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Effects of macroalgae invasive species and temperature on estuarine sediments microbial communities and nitrogen biogeochemistry

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DISSERTAÇÃO DE MESTRADO EM CIÊNCIAS DO MAR – RECURSOS MARINHOS

2014

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Dissertação de Candidatura ao grau de Mestre em
Ciências do Mar – Recursos Marinhos submetida ao
Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto
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Dedicado às duas pessoas que me fizeram chegar até aqui,

Aos meus Avós

Agradecimentos

Deixo um desmedido obrigado:

À minha orientadora, a Dr.ª Catarina Magalhães por ter sido incansável e estar sempre disponível a qualquer hora. Pela sua paciência nos meus atrasos e pela contínua aprendizagem que me proporcionou. Pelo facto de ser uma referência de profissionalismo que terei em mente ao longo da minha jornada profissional. Claramente, este trabalho não seria possível sem ela. Muito Obrigada.

Ao meu co- orientador, o Dr. Francisco Arenas pela transmissão dos seus conhecimentos de estatística e fornecimento de ferramentas de trabalho.

Ao diretor do Mestrado em Ciências do Mar – Recursos Marinhos, Prof. Eduardo Rocha, que mostrou-se sempre disponível para me auxiliar nas tarefas mais burocráticas.

Aos meus colegas do ECOBIOTEC pelo acolhimento, pelos conselhos sábios, pela ajuda em qualquer questão laboratorial e não só. Principalmente pela boa disposição que proporcionava um ótimo ambiente de trabalho.

Aos meus colegas de mestrado. Estamos todos no mesmo barco e foram uma grande ajuda em todo o processo, tanto em termos de interajuda no processo da dissertação, mas por todos os trabalhos que realizamos juntos e que contribuíram para amadurecer conhecimentos. Pelos momentos de desespero e pelos momentos de festejo que partilhamos.

À minha família que esteve sempre lá, para encorajar, para chamar a atenção, pela constante preocupação para que eu chegasse ao fim de mais uma etapa. À minha mãe, aos meus tios e aos meus primos: Família não se escolhe, mas com esta eu não queria mais nenhuma.

Aos meus amigos/as, ora porque são eles a família que eu acrescentei à minha vida. Obrigada a todos por fazerem parte do meu dia- a- dia, por contribuírem a construir a pessoa que eu sou. Pelas conversas, pelos conselhos, pelos momentos de descontração e diversão, pelas nossas noites sagradas de dança e por estarem sempre comigo quer seja física ou espiritualmente.

Especialmente à minha irmã, ao meu ser semelhante, à pessoa que está mais próxima de mim desde que tenho noção de mim. Por me teres trazido até aqui, pelo que passamos juntas que

é incontável e imensurável, por acreditares em mim mais do que ninguém e por seres uma irmã simplesmente fenomenal, devo-te todas as minhas conquistas e mais esta.

Finalmente, este trabalho foi financiado por Fundos Nacionais através da FCT – Fundação para a Ciência e a Tecnologia no âmbito do projeto, Project PTDC/AAC-AMB/102866/2008, e o Projeto PTDC/AAC-AMB/113973/2009 bem como o PEst-C/MAR/LA0015/2013.

Resumo

É geralmente aceite que os efeitos das mudanças climáticas e espécies invasoras podem ter impacto sinérgico nos ecossistemas costeiros, no entanto existem poucas evidências quantitativas acerca de como é que estas alterações irão modificar a estrutura e função das comunidades naturais. Neste estudo investigamos o efeito interativo da decomposição de macroalgas exóticas/invasoras e da temperatura no ciclo biogeoquímico do azoto em sedimentos estuarinos e na estrutura das comunidades microbianas bentónicas. Foi montado um conjunto de microcosmos com sedimento estuarino a fim de se manipular a temperatura (15ºC e 20ºC) e a decomposição de macroalgas exóticas/invasoras (*Gracilaria vermiculophylla* and *Sargassum muticum)* e nativas (*Ulva lactuca*, *Fucus vesiculosus*, *Ascophyllum nodosum)* e a erva marinha nativa *Zostera marina.* Nestes tratamentos foram estimados os fluxos líquidos de amónia (NH_4^+), nitratos (NO_3^-) e nitritos (NO_2^-) entre o sedimento e a coluna de água, e avaliadas as alterações na estrutura e diversidade bacteriana do sedimento, através da utilização da técnica de ARISA, Automated rRNA Intergenic Spacer. Foi utilizada a análise de variâncias e o particionamento de variâncias para analisar o efeito da temperatura e a presença de algas invasoras/os nos fluxos de N. Apenas o fluxo líquido de NO_3^- demonstrou ser significativamente afetado pela Origem, isto é, nativa versus invasora. Os nossos resultados demonstraram que a decomposição das algas estimulou as taxas de efluxo de NH_4^+, provavelmente causada pelo aumento das taxas de amonificação. Além disso, os resultados revelaram um impacto significativo da temperatura nos processos envolvidos na reciclagem do azoto, visto que temperaturas elevadas promoveram um aumento de libertação de NO_3^- e NO_2^-, e logo uma maior disponibilidade destes nutrientes na coluna de água. Apesar de não se ter verificado alterações na estrutura das comunidades bacterianas nos tratamentos a diferentes temperaturas e com macroalgas e ervas marinhas nativas e invasoras, ocorreu uma seleção na comunidade microbiana nos sistemas com *Fucus vesiculosus* e *Ascophyllum nodosum* (R: 0.34, nível de significância: 1%). Estas duas macroalgas pertencem à mesma família Fucaceae, apresentando semelhanças químicas e fisiológicas que podem justificar a seleção de grupos bacterianos especializados na sua degradação. Os resultados sugerem que elevadas temperaturas podem causar um decréscimo na eficiência dos processos do ciclo do azoto que são responsáveis pela reciclagem destes compostos no ecossistema; promovendo um possível crescimento descontrolado de macroalgas, conduzindo a uma disfunção dos ecossistemas estuarinos.

Abstract

It is generally accepted that the effects of climate change and invasive species may simultaneously impact coastal ecosystems; however there are limited quantitative evidences about how it will modify the structure and function of natural communities. In this study we investigated the interactive effect of exotic macroalgae degradation and temperature on estuarine sediments nitrogen biogeochemistry and on the structure of benthic microbial communities. One experiment using microcosms with estuarine sediments were set up in order to examine the effects of temperature (15ºC and 20ºC) on the decomposition rates of invasive (*Gracilaria vermiculophylla* and *Sargassum muticum*) and native (*Ulva lactuca*, *Fucus vesiculosus*, *Ascophyllum nodosum*) and in the native seagrass *Zostera marina*. In those treatments changes in inorganic nitrogen compounds were evaluated over time by measuring the net fluxes of NH_4^+, NO_3^- and NO_2^-, and the ARISA technique was used to evaluate shifts on sediment microbial composition. We used analysis of variance and variance partitioning techniques to examine the effect of temperature and the presence of native vs invasive species in N fluxes. Only net flux of NO_3^- was found to be significantly affected by the origin of the macroalgae/seagrass, i.e. native versus invasive. Our results showed that algae decomposition increased rates of NH_4^+ effluxes probably caused by a stimulation of the amonification rates. In addition, our findings revealed a significant impact of temperature on the processes involved on nitrogen recycling, since high temperatures promoted an increase on the release of NO_3^- and NO_2^- to the water column. While, no changes in the microbial communities were observed within the different temperature and macroalgae type (native and invasive) treatments, a microbial community selection occur where *Fucus vesiculosus* and *Ascophyllum nodosum* were present (Global R: 0.34, Significance level: 1%). These two macroalgae belong to the family Fucaceae, presenting chemical and physiological similarities that may explain these observations. Our results suggested that high temperatures may cause a decrease on the efficiency of the nitrogen cycle processes that are responsible for the ablation of these compounds within natural impact systems, promoting a possible uncontrolled growth of algae and consequently a dysfunction of the estuarine ecosystems.

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The elaboration of this dissertation benefited from a scientific paper yet to be finished and two oral communications:

Monteiro, M.A. Rábano, O., García C., Salgado, P., Silva, J., Mucha, AP, Arenas, F., Magalhães, C. Effects of macroalgae invasive species and temperature on estuarine sediments microbial communities and nitrogen biogeochemistry. To be published

Monteiro, M.A. Rábano, O., García C., Salgado, P., Silva, J., Mucha, AP, Arenas, F., Magalhães, C. Effects of macroalgae invasive species and temperature on estuarine sediments microbial communities and nitrogen biogeochemistry. ECSA 54 Conference. 12-16 May 2014 Sesimbra, Portugal.

Monteiro, M.A. Rábano, O., García C., Salgado, P., Silva, J., Mucha, AP, Arenas, F., Magalhães, C. Effects of macroalgae invasive species and climate-driven stressors on estuarine sediments nitrogen biogeochemistry. IJUP- 7º Encontro de Investigação de Jovens da Universidade do Porto. 12 February 2014, Porto, Portugal.

CHAPTER 1

1. Introduction

1.1. Macroalgae invasion

There is strong scientific consensus that coastal marine ecosystems are threatened by several factors such as pollution, habitat destruction, eutrophication, overfishing, and climate change (e.g. increasing sea temperatures, changing circulation patterns, increased storminess and ocean acidification) or by introduction of non-indigenous species. Non-indigenous species may have serious consequences not only on biodiversity, but also on the management and restoration of natural ecosystems. By disrupting ecosystem structure and function, invasive species alter the ability of natural systems to provide high-value goods and services (Stachowicz et al. 2002). Adding to the threat posed by non-indigenous species, predicted climate change scenarios are accelerating changes in the structure and function of coastal marine systems with unprecedented ecological, economic and social implications (Harley and Hughes 2006). While it is generally accepted that the effects of climate change and invasive species may simultaneously impact coastal ecosystems, there are limited quantitative evidences about how it will modify the structure and function of coastal communities (Viejo 1997; Staehr et al. 2000; Wilson 2001; Britton-Simmons 2004; Olabarria et al. 2009). Followed by loss of habitat, invasive species appear as the second leading cause of biodiversity loss, particularly in freshwater ecosystems (Vitousek et al. 1997; Mack and Simberloff, 2000; Ross et al. 2004). According to Williams and Smith (2007), marine macroalgae are a significant component of invasion counting with a total of 277 invasive species recorded.

Introduced species have the potential to substantially modify native communities (Walker and Kendrick,1998; Levin et al. 2002). This capacity relies both in the capacity of the introduced species to invade, usually referred as invasiveness, and in the resistance of native assemblages to invasion, i.e. invasibility. Community invasibility depend mostly on the resource availability within recipient communities, which usually hinges on the number and functional diversity of existing species (Arenas et al. 2006). For example, the Japanese seaweed *Codium fragile subsp. tomentosoides* was documented to have detrimental effects on subtidal kelp communities in the northeast Atlantic Ocean, while on the European intertidal shores it occurs in low abundances, with no effects on native assemblages (Buschbaum et al. 2006). In seagrass habitats, invasive species are documented to decrease oxygen availability and consequently an increased in sulphide accumulation (Holmer et al. 2007), causing an

acceleration of the processes involved on seagrass decline in several natural ecosystems (Martínez-Lüscher and Holmer, 2010). The two selected invasive species of macroalgae, natives from Asia that have been introduced in the North of the Iberian Peninsula several decades ago.

