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Salinity as a key selector on the activity and diversity of ammonia oxidizers in estuarine systems

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“The only source of knowledge is experience”

Albert Einstein

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Resumo

Estuários são interfaces entre ambientes de água doce e marinhos tornando-se em zonas extremamente dinâmicas. A mistura de água doce e salgada, juntamente com a geomorfologia, ventos e alturas das marés criam gradientes físico-químicos que são acompanhados por mudanças nas comunidades microbianas residentes. Além disso, os impactos antropogénicos continuam a causar alterações nos sistemas estuarinos. Uma destas alterações é o "input" excessivo de azoto, o qual é determinado pelas comunidades microbianas pertencentes ao ciclo do azoto. A salinidade é conhecida por desempenhar um papel importante na regulação destas comunidades nos ambientes estuarinos. Além disso, investigações anteriores efetuadas no estuário do Rio Douro demonstraram variações sazonais, e uma influência positiva no processo de nitrificação por parte de salinidades intermédias.

Neste estudo avaliámos a influência de um gradiente de salinidade (efeito direto) assim como o efeito de flutuações de salinidade (efeito a longo termo) em sedimentos de dois locais distintos de um sistema estuarino. Os resultados revelaram que as comunidades microbianas estuarinas adaptadas a regimes distintos de salinidade apresentam diferentes respostas em termos de atividade nitrificante quando sujeitos a mudanças de salinidade. Complementarmente, os nossos resultados demostraram que a salinidade é uma importante variável ao controlar a seleção dos grupos de organismos (bactérias e archaeas oxidantes de amónia) que medeiam o processo de nitrificação nos sistemas estuarinos. Para além disso, os resultados sugerem que as bactérias oxidantes de amónia exibem uma maior plasticidade quando sujeitas a um tratamento de flutuações de salinidade. Por fim, em locais de baixa salinidade, as espécies pertencentes ao género *Nitrospira*, capazes de realizar os dois passos da nitrificação (commamox) aparentam deter um papel principal no processo de nitrificação.

Acreditámos que os resultados obtidos neste estudo são cruciais para compreender a dinâmica do processo de nitrificação sob gradientes de salinidades, assim como quando sujeitas a flutuações de salinidades, proporcionando uma melhor compreensão sobre como as comunidades nitrificantes respondem a variações de salinidades.

Palavras-chave: nitrificação, bactéria, archaea, amónia oxidantes, gradiente de salinidade, flutuações de salinidades, *amoA.*

Abstract

Estuaries are the interfaces between freshwater and marine environments being extremely dynamic zones. The mixing of freshwater and saltwater along with the geomorphology, winds and tidal heights creates physical-chemical gradients that are accompanied by shifts in the resident microbial communities. In addition, anthropogenic impacts keep driving alterations to estuarine systems. Nitrogen input is one consequence of these anthropogenic actions and the fate of excess nitrogen in estuaries is determined by the microbial-driven nitrogen cycle.

Salinity has been shown to play an important role in regulating these communities in environmental systems. In addition, previous research in the Douro River estuary demonstrated seasonal variances and a positive influence of intermediate salinities on nitrification.

In this study, we evaluated the influence of a salinity gradient (short-term effect) as well the fluctuation of salinity effect (long-term effect) on two distinct sediment sites from an estuarine system. Results revealed that microbial communities adapted to distinct estuarine salinity regimes show different responses in terms of nitrification activity when facing salinity changes. In addition, our findings demonstrated that salinity is an important variable in controlling the selection of the group of organisms (archaea and bacteria ammonia-oxidizers) that mediated the nitrification processes in estuarine systems. Furthermore, results suggested that Bacteria ammonia-oxidizers display higher plasticity when subjected to a salinity fluctuation treatment. Lastly, it seems that under the low salinity site, the *Nitrosospira* species able to perform both steps of nitrification (commamox) display a main role on the nitrification process.

We believe that results from this study are critical to understand the dynamics of the nitrification processes under a gradient of salinities as well under salinity fluctuations, providing better insights on how nitrifier communities respond to salinity changes.

Key-words: nitrification, bacteria, archaea, ammonia-oxidizers, salinity gradient, salinity fluctuations, *amoA*.

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List of accomplishments

During the elaboration of this dissertation, the study was presented in the following national and international meetings and a parallel work has been submitted to an international journal:

- Ribeiro H, Sousa T, **Santos J**, Teixeira C, Salgado P, Mucha AP, Almeida CMR, Magalhães C. Potential for naphthalene and fluoranthene degradation under dissimilatory nitrate reducing conditions - shifts on an indigenous estuarine prokaryotic consortium. 2016, Submitted to Frontiers in Microbiology

- **Santos J**., Monteiro M., Mendes D., Ribeiro H., Borges T., Magalhães C. 2015. The role of an estuarine salinity gradient on key nitrogen processes. BioMicroWorld VI - International Conference on Environmental, Industrial and Applied Microbiology, 28-30 October, Barcelona, Spain. (Oral communication)

- **Santos J.**, Magalhães C, Ribeiro H., Padeiro A., Canário J. 2015. Shifts on bacterial community structure in Antarctica Fildes Peninsula soils with different levels of metal concentrations. BioMicroWorld VI - International Conference on Environmental, Industrial and Applied Microbiology, 28-30 October, Barcelona, Spain. (Poster communication)

- **Santos J**., Monteiro M., Mendes D., Ribeiro H., Borges T., Magalhães C. 2015. The role of salinity on estuarine ammonia oxidizing activity. IJUP – Encontro de Jovens Investigadores da Universidade do Porto, 13 – 15 May, Porto, Portugal (Oral communication)

- **Santos J.**, Monteiro M., Mendes D., Ribeiro H., Borges T., Magalhães C. 2016. Salinity fluctuation effect on estuarine key nitrogen processes. ECSA56 - Coastal systems in transition: From a 'natural' to an 'anthropogenically-modified' state. 4 – 7 September 2016, Bremen, Germany. (Oral communication)

- **Santos J.**, Magalhães C, Ribeiro H., Padeiro A., Canário J. 2016. Shifts on bacterial community diversity in Antarctica Fildes Peninsula soils with different levels of metal concentrations. ECSA56 - Coastal systems in transition: From a 'natural' to an 'anthropogenically-modified' state. 4 – 7 September 2016, Bremen, Germany. (Poster communication)

- Ribeiro H, Sousa T, **Santos J**, Teixeira C, Salgado P, Mucha AP, Almeida CMR, Magalhães C. 2016. Naphthalene and fluoranthene degradation under nitrate reduction conditions - key shifts on indigenous estuarine microbial consortium. ECSA56 - Coastal systems in transition: From a 'natural' to an 'anthropogenically-modified' state. 4 – 7 September 2016, Bremen, Germany. (Poster communication)

List of abbreviations

®	Registered trademarker
µL	Microliter
µM	Micromolar
‰	Parts per thousand
$^{14}NH_4Cl$	Ammonium chloride
^{15}N	Nitrogen element with isotope 15
$^{15}NH_4Cl$	^{15}N labelled ammonium chloride
16S rRNA	16S ribosomal RNA
A	Constant salinity treatment in Afurada sediments
ADP	Adenosine diphosphate
$AgNO_3$	Silver nitrate
AMO	Ammonia monooxygenase enzyme
amoA	Alpha sub-unit of the ammonia-monooxygenase enzyme
AO	Ammonia-oxidizers
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOM	Ammonia-oxidizing microorganisms
ATP	Adenosine triphosphate
bp	Base pair(s)
C	Constant salinity treatment in Crestuma sediments
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
CG	Crenarchaeotal group
cm	Centimetre
DA_1	NGS ID of replica 1 from salinity fluctuation treatment in Afurada
DA_2	NGS ID of replica 2 from salinity fluctuation treatment in Afurada
DC_1	NGS ID of replica 1 from salinity fluctuation treatment in Crestuma
DC_2	NGS ID of replica 2 from salinity fluctuation treatment in Crestuma
DGGE	Denaturing gradient gel electrophoresis

DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonium
Fe	Iron element
g	Gram
GOGAT	Glutamine-2-oxoglutarate amino transferase
GS	Glutamate synthase
GS-GOGAT	Enzymatic system CG-GOGAT
h	Hour
H_2	Hydrogen
H_2S	Hydrogen sulfide
HAO	Hydroxilamine oxidoreductase
IP	Iberian Peninsula
k_m	Michaelis constant
L	Litre
LSU	Large subunit
mM	Milimolar
Mo	Molybdenum
mRNA	Messenger RNA
N	Nitrogen element
N_2	Nitrogen gas
N_2O	Nitrous oxide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAP	Periplasmic nitrate reductase enzyme
napA	Gene coding for a specific nitrate reductase
NAR	Respiratory nitrate reductase enzyme
narG	Gene coding for a specific nitrate reductase
NAS	Assimilatory nitrate reductases enzyme
ng	Nanogram
NGS	Next-generation sequencing
NH_2	Amine

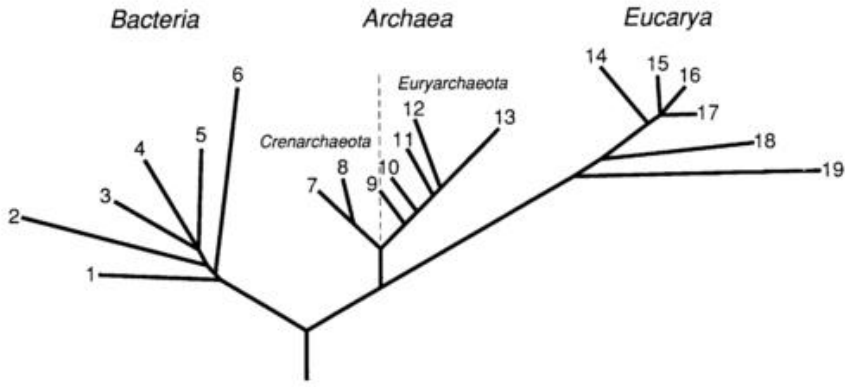
NH_3	Ammonia
NH_4^+	Ammonium
NIR	Siroheme-dependent nitrite reductase enzyme
nirK	Gene coding for a specific nitrite reductase
nirS	Gene coding for a specific nitrite reductase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite-oxidizing bacteria
norB	Gene coding for nitric oxide reductase
nosZ	Gene coding for nitrous oxide reductase
NRF	Nitrite reductase enzyme
NXR	Nitrite oxireductase enzyme
nxrA	Gene coding for alpha subunit of nitrite oxidoreductase
nxrB	Gene coding for beta subunit of nitrite oxidoreductase
ºC	Celsius degrees
OTU	Operational Taxonomic Unit
PCR	Polymerase chain reaction
pg	Picogram
Pi	Inorganic phosphate
rpm	Rotation per minute
rRNA	Ribosomal RNA
S_2^-	Sulfide
SD	Standard deviation
SSU	Small subunit
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
TNM	Total nitrifier microorganisms
V	Vanadium
vs	Versus
ΔA	Salinity fluctuations treatment in Afurada sediments
ΔC	Salinity fluctuations treatment in Crestuma sediments

Introduction

1. Role of microorganisms on earth

The planet Earth is a multi-complex system harbouring millions of species continuously interacting in the multi trophic chains. Within these species, microorganisms are the most ubiquitous, simple and widespread forms of life, from the most mesophilic to the most extreme environment (Whitman et al. 1998). However, even with their wide distribution across ecosystems (Prosser et al. 2007), it is estimated that less than 1% has been identified (Hugenholtz 2002, Choffnes et al. 2013).

