl viologen. All material and reagents were purged with pure $N_2$. The $N_2OR$ activity was measured based on the Beer-Lambert law and expressed as µmol $N_2O$ reduced per min per mg of protein.

### 4.2.7. **Data analysis**

Normality of the $N_2O$ production data from the different MeSH treatments was checked by performing the Shapiro-Wilk test (Shapiro and Wilk, 1965). Equality of variances was confirmed with the Levene test (Levene, 1960), followed by a Wilcoxon Signed-Rank Test ($p < 0.001$) to evaluate the statistical differences between the different MeSH amendments. For the qPCR transcript abundance and relative expression, the different treatments were compared for significant ($p < 0.001$) changes by a one-way analysis of variance (ANOVA). Statistical analysis was performed using the R version 3.3.3 (2017-03-06).

## 4.3. **Results**

Higher additions of MeSH yielded higher $N_2O$ accumulation by *R. pomeroyi* DSS-3 cell cultures, with the progressive increase along the different treatments significantly different ($p < 0.001$; **Figure 4.2**), suggesting an inhibition on the *nosZ* transcription or $N_2OR$ activity. In the control (without MeSH), the production of nitrous oxide was very low (36.97 ± 2.39 pmoles $N_2O$ $h^{-1}$ $ml^{-1}$) in anoxic cell suspensions, indicating an active denitrifying activity with almost complete $N_2O$ reduction. Furthermore, $N_2O$ formation increased progressively with the level of MeSH amendment, reaching 16,043 ± 3,781 pmoles $N_2O$ $h^{-1}$ $ml^{-1}$ in *R. pomeroyi* cell suspensions. However, the highest MeSH addition produced less $N_2O$ (7,224 ± 499 pmoles

$N_2O$ $h^{-1}$ $ml^{-1}$ cell suspension), possibly indicating overall saturation of the cell at high MeSH concentrations.

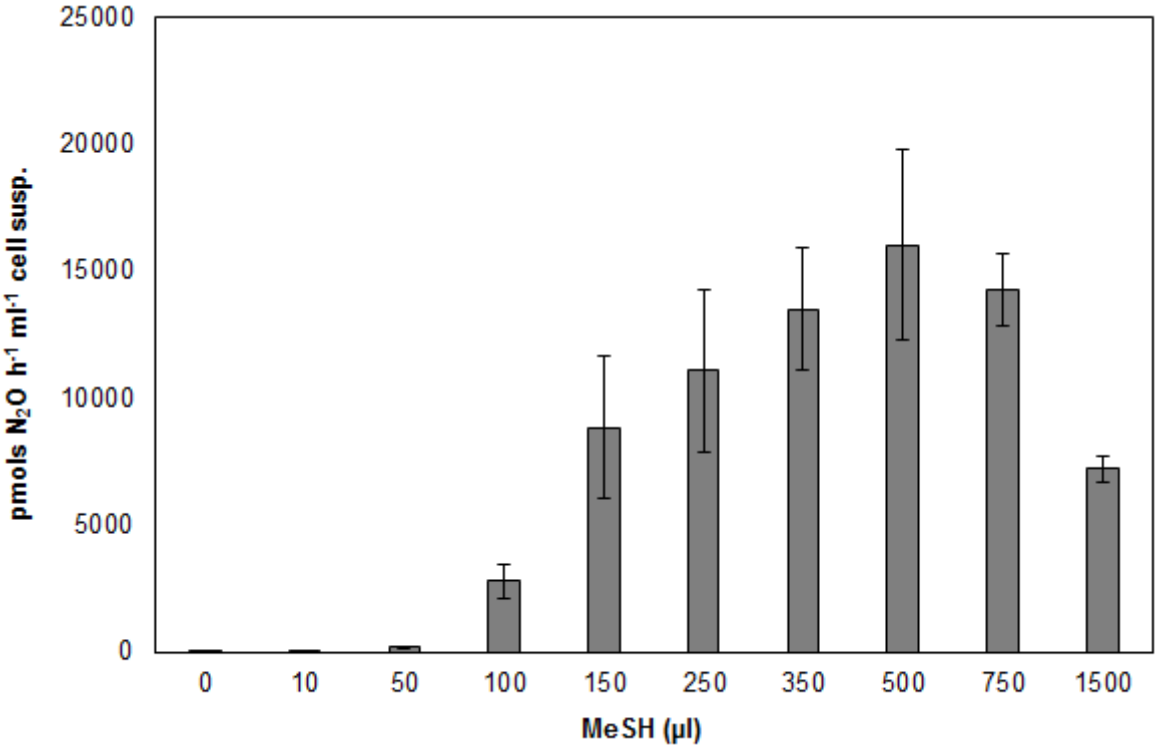


**Figure 4.2.** Influence of progressively higher MeSH additions on $N_2O$ accumulation rates in *R. pomeroyi* DSS-3 cell suspensions.

Our first hypothesis regarding the mechanism(s) responsible for influencing $N_2O$ accumulation was an effect on expression of denitrification genes, and specifically the MeSH inhibition of transcription of the nitrous oxide reductase gene. Therefore, we evaluated the expression levels of three functional genes involved in denitrification: *nirS* (nitrite reductase), *norB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase), through quantification of transcripts in *R. pomeroyi* DSS-3 cultures exposed to the different levels of MeSH amendment. It was found that neither *nirS* nor *norB* exhibited significant ($p > 0.05$; **Figure 4.3a** and **b**) differences in transcript abundances among the different amendments of MeSH. Furthermore, the expression of *nirS* ranged from 18.99 x $10^{12}$ to 21.24 x $10^{12}$ gene copies, while *norB* ranged from 78.13 x $10^{11}$ to 97.97 x $10^{11}$ gene copies. Accordingly, the expression of *nirS* and *norB* relative to control samples (without MeSH amendments) showed non-significant ($p > 0.05$) changes along the different MeSH treatments, with very low ratios (0.3 – 1.7-fold change; **Figure 4.4a** and **b**).

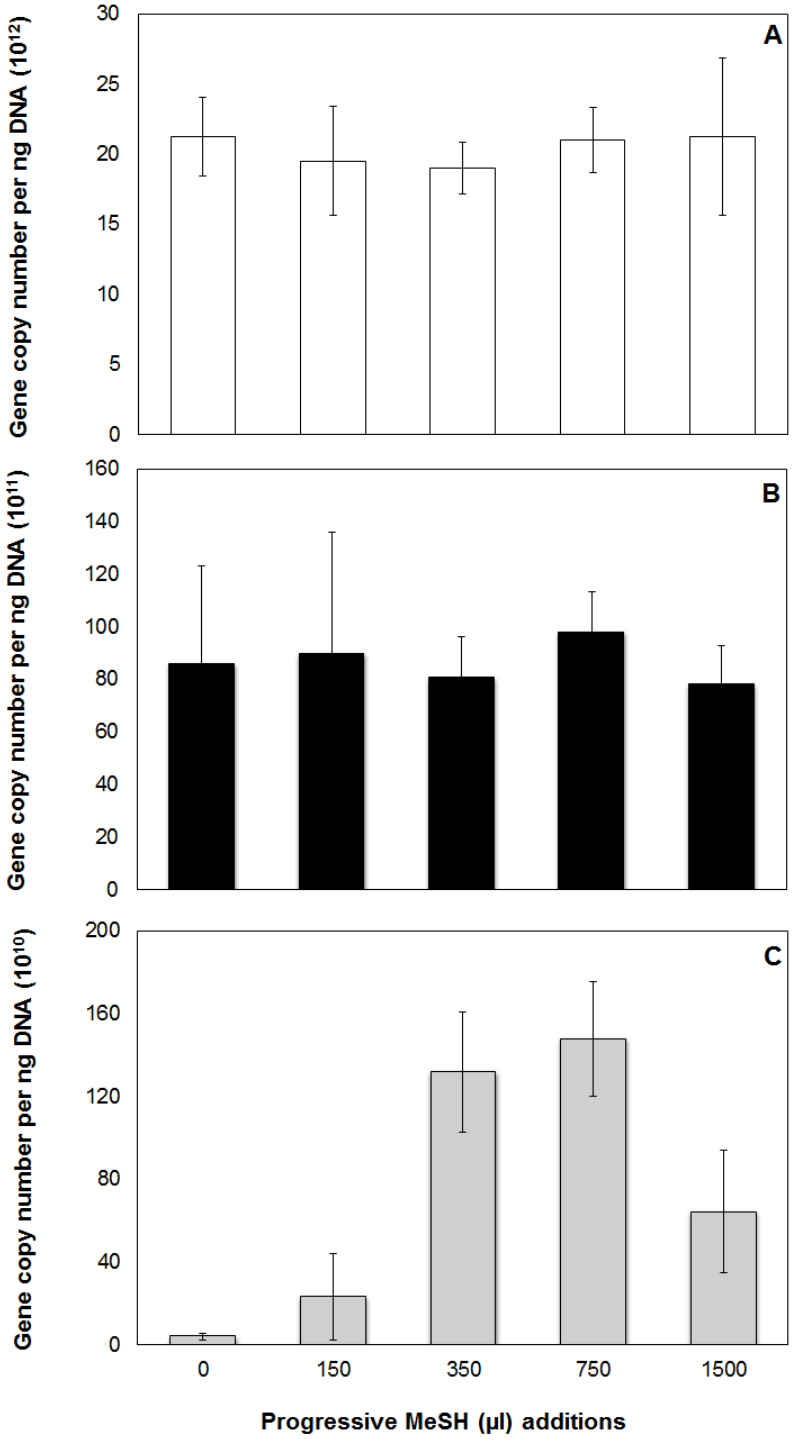


**Figure 4.3.** Transcript abundance of (a) *nirS*, (b) *norB* and (c) *nosZ* in treatments with progressively higher MeSH additions in *Ruegeria pomeroyi* DSS-3 cell suspensions.

