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INFLUENCE OF SALINITY ON DIMETHYL SULFIDE AND METHANETHIOL FORMATION AND ITS SIDE EFFECT ON NITROUS OXIDE EMISSIONS

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Orientador – Doutora Catarina Maria Pinto Mora Pinto de Magalhães

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À memória do meu avô,

o meu amigo, o meu anjo…

Para ler **antes**

(… cada nova possibilidade que a existência possui,

incluindo a menos provável, transforma a existência inteira.

Milan Kundera),

durante

(*It is not the strongest of the species that survive, nor the most intelligent,*

but the one most responsive to change.

Charles Darwin)

… e **depois**

(*Escritas de luz investem pela sombra, mais prodigiosas do que meteoros.*

A alta cidade irreconhecível cresce sobre o campo.

Certo da minha vida e da minha morte, olho os ambiciosos e queria entendê-los.

O seu dia é ávido como o laço no ar.

A sua noite é a trégua da ira no ferro, rápido ao atacar.

Falam de humanidade.

A minha humanidade está em sentir que somos vozes da mesma penúria.

Falam de pátria.

A minha pátria é um ganido de guitarra, alguns retratos e uma velha espada,

a clara prece do salgueiral nos entardeceres.

O tempo está a viver-me.

Mais silencioso do que a minha sombra, cruzo o tropel da sua excitada cobiça.

Eles são imprescindíveis, únicos, merecedores do amanhã.

O meu nome é alguém e qualquer um.

Passo com lentidão, como quem vem de tão longe que não espera chegar.

Jorge Luis Borges)

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Existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis.
Fernando Pessoa

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Resumo

Emissões de sulfureto de dimetilo (DMS) para a atmosfera influenciam a química atmosférica e potencialmente o clima terrestre. A libertação de DMS advém da clivagem do dimetilsulfoniopropionato (DMSP), um composto sulfuroso orgânico reduzido bio-sintetizado pelo fitoplâncton marinho, macroalgas e determinadas plantas superiores. Esta via catabólica compete directamente com a alternativa dimetilação/dimetiolação que produz metanotiol (MeSH) e que pode ser subsequentemente incorporado em biomoléculas (e.g. metionina). Existe, contudo, escassa informação relativamente ao controlo de factores ambientais sobre estas vias de degradação do DMSP, mediadas por microorganismos, que seleccionam a formação de DMS e/ou MeSH (clivagem *vs.* dimetilação, respectivamente). Assim, este trabalho abordou o impacto da salinidade na produção de DMS e MeSH e na potencial interacção com o último passo da desnitrificação (i.e. redução de óxido nitroso – N_2O – para azoto atmosférico – N_2), em sedimentos estuarinos e numa cultura pura de *Ruegeria pomeroyi* DSS-3, uma estirpe capaz de degradar o DMSP sob ambas as vias da clivagem e dimetilação. Na primeira abordagem a produção líquida de DMS e MeSH foi analisada por cromatografia gasosa usando fotometria de chama pulsante (GC/P-FPD) e o N_2O foi avaliado com um detector de captura de electrões (GC/ECD) após incubação de *slurries* preparados com sedimentos recolhidos ao longo do gradiente de salinidade do Estuário do Rio Ave (NW, Portugal) e submetidos a salinidades diferentes (0, 15, 30 ppt), com e sem adições de DMSP (0 – 50 µM) ou metionina (0 – 500 µM), um precursor do MeSH. Os resultados evidenciaram uma resposta oposta ao longo das diferentes salinidades (0, 15, 30 ppt), onde as maiores salinidades (30 ppt) inibiram as acumulações de DMS em ambas as incubações aeróbias e anaeróbias, enquanto que as taxas de acumulação de MeSH foram tendencialmente mais elevadas nas salinidades superiores (15 e 30 ppt). Adicionalmente, foi avaliada a interferência do MeSH na produção do N_2O, um potente gás com efeito-de-estufa, ao longo do gradiente de salinidade simulado. Consequentemente, os *slurries* incubados com maiores concentrações de metionina confirmaram uma modulação pela salinidade nos fluxos de N_2O através do efeito na produção líquida de MeSH. Dum modo geral, estes resultados sugerem que mudanças na salinidade podem ter um papel de regulação importante na produção líquida de DMS, MeSH e N_2O. Contudo, os vários processos que ocorrem em simultâneo nas complexas comunidades microbianas dos sedimentos não fornecem uma resposta acerca de quais os processos que estarão envolvidos na produção de DMS e MeSH quando as comunidades são sujeitas a alterações nas amplitudes de salinidade. Deste modo e de

forma a evitar as complexidades dos sedimentos, o efeito da salinidade na regulação das duas vias catabólicas do DMSP foi testado numa cultura pura de *R. pomeroyi* DSS-3, repicada para um meio basal mínimo (MBM) e para o meio ½ YTSS com diferentes salinidades (10, 20, 30 ppt). Suspensões celulares de *R. pomeroyi* DSS-3 foram incubadas com diferentes adições de DMSP (0, 50, 500 µM) em condições de aerobiose e anaerobiose e, posteriormente, os compostos orgânicos sulfurosos voláteis (DMS, MeSH) foram monitorizados por GC/ P-FPD. Os resultados confirmaram o mesmo padrão antagónico relativamente à produção de DMS e MeSH, previamente demonstrada nos *slurries*, entre as diferentes salinidades (10, 20, 30 ppt) sob condições anaeróbias onde a acumulação de DMS foi significativamente maior nas salinidades menores (10 ppt; $p < 0.001$) em ambos os meios e, por outro lado, a acumulação de MeSH foi amplificada nas salinidades maiores (20, 30 ppt). Em conclusão estes resultados sugerem que a salinidade pode efectivamente ter uma função relevante na selecção das vias enzimáticas de degradação do DMSP e também na modulação da interacção inibitória entre o MeSH e a actividade da enzima N_2O reductase, com um potencial impacto nas emissões do DMS, MeSH e N_2O para a atmosfera.

Abstract

Dimethylsulfide (DMS) emissions to the atmosphere represent a major influence on atmospheric chemistry and potentially to the global climate. Release of DMS derives from cleavage of dimethylsulphoniopropionate (DMSP), a reduced organic sulfur compound biosynthesized by marine phytoplankton, macroalgae and a few higher plants. This catabolic pathway directly competes with an alternative demethylation/demethiolation which yields methanethiol (MeSH) that can be subsequently incorporated into biomolecules (e.g. methionine). There is, however, little information on how environmental factors control these microbial mediated DMSP degradation routes that select the formation of DMS and MeSH (cleavage *vs* demethiolation, respectively). Therefore, this work focused on the impact of salinity changes on DMS and MeSH production and the potential interaction with the nitrous oxide (N_2O) reductase step of the denitrification pathway, in estuarine sediments and in a pure culture of *Ruegeria pomeroyi* DSS-3, a strain that is able to perform both the cleavage and demethylation pathways for DMSP degradation. In a first trial DMS and MeSH net production were analyzed by gas-chromatography with a Pulsed-Flame Photometric Detector (GC/P-FPD) and N_2O was measured with an Electron-capture detector (GC/ECD) after slurries incubations prepared with sediments collected along the salinity gradient of the Ave River Estuary (NW, Portugal) and exposed to different salinities (0, 15, 30 ppt), with and without amendments with DMSP (0 – 50 µM) or methionine (0 – 500 µM), a MeSH precursor. Results evidenced an opposite response of DMS and MeSH production along the different salinities (0, 15, 30 ppt). While increasing salinities (30 ppt) inhibited DMS accumulations under both oxic and anoxic incubations, MeSH tended to accumulate to higher concentrations at higher salinities (15 and 30 ppt). Additionally, it was evaluated the MeSH interference on N_2O production, a potent greenhouse gas, along the simulated salinity gradient. Consequently, the slurries incubated with increasing methionine confirmed a salinity modulation on N_2O fluxes through its effect on MeSH net production. Overall, these findings suggest that changes in salinity may have an important regulatory role in net production of DMS, MeSH and N_2O. However, the simultaneous processes involved in sediments complex communities do not provide a response on which specific processes were involved on the DMS and MeSH net fluxes measured. Therefore, in order to avoid the complexities of sediments, the effect of salinity on the regulation of the two competing DMSP catabolic pathways was tested in a pure culture of *R. pomeroyi* strain DSS-3, harvested into a modified Marine Basal Medium (MBM) and half strength YTSS (½ YTSS) medium at different salinities (10, 20, 30 ppt). These salinity treatments were

incubated with different DMSP amendments (0, 50, 500 μM) under oxic and anoxic conditions and volatile organosulfur compounds (DMS, MeSH) were monitorised by GC / P-FPD. Results confirmed the same antagonic pattern in DMS and MeSH production, previously demonstrated in sediment slurries, between the different salinities (10, 20, 30 ppt). Under oxic conditions DMS accumulation was significantly higher in low salinity treatments (10 ppt; $p < 0.001$) in both media and MeSH accumulation, on the other hand, was enhanced in higher salinities (20, 30 ppt). Altogether, these findings suggest that salinity may indeed have an important regulatory role on the selection of the DMSP enzymatic degradation routes and also in modulating the inhibitory interaction between MeSH and nitrous oxide reductase enzyme activity, with a consequent potential impact on DMS, MeSH and N_2O emissions into the atmosphere.

List of publications

The elaboration of this dissertation benefited from the following published or submitted scientific papers and additional conference presentations with a relevant contribution:

- Magalhães C., Salgado P., Kiene R. P. & Bordalo A. A. (2012) Influence of salinity on dimethyl sulfide and methanethiol formation in estuarine sediments and its side effect on nitrous oxide emissions. Biogeochemistry 110: 75-86
- Salgado P., Kiene R. P., Wiebe W. J. & Magalhães C. Salinity as a key regulator of DMSP catabolism pathways in *Ruegeria pomeroyi* DSS-3. Submitted to *Aquatic Microbial Ecology*
- Salgado P., Kiene R. P., Bordalo A. A. & Magalhães C. Influence of salinity on dimethyl sulphide, methanethiol and nitrous oxide oxide emissions. Congresso de Jovens Investigadores de Universidade do Porto (IJUP), Porto, Portugal, February 2011.
- Salgado P., Bordalo A. A., Kiene R. P. & Magalhães C. Influence of salinity on DMSP and methionine degradation and its regulatory effect on the nitrous oxide reduction step of denitrification. 4th Congress of European Microbiologists FEMS 2011, Geneva, Switzerland, 26-30 June 2011
- Salgado P., Kiene R. P., Bordalo A. A. & Magalhães C. Salinity as a key regulator of DMSP catabolism pathways in estuarine sediments and in a *Ruegeria pomeroyi* pure culture. First EMBO Conference on Aquatic Microbial Ecology: SAME13, Stresa, Italy, 8-13 September 2013
- Magalhães C., Salgado P., Buchan A., Machado A., Wiebe W. J., Kiene R. P. How dimethylsulfoniopropionate degradation compounds may control the efficiency of denitrification pathway. First EMBO Conference on Aquatic Microbial Ecology: SAME13, Stresa, Italy, 8-13 September 2013

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DMSP – dimethylsulfoniopropionate

DMS – dimethyl sulfide

MeSH – methanethiol

GC – gas-chromatography

P-FPD – Pulsed-Flame Photometric Detector

ECD – Electron-Capture Detector

MBM – marine basal medium

½ YTSS – half strength yeast extract-tryptone-sea salt

VOSC – Volatile Organic Sulfur Compounds

CLAW – Charlson, Lovelock, Andreae, Warren

CCN – cloud condensation nuclei

$DMSP_d$ – dissolved dimethylsulfoniopropionate

CHAPTER 1

Introduction

1.1. Motivation

Sulfur's biogeochemical properties have been studied for decades. But only, more recently, researchers have acknowledged the environmental and ecological significance of **V**olatile **O**rganic **S**ulfur **C**ompounds (VOSC's) in the global sulfur cycle. An effort has been subsequently made to understand the interactions between all the biological, geological and biochemical processes involved in the complex marine sulfur cycle, namely the dimethylsulfoniopropionate (DMSP) uptake by heterotrophic bacteria or phytoplankton, the photochemical production of volatile compounds (e.g. carbonyl sulfide – COS), oxidation of dimethyl sulfide (DMS) or precipitation and sedimentation of iron sulfides, and on the influence of physical parameters (e.g. temperature, solar radiation, salinity) on the dynamics and distribution of VOSC´s. All these processes may have a direct or indirect influence on DMS emissions which, on the other hand, can counteract the anthropogenic rising gas emissions into the atmosphere with implications on the climate change, a current topic in today's concerns (Vallina & Simó 2007).

It all started with the confirmation of ubiquitous biogenic gases, such as DMS, in Atlantic surface waters making it a probable candidate in the natural sulfur transfer (Lovelock et al. 1972). Then, Lovelock & Watson (1982) launched the perspective that planet Earth was *alive* through "The Gaia Theory" where "organisms and their environment evolve as a single, self-regulating system". And it continued with the discovery of a link through the compound DMS between the ocean and atmosphere sulfur flux. This was known as the CLAW hypothesis, proposed by Charlson et al. (1987), which suggested a climate self-regulation through a negative feedback between the solar irradiance and temperature on the phytoplankton and consequently the cloud's coverage, affecting Earth's radiation budget. Nowadays, with the advance of technology and several genomic tools, microbiological investigation was taken into new and all different levels. Specifically, DMSP microbial catabolism that generates DMS and related compounds was the focus of a recent researching "boom" regarding the diverse and complex biochemical pathways and consequently its impact on the Earth's climate (e.g. Howard et al. 2008, Curson et al. 2011b, Reisch et al. 2011, Moran et al. 2012). Indeed, the release of DMS to the

atmosphere significantly affects atmospheric chemistry as well as the global climate system because DMS is oxidized to sulfate aerosols which act to reduce the amount of solar radiation through cloud's coverage (Charlson et al. 1987; Vallina & Simó 2007). The knowledge of the functioning and regulation of these mechanisms could represent a small step to understand and counteract the, today's even more concerning, global warming much related to the increase of atmospheric greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O; Dickinson & Cicerone 1986).

On the other hand, previous work have projected a new perspective between the marine organic sulfur and nitrogen biogeochemical cycles through the discovery of a new inhibitory interaction between organic sulfur degradation compound(s) and the last step of denitrification (Magalhães et al. 2011, 2012). These studies demonstrated that DMSP degradation product(s) interfere with the nitrous oxide reductase step of denitrification, limiting nitrogen loss through N_2 and enhanced nitrogen loss via N_2O, a greenhouse gas involved in the destruction of stratospheric ozone (Dickinson & Cicerone 1986). Thus, this newly discovered interaction revealed that DMSP decomposition could cause offsetting effects on climate change by promoting emissions of climate cooling DMS (Charlson et al. 1987; Lawrence 1993; Vallina & Simó 2007) and climate warming N_2O (Dickinson and Cicerone 1986, Ravishankara et al. 2009) compounds to the atmosphere.
The present study was motivated by the results obtained in these previous researches and intends to understand the influence of specific environmental parameters (salinity) on the regulation of DMSP catabolism and, consequently understanding how it modulates N_2O fluxes through its effect in controlling organic sulfur compounds formation.

1.2. Background

1.2.1. Sulfur Biogeochemistry

1.2.1.1. Inorganic Sulfur

Environmental balance on Earth is dependent of the major elemental biogeochemical cycles, such as carbon, sulfur, nitrogen and phosphorus. This vulnerable stability is supported by a flexible feedback involving atmosphere and physical, chemical and biological components and therefore is an important topic on several research studies concerning, namely, climate change (Cess 1976, Charlson et al. 1987, Bony et al. 2006, Davidson & Janssens 2006, Denman et al. 2007, Jeppesen et al. 2009, Chen et al. 2013).

The natural sulfur cycle has long been affected by human activities mainly after industrialization, by increasing sulfur emissions to the atmosphere. These compounds disperse through the atmosphere and are lately deposited, mainly as sulfate (SO_4^{2-}) and sulfur dioxide (SO_2), a process deleterious to natural ecosystems commonly known as "acid rain" (Likens & Bormann 1974).