Microorganisms are known for their continuous exchange in energy fluxes as well chemical elements in the environment, being key operators in many biogeochemical cycles (like nitrogen, sulphur and carbon cycle) and environmental processes, through several aerobic and anaerobic respiration processes, fermentations, as well as photosynthetic processes (aerobic and anaerobic photosynthesis), making them crucial to the functionality and sustainability of the ecosystems (Prosser et al. 2007, Graham et al. 2016). These microorganisms are included by prokaryotic and eukaryotic organisms which are distributed in the tree domains of life (Archaea, Bacteria, Eukarya) as illustrated in Figure 1 (Woese et al.



1990, Cavicchioli 2011).

Figure 1. Illustrative phylogenetic tree used to demonstrate the proposal of three phylogenetic domains in 1990 (Pace et al. 2012).

After the proposal of the tree of life by Woese *et al.*, (1990), the increase interest of the scientific community in the phylogenetic relationship between the countless organisms throughout many ecosystems (Wu et al. 2009) enabled the introduction of the culture independent methods (Fischer and Lerman 1980, Mullis et al. 1986, Muyzer et al. 1993) which allowed for the first time the direct amplification and sequencing of 16S rRNA genes from environment samples (Giovannoni et al. 1990). The advances on culture independent methods focusing on the nucleic acid information (Torsvik and Øvreås 2002), functional genes (Purkhold et al. 2003) and synthesized proteins (Karlsson et al. 2009), drastically increased our understanding on the phylogeny, physiology and functionality of microorganisms (Purkhold et al. 2000, Treusch et al. 2005, Bayer et al. 2008, Walker et al. 2014).

Nowadays, with the application of next-generation sequencing (NGS) technologies, millions of microbial sequences are analysed and uploaded to web databases, which allow comparisons from microbial data from all over the world (MacLean et al. 2009, Metzker 2010, DePristo et al. 2011).

2. The nitrogen element

Nitrogen (N) is the seventh element in the periodic table with an atomic mass of 14 (Zehr and Kudela 2011). It is recognized as an essential element for the functionality and sustainability of ecosystems since it is fundamental to maintain the microbial metabolism that sustains global biogeochemical cycles (Capone and Knapp 2007, Francis et al. 2007). Additionally, it can be found in all biological materials once it is a structural component of nucleic acids (DNA, RNA), proteins (where nitrogen constitutes 18% of proteins), and in certain polysaccharides such as chitin (shell of arthropods) or peptidoglycan in the membrane wall of bacteria (Sinden 1994, Strock 2008, Bertrand et al. 2015). It is estimated that around $1.5x10^{15}$ tons of N exist in the total biological materials on earth (Postgate 1982, Prosser et al. 2007).

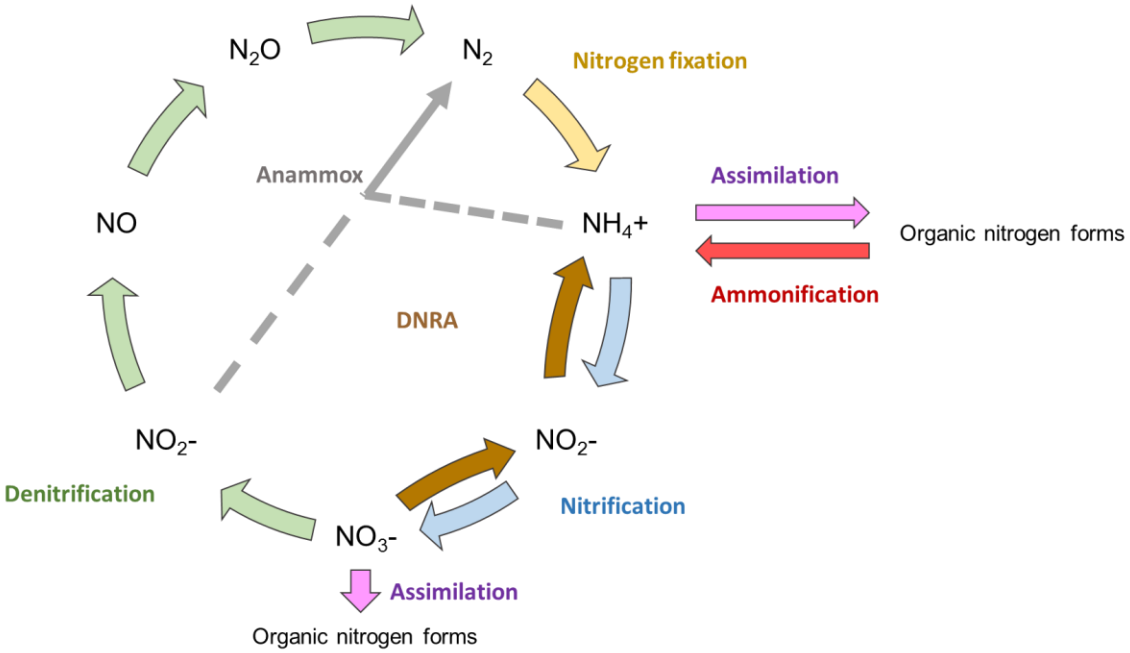
Although 78% of the atmosphere is composed by triple-bonded N_2 gas, it cannot be directly used by living organisms (Zehr and Kudela 2011). It has to be firstly fixed by specialized organisms which can convert N_2 into NH_3 (see the “Nitrogen Fixation” section).

Ammonia (NH_3) plays a major role in primary productivity where N is normally a limiting nutrient along most temperate estuarine systems as well as other marine coastal systems (Howarth and Marino 2006, Bernhard and Bollmann 2010a). Additionally, N is directly connected to eutrophication and subsequently to the degradation of coastal marine systems (Howarth and Marino 2006), which is normally caused by high N inputs derived from anthropogenic activities (Gruber and Galloway 2008, Statham 2012). Furthermore, some nitrogen compounds like nitrous oxide (N_2O) and nitric oxide (NO) are strong greenhouse gases directly involved in climate change (Fields 2004).

In nature, nitrogen undergoes transformations as an electron acceptor or donor in a wide range of oxidation/reduction states, from +5 (NO_3^-) to -3 (NH_4^+), which are primarily mediated by bacteria, archaea and some specialized fungi (Bothe et al. 2006). These transformations are described below allowing a better understanding of the oxidation/reduction reactions.

3. The biogeochemical nitrogen cycle

The inorganic biogeochemical nitrogen cycle comprises 7 main pathways (Figure 2): nitrogen fixation, ammonification, nitrogen assimilation, anammox, denitrification,



dissimilatory nitrate reduction to ammonium (DNRA) and nitrification.

Figure 2. Scheme of the inorganic nitrogen cycle showing the 7 key pathways of the nitrogen biogeochemical cycle: nitrogen fixation (yellow), ammonification (red), nitrogen assimilation (purple), anammox (gray), denitrification (green), DNRA (brown) and nitrification (blue).

These processes are mediated by a wide range of microorganisms (autotrophs and heterotrophs) and occur under different oxic conditions (aerobic and anaerobic). A description of each pathway regarding the microorganisms involved, their functionality and importance in estuarine systems are presented on the following topics.

3.1 Nitrogen fixation

Most of the atmosphere is composed by N_2, however it cannot be used directly by the majority of the organisms, unless it is fixed through nitrogen fixation in which the atmospheric N_2 gas is reduced into ammonia. The biological nitrogen fixation includes the production of two moles of ammonia from one mole of nitrogen gas, followed by the conversion of 16 molecules of adenosine triphosphate (ATP) to 16 molecules of adenosine diphosphate (ADP) and the release of one molecule of hydrogen (H_2) and 16 molecules of inorganic phosphate (Pi) as by-products (Zehr and Kudela 2011). This process is performed by phylogenetically diverse microorganisms within the Bacteria and Archaea, which are called diazotrophs (Hartmann and Barnum 2010).

The enzyme responsible for the biological N_2 fixation is the nitrogenase (McGlynn et al. 2013), which depending on the metal constitution of the active site (molybdenum (Mo), vanadium (V), or iron (Fe)) can be subdivided: Nif, Vnf, and Anf (McGlynn et al. 2013). However, despite differences in their metal content, these nitrogenase sub-types are structurally, mechanistically, and phylogenetically related. Their catalytic components include two distinct proteins: dinitrogenase (comprising the D and K component) and dinitrogenase reductase comprising the H component (Dos Santos et al. 2012). The structural components of molybdenum-dependent (Mo-dependent) nitrogenase is encoded by *nifH, nifD*, and *nifK* genes. The two other sub-types of nitrogenase, which are known as alternative nitrogenases, are enzyme homologs with the exception of an additional subunit (G) in the dinitrogenase component and the absence of the heteroatom Mo. The vanadium-dependent nitrogenases is

encoded by *vnfH, vnfD, vnfG*, and *vnfK* genes. The members of the third nitrogenase sub-type, the iron-only nitrogenase, devoid of Mo and V, is encoded by *anfH, anfD, anfG*, and *anfK* genes (Dos Santos et al. 2012). On the top of all N fixation organisms, cyanobacteria are considered as the most important and abundant N fixers on Earth (Vitousek et al. 2002, Zehr 2011).

Nitrogen fixation can also occur without the intervention of microorganisms throughout photochemical fixation in the atmosphere (Banerjee et al. 2015) or through the Haber–Bosch process used in the manufacture of commercial fertilizers (Strock 2008).

Although nitrogen fixation represents an important process by making N available for other organisms, in estuarine systems this process tends to be not relevant for nitrogen availability (Santoro 2009), due to high nitrogen inputs mostly derived from anthropogenic action (Zehr et al. 2007)**.**

3.2 Nitrogen mineralization and nitrogen assimilation

As mentioned before, the majority of biological materials contain N compounds such as proteins, polyamine sugar, nucleic acids and others (Cooper and Hausman 2000). The process that allows the assimilation of nitrogen into biomass is called the nitrogen assimilation. This process is performed by eukaryotic and prokaryotic microorganisms that can assimilate simple inorganic N (nitrate, nitrite and ammonia) into biomass.

In the case of NO_3^- and NO_2^- the assimilation process is an energy-consuming reaction which is carried out by the enzymes nitrate reductase (NAS enzyme; (Luque-Almagro et al. 2011)), and the siroheme-dependent nitrite reductase (NIR enzyme; (Bothe et al. 2006)). Although in the case of NH_4^+ assimilation the process is not energy-consuming and, the main enzymes involved are the glutamate synthase (GS) and the glutamine-2-oxoglutarate amino transferase (GOGAT) which form the enzymatic system GS-GOGAT (Miflin and Habash 2002, Mokhele et al. 2012). Where the first enzyme (GS, glutamine synthetase) adds a group NH_3 to a molecule of glutamate. The resulting glutamine transfers one of its nitrogenous groups to α-ketoglutarate under the action of the second enzyme GOGAT (glutamine α-ketoglutarate aminotransferase). This transaminase allows the transfer of a group ammonia to another molecule of α-ketoglutarate to form two molecules of

glutamate (Glutamate serves as a template for the synthesis of most amino acids or for the reactions of transamination with keto acids (Bertrand et al. 2015)).