On the other hand, it was found a distinct transcript profile in the abundance of *nosZ,* although it was in the opposite direction from our initial hypothesis, which proposed a reduction in *nosZ*

expression with progressive additions of MeSH. Instead, *nosZ* transcription had an increase significantly ($p < 0.001$; **Figure 4.3c**) different with increasing MeSH additions, ranging from 4.18 x $10^{10}$ (on the control samples) to 147.87 x $10^{10}$ gene copies (with addition of 750 µl MeSH). Consistent with the biogeochemical profile of $N_2O$ accumulation (**Figure 4.2**), the highest MeSH amendment resulted in fewer transcripts (64.27 ± 29. x $10^{10}$ gene copies) than expected in the progression. When evaluating the relative expression of *nosZ* compared to the control, it was observed a significantly ($p < 0.001$) higher expression ratio along the MeSH treatments, namely with 350 µl of MeSH addition in DSS-3 cell cultures (~104-fold change; **Figure 4.4c**). However, the highest MeSH additions indicated a reduction of the denitrifying activity in *R. pomeroyi* DSS-3 cell suspensions, clearly demonstrated by a decrease in expressions ratios (**Figure 4.4c**).

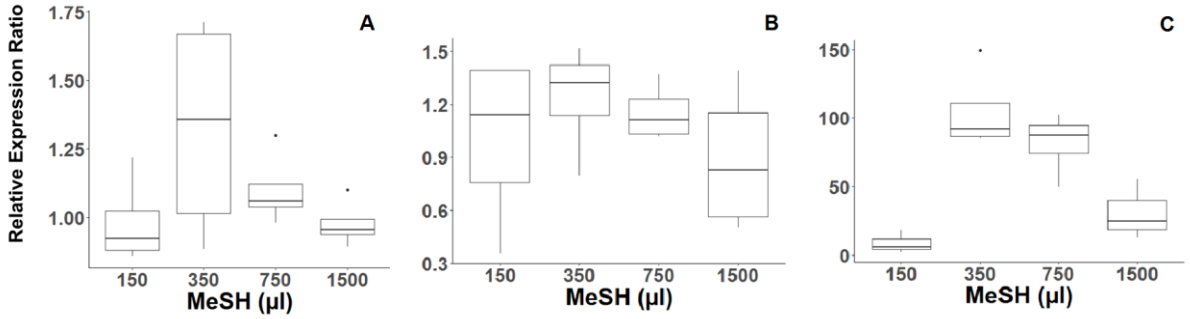


**Figure 4.4.** Relative expression ratio of denitrification (a) *nirS*, (b) *norB* and (c) *nosZ* genes, in treatments with progressively higher MeSH additions in *Ruegeria pomeroyi* DSS-3 cell suspensions. The relative expression ratio of each gene was calculated relative to the control samples.

After evaluating the gene expression, a preliminary study of the $N_2$OR activity in *R. pomeroyi* DSS-3 cell suspensions under denitrifying conditions was assessed, by monitoring the oxidation of the methyl-viologen (the electron donor), through the decline of the absorbance. When measuring the enzyme activity of whole cells, the positive control without MeSH showed a decrease in the oxidation of methyl viologen (compared to the negative control; **Figure 4.5a**), reflecting the indirect $N_2O$ reduction which resulted in an enzyme activity of 1.9 µmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ of protein (**Figure 4.5b**). However, similar activity was also observed after treatment with 4 mM of MeSH, with an activity of 1.7 µmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ protein (**Figure 4.5b**). The nitrous oxide reductase in cultures of *R. pomeroyi* DSS-3 remained active under denitrifying conditions even after exposure to MeSH.

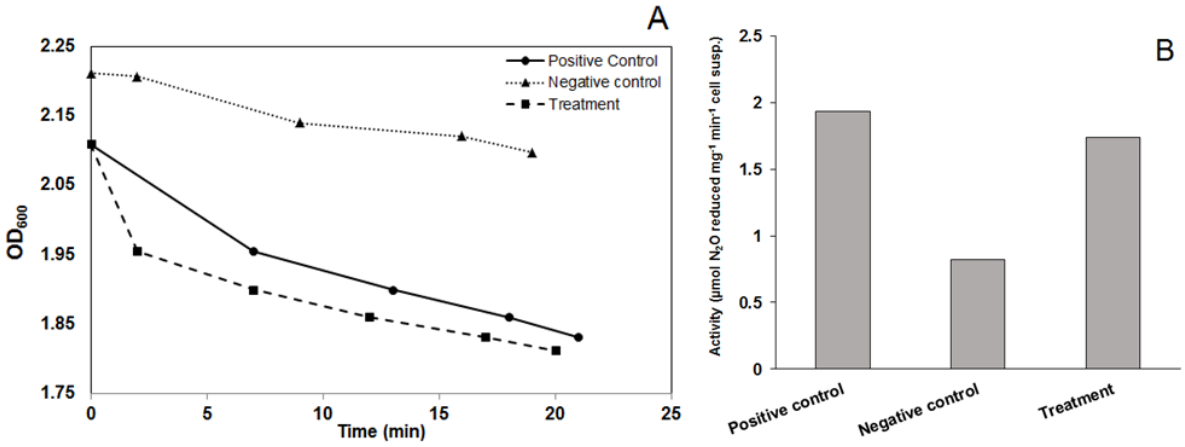


**Figure 4.5.** Nitrous oxide reduction activity was measured in whole cells lysate of *Ruegeria pomeroyi* DSS-3 using the methyl viologen-linked assay, in a positive control without MeSH addition, a negative control without the substrate [$N_2O$] and a treatment in which 3 µM of MeSH was added to the reaction mixture. (a) The absorbance was monitored at 600 nm, and (b) the activity was calculated based on the rate of oxidation after $N_2O$ addition.

## 4.4. **Discussion**

The $N_2O$ reduction is the last step of complete denitrification, an autonomous respiratory process concomitant with energy conservation that allows bacterial growth under low oxygen conditions (Zumft, 1997). Although, $N_2O$ emissions in marine ecosystems due to microbial metabolism represent a major source in the biological $N_2O$ budget, there are few studies investigating the factors regulating the underlying microbial activity. The most important factors addressed in modeling studies is the concurrent occurrence of low availability of $O_2$ and high availability of nitrogen leading to denitrification, and $N_2O$ accumulation (Jørgensen et al., 1984, Naqvi et al., 2000, Codispoti, 2010, Naqvi et al., 2010). To improve estimates of $N_2O$ emissions, further research is needed focusing on the mechanisms and controls of microbial $N_2O$ production.

The linkage between dissolved DMSP and denitrification in which the presence of MeSH exacerbated $N_2O$ accumulation has been previously identified (Magalhães et al., 2011, Magalhães et al., 2012a). This interaction was initially evaluated in estuarine sediment slurries, where the influence of increasing MeSH concentrations resulted in a proportional $N_2O$ increase. This same response was subsequently confirmed in cell suspensions of a pure culture of *R. pomeroyi* DSS-3 (Magalhães et al., 2011). However, the reason behind the increased $N_2O$ accumulation and the point at which the mechanism acted, remained unanswered. In the present study, not only the influence of MeSH on the $N_2O$ production in

DSS-3 cell suspensions was confirmed, but different MeSH amendments generated different rates of $N_2O$ production, which initially suggested an inhibition at the transcription level.

The initial hypothesis was that the *nosZ* transcription could be inhibited by MeSH, resulting in an inefficient reduction of $N_2O$ releasing it. An alternative hypothesis was that the higher transcription of *nirS* or *norB* could cause a buildup of downstream intermediates, resulting in more $N_2O$ accumulation. However, the non-significant ($p > 0.05$) results of *nirS* or *norB* among the different amendments of MeSH, ruled out the possibility that changes in expression of nitrite or nitric oxide reduction genes were responsible for higher $N_2O$ accumulation. Additionally, when evaluating the *nosZ* transcription, the first hypothesis was rejected since the gene expressed an upregulation, instead of an inhibition as expected, with the MeSH amendments. Hence, these results indicated that MeSH does not act as an inhibitor on the transcription of *nosZ,* but apparently is a promotor. In fact, the gene is being upregulated possibly in response to the accumulation of the product $N_2O$ i.e. not being reduced to $N_2$. On the other hand, NO can signal the upregulation of *nosZ* in organisms of the genera *Pseudomonas* (Vollack and Zumft, 2001, Arai et al., 2003). In this study, however, neither the expression of *nirS* (the gene converting $NO_2^-$ to NO) nor the *norB* was not afected by MeSH addition, indicating that NO is not likely to be responsible for the observed increase in *nosZ* transcription. Instead, it appears that *nosZ* may be positively regulated in *R. pomeroyi* DSS-3, based on the level of $N_2O$ accumulation.

Several studies have addressed the mechanisms of RNA regulation and consequent adaptation in bacteria in response to variable environmental conditions (Cases et al., 2003, Shi et al., 2009, Waters and Storz, 2009). Recently, studies of small RNAs (sRNAs) in *R. pomeroyi* DSS-3 during DMSP metabolism have shown the importance of these regulatory RNAs (Burns et al., 2016, Rivers et al., 2016). On the preliminary electropherogram profiles from DSS-3 cell suspensions amended with MeSH (data not shown), the expression of sRNAs is higher. Therefore, it is possible that this type of feedback might occur, when cell cultures of *R. pomeroyi* DSS-3 encountered elevated $N_2O$ concentrations, with some sRNA acting post-transcriptionally by modulating protein activity, since sRNAs are produced faster than proteins, and act post-transcriptionally (Shimoni et al., 2007, Mehta et al., 2008, Van Assche et al., 2015). Thus, it is possible that a more complex regulatory scheme involving sRNAs acting in a post-transcriptional regulation could explain the link between MeSH and $N_2O$ accumulation in *R. pomeroyi* DSS-3. However, the fact that $N_2O$ accumulation was initially discovered in studies of diverse marine microbial communities suggests that MeSH effects are not unique to a particular regulatory arrangement in a particular bacterium.