1.1.1. Sargassum muticum

Sargassum muticum is native to SE Asia (Yendo 1907), but its present distribution as an invasive species is widespread, including Europe, Mediterranean Sea and the west coast of North America (Britton-Simmons 2004). In the Iberian Peninsula, it was first recorded in the 80s both in the Basque Province and in the Galician coast (Pérez-Cirera 1989) reaching recently the southern coast of Spain (Bermejo et al. 2012). Some studies support that the successful colonization of *S. muticum* in Northern Spain is likely due to the large production of embryos (Arenas et al. 1995), its fast growth and large thallus size (>3 m). It also has a very successful reproductive strategy by self fertilization (Fletcher 1975; Norton 1976; Norton 1977) and several dispersal mechanisms (including floating thalli), which have turn this algae highly invasive by out-compete with native algal species and the organisms associated with them (Buschbaum et al. 2006). While studies done on intertidal shores have documented little (Buschbaum et al. 2006; Harries et al. 2007;) or no impact of *S. muticum* (De Wreede 1983; De Wreede and Vandermeulen 1988; ; Viejo 1997; Wilson 2001; Sanchez and Fernandez 2005), studies in subtidal habitats have indicated relatively strong impacts (Ambrose and Nelson 1982; Staehr et al. 2000; Britton-Simmons 2004). Indeed, competition with *S. muticum* reduced the abundance of native canopy algae by approximately 75% and native understory algae by about 50% on San Juan Island; changed the relative abundance of the two most common native kelp species, *Laminaria bongardiana* and *Agarum fimbriatum*. *L. bongardiana* (Britton-Simmons 2004). The invasion of *Sargassum muticum* in Limfjorden, a shallow Danish estuary, was found to affect the local algal community through competition with members of the thick leathery and coarsely branched algae (Staehr et al. 2000).

Although, Cacabelos et al. (2013) highlighted that the variation in *S. muticum* invasion was found to occur at small-scale suggesting a major role of diverse local drivers in the invasion process. Environmental factors acting at large and/or meso-scales, such as seawater and air temperature, variability in hydrodynamic conditions or topography, wave exposure and sedimention rates (Incera et al. 2009; Olabarria et al. 2009) are key factors driving its reproductive patterns and dispersal mechanisms (Incera et al. 2011).

In the process of a successful invasion and settlement recent findings have pointed out that the natural diversity of functional groups (encrusting, turf, sub canopy, and canopy species) in a

certain ecosystem is more decisive than the species richness in determining the resistance of marine macroalgal assemblages against invasion (Arenas et al. 2006).

1.1.2. Gracilaria vermiculophylla

Gracilaria vermiculophylla is of particular interest because is originated from the northeast Pacific but has been accidentally introduced to both the East and West Atlantic where it can form large mono-specific drift mats in low-energy shallow estuaries (Thomsen. et al 2006; Thomsen and McGlathery, 2006b). Algal mats of this specie can also form physical barriers for settling larvae, decrease light intensity, increase the likelihood of anoxia and change water movement patterns, which in turn affects sedimentation rates and thus food availability for deposit feeders (Nyberg 2009).

The invasion by these algae has been demonstrated to have negative effects on native seagrass beds of *Zostera marina* by decreasing net leaf photosynthesis and survival rates (Martínez-Lüscher and Holmer 2010a). Negative effects on seagrass are even greater at higher temperatures, suggesting that impacts could increase with future ocean warming (Martínez-Lüscher and Holmer 2010a). In some areas such as Hog Island Bay in Virginia, *G. vermiculophylla* dominate algal assemblages, in all seasons and elevation levels (Thomsen and McGlathery, 2006a). *Gracilaria vermiculophylla* is still in an early invasion phase but is likely to invade protected estuaries characterized by abundant seagrass beds as seen for *Gracilariopsis andersonii* in Californian seagrass beds (BE 2008).

Additionally, the water advection, accumulation and decomposition of *G. vermiculophylla* are likely to have important implications in nutrient cycling processes and trophic dynamics in the *Separtina alterniflora* dominated low marsh (Thomsen et al. 2009).

1.2. Aquatic plants decomposition

Deposition of organic matter in these environments can result from episodic events such as the rapid sedimentation of phytoplankton or benthic macroalgal blooms, or, in systems where rooted macrophytes are dominant and deposition of dead plant material can occur throughout the year.

The arrival of detrital invasive seaweeds to new ecosystems that can appear mixed to native species or in isolation will ultimately alter the carbon and nitrogen provision and consequently modify both benthic community structure and carbon or nitrogen cycling (Rossi et al. 2011). Understanding the decomposition of aquatic plants allows for the determination of their role in providing organic matter to detrital food chains, or alternatively being a source of regenerated inorganic nutrients for autotrophic assimilation (Twilley et al. 1986). In intertidal sediments,

burial and decomposition of macroalgae detritus can fuel the sediment of carbon (C) and nitrogen (N), which can be either promptly mineralized or assimilated to enter in the food web (Rossi 2007). Detached seaweeds often deposit as detritus on the substratum of marine intertidal zone where they can be locally buried by sediment reworking and start decomposing (Ford et al.1999; Kelaher and Levinton, 2003; Rossi 2007). When buried they can represent a relevant supply of organic matter for the benthos (Rossi 2006). The bacterial respiration can increase at higher levels of organic matter (Fontaine et al. 2004). However, the quantity, quality and spatial distribution of the deposited organic matter in the sediment regulate the rates of benthic nutrient regeneration. For example, in macroalgal dominated sediment, mineralization can be accelerated in relation to systems dominated by vascular plants, because macroalgae have little structural material and decompose rapidly (Enoksson 1993; Duarte 1995). However, the high macroalgal requirements for dissolved inorganic nitrogen influences the flux of dissolved inorganic nitrogen between sediment and overlying water, and thus the dissolved inorganic nitrogen produced will supply the macroalgal N requirements (Trimmer et al. 2000). In fact, a large percentage of the NH_4^+ produced during mineralization (40 to 60%) of organic nitrogen in sediments can also be lost from the ecosystems as N2; essentially the NH_4^+ produced in the sediments is nitrified and subsequently denitrified (Seitzinger 1990; Rossi 2007).

The accumulation of excess nutrients into the system resulted from massive decomposition caused by episodic events such as the rapid sedimentation of macroalgal blooms, will lead to eutrophication with a consequent depletion of dissolved oxygen (hypoxia and anoxia), responsible for the loss of important habitat such as seagrass beds and corals, changes in marine biodiversity and distribution of species (with impacts on commercial fisheries), and associated die-offs of marine life (Howarth 2000).

Some studies have demonstrated that macroalgae degrade faster than seagrasses and higher plants (likely due to their higher N-content), releasing both inorganic nutrients and organic N (Williams 1984; Buchsbaum et al. 1991; Bourguès et al. 1996). Fast-growing plants tend to have high nutrient concentrations (Chapin et al. 1987), and also decompose fast because of the adequacy of their litter as substrate for microbial growth (Enriquez et al. 1993). Direct comparisons of leaf decomposition, on tree leaves and macrophytes in lakes, streams, and wetlands showed that in cases of nutrient-poor versus nutrient-rich systems have indicated faster breakdown in nutrient-rich systems and higher temperatures (Brock et al. 1985; Webster and Benfield 1986;). Nevertheless, each algal species has different characteristics that will affect the rate of its decomposition depending on the biochemical and morphological composition. Therefore, decomposition of algae provides a potentially important supply of

organic and inorganic compounds to the water column where they can be recycled rapidly (Gabrielson et al. 1983; Twilley et al. 1986; Paalme et al. 2002)

Temperature is regarded as one of the key factors responsible for controlling decomposition pathways (Paalme et al. 2002). Thus, it is expected that lower temperatures (e.g. 15°C) strongly decelerate decomposition of all macrophyte species (Paalme et al. 2002). However, as the temperature increases towards 30°C a tendency for rapid degradation of algal populations is documented, with regards to biomass loss (Hanisak 1993; Paalme et al. 2002;). The decomposition process is initiated with an enzymatic breakdown of cellular constituents, such as cell walls, which ultimately increases its susceptibility for bacterial activity (Gabrielson et al. 1983). As a result, in macroalgae degradation experiments, it is important to use living algal material, with intact cell walls, as this ensures greater accuracy when determining the 'actual' extent of inorganic nutrient release (Gabrielson et al. 1983; Paalme et al. 2002;).

1.3. Marine Nitrogen cycle

Nutrient availability is one of the key factors regulating the main physiological responses of seaweeds, with nitrogen (N) being the most likely to limit their growth in temperate waters (DeBoer et al. 1978; Lobban and Harrison, 1994;). Nitrogen is the most abundant chemical on Earth's atmosphere but it can only be used by N_2-fixing specialized microorganisms, which have the ability to reduce it to ammonium and integrate it into biomass. The other prokaryotes and all eukaryotes can only use fixed nitrogen in the form of nitrate, ammonium or organic nitrogen, which represents less than 0.1% of the total N in earth (Thamdrup 2012). The general unavailability of N_2 for marine organisms makes the conversion of N_2 to organic nitrogen (N-fixation), and the conversion of fixed nitrogen (NO_3^-) to N_2 (denitrification), particular important processes in controlling the N availability in natural ecosystems (Capone et al. 2008).

Biogeochemical cycling of inorganic nitrogen in marine environments has received much attention over the last several decades, mostly because of problems associated with excess nitrogen loadings, being a main concern eutrophication of aquatic systems (Gruber and Galloway 2008). Indeed artificial N inputs due to industrial fertilizer production (140 $TgNyear^{-1}$) exceed natural N sources (110 $TgNyear^{-1}$) (Canfield et al. 2010). In this way, human activities have shifted the balance between N_2 fixation and its complex recycling processes to the extreme (Thamdrup 2012). Because nitrogen is required by all plants, animals, and microorganisms, changes in the fluxes of this element can alter the rates of basic processes. To understand the consequences of this instability a detailed understanding on how nitrogen in cycling over the different ecosystems is required. However, while there is a worldwide marine literature available for most nitrogen biogeochemical transformations and on the

microorganisms involved (Zumft 1997; Valiela 1997; Jickells 1998; Seitzinger and Harrison 2006; Prosser and Nicol 2008;) our understanding of how N is cycled on Earth has changed greatly in the last few years. First by the discovery of anaerobic ammonium oxidation in natural ecosystems (Thamdrup 2012) and secondly by the demonstration of aerobic ammonia oxidation within the domain Archaea (Treusch et al. 2005; Könneke et al. 2005;).

The complexity of the nitrogen cycle is demonstrated in Figure 1, which shows that nitrogenous compounds undergo a series of oxidation/reduction reactions mediated by a metabolically diverse range of autotrophic and heterotrophic organisms. These biogeochemical conversions are either energy-yielding (e.g. nitrification and denitrification) or energy-demanding (e.g. nitrogen fixation) and are fundamental processes in microbial biosynthesis and bioenergetics (Madigan et al. 2004).