There are several sulfur sources (e.g. burning fossil fuels, decomposition and combustion of organic matter, sea salts over the oceans, volcanoes) which emit reduced forms of sulfur like DMS and hydrogen sulfide (biogenic H_2S) or oxidized sulfur dioxide (anthropogenic and volcanic SO_2) which are then transported and mixed in the atmosphere by winds and turbulence (Kellogg et al. 1972, Faloona 2009). Some estimates of annual sulfur fluxes of natural and anthropogenic sources have been made (see Ivanov 1981 and Möller 1984), whence anthropogenic emissions (e.g. fossil fuel combustion) and oceanic biological activity seem to be the most conspicuous (Kwint 1997, Liss et al. 1997). Nevertheless, Faloona (2009) compiled a number of references to establish an average of the global budgets of the main sulfur species. In light of this review, the significant biogenic sulfur source has an estimated global budget of 19.4 Tg S yr^{-1} (1 Tg = 10^9 kg) while the anthropogenic emissions (67.2 Tg S yr^{-1}) exceed the natural ones, mainly through SO_2.

Sulfur is a vital component for all life forms and regulates several microbial processes essential to maintain life on Earth (Malin 2006).

Table 1 – Oxidation numbers of Sulfur compounds (adapted from Bentley et al. 2002)

Inorganic Sulfur	Organic Sulfur	Valence State
SO_3, SO_4^{2-}	—	+6
SO_2, $H\text{-}SO_3^-$, SO_3^{2-}	CH_3SOH,	+4
SO	$(CH_3)_2SO_2$, CH_3SO_2H	+2
S	$(CH_3)_2SO$, CH_3SOH	0
—	$(CH_3)_2S_2$	-1
HS^{1-}, H_2S	$(CH_3)_2S$, CH_3SH	-2

This element embraces a variety of valence states (Table 1), ranging from –2 (e.g. sulfide) to +6 (e.g. sulfate, SO_4^{2-}) from which most of them are emitted to the atmosphere in the reduced form (Andreae 1990). These chemical configurations embrace a variety of

inorganic and organic forms, which are subject of abiotic and biotic transformations making sulfur a key element of several important biogeochemical transformations such as sulfate reduction, atmospheric sulfur emissions, sulfur assimilation into amino acids, etc. (Cooper 1983, Luther et al. 1986, Bentley et al. 2002, Chasteen & Bentley 2004, Likens et al. 2002). Majority of these processes are mediated by microorganisms belonging to Bacteria and Archaea in natural environments (Andreae 1990, Barrie Johnson & Hallberg 2008, Ghosh & Dam 2009). Some sulfur inorganic compounds participate in a series of oxidation-reduction reactions (Fig. 1; Lomans et al. 2002), where SO_4^{2-}, the most oxidized inorganic form, represents the most abundant sulfur in global ocean waters (Kiene et al. 1999). In addition, SO_4^{2-} plays a central role in the mineralization of organic matter in anaerobic coastal marine and salt marsh sediments, as a terminal electron acceptor in the respiratory metabolism, where sulfate-reducing bacteria convert sulfate to sulfide (Jørgensen 1977a, Jørgensen 1982, Andreae 1990).

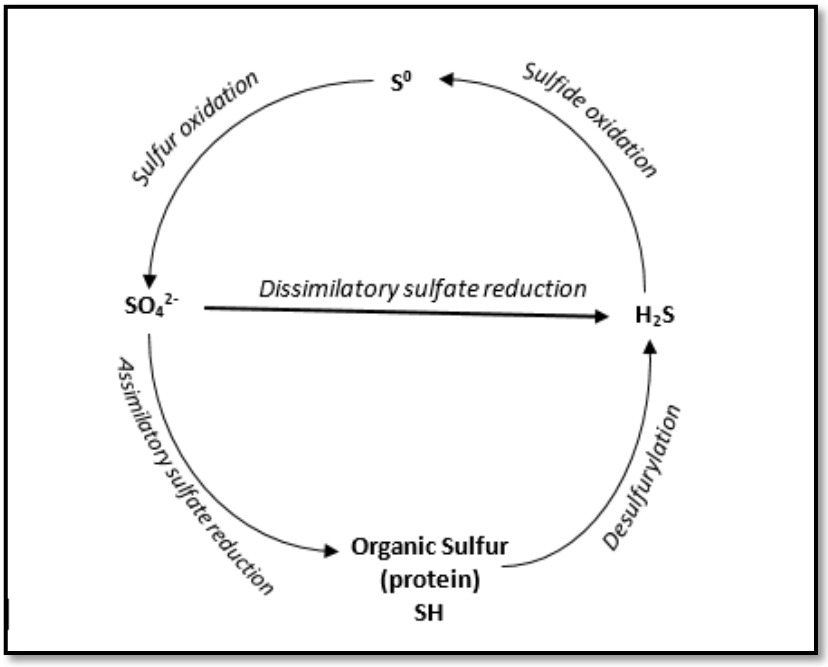

Figure 1 - Redox sulfur cycle (adapted from Lomans et al. 2002)

In sediments much of the converted sulfide precipitates with metal ions, namely iron, to form iron monosulfides (FeS) or ultimately pyrite (FeS_2) or sulfide is oxidized back to sulfate (Jørgensen 1977a, Howarth & Merkel 1984). On the other hand, assimilative sulfate reduction leads to the biosynthesis of especially sulfur-containing amino acids which, in turn, represent a fundamental component in multi-step biochemical transformations for several biological materials (Howarth 1984, Andreae 1990, Jasińska et

al. 2012). Thus, SO_4^{2-} is an essential compound in several assimilatory and dissimilatory pathways occurring in soils, sediments and water, participating in the sulfur ocean-land transfer (Kwint et al. 1996). Nevertheless, sulfate oxidized sulfur, bio-energetically, represents a disadvantage in what constitutes the biochemical processes of sulfur incorporation into amino acids, requiring a considerable amount of energy on assimilatory reductions (Likens et al. 2002). Therefore, the availability of organic reduced sulfur compounds such as DMSP benefits the above mentioned biochemical transformations through a less energy yield leading, subsequently, to a quick assimilation into bacterial biomass (Kiene et al. 1999).

1.2.1.2. Organic Sulfur

Oceanic sulfur flux constitutes one of the most important reservoir for sulfur emissions where the organic compound DMS, among other volatile sulfur gases (e.g. methanethiol, MeSH; carbon disulfide, CS_2; hydrogen sulfide, H_2S; carbonyl sulfide, COS), stands for the most abundant biogenic fraction that contributes to maintain an atmospheric balance (Charlson et al. 1987, Andreae 1990, Bates et al. 1992, Visscher et al. 2003, Vallina & Simó 2007).

In 1972 Lovelock and coauthors emphasized for the first time the importance of the biogeochemical cycle of sulfur through the oceanic DMS emissions to the atmosphere, later estimated to represent a flux of ca. 30% biogenic sulfur, with a contribution of 14% by the Northern Hemisphere and 67% from the Southern Hemisphere (Kloster et al. 2006). After being accepted that DMS was the “missing link” between the ocean and atmosphere sulfur transfer and, consequently, the dominant oceanic sulfur compound emitted to the atmosphere, DMS became the topic of several ecological and biogeochemical studies concerning aquatic systems. Therefore, aquatic environments such as salt marshes/estuarine systems, coastal wetlands and oceans, which emitted high concentrations of volatile organic sulfur, were recognized as important contributors to the global sulfur cycle and, consequently, in shaping earth’s atmosphere and climate (Steudler & Peterson 1984; Kiene & Visscher 1987; Aneja & Cooper 1989). DMS is the most important source of biogenic sulfur derived from DMSP biosynthesized by phytoplankton (among other sources, as described below). DMS has a low residence time and is readily emitted to the atmosphere where it reacts with other species to produce a particulate-phase containing SO_4^{2-} which, in turn, composes most of the cloud condensation nuclei – CCN (Sievert et al. 2007). Hence, an increase in marine DMS

emissions would result in a CCN increase and clouds albedo, with a consequent decrease in incoming solar radiation to Earth's surface (Charlson et al. 1987). However, recent scientific studies have acknowledged the complexity of the CLAW hypothesis and there are several uncertainties relating to this hypothetical climate feedback between DMS and plankton. Quinn and Bates (2011) have reviewed several studies that tried to support the DMS-climate feedback loop theory and verified that CCN has other important sources such as wind-driven sea-salt particles and organic matter and it also depends on particle growth.

Besides its influence on global climate, biogenic sulfur compounds play a fundamental role in microorganisms' metabolism mainly through the assimilation of organic sulfur into biomolecules such as methionine and cysteine (Cooper 1983, Lomans et al. 2002). In fact, inorganic sulfates, which are the most stable and abundant sulfur source in seawater, were considered for a long time an important constituent in the assimilatory pathway by aquatic microorganisms (Jørgensen 1977a, b, Middleton & Lawrence 1977, Widdel & Pfennig 1981, Cuhel et al. 1982, Sievert et al. 2007). However, as abovementioned, this inorganic sulfate yields a high energetic cost for microorganisms due to its sulfur in the most oxidized form (+6; Kiene et al. 1999). Therefore, DMSP, an organic sulfur compound produced in large amounts by numerous species of marine phytoplankton (e.g. Stefels 2000), seaweeds (Reed 1983) and salt marsh grasses (Otte et al. 2004), represents an advantage due to its low and reduced molecular weight (Kiene et al. 1999, Kiene et al. 2000). This organic sulfur compound, also abundant in seawater (an average of < 2.8 nM, Kiene & Slezak 2006), is widely used as a growth substrate by bacterioplankton (Ledyard & Dacey 1994) which rapidly metabolize it and incorporate sulfur into biomass.

1.2.1.3. Organic Sulfur Catabolism

The metabolism of organic sulfur represents an important component on the global sulfur cycle. In particular, the catabolism of DMSP which constitutes ca. 24% of the total organic sulfur in surface seawater (Bates et al. 1994) and has now been thoroughly studied namely due to its role as a precursor of DMS and MeSH (Groene 1995, Kiene 1996, Kiene et al. 2000). The DMSP biosynthesis was a process initially approached in higher plants (Greene 1962, Hanson et al. 1994, Trossat et al. 1996) and, later on, elucidated in marine algae (Gage et al. 1997, Summers et al. 1998, Stefels 2000), which confirmed methionine as the precursor protein for DMSP production through different initial routes

(transamination *vs.* methylation) according to the respective organism (Otte et al. 2004). DMSP physiological functions are still yet to be fully defined, however its high intracellular concentrations and compatibility with cell metabolism confer to DMSP a main osmolytic function (Dickson & Kirst 1987, Stefels 2000). Additionally, this solute serves as antioxidant (Sunda et al. 2002, Husband et al. 2012), cryoprotectant (Karsten et al. 1990, Kirst et al. 1991) and herbivore deterrent (Van Alstyne et al. 2001, Strom et al. 2003, Fredrickson & Strom 2009). Also, Stefels (2000) hypothesized an additional buffering capacity of DMSP when reduced sulfur exceeds cell's ability to convert it into amino acids. More recently, Geng & Belas (2010) suggested DMSP as a trigger molecule under chemotactic response acting in benefit for the phytoplankton-*Roseobacter* symbiosis.

Higher plants and marine algae release particulate DMSP into the extracellular environment by several processes which include viral lysis (Hill et al. 1998), physiological stress (Mulholland & Otte 2002), algal senescence (Yoch 2002 and references therein), zooplankton grazing upon phytoplankton blooms (Wolfe et al. 1997, Archer et al. 2003), exudation (Laroche et al. 1999) and cell lysis (Nguyen et al. 1988, Simó 2004). Consequently, dissolved DMSP ($DMSP_d$) assimilation and its degradation compounds (e.g. DMS, MeSH) production might be enhanced, since this labile organic sulfur compound is now available for marine prokaryotes, representing an important ecological role in the marine carbon and sulfur cycles (Kiene and Bates 1990; Kiene et al. 2000; Kiene and Linn 2000a, b, Yoch 2002). DMSP degradation undergoes a rapid turnover by essentially aerobic microorganisms (Kiene 1990, Taylor & Gilchrist 1991, Visscher et al. 1992, Visscher & Taylor 1994, Zubkov et al. 2001), without excluding its catabolism in alternative anoxic marine communities (Kiene & Taylor 1988, Van Der Maarel et al. 1993, Jonkers et al. 1998). Recent scientific research has focused on two main DMSP degradation pathways – cleavage *vs.* demethylation/demethiolation (Kiene et al. 2000, Yoch 2002), due to its ecological and biogeochemical relevancy. In fact, cleavage presents itself a considerable enzymatic process resulting in the production of DMS by $DMSP_d$ bacterial turnover (Curson et al. 2011b) and posterior liberation of DMS into the atmosphere contributing for the regulation of global climate (Fig. 2).

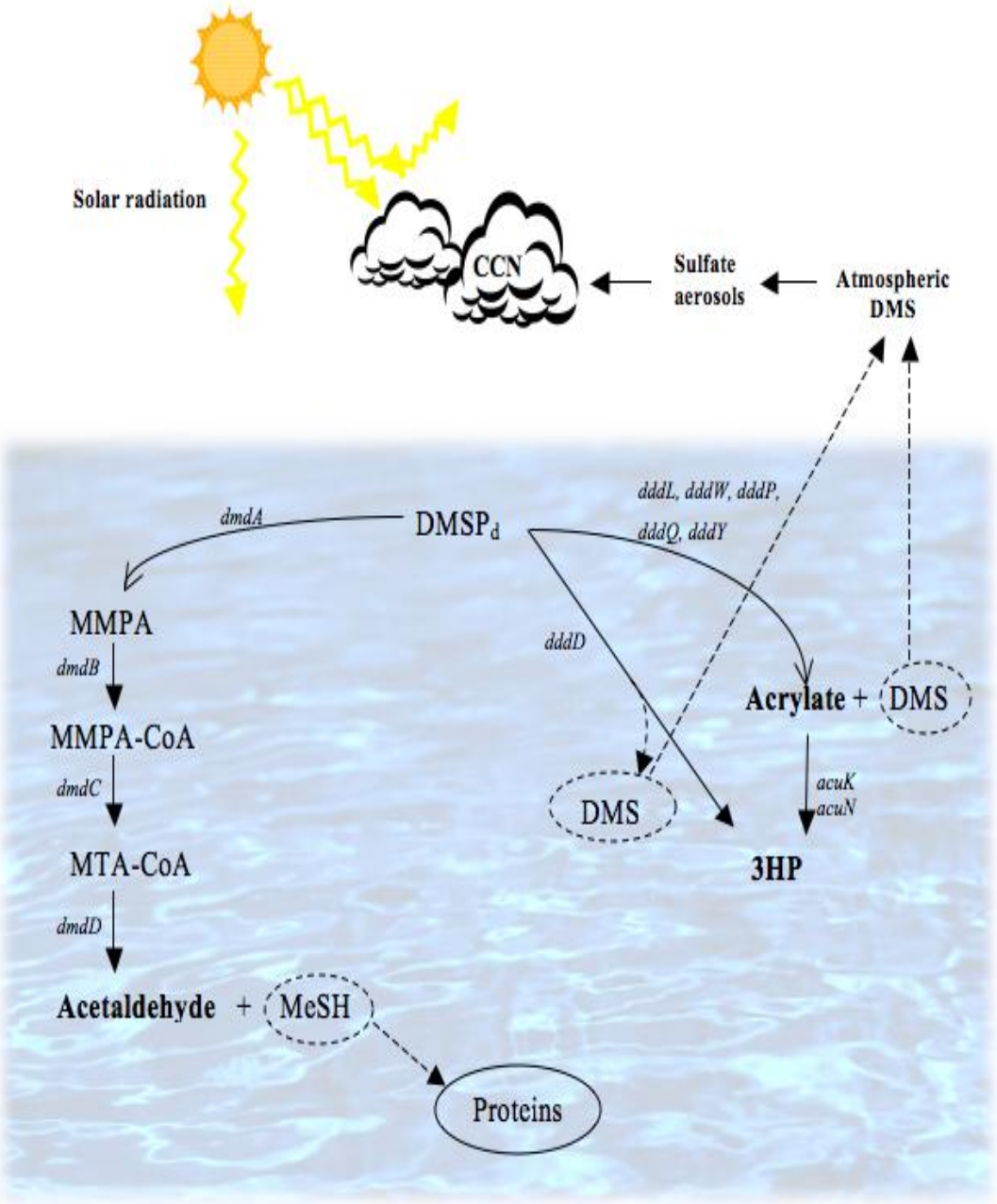

Figure 2 - Representative scheme for the microbial mediated DMSP catabolism and cycling involving the two main biochemical pathways: the demethylation/demethiolation (**left**) whose final product is acetaldehyde liberating MeSH which can be incorporated into bacterial sulfur-containing amino acids; and cleavage (**right**) with the hypothetical direct formation of 3HP mediated by lyase DddD, or DMSP can alternatively be converted into acrylate which in turn can be further catalized into 3HP by the AcuN and –K transferases, also releasing DMS with posterior liberation to the atmosphere. DMSPd – dimethylsulfoniopropionate, DMS – dimethyl sulfide, 3HP – 3-hydroxypropionate, MMPA – 3-methiolpropionate, MTA-CoA – methylthioacryloyl-CoA, MeSH – methanethiol. Adapted from Curson et al. (2011b).