Given the gene expression data, it was developed a second hypothesis that posited MeSH inhibition of the ability of the NosZ enzyme to reduce $N_2O$. Recent transcriptomic analyses

demonstrated the importance of copper in regulating expression of the *nos* operon at the level of gene expression (Gaimster et al., 2017) and $N_2OR$ requires copper for its activity (Pomowski et al., 2011, Sullivan et al., 2013). $N_2O$ reductase goes through a conformational change when the cytochrome *c* subunit, which participates in electron transport during $N_2O$ reduction to $N_2$, forms a copper-sulphur cluster that enables enzymatic activity (Rasmussen et al., 2000, Pomowski et al., 2011). Consequently, a possible cause for nitrous oxide accumulation could be hindrance by MeSH of the enzyme assembly or active site access, preventing the formation of the cytochrome *c* - nitrous oxide reductase complex. Alternatively, Manconi et al. (2006) suggested that the presence of sulfide causes copper sequestration from the metalloenzyme $N_2OR$ in *Pseudomonas aeruginosa* and consequently eliminates $N_2O$ reduction activity. However, no differences on the $N_2OR$ activity with or without MeSH addition were found, with both samples representing the reduction of $N_2O$. The values of enzyme activity agreed with other studies, with different organisms, where the enzyme specific activity was ~0.1 – 2.5 µmol $N_2O$ reduced $min^{-1}$ $mg^{-1}$ of protein, which indicates that the $N_2OR$ in DSS-3 is actively denitrifying (Jones et al., 1992, Nunes, 2013). Additionally, it is possible that not enough MeSH was added to inhibit the enzyme. Nevertheless, the nitrous oxide reductase in cultures of *R. pomeroyi* DSS-3 remains active under denitrifying conditions even after exposure to MeSH. The absence of inhibition of the enzyme leads to reconsider the bacterial whole-transcriptome analysis to study the mechanisms underlying the $N_2O$ reduction.

Transcription of the full *nosRZDFYLX* operon, which includes genes for additional enzymes required to catalyze the reduction of nitrous oxide in denitrifying bacteria (Chan et al., 1997, Honisch and Zumft, 2003, Velasco et al., 2004), can be controlled by regulatory proteins such as NosR (an iron-sulfur flavoprotein), NasT or $FixK_2$ (a CRP/FNR-like transcriptional regulator) in response to oxygen or N-oxides (Cuypers et al., 1992, Arai et al., 2003, Wunsch and Zumft, 2005, Sánchez et al., 2017, Torres et al., 2017). Zheng et al. (2014) analyzed the inhibition of $N_2O$ reduction rates and the transcriptional profiling by zinc oxide nanoparticles, in *Paracoccus denitrificans* cultures, and demonstrated a down-regulation of the *nos* gene cluster. On the other hand, Beauchamp et al. (1989) and Zumft (1997) observed a decrease in gene expressions of key enzymes involved in the metabolism of organic matter, responsible for the electrons supply necessary to complete denitrification. These studies demonstrated that *nosZ* expression was substantially regulated along with the rest of the genes in the *nos* operon, unlike the present results that showed a significantly different upregulation only of *nosZ*.

## 4.5. **Conclusions**

Overall, this study presents a mechanistic study about the probable reason involved in the reduction of $NO_2^-$ to $N_2O$ rather than $N_2$, in a marine denitrifying bacterium. Results from this study demonstrate that the presence of marine organic sulfur can exacerbate $N_2O$ emissions in low oxygen conditions and these interactions represent an important factor when making estimations about marine $N_2O$ cycling. Ocean model simulations account nitrification as the dominant pathway of $N_2O$ production, however, in suboxic conditions, marine $N_2O$ flux appears to be higher in magnitude than predicted due to an incomplete denitrification. Sometimes this outcome is neglected in global climate models, leading to an underestimation of $N_2O$ budget.

Our experiments demonstrated first that MeSH-induced $N_2O$ accumulation in *R. pomeroyi* DSS-3 cultures was not attributable to inhibition of *nosZ* transcription, and transcription rates may instead be upregulated by accumulating $N_2O$. Also, we hypothesize that MeSH does not hinder the function of NosZ through either direct (e.g. interfering with enzyme assembly) or indirect (e.g. sequestering a metal needed for enzyme activity) methods. To look more broadly at potential mechanisms, future efforts will investigate genome-wide transcriptional patterns in *R. pomeroyi* in response to MeSH.

# CHAPTER 5

Final considerations and future directions

# 5.1. Final considerations

While often overlooked within the scope of climate change, the ocean microbiota is of paramount importance for the sustainability of life on Earth (Falkowski et al., 2008). Microorganisms are pivotal on the regulation of global biogeochemical cycles and have been the focus of a growing interest in the study of climate change biology (Cavicchioli et al., 2019). Improved knowledge about the microbial community responses to environmental change, the factors and mechanisms that regulate microbially mediated biogeochemical processes and their interactions is fundamental for understanding the functionality of marine ecosystems.

Nitrogen and sulfur are key elements required for all living organisms. Marine N and S biogeochemical cycles are instrumental to maintain and regulate the stability of the biosphere, namely through the production and consumption of the Earth climate-regulating gases, such as $N_2O$ and DMS (Charlson et al., 1987, Ravishankara et al., 2009). However, the growing anthropogenic pressures (e.g. acidification, deoxygenation), are impacting the nitrogen and sulfur inputs in the marine environment, changing ocean biogeochemistry and, consequently, influencing the atmosphere stability (Rees, 2012a).

Numerous studies have made a concerted effort to evaluate the factors that influence the N cycle processes and, more importantly, trace all the sources and sinks of such important climate active gas as $N_2O$. Furthermore, assessing if $N_2O$ derives from only one N-cycling pathway or results from the interaction of coupled mechanisms, is an ongoing open challenge.

To the present, the known biotic pathways for $N_2O$ production are denitrification (Zumft, 1997), hydroxylamine oxidation (Otte et al., 1999), dissimilatory nitrate reduction to ammonia (Bleakley and Tiedje, 1982), and nitrifier denitrification (Poth and Focht, 1985). However, from **CHAPTER 1**, it was clear that there is a far more complex network of microbial processes and pathways that mediate $N_2O$ flux in the marine environment. Oxygen is a key factor regulating the two main N cycle processes - nitrification and denitrification, in marine environments, with repercussions on the $N_2O$ emissions (Jørgensen et al., 1984, Codispoti and Christensen, 1985, Rysgaard et al., 1994, An and Joye, 2001, Qin et al., 2017). Of particular concern is the effect of ocean deoxygenation caused by climate change on N cycle and, especially, on the release of the powerful greenhouse gas, $N_2O$, into the atmosphere (Naqvi et al., 2000, Codispoti, 2010, Keeling et al., 2010, Arévalo-Martínez et al., 2019). Indeed, a predicted consequence of anthropogenic pressure on the open ocean is the expansion of OMZs, that are hotspots for the occurrence and/or coupling of anaerobic metabolisms such as denitrification, DNRA and anammox (reviewed in Paulmier and Ruiz-Pino, 2009, Wright et al., 2012, Bertagnolli and Stewart, 2018, Kuypers et al., 2018). The decline in oceanic dissolved $O_2$ will likely increase

$N_2O$ production by several pathways, as reviewed in **CHAPTER 1**, acting as a positive feedback factor and potentially amplifying global warming (Gruber, 2008, Wuebbles, 2009). However, there are numerous biogeochemical feedbacks with unknown effects on the marine N inventory, to help stabilize against anthropogenic perturbations, and unpredictable consequences in $N_2O$ production and consumption (Landolfi et al., 2017, Battaglia and Joos, 2018). The large uncertainties in future marine $N_2O$ emissions require further studies focusing on the biogeochemistry and interactions occurring in natural environments, aiming to assess a deeper knowledge about the complexity of N cycle feedbacks. Therefore, the present thesis starts with a broad analysis of the two main N cycle processes - nitrification and denitrification, in an estuarine system, where the river meets the sea; then, narrows the study to the interaction between denitrification and DMSP catabolism; and, finally, focus on the specific effect of MeSH on the $N_2O$ reduction step.

In **CHAPTER 2**, an overview about the environmental influence on the spatial-temporal distribution of N-cycle microbial communities in the water column of the Douro estuary is presented. The knowledge about the ecology and distribution of microorganisms is crucial to understand the marine ecosystems metabolism and is required to predict how they may respond to global environmental changes (Cavicchioli et al., 2019). This chapter showed seasonal patterns associated with more abundant denitrifying *nirS*, bacterial and archaeal *amoA,* and the recently discovered non-denitrifying $N_2O$ reducers (*nosZ*II) during winter/spring. These seasons were characterized namely by high levels of dissolved oxygen and turbidity. The latter was a key factor controlling the distribution of functional genes, mainly *nirS* and bacterial and archaeal *amoA*, and possibly reinforcing the relationship between microorganisms, suggesting the potential coupling between denitrification and nitrification. Oxygen is a major factor controlling the partitioning between anaerobic N transformation processes (denitrification, DNRA and anammox; Kalvelage et al., 2011, Cojean et al., 2019) and influencing the co-existence of N-cycle pathways (Rysgaard et al., 1994, An and Joye, 2001, Zakem et al., 2020). Particularly, the co-occurrence of coupled nitrification-denitrification was suggested in this chapter because of a strong correlation between denitrifying and AO genes, also associated with turbidity. These two processes typically occur in the oxic-anoxic interface, with a very close spatial coupling of nitrification and denitrification (Jenkins and Kemp, 1984, Marchant et al., 2016). However, they can also take place in oxic conditions of aquatic environments with denitrification occurring inside of suspended particulate matter, rather abundant in turbid environments, which provide an anoxic microenvironment, and with nitrification occurring at the surface layer (Bianchi et al., 1992, Zhang et al., 2014, Xia et al., 2017, Bianchi et al., 2018, Zhu et al., 2018, Fuchsman et al., 2019). The chapter contributes to expand the knowledge about N cycle microbial communities in the water column, which is

considerably understudied compared to benthic microbes. Despite being a DNA-based analysis study, yielded surprising associations between functional genes, determined by specific environmental conditions, suggesting a potential coupling of processes.