However, when living beings decease, the organic nitrogen available is mineralized by the action of heterotrophic bacteria that convert organic nitrogen into inorganic forms. Mineralization is a two-step process that begins with aminization, followed by ammonification (Strock 2008). In the first step, aminization microorganisms (such as prokaryotes and fungi) break down complex proteins into simpler amino acids, amides, and amines (Power and Prasad 1997, Jorgensen and Fath 2014). And, in ammonification the NH_2 groups are converted into ammonia or its ionic form, ammonium (NH_4^+), as an end product (Power and Prasad 1997).

This process plays an important role on recycling nitrogen in marine, coastal and estuarine environments (Sohm et al. 2011), by allowing primary producers to use the recycled inorganic nitrogen forms unavailable in the organic nitrogen forms.

3.3 Anaerobic ammonium oxidation (Anammox)

Anammox is a biological anaerobic process by which chemoautotrophic bacteria in the presence of nitrite (NO_2^-) can oxidize ammonia into nitrogen (Mulder 1992). This process, after being firstly identified on a wastewater treatment plant (Mulder 1992) was identified in marine sediments (Thamdrup and Dalsgaard 2002), arctic marine sediments (Rysgaard et al. 2004), tropical freshwater system (Schubert et al. 2006), anoxic waters in the Black Sea (Lam et al. 2007) and in many other environments.

This process is mediated by 5 bacteria' genera forming the monophyletic order of the Brocadiales belonging to the phylum Planctomycetes (van de Vossenberg et al. 2013, Oshiki et al. 2016). Through *in silico* analysis of the metagenome of an anammox bacterium from an enrichment culture of "*Candidatus Kuenenia stuutgartiensis*" a theory about anammox redox reactions was postulated suggesting the existence of three different reactions for anammox catabolism (Strous et al. 2006, Kartal et al. 2011). These reactions include the reduction of nitrite to nitric oxide via a cd1 nitrite reductase encoded by *nirS* gene (which is distinct from denitrifier *nirS*) (Lam et al. 2009), the condensation of ammonium and nitric oxide into hydrazine via a hydrazine synthase encoded by *hzs* gene, and the oxidation of hydrazine into

di-nitrogen gas via a hydrazine oxidoreductase encoded by the *hzo* gene (van de Vossenberg et al. 2013).

In marine nitrogen budgets, the benthic contribution for N_2 production is on average 28%, and despite the assumption that in estuarine systems it shows a minor importance (Thamdrup 2012), recent studies strengthen the importance of the anammox reaction in removing fixed nitrogen from estuarine systems. Previous studies in the Douro River estuary showed an important contribution of anammox to N_2 production which reached 54%, with an average of 25% (Teixeira et al. 2012, Teixeira et al. 2014).

3.4 Denitrification

Denitrification is responsible for input of nitrogen gas into atmosphere through a series of reduction pathways mediated by microbial communities under anaerobic conditions, where the final product is nitrogen gas (Thamdrup 2012).

The denitrification process consists in the dissimilatory reduction of nitrate (NO_3^-) to nitrite (NO_2^-), its subsequent reduction to nitric oxide (NO), then to nitrous oxide (N_2O) and finally to nitrogen gas (N_2) (Thamdrup 2012). These reactions are closely coupled and thus nitrite, nitric oxide and nitrous oxide, rarely accumulate in large amounts in the environment.

These pathways are mediated by organisms from all domains which normally are involved in several steps of the denitrification process, and capable of different respiratory pathways including oxygen respiration (Zhang et al. 2014). The enzymes and genes responsible for these pathways are the nitrate reductase encoded by *narG* or *napA* gene (Smith et al. 2007), the nitrite reductase encoded by the *nirK* or *nirS* gene (Theerachat et al. 2011, Chen et al. 2014b), the nitric oxide reductase encoded by the *norB* gene (Casciotti and Ward 2005), and the nitrous oxide reductase encoded by the *nosZ* gene (Orellana et al. 2014).

Denitrification plays an important role in nitrogen enriched estuarine systems since it permanently removes nitrogen from the system counteracting eutrophication (Howarth 1998, Thamdrup and Dalsgaard 2002).

3.5 Dissimilatory nitrate reduction to ammonium (DNRA)

Dissimilatory nitrate reduction to ammonium (DNRA) consists on the transference of eight electrons (Woods 1938, Cole and Brown 1980, Gardner et al. 2006, Kraft et al. 2011). This process can occur under two different pathways, the fermentative and the sulphur pathway (Burgin and Hamilton 2007, Roberts et al. 2014).

The fermentative pathway, as the name suggests, consist on fermentative reactions by coupling electron flow from organic matter to the reduction of nitrate. Substantially different from the fermentative pathway, the sulphur pathway involves chemolithoautotrophic organisms (some able to perform both DNRA and denitrification) that are capable to combine the reduction of nitrate with the oxidation of reduced sulphur forms like H_2S and S_2^- (Gu et al. 2012) as well elemental sulphur (Kraft et al. 2011).

The dissimilatory nitrate reduction to ammonium involves two sub steps, the reduction of nitrate to nitrite, catalysed by the membrane bound NAR or NAP enzymes, where NAR has been shown to be most effective under high nitrate conditions, and NAP under low nitrate conditions (Potter et al. 1999). The nitrite reduction to ammonium is catalysed by the nitrite reductase, NRF enzyme (Kraft et al. 2011, Papaspyrou et al. 2014).

The DNRA represents an important process for coastal nutrient budgets, since it reduces nitrate into a more available form of nitrogen (ammonium) in the system (Santoro 2009).

3.6 Nitrification

Nitrification is an aerobic process carried out mainly by chemolithoautotrophic microorganisms, which are strictly prokaryotes (bacteria and archaea). It represents the oxidative part of the nitrogen cycle, completing the redox cycle from the most reduced (NH_3) to the most oxidized form (NO_3^-) (Bothe et al. 2006).

The process of nitrification involves two steps where first, the ammonia is oxidized to nitrite and the secondly the nitrite is oxidized to nitrate. The oxidation of ammonia to nitrite does not occur directly, being also a two-step process, where ammonia is oxidized to hydroxylamine, shown in equation 1, which is catalysed by the ammonia monooxygenase,

AMO enzyme (Norton et al. 2002, Junier et al. 2010b, Daims et al. 2016), and subsequently hydroxylamine is oxidized to nitrite, shown in equation 2, which is catalysed by the hydroxylamine reductase, HAO enzyme (Junier et al. 2010a). This is a rate-limiting step due to the fact that NO_2^- rarely accumulates, and therefore critical for global nitrogen cycle (Kowalchuk and Stephen 2001, Martens-Habbena et al. 2009, Cortés-Lorenzo et al. 2015). The oxidation of nitrite to nitrate, shown in equation 3, is catalysed by the nitrite oxidoreductase, NXR enzyme (Pester et al. 2014).

$$NH_3 + \frac{1}{2}O_2 \longrightarrow NH_2OH \quad (\mathbf{1})$$

$$NH_2OH + O_2 \longrightarrow NO_2^- + H_2O + H^+ \quad (\mathbf{2})$$

$$NO_2 + \frac{1}{2}O_2 \longrightarrow NO_3^- \quad (\mathbf{3})$$

The energy yield by ammonia-oxidizing microorganisms is low, reflecting on a slow growth rate of these organisms with generation times varying between 0.34 to 2.2 day^{-1} for AOB (Blackburne et al. 2007) and 0.2 to 0.8 day^{-1} for AOA (Stieglmeier et al. 2014). Consequently, when cultivated it can take several months until they reach a pure culture (Horz et al. 2000).

Nitrification is carried out by a limited group of prokaryotes. It was believed to be exclusively performed by bacteria, the ammonia-oxidizing bacteria (Kowalchuk and Stephen 2001) and the nitrite oxidizing bacteria. However, new discoveries proved that archaea also played an important role in the oxidation of ammonia to nitrite (Francis et al. 2005, Konneke et al. 2005).

3.6.1 Ammonia-oxidizing bacteria

In 1890, it was reported for the first time the isolation of an ammonia-oxidizing bacterium (Winogradsky 1890), and with it the chemoautotrophic concept, characterized by

the capacity of oxidizing inorganic compounds with molecular oxygen to acquire energy (Winogradsky 1890). Since then, numerous studies have been performed focusing on this specific group of organisms (Purkhold et al. 2000, Magalhães et al. 2007, Chen et al. 2014a, Monteiro et al. 2014).

Nitrosomonas europaea (Winogradsky 1890) was the first AOB isolated and became one of the most studied ammonia-oxidizing species regarding its physiology, phylogeny and biochemistry (Chain et al. 2003, Monteiro et al. 2014). These organisms are difficult to isolate, due to their low energy yield from ammonia oxidation, reflecting in a slow growth and consequently low biomass (Head et al. 1993, McCaig et al. 1994, Utaker and Nes 1998). Thus, these organisms can take several months to develop into small colonies that are highly vulnerable to heterotrophic contaminations (McCaig et al. 1994, Pommerening-Röser et al. 1996). Despite these limitations, based on morphological and ultrastructural characteristics, it was possible to discriminate 5 genera of AOB cultured representatives: the *Nitrosomonas*, the *Nitrosococcus*, the *Nitrosospira*, the *Nitrosolobus* and the *Nitrosovibrio* (Koops et al. 2006).

With the application of culturing independent tools, AOB distribution, diversity and phylogeny start to be reported in many ecosystems such as lakes (Jiang et al. 2009, Bollmann et al. 2014), rivers (Cebron et al. 2004), estuaries (Bernhard et al. 2010b), oceans (Bano and Hollibaugh 2000) and terrestrial soils (Sims et al. 2012), which greatly improve our knowledge and understanding of the level of importance of the different AOB to the ecosystem (Rotthauwe et al. 1997, Purkhold et al. 2003).

Nowadays, based on their 16S rRNA, AOB are classified in two monophyletic lineages included in Gammaproteobacteria and Betaproteobacteria (Junier et al. 2010b). The Gammaproteobacteria is represented by the *Nitrosococcus* genus including 3 species, *Nitrosococcus halophilus* (Ward and O'Mullan 2002), *Nitrosococcus oceani* (Ward and O'Mullan 2002) and the most recent discovered *Nitrosococcus watsonii* sp. (Campbell et al. 2011). The AOB Betaproteobacteria can be subdivided into two lineages, the *Nitrosospira*-lineage and the *Nitrosomonas*-lineage (Dang et al. 2010, Cao et al. 2012). Based on the analyses of 16S rRNA and *amoA* gene sequences, the *Nitrosomonas*-lineage can be subdivided into 5 sub-lineages (Figure 3) including the *Nitrosomonas Oligotropha*, *Nitrosomonas marina*, *Nitrosomonas europaea / Nitrosococcus mobilis*, *Nitrosomonas*

commnunis and *Nitrosomonas* sp. NM 143 (Purkhold et al. 2003). Besides these 5 sublineages, the *Nitrosomonas crytolerans* seems to form an independent sublineage (Purkhold et al. 2003, Dang et al. 2010, Cao et al. 2012). The *Nitrosospira*-lineage which comprehends the genus *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* can be subdivided into 4 clusters (Purkhold et al. 2003, Dang et al. 2010, Cao et al. 2012).