On the other hand, DMSP catabolism can be switched to another biological transformation in which the end product, MeSH, is ca. 75% assimilated by bacteria for subsequent incorporation into amino acids (Kiene & Linn 2000a). This latter process, mediated by marine bacteria, seems to be the preferential enzymatic pathway in seawater (Kiene 1996, Kiene & Linn 2000b), but what controls the "bacterial switch" between the two main pathways of DMSP degradation still remains unanswered and reveals itself as a quest for a better determination of surface ocean DMS concentrations. The progress of genomic and transcriptomic technologies have contributed to unveil some of the complex steps implicated on DMSP catabolism, namely through genes identification (Todd et al. 2007, Todd et al. 2010, Varaljay et al. 2010, Curson et al. 2011a, Todd et al. 2011, Todd et al. 2012, Howard et al. 2011, Moran et al. 2012, Varaljay et al. 2012).

1.2.2. Organic sulfur catabolism *vs* environmental controls

Potential variability in the dynamics of coastal and oceanic ecosystems is likely a consequence of alterations in several ecological features. Specifically, shifts in abiotic factors can lead to redefinitions of chemical and microbiological patterns as well as community composition (Bouvier & del Giorgio 2002, Herlemann et al. 2011, Shah & Shah 2011). Several studies have focused on the influence of different biotic and abiotic factors on phytoplankton and macroalgae DMSP intracellular concentrations (Gage et al. 1997, Sunda et al. 2002, Malmstrom et al. 2004, Pinhassi et al. 2005, Van Alstyne & Puglisi 2007, Kumar et al. 2009, Yang et al. 2011, Husband et al. 2012). Since several algae can accumulate low molecular weight organic solutes, osmotic stress affects the physiology of phytoplankton and macroalgae and their intracellular DMSP accumulation (Kirst 1990). In fact, salinity has been identified as a relevant factor on the physiology of phytoplankton and macroalgae reflecting an increase of intracellular DMSP by the osmotic potential of the cell (Vairavamurthy et al. 1985, Stefels 2000, Yang et al. 2011). While there are relevant studies about the physiological behaviour of phytoplankton and macroalgae and subsequent influence of biotic and abiotic factors on DMSP intracellular concentrations (e.g. Vairavamurthy et al. 1985; Van Alstyne et al. 2003; Stefels et al. 1996, Yang et al. 2011, Husband et al. 2012), relatively few studies have addressed the environmental controls on the pathways of DMSP degradation. It is, however, expected that numerous environmental and biological factors may control DMSP catabolism to DMS (cleavage) versus MeSH (demethylation/demethiolation), with certainly implications on the different production ratios of DMS and MeSH in marine environments. Indeed, there are evidences

that the type of microbial species assemblage, seems to be linked to the dynamics of phytoplankton blooms, which then affects DMSP turnover (Jonkers et al. 1998, Zubkov et al. 2001, Pinhassi et al. 2005). Nevertheless, apart from work from the present research team and two other studies that hypothesized salinity as a relevant abiotic factor controlling DMSP catabolism in natural systems (Visscher et al. 2003; Niki et al. 2007, Magalhães et al. 2012), there is still a gap about the environmental controls on the pathways of bacterial degradation of $DMSP_d$ to DMS (cleavage) versus MeSH (demethylation/demethiolation).

1.2.3. Interactions between sulfur compounds and N_2O emissions

Nitrous oxide (N_2O) is partially responsible for the destruction of the stratospheric ozone through the reaction with the atomic oxygen, which consequently potentiates the increase of harmful UV-B radiation (Krupa & Kickert 1989, Caldwell & Flint 1994, Nevison & Holland 1997) and was recently identified as the dominant ozone-depleting substance (Ravishankara et al. 2009). Nitrous oxide acts also as a potent greenhouse agent in the atmosphere (300 times more potent than CO_2) and represents a particular environmental problem due to its long atmospheric lifetime of 114 years (Dickinson and Cicerone, 1986). Soils and oceans represent the largest sources of N_2O emissions, however, anthropogenic sources, such as agriculture, sewage treatments, or combustion of fossil fuel, account for almost two-thirds of the total emissions (Denman et al 2007). Different microbial nitrogen transforming processes, such as microbial nitrification and heterotrophic denitrification, contribute to the formation of N_2O and soils represent major sources (Seitzinger 2000, Frame & Casciotti 2010). Therefore, nowadays concern is focused namely on the consequences of climate change aggravated by greenhouse gases and the demand for mitigation measures.

Interestingly, a previous study, motivated by the observed production of nitric and nitrous oxides in the redox transition zone of a coastal marine sediment [by denitrifying bacteria] near the sulfide maxima concentration (Sørensen 1978), lead to the analysis and consequent corroboration of a sulfide inhibition on the nitrous oxide reduction pathway of denitrification (Sørensen et al. 1980). This interaction represented a novel quest to better understand the nitrogen and sulfur biogeochemical processes inherent to marine and freshwater environments. Later, Joye & Hollibaugh (1995) also identified a sulfide inhibition of nitrification through experiments with HS^- additions within a range to estuarine

sediments, enhancing a link between both sulfur and nitrogen cycles. The apparent regulation of the N cycle opened a precedent for researching new and possible sulfur compounds which could influence the nitrogen biogeochemical pathways. In fact, more recently (Magalhães et al. 2012) it was detected a novel interaction between both sulfur and nitrogen biogeochemical cycles, specifically through an interference on the last reductase step of denitrification (reduction of N_2O to N_2) triggered by a DMSP breakdown product – MeSH (Magalhães et al. 2011).

1.3. Goals

The main objective of this study was to investigate the influence of an environmental stressor, salinity, in selecting DMSP degradation routes and to understand how salinity could modulate N_2O fluxes through its effect on MeSH and DMS net production. In order to achieve this goal, two chapters (Chapter 3 and 4) are presented with the following detailed objectives:

In **Chapter 3** we studied the influence of salinity on the DMS *vs* MeSH net fluxes in sediment microbial communities along the salinity gradient of Ave River estuary; the interaction between sulfur volatiles accumulation (MeSH and DMS) and N_2O fluxes, was also evaluated is these natural microbial communities.

In **Chapter 4** we focused in a more specific evaluation of osmotic pressures onto DMSP catabolism and consequent DMS and MeSH production using a model organism – *Ruegeria pomeroyi* DSS-3. Here we aim to evaluate the salinity influence on the bacterial preference between the two main DMSP enzymatic pathways – cleavage and demethylation/demethiolation – expressed by the proportion of DMS *vs.* MeSH net fluxes.

This dissertation is integrated in a research project untitled **NITROSUL – Novel interaction between marine biogeochemical nitrogen and sulfur cycles: characterization and ecological implications** and it represents a follow-up of previous research focusing on the inhibitory interaction between the DMSP degradation products and the last reductase step of denitrification (conversion of N_2O to N_2).

CHAPTER 2

Material and Methods

Methodology was organized taking into account the sequence of the experiments performed in order to first evaluate the influence of salinity on DMS and MeSH emissions in estuarine sediments from Ave estuary, and secondly to restrict a more detailed characterization of the salinity effect into DMS and MeSH formation in cell cultures of *Ruegeria pomeroyi* DSS-3.

2.1. Environmental survey

2.1.1. Characterization of sampling area

Ave River is a mesotidal estuary located on the northern coast of Portugal with a 1391 km^2 drainage basin, a 40 m^3/s average discharge, lithologically composed by granitic (quartz and feldspars) and schist rocks and by silty-clay sediments, with mainly marine origin organic matter (Mil-Homens et al. 2006). Ave River is 94 km (E-W) long, from the spring – Cabreira mountains – till the mouth located at the south of Vila do Conde and its catchment presents a variable annual precipitation between 900 – 3900 mm, which 73% occurs during the wet season (October – March; ARHNorte). The main tributaries are the Este River, on the right edge, and Vizela River, on the left edge, draining 247 km^2 and 340 km^2, respectively. The Ave estuary is bordered by riparian vegetation dominated by *Eucalyptus globulus*, *Juncus* sp., *Alnus glutinosa*, *Populus* sp. (Pascoal et al. 2005).

Ave River basin covers 15 locations and its estuary is described as one of the most contaminated on the northwest Portuguese region, mainly due to domestic sludge and textile, leather and paper industries which directly discharged their untreated effluents into the streams (Soares et al. 1999; Cunha et al. 2005). Since 1985, several infra-structures were created with the purpose of watershed rehabilitation through e.g. an indicator system of water quality (Oliveira et al. 2005). Also, Ave River basin has been the focus of some heavy metal assessment studies which have corroborated industrial effluent contamination (e.g. Araújo et al. 1998; Soares et al. 1999; Cunha et al. 2005; Mil-Homens

et al. 2006). Water column of Ave River estuary ranges a salinity gradient from < 2 ppt upstream, an oligohaline zone, to almost 35 ppt downstream (Da Silva 2011).

2.1.2. Sampling strategy

Sampling was performed in September 2010 during low tide, covering the north margin of Ave estuary, at four stations along the riparian zone of Vila do Conde (Fig. 3); the sampling stations were meticulously chosen so that it could be representative of the estuarine salinity gradient. At each site, a total of twenty cores (3 cm diameter and 8 cm long) were collected within 50 cm of each other, and all within of approximately 3 m^2. The twenty cores collected from each site were homogenized and used as a composite sample. Also, 0.5 L of sub-superficial water from each sampling station and an additional 5 L sample of freshwater (0 ppt) from an upstream location were collected in acid-cleaned polyethylene bottles. The Ave River freshwater was used for the salinity treatments. All the samples were kept in the dark and transported in refrigerated isothermal containers for posterior processing.

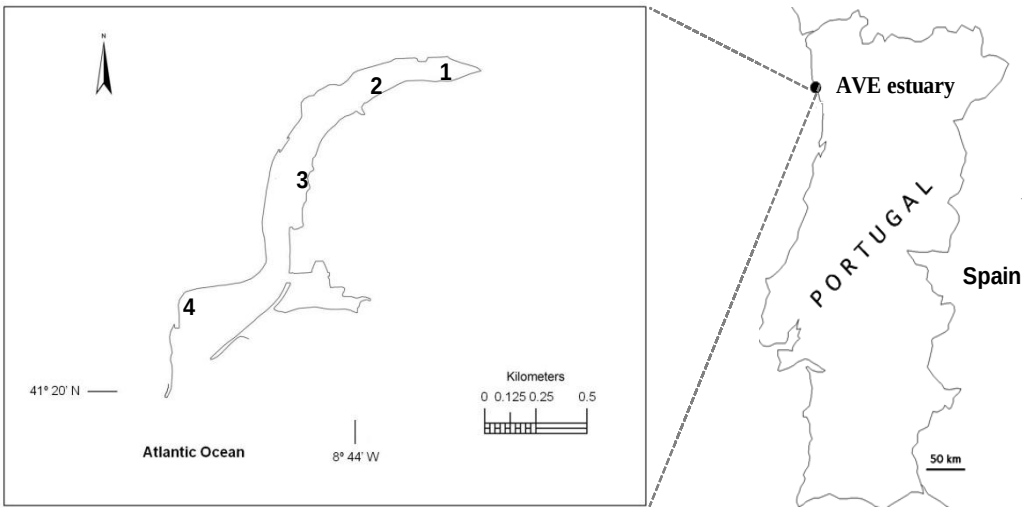

Figure 3 - Ave River estuary and location of sampling sites.

Additionally, salinity and temperature were measured *in situ*, at the specific sampling site, with YSI Model 30 probe. At the laboratory, all the water samples were immediately filtered through 0.45 µm pore diameter (Φ) membrane filter (GF/F glass fiber filters,

Whatman) and a sub-sample stored at -20ºC for nutrient analysis. Sediment samples were stored at 4ºC and used after no more than 4 days after collection for the slurry experiments and sediment analysis.

2.1.3. Slurry experiments

Salinity simulations (15 and 30 ppt) were prepared by, initially, adding artificial sea salts to Ave estuary freshwater (0 ppt) according to Cavanaugh's formula (1975). This way, it reassures salinity as the only variable in slurry treatments, preserving water chemistry.

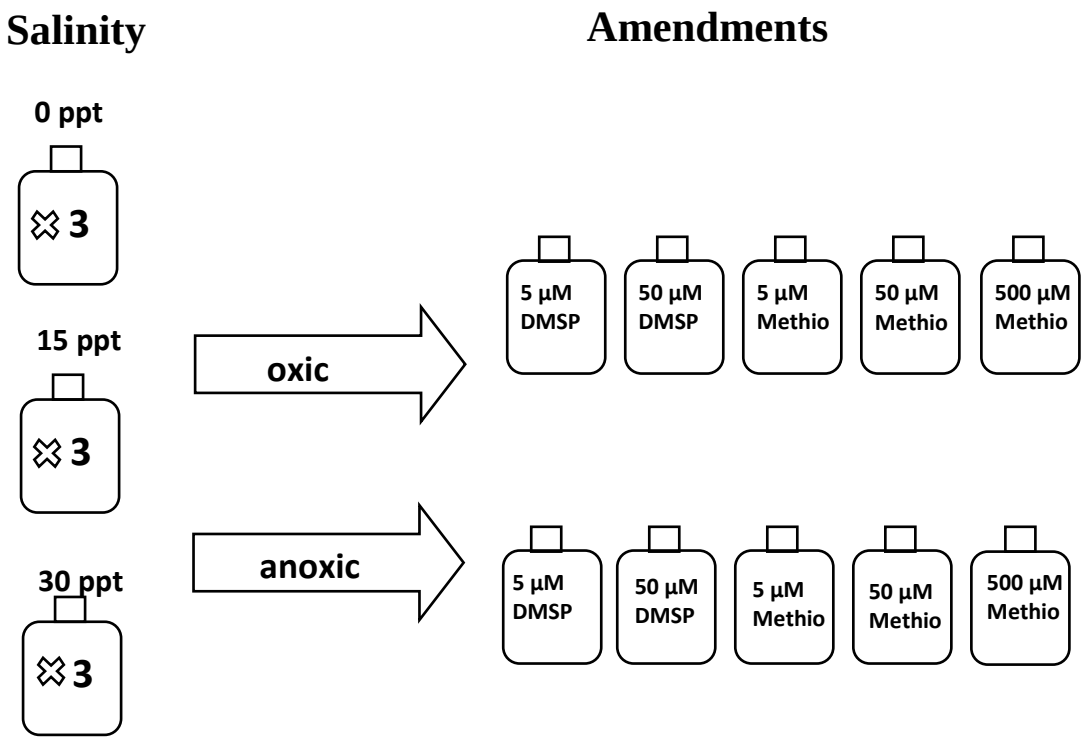

Figure 4 - Graphic design of slurry experiments. Replicates of salinity treatments (0, 15, 30 ppt) were incubated under oxic and anoxic conditions and amended with different DMSP and methionine concentrations. DMSP- dimethylsulphoniopropionate; Methio – methionine.