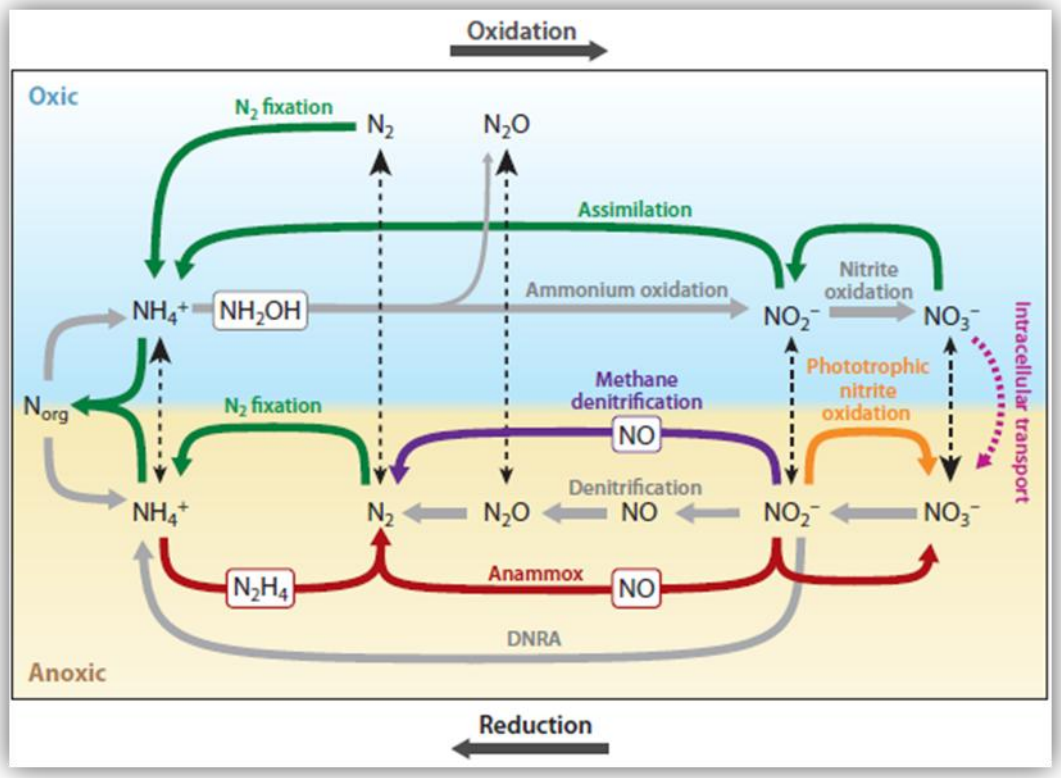

Figure 1- Schematic representation of the nitrogen cycle. Metabolic transformations are shown as thick arrows. It shows the classical processes of assimilation (*green*) and dissimilation (*gray*) as well as recently discovered pathways (*colored*). Aerobic and anaerobic processes are separated, and dashed vertical arrows indicate exchange or transport between oxic and anoxic environments, with the relative size of arrowheads indicating the dominant direction of transport. Abbreviation: DNRA, dissimilatory nitrate reduction to ammonium (Thamdrup 2012).

There are two dissimilatory microbial processes that convey the recycling of ammonium, as generated by the decomposition of organic N (ammonification): (1) nitrification, the aerobic oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-), with each step performed by a specialized group of prokaryotes, and (2) denitrification, the respiratory reduction of nitrate

(NO_3) and nitrite (NO_2^-) reductions to nitric oxide (NO), nitrous oxide (N_2O) and N_2 (Thamdrup 2012). One of the reactions that link the nitrogen and the carbon cycle is performed by the autotrophic nitrifying bacteria, which use some of the electrons from oxidation of ammonium and nitrite to reduce CO_2 and create biomass. The second mechanism for reducing nitrate involves nitrate-reducing bacteria that mediate a process termed dissimilatory nitrate reduction to ammonium (DNRA). In contrast to denitrification where nitrogen is lost from the ecosystem, DNRA results in the conservation of fixed nitrogen within the system (Magalhães 2005).

All microbial mediated N transformations are strongly regulated by the prevailing environmental physico-chemical conditions. Thus, environmental specificities of each marine ecosystem can affect the complex interactions of the several recycling nitrogen pathways, and the significance of the N processes can vary according to the specific characteristics and anthropogenic pressures of each habitat (Lobban and Harrison, 1994). In nutrient enriched aquatic systems, blooms of macroalgae are followed by dystrophic events during which the bloom crashes releasing large amounts of particulate and dissolved organic matter, dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) (Tyler et al. 2001; Sundbäck et al. 2003). Respiration of the organic matter, released during micro- and macroalgal senescence, often results in severe oxygen depletion (hypoxia) in both sediments and the water column. The shift to hypoxia or anaerobic conditions facilitates fixed nitrogen removal from the environment via denitrification (Figure 1; Seitzinger 1988; Ogilvie et al. 1997; Seitzinger 2000).

1.4. Global warming consequences

Global warming has been increased along the decades, especially because of human activities that increased the emissions of carbon dioxide, methane and nitrous oxide and toxic compounds. This emissions generated an increase of 0.6ºC (0.4ºC-0.8ºC) global mean surface temperature over the last 100 years (Gitay 2002) and from 1901 to 2013, temperatures rose at an average rate of 0.13°F per decade (Spring 2001) (Figure 2).

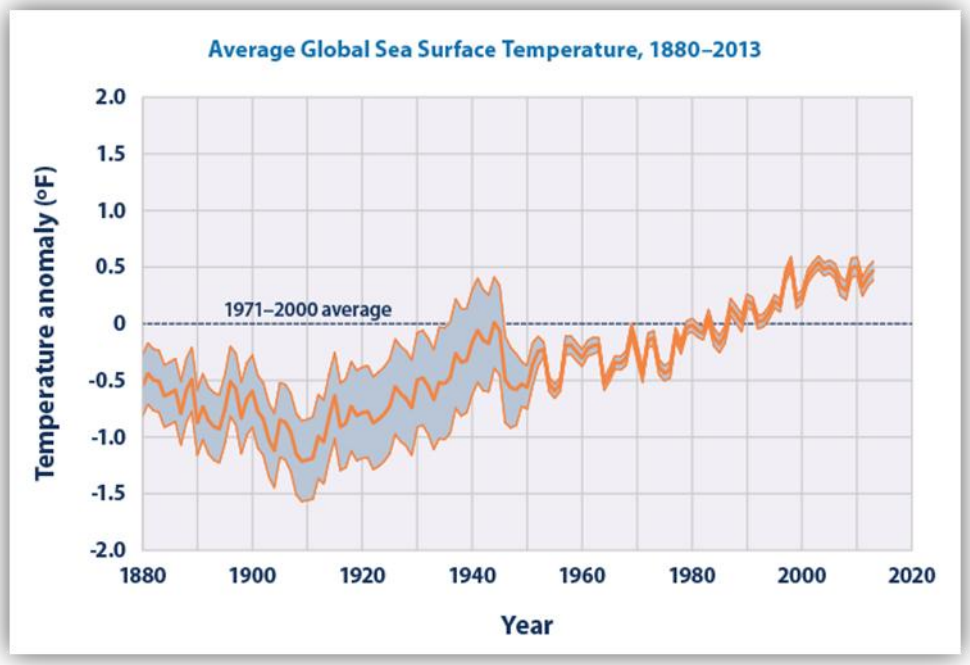

Figure 2 - High temperatures are accompanied by high concentrations of CO_2, and this increased became clearly evident since the 80s and continues to the present days (EPA 2014)

This issue took great relevance because of the social economical and environmental problems already caused and predicted in the near future, including loss of seagrass beds, macroalgal beds, and changes in coral reefs (Howarth et al. 2000). Although most reactive nitrogen is eventually denitrified to N_2 within the coastal ecosystems and associated shelf, reactive nitrogen pollution has significant and widespread impacts on various ecosystems and in human health (Galloway et al. 2003).

A relevant fact is the interaction of nitrogen with other biogeochemical cycles, like the carbon cycle that has particular relevance, because of the central role of atmospheric CO_2 in controlling climate (Sarmiento and Gruber, 2002) and with nitrogen having a crucial role in controlling key aspects of this cycle (Gruber and Galloway, 2008). As a result of the burning of fossil fuels and carbon emissions from land-use change, atmospheric CO_2 has increased to levels that are more than 30% above those of pre-industrial times (Gruber and Galloway, 2008).

These biogeochemical cycles are linked to each other and the process consists on the efficiency of the atmosphere in spreading the nitrogen oxides and ammonia emitted as a result of energy and food production and the deposition of nitrogen in the ground that is already available for plants, allowing the production and improving the uptake of CO_2 from the

atmosphere. The global oceans, freshly produced algae in the ocean surface typically have a carbon to nitrogen ratio of about 4 to 10 (Meyers 1994).

1.5. Goals

In this study we investigated the interactive effect of exotic macroalgae degradation and temperature on benthic inorganic nitrogen fluxes (ammonia, nitrate and nitrite) and on the diversity of benthic microbial communities.
In this study we hypothesized that temperature is a key factor in controlling rates of macroalgae and seagrass decomposition with a direct impact on N inorganic flux dynamics and that differences between indigenous (*Ascoplyllum nodosum, Fucus vesiculosus, Ulva lactuca, Zostera marina)* and non indigenous species (*Gracilaria vermiculophylla* and *Sargassum muticum*) will affect rates of N recycling.

CHAPTER 2

2. Materials and methods

2.1. Site description and sample collection

Sampling program was conducted in the Ria de Vigo situated in the northeast Iberian Peninsula, in the province of Pontevedra (Galiza). Ria de Vigo has 35 km length and its large bathymetry is reached in Arcade. Extends itself from southwest to northeast protected in the entry by Islas Cíes, draining in the Atlantic Ocean (Figure 3). The averaged values of surface temperature ranged between 13ºC, from January to early May, and 18ºC, during July and August (Nogueira et al. 1997). The North East Atlantic Central Water (NEACW) is characterized by an almost linear relationship between salinity and temperature, with salinities ranging from 36.0 to 35.6 and temperatures from 15 to 11 º C, at 50-75m and 400 m depth, respectively (González-Garcés Santiso et al. 2011). Salinity surface waters range from 35.5 to 35.7, showing an homogeneous water column (De Castro et al. 2006). The sampling was conducted at low tide and the sediment was collected from an intertidal muddy sediments bank.

Figure 3- Map of the study site area. Ria de Vigo is located on the northeast Iberian Peninsula. Samples were collected on the intertidal banks at the entry of the estuary.

The non-indigenous species *Gracilaria vermiculophylla* and *Sargassum muticum,* and the native species *Ascophyllum nodosum, Fucus vesiculosus, Ulva lactuca* and the native seagrass *Zostera marina*, were collected at different intertidal banks of Ria de Vigo.