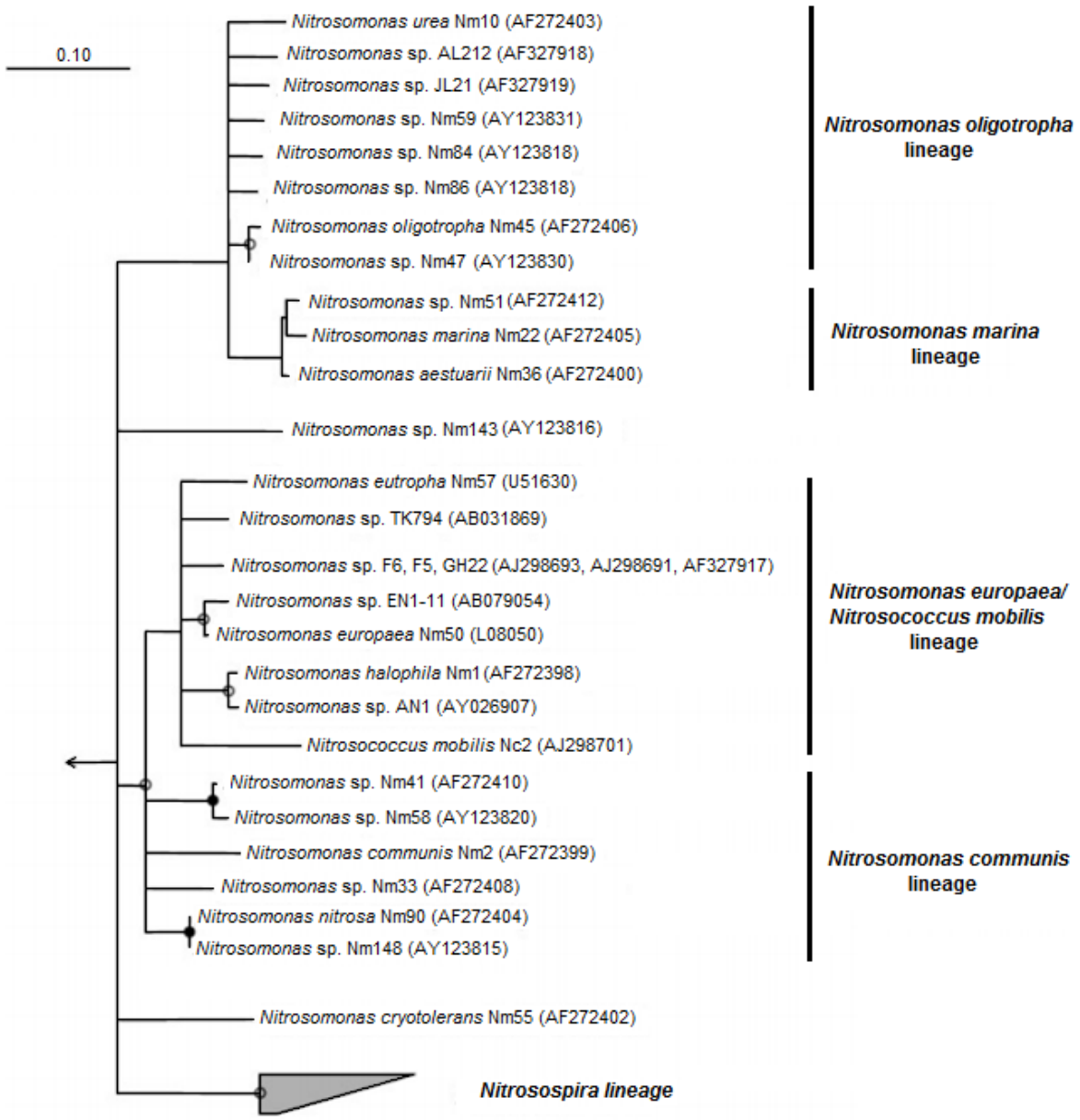


Figure 3: *AmoA*-based phylogenetic tree of the Betaproteobacterial AOB adapted from Purkhold et al. (2003). The 453 bp *amoA* gene fragment (Rotthauwe et al., 1997) was used for phylogeny inference. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90 and 70 %, respectively.

3.6.2 Ammonia-oxidizing archaea

A decade ago, pioneering studies on metagenomic libraries from seawater (Venter et al. 2004) and soils (Treusch et al. 2005) revealed the existence of putative genes involved in ammonia oxidation in genomic fragments derived from uncultivated archaea belonging to the Crenarchaeota phylum. The final proof of the discovery of archaea ammonia oxidation was obtained by the cultivation of an ammonia-oxidizing archaea (AOA) named *Candidatus Nitrosopumilus maritimus* (Konneke et al. 2005). Afterwards, other successful enrichment and cultivation studies of AOA demonstrated ammonia oxidation aptitude within the Archaeal domain, including *Nitrosocaldus yellowstonii* (de la Torre et al. 2008), *Nitrososphaera gargensis* (Hatzenpichler et al. 2008), *Nitrosoarchaeum limnia* (Blainey et al. 2011), *Nitrosotalea devanaterra (Lehtovirta-Morley et al. 2011)*, *Nitrosphaera viennensis* (Tourna et al. 2011), *Nitrosoarchaeum koreensis* (Jung et al. 2011b) and *Nitrosopumilus salaria* (Mosier et al. 2012a).

These mesophilic archaea were placed initially as a sister group of the Crenarchaeota and named as "non-thermophilic Crenarchaeota" (Pester et al. 2011). More recently, it was suggested that the non-thermophilic Crenarchaeota constituted a separate phylum of Archaea that branched off before the separation of Crenarchaeota and Euryarchaeota (Brochier-Armanet et al. 2008). The Thaumarchaeota (the Greek "Thaumas", meaning wonder) was therefore proposed as a novel third Archaeal phylum (Brochier-Armanet *et al.*, 2008). Additionally to phylogenetic analysis, the presence of the lipid crenarchaeol in all AOA is also consistent with a separate placement of this group on the archaeal tree (Pester et al. 2011).

Nowadays, all known AOA from the Thaumarchaeota phylum can be subdivided into 4 lineages (Figure 4) from which, each lineage name is derived from the genus name of the first cultured representative of each respective group such as the marine Group 1.1a (represented by the *Nitrosopumilus* cluster) and 1.1a-associated (represented by the *Nitrosotalea* cluster), the soil group 1.1b (represented by the *Nitrososphaera* cluster) and the ThAOA group represented by thermophilic *Nitrosocaldus* cluster (Pester et al. 2011, Hatzenpichler 2012, Pester et al. 2012, Stahl and de la Torre 2012, Marusenko et al. 2013, Zhou et al. 2015).

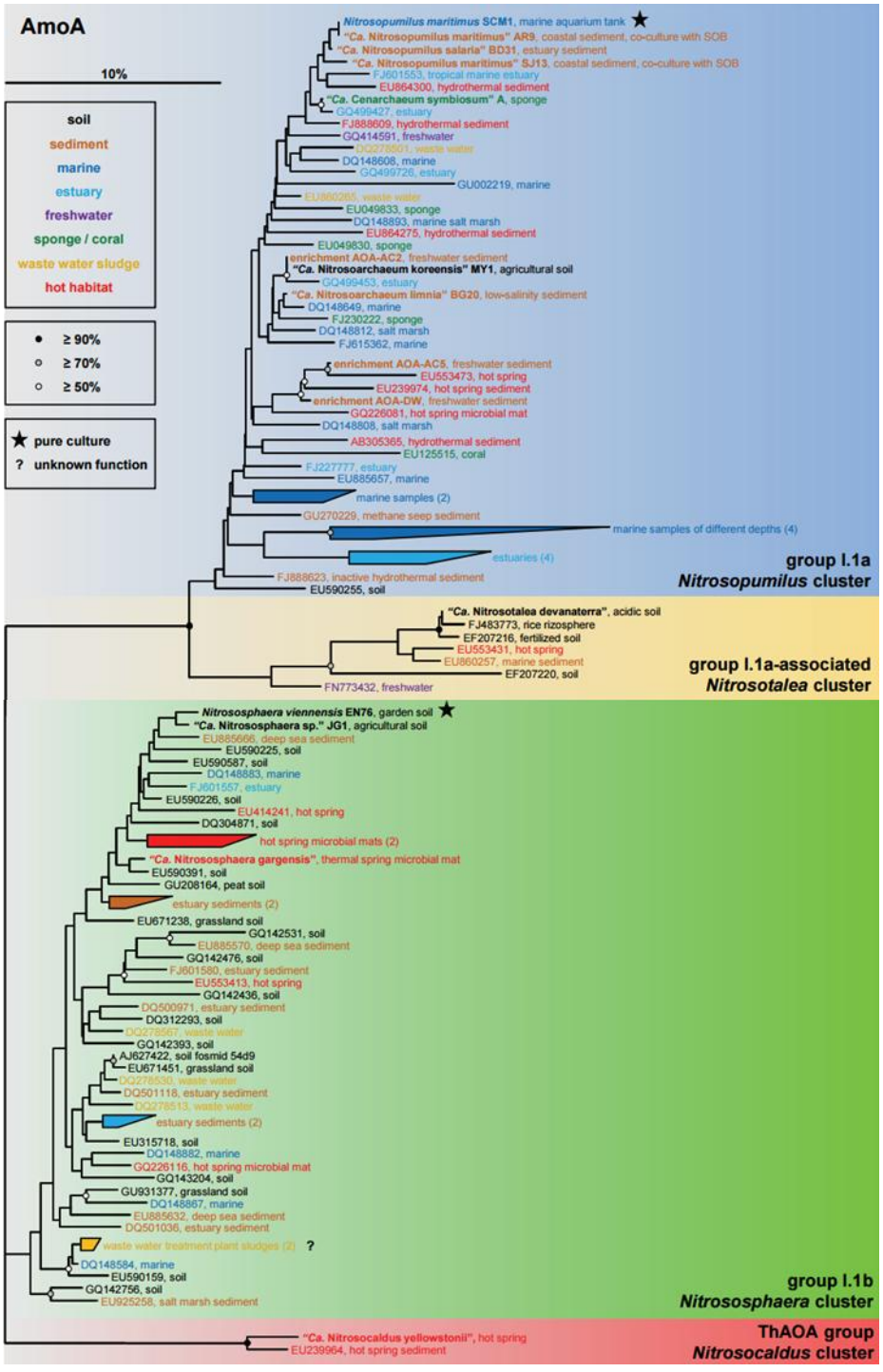


Figure 4: Phylogenetic analysis of a representative selection of archaeal ammonia monooxygenase subunit A (*amoA*)-like sequences demonstrating the major lineages of archaea ammonia-oxidizers (Hatzenpichler, 2012). Group names refer to the traditional names adopted from 16S rRNA phylogeny.