Sediment slurries consisted in a mix of 5 g of sediment from each sampling site, and 10 ml of overlying water with the different simulated salinities (0, 15 and 30 ppt) in 30 ml serum bottles, as previously described (Magalhães et al. 2011, 2012). For each salinity

treatment (0, 15 and 30 ppt), a set of triplicate slurries was incubated in the dark, under oxic (air headspace) and anoxic (purged with N_2 for 15 min) conditions. Within those treatments, slurries were incubated with no amendments, or with either methionine (5, 50, 500 µM) or DMSP additions (5, 50 µM). Slurries were incubated for 4 h at constant temperature (20 ºC) with rotary shaking (80 rpm). Sulfur volatiles compounds (DMS and MeSH) and N_2O accumulated in the slurries were monitored by headspace analysis (Magalhães et al. 2011, 2012) at the beginning (time 0 h) and end (time 4 h) of the incubation, as described below. Potential chemical production of N_2O, DMS and MeSH was evaluated at Site 1, in parallel experiments where biological activity was blocked by the addition of $ZnCl_2$ (2.5 M final concentration).

2.1.4. Water and Sediment characteristics

For DMSP determination, sediments triplicate subcores (1 cc) were placed in separate 12 ml serum bottles, submitted to cold alkaline treatment (pH ~13), turning DMSP to DMS, by addition of 1 ml of 10 N NaOH (Challenger et al. 1957, White 1982) and left for 24 H to hydrolyse. The final DMS accumulated in the vials was measured by gas-chromatography as described below. These data must be interpreted with caution once DMS final concentrations might be overestimated due to other possible DMS precursors present in sediments (Kiene 1996, Russell & Howard 1996).

Sediment subsamples were also collected in triplicate for chlorophyll *a* (Chl *a*) determination purposes. Chl *a* extraction was performed using 10 ml of a mixed solution of acetone, methanol and water (45:45:10) (Joye et al. 1996) and incubated for 24 h at 4 ºC in the absence of light. The water content in the sediment was previously measured by the difference between dry and moist weight in separate samples, in order to maintain the solvent proportions. After incubation, the solvent was centrifuged at 4000 rpm for 5 min. and Chl *a* concentration in the extracts was determined spectrophotometrically using standard equations (Strickland & Parsons 1972).

The percent organic matter content of sediment was determined by drying the sediment at 60 ºC to a constant weight, followed by ignition in a muffle furnace at 550 ºC for 4 h for the organic removal and reweighting. Sediment particle size distribution was determined by sieving the pre-combusted sediments in a sieve shaker device (< 0.063 mm, >0.063 mm; >0.125 mm; >0.25 mm; >0.5 mm; >1 mm; >2 mm).

At each sampling site, water column ammonium (NH_4^+) and nitrite (NO_2^-) were quantified using methods described in Grasshoff et al. (1983). Nitrate (NO_3^-) was assayed using an adaptation of the spongy cadmium reduction technique (Jones 1984), with the NO_2^- value subtracted from the total.

2.2. *Ruegeria pomeroyi* DSS-3 cell cultures experiments

2.2.1. Bacteria strain

Ruegeria pomeroyi DSS-3 was previously isolated from coastal Georgia (USA) seawater in a media with 14 ppt (González et al., 1999). *Ruegeria pomeroyi* DSS-3, among more than 40 strains, is a member of the *Roseobacter* lineage (Moran et al. 2012) abundant in marine environments and salt marsh-estuarine ecosystems (Gonzaléz et al. 1999, Buchan et al. 2005). This group retains several strategies and characteristics for adaptations in marine environments (Moran et al. 2004, Christie-Oleza et al. 2012) which is why this organism is able to cope with salinity fluctuations. In addition, Georgia salt marshes can vary between 20 to 30 ppt, reaching 12 ppt in seasons of heavy rain (Teal 1962). Effectively, DSS-3 in culture possesses a NaCl requirement but with a high tolerable salinity range of 100 – 400 mM (~6 – 23 ppt; Gonzaléz et al. 2003). Moreover, the *Roseobacter* clade is represented by several microorganisms that are key participants in DMSP assimilation (Malmstrom et al. 2004) and organic sulfur biogeochemical fluxes (Kiene et al. 1999, González et al. 2000, Zubkov et al. 2001). *R. pomeroyi*, formerly *Silicibacter pomeroyi* (Yi et al. 2007), holds diverse genes capable of both cleavage and demethylation enzymatic processes (Newton et al. 2010, Curson et al. 2011b, Moran et al. 2012). After *R. pomeroyi*'s whole genome sequenced (Moran et al. 2004), this model organism represented a shortcut for the understanding of DMSP catabolism.

For this study *R. pomeroyi* DSS-3 strain was obtained from the Spanish Type Culture Collection (CECT) from University of Valencia, Spain and was stored on half strength YTSS (½ YTSS) solid medium containing 2 g yeast extract (Merck Millipore), 1.25 g tryptone peptone (OXOID), 20 g Sea salts (Sigma) and 15 g Agar powder (HIMEDIA) per Liter of medium.

2.2.2. *Ruegeria pomeroyi* DSS-3 grow conditions

Ruegeria pomeroyi DSS-3 cells were inoculated into two different media, a modified Marine Basal Medium (MBM; Baumann & Baumann 1981) containing 50 mM Tris-HCl pH 7.5, 0.33 mM $K_2HPO_4.3H_2O$ with addition of 0.1 mM $FeSO_4$ instead of FeEDTA, according to Gonzaléz et al. (1999), and ½ YTSS both with different sea salts additions to simulate salinities of 10, 20 and 30 ppt. All growth cultures were amended with 10 mM Glucose and incubated at 26 ºC under aerobic conditions with rotary shaking (80 rpm). Readings (OD_{540}) of each independent salinity cell suspension replicate (n = 2) were performed at intervals of averaged 10.5 h until a total of 60 h of incubation.

2.2.3. Salinity treatments in *Ruegeria pomeroyi* DSS-3 cell suspensions

The salinity effect on volatile organosulfur compounds (DMS, MeSH) accumulation was evaluated in *R. pomeroyi* DSS-3 cells harvested into 30 ml of liquid ½ YTSS and MBM media at different salinities (10, 20, 30 ppt) by modeling the quantity of sea salts in each medium and measuring the correspondent salinity with a YSI Model 30 probe. Cells were grown at the different salinities overnight (16 h) at 26 ºC in rotary shaking (80 rpm) until the end of the exponential phase (OD_{540}~1.3). Consecutively, 3 ml of *R. pomeroyi* DSS-3 culture were added to a 12 ml crimp-toped serum vial and sealed with a Teflon stopper. Each salinity treatment was incubated in triplicate for 4 h at 26 ºC, with constant rotary shaking (80 rpm), under oxic (air headspace) and anoxic (purged with N_2 for 15 min) conditions and with different DMSP amendments (0, 50, 500 µM). Sulfur volatiles compounds (DMS and MeSH) were monitored in the headspace at the beginning (time 0 h) and end (time 4 h) of the incubation and analyzed by pulsed flame-photometric detection (GC / P-FPD) as described below. Negative controls with only ½ YTSS and MBM media and DMSP amendments were run in triplicate for the different salinity treatments performed.

2.3. Analytical techniques

DMS and MeSH were measured by removing a headspace subsample from the incubation vials with a glass gas-tight syringe and analyzed in a Varian gas chromatograph (CP-3800). Each volatile sulfur gas was separated with Mega-Bore silica plot column at 189 ºC and detected with a P-FPD where nitrogen was the carrier gas at 3 ml min^{-1}. The retention times for MeSH and DMS were 1.55 min and 2.75 min, respectively. DMS concentrations were calculated by using standards generated from DMSP conversion (> 95 %) into DMS after alkaline hydrolysis with a 5 M NaOH solution (Kiene & Service 1991) and MeSH concentrations estimated based on the same standard curve, once it was previously demonstrated to agree (Kiene 1996). Detection limit for both sulfur gases was 10 nM. DMS and MeSH concentrations in solution were determined from measured headspace concentrations and empirical distribution coefficients of the selected organosulfur compounds according to respective salinity (Przyjazny et al. 1983).

In the slurries experiments nitrous oxide was quantified using a Varian gas chromatograph (CP-3800) equipped with an electron-capture detector (ECD) with two Hay Sep D columns according to Magalhães et al. (2005), with a retention time of 1.62 min and detection limit was 15 nM. N_2O concentration was calculated using a standard curve generated from certified gas standards (N_2O in He, Scott Specialty Gas).

2.4. Statistical analysis

Before testing the statistical significance of data, the Leven's test for the equality of variances and Kolmogorov-Smirnov test for normality was applied in all salinity experiments data. After accomplish homoscedasticity and normality, the one-away ANOVA was used to evaluate the statistical differences between salinity treatments. All data analysis were performed at the 95% confidence level ($p < 0.05$), unless otherwise stated. QI Macros SPC Software 2013 was applied for statistical analysis.

CHAPTER 3

The regulatory effect of salinity on dimethyl sulfide and methanethiol formation in estuarine sediments and subsequent interaction on nitrous oxide production

3.1. Abstract

It was investigated the regulatory effect of salinity on the production of DMS and MeSH in estuarine sediments and the potential interactions with the N_2O reductase step of the denitrification pathway. This was achieved by monitoring DMS, MeSH and N_2O accumulation in sediment slurries from a temperate estuary (Ave, NW Portugal). Treatments were performed without and with additions of potential sulfur gas precursors, DMSP (0 – 50 µM) or methionine (0 – 500 µM) at different salinities (0, 15 and 30 ppt). Experimental increases of salinity inhibited DMS accumulation under both oxic and anoxic incubation conditions, and the pattern was observed whether DMSP or methionine was added or not, i.e. lower salinities stimulated DMS net production. On the other hand, MeSH tended to accumulate to higher concentrations in higher salinity treatments (15, 30 ppt). N_2O production followed the pattern of MeSH accumulation in methionine treatments, with higher N_2O accumulation rates in treatments where MeSH production was higher (15 ppt), a finding consistent with previously-reported inhibition of nitrous oxide reductase activity by MeSH. Overall, these results suggest that changes in salinity may have an important regulatory role in net production of DMS, MeSH and N_2O and their potential emissions to the atmosphere.

3.2. Background

Organic sulfur compounds are widespread in aquatic environments. These compounds are released to the external environment by a variety of processes like the death of organisms, physiological stress and cell senescence (c.f Kiene et al. 2000; Stefels 2000). Once released into the dissolved phase, organic sulfur compounds rapidly disappear

because of microbial degradation (Kiene and Bates 1990; Kiene et al. 2000; Kiene and Linn 2000a, b). In marine waters, organic sulfur compounds can satisfy a substantial part of the sulfur and/or carbon demand for many marine prokaryotes (Kiene et al. 2000; Zubkov et al. 2002).

DMSP, an organic sulfur compound, has been studied extensively because of its role as a precursor of DMS and MeSH, a climatically important gas and an energetically favourable assimilation compound, respectively (Charlson et al. 1987, Kiene 1996, Schlessinger 2013, Kiene et al. 1999, Kiene et al. 2000, González et al. 2003).

Several previous studies have addressed the effect of environmental controls on DMSP-producers physiology (Karsten et al. 1990, Kiene & Service 1991, Kirst et al. 1991, Karsten et al. 1992, Van Duyl et al. 1998, Laroche et al. 1999, Tang et al. 1999, Stefels 2000, Otte et al. 2004, Van Alstyne & Puglisi 2007, Yang et al. 2011). However, there's a hiatus relating to the microbial mediation upon the two competing DMSP degradation pathways and what controls the fluxes of DMSP breakdown products. The influence of abiotic factors on the net fluxes of these DMSP degradation compounds have been only addressed by few studies which indicate that DMS production is a function of salinity, temperature and dissolved oxygen in hypersaline microbial mats (Visscher et al. 2003), and that salinity affects water column DMSP and DMS dynamics in a coastal environment (Niki et al. 2007). In this study, it was hypothesized that because the formations of these volatile organic sulfur compounds are mainly regulated by biological pathways, environmental factors may exert strong constraints on the emissions/distributions of these VOSCs. Therefore, this study was focused on the impact of salinity changes on DMS and MeSH net production in slurries prepared from sediments collected along the salinity gradient of the Ave River Estuary (NW, Portugal). The present experiments included non-amended sediments and sediments that were amended with DMSP or methionine. It was used methionine as a main precursor for the production of the methylated sulfur compound MeSH (Kiene and Visscher 1987). While MeSH is a major thiol formed during the anoxic degradation of DMSP though the demethylation/demethiolation pathway, in the locations under study the methanethiol accumulation from DMSP degradation was found to be of minor importance (Magalhães et al. 2011, 2012). The previous studies demonstrated that MeSH interfered with the N_2O reductase step of denitrification, potentially leading to the accumulation of N_2O, a potent greenhouse gas in the atmosphere (Magalhães et al. 2011). Thus, in this study it was also evaluated how salinity could modulate N_2O fluxes through its effect on MeSH net production.

3.3. Results

3.3.1. Environmental characterization

Salinity between the sampling sites comprised values between 2.1 ppt and 15.6 ppt, upstream and downstream Ave's estuary, respectively (Table 2; Fig. 3). The granulometry analysis revealed a tendency towards a sandy sediment (> 2000 µm) downstream's estuary. Among the inorganic nutrients, NO_3^- revealed a substantial variation (89.2 ± 4.0 to 160.3 ± 12.1 µM; Table 2), with the highest concentration registered at A-1 (160.3 ± 12.1 µM; Table 2), an upstream sampling site.

DMSP concentrations in sediment correlated to Chl *a* ones ($r = 0.97$, $p < 0.05$), where A-1 and A-4 presented the highest DMSP values (16.0 ± 2.9 moles L^{-1} wet sed and 35.5 ± 6.7 moles L^{-1} wet sed, respectively).

Table 2 - Physical and chemical characteristics of sediments and overlying water at the sampling sites in the Ave Estuary.

		Characteristics of overlying water				Characteristics of sediments				
Dates	**Sites code**	**Salinity (ppt)**	**N inorganic (µM)** NO_3^-	NH_4^+	NO_2^-	**Chl *a* (mg g^1wet sed)**	**DMSP (µmoles L^{-1} wet sed)**	**OM (%)**	**Grain Size (% dry weight)** < 63µm	> 2000 µm
09/21/2010	1	2.1	160.3 ± 12.1	12.2 ± 0.7	6.0 ± 0.1	10.0 ± 2.1	16.0 ± 2.9	2.5 ± 0.2	8.3	15.5
09/21/2010	2	5.2	100.0 ± 3.1	12.5 ± 0.7	4.7 ± 0.0	7.1 ± 0.5	3.8 ± 0.5	2.3 ± 1.0	6.9	28.5
09/21/2010	3	12.4	77.4 ± 1.4	12.4 ± 0.8	3.7 ± 0.0	3.1 ± 0.3	2.6 ± 0.6	1.8 ± 0.2	3.2	20.6
09/21/2010	4	15.6	89.2 ± 4.0	8.1 ± 5.4	4.0 ± 0.1	18.9 ± 2.6	35.5 ± 6.7	1.2 ± 0.0	0.8	33.6

3.3.2. DMS *vs.* MeSH production

Generally, DMS production exceeded MeSH in all sampling sites submitted to different salinity treatments in both oxic and anoxic conditions (Fig. 5, 6).