Algae, seagrass and sediment were collected by hand and with shovels, placed in bags and plastic boxes and transported to the lab in ice chests.

2.2. Experiment Set Up

A set of microcosm experiments with sediments together with invasive (*Gracilaria vermiculophylla* and *Sargassum muticum*) or native macroalgae (*Ulva lactuca*, *Fucus vesiculosus*, *Ascophyllum nodosum*) and native seagrass (*Zostera marina)* were set up under two temperature conditions (15ºC and 20ºC).

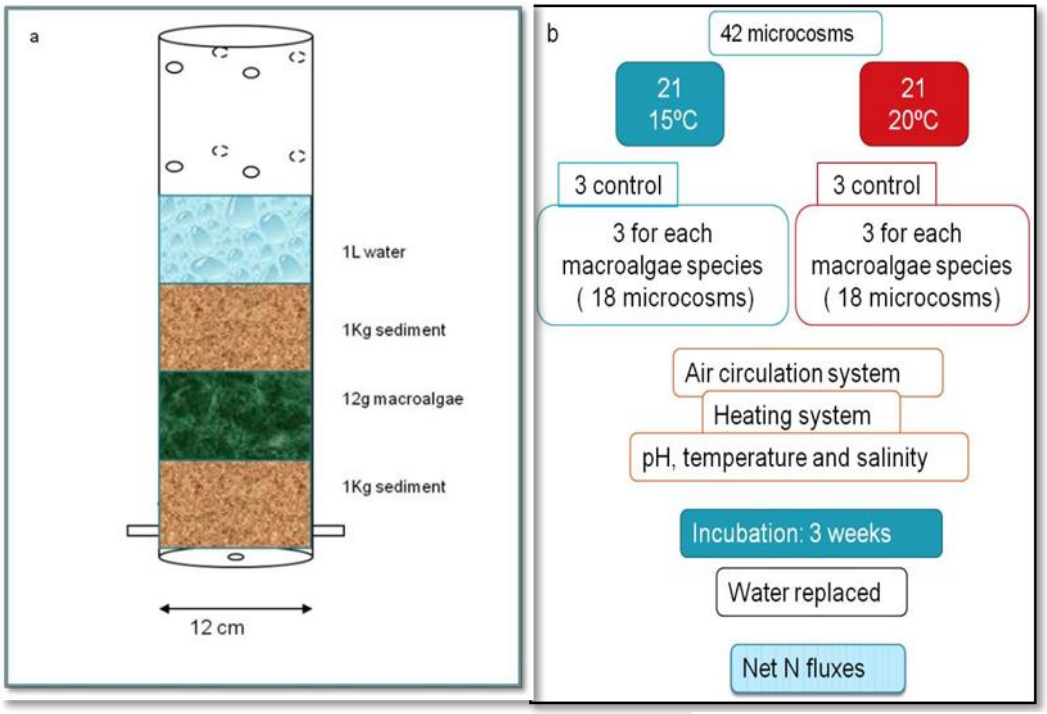

Figure 4- a) Microcosm set up schematics. Each microcosm was composed by 1kg sediment in the base, followed by 12g of macroalgae/seagrass, another 1kg of sediment and 1L of saline water (35) b) Scheme of the experiment set up. From the total of 42 microscosms, 21 were incubated at 15ªC and other 21 at 20ºC.

As shown in Figure 4, the experiment set up consisted into six replicates of sediment microcosms with the studied macroalgae species, seagrass, and only sediment (control). Triplicate microcosms of each treatment were incubated at 15ºC and the other set of triplicate microcosms at 20ºC. The whole experiment was composed by two large tanks (15ºC and 20ºC) including a total of 42 microcosms. In each microcosm was introduced 1kg of homogenized sediment, followed by 12g of the respective macroalgae or seagrass, followed by another 1 kg of sediment to bury macroalgae into the sediment. 1L of overlying water was then added to each microcosm.

The set up provided an air circulation system, and controlled heating system to maintain the required temperatures in each tank.

Figure 5 - a) Images from microcosm's installation b) Images from invasive (*Gracilaria vermiculophylla* and *Sargassum muticum*) native macroalgae (*Ascophyllum nodosum, Fucus vesiculosus and Ulva lactuca*) and native seagrass (*Zostera marina).* Images from, imgarcade.com; www.seaweed.ie; www.onlyfoods.net; medalimenta.blogspot.com; protistaproject.weebly.com; www.oceanario.pt .

2.3. Macroalgae and Seagrass Carbon and Nitrogen content

At the end of the experiment, the remaining macroalgae/seagrass buried in the sediment was collected from each microcosm, dried at 60°C for 48 h and grounded to a fine powder. Total carbon and nitrogen content were determined using a CHN elemental analyzer (Olabarria et al. 2013). A conventional elemental analyzer operates by flash combustion of the specimen encapsulated in tin cups or disks where the specimen was completely combusted. The resulting gases are chemically scrubbed of halogens and of sulfur and separated in a GC column. Total C, H and N are calculated with a precision of 0.1g (Baldino 1995).

2.4. Inorganic Nitrogen Determinations

The analysis of ammonia, nitrites and nitrates was done in triplicate for each water sample. All species of inorganic N quantification were obtained by performing daily calibration curves constructed from standard solutions with known concentrations. The inorganic nitrogen compounds were quantified by spectrophotometric methods that differ according to the nutrient. The method used for nitrites was described by Grasshoff et al. (1983). It's based in the reaction of nitrite with an aromatic amine (sulfanilamide) that result in a diazotized compound that connects to a second aromatic amine (N-(1-naphthyl)-ethylenediamine) creating a pink complex which intensity depends on the quantity of nitrite. Nitrite concentration was calculated based on a standard curve plotting standard concentration in the Y axis and absorbance in the X axis, according with the following formula:

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

NO_2^- - concentration of dissolved orthophosphates (µM)

D – Slope of the standard curve

A_a- value of the absorbance obtained for the sample

A_b – value of the absorbance obtained in the blank

To obtain the concentration of nitrates the method described by Jones (1984) and adapted by Joye, S.B. & Paerl (1993) was used.

For NO_3^- quantification, a chemical reduction was performed. This reduction was triggered by mixing the sample with spongy cadmium (Cd) (s) (reducing agent) in the presence of a buffer, ammonium chloride, (an alkaline solution that complexes the oxidized Cd^{2+}). Nitrate was reduced into NO_2^- and from this step the methodology was the same as previously described for NO_2^- quantification. Nitrate concentration was calculated based on a standard curve plotting standard concentration in the Y axis and absorbance in the X axis, according with the following formula:

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

NO_3^-- Concentration of dissolved nitrates (µM)

D – Slope of the standard curve

Aa – value of absorbance obtained for the sample

Ab – value of the absorbance obtained in the blank

NO_2^- - Concentration of nitrites in the solution

C –Dilution factor of ammonium chloride

To obtain the concentration of ammonium (NH_4^+ and NH_3) was used the Koroleff method (Grasshoff et al. 1983). The method is based on the fact that this nutrient, when present in solution in alkaline conditions, reacts with hypochlorite forming the compound monochloramine which in turn, in the presence of the catalyser (sodium nitroprusside), phenol and excess of hypochlorite produces a blue complex of indophenol. The reaction takes 6 hours to complete and the incubation is at room temperature in the dark. During samples processing was added magnesium to cause precipitation of magnesium hydroxide present in salt water. This absorbs the suspended matter and the humic acids, avoiding interferences. The absorbance was read spectrophotometrically at a wavelength of 630 nm. Ammonium concentration was calculated based on a standard curve plotting standard concentration in the Y axis and absorbance in the X axis, according with the following formula:

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

$NH_4^+ + NH_3$ -concentration of ammonia and ammonium in water (µM)

D – Slope of the standard curve

Aa – absorbance value of the sample

Ab – absorbance value for blank

2.5. Inorganic N in the interstitial water

At the end of the experiment and after removing the overlying water from each system, 5 ml of sediment were collected from each microcosm. Sediments were mixed with 8 ml of miliQ water by vortex it during 1 min, and water was filtered (0.45 µM membrane filters) and stored at -20ºC for later analysis of NO_3^-, NO_2^- and NH_3/NH_4^+, according with the analytic methods previous described. Dilution was made by adding 8 ml of miliQ water was taking into account in the calculations.

2.6. Net Inorganic Nitrogen Fluxes

At the end of the incubation and thirteen minutes after refilling the overlying water (to stabilize the system), a T_0 and T_{3h} samples (20 ml) were collected from each microcosm after gently stirred. All water subsamples were immediately syringe-filtered through 0.45 µm membrane filters and stored at -20 ºC in acid-cleaned, polyethylene flasks until analysis. Fluxes of inorganic nitrogen compounds (NO_3^-, NO_2^- and NH_3/NH_4^+) were calculated using the slope of the linear relationship between the change in the nutrient concentration in the water chamber versus the time of incubation (e.g. Barbanti et al 1992), following the equation:

$$Fn = \left(\frac{T3h - T0}{A} \right) \times 10^4 / 3$$

Where *Fn* is the flux of each inorganic nutrient in µmol m^{-2} h^{-1}, T_{3h} is the concentration of N compound at the end of the incubation, T_0 is the concentration of the N compound at the beginning of the incubation, *A* is the sediment surface area in cm^2 and 10^4 is the conversion factor from cm^2 to m^2, 3 refers to the conversion to 1h.

2.7. DNA extraction and Automated rRNA Intergenic Spacer Analysis (ARISA)

Total DNA was extracted from 0.5 g of wet homogenized sediment samples collected from each microcosm at the end of the experiment, using a modification of the CTAB (bromide-polyvinylpyrrolidone-b mercaptoethanol) DNA extraction protocol (Dempster et al. 1999) described by Barrett et al. (2006). Quality of extracted DNA was evaluated by visualization on 1.5% agarose gel.

PCR reactions were carried out using Ready-to-Go PCR Beads (Amersham Biosciences, Buckinghamshire, UK) in 25 µl reactions containing 1-5 ng of DNA template and 20 pmol/µl of each primer (see below). A PCR reaction mixture with all reagents except template DNA served as a negative control. Extracted DNA was amplified using ITSF (5-GTCGTAACAAGGTAGCCGTA-30) and ITSReub (50-GCCAAGGCATCCACC-3) primers set (Cardinale et al. 2004), which amplifies the ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson and Fuhrman 2004). ITSReub was labeled with the phosphoramidite dye 6-FAM (6-carboxyfluorescein). PCRs were performed in duplicate 25 ml volumes containing between 10 and 50 ng of DNA, 400 nM of primers, 200 mM dNTPs, 3 x Taq PCR buffer, 2.5 U Taq DNA polymerase, 2.5 mM $MgCl_2$ and 1 mg bovine serum albumin. The PCR mixture was held at 94 ºC for 2 min, followed by 30 cycles of 94ºC for 45 s, 55ºC for 30 s, 72ºC for 2 min, and a final extension at 72 ºC for 7 min. Duplicate PCR products were combined, examined on 1.5% agarose gel, purified using a GFX PCR DNA purification kit (GE-Healthcare) and eluted in 30 µL. Purified product was quantified using the Quant-it dsDNA assay kit and the Qubit fluorometer (Invitrogen), and a standardized amount of the purified PCR product was diluted 1 in 5 and mixed with 0.5 µL of ROX-labeled genotyping internal size standard (ROX 1000, Applied Biosystems). The sample fragments were run on an ABI3730 XL genetic analyzer at STABVIDA sequencing facilities (Lisbon, Portugal).