Higher rates of $N_2O$ production were previously observed in the more urbanized stretches of the Douro estuary (Teixeira et al., 2010), which are affected by sewage discharge and untreated wastewater (Azevedo et al., 2006, Azevedo et al., 2008). The excessive loads of nitrogen in coastal ecosystems, which have been paralleled by human population growth, drive eutrophication and can lead to algal blooms (Paerl, 1997, Conley et al., 2009, Paerl and Scott, 2010), the DMSP producers. Consequently, the collapse of these blooms, also followed by an increase in dissolved DMSP concentrations (Gibson et al., 1996, Levasseur et al., 1996, Van Duyl et al., 1998, Yoch, 2002), results in dissolved oxygen depletion in sediments and water column, promoting denitrification and the potential release of $N_2O$ to the atmosphere. $N_2O$ emissions can be enhanced when microorganisms lack the *nosZ* gene, encoding the $N_2O$-reductase (Zumft, 1997), or alternatively when the final step of denitrification ($N_2O \rightarrow N_2$) is inhibited. Indeed, an inhibitory interaction between DMSP degradation pathway and the final step of denitrification, in estuarine sediments from Douro estuary and cell suspensions of the marine bacterium *Ruegeria pomeroyi*, resulting in $N_2O$ accumulation is known (Magalhães et al., 2011, Magalhães et al., 2012a). This impact on $N_2O$ production catalyzed the goal of surveying the possible mechanism(s) involved in this linkage between marine organic S and N cycles.

Therefore, **CHAPTER 3** aimed to evaluate the inhibitory interaction between DMSP metabolism and the denitrification process in cell suspensions of different Roseobacter strains, that are representative of a bacterial group associated with phytoplankton blooms (Zubkov et al., 2001, Onda et al., 2015), to uncover the missing links that shape $N_2O$ outcome. Indeed, the obtained results showed that $N_2O$ production was affected by DMSP or its degradation compounds, through the regulations of the denitrifying genes. When DMSP is released into the organic matter pool by viral lysis, algal senescence, zooplankton grazing upon phytoplankton blooms or physiological stress (Hill et al., 1998, Laroche et al., 1999, Kiene et al., 2000, Mulholland and Otte, 2002), its dissolved form is rapidly metabolized by microorganisms via two competing catabolic pathways: cleavage and/or demethylation/demethiolation (Kiene et al., 2000, Yoch, 2002). Hitherto, it is still not clear what environmental controls and regulatory processes drive the utilization of one DMSP degradation route over the other. It was expected a *nosZ* inhibition because of DMSP influence, however, it was observed distinct *nosZ* responses possibly due to different microbial regulation of the gene expression. Recently, it was verified in *R. pomeroy* experiments, that if the ambient concentration of DMSP is >10 µM, being the bacterial sulfur demand completely satisfied, it

shifts DMSP degradation towards the cleavage route releasing excess sulfur as DMS (Gao et al., 2020). The results observed in this chapter show a downregulation in *nosZ* at 50 µM DMSP in *R. pomeroy* cultures, paired with an upregulation of *dddW* and *dmdA*, suggesting that DMSP metabolism regulates *nosZ* expression. In *R. pomeroy* cultures, the downregulation of *nosZ* was expected to persist across DMSP concentrations. However, at the highest concentration of DMSP (500 µM), the expression of *dmdD*, the gene encoding the hydratase (DmdD) that degrades methylthioacryloyl-CoA and releases MeSH (**Figure 3.2**), plateaued and *nosZ* expression was marginal. At the highest DMSP concentration, MeSH accumulation, the potential inhibitor of *nosZ* transcription (Magalhães et al., 2011), decreased probably due to the saturation of the cell or the gene expression machinery, suggested in (Gao et al., 2020), and also confirmed by the plateau on the *dmdD* expression. On the other hand, in *R. denitrificans* cultures, *dmdA* had the same expression as *nosZ* with a downregulation at the 500 µM DMSP concentration, indicating that *R. denitrificans* has a lower tolerance to DMSP at those high concentrations.

This study indicated that microorganisms respond differently to the surrounding conditions, and that the interference in $N_2O$ production from DMSP-producers may be indirectly controlled by the availability of dissolved DMSP. Nevertheless, it was evident that the accumulation of MeSH, a DMSP degradation compound, was likely controlling the *nosZ* transcription before the $N_2O$ reduction. After confirming the effects of DMSP addition on $N_2O$ reduction via regulation of bacterial gene expression, a mechanistic study was performed to explore and understand the mechanisms underlying the interaction between organic S and N cycles.

In **Chapter 4**, it was evaluated the direct influence of MeSH on the molecular and enzymatic mechanisms of $N_2O$ production in *R. pomeroyi* cultures. Surprisingly, it was confirmed a distinct upregulation of *nosZ* abundance, indicating that the increase in $N_2O$ accumulation was not a consequence of an inhibition on the *nosZ* transcription by MeSH, as expected. This was a different reaction from *R. pomeroyi* than that of **CHAPTER 3**, where *nosZ* transcription was downregulated at the 50 µM DMSP treatment. Additionally, the $N_2OR$ also remained active after exposure to MeSH, which indicated that the MeSH presence may affect other regulatory or enzymatic mechanisms required for microbial denitrification (Zumft, 1997, Zheng et al., 2014), and exacerbate $N_2O$ emissions. The different concentrations of DMSP influence the expression of the microbially-mediated DMSP degradation pathways and, consequently, control the differential production between DMS and MeSH (González et al., 1999, Gao et al., 2020). MeSH exerts a de facto control on $N_2O$ accumulation (**CHAPTER 4**), but the occurrence of MeSH depends on the external availability of DMSP, and the bacteria tolerance towards DMSP (**CHAPTER 3**), which regulates the relative expression of demethylation and cleavage. In phytoplankton blooms, where DMSP concentrations can go up to thousands of nanomolar

(4,240 nM; Kiene et al., 2019), dissolved DMSP is rapidly scavenged by bacteria to satisfy up to 95% of S demand (Kiene et al., 1999, Kiene and Linn, 2000a, Zubkov et al., 2001, Pinhassi et al., 2005). Both DMSP degradation pathways are activated (Gao et al., 2020), with 75% of DMSP initially being metabolized via demethylation to yield MeSH (Kiene and Linn, 2000b). But demethylation is halted when the bacterial S requirements are achieved, therefore diverting S to cleavage, releasing DMS to atmosphere, and annulling the influence of MeSH in $N_2O$ production in marine denitrifying bacteria. From this study, it was evident that the sensitivity towards DMSP and the sulfur requirements are different within different microorganisms. This leads to the concluding hypothesis that the DMSP availability will distinctly influence the inhibitory interaction between DMSP metabolism and denitrification and, ultimately, the fate of DMS and $N_2O$ in the ocean.

## 5.2. **Future directions**

This thesis contributed to the general knowledge of how the N-cycling microbial communities are influenced by environmental factors, on a spatial but also temporal scale, addressing an important and genetically unexplored interaction between N and organic S cycles, with potential implications on $N_2O$ emissions, a powerful greenhouse gas. It is imperative to understand how microbial communities regulate the N biogeochemical cycle, as global environmental conditions continue to change. Therefore, future research should focus on:

i. studying the N-cycle coupled processes and the interactions with other biogeochemical cycles, that control $N_2O$ production in different marine environments, expanding the knowledge and data on N biogeochemistry for further analysis when using biogeochemical and ecological modelling to predict ecosystem services responses;
ii. exploring the metabolism and ecology of microorganisms from the less-studied DNRA and anammox processes and how it influences $N_2O$ emissions, in marine ecosystems;
iii. reinforcing the research of N-cycle processes in the water column, namely the ecosystem dynamics of deep sea, which is still scarcely studied, supporting the knowledge about $N_2O$ dynamics in the open ocean;
iv. and, identifying the environmental factors which have a selective pressure on the occurrence or coupling of N-cycle processes and understand the response and dynamics of natural microbial communities, which may not always follow the thermodynamics predictions.

*…organisms and their material environment evolve as a single coupled system, from which emerges the sustained self-regulation of climate and chemistry at a habitable state for whatever is the current biota.* (Lovelock, 2003)

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# APPENDIX

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# Spatial-temporal dynamics of N-cycle functional genes in a temperate Atlantic estuary (Douro, Portugal)

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ABSTRACT: Understanding the spatial and seasonal dynamics of nitrogen (N)-cycle microbial communities is pivotal for the knowledge of N biogeochemistry. The present study addressed the spatial-temporal variability of nitrification (bacterial and archaeal amoA) and denitrification (nirS, nirK, and nosZI) key genes, as well as of non-denitrifying nitrous oxide ($N_2O$) reducers (nosZII), coupled with key environmental variables, in an estuarine ecosystem (Douro, NW Portugal). Samples were collected on a monthly basis over 1 yr, key physical-chemical parameters were measured, and specific functional gene abundances were assayed. The results revealed a clear seasonality for nirS, nosZII, and bacterial and archaeal amoA abundance, with an increase during the winter/spring seasons. This period was especially characterized by high levels of dissolved oxygen, low temperature, low salinity, and increased turbidity. Indeed, turbidity emerged as the key factor controlling the distribution of nirS, nosZII bacterial, and archaeal amoA abundance. In contrast, the abundance of nosZI increased during the summer, while nirK abundance was enhanced from the fall to late spring. Additionally, the availability of dissolved inorganic nitrogen nutrients had no commensurable effect on N-cycle functional genes. This study of the annual variation of N-cycle functional genes in a temperate Atlantic estuary provides a major contribution to the understanding of how environmental factors potentially influence the distribution and abundance of N-cycle microbial communities.

KEY WORDS: Nitrogen cycle · Environmental factors · Denitrifier · Ammonia-oxidizing Archaea · AOA · Ammonia-oxidizing Bacteria · AOB · Nitrogen functional genes · Estuary

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# Spatial–temporal dynamics of N-cycle functional genes in a temperate Atlantic estuary (Douro, Portugal)

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ABSTRACT: Understanding the spatial and seasonal dynamics of nitrogen (N)-cycle microbial communities is pivotal for the knowledge of N biogeochemistry. The present study addressed the spatial–temporal variability of nitrification (bacterial and archaeal *amoA*) and denitrification (*nirS*, *nirK*, and *nosZ*I) key genes, as well as of non-denitrifying nitrous oxide ($N_2O$) reducers (*nosZ*II), coupled with key environmental variables, in an estuarine ecosystem (Douro, NW Portugal). Samples were collected on a monthly basis over 1 yr, key physical-chemical parameters were measured, and specific functional gene abundances were assayed. The results revealed a clear seasonality for *nirS*, *nosZ*II, and bacterial and archaeal *amoA* abundance, with an increase during the winter/spring seasons. This period was especially characterized by high levels of dissolved oxygen, low temperature, low salinity, and increased turbidity. Indeed, turbidity emerged as the key factor controlling the distribution of *nirS*, *nosZ*II bacterial, and archaeal *amoA* abundance. In contrast, the abundance of *nosZ*I increased during the summer, while *nirK* abundance was enhanced from the fall to late spring. Additionally, the availability of dissolved inorganic nitrogen nutrients had no commensurable effect on N-cycle functional genes. This study of the annual variation of N-cycle functional genes in a temperate Atlantic estuary provides a major contribution to the understanding of how environmental factors potentially influence the distribution and abundance of N-cycle microbial communities.