3.6.3 Nitrite-oxidizing bacteria

The nitrite-oxidizing bacteria (NOB) play a key role in nitrification by oxidizing nitrite (NO_2^-) to nitrate (NO_3^-). This group of bacteria are widely distributed in nature, and have adapted to variable environmental conditions, and therefore be found on moderate aquatic and terrestrial ecosystems but also in extreme environments such as acidic habitats (González-Toril et al. 2003), geothermal springs (Lebedeva et al. 2011) and permafrost soils (Alawi et al. 2007).

The NOB are phylogenetically a relatively heterogeneous group being composed by seven genera of aerobic chemolithoautotrophic bacteria, such as *Nitrobacter*, *Nitrotoga*, *Nitrococcus*, *Nitrospira*, *Nitrospina*, *Nitrolancea*, and '*Candidatus Nitromaritima'*. Whereas *Nitrobacter* is an Alphaproteobacterium (Woese et al. 1984), *Nitrococcus* is a Gammaproteobacterium (Woese et al. 1985, Teske et al. 1994). *Nitrospina* was initially classified as a Deltaproteobacteria (Teske et al. 1994), but analyses using larger 16S rRNA gene sequence databases have suggested that *Nitrospina* belongs to a distinct bacterial phylum, the phylum "Nitrospinae" (Schloss and Handelsman 2004, Spieck et al. 2014). The genus *Nitrospira* occupies its own deep-branching lineage, and therefore is a major lineage of the bacterial phylum, Nitrospirae (Watson et al. 1986, Ehrich et al. 1995, Spieck and Bock 2005). The genus *Nitrotoga* is a member of the Betaproteobacteria (Alawi et al. 2007); the genus *Nitrolancetus* is a member of the phylum Chloroflexi (Sorokin et al. 2012); and lastly the new candidate genus of uncultured marine NOB, '*Candidatus Nitromaritima*', which is related to *Nitrospina* and was proposed on the basis of comparative analyses of single-cell amplified genomes as a new candidate genus of uncultured marine NOB (Ngugi et al. 2016).

The key enzyme of NOB is the nitrite oxidoreductase, NXR enzyme (Daims et al. 2016). Most biochemical investigations have been performed in *Nitrobacter* (Vanparys et al. 2007), where NXR is a membrane-bound enzyme containing one large (alpha) and one small (beta) subunit that are encoded by the genes *nxrA* and *nxrB,* respectively (Kirstein and Bock 1993, Poly et al. 2008). Moreover, the *nxrB* gene is a powerful functional phylogenetic marker to detect and identify uncultured NOB (Pester et al. 2014).

In spite of the importance of NOB on ecosystems, our current knowledge of their biology is still very incomplete (Daims et al. 2016). Nevertheless, recently new approaches

such as metagenomics, genomics, single-cell isotope labelling and improved cultivation methods have been applied on the study of this group of bacteria (Stepanauskas 2012, Albertsen et al. 2013, Wilmes et al. 2015). These studies culminated with the discovery that nitrite oxidizing bacteria are able to reduce cyanate to ammonia, and therefore, used by ammonia oxidizers to form nitrite in a "reciprocal feeding" pattern (Palatinszky et al. 2015). Additionally, it was described for the first time that two species of *Nitrospira* bacteria able to perform both steps of nitrification (van Kessel et al. 2015, Daims et al. 2016), this capacity has been nominated as comammox (complete ammonia oxidation).

3.6.4 Environmental controls on ammonia-oxidizing microorganisms

It is known that microorganisms are affected by environmental and chemical parameters, which are constantly interacting with their metabolism. These parameters model the abundance, distribution, composition and activity of microbial communities, and therefore understanding the effect of environmental variables is crucial to comprehend the natural dynamics of the microbial communities involved in the different biogeochemical pathways. Over the past decades several variables have been identified as key controllers of niche differentiation, abundance and activity of ammonia-oxidizing microorganisms (Strauss et al. 2002, Hatzenpichler 2012, Xing et al. 2015). Here we will describe some of the principal environmental factors shaping these group of organisms in natural habitats.

The effect of light has been studied on AOB cultures over the years (Lipschultz et al. 1985, Guerrero and Jones 1997) suggesting that light act as a photoinhibitor of nitrification in many ecosystems, and that this inhibition acts on the first step of the nitrification (Hyman and Arp 1992). Studies about the distribution of archaeal *amoA* genes under light and dark conditions (Merbt et al. 2011) as well as along a vertical profile in the Atlantic Ocean (Church et al. 2010) suggested that AOA could also be sensitive to light. As a result of these conclusions, Merbt et al. (2012) performed a study regarding the effect of different light intensities on bacterial and archaeal ammonia oxidation with the support of several laboratory AOA and AOB cultures. Results showed that AOA were more sensitive to light than AOB, and

recovered slower after light exposure. Therefore, light acts as a photoinhibitor of AOA and AOB and thus may contribute to AOB and AOA niche differentiation.

Nitrification is an aerobic process and as a result, oxygen plays an important role in nitrification by acting as a substrate for the AMO enzyme, and acting as a terminal electron acceptor (Zhalnina et al. 2012). Studies regarding the effect of oxygen levels on ammonia-oxidizing organisms have shown that AOA are more abundant than AOB under low oxygen estuarine environments (Abell et al. 2011). Moreover, Jung et al. (2011b) using kinetic respirametry assays showed that the archaea strain *Candidatus Nitrososphaera gargensis* MY1 have much higher affinities for oxygen than ammonia-oxidizing bacteria. In addition, according with several studies (Hatzenpichler 2012), some marine AOA might display preference for regions of low dissolved oxygen.

Temperature is one of the most significant factors affecting nitrification, however studies have shown different effects on ammonia oxidizers microorganisms (Tourna et al. 2008, Zeng et al. 2014), and that AOA and AOB display different ranges in temperature tolerance. In fact, in a study from Zeng et al. (2014), highest diversity of bacterial *amoA* gene was found at 25ºC, as well that significant growth of AOB could be found between 15ºC and 25ºC. Additionally, no AOB were found in environments where temperature was continuously above 40ºC (Hatzenpichler 2012), and therefore, it might represent their maximum temperature limit. Concerning their lower limit, a study reported that *Nitrosomonas cryotolerans* could grow under -5ºC (Koops et al. 2006).

In the case of archaea, most of the AOA identified on marine and soil environments are non-thermophilic comprehending usually temperatures between 22ºC and 37ºC (Konneke et al. 2005, Jung et al. 2011a, Tourna et al. 2011). In fact, Cao et al. (2011) performed a study on AOA and AOB *amoA* genes in surface sediments where temperature ranged from 2.9ºC to 21.3ºC and found no significant correlation between temperature and the abundances of both archaeal and bacterial *amoA* genes. In agreement, Wu et al. (2013a) found that abundances of archaeal *amoA* gene were comparable in the 15ºC and 25ºC treatment groups, however, a significant decline in the abundance of archaeal *amoA* gene was found in the 37ºC treatment. Different AOA ecotypes might have different responses to temperature, since it has been demonstrated that community structure of active AOA clearly

changed in soil microcosms incubated under different temperatures, 10ºC to 30ºC (Tourna et al. (2008).

However, not all AOA are non-thermophilic and even those have different responses to temperature. Archaeal *amoA* gene have been detected in Artic coastal waters with AOA *amoA* levels and nitrification rates higher in winter (−1.7ºC) (Christman et al. 2011); and under hot springs in Iceland with 97ºC (Reigstad et al. 2008). Thus, distinct tolerances within AOA species have been reported across many environments suggesting a specific adaptation of each group. For example, Zhao et al. (2011) found that AOA richness indexes in hot springs (42ºC to 87ºC) were higher at temperatures below 75ºC, indicating that AOA may be favoured in moderately high temperature environments. Indeed, the moderate thermophile AOA, *Candidatus Nitrososphaera gargensis*, has an optimum growth temperature of about 46ºC (Hatzenpichler et al. 2008). Furthermore, AOA strains that tolerate higher temperatures have been isolated from geothermal habitats and can grow at temperatures as high as 74ºC (de la Torre et al. 2008) and 97ºC (Reigstad et al. 2008).

Another well-known major driver of niche segregation among ammonia oxidizers is pH. Several studies have focused on the *amoA* gene abundance of these two groups of organisms (AOA and AOB) under different pH environments such as acidic soils (Gubry-Rangin et al. 2010, Zhang et al. 2012), sediments (de Gannes et al. 2014, Xing et al. 2015) and alkaline soils (Prosser and Nicol 2012). These results showed that AOA are more tolerant to low and high pH levels than AOB. The *Candidatus Nitrosotalea devanaterra* is an example of an obligate acidophilic AOA with the ability to grow under extremely low concentration of ammonia under very low pH between 4.0 to 5.5 (Lehtovirta-Morley et al. 2011). This behaviour might be due to the high affinity for ammonia by AOA that allows certain ecotypes to grow under low concentrations of ammonia (Zhalnina et al. 2012), as well the fact that at low pH levels the reduction of NH_3 into NH_4^+ occurs and therefore the energy necessary to use NH_4^+ by these organisms increases (Nicol et al. 2008). Contrary to AOA, most cultivated bacterial ammonia-oxidizers do not grow below pH 6.5 (Jiang and Bakken 1999). Therefore, in acidic conditions archaeal ammonia oxidation has advantages to outcompete with AOB (He et al. 2012).

Ammonia is the substrate of ammonia oxidation, and therefore its concentration is expectable to be an essential factor in ammonia oxidizers niche segregation. Several studies have reported AOA dominance under low ammonia environments vs AOB dominance in high ammonia environments. For instance, Höfferle et al. (2010) reported that AOA were highly active and able to grow well, if the concentration of ammonia was relatively low or ammonia was directly supplied through mineralization of organic matter. This phenomenon might be explained due to the higher affinity of AOA to ammonia compared with AOB (Martens-Habbena et al. 2009). Contrary, under high ammonia levels, it was reported that AOB were more competitive and outnumbered the AOA (Di et al. 2009, Verhamme et al. 2011). The advantage selection of the different groups of ammonia oxidizers under different NH_3/NH_4^+ concentration conditions is explained by the K_m of AOA for NH_3/NH_4^+ to be nearly 1,000 times lower than the K_m of AOB (Martens-Habbena et al. 2009, Jung et al. 2011b).