2.8. Statistical analysis

Automated rRNA intergenic spacer analysis fragment lengths (ARISA - AFLs) were analyzed by Peak Scanner™ Software v.1.0 (Apllied Biosystems).The AFLs that differed by less than or equal to 2 bp were considered identical and AFLs with fluorescence units below 200 bp were considered “background noise”. Fragments of less than 200 bp were removed since were considered to be too short ITSs for bacteria. All ARISA-AFLs data were used to create a matrix imported to the PRIMER 6 software package (version 6.1.11) (Clarke, KR & Gorley 2006) and transposed to presence/absence prior statistical analyses. Since ARISA is a PCR-based method it is not correct to use the relative fluorescence of individual peaks as a proxy of relative abundance of each phylotype. Multivariate analysis from all treatments was performed using multidimensional scaling (MDS) and hierarchical cluster (HC) based on Bray–Curtis similarities to detect inter-site differences and/or similarities in bacteria diversity within the different treatments performed (5% significance, mean number of permutations, 1000; number of simulations, 999) (Ter Braak and Smilauer 2002). ANOSIM analysis (Clarke, KR and Gorley 2006) was also performed to test whether differences in assemblage grouping in the MDS and hierarchical cluster analysis were significant; the values of the R statistic were an absolute measure of how well the groups separated and ranged between 0 (indistinguishable) and 1

(well separated). To examine the effects of temperature and origin (native *versus* invasive) on the degradation rates and on nutrient fluxes we used analysis of variance. We considered both factors (temperature and origin) as fixed and orthogonal (crossed) factors. Analyses were performed using GMAV5 (University of Sydney 1998). Cochran's test was used to check the assumption of homogeneity of variances prior to testing hypotheses concerning mean differences.

In the case of nutrient fluxes to compare the effects of native versus invasive species we use a variance partitioning technique for those sources of variation where identity was significant.

The method used here, described in Underwood (1993), allowed for example when the p-values of the interaction temperature X identity were significant to perform a partitioning of the sum of squares (SS) and examine whether the interactions temperature X native, temperature X invasive or the most relevant interaction temperature X origin were significant.

CHAPTER 3

3. Results

3.1. Macroalgae and Seagrass Carbon and Nitrogen Composition

As we can observe in the Figure 6 and 7, the percentages of carbon on macroalgae/seagrass tissues were always higher than the nitrogen percentage. Results showed also a tendency for higher percentages of carbon values at the 20ºC treatments; although differences between the two temperatures tested (20ºC and 15ºC) were not statistically supported (p>0.05 in all the tests).

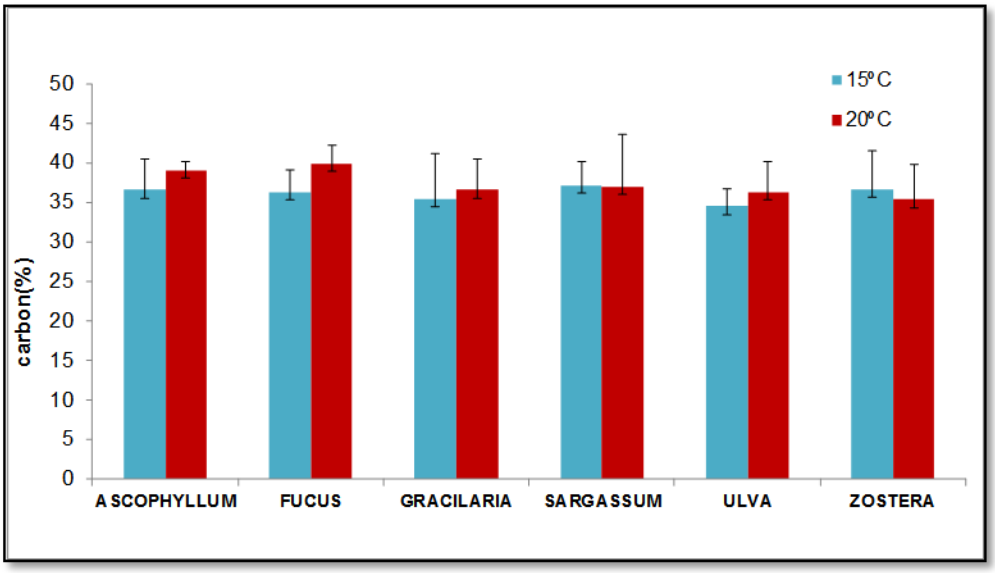

Figure 6 - Carbon Percentage Chart (%, mean, standard deviation). Blue bars represent values at 15ºC; Red bars represent the values at 20ºC.

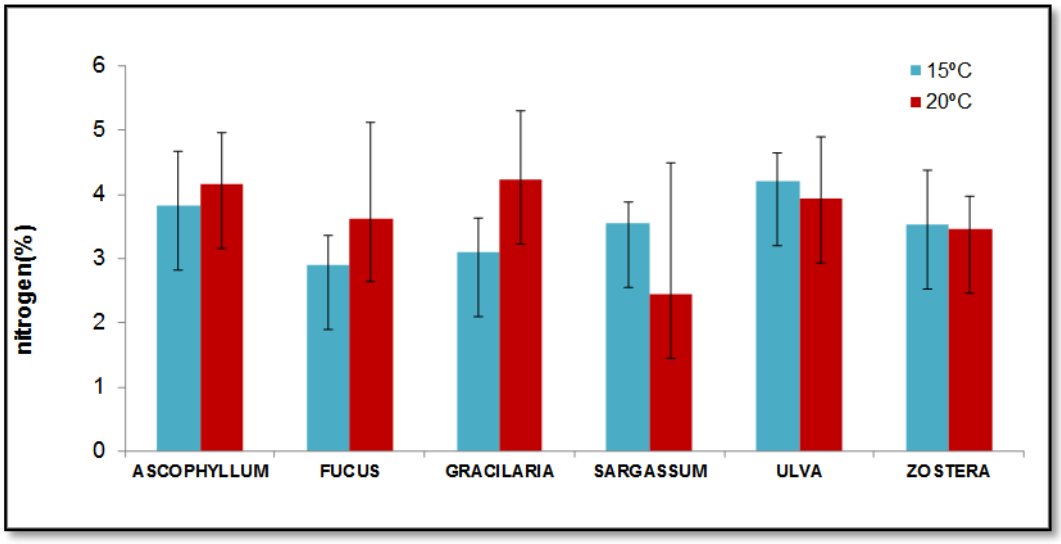

Figure 7 - Nitrogen Percentage Chart (%, mean, standard deviation). Blue bars represent values at 15ºC; Red bars represent the values at 20ºC.

According to figure 8, the C: N ratios measured at the end of the experiment in the remaining macroalgae/seagrass, were different between species. *Sargassum muticum* presented the highest C: N differences between the temperature treatments (Fig. 8), with a C: N at 15ºC of 10 and much higher (15) at 20ºC. Although not statistically significant, there was a major difference (almost 5) on the composition of carbon vs. nitrogen when exposed to different temperatures. *Gracilaria vermicullophylla* presented also C: N differences between treatments, but an opposite pattern was observed, with higher C: N at 15ºC.

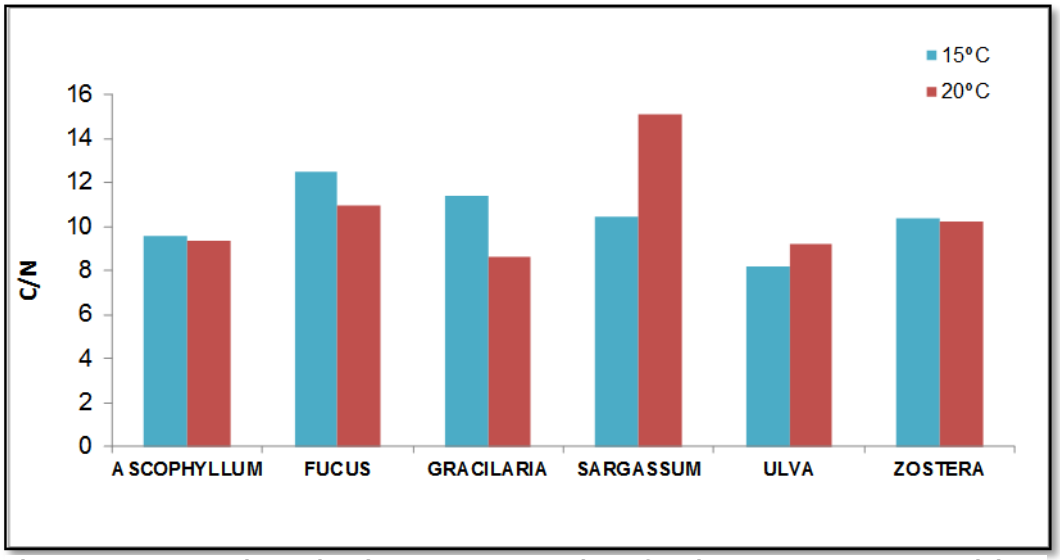

Figure 8 - C: N ratios. Blue bars represent values for the 15ºC treatment; Red bars represent the values for the 20ºC treatment

3.2. Biomass Degradation

The macroalgae/seagrass biomass degradation was determined at the end of the experiment by deducting the final fresh weight to initial fresh weight (12g).
According to the ANOVA analysis, the biomass degradation was not significantly different between the different temperature treatments for all the macroalgae and seagrass analyzed values ($p>0.05$). However, a clear tendency for higher biomass loss was observed in the higher temperature treatment (20ºC).

Differences among species were significant ($p<0.05$). The specie that presented the minimum biomass loss was the seagrass *Zostera marina* with two replicates reaching almost no degradation at 15ºC. The macroalgae *U. lactuca* was the one that had higher biomass degradation loosing on average 11.11g of biomass at 15ºC and 11.54g at 20ºC. Similarly, *F. vesiculosus* and *A. nodusum* presented similar degradation values at both temperatures. *Fucus vesiculosus* lost 9.16g and 10.01g and *A. nodusum* 8.02g and 9.61g, respectively at 15ºC and 20ºC. *Gracilaria vermiculophylla* and *S. muticum* were the two species of macroalgae that showed higher differences between temperatures. *Gracilaria's* biomass loss was of 5.20g at 15ºC (lower comparatively with the others) and 8.28g at 20ºC. *Sargassum's* biomass degradation was 6.33g at 15ºC and 8.79g at 20ºC. Although not statistically significant, differences between temperatures in these last two invasive species were graphically obvious.