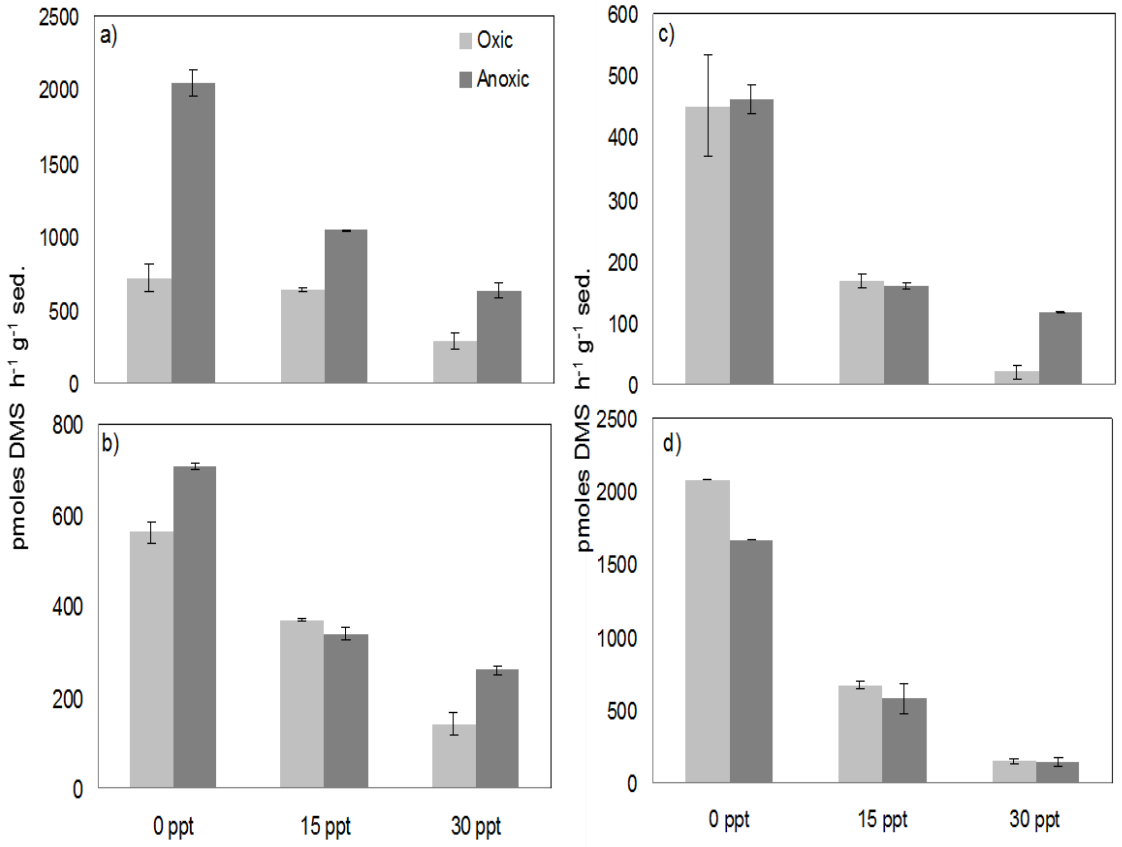

Figure 5 - Net DMS production rates in oxic and anoxic sediment slurries from sites 1 (a), 2 (b), 3 (c) and 4 (d) in the Ave Estuary at three different salinities. For the salinity treatments, sediments from each site were slurried with either Ave River freshwater (0 ppt) or Ave River freshwater amended with different concentrations of sea salts to reach 15 ppt and 30 ppt, respectively. The *in situ* salinities at each station are given in Table 1.

DMS accumulations between incubations with and without oxygen were variable according with to site and the salinity treatment. MeSH accumulations occurred with more extent under anoxic conditions but its net production wasn't observed in sampling site A-1, upstream Ave's estuary, regardless of the experimental salinity (Fig. 6a).

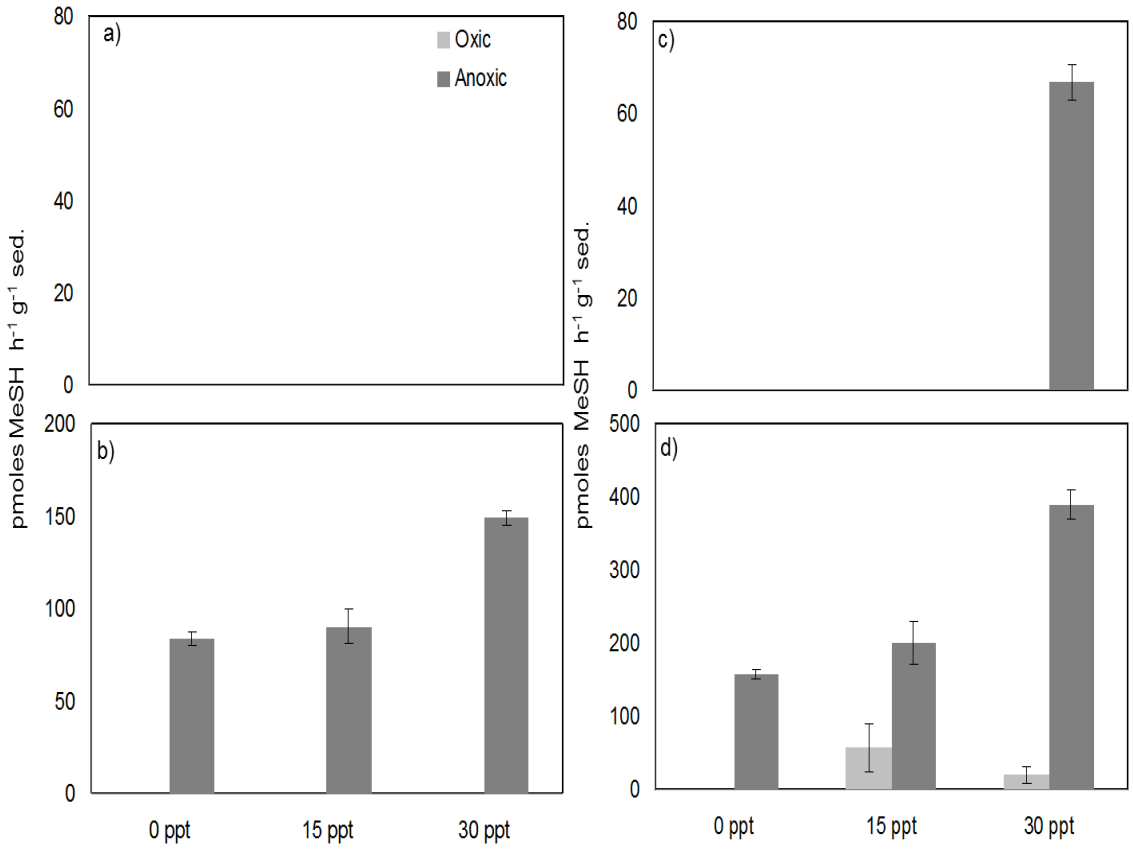

Figure 6 - Net MeSH production rates in oxic and anoxic sediment slurries from sites 1 (a), 2 (b), 3 (c) and 4 (d) in the Ave Estuary at three different salinities. For the salinity treatments, sediments from each site were slurried with either Ave River freshwater (0 ppt) or Ave River freshwater amended with different concentrations of sea salts to reach 15 ppt and 30 ppt, respectively. The *in situ* salinities at each station are given in Table 1. MeSH accumulation was not detected in any of the treatments in slurries from Station 1, the station with the lowest salinity.

These salinity manipulation experiments revealed an opposite pattern between DMS and MeSH accumulations i.e., while the production of DMS is enhanced in the freshwater treatments (0 ppt) MeSH seems to be utmost expressed at highest salinities (30 ppt). Isolated, all sediment sampling sites demonstrated a consistent salinity effect on DMS net accumulations, regardless of the *in situ* salinity of the station (Fig. 5, Table 2). Therefore, higher DMS net accumulations were always observed in freshwater treatments (0 ppt) decreasing its production rates towards the highest salinity treatments and this was a coherent pattern in both oxic and anoxic conditions (Fig. 5). In contrast, net MeSH production was considerably lower in oxic conditions and was not detected in majority of the sampling sites (Fig. 6). Nevertheless, anoxic manipulation experiments showed a general net MeSH accumulation, with high rates observed at the highest salinities (30 ppt) and less net effluxes of MeSH in freshwater treatments (0 ppt; Fig. 6).

Regarding the substrate amendments (DMSP and methionine), both sulfur volatiles breakdown products (DMS and MeSH) demonstrated the same opposite pattern as determined on the non-amended incubations above mentioned. In all DMSP additions DMS displayed lower production in the highest salinity treatments (30 ppt) and highest net accumulation in the lowest salinities (0 ppt; Fig. 7). This same pattern was found to be coherent for all sediments, collected in different stations, incubated with or without oxygen (Fig. 7). For the methionine amendments, sediment slurries incubated in anoxic conditions confirmed the same opposite pattern previously mentioned (Fig. 8a, b, d); all sampling sites, except for A-3 (Fig. 8c), presented the higher MeSH net accumulation in the highest salinity treatments (30 ppt) and lowest production in 0 ppt treatments.

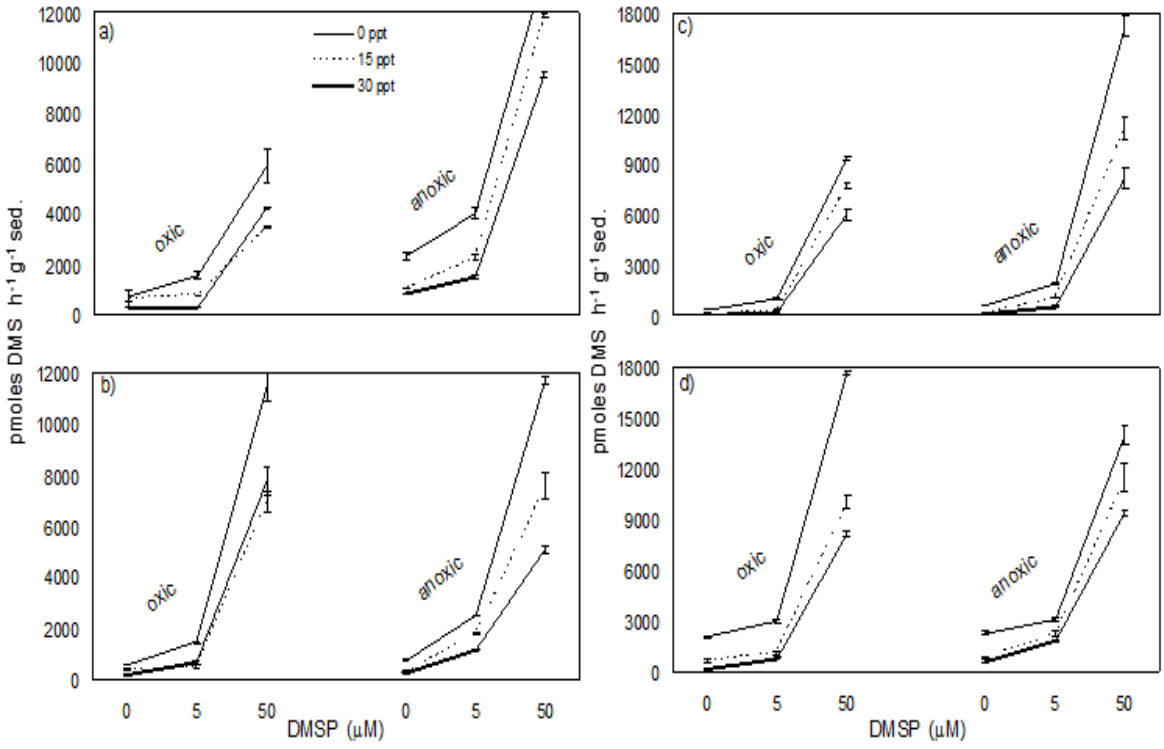

Figure 7 - The effects of DMSP additions and salinity treatments (0, 15 and 30 ppt) on net DMS accumulation rates in oxic and anoxic sediment slurries from sites 1 (a), 2 (b), 3 (c) and 4 (d) of the Ave Estuary. For the salinity treatments, sediments from each site were slurried with either Ave River freshwater (0 ppt) or Ave River freshwater amended with different concentrations of sea salts to reach 15 ppt and 30 ppt, respectively.

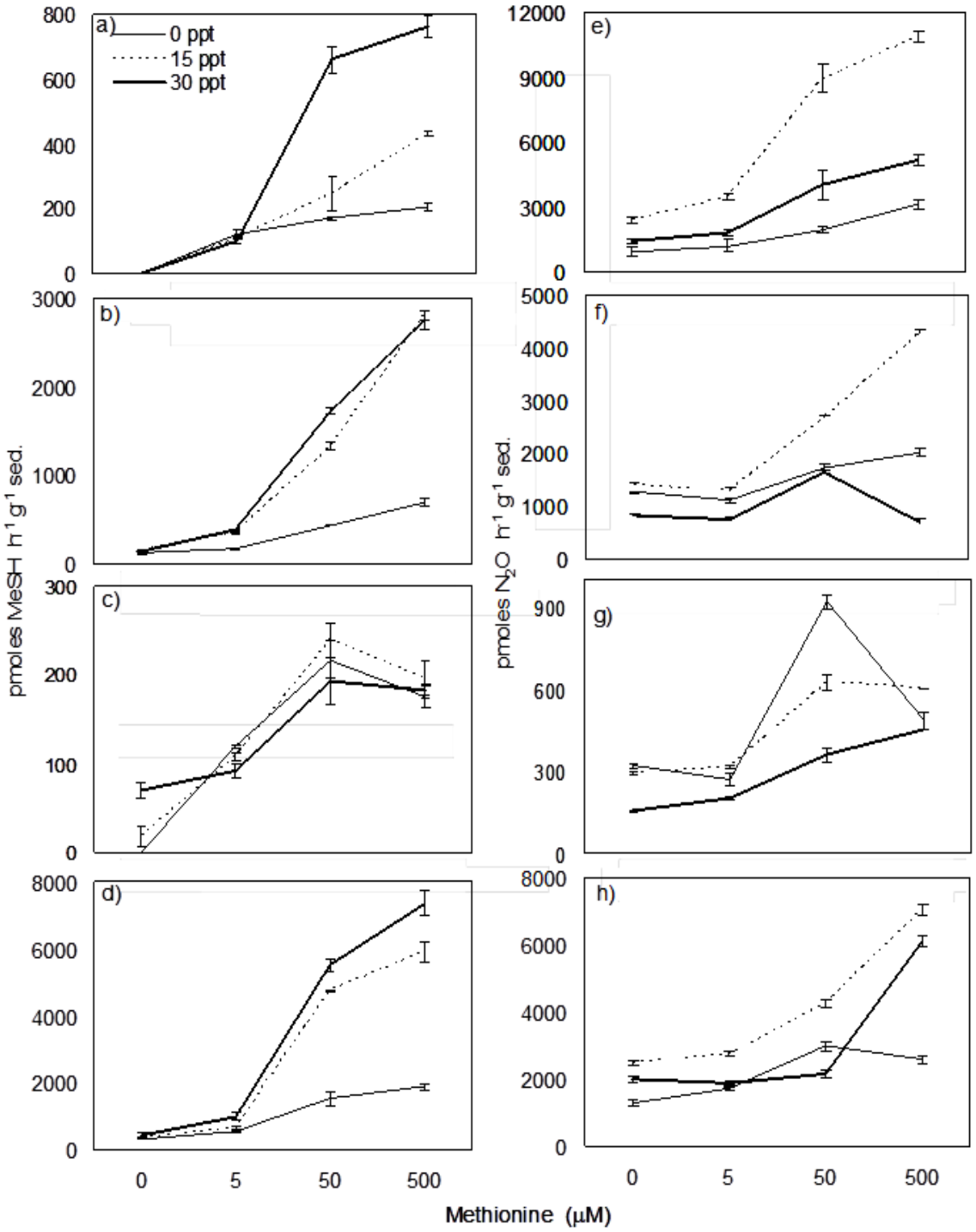

Figure 8 - The effects of methionine additions and salinity treatments (0, 15 and 30 ppt) on net MeSH and N_2O accumulation rates in anoxic sediment slurries from sites 1 (a, e), 2 (b, f), 3 (c, g) and 4 (d, h) of the Ave estuary. For the salinity treatments, sediments from each site were slurried with either Ave River freshwater (0 ppt) or Ave River freshwater amended with different concentrations of sea salts to reach 15 and 30 ppt, respectively.