KEY WORDS: Nitrogen cycle · Environmental factors · Denitrifier · Ammonia-oxidizing *Archaea* · AOA · Ammonia-oxidizing *Bacteria* · AOB · Nitrogen functional genes · Estuary

## 1. INTRODUCTION

Understanding how environmental factors control the abundance and distribution of microorganisms is fundamental to microbial ecology studies and to evaluating the impact on global biogeochemical cycles (Fuhrman et al. 2006, Steele et al. 2011). Estuaries are dynamic ecosystems with distinct spatial and temporal environmental variations (McLusky & Elliot 2004) that may shape the structure of microbial communities. These transition systems receive increased loads of nutrients, particularly nitrogen which can be transformed by microbes and promote critical biogeochemical functions, leading eventually to its removal (Damashek & Francis 2018). Denitrification and ammonia oxidation are major microbially mediated processes that play a key role in the global nitrogen (N) cycle (Voss et al. 2013). Canonical denitrification occurs under low oxygen conditions, and is a metabolic respiratory process which consists of the dissimilatory stepwise reduction of nitrate ($NO_3^-$) through nitrite ($NO_2^-$), nitric oxide (NO), nitrous

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oxide ($N_2O$) to dinitrogen gas ($N_2$; Knowles 1982, Zumft 1997). The reduction of $N_2O$ to $N_2$ is catalyzed by a metalloenzyme encoded by the clade I type *nosZ* (hereinafter *nosZ*I) gene (Pauleta et al. 2013). Denitrification represents an ecologically important process. On the one hand, it promotes the biological removal of nitrogen from natural or anthropogenic systems (Seitzinger et al. 2006), reducing the eutrophication risk. On the other hand, it can affect the global climate due to the potential emission of the greenhouse gas $N_2O$ (Philippot et al. 2011). Moreover, a newly recognized and abundant phylogenetic clade of $N_2O$ reducers, which generally have an atypical NosZ protein (encoded by the clade II type *nosZ*, hereinafter *nosZ*II), act as effective $N_2O$ consumers (Sanford et al. 2012, Jones et al. 2013, Hallin et al. 2018).

Nitrification is an autotrophic, aerobic process in which microorganisms obtain energy by converting ammonia ($NH_3$) to $NO_3^-$ (Bock & Wagner 2013). The first oxidation step of nitrification is the transformation of $NH_3$ into $NO_2^-$ (nitritation), catalyzed by ammonia monooxygenase, for which most environmental studies had originally focused on the ammonia-oxidizing *Bacteria* (AOB), belonging to the *Betaproteobacteria* (Kowalchuk & Stephen 2001). The discovery of ammonia monooxygenase genes associated with archaeal scaffolds within marine metagenomic datasets (Venter et al. 2004) subsequently confirmed the existence of marine ammonia-oxidizing *Archaea* (AOA; Könneke et al. 2005), usually dominating ocean nitrification (Francis et al. 2005, Wuchter et al. 2006, Santoro et al. 2011). The last oxidation step results in the transformation of $NO_2^-$ to $NO_3^-$ (nitratation), by nitrite-oxidizing bacteria, eventually supplying denitrification with the required $NO_3^-$. Microbial ecology molecular studies use *nirS*, *nirK*, and *nosZ* as marker genes of denitrifying bacteria in order to quantify the functional community in several environments (Henry et al. 2006, Kandeler et al. 2006). The bacterial and archaeal *amoA*, that encodes the ammonia monooxygenase-α subunit, serve as a proxy for the quantification of ammonia oxidizers (AO; Rotthauwe et al. 1997, Mincer et al. 2007).

With increasing awareness about the vital role of microbial communities to ecosystem functions, and how they may be impacted by climate change (Cavicchioli et al. 2019), the need to comprehend the feedback response of microorganisms to environmental change constitutes a fundamental quest in microbial research. In estuaries, some studies advocate that the AO and the denitrifying communities are strongly influenced by salinity fluctuations (Bernhard et al. 2010, Francis et al. 2013). Additionally, ammonium concentration seems to be a relevant factor for the niche separation between AOB and AOA (Urakawa et al. 2014). Nitrate can influence the selection between denitrification and dissimilatory reduction to ammonia, coupled with nitrate affinity or the availability of other elements (e.g. sulfide, $Fe^{2+}$), that may favor nitrate ammonifiers (Brunet & Garcia-Gil 1996, Kessler et al. 2018).

Since habitat-specific features of ecosystems determine the diversity and distribution of microbial communities, we hypothesized that the seasonal variability of key environmental parameters may influence the distribution of N-cycle microbial communities in a temperate estuary. Using real-time PCR, we assessed and characterized the spatial–temporal variations of key functional genes of AOB and AOA (*amoA*), of denitrification (*nirS*, *nirK*, *nosZ*I), and of the novel non-denitrifying $N_2O$ reducers (*nosZ*II). In addition, the relationships between key environmental parameters and functional gene abundance were also assessed to determine the potential environmental drivers of the distribution of N-cycle microbial communities.

## 2. MATERIALS AND METHODS

### 2.1. Study area and water sampling

Water samples were retrieved from the Douro River estuary (NW Portugal, Fig. 1). The Douro River drains an international watershed of 97 682 $km^2$, and the estuary is limited by a hydroelectric power dam located 21.6 km from the mouth (Bordalo & Vieira 2005). Mean annual flow reaches 505 $m^3 s^{-1}$, ranging from 0 to >13 000 $m^3 s^{-1}$ during the summer and winter, respectively (Vieira & Bordalo 2000, Azevedo et al. 2010). The water is contaminated by treated and untreated sewage disposal (Ribeiro et al. 2018). The lower, middle, and upper estuarine sectors are based on the salinity gradient, according to Vieira & Bordalo (2000).

Monthly sampling surveys were performed for 1 yr (from July 2009 to June 2010), at low tide, at 3 sampling sites (lower, middle, and upper stretches), covering the present full length of the estuary (Fig. 1). The exact position of each sampling site was obtained by means of GPS (Magellan 600). Key physical and chemical parameters, namely temperature, salinity, pH, oxygen saturation, and turbidity, were measured *in situ*, using a YSI 6920 CTD multi-

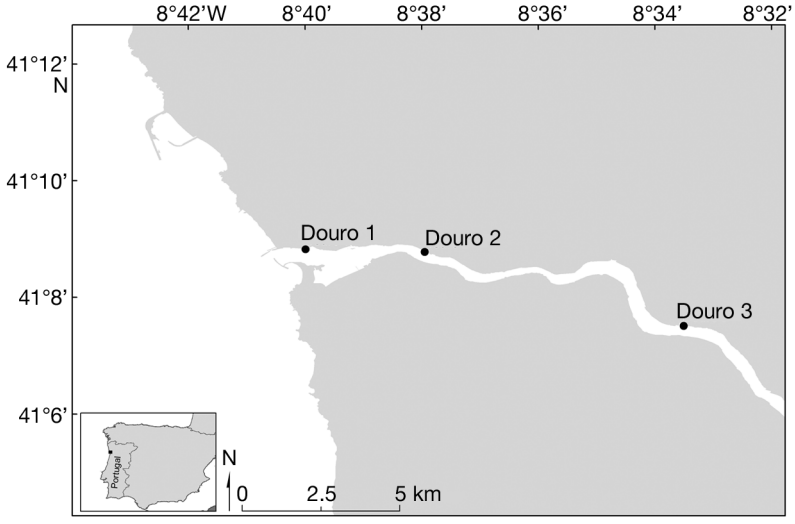


Fig. 1. Sampling sites in the Douro estuary, corresponding to the lower, middle, and upper stretches of the estuary (from west to east), according to Vieira & Bordalo (2000)

parameter probe. Surface water samples were collected using 500 ml acid-cleaned polyethylene bottles for chemical parameters analysis, and sterile bottles for microbiology analysis. All samples were kept in the dark, refrigerated in ice chests until further analysis.

Nutrient concentration of phosphate ($PO_4^{3-}$), $NO_2^-$, $NO_3^-$, and ammonium ($NH_4^+$) were determined colorimetrically using methods described in Grasshoff et al. (1983). Chlorophyll *a* (chl *a*) concentration was assayed according to Parsons et al. (1984). Particulate organic matter (POM) was measured as percentage of weight loss on ignition (500°C, 2 h) (APHA 1992). Determination of dissolved total carbon (TC), organic carbon (DOC), and total nitrogen (TN) was performed by high-temperature catalytic oxidation with a TOC-VCSN analyzer coupled to a total nitrogen-measuring unit (Shimadzu Instruments), according to the manufacturer's instructions.

### 2.2. Nucleic acids isolation

Water samples were concentrated onto cellulose nitrate membranes (0.22 µm pore size, 0.47 mm diameter, Whatman, GE Healthcare), and total DNA of each sample was extracted using the DNeasy PowerSoil Kit (QIAGEN) following the manufacturer's instructions. DNA quality was checked on 2% (w/v) agarose gel, and quantified on a Qubit fluorometer (Life Technologies), using the Quant-iT dsDNA assay.