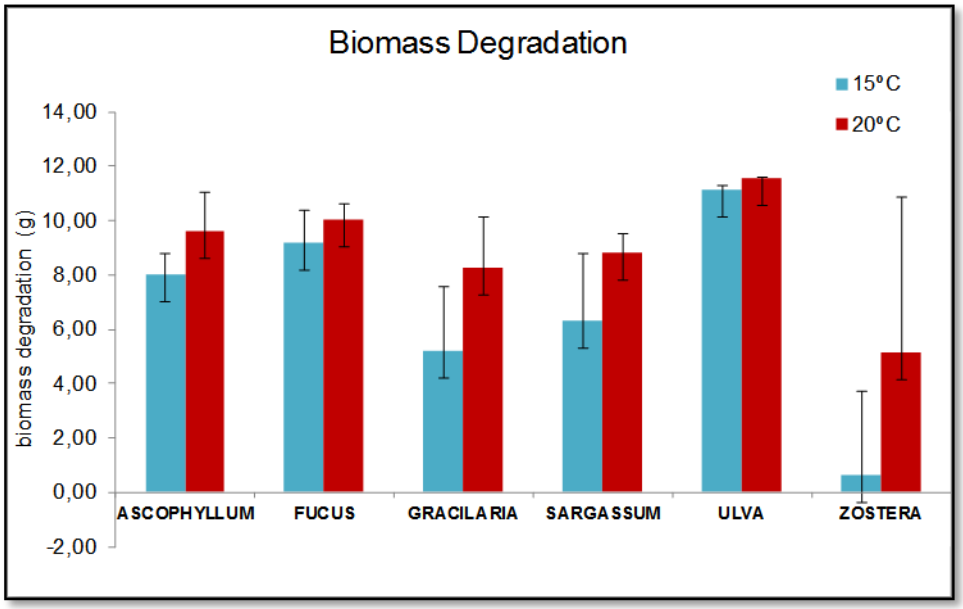

Figure 9- Biomass degradation of macroalgae/seagrass species during the period of incubation expressed in grams (mean ± standard deviation).

3.3. NO_3^-, NO_2^- and NH_4^+ accumulation in Interstitial Water

As stated in the methods section, at the end of the experiment the concentrations of inorganic nitrogen compounds present in the interstitial water were evaluated in each microcosm (Figure 10).

The concentrations of NO_3^- seemed to be different between species and also between temperatures (Figure 10a) however, ANOVA analysis revealed only significant effects of temperature on the NH_4^+ concentration (table 1)

The highest NO_3^- concentration in the interstitial water were registered in the *G.vermiculophylla* (41; 02 ± 11; 06µM/m^2 at 15ºC), *F.vesiculosus* (37; 37 ± 2; 79µM/m^2 at 20ºC) and *A.nodusum* (36; 67 ± 7; 49µM/m^2 at 15ºC). The lowest concentrations occurred in the control (14; 19 ± 4; 63µM/m^2 at 20ºC) and in the systems with *A.nodusum* (22; 18 ± 3; 70µM/m^2 at 20ºC).

Like the NO_3^- results, the NO_2^- concentrations in interstitial water didn't present significant differences between treatments. However, in the case of *Fucus, Sargassu*m and *Zostera marina*, higher NO_2^- accumulations in the pore water were registered in the lower temperature treatments (15ºC). In general the quantity of nitrites was low, with the exception of *S.muticum* (1; 81 ± 0; 87µM/m^2 at 15ºC) and *Z.marina* (1; 65 ± 0; 11µM/m^2 at 15ºC) (Figure 10b). In what NH_4^+ concentrations are concerned values in the interstitial water were found to be much higher than nitrates and nitrites*. Gracilaria vermiculophylla (*1482, 60 ± 318, 34µM/m^2 at 15ºC)*, A. nodosum (*1410; 62 ± 408; 70µM/m^2 at 15ºC) *and F. vesiculosus (*1278; 39 ± 287; 72µM/m^2 at 15ºC) registered the higher NH_4^+ concentrations in the pore water at the end of the experiment (Figure 10c). The lower concentrations were observed in control (91; 95 ± 159; 26µM/m^2) and *A.nodusum (*235; 40 ± 72; 53µM/m^2) at 20ºC.

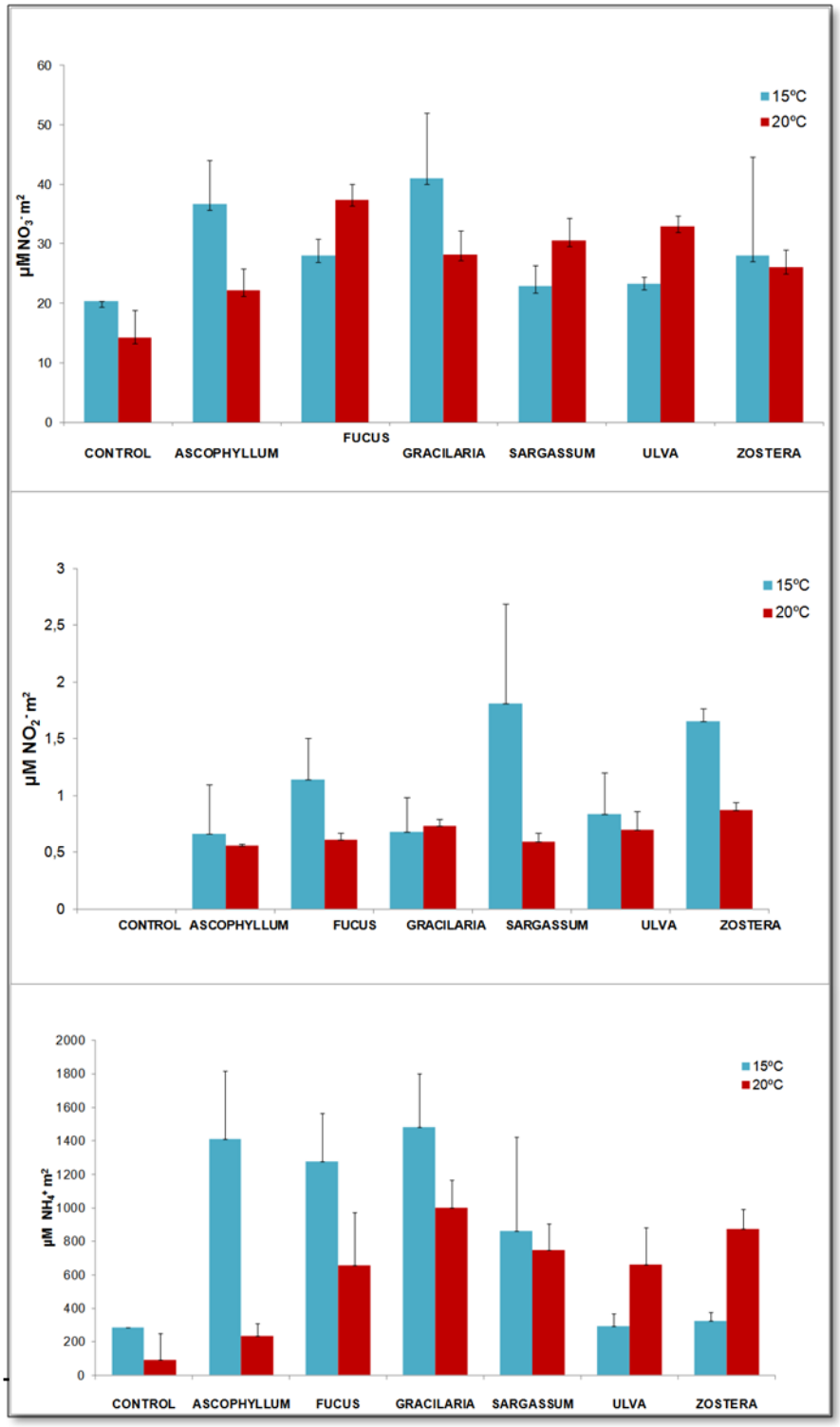

Figure 10 - NO_3^- (a),NO_2^- (b) and NH_4^+ (c) interstitial water concentrations, measured in triplicate treatments with the six species of macroalgae and the seagrass after 3 weeks of incubation (µM) mean and standard deviation).

Table 1 - ANOVA analysis between temperature (fixed effect) and identity (random effect) on N in interstitial water Concentrations. None of the results was significant in relation to the different temperatures (p>0.05)

	Slurries Concentrations								
	Nitrates			Nitrites			Ammonia		
Source	F	P	F versus	F	P	F versus	F	P	F versus
Temperature	0.37	0.5692	teXid	0.22	0.6585	teXid	65.41	0.0005	teXid
Identity	1.24	0.3201	RES	0.23	0.9477	RES	0.95	0.4669	RES
Temperature X Identity	0.72	0.6122	RES	0.69	0.635	RES	0.08	0.9952	RES

3.4. Net Fluxes of NO_3^-, NO_2^- and NH_4^+

According to figure 11, at 15ºC, an average negative NO_3^- net flux were observed meaning a clear absorption of NO_3^- by the water column. Net NO_3^- fluxes presented significant differences relatively to the temperature treatments according to the ANOVA analysis (Table 1). An opposite pattern was observed for the 20ºC treatment, where the net fluxes of NO_3^- were positive in most cases. More importantly the significant interaction between temperature and origin (TempxOrigin, $p<0.05$) suggest that temperature effects were different depending on the origin of the species.

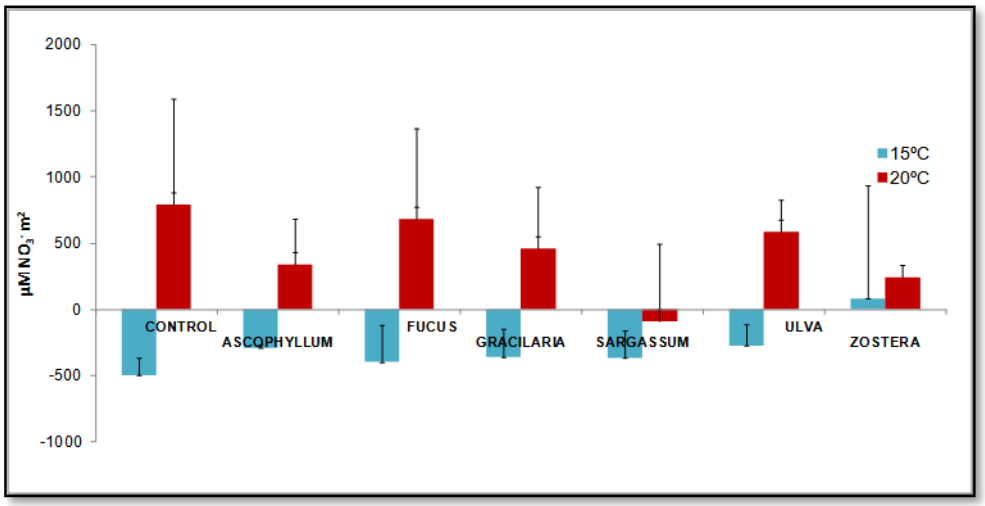

Figure 11 - NO_3^- net flux for all the treatments performed at 15ºC and 20ºC (µmol $NO_3^- m^{-2}$, mean, standard deviation). Positive values represent release of the nutrient to the water column and negative values represent uptake of the nutrient by the sediments. Blue bars represent values at 15ºC; Red bars represent the values at

Net NO_2^- fluxes were relatively lower when compared with NO_3^- net fluxes and the lowest values were registered in the controls. Temperature seemed to have effects, contributing to higher release of NO_2^- to the water column at 20ºC. At 15ºC the fluxes were null or negative in most of the cases, with the exception of *Zostera marina* and *Fucus vesiculosus,* where effluxes of NO_2^- were also registered at 15ºC. However none of the treatments were significant.