3.3.3. N_2O net accumulation along methionine additions

For non-amended sediment slurries incubated under anoxic conditions, generally N_2O accumulated to a greater extent in the 15 ppt salinity treatments compared to the freshwater (0 ppt) treatment (Fig. 9).

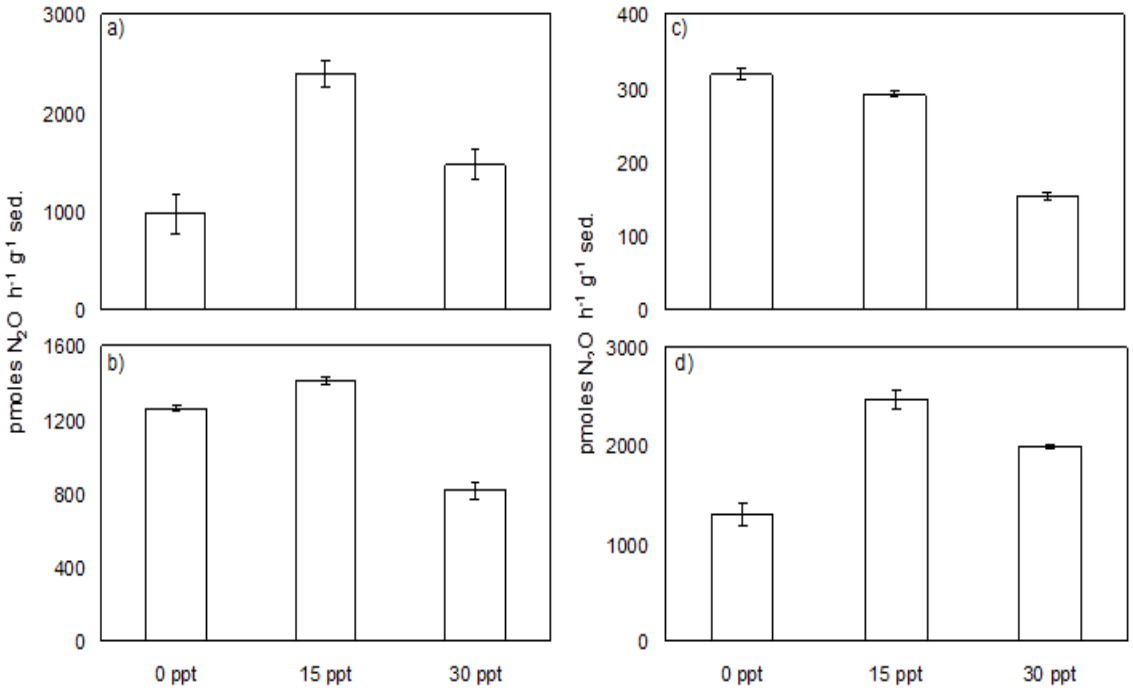

Figure 9 - Net N_2O production rates in anoxic sediment slurries from sites 1 (a), 2 (b), 3 (c) and 4 (d) in the Ave Estuary at three different salinities. For the salinity treatments, sediments from each site were slurried with either Ave River freshwater (0 ppt) or Ave River freshwater amended with different concentrations of sea salts to reach 15 ppt and 30 ppt, respectively. The in situ salinities at each station are given in Table 1.

Also, the higher salinity (30 ppt) treatments tend to reduce potential N_2O net accumulation compared to 15 ppt slurries (Fig. 9). In treatments where methionine was added, results showed high N_2O accumulations in the intermediate salinity treatments (Fig. 8e, f, g, h), which is in agreement to the results observed in the non-amended sediment slurries (Fig. 9).

3.4. Discussion

3.4.1. DMS and MeSH production

In natural environments DMSP profiles can vary with seasonality, DMSP-producers composition, phytoplankton blooms (Yoch 2002, Simó et al. 2009). DMSP and its related thiols present higher concentrations associated to sediments than to water column, and Chl *a* concentrations usually correspond to DMSP (Kiene 1991, Nedwell et al. 1994; Jonkers et al. 1998, Trevena et al. 2000). This correlation is an indication of an algal biomass source for DMSP, an osmolyte mainly synthesized by several photoautotrophic primary producers in marine, estuarine and salt-marsh systems (Karsten et al. 1990, Kirst et al. 1991, Stefels & Van Boekel 1993, Stefels 2000, Van Alstyne et al. 2003, Yang et al. 2011). Results from this study show higher rates of net DMS production at sites where higher DMSP concentration were measured, an observation that is likely due to the fact that DMSP is a major precursor of DMS (Kiene and Bates 1990). In fact, in marine environments DMSP is perceived as the main source of DMS (Stefels & Van Boekel 1993, Van Alstyne & Puglisi 2007), a metabolism which involves different enzymes (Fig. 2; Todd et al. 2009, Todd et al. 2010, Curson et al. 2011b, Moran et al. 2012, Todd et al. 2012). On the other hand, several other studies have addressed alternative sources for DMS formation; DMS can also be produced through a respiratory reduction of DMSO (Griebler 1997, López & Duarte 2004), methylation of MeSH (Kiene & Hines 1995) and anaerobic degradation from methoxylated aromatic compounds under high sulfide concentrations (Bak et al. 1992, Lomans et al. 1997). Even though the present study is unable to differentiate what processes are specifically involved in these estuarine sediments of Ave River, the enzymatic degradation of DMSP represents a relevant biological process, mediated by bacteria, leading to DMS production.

Steady-state concentrations and fluxes of DMS and MeSH in the sediment and surface waters depend on complex microbial formation and degradation processes (Kiene 1996, Lomans et al. 1997, Kiene et al. 2000), as well as on complex physicochemical interactions between the different natural elements (Mopper and Taylor 1986). In the sediment slurries from the Ave estuary, the production of DMS always exceeded consumption, resulting in net DMS accumulation. In contrast, MeSH accumulations under oxic conditions were much less important than under anoxic conditions. Actually, MeSH is likely to be subject to oxidation in the presence of O_2 (Suylen et al. 1987), but its oxidative metabolism is poorly understood. The absence of MeSH accumulation in oxic treatments has been suggested to be a result of rapid MeSH oxidation to dimethyl disulfide (DMDS)

in the presence of oxygen (Visscher et al. 2003) and also, in anaerobic lake sediments, microbial populations can metabolize MeSH into methane and carbon dioxide (Zinder & Brock 1978). Thus, it remains unclear whether lack of production or simply high rates of consumption were responsible for the minimal MeSH accumulations obtained. MeSH accumulation in anoxic treatments was not related to DMSP availability in the sediments suggesting that other pathways of MeSH formation besides DMSP demethylation/demethiolation (Kiene 1996; Kiene et al. 2000) might be important. A multitude of MeSH production pathways may contribute to the MeSH fluxes in our anoxic incubations, including methylation of hydrogen sulfide (Mopper and Taylor 1986, Lomans *et al.*, 1997; 1999), sulfide methylation by methoxylated aromatic compounds (Lomans et al. 2001) or by degradation of sulfur-containing amino acids (Lomans et al. 1997; Lomans et al. 1999).

3.4.2. Salinity effect on DMS and MeSH potential emissions

Higher DMS production rates were observed in freshwater treatments (0 ppt), while higher salinity treatments resulted in the lowest rates of DMS accumulation. This pattern of DMS accumulation in the different salinity treatments was found to be coherent in the oxic and anoxic treatments. The role of salinity in regulating DMS flux to the atmosphere has been identified in previous research performed on microbial mats grown in hypersaline ponds with different salinities (Visscher et al. 2003). Higher DMS net accumulation was found in a lower salinity pond (90 ppt) compared with a higher salinity pond (115 ppt) (Visscher et al. 2003). While such high salinities were not tested in our study, our results are in agreement with these previous findings, suggesting that lower salinities stimulated DMS production rates. Salinity fluctuations have a direct impact on intracellular DMSP concentrations of micro- and macroalgae inhabiting intertidal estuarine environments, where the osmotic function of DMSP has been described (Edwards et al. 1987). Intracellular concentrations of DMSP tend to decrease in response to a decrease in salinity and DMSP biosynthesis increase in higher salinities (Vairavamurthy et al. 1985; Dickson and Kirst, 1987; Edwards et al. 1987). Thus, we hypothesize that the rapid release of DMSP from cells at lower salinities may increase the availability of DMSP to the DMSP-degrading microbial communities, resulting in high levels of DMS accumulation. In fact, Van Bergeijk et al. (2003) described DMSP excretion into the medium by the marine benthic diatom *Cylindrotheca closterium* after salinity down-shock, which means that there's an enhancement in extracellular DMSP resulting in high levels of DMS

accumulation through the cleavage pathway. Niki et al. (2007) also identified salinity as an important factor controlling the production of DMS in the water column of a coastal bay of Japan. In agreement with the present results, it has been found that low-salinity shock leads to an increase in potential DMS production to the environment (Niki et al. 2007). In addition, DMSP-lyases aren't exclusive bacterial isozymes and have already been identified in several marine micro- and macroalgae (De Souza & Yoch 1996, Stefels & Dijkhuizen 1996, Steinke et al. 1996, Steinke et al. 1998), representing a further contribution for DMS formation, besides bacterial production (Niki et al. 2000). In fact, low salinity was found to enhance algal DMSP lyase activity (c.f. Stefels 2000; Steinke et al. 2002), which can increase the relative contribution of algal DMS production compared to bacterial production of DMS. However, optimal performance of the different marine algae lyase activity requires specific chloride concentrations (Steinke & Kirst 1996). These data on net potential DMS productivity do not allow to discriminate between algal *vs* bacterial DMS production, but in light of the high Chl *a* levels found, the algal role in DMS production may be relevant in Ave estuarine sediments and should be considered in future work.

DMSP additions at different salinity treatments showed a progressive increase in DMS accumulation with the same pattern of salinity effects observed for the non-amended sediments; i.e. higher rates of DMS accumulation in the freshwater treatments. These results together suggest that salinity alone influences the degradation rates of extracellular DMSP and methionine. The inverse relationship between DMS accumulation rate and salinity can be attributed to either enhanced production of DMS from DMSP and methionine or suppressed decomposition of DMS at lower salinities. Salinity also affected net MeSH production but in the opposite sense from that observed with DMS. MeSH accumulation was not detected at all Ave Estuarine sites, but when rates were measurable, higher values were registered for the higher salinity treatments. These results are not in agreement with a previous study (Visscher et al. 2003), which found an increase in MeSH flux resulting from decreased salinity in hypersaline microbial mats. However, the similar regulatory effect of salinity on MeSH accumulation observed in slurries without and with added methionine suggests that sulfur containing amino acids can be potential precursors of MeSH in intertidal Ave Estuary sediments. Other factors may promote MeSH accumulation in these sediments, such as higher sulfide concentrations and lower iron oxide availability (Mopper and Taylor 1986). Higher sulfide levels in more saline sediments would be expected due to the very high sulfate concentrations in seawater (Capone and Kiene, 1988).

3.4.3. Salinity effect on N_2O production during MeSH accumulation

In a previous study it was demonstrated that MeSH interferes with the N_2O reductase step of denitrification (Magalhães et al. 2011) and leading to the accumulation of N_2O and its potential emission to the atmosphere. In the present study, it was hypothesized that salinity may modulate N_2O fluxes through its regulatory effect on the magnitude of MeSH accumulation. For sediment slurries incubated under anoxic conditions N_2O accumulated to a greater extent in the 15 ppt salinity treatment compared to the freshwater (0 ppt) treatment. Since MeSH accumulated to a much greater extent from methionine additions at higher salinities (15 and 30 ppt), these findings are consistent with MeSH playing a role in the salinity effect on N_2O accumulation (Magalhães et al. 2011). The interactions between salinity, MeSH and N_2O accumulation may be complex, as it was found that N_2O accumulation rates were lower at 30 ppt compared to those at 15 ppt. These results are attributed to the direct effect of the higher salinity (30 ppt) in reducing potential N_2O net accumulation from non-amended slurries compared to 15 ppt slurries. This was particularly evident at site 3 where no differences were observed in MeSH fluxes for different salinities tested, and lower N_2O accumulation rates were registered at the higher salinity treatment. N_2O accumulations measured in anoxic incubations were likely to be a function of the rates of denitrification in our sediments. Previous studies evaluating the regulatory effect of salinity on denitrification pathway have found that tolerance to salinity may differ within denitrifier communities from different coastal and estuarine systems (Seitzinger et al. 1991, Rysgaard et al 1999, Magalhães et al. 2005, Fear et al. 2005). For example Rysgaard et al. (1999) showed that denitrification decreased with increasing salinities in estuarine sediments where *in situ* salinities ranged from 3 to 13 ppt. However, Magalhães et al. (2005) reported that denitrification rates were independent of salinity in estuarine sediments exposed to a wide range of salinities (0 to 35 ppt). Other studies performed in estuarine sediments (Kana et al. 1998; Fear et al. 2005) indicated that denitrifying communities may adapt to the range of salinities inherent to a given ecosystem and that salinity becomes a driving force in selecting halotolerant or halophilic denitrifier communities. MeSH is typically present at nanomolar to low micromolar concentrations in sediment pore waters (Mopper and Taylor 1986; Kiene 1991). In these experiments, N_2O started to accumulate at MeSH concentrations between 0.1 to 6.7 µM depending on the different sampling sites. This is in agreement with a previous study where it provides evidences that MeSH concentrations typically found in marine sediments can be sufficient to cause inhibition of the nitrous oxide step of denitrification (Magalhães et al. 2011).

In this study salinity was identified as an important environmental factor regulating natural DMS and MeSH net accumulations in a temperate estuarine system. These results indicated that salinity changes affected the magnitudes of DMS and MeSH accumulations in an opposite pattern; while DMS production rates were stimulated in freshwater treatments (0 ppt), higher salinity treatments (15, 30 ppt) resulted in higher rates of MeSH accumulation. These findings may reveal that different communities/processes are implicated in the formation of these volatile sulfur compounds. In addition, salinity was suggested to be an important factor modulating the previously identified inhibitory relationship between MeSH and nitrous oxide reductase enzyme activity in estuarine sediments (Magalhães et al. 2011). Thus, salinity fluctuations that are typical of estuarine systems could play an important role in regulating DMS, MeSH and N_2O emissions to the atmosphere, with potential effects on the global climate balance (Dickinson and Cicerone 1986; Charlson et al. 1987; Visscher et al. 2003; Kiene et al. 2000; Vallina and Simó, 2007).

CHAPTER 4

Salinity as a key regulator of DMSP catabolism pathways in *Ruegeria pomeroyi* DSS-3

4.1. Abstract

Dimethylsulfoniopropionate (DMSP) is an important reduced organic sulfur compound to marine bacterial communities, and a main precursor of dimethylsulfide (DMS), a gas that influences atmospheric chemistry and potentially the global climate. In nature DMSP bacterial catabolism may yield different proportions of DMS and methanethiol (MeSH), but yet relatively little is known about the factors controlling the pathways of bacterial degradation that select the formation of DMS and MeSH (cleavage *vs* demethiolation). In this study, we carried out a series of experiments to evaluate the influence of salinity in selecting the routes of DMSP catabolism in *Ruegeria pomeroyi* DSS-3 strain. We monitored DMS and MeSH accumulation in cell suspensions grown in a range of salinities (10, 20, 30 ppt) and with different DMSP amendments (0, 50, 500 μM). Results revealed significant higher DMS accumulation in low salinity treatments (10 ppt; $p < 0.001$) in both MBM and ½ YTSS media, with a 47.1% and 87.5% decrease of DMS accumulation (10 to 20 ppt) in MBM and ½ YTSS media, respectively. On the other hand, MeSH followed a converse pattern with enhanced accumulations in higher salinities (20, 30 ppt), with a 90.6% increase of MeSH accumulation from the 20 to the 30 ppt salinity treatments. These findings with the single bacterial species in culture are in agreement with those found with estuarine sediments and suggest that salinity has a modulating influence on the selection of the DMSP enzymatic degradation routes with a consequent potential impact on DMS and MeSH liberation into the atmosphere.