### 2.3. Quantification of N-cycle functional genes

All qPCR reactions were carried out in duplicate, containing 30–180 ng (for *nirS*, *nirK*, and *nosZ*I amplification; Table S1 in the Supplement at www.int-res.com/articles/suppl/a084p205_supp.pdf), or 5–60 ng (for *nosZ*II and *amoA* from AOB and AOA amplification; Table S1) of DNA template, and Power SYBR® Green PCR Master Mix (2×), that included ROX as a passive reference, to a final 20 µl reaction volume. All reactions were performed in MicroAmp Fast Optical 96-Well Reaction Plates (Bio-Rad), using optical adhesive cover, and performed on a CFX96™ Real-Time System/C1000™ Thermal cycler (Bio-Rad). A no-template control was included for each run. Gene-specific primer sequences and thermal cycling conditions are detailed in Table S1. A composite sample was prepared by adding equal volumes of all the extracted DNA samples from the Douro estuary. This composite sample was used as a template for the amplification of each gene, and the resulting PCR amplicon was analyzed by agarose gel electrophoresis, and subsequently quantified by fluorometry. Quantification of functional gene abundance was achieved through calibration curves for each target gene by serially diluting (10-fold) the PCR amplicon. Gene copy numbers were determined based on the calibration curves, assuming 1 gene copy per genome (except the AOB, carrying an average of 2.5 copies), and normalized against the mass (ng) of the extracted DNA. To evaluate qPCR reaction specificity, a melting curve analysis was performed at the end of each reaction, and all PCR products were analyzed on 2% (w/v) agarose gel to ensure that only the expected target genes were amplified without artifacts. qPCR results were analyzed with the software Bio-Rad CFX Manager 1.6.

### 2.4. Data analysis

Statistical analysis was performed with the open-source software R, version 3.3.3 (R Development Core Team 2013). The proportion of N-cycle functional genes and total microbial community was estimated by normalizations against the RNA polymerase subunit gene (*rpoB*; Dahllöf et al. 2000, Vos et

al. 2012). Significant changes in the ratios and abundance of each functional gene were analyzed by means of the Mann-Whitney *U*-test ($p < 0.001$), after examining the distribution and homoscedasticity of the data using the Shapiro-Wilk and Levene tests, respectively (Levene 1960, Shapiro & Wilk 1965). For the ordination analysis, non-normally distributed data were log transformed. Temporal associations between the environmental parameters and the N-cycle functional genes were analyzed with the standardized data (to zero mean and unit variance), through a constrained redundancy analysis (RDA) performed with the vegan package (Oksanen et al. 2018). Collinearity between environmental variables was evaluated using the variance inflation factor (VIF). Collinear variables with a VIF > 10 were excluded from the RDA model. General associations between environmental parameters and functional gene abundances were further explored with the Spearman's rank correlation coefficient, with the correlation p-values calculated by means of the corrplot and Hmisc packages (Wei & Simko 2016, Harrell 2019).

## 3. RESULTS

### 3.1. Environmental characterization

Temperature ranged from lower values in the winter months (8.0–10.9°C), to higher values in the summer (21–24.7°C, Fig. 2a). Salinity decreased from November to May (0.1–4.8), due to higher river runoff (Fig. S1 in the Supplement), while September displayed the highest salinity, in the range 13.9–29.5 (Fig. 2b). The lowest dissolved oxygen (DO) concentrations were observed in July (2.0 mg $l^{-1}$, lower estuary), while higher oxygenation was found during the winter season (10.8–15.0 mg $l^{-1}$, Fig. 2c). Phytoplankton chl *a* peaked in July (9.8 µg $l^{-1}$, upper estuary), and August (10.1 µg $l^{-1}$, lower estuary), corresponding to the summer phytoplankton bloom (Fig. 2d). During the winter months, turbidity increased, namely in January at the lower estuary (33.5 NTU), and in March at the upper stretch (33.2 NTU, Fig. 2e). TN ranged from 0.017 to 3.21 mg $l^{-1}$ (Fig. 2f), with overall higher concentrations in the winter, while POM levels fluctuated during the sampling surveys (Fig. 2g). The annual distribution of ammonium and nitrite concentrations ranged from 0.8 to 134.8 µM (Fig. 2h), and from 0.2 to 2.8 µM (Fig. 2i), respectively, with a peak in September in the upper estuary. Nitrate was the most abundant inorganic nitrogen form (24.6–373.1 µM, Fig. 2j), with the highest concentrations observed in August in the lower stretch.

### 3.2. Relationships between environmental variables and functional genes

The Douro estuary presented environmental gradients associated with seasonality, a characteristic of temperate ecosystems. After selecting the studied environmental variables, we used a subset of the matrix for the constrained RDA in order to determine which environmental variables best correlated with the variance of N-cycle functional genes. As a result, the entire microbial community variation could be explained by the environmental variables (90.1%, Fig. 3), which significantly ($R^2 = 0.507$; $p < 0.05$; 999 permutations) influenced the N-cycle functional genes. Overall, all N-cycle functional genes in the Douro estuary were abundant in well-oxygenated waters, being negatively influenced by temperature and salinity. In particular, *nirS*, *nirK*, and bacterial and archaeal *amoA* presented higher abundances in the fall and winter months when temperature and salinity were lowest (Fig. 2), and displayed significant correlations with DO ($p < 0.01$, Fig. 4, Table S2 in the Supplement). TN was significantly linked to the abundance of *nosZ*II and *nirK* ($p < 0.05$), and *nirS* and *nosZ*I ($p < 0.01$, Fig. 4, Table S2). Curiously, both AO were not associated with the availability of ammonium, typically the substrate used by ammonia oxidizers. Instead, AO were more related to turbidity, which was the explanatory variable most strongly related with the first RDA axis (Fig. 3). Turbidity in the estuary was strongly linked to DO ($p < 0.001$, Figs. 3 & 4). As expected, the seasonal profiles of turbidity and river flow were similar, with an increase during winter and spring (Fig. 2e, Fig. S1). In addition, linear regression analysis suggested that 37–47% of the variability in *nirS*, *nirK*, and archaeal *amoA* abundance (Fig. S2 in the Supplement) was significantly explained by river flow ($p < 0.05$).

The RDA showed that archaeal and bacterial *amoA* and *nirS* were mostly associated with the winter and spring seasons, also evidenced by the temporal profiles of the functional gene abundances which were clearly enhanced in those seasons (see Fig. 5), when the water was well oxygenated and murky. Additionally, *nirS* was significantly correlated with all functional genes ($p < 0.001$), mainly with the bacterial *amoA*, which was also demonstrated by the similar spatial–temporal gene abundance patterns (Fig. 3).

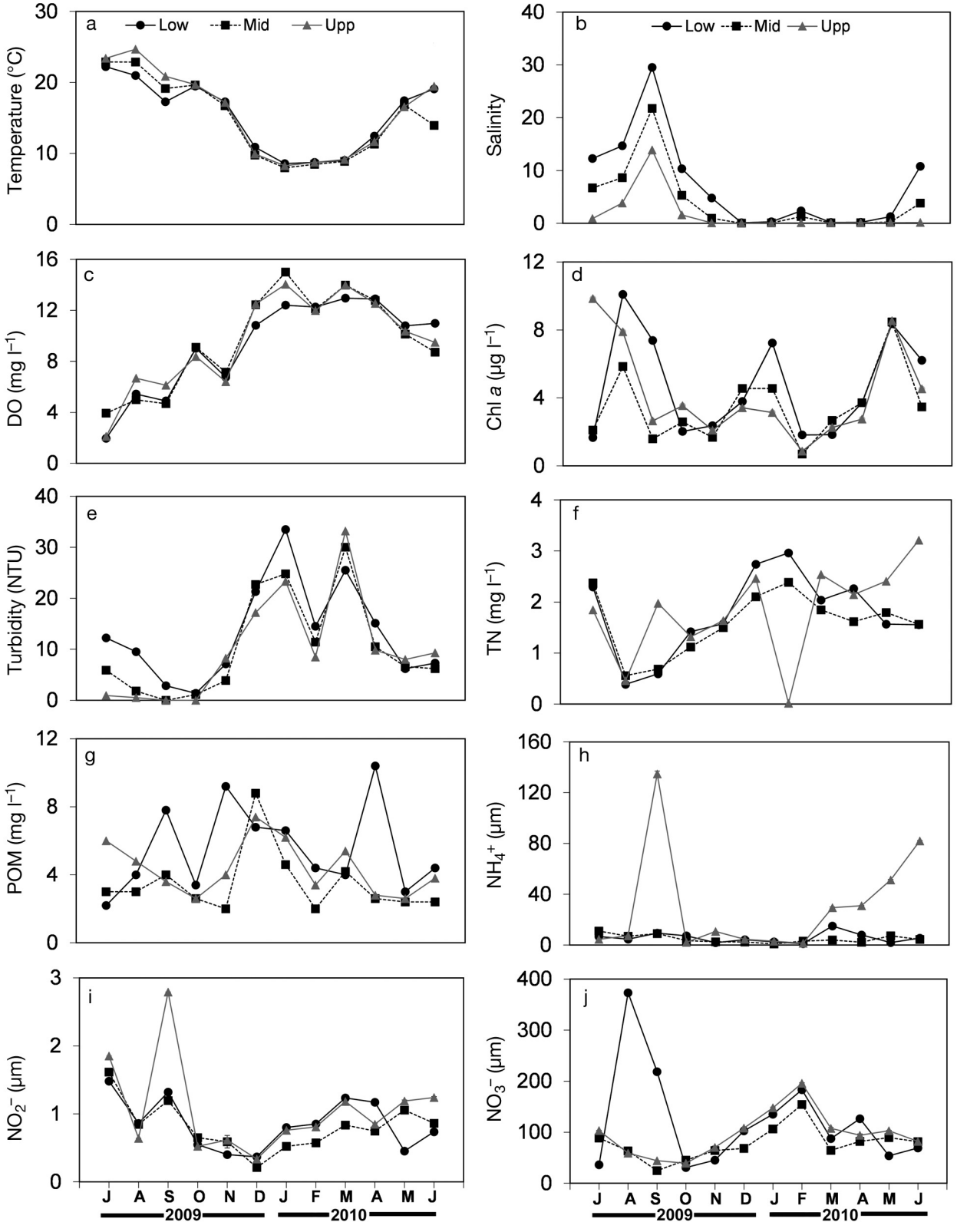


Fig. 2. Seasonal variability of (a) temperature, (b) salinity, (c) dissolved oxygen (DO), (d) chl *a*, (e) turbidity, (f) total nitrogen (TN), (g) particulate organic matter (POM), (h) $NH_4^+$, (i) $NO_2^-$, and (j) $NO_3^-$ (mean ± SD, 3 replicates; small SDs are hidden behind symbols), in the lower (low), middle (mid), and upper (upp) stretches of the Douro river estuary, from July 2009 to June 2010