In the higher temperature treatments (20ºC), rates of NO_2^- release were higher in the microcosm with the different macroalgae, comparing with the controls (only sediment), where almost null net fluxes of NO_2^- were registered. Lower NO_2^- net fluxes were also verified in the *Sargassum muticum* treatment. The higher release rates were observed at in *Ascophyllum nodosum* and *Fucus vesiculosus* at the 20ºC treatment (Figure 12).

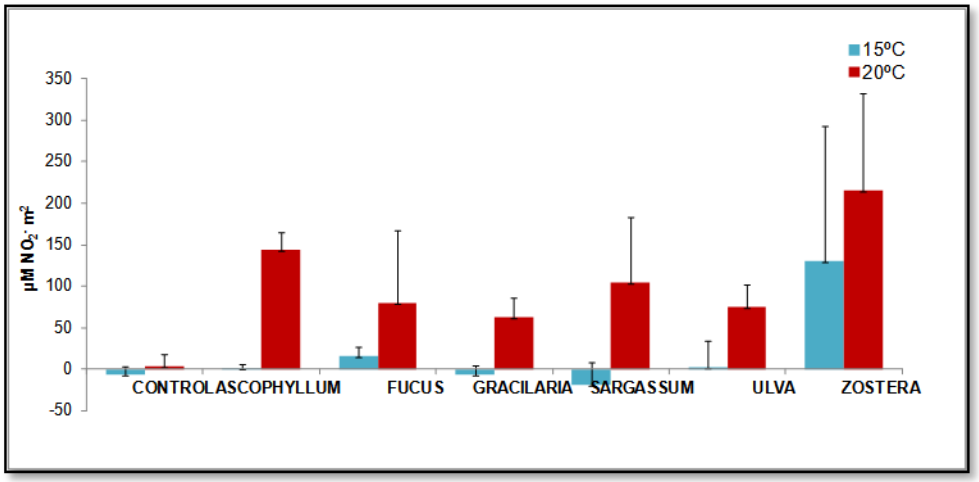

Figure 12 - NO_2^- net flux for all the treatments performed at 15ºC and 20ºC (µmol NO_2^- m^{-2}, mean, standard deviation). Positive values represent release of the nutrient to the water column and negative values represent uptake of the nutrient by the sediments. Blue bars represent values at 15ºC; Red bars represent the values at 20ºC.

In general, the lower temperature (15ºC) seemed to stimulate the net NH_4^+ release to the water column, with the exception of *Gracilaria vermiculophylla* and *Sargassum muticum*. This temperature effect was not significant but *p* values were relatively low ($p<0.1$). With respect to NH_4^+ net fluxes different results seemed to occur according to the macroalgae/seagrass species identity, however these differences were not significant (Table 2) (Figure 13).

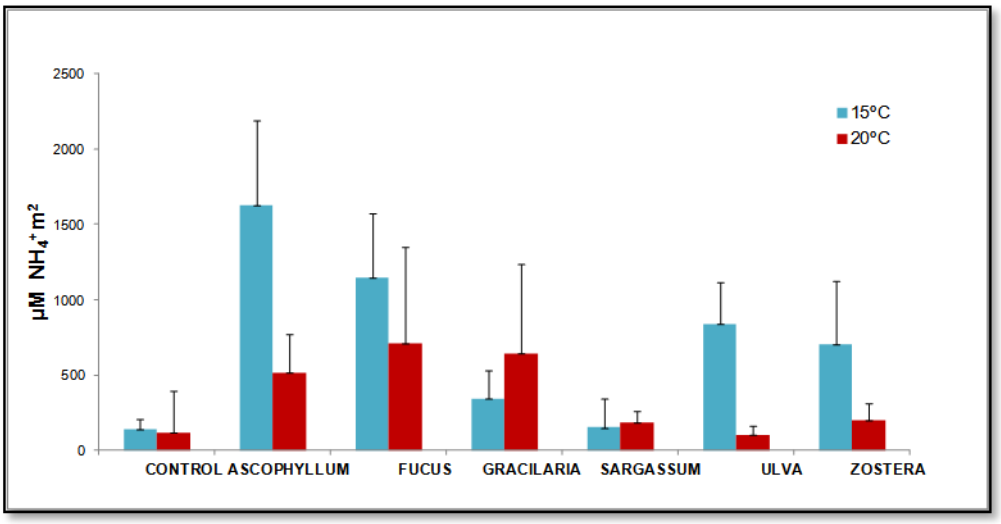

Figure 13 - NH_4^+ net flux for all the treatments performed at 15ºC and 20ºC (µmol NH_4^+ m^{-2}, mean, standard deviation). Positive values represent release of the nutrient to the water column and negative values represent uptake of the nutrient by the

Table 2 - ANOVA analysis between temperature (fixed effect) and identity (random effect) on N Net Fluxes. Significant values (p <0,05) are presented on NO_3 Net Fluxes (red bold). Identity has an influence on the net fluxes (p = 0, 03) between native species (p = 0, 13) allowing to obtain the f and p values for temperature X origin (p = 0, 0095).

	N Net Fluxes								
	Nitrates			Nitrites			Ammonia		
Source	**F**	**P**	**F versus**	**F**	**P**	**F versus**	**F**	**P**	**F versus**
Temperature	8.15	0.029	teXid	0.69	0.4372	teXid	3.92	0.0951	teXid
Identity	0.36	0.8971	RES	0.56	0.7608	RES	0.77	0.6007	RES
Temperature X Identity	**3.14**	0.029	**RES**	1.29	0.2949	RES	0.79	0.5845	RES
Temperature X IdNative	1.94	0.130	RES						
Temperature X IdInvasive	3.36	0.077	RES						
Temperature X Origin	7.74	0.00096	RES						

4. Bacterial Diversity

DNA profiling of the bacterial communities in the sediments of all treatments was performed by Automated rRNA Intergenic Spacer analysis fragment lengths (ARISA-AFLs). The distribution of the different phylotypes among the different samples corresponds to differences in their genetic structures. We performed a multidimensional scaling analysis based on the bacteria ARISA-AFLs profiles obtained for all treatments (Figure 14). Results showed no significant differences in the structure of bacteria communities within the different temperature treatments (15ºC and 20ºC), confirmed by the ANOSIM test (R = -0,009; Significance level = 53, 8%) and between macroalgae types (invasive and native) according to the ANOSIM test (R= -0,007; Significance level = 45, 6%). Although according to our analysis is clear the occurrence of a bacterial community selection in *Fucus vesiculosus* and *Ascophyllum nodosum* (R= 0.34; Significance level= 0.1%), since MDS analysis showed a clear differentiation in the microbial community structure in samples collected in the *F.vesiculosus* and *A.nodusum* treatments, both at 20ºC and 15ºC.

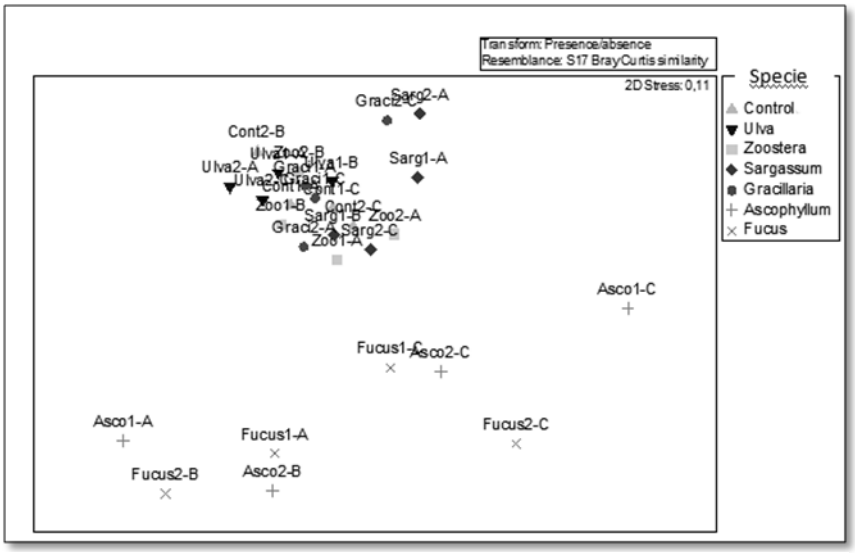

Figure 14 -Multidimensional scaling (MDS) ordination based on Bray–Curtis similarities on the presence/absence matrix obtained from ARISA fingerprints of bacterial communities. The numbers (1 and 2) that appeared next to the species names are referred to 15ºC and 20ºC, respectively.

CHAPTER 4

4. Discussion

Stressors are expected to exert complex effects that cannot be easily inferred using single-stressor studies because synergistic or antagonistic interactions may occur (Folt and Chen 1999). For instance, some evidence suggests that interactions between climate change and biological invasions are likely to have widespread and unexpected effects on coastal ecosystem dynamics (Harley and Hughes 2006). Nonetheless, despite the high research effort dedicated to climate change and non-indigenous species in the marine realm, empirical studies linking these stressors are limited and mostly are observational studies preventing any prediction of future scenarios. Rossi (2007) investigated the transfer of algal-derived C and N to the sediment and to the infauna feeding and discovered that the transfer of C and N to the sediment and to the surface deposit-feeders can be a relevant mechanism to remove the excess of detritus. The relationship between species diversity and ecosystem functioning in natural communities after severe environmental stress were studied by Rossi et al. (2009), who demonstrated that the carbon that flow within the system is highly dependent on the dominant species that aren't replaced during succession, becoming a keystone to the stability of ecosystem functioning under environmental disturbances.

4.1. Influence on inorganic N biogeochemistry

Studies in *Sargassum muticum* invasion showed different conclusions (Wilson, 2001; Britton-Simmons, 2004; Arenas et al. 2006; Olabarria et al. 2009; Cacabelos et al. 2013). While some demonstrated no impact of *S. muticum* on the native species abundance*,* others showed effect at small-scales and with influence in reducing the abundance of native canopy algae. Relatively to *Gracilaria vermicullopylla,* these previous findings demonstrated that the invasion of this algae have negative effects on native seagrass beds of *Zostera marina* by decreasing net leaf photosynthesis and survival rates (Martínez-Lüscher and Holmer 2010b) and affecting the ecosystem nutrients cycling (Thomsen et al. 2009). In agreement, our results clearly showed an impact of the macroalgae degradation buried in the sediments, into the net fluxes of NO_2^-, NO_3^- and NH_4^+ to the water column, however there was no differentiated effect of *S. muticum* and *G. vermicullophylla* when compared with all the other native macroalgae/seagrass native species.