4.2. Background

Sulfur, in its biogenic form, is a vital element in microorganisms' metabolism. Besides the assimilation of this compound into biomolecules such as methionine (Cooper 1983), marine algae incorporate sulfur into dimethylsulfoniopropionate (DMSP; Stefels 2000). DMSP is an osmolyte and precursor of volatile DMS which is a major contributor to

organic sulfur emissions from salt marshes, coastal wetlands and oceans (Steudler & Peterson 1984, Aneja & Cooper 1989, Liss et al. 1997). Lovelock et al. (1972) were the first to propose that DMS, and not H_2S, was the major agent transferring sulfur between the ocean and land to complete the sulfur cycle. DMS is oxidized in the atmosphere to sulfate and methanesulfonate which contribute to aerosol formation and growth and can influence the climate (Charlson et al. 1987). However, DMS connection to earth's climate is currently under debate and was recognized to be more complex than previously believed (Quinn and Bates 2011).

DMSP is produced by microalgae, macroalgae and a few higher plants (Van Rijssel & Gieskes 2002, Otte et al. 2004, Van Alstyne 2008). This osmolyte and cryoprotectant (Kirst et al. 1991, Tang et al. 1999) compound is released into the extracellular environment 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 & Otte 2002). Dissolved DMSP is a labile substrate and it represents an important carbon sulfur source for marine heterotrophic bacteria (Simó et al. 2009). DMSP catabolism is essentially a microbial mediated process (Kiene 1990, Taylor & Gilchrist 1991, Visscher et al. 1992, Visscher & Taylor 1994) with a rapid turnover under oxic conditions (Zubkov et al. 2001). The $DMSP_d$ in seawater can be rapidly turned by two main pathways: cleavage and/or demethylation/demethiolation (Yoch 2002). Although cleavage represents a considerable source of DMS (Curson et al. 2011b) demethylation/demethiolation, which final product is MeSH, appears to be the preferential enzymatic pathway in oceans from bacterial $DMSP_d$ turnover (Kiene 1996, Kiene & Linn 2000b). This biogeochemical finding is consistent with the high frequency of DMSP – demethylating cells registered in a most recent analysis of oceanic metagenomic data (Howard et al. 2008).

Numerous environmental and biological factors may control the pathways of degradation of $DMSP_d$ to DMS (cleavage) versus MeSH (demethylation/demethiolation), with certainly implications on the different production ratios of DMS and MeSH in marine environments. There is evidence that the type of microbial species assemblage, influences the magnitudes of DMS emissions (Jonkers et al. 1998, Zubkov et al. 2001). The diversity of these microbial assemblages seems to be linked to the dynamics of phytoplankton blooms, which then affects DMSP turnover and, subsequently, on the amount of DMS emitted to the atmosphere (Jonkers et al. 1998, Zubkov et al. 2001, Pinhassi et al. 2005). Relatively few studies have addressed the environmental controls on the pathways of DMSP degradation and DMS/MeSH formation in natural systems (Visscher et al. 2003; Niki et al. 2007, Magalhães et al. 2012), and knowledge about the main environmental

regulators on DMSP metabolism and on the distribution of its catabolic products remain scarce.

This study was motivated by the results obtained in the previous work (Chapter 3) in which it was evaluated the impact of salinity changes on DMS and MeSH net accumulation in sediments collected along an estuarine salinity gradient. In this previous study it was identified salinity as an important environmental factor regulating ratios of natural DMS and MeSH emissions, however, it was not possible to discriminate which specific processes were involved on the DMS and MeSH net fluxes measured in those complex communities, where several biological and chemical processes of DMS and MeSH production may operate simultaneously.

In the present study it was avoided the complexities of sediments by investigating the effect of salinity on the regulation of the two competing DMSP catabolic pathways in a pure culture of *R. pomeroyi* strain DSS-3, an organism that possesses both the cleavage and demethylation pathways for DMSP degradation (González et al. 2003, Newton et al. 2010). In the present study is hypothesized that salinity is a key factor in selecting the two competing pathways of bacterial DMSP degradation, and thus in controlling the relative production of DMS and MeSH within the DMSP catabolism.

4.3. Results

4.3.1. *R. pomeroyi* growing curves at different salinities

The growth behavior of *R. pomeroyi* DSS-3 strain in MBM and ½ YTSS media at different salinities (10, 20, 30 ppt) showed a typical exponential growth in both media, however the initial lag phase was more pronounced in MBM (Fig. 10a) compared with ½ YTSS medium (Fig. 10b). In ½ YTSS media the lag phase was undetectable; the culture had a rapid development to exponential phase. While *R. pomeroyi* DSS-3 growth curves were found to be different for the type of media used, the growth curves were found to be similar to each other for each specific salinity within each type of media. These results confirmed that the range of salinities tested (10, 20, 30 ppt) did not substantially affect *R. pomeroyi* DSS-3 growth performance in MBM and YTSS media.

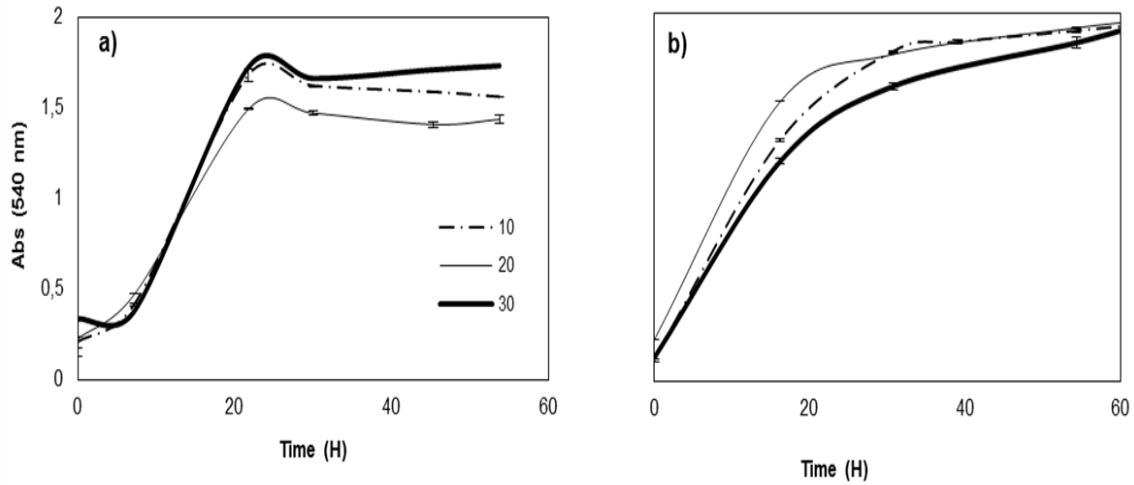

Figure 10 - Growth curves for *R. pomeroyi* DSS-3 cell culture in MBM (a) and ½ YTSS (b) media of different salinities (10, 20 and 30 ppt). Values were adjusted according to replicates average and the range bars indicate standard deviations.

4.3.2. Salinity effect on *R. pomeroyi* DSS-3 DMSP cleavage and demethylation catabolism

As expected, the higher concentrations of DMSP added to cultures of *R. pomeroyi* DSS-3 resulted in proportionally higher DMS accumulations in all treatments (Fig. 11). However, changes in salinity clearly affected the magnitude of DMS accumulated in both media. Increases of salinity caused progressively less DMS accumulation ($p < 0.001$) which was

more evident in the 500 µM DMSP amendment. Thus, the highest DMS accumulations were always registered for the lowest salinity tested in both media and for any given concentration of DMSP. In MBM and with the 500 µM DMSP amendment, 89.1% more DMS was accumulated in the 10 ppt compared with the 20 ppt salinity treatment (Fig. 11a). Additionally, in ½ YTSS medium, amended with 500 µM DMSP, 77.3% lower DMS accumulation was observed in the 20 ppt compared to the 10 ppt salinity treatments and 57.9% less DMS accumulation was registered in the 30 ppt salinity treatment compared to the 20 ppt (Fig. 11b) media at the highest DMSP amendment. In the MBM, MeSH was detected only in the 500 µM DMSP amendment (Fig. 12a), with the highest value occurring in the 30 ppt salinity treatment (568.5 ± 47.9 pmoles h^{-1} ml^{-1} cell susp, $p < 0.001$). There was an increase of 90.6% from the 20 ppt to the 30 ppt salinity treatments and no detected production at the lowest salinity tested (10 ppt) (Fig. 12a).

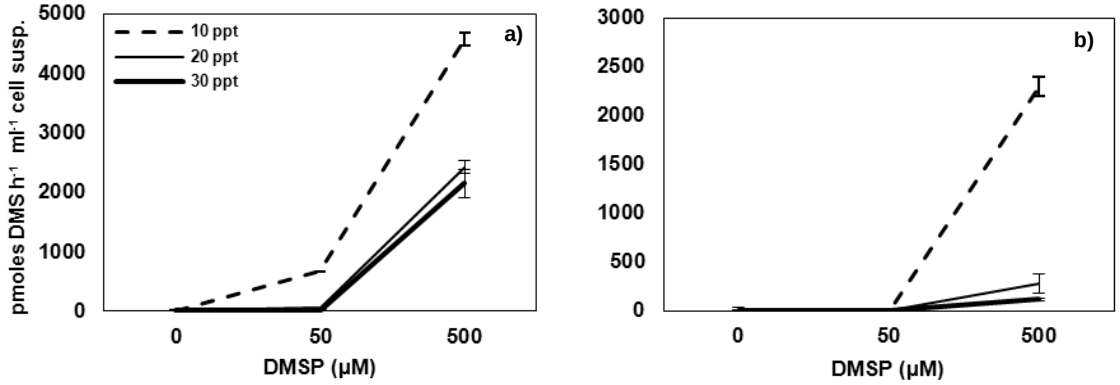

Figure 11 - Net DMS production rates in *R. pomeroyi* DSS-3 cell suspensions submitted to oxic conditions and simulated gradual salinities in MBM (a) and ½ YTSS (b) media. The salinity treatments (10, 20, 30 ppt) were amended with 10 mM Glucose and different DMSP concentrations.

Regarding the ½ YTSS medium (Fig. 12b), while it stimulated a high level of MeSH accumulation, smaller increases of MeSH accumulation were observed as salinity increased; MeSH accumulation increased 33.9% between 10 ppt to 20 ppt salinity treatments amended with 500 µM DMSP ($p < 0.005$). For treatments amended with 50 µM DMSP ($p > 0.05$; Fig. 12b) there were no significant differences in MeSH accumulation between the different salinities. Interestingly, increasing salinity had the opposite effect on MeSH accumulation, with higher magnitudes of MeSH accumulation in treatments with higher salinities for MBM and ½ YTSS media at the highest DMSP amendment (Fig. 12).

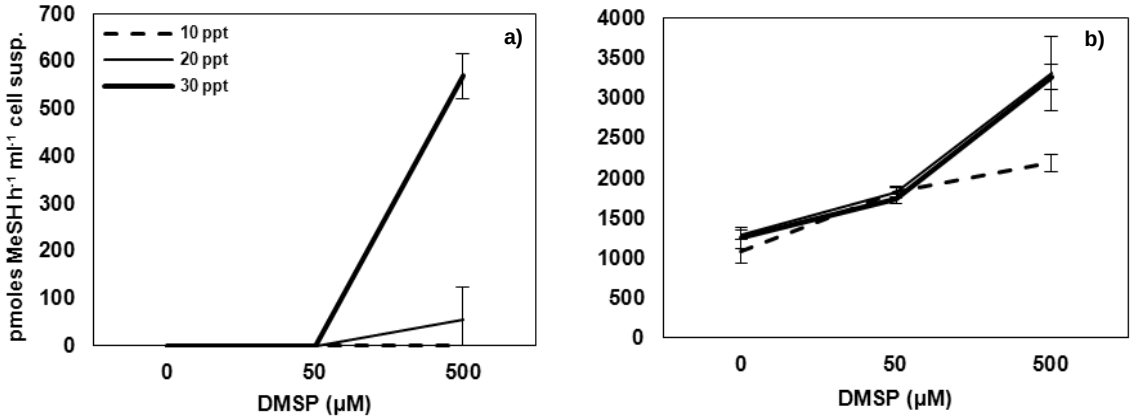

Figure 12 - Net MeSH production rates in *R. pomeroyi* DSS-3 cell suspensions submitted to oxic conditions and simulated gradual salinities in MBM (a) and ½ YTSS (b) media. The salinity treatments (10, 20, 30 ppt) were amended with 10 mM Glucose and different DMSP concentrations.

In MBM medium net DMS accumulation rates were significantly higher than MeSH accumulations in all treatments (Fig. 13a). On the other hand, in ½ YTSS medium MeSH production was higher than that of DMS in almost all the simulated conditions (Fig. 13b) excluding the 10 ppt where DMS net accumulation rates were comparable with 2301.1 ± 101.9 pmoles DMS h^{-1} ml^{-1} cell susp. and 2192.0 ± 110.0 pmoles MeSH h^{-1} ml^{-1} cell susp. The fact that DMS and MeSH production had opposite production patterns in both media (Fig. 13a, b), revealed that salinity had consistent effects on the different pathways of DMSP degradation in both media.

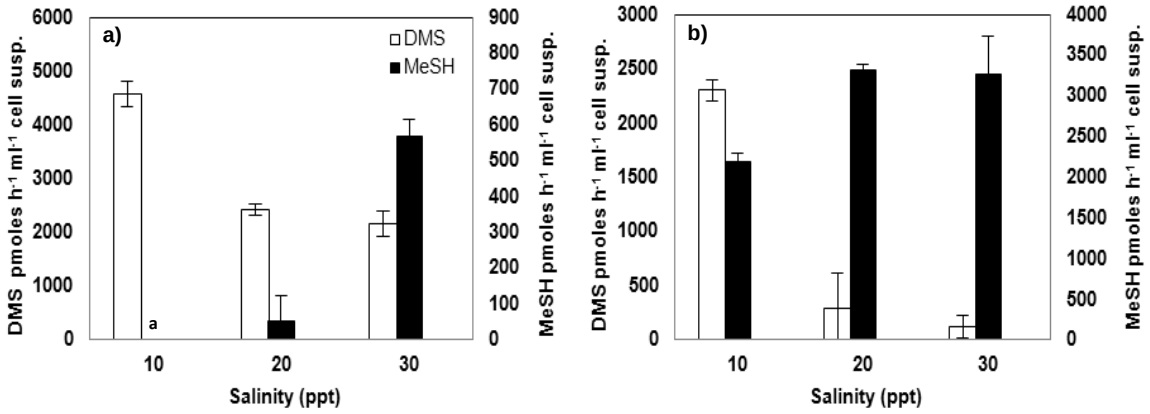

Figure 13 - Net DMS and MeSH accumulation rates for the different salinity treatments (10, 20, 30 ppt) amended with 500 µM DMSP in *R. pomeroyi* DSS-3 cell suspensions under oxic conditions on MBM (a) and ½ YTSS (b) media with 10 mM Glucose addition. [a] MeSH production was not detected.