The effect of salinity on nitrification rates was first described in 1884, when Warington (1884) realized that the presence of 0.032% of sodium bi-carbonate reduced the nitrification process, and that in the presence of 0.096% nitrification was considerably decreased. Since then, numerous approaches have been undertaken to study the role of salinity on these communities, particularly studies (e.g. Sánchez et al. 2005, Magalhães et al. 2005b, Sebilo et al. 2006, Zhao et al. 2014) focusing on the potential nitrification activity under different salinities. Additionally, the use of potential AOA and/or AOB inhibitors allows the distinction of AOA vs AOB activity (Shen et al. 2013, Sonthiphand and Neufeld 2014, Hu et al. 2015, Martens-Habbena et al. 2015). Another approach to study the effect of salinity in nitrifiers is the assessment of the *amoA* gene expression and/or abundance (Bernhard et al. 2007, Magalhães et al. 2009, Bernhard et al. 2010b, Fukushima et al. 2012); or through the 16S rRNA genes and/or *amoA* rRNA genes diversity (Nicolaisen and Ramsing 2002, Magalhães et al. 2007, Cao et al. 2012). The *amoA* transcripts are used as an indicator of the microbial community responsible for ammonia oxidation, the AOA and AOB (Santoro 2009). Furthermore, the more complete method is the combination of the previous approaches (Cortés-Lorenzo et al. 2015, Smith et al. 2015, Zhang et al. 2015). However, despite all the efforts on responding questions about these microorganisms, there are still significant

questions about the contribution of AOA and AOB to *in situ* nitrification rates (Martens-Habbena et al. 2015).

Salinity plays an important role on nitrification by limiting the distribution and functionality of the ammonia oxidizers (Magalhães et al. 2005a, Santoro et al. 2008, Li et al. 2015), since it might affect nitrifiers directly due to osmotic pressure, affecting their activity, abundance and/or diversity (Bassin et al. 2011). However, it is important to realize that ammonia oxidizers are composed by two different groups of organisms, the AOA and AOB, and therefore different responses might be expected (Zhang et al. 2015) as well as some species of AOB are limited to a narrow habitat, while others tolerate a much wider range of environmental conditions (Sahan and Muyzer 2008). These findings are supported by Cortés-Lorenzo et al. (2015) that through the sequencing of *amoA* genes found that some OTUs from the control experiment were still present at the NaCl treatment, suggesting a wide tolerance range to salinity. Moreover, the same study showed that concentrations of 24.1 and 44.1 g of NaCl/L promoted the emergence of new OTUs phylogenetically related to AOB already described for saline environments.

Even though salinity affects ammonia-oxidizing organisms, the majority of the results are not consensual. For example, while in Plum Island Sound estuary AOA were always greater than AOB along the salinity gradient (Bernhard et al. 2010b), studies performed in Colne estuary and in other estuaries showed higher abundances of AOB at higher salinities comparing to the constant abundances of AOA along the estuary (Santoro et al. 2008, Magalhães et al. 2009, Li et al. 2015). [One reason for that is the marked gradient of NH_4^+ concentrations, temperature or O_2 levels which, along with salinity, that are known to characterize the estuarine systems (Mosier and Francis 2008)]. Many estuaries have shown shifts in AOA and AOB communities along the salinity gradient (Beman and Francis 2006, Freitag et al. 2006, Mosier and Francis 2008, Santoro et al. 2008, Li et al. 2015), meaning that the different groups of organisms involved in ammonia oxidation have different levels of adaptation to salinity and niche specialization related with specific abiotic parameters (Beman & Francis 2006, Erguder *et al.* 2009; Stahl and de la Torre 2012; Hugoni *et al.* 2015) and therefore, salinity might be a significant factor in determining AOA and AOB communities diversity, distribution and activity.

4. Goals of this study

As previously mentioned, nitrogen (N) is a key element for the functionality of the estuarine systems once primary production is limited by nitrogen supplies. Nitrification plays an important role in the nitrogen cycle, providing the key link between mineralization of organic matter and the loss of fixed nitrogen by denitrification. Furthermore, the importance of environmental factors in modulating the abundance, diversity and activity of nitrifying microorganisms are crucial when studying this group of organisms. From those factors, salinity has been shown to play an important role in regulating these communities in environmental systems. In addition, previous research in the Douro River estuary demonstrated seasonal variations and a positive influence of intermediate salinities on nitrification (Magalhães *et al.* 2005a). Additional quantifications of ammonia-oxidizing communities at the mouth of the Douro estuary suggested higher abundances of AOB over AOA, which could indicate AOB as the main contributors for nitrification (Magalhães et al. 2009). Nonetheless, we believe that the marked salinity fluctuations on an estuarine system is a key element in defining the plasticity of AOA and AOB to cope with salinity changes in short time scales. Thus, in this thesis project, we conducted a series of experiments to investigate shifts on AOA and AOB, to assess the effect of salinity gradients and fluctuations on their structure, abundance and activity. For that purpose, we organize this thesis in two chapters.

In Chapter I, the effect of a saline gradient in selecting bacteria and archaea ammonia oxidizers from two distinct locations in Douro River estuary was evaluated. For that purpose, a controlled experiment was designed where microbial communities from Douro estuarine sediments were exposed to three levels of salinity (0‰, 15‰ and 30‰). Fingerprinting techniques of AOA and AOB *amoA* gene transcription and isotopic measurements of nitrification rates using specific inhibitors to access the activity of AOA and AOB separately were used to link the biotic communities with their biogeochemical signatures.

In Chapter II, an experiment was designed to evaluate the AOA and AOB response to a salinity fluctuations regime vs a constant salinity regime during 36 days of exposure. The plasticity of AOA and AOB to cope with salinity fluctuations was measured by recording AOA, AOB and total nitrification rates using isotope paring techniques as well as fingerprinting techniques of AOA and AOB *amoA* transcripts, and sequencing of the 16S rRNA gene using next-generation sequencing (NGS) analysis.

Material and Methods

1. Description of the sampling area

The selected area for this study was Douro River estuary (Figure 5). The Douro River is one of the major rivers of the Iberian Peninsula (IP) with 930 km length. Douro River has the largest watershed (97682km^2) of IP, covering about 17% of the peninsula (Vieira and Bordalo 2000). It drains into the Atlantic Ocean (41º08'N 8º40'W) between Porto and Vila Nova de Gaia cities. It is a river marked by several dams, in 1985 it was constructed the last dam in Crestuma, 21.6 km from the mouth, becoming the artificial upstream limit of the Douro River estuary, with an impact on the river discharges nearby 48% (Vieira and Bordalo 2000). As a result of the construction of these dams, river inflow is characterized by pulse discharges instead of continuous flow of freshwater, particularly during summer where in a

matter of minutes, flow can rise from 0 to 1000 m^{3}s^{-1}.

Figure 5. Douro River estuary with, Afurada (bottom left image) and Crestuma (top right image) photos of each sampling location.

According with Vieira and Bordalo (2000), the average spring tidal range is 2.8 m at the mouth and 2.6 m at the head of the estuary. The estuary has 3 stretches, the lower

estuary that extends 3 km from the mouth and present an average width and depth of 645 m and 7.8 m respectively; the middle estuary with 271 m and 10 m respectively; and the upper part of the estuary with 333 m and 7 m, respectively.

Geologically, it is characterized by the predominance of granite, highly deformed metasedimentary units and cenozoic sediments (Balsinha et al. 2009). Nonetheless, it is possible to establish a classification of the sediment deposits variation along the estuary. Sandy gravel deposits characterize the upstream part of the estuary, and the downstream part is characterized by silts, clays, sandy gravel and coarse sand (Mucha et al. 2005, Balsinha et al. 2009).

Bathymetry is characterized by a regularly dredged navigation channel of 6 m deep and a V-shaped cross-section with asymmetric slopes. Daily freshwater discharges by the Douro River ranges from 0 to 13000 m^3s^{-1}, with an average of 505 m^3s^{-1} (Azevedo et al. 2010, Azevedo et al. 2014). Complementary, along the estuary there are 13 small tributaries supplying freshwater with an average flow around 0.1 to 5 m^3s^{-1} (Azevedo et al. 2010).

The Douro estuary is also characterized as a salt wedge due to the sea water excursion during flood and/or under low river flow (less than 800 m^3s^{-1}). This salt wedge can eventually reach the head of the estuary where the tidal excursion is halted by the Crestuma dam, remaining in the estuary till the next ebb (Vieira and Bordalo 2000, Azevedo et al. 2006).

The last 8 km are populated by more than 700,000 inhabitants around the estuarine area, with eight wastewater treatment plants that drains into the estuary (Azevedo et al. 2008, Delibes-Mateos et al. 2014). The lower part is also characterized as heavily polluted where metals such as Zinc, Copper, Lead, Cadmium and Chromium can be found which affect the communities' structure (Mucha et al. 2005)

The Douro River estuary is an heterotrophic estuary, yet some benthic intertidal sediments are autotrophic being important areas for nutrient transformation and/or sequestration which consequently play an important role in the removal of inorganic nutrient from the water column (Magalhães et al. 2005a).

2. Sample collection

In order to accomplish our goal, two distinct sites along the salinity gradient of the Douro River estuary were selected (Afurada and Crestuma). While Afurada is located in the mouth of the estuary (downstream estuary), a highly dynamic site with marked salinity changes, Crestuma is located upstream of the estuary, highly influenced by the river water and thus with lower salinities.

Surface sediments (2 cm depth) were collected at each site and homogenized in sterile plastic bags (1 L) and plastic boxes (5 L) for the experiments of Chapter I and Chapter II respectively, and stored in refrigerated ice chests. In laboratory, sub-samples were collected in sterile plastic bags and stored at -80ºC for later DNA extraction.

Water was collected from each site into cleaned polyacrylamide bottles and stored in refrigerated ice chests. In the laboratory, it was filtered through 0.7 µm GF-F glass microfiber filters (Whatman®) and stored at -20ºC for later nutrient analyses. Water temperature and salinity were also measured from each local with the conductivity meter CO 310 (VWR® Collection).

3. Salinity gradient experiment

The experimental design consisted of exposing the two types of sediments to three different salinities (0‰, 15‰ and 30‰), all in triplicates. Twenty milliliters of sediment were introduced in each 50 mL serum bottle and weighted. Water previously collected from Douro River (0‰) was adjusted for the desired final salinities 0‰, 15‰ and 30‰ with marine salts (Trophic Marin®). Forty milliliters of water with 100 µM of $^{14}NH_4^+$ were added to each bottle and left in the dark overnight, representing the acclimatization period. Inhibitor treatments for bacteria (sulfathiazole) and archaea (PTIO) were also applied to distinguish nitrification activity between bacteria and archaea ammonia oxidizers (Figure 6). Inhibitor solutions with the desired concentration were prepared before the experiments with $^{14}NH_4Cl$ (≥ 99.5%,

Aldrich) solution 10 mM, sulfathiazole (99%, Alfa Aesar) solution 100 mM and PTIO (2-phenyl-4, 4, 5, 5-tetramethyl imidazoline-1-oxyl 3-oxide; > 98%, Aldrich) solution 10 mM.