Lemley et al. (2014) studied the effect of different temperature regimes on the rate of decomposition of three macrophyte species and evaluated the extent of inorganic nutrients released. Their results showed that the release of inorganic nutrients was greatest at higher

temperatures (i.e. 25°C and 30°C), due to the reduced bacterial activity at lower temperatures (i.e. 15°C). Our results of net fluxes of NO_2^- and NO_3^- are in agreement with this previous study, since we found that the higher temperature tested generally shift the net fluxes of these compounds from an uptake by the sediments at 15ºC to a release to the overlying water at 20ºC. However, a different picture emerges in the case of NH_4^+ net fluxes, since higher release was almost always registered in the lower temperature treatment. While the processes responsible for the nitrogen transformations were not evaluated in our study, previous findings could be explained by the fact that microbial communities that use NH_4^+ (Nitrifiers) where stimulated at higher temperatures reducing the amount of the NH_4^+ available and consequently lower release to the water column. In fact, results on the concentrations of NH_4^+ in the interstitial water are in agreement, since higher NH_4^+ concentrations in the pore water were in almost of the cases obtained for the lower temperature treatment (15ºC). The decomposition of algae therefore provides a potentially important supply of organic and inorganic compounds to the water column where they can be recycled rapidly (Gabrielson et al. 1983; Twilley et al. 1986; Paalme et al. 2002).

According to our statistical analysis, NO_3^- net fluxes, were the only flux significantly affected by temperature, however no significant differences were observed among natives ($p=0.13$, $p>0.05$) or invasive ($p=0.07$. $p>0.05$) species. Nevertheless, when using the method of combining the sum of squares values from separate analyses of variance we reached a significant value between temperature and origin ($p =0.0095$, $p<0.05$) meaning that being native or invasive make difference on the assimilation or release of nitrates at the different temperature treatments. Although not statistically significant, invasive species present an approximated *p* value to 0.05 ($p = 0. 07$), thus is more likely to have a greater effect in on the nitrogen cycle between the two invasive species than native ones. Net NO_3^- fluxes were always negative for the 15ºC treatment, with the exception of *Sargassum muticum* and *Zoostera marina*, where a release of NO_3^- to the water column was observed. These results suggested that the lower temperature stimulates the benthic processes involved in NO_3^- consumption, i.e. denitrification, anammox, and dissimilatory nitrate reduction to ammonia (DNRA) (Jensen et al. 1990; Kemp et al. 1990; Seitzinger 1990). An explanation for the exceptions that occurred on NO_3^- fluxes are the fact that *Sargassum muticum* is an invasive species with different behavior at low temperatures than the other species (Allison 2004; Harley and Hughes 2006), contributing to the adsorption or consumption of nitrates by the sediments. However, *Zostera marina*, which is a seagrass, has different metabolic patterns relatively to macroalgae (Hemminga and Duarte 2000).

On the other hand treatments at 20ºC, showed positive net NO_3^- fluxes with the exception of *Sargassum muticum*, where an adsorption of NO_3^- by the sediments were registered. Taking in

to account the processes involved in the N cycle, we can hypothesized that the NO_3^- reduction processes, like denitrification and annamox are favorable by low temperatures. It could be also the case that those processes are not affected, but temperature could stimulate nitrification (oxidation of NH_4^+ to NO_3^- and to NO_2^-), resulting in a higher release of NO_3^- and NO_2^- to the overlying water at the 20ºC treatments (Lemley et al. 2014). The nitrite fluxes at 15ºC were close to zero, and only *Zostera marina* presented a clear absorption of nitrites. In deed NO_2^- and PO_4^{3-} net effluxes are normally expected to be lower than those of NO_3^- and NH_4^+ (García-Robledo et al. 2008). At 20ºC, was observed a general release of nitrite to the water column.

Net fluxes of NH_4^+ were always positive, so there is a general release of ammonia to the water column, probably because macroalgae/seagrass degradation stimulated bacterial degradation / ammonification by the large input of organic matter into the sediments. The higher rates of NH_4^+ effluxes registered at the 15ºC treatments could not just be explain by a stimulation of ammonification but also on the microbial communities involved on the DNRA.

Rossi et al. (2011) emphasized the importance of detrital diversity and non-native seaweeds in benthic nitrogen cycling showing that the detrital mixing of *S. muticum*, *F. vesiculosus* and *U.lactuca* provided more ^{15}N-nitrogen to sediments and to the macrofauna due to the high composition of polyphenols in *S. muticum* and *F. vesiculosus*, and the large amounts of nitrogen content in *U. lactuca*. Interesting, in our experiments the systems with *F. vesiculosus* presented the highest accumulation of NO_2^- in the interstitial water, when compared with the other macroalgae/seagrass treatments. According to the ANOVA analysis, the inorganic N concentrations in the interstitial water were not affected by the different temperatures (Table 2). Although a significant increase of NO_2^-, NO_3^- and NH_4^+ in pore water occurred in all treatments with macroalgae/seagrass compared with the controls (only sediment), suggesting that the degradation of these species stimulated N accumulation/availability in the interstitial waters. In agreement to our results, a study performed in the region of Puck Bay also showed that the increased concentrations of organic matter were followed by higher NH_4^+ concentrations in interstitial waters (Bolałek and Graca 1996). In fact these results are expected since the concentration of inorganic nitrogen compounds in interstitial waters tend to be proportional related to the organic matter content in the sediments which in turn stimulated ammonification, with the formation of ammonium (Bolałek and Graca 1996).

4.2. C: N Ratios

The C: N elemental ratios in the macroalgae tissues often acts like an indicator of the predominant sources of organic matter in the aquatic ecosystems (Rivers et al. 1990; Thornton and McManus 1994; Andrews et al. 1998)**.** In our study C: N ratios at 15ºC varies between 8 and 12 and at 20ºC between 8 and 15. According to Liu et al. (2006), the C: N ratios of sedimentary organic matter in the intertidal flats have the range of 8.01-12.30, thus our C: N ratios were within the range of values typically found with the exception of *S. muticum*. The potential sources of organic matter in intertidal ecosystems are generally expected to include terrestrial detritus, marine material, *in situ* primary producers (e.g. benthic algae) and sewage (Liu et al. 2006).

The arrival of detrital invasive seaweeds that can appear mixed to native species or in isolation could alter the carbon and nitrogen provision and modify both benthic community structure and carbon or nitrogen cycling (Rossi et al. 2011). This could be the case observed on *S. muticum* but not on *G. vermiculopylla* which C: N ratios were 11 and 9, at 15ºC and 20ºC, respectively. Indeed, the only C: N ratio that was in the high range of the typical C: N values belongs to *Sargassum muticum* (15). When organic matter decays, the carbon is dissipated more rapidly than the nitrogen, thus bringing down the C: N ratio (Miller 2000). In the case of *S. muticum* is the opposite, although it did not present a very high C: N ratio, compared to the other species, contains more carbon on its composition and consequently it will decompose slower. The ratios of native species and seagrass stayed between the expected ranges cited previously, noticing that different temperatures didn't affect the composition of the macroalgae and seagrass C: N ratios.

4.3. Macroalgae Decomposition

When comparing the decomposition characteristics of each macrophyte species, it is important to realize that the rate and extent of decomposition is largely dependent on the biochemical composition of each specific macrophyte (Buchsbaum et al., 1991; Hanisak, 1993; Bourguès et al., 1996). Comparing the C: N ratios and biomass degradation we concluded that high C: N ratios correspond to lowest biomass degradation. *S. muticum* was a good example*,* presenting the highest C: N ratio (15) and the lowest biomass degradation of all the tested macroalgae species.

In our study we observed a tendency for higher macroalgae/seagrass degradation in high temperature (20ºC), with almost a total loss of *Ulva lactuca*. Ephemeral species such as Ulva sp., showed the fastest rate of decomposition due to the low content of refractory and phenolic

compounds in comparison to brown and perennial algae (Buchsbaum et al. 1991). Overall ephemeral green seaweeds such as Ulvaceae have high nitrogen content, decompose rapidly and serve as food for grazers and detritivores. Conversely, brown seaweeds such as Fucales have great lignin content decompose relatively slowly and produce phenol compounds that are usually associated with a chemical defense against grazers (Buchsbaum et al. 1991a; Pedersen et al. 2005; Rossi et al. 2010). Fast growing marine algae have higher N requirements than slow growing perennial species such as *Fucus sp*. and other benthic macrophytes, like seagrasses. Thus, fast growing species are positively affected by increased nutrient availability (Pedersen & Borum, 1996; Rosenberg & Ramus, 1982).

4.4. Effects on Bacterial diversity

Shifts on bacterial community composition within the different treatments were analyzed by ARISA, a DNA fingerprinting technique that allows the rapid assessment of the genetic structure of complex communities in diverse environments (Hewson and Fuhrman 2004; Ranjard et al. 2006; Danovaro et al. 2009). Multidimensional scaling analysis allowed the detection of differences in the benthic bacterial communities in two macroalgae species, by confirming in terms of microbial community selection in the sediments where *F. vesiculosus* and *A. nodosum* were decomposing. This selection could be explained by the fact that this two species belong to the same family Fucaceae (Johnson and Scheibling 1987), sharing physiological and morphological characteristics and therefore similar biochemical structure.

CHAPTER 5

5. Conclusion

In this study we confirmed the hypothesis, that temperature affects the natural course of nitrogen biogeochemistry when the levels of macroalgae/seagrass biomass increased within the benthic compartment. Indeed our study suggested that temperature is a key factor responsible for controlling benthic macroalgae/seagrass decomposition processes. A clear shift in NO_3^- and NO_2^- net fluxes was demonstrated to occur when temperature changed from 15ºC to 20ºC, with a clear release of these inorganic nutrients to the water column at higher temperatures (20ºC). Interestingly, an opposite pattern was registered for NH_4^+ net fluxes, where a higher release of this compound was registered at low temperatures (15°C).

Although in our study there is a N net flux (NO_3^-) that is influenced by the origin of the macroalgae and seagrass (native or invasive), we cannot infer that invasibility by *S. muticum* and *G. vermiculophylla* contribute as an addictive factor to promote changes in the nitrogen cycle.

Further future studies should improve the methodologies to access clearly detailed conclusions regarding invasibility and the processes and microbial communities involved on the nitrogen recycling in the benthic compartment. We believe that this study generated important results to predict future environment disturbances with the possibility to avoid economic costs to an area that suffers high pressures of intensive aquaculture. Indeed, aquaculture in the Galician Rias is a growing industry and in 2000 the Rias supported 3386 mussel rafts producing 2.5 x 10(8) kg year (-1), i.e. 40% of European Union total seafood production (Prego and Cobelo-Garcıa 2003). From a management point of view, addressing the impacts of both climate change stressors and invasive species our study provide basic information to design efficient and innovative mitigation plans in coastal areas. Providing quantitative experimental information on these effects will help increasing the precision and accuracy of ecological models and predicting how marine ecosystems will change in the near future.

CHAPTER 6

6. Bibliography

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