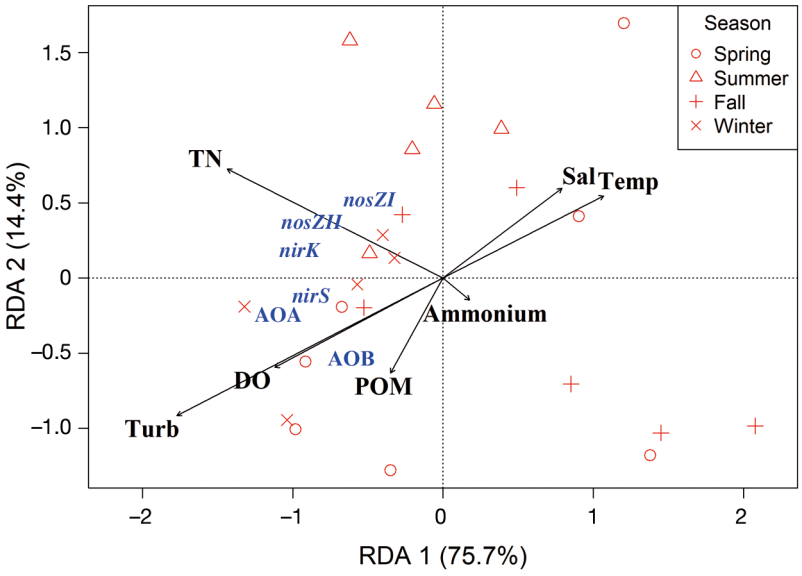


Fig. 3. Ordination triplot of redundancy analysis (RDA) between physical-chemical parameters and seasonal distributions of ammonia oxidizers (bacterial [AOB] and archaeal [AOA] *amoA* genes), denitrifying (*nirS*, *nirK*, *nosZ*I), and non-denitrifying (*nosZ*II) functional gene abundance, in the Douro estuary. Each ordination axis represents the proportion of variance explained by environmental variables. DO: dissolved oxygen; POM: particulate organic matter; Sal: salinity; Temp: temperature; TN: total nitrogen; Turb: turbidity

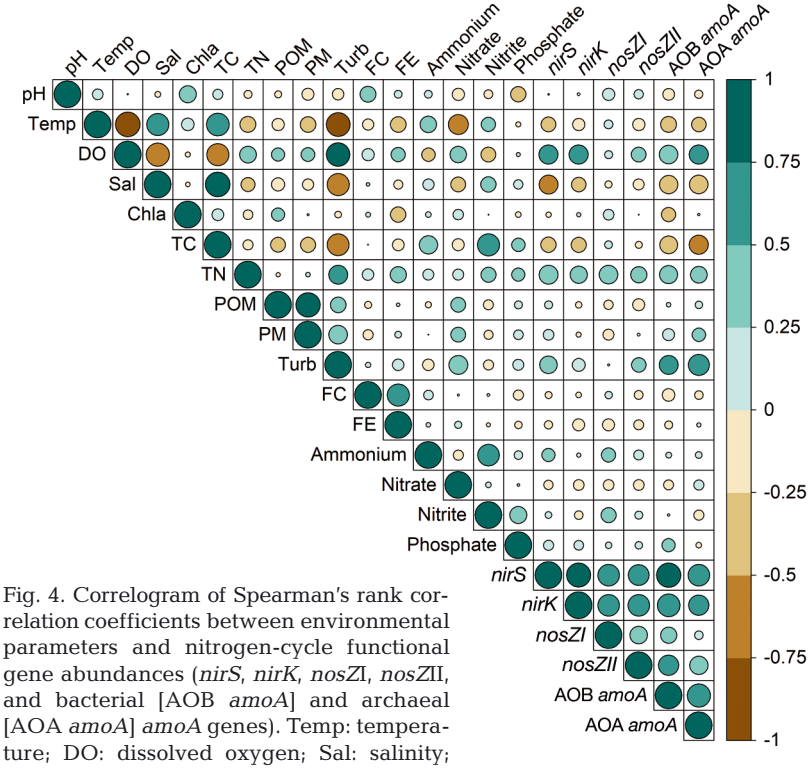


Fig. 4. Correlogram of Spearman's rank correlation coefficients between environmental parameters and nitrogen-cycle functional gene abundances (*nirS*, *nirK*, *nosZ*I, *nosZ*II, and bacterial [AOB *amoA*] and archaeal [AOA *amoA*] *amoA* genes). Temp: temperature; DO: dissolved oxygen; Sal: salinity; TC: total carbon; TN: total nitrogen; POM: particulate organic matter; PM: particulate matter; Turb: turbidity; FC: fecal coliform; FE: fecal enterococci

The bacterial community (based on *rpoB* gene abundance) revealed a different temporal profile, with higher values occurring mainly in the fall (Fig. S3 in the Supplement). On the other hand, the proportion of the bacterial N-cycle functional genes in ratio to *rpoB* gene abundance yielded a significant seasonal variability ($p < 0.01$; Fig. S4 in the Supplement) in the 3 sections of the estuary. The highest ratio was observed in August, occurring at the same time as the highest chl *a* concentration (Fig. 2d).

### 3.3. Distribution of N-cycle functional genes

Overall, the results displayed distinct patterns of N-cycle functional gene abundance (Fig. 5). Archaeal *amoA* peaked in mid-summer (July) in the middle estuary, to recover again during the winter months (Fig. 5a). Bacterial *amoA* abundance also increased during late winter (February–March), at the upper and lower stretches of the estuary, while the middle estuary showed a lower abundance ($2.6 \times 10^6$ gene copies $ng^{-1}$ DNA, Fig. 5b). The denitrifying communities had different temporal distributions throughout the year. For instance, *nirK*-type denitrifiers' abundance varied from $4.4 \times 10^5$ to $3.0 \times 10^7$ gene copies $ng^{-1}$ DNA (Fig. 5c), with higher concentrations in early fall (October) within the estuary, decreasing towards late winter (February). The abundance of *nirS* was higher upstream in mid-fall (November), expanding throughout the estuary during winter (Fig. 5d). The *nosZ*II abundance, related to the non-denitrifying $N_2O$ reducers, increased during the winter period (December–March), particularly in the middle estuary (Fig. 5e). The *nosZ*I denitrifying communities remained low throughout the year ($1.1 \times 10^5$ to $3.7 \times 10^6$ gene copies $ng^{-1}$ DNA), with a seasonal profile different from *nirS* and *nirK*; increased

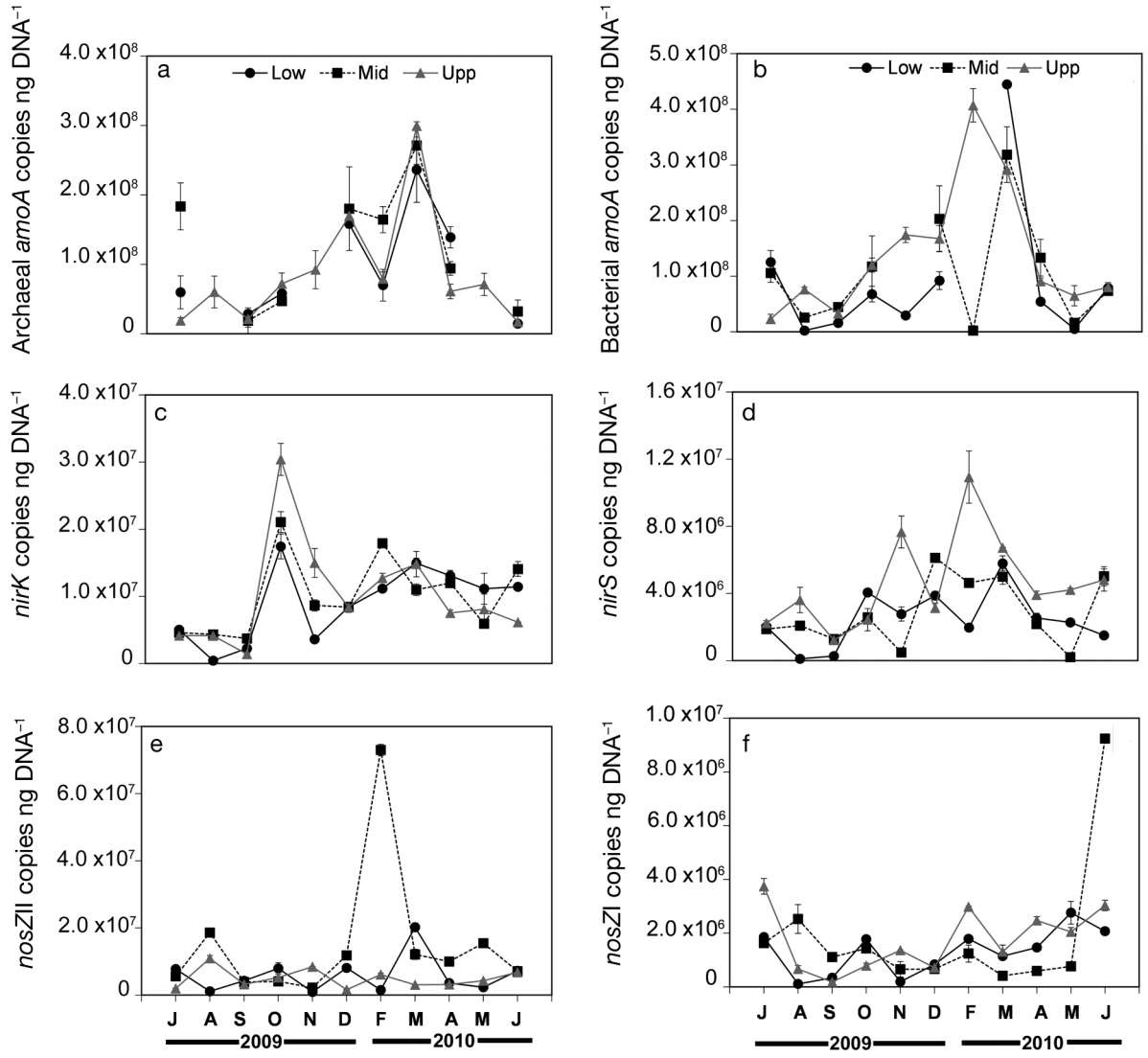


Fig. 5. Spatial–temporal distribution of (a) archaeal *amoA*, (b) bacterial *amoA*, (c) *nirK*, (d) *nirS*, (e) *nosZ*II, and (f) *nosZ*I, in water samples from the lower (low), middle (mid), and upper (upp) stretches of the Douro estuary, from July 2009 to June 2010. Relative abundance of each functional gene expressed as gene copies normalized to ng of extracted DNA. Error bars: SD of the mean. Samples from January 2010 were missing, and that month was not included in the analysis

abundance was observed at the onset of the summer (June), within the middle estuary (Fig. 5f).