4.3.3. Side effect of oxic *vs.* anoxic conditions

Once the cell suspensions were submitted to anoxic conditions (purged with N_2 for 15 min), DMSP was still converted to DMS and MeSH in both media but accumulation rates of these sulfur compounds were found to be lower (Fig. 14) than in oxic conditions (Fig. 13). In MBM under anoxic conditions, DMS had higher net accumulation rates in the lowest salinity treatment, and a progressive decrease in higher salinities ($p < 0.05$; Fig. 14a), as observed under oxic conditions. Under anoxic conditions MeSH accumulation rates were higher in the high salinity treatments, as observed in the oxic conditions, but the differences were not statistically significant ($p > 0.05$; Fig. 14a). Surprisingly, in anoxic ½ YTSS medium, MeSH accumulation among the salinity treatments differed in the sense that MeSH tended to accumulate less in the highest salinity treatment (30 ppt; Fig. 14b). Also, DMS accumulation was detected only in the 20 ppt salinity treatment and only at a low level (8 pmol DMS h^{-1} ml cell suspensions^{-1}; Fig. 14b). Thus, these results suggested an oxygen influence in the pattern of salinity regulation of DMSP catabolism when cells are grow in ½ YTSS medium.

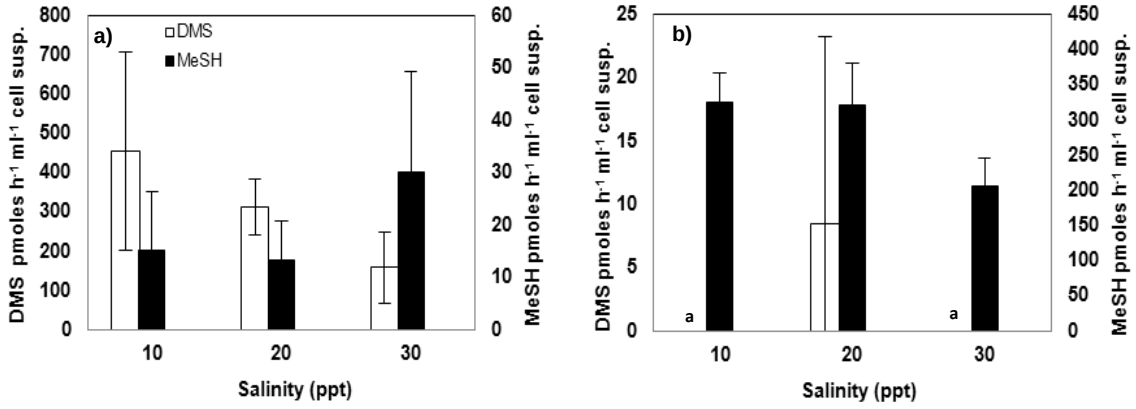

Figure 14 - Net DMS and MeSH accumulation rates for the different salinity treatments (10, 20, 30 ppt) amended with 500 µM DMSP in *R. pomeroyi* DSS-3 cell suspensions under anoxic conditions on MBM (a) and ½ YTSS (b) media with 10 mM Glucose addition. [a] DMS production was not detected.

4.3.4. Potential chemical production of DMS and MeSH

There was no detectable DMS and MeSH accumulation in parallel experiments incubated without cells indicating the production of sulfur gases was due to the presence of the cells (data not shown). These trials were prepared according to the aforementioned conditions between 10, 20 and 30 ppt salinities and with 50 µM DMSP and 500 µM DMSP amendments, in both MBM and ½ YTSS media.

4.4. Discussion

Methylated sulfur compounds such as DMS and, to a much lesser extent, MeSH comprise an important contribution to the sulfur transfer between aquatic environments and atmosphere (Charlson et al. 1987, Aneja & Cooper 1989, Howard et al. 2006). But their production seems to be controlled by diverse marine bacterioplankton taxa mainly through a "bacterial switch" between the competitive DMSP catabolic pathways cleavage and/or demethylation (Jonkers et al. 1998, Simó 2001, Zubkov et al. 2001, Moran et al. 2012). Consequently, the structure of bacterioplankton communities play a key role in controlling the yield of DMS from DMSP, which can range from 5 to ~100% in the ocean water column (Simó et al. 2000, Zubkov et al. 2001, Pinhassi et al. 2005).

In this study, we addressed the importance of salinity as a modulating factor on the magnitude of DMS and MeSH accumulation during DMSP catabolism and tested our hypothesis in a simplified biological model system consisting of a pure culture of *R. pomeroyi* DSS-3. Our results showed that salinity modulated the prevalence of the two different DMSP degradation routes within *R. pomeroyi* DSS-3 cultures. Overall, our findings suggested an enhanced MeSH accumulation in higher salinities opposed to higher DMS production in the lowest salinity treatments. These results together suggested that variability in salinities may select the preferential route of DMSP degradation pathway within bacterial communities.

The pattern of higher DMS accumulations at lower salinities corresponded to what was observed in estuarine sediment slurries, where the highest net DMS accumulation rates were found in freshwater treatments (Magalhães et al. 2012). Magalhães et al. (2012) hypothesized that lower salinities might increase DMSP release from micro- and macroalgae cells within the sediment and, therefore, enhance DMS production from more

available $DMSP_d$ degraded by the diverse microbial community. In fact, Yang et al. (2011) confirmed that within axenic cultures of a DMSP producing diatom, *Skeletonema costatum*, the liberation of extracellular DMSP increased in lower salinities and, consequently, the increase in DMSP availability had a direct correlation with DMS accumulation. While it's still unknown if diatoms produce DMSP-lyase, DMS production was already attributed to algal DMSP-lyase activity (Yang et al. 2011; Stefels 2000; Steinke et al. 2002). Previous studies have also reported production of DMS in *Skeletonema costatum* cultures (Vetter & Sharp 1993, Matrai et al. 1995). Furthermore, high DMSP liberation into the extracellular environment, after a phytoplankton bloom, was reported to be followed by DMS peaks related to high bacterial activity (Levasseur et al. 1996). Therefore, there is a strong connection between $DMSP_d$ liberation from phytoplankton cells under lower salinity leading to an increase of available DMSP to the DMSP-degrading microbial communities which, according to their composition, may modulate the levels of DMS and MeSH accumulation (Pinhassi et al. 2005). In agreement, other studies with natural communities suggested that DMS production was amplified in lower salinities (Visscher et al. 2003, Niki et al. 2007, Yang et al. 2011). Visscher et al. (2003) verified that DMS flux from a hypersaline microbial mat increased upon low salinities, even though the lowest salinity treatments tested (85 – 95 ppt) where higher than our salinity range. Additionally, Niki et al. (2007) found that low-salinity shock enhanced DMS production as a result of algal $DMSP_d$ lyase stimulation. These previous studies converge to the general hypothesis that salinity does influence the accumulation of DMS in water samples and microbial mats slurries. However, they don't provide a mechanistic explanation for why salinity influenced DMS production. We believe that our present study demonstrated that salinity indeed influences the relative prevalence of the different $DMSP_d$ degradation pathways, by benefiting demethylation/demethiolation over the cleavage catabolic pathway with a significant influence on net accumulation of MeSH *vs* DMS.

It is also well established that the dominant process in ocean waters is demethylation/demethiolation most likely due to the relevant incorporation of MeSH into methionine, by bacterioplankton (Kiene et al. 1999, Simó et al. 2000). This was also supported by the high frequency of DMSP-demethylating cells demonstrated to occur in oceanic metagenomic data (Howard et al. 2008). Our experiments confirmed this trend for high MeSH production in *R. pomeroyi* DSS-3 cell cultures growth in high salinity treatments. The demethiolation of methylmercaptopropionic acid (MMPA; a transient DMSP degradation compound), leads sequentially to MeSH production, and seems to be favored in the highest salinity treatments (30 ppt) which is in agreement with the previous

studies that mentioned a bacterial preference (> 50%) for the MeSH-producing pathway in most high salinity environments (Kiene & Linn 2000b, Kiene et al. 2000, Howard et al. 2008). Moreover, Kiene & Linn (2000b) averaged quantitative ^{35}S-DMSP$_d$ partitioning flows by using ^{35}S-DMSP tracer and found that in seawater samples, where salinity was 29, MeSH was the dominant sulfur volatile product (~75%) of initial DMSP metabolism. Additionally, this pathway has been proposed to be the dominant one in ocean waters probably due to an acquired ecological advantage in consequence of shifting ecological conditions such as phytoplankton blooms, which can alter DMSP supply, and benefit *dmdA* community pool (Howard et al. 2011). In our salinity trials we can corroborate that demethylation/demethiolation is enhanced at higher salinities in *R. pomeroyi* DSS-3 cell suspensions. MeSH is also a potential degradation product of DMS and can be produced through biological methylation of hydrogen sulfide and conversion of methionine in natural environments (Bak et al. 1992, Lomans et al. 1997, Visscher et al. 2003). Nevertheless, González et al. (1999) did not detected MeSH in *R. pomeroyi* DSS-3 cell suspensions grown in the presence of DMS, which means that in our cell suspensions the biological formation and subsequent detection of MeSH most likely was derived by demethylation/demethiolation of DMSP. Although, higher MeSH accumulations in the ½ YTSS compared with MBM were registered in all salinity treatments which may be originated from degradation of its organic sulfur compounds, as Bürgmann et al. (2007) noticed on their control samples. The yeast extract-tryptone complex in ½ YTSS medium can provide as abovementioned some conversion of methionine leading to an additional MeSH accumulation.

Results from this study revealed that high salinities benefit the MeSH net accumulation through the demethylation/demethiolation route which might be also energetically favorable for marine bacteria and, plus, *R. pomeroyi* demethylase genes may be enzymatically optimized for these osmotic conditions. Furthermore, the formation of MeSH involves more enzymatic conversion steps than the cleavage route (Curson et al. 2011b, Moran et al. 2012). In low salinities, where bacteria have to prevent at the same time the cellular lysis, the demethylation/demethiolation process might be energetically less costly to provide all sulfur and methyl groups available for microorganisms. On the other hand, the low salinities might inhibit the bacterial sulfur demand which might be a probable cause to favor DMS production through the cleavage pathway (Kiene et al. 2000) and, additionally, the DMSP-derived C and S tend to be “lost by diffusion of DMS through the bacterial cell membrane” (Moran et al. 2012).

In our measurements, independently of the salinity treatments, DMS accumulations were favored in detriment of MeSH in MBM. In agreement, other studies have measured very

low concentrations of MeSH as a DMSP degradation product due to its rapid turnover through continuous demethylations or conversions (Suylen et al. 1987, Bürgmann et al. 2007, Dickshat et al. 2010, Reisch et al. 2011) what might explain the possible underestimation of net MeSH accumulation rates in our results. The free iron in MBM (from $FeSO_4$) could also bind to MeSH and, therefore, reduce the final MeSH concentrations (Butler et al. 1992). Additionally, *R. pomeroyi* DSS-3 is able to degrade MeSH up to 40% after some hours of incubation (González et al. 1999) and an excess of available $DMSP_d$ might lead to a shifting from demethylation to cleavage, once bacterial sulfur demand is fulfilled, which in turn leads to later DMS liberation (Kiene 1996, González et al. 1999, Kiene & Linn 2000b).

While we addressed the importance of salinity as a crucial modulating factor on the magnitude of DMS and MeSH fluxes by *R. pomeroyi* DSS-3 cell suspensions, it is however expected that salinity is not a single acting factor in regulating DMSP catabolism. Actually, when our incubation conditions were changed to anoxic, previously demonstrated to yield DMS and MeSH via enzymatic cleavage or successive demethylations respectively (Kiene & Taylor 1988), the effect of salinity differed for the ½ YTSS medium. While in MBM medium results suggested that the influence of salinity on DMS and MeSH accumulations is oxygen independent, a shift in the trend of MeSH *vs.* DMS accumulation with salinity was observed in our anaerobic trials performed with ½ YTSS medium. Probably in ½ YTSS there might be other organic compatible solutes than glucose or its derivatives which might enable microorganisms to cope with salinity stress with less effort and, consequently, DMSP might be left out suspending its inherent catabolic processes in anoxic conditions. On the other hand, MeSH can also result from methoxylated aromatic compounds (Bak et al. 1992) or methylation of H_2S (Lomans et al. 1997). Actually, Lizotte et al. (2012) suggested some mediation by substrate availability in the microbial transformations of DMSP, promoting the conversion to DMS. Therefore, if there isn't limitation of C and S supply and available DMSP is in excess for bacterial sulfur demand, a larger fraction of the DMSP could be degraded to DMS (Kiene et al. 1999). Moreover, if DMSP concentrations are too low it will probably be most or entirely assimilated (Hatton et al. 2012). In the majority of our treatments with lower DMSP addition (50 µM), undetectable DMS and MeSH accumulations were registered. Actually, *R. pomeroyi* DSS-3 strain is known to rapidly degrade DMSP and incorporating most of the sulfur into stable macromolecules, like proteins, required for bacterial growth (González et al. 1999, Kiene et al. 1999, Kiene & Linn 2000a). At the high biomass of cells in the culture samples, it was likely that 50 µM DMSP represented a low "DMSP availability" relative to sulfur demands, thereby leading to low DMS and MeSH liberation.

The present results with different salinity conditions and changes in the presence of O_2, confirmed that *R. pomeroyi* DSS-3 strain is flexible in DMSP metabolism when facing unpredictable growth conditions. These findings are consistent with the conclusion that *R. pomeroyi* DSS-3 possesses several strategies and characteristics for adaptations in marine environments (Moran et al. 2004, Christie-Oleza et al. 2012). As previous studies found an influence of salinity on the magnitudes of release of DMS and MeSH in natural estuarine sediments along a salinity gradient, the present study confirmed that salinity indeed acts as a modulating factor on the bacterial switch between the alternative DMSP degradation pathways. Thus, abiotic factors might influence the proportion of these DMSP degradation products released to the atmosphere. Further information is needed concerning other factors which might be involved on the control of DMSP degradation products and gene expression. This complement will probably lead to a more reliable determination on the magnitude of the salinity effect on DMSP catabolism and on the subsequent enzymatic processes.

CHAPTER 5

Conclusions and future research

Shifts on abiotic factors, such as salinity, may indeed induce an ecological adaptation in microbial communities through alterations in their chemical and microbiological patterns. This work reflects a brief perception of the effect of salinity on the dynamics of organic sulfur compounds accumulation by intertidal microbial communities and particularly by one representative model organism of *Roseobacter* (*Ruegeria pomeroyi* DSS-3), an ubiquitous group in marine environments and key participants in DMSP assimilation. The obtained results from natural microbial communities revealed that salinity changes lead to an opposite pattern on the magnitudes of DMS and MeSH accumulations with a consequent modulation on the inhibitory interaction between MeSH and nitrous oxide reductase enzyme activity in estuarine sediments. While these findings do not allowed us to discriminate between the processes involved in the DMS and MeSH production, *R. pomeroyi* DSS-3 experiments gave us some insights on the potential effect of salinity on the two DMSP degradation pathways (cleavage and demethylation/demethiolation). Indeed, our results suggested that variability in salinities select the preferential route of DMSP degradation pathway within bacterial communities. In agreement to what was observed in sediment slurries, suspensions of *R. pomeroyi* DSS-3 cells showed an enhanced MeSH accumulation in higher salinities opposed to higher DMS production in the lowest salinity treatments. These results together provide a mechanistic explanation for how salinity influenced DMS and MeSH production by demonstrating that salinity indeed influences the relative prevalence of the different $DMSP_d$ degradation pathways, with a significant influence on net accumulation of DMS *vs.* MeSH. Finally, this study represents an important contribution to understand the importance of salinity as a key regulator on DMS, MeSH and N_2O emissions to the atmosphere, with potential effects on the global climate balance. Nowadays, with progressive genomic methods it is possible to take a next step forward and expand the knowledge about DMSP biogeochemistry. The use of molecular biology and genetic approaches can give access to information concerning other environmental and biological factors which might be involved on the control of DMSP degradation products and gene expression. In addition, the dissection of DMSP transformations and consequent gene identification on both cleavage and demethylation pathways can reveal a useful insight of DMSP metabolism by allowing to understand if the influence on its enzymatic processes occurs at a genomic or proteomic

level. Further research must be developed in order to identify the effects of a possible gene inhibition or induction by salinity. Future work may also represent a significant contribution to understand the environmental significance and implications of regulating environmental and biological factors on marine organic sulfur and nitrogen biogeochemical interactions.

CHAPTER 6

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