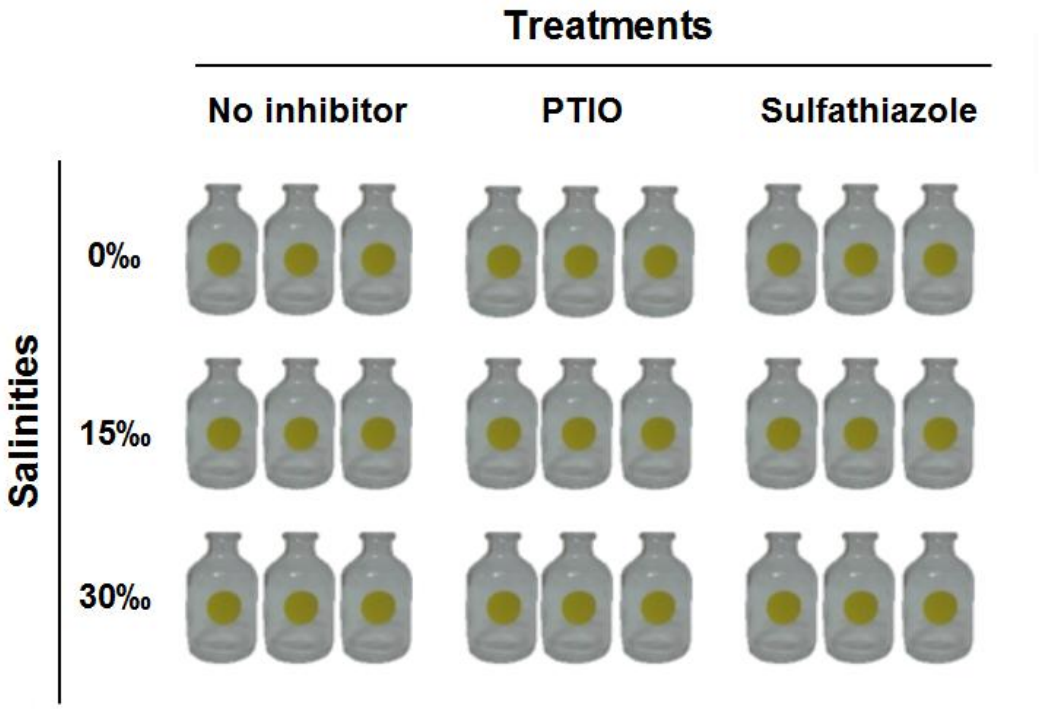


Figure 6. Scheme of the batch experiment with the different salinities and inhibitors treatments in triplicate.

On the following day, water was removed from the serum flasks. Forty milliliters of water enriched with ammonium (70 µM of $^{15}NH_4^+$ and 30 µM of $^{14}NH_4^+$) were added. PTIO and sulfathiazole was added to the respective treatments at concentrations of 100 µM and 1500 µM, respectively. All flasks were mixed properly previously starting the incubation. The incubation period was 3 hours, and at each hour of incubation (0h, 1h, 2h and 3h), 2 mL of overlying water was collected, centrifuged at 2500 rpm and stored at -20ºC for later nutrient analyses. Additionally, at time 0h and 2h, 8 mL of overlying water was collected, centrifuged at 2500 rpm, filtered at 0.45 µm and stored at -20ºC for later isotopic analyses.

At the end of the incubation period, composed sediment samples from treatments without inhibitors at different salinities were collected for total RNA and DNA analyses.

4. Salinity fluctuations experiment

The salinity fluctuations experiment consisted of two treatments per site (Afurada and Crestuma) performed in triplicate. The salinity fluctuations were achieved by an oscillation between 0‰, 15‰ and 30‰, and a constant salinity treatment characterized by a constant salinity of 0‰ (Crestuma) and 15‰ (Afurada), as shown in Figure 7.

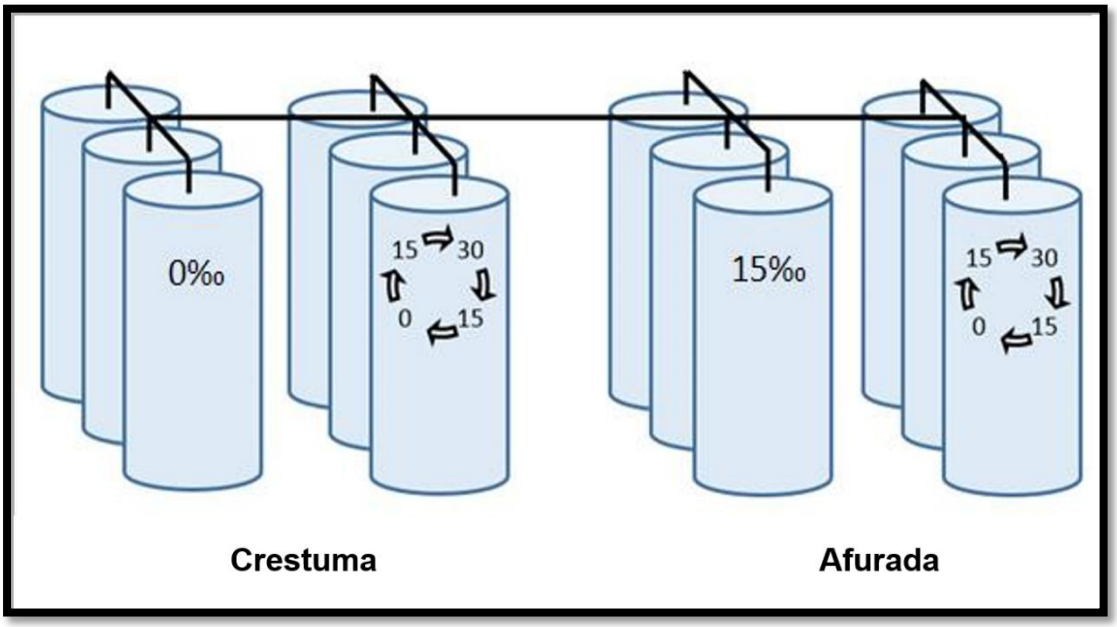


Figure 7. Scheme of the salinity fluctuations experiment (constant and salinity fluctuations treatments) for Afurada and Crestuma sediments in triplicate.

Sediments collected in Afurada and Crestuma were added to each incubation core (708.47 cm^3, 10 cm depth). Then, 1.2 L of water previously collected from Douro River (0‰) and adjusted for the desired final salinities (0‰, 15‰ and 30‰) with marine salts (Trophic Marin®), were added along with 20 µM of $^{14}NH_4^+$ to each core according to Figure 7. Every twelve hours, water with 20 µM of $^{14}NH_4^+$ was exchanged with respective salinity, simulating the salinity fluctuations. Salinity and water temperature were monitored before the change of the water using a conductivity meter CO 310 (VWR® Collection).

Cores used in the experiment were made from acrylic with 14 cm diameter and 33 cm height. An autonomous water agitation system was set up for the 12 cores (Figure 8), with a velocity set at 32 rotations per minute to maintain water homogenized and oxygenized. The photoperiod was simulated, starting at 7:30 and switching off at 19:30.

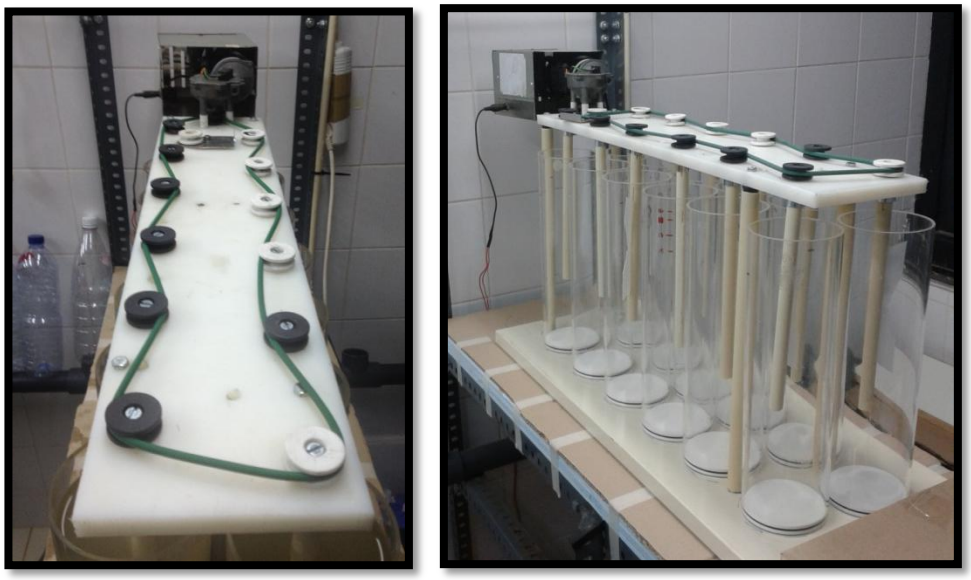


Figure 8. Images from the agitation system with at left the engine and at right the full system set up.

The cores as well the agitation pipes were acid cleaned (10% HCl) before the beginning of the experiment. Sediments were exposed for the salinity fluctuations experiment during 36 days. Sediment samples were collected before and at the end of the experiment, and stored at -20 ºC for later DNA analysis. Moreover, RNA was extracted in the end of the experiment from each core. At the end of the exposed period (36 days), inorganic nitrogen (NO_3^-, NH_4^+, NO_2^-) fluxes and nitrification rates were measured in the undisturbed sediment cores subjected to constant and salinity fluctuations treatments. Before those measurements, the overlying water of each core was renewed (with 20 µM of $^{15}NH_4^+$) and water samples were collected at 4 time periods (T0, T1h, T2h and T3h). Overlying water samples were taken from Crestuma treatments when salinity was at 0‰ on both treatments (C and ΔC), and from Afurada sediments were taken when salinity was at 15‰ on both treatments (A and ΔA). Time zero was collected after 15 minutes of exchanging water, to allow sediment deposition. Samples were collected, filtered through disposable 0.2 µm sterile syringe filters (VWR®

Syringe Filters) and stored at -20ºC for future nutrient and isotope analyses. At the end of the experiment composed sediments from each treatment (A, C, ΔA and ΔC) were collected for RNA and DNA analyses.

In those samples, it was also tested the effect of salinity fluctuations in selecting ammonia-oxidizing bacteria and archaea activity, through a batch experiment similar to the previously described in salinity gradient experiment. Sediments exposed to a constant (C, A) and salinity fluctuations (ΔA and ΔC) were incubated in 100 mL serum bottles in treatments with PTIO, with sulfathiazole, and without inhibitor (Figure 9). Twenty milliliters of sediment were weighted and introduced in each 100 mL serum bottle. Concentrations of 70 µM of $^{15}NH_4^+$ and 30 µM of $^{14}NH_4^+$ were added to the different treatments, and 100 µM of PTIO and 1500 µM of sulfathiazole to the respective treatments (Figure 9). The incubation period was 3h, and at each hour of incubation (0h, 1h, 2h and 3h), 2 mL of water was collected, centrifuged at 2500 rpm and stored at -20ºC for later nutrient analyses. Additionally, at time 0h and 2h, 8 mL of water was collected, centrifuged at 2500 rpm, filtered at 0.45 µm and stored at -20ºC for later isotopic analyses. Between each interval, serum flasks were hold in a dark room with constant agitation at 100 rpm (Figure 10).