## 4. DISCUSSION

In the Douro estuary, the microbial N-cycle gene abundances had distinct seasonal patterns, constrained by environmental factors. All studied N-cycle functional genes were generally more abundant in well-oxygenated, lower-temperature and -salinity waters, typical of the winter season. Therefore, the distribution of denitrifying *nirS*, *nirK*, *nosZ*II, and bacterial and archaeal *amoA* was influenced by the seasonality of the environmental factors. Previous studies (Santoro et al. 2006, Jones & Hallin 2010, Francis et al. 2013) have demonstrated that the diversity and abundance of denitrifying communities can be governed by environmental variables, responding differently to estuarine salinity and temperature gradients. Likewise, Lee & Francis (2017) observed in San Francisco Bay that *nirK* had a higher relative abundance with low temperatures, while *nirS* was enhanced with high nitrate concentrations. But in the Douro estuary, *nirS* had higher gene abundance mainly during the winter season, with low temperature and salinity, highlighting the specificities of different estuaries. The denitrifying enzymes NirK or NirS

(encoded by the *nirK* and *nirS* genes, respectively) are structurally different and believed to be non-homologous, responding differently to environmental gradients (Jones & Hallin 2010). Additionally, the distribution of the denitrifying *nosZ*I and non-denitrifying *nosZ*II genes was predominantly related to DO, an important environmental factor that leads to niche partitioning between *nosZ* clades I and II, as demonstrated by Wittorf et al. (2016). Nevertheless, all studied N-cycle genes, including the denitrifying ones, were abundant in highly oxygenated estuarine waters. Although denitrification and $N_2O$ reduction are anaerobic processes, the occurrence of these functional genes in well-oxygenated waters can be derived from the presence of microorganisms that possess both nitrifying and denitrifying genes.

The present study evaluated the microbial community by DNA analysis, but the presence of functional genes does not necessarily indicate the presence of the expected activities. However, certain organisms can possess and express denitrification genes under aerobic growth, i.e. the activity is stimulated when organisms face a switch between anoxic to oxic conditions (Ka et al. 1997, Morley et al. 2008, Marchant et al. 2017). On the other hand, denitrification can occur in the presence of low levels of oxygen when microorganisms are metabolizing in anoxic microenvironments, such as within suspended particles (reviewed by Brezonik 2013). Estuaries are dynamic bodies of water that receive freshwater inputs resulting in high inorganic nutrient and phytoplankton fluxes, stimulating primary production (Joint & Pomroy 1981). Additionally, the freshwater runoff delivers dissolved and particulate materials that foster turbidity in the estuary. Indeed, in the Douro estuary, turbidity played a major role in the distribution of denitrifying *nirS*, the non-denitrifying $N_2O$ reducers (*nosZ*II), and the AO, which may be an indication of particle-attached microorganisms that are also capable of ammonia oxidation (Jing et al. 2018, Cai et al. 2019). Indeed, the occurrence of denitrification activity has been associated to small and large particles in coastal, river, and estuarine waters (Michotey & Bonin 1997, Liu et al. 2013). The existence of anoxic microenvironments inside suspended or sinking particles, associated with high turbidity, is evidence of hotspots for POM remineralization and a niche for the occurrence of the heterotrophic denitrification process in the water column (Simon et al. 2014, Bianchi et al. 2018, Zhu et al. 2018). Still, since denitrifiers are also capable of aerobic respiration, the presence of denitrifying genes is not necessarily an indication of denitrification activity.

The Douro estuary is highly dynamic, influenced by the river flow (Vieira & Bordalo 2000). The increased abundance of archaeal and bacterial *amoA* in the winter season (December–March) was strongly associated with turbidity when the river flow peaks. Indeed, high nitrification rates have been observed in turbid estuarine waters, with strong correlations with suspended particulate matter, possibly influenced by resuspension of benthic nitrifiers or abiotic ammonium release from sediment (Damashek et al. 2016, Sanders & Laanbroek 2018). On the other hand, the higher turbidity in the lower estuary may also be related to sewage discharge, associated with increased levels of ammonium and fecal indicators (Azevedo et al. 2006, 2008). However, the abundance of the fecal indicators and the levels of ammonium were not correlated with any of the studied N-cycle functional genes, suggesting that sewage does not seem to be a relevant source for those genes in the Douro estuary. Additionally, river flow can act to transport biogenic matter and shape the estuarine community, as well as influence the physical-chemical characteristics of estuaries, changing the environmental conditions (de Oliveira & Margis 2015, Fortunato & Crump 2015, Smith et al. 2019). Although river runoff in the Douro estuary statistically explains the variability of the *nirS*, *nirK*, and archaeal *amoA* abundances, river flow fails to show significant ($p > 0.05$) relationships with the abundance of N-cycle genes. Therefore, its importance is not evident for explaining the spatial–temporal dynamics of the abovementioned functional genes. During the freshet in the winter season, residence time in the water column decreases (Vieira & Bordalo 2000). Consequently, during that period, the river runoff continuously exports microbial populations towards nearshore and/or until they reach a 'hotspot' with extended water retention which gives enough time to increase the relative growth rate (Crump et al. 2004).

Ammonia oxidation kinetics can influence the niche separation between AOB and AOA (Martens-Habbena et al. 2009). Surprisingly, in the Douro estuary, archaeal and bacterial *amoA* were not correlated with ammonium. Although AO are known to use $NH_3$ rather than $NH_4^+$ as a substrate for their chemotrophic growth (Suzuki 1974), the presence of ammonium in the water column, independent of its origin, is indicative of a nitrification potential (Sanders & Laanbroek 2018). The high inorganic nutrient loads may not be limiting the nitrifying and denitrifying activity, which may explain the absence of correlation to N-cycle

functional genes. Environmental parameters other than ammonium did influence the AO abundances. Both AOB and AOA may exhibit a wide adaptive range of tolerance to environmental gradients and adapt to different estuarine characteristics (Bernhard et al. 2007, Gao et al. 2018). Salinity gradients have been associated with changes in the community structure and nitrification activity, but without consistent results for AO community shifts and distribution patterns (de Bie et al. 2001, Bernhard et al. 2005, 2007, Santoro et al. 2008). Although Magalhães et al. (2009) observed that the abundance of AOB in sandy sediments of the Douro estuary dominated over AOA, and positively correlated with salinity, the present study found a different pattern in the water column. Indeed, the AO abundance was more associated with low-temperature and low-salinity waters, as well as with high concentrations of DO and high levels of turbidity, usually occurring during the winter months.

Although the normalization against total DNA is a quantification approach used in molecular studies to measure the potential for N-cycle processes in the ecosystem (e.g. Christman et al. 2011, Wakelin et al. 2011, Soper et al. 2018), it is possible that on a temporal and spatial scale, changes in non-bacterial DNA contents could have biased the results. Additionally, because ecological data, especially from estuarine studies, are complex and explanatory variables are often correlated among each other (multicollinearity), caution must be exercised when interpreting results and making inferences. The co-occurrence between the functional genes can potentially represent an interaction between microorganisms, which reflects the influence of the environment on their distribution (Faust & Raes 2012, Graf et al. 2014). In the present study, the strong relationship between *nirS* and bacterial and archaeal *amoA* indicated that these genes can be correlated with the same environmental variables. Nevertheless, the similar spatial–temporal distribution pattern between these genes, and the association with turbidity, may also suggest the potential coupling between nitrification and denitrification processes, namely for particle-associated assemblages (Zhang et al. 2014, 2019, Zhu et al. 2018). AO can remineralize the available organic matter while fueling the oxidation of the reduced inorganic nitrogenous compounds to nitrite/nitrate, making it available for denitrifiers to reduce (Zhu et al. 2013). Surprisingly, a noticeable association between *nirK* abundance and the *nosZ*II group was observed. Denitrifying organisms that harbor *nirK* instead of *nirS* are likely to have a truncated denitrification lacking the last step of $N_2O$ reduction (Graf et al. 2014). This link can hypothetically represent the interaction of microorganisms that perform single or isolated steps of each denitrification process, working as a co-op community through metabolic handoffs, and the final product of one organism is the substrate of the other (Hug & Co 2018). This cooperation represents an interesting topic to be further explored, since there is a growing awareness of the interactions between organisms and how these networks shape and link biogeochemical cycles (Anantharaman et al. 2016).

## 5. CONCLUSIONS

Few studies have addressed how N-cycle processes and microbial communities respond to environmental change in the water column. Our research contributes to an improved understanding of how the microbial communities involved in the N-cycle are influenced by environmental factors in the waters of an estuarine system, and the potential implications on the microbial structure and N-biogeochemical cycle. The Douro river estuary was found to have a specific environmental fingerprint that influenced the distribution and abundance of N-functional genes, with potential biogeochemical feedbacks. Overall, temperature, salinity, turbidity, and DO exerted a stronger influence on the variability of the genes encoding nitrite reductase (*nirS* and *nirK*), the non-denitrifying *nosZ*II, and bacterial and archaeal ammonia monooxygenase-α subunit (*amoA*). To some extent, the environmental patterns benefited the relationships between the N-cycle functional genes. Therefore, the obtained results support the assumption that the combination of habitat-specific environmental variables, and not an isolated factor, may shape the structure of microbial communities, and control their abundance and distribution. Overall, the present study provides insight into the potential dynamics of N-cycle microbial communities responding to environmental change in a temperate estuary, and into the regulation of the newly recognized marine non-denitrifying $N_2O$ reducers.

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