Solutions with the desired concentration were prepared before the experiments: 10 mM of $^{14}NH_4Cl$ and $^{15}NH_4Cl$ (98 atom % ^{15}N, Aldrich); 100 mM of sulfathiazole; 10 mM of PTIO.

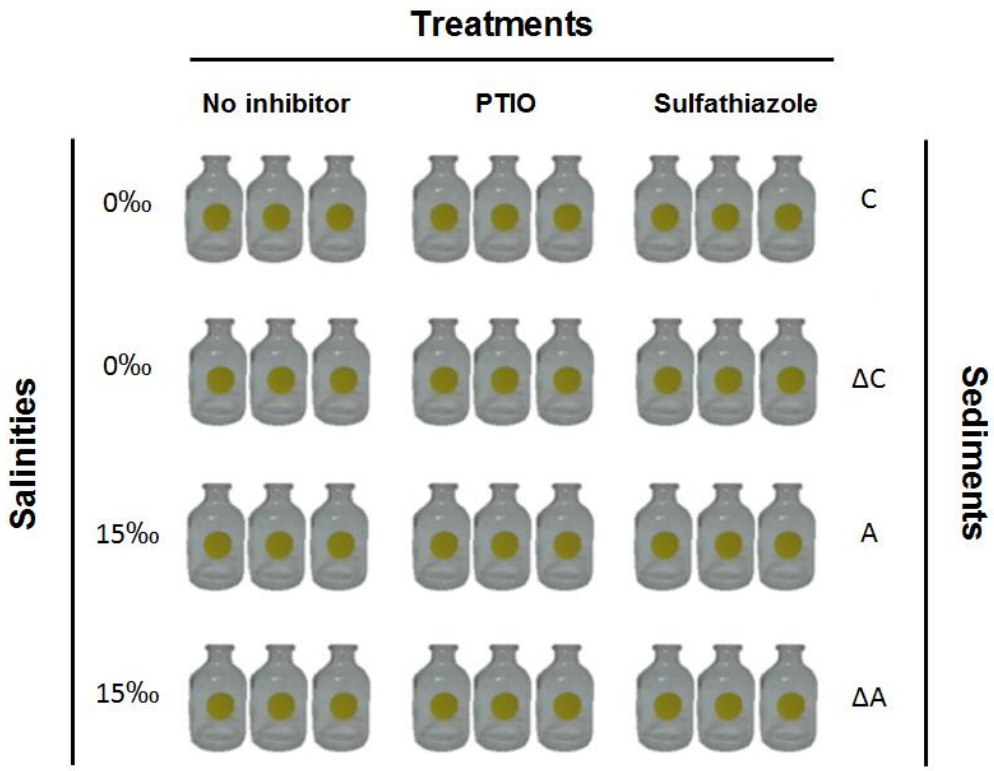


Figure 9. Scheme of the incubation batch experiment to measure AOA and AOB ammonia oxidation activity in sediments exposed 36 days to salinity fluctuations (ΔA and ΔC) and constant salinity (A and C) treatments.

Figure 10. Picture with slurries during the incubation experiment shown in Figure 9 at a constant agitation of 100 rpm.

5. Biogeochemical analyses

5.1 Nitrogen fluxes

Net fluxes of inorganic nitrogen compounds (NO_3^-, NO_2^- and NH_4^+) were calculated using the slope of the linear relationship between changes in the nutrient concentration in the water chamber versus the time of incubation according to Magalhães et al. (2003), following the equation (4):

$$F_N = \left(\frac{\alpha}{A}\right) \cdot 10^{-4} \qquad (\mathbf{4})$$

Where *Fn* is the flux of each inorganic nutrient in µmol m^{-2} h^{-1}, *α* is the slope of the linear relationship between time and the evolution of nutrient concentration, *A* is the sediment surface area in cm^2 and 10^4 is the conversion factor from cm^2 to m^2.

The analysis of ammonia, nitrite and nitrate were 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. All measurements were performed using a V-1200 spectrophotometer (VWR) with a Tungsten Halogen lamp, ≤ 0.3% T and cuvettes PS semi-micro (VWR). All reagent solutions were prepared in the laboratory (see Appendix 2 for further details). The following basic rules for nutrient analyses were considered for measurements: i) the standard curve should include the concentrations of the samples being analysed; ii) the standard should be performed at the same time as the samples and treated in an identical way to the samples analysed; and iii) standards should be prepared in a matrix that matches the matrix of the samples.

The method used for NH_4^+ and NH_3 quantification was adapted from Koroleff (1983) which is based on the Berthelot reaction. The Berthelot reaction is a colorimetric reaction where ammonia reacts with hypochlorite (in this study Trione was used, alternatively) to form a monochloriamine which subsequently in the presence of phenol, catalytic amounts of nitroprusside ions and excess of hypochlorite, giving an indophenol blue colour. The reaction

requires a catalyst or elevated temperatures to achieve the sensitivity needed. In our case, we used nitroprusside which produce an azo dye measured on the spectrophotometer at λ = 630 nm. The formation of a stable indophenol blue takes between 6 to 30 hours at room temperature. For samples with low salinity (< 5‰) with high humic acids, magnesium sulphate solution was added to precipitate the humic acids. Ammonium and ammonia concentration were quantified according with the following equation:

$$NH_4^+ + NH_3 = D \times (A_a - A_b) \times FS \times Dilution \quad (\mathbf{5})$$

Where, NH_4^+ and NH_3 is the ammonium and ammonia concentration in water; *D* is the standard slope; A_a is the absorbance value obtained for the sample; A_b is the absorbance value with only water; *FS* is salinity factor according Koroleff (in Grasshoff et al. (2009)) and; *Dilution* correspond to the dilution used on the sample.

The determination of nitrite concentrations in the water sample was based on Grasshoff et al. (2009). The methodology includes the reaction of nitrite with an aromatic amine, forming a diazominium compound which couples with a second aromatic amine, the sulphanilamide and N(1-naftil)etilodiamine, respectively. The final product results in a pink azo dye which is measured on the spectrophotometer at λ = 540nm. The nitrite concentration is calculated using equation 6.

$$NO_2^- = D \times (A_a - A_b) \times Dilution \quad (\mathbf{6})$$

Where, NO_2^- is the nitrite concentration (µM) on the water sample; *D* is the standard slope; A_a is the absorbance value obtained for the sample; A_b is the absorbance value with only water and; *Dilution* correspond to the dilution used on the sample.

The method used for NO_3^- quantification was adapted from Jones (1984) where instead of cadmium columns, it uses spongy cadmium with the advantage of increasing the surface area as well as a bigger stability in reduction efficiency. This method measures the

total nitrate and nitrite concentration of the samples in the spectrophotometer at λ = 540nm, where nitrate concentration is obtained by subtracting NO_2^- concentration to the total concentration of NO_2^- + NO_3^-.

$$NO_3^- = \left(\left(D \times (A_a - A_b)\right) \times C \times Dilution\right) - NO_2^- \quad (7)$$

Where, NO_3^- is the nitrate concentration dissolved in water; *D* is the standard slope; A_a is the absorbance value obtained for the sample; A_b is the absorbance value with only water; NO_2^- is the nitrite concentration in the water; *C* is the dilution factor of ammonium chloride reagent and; *Dilution* correspond to the dilution used on the sample.

5.2 Nitrification rates

Currently there are few techniques to measure nitrification rates, however a very useful technique is the ^{15}N tracer that traces nitrification directly without the requirement of inhibitory techniques (Jantti et al. 2012). The ^{15}N tracer technique that is normally used to study sediment nitrification rates is the isotope pairing technique.

In the present work, we used the $^{15}NH_4^+$ oxidation technique, which fundamentally involves the amendment of the samples with $^{15}NH_4^+$, and the measurement of $^{15}NO_3^-$ produced over time, providing an estimation of the uncoupled nitrification that is producing NO_3^- to the water column (Jantti et al. 2012). Formed $^{15}NO_3^-$ was then converted into gas (N_2) in order to be quantified by an isotope-ratio mass spectrometer, IRMS (Jantti et al. 2012, Ryabenko 2013). In this study the total $^{15}NO_2^-$ and $^{15}NO_3^-$ produced in the samples was analysed as the two products of the nitrification process.

Firstly, $^{15/14}NO_3^-$ was reduced to $^{15/14}NO_2^-$ via the spongy cadmium method (Jones 1984) as previously described. To assure an efficient reduction (equation 8) of NO_3^- to NO_2^- by Cd as well a stable $\delta^{15}N$ values that closely matched expected values for standards, 5M NaCl was added (Ryabenko et al. 2009) to our sample in a proportion of 2.25:2.25 (v/v) in a

15 mL falcon tube with 0.5 g of activated spongy cadmium. The falcon tubes were left in dark conditions overnight with constant agitation (100 rpm).

$$NO_3^- + Cd + H_2O = NO_2^- + Cd(OH)_2 \quad (8)$$

Then, the tubes were centrifuged during 8 minutes at 4000 rpm, and 4 mL of sample were transferred to a 6 mL exetainer (Vacuette®) and properly closed in order to avoid possible air contaminations. All exetainers were purged with Argon (≥ 99.9%, Air Liquid®) during 10 minutes in order to remove background N_2 (Holtappels et al. 2011), according to Figure 11.

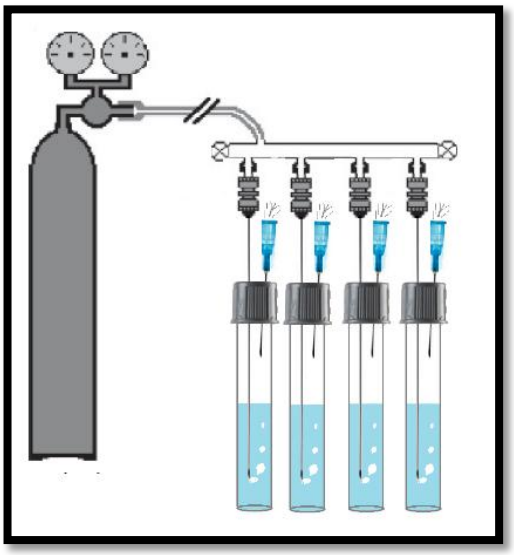


Figure 11 Scheme of exetainers being purged with argon.

Nitrite in the sample was converted to N_2 by adding 50 µL of 4% sulfamic acid (98%, Panreac AppliChem) with a SGE Gas Tight Syringe, followed by vortex to mix the acid and to transfer the formed dissolved $^{28}N_2$ and $^{29}N_2$ to the headspace.

After an incubation in the dark of at least one hour, the samples were carefully vortexed in order to release the N_2 in solution to the headspace. With a 250 µL GASTIGHT® syringe (1700 series, PTFE luer lock, Hamilton®) samples were injected in to the inlet IRMS system (Figure 12) to measure $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ in our gas sample. For supplementary details on the process, see Appendix 5.

Figure 12. Left - Syringe inserted into the exetainer, pulling 250 µL of air; Right - IRMS system used to measure $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ in gas samples;

The results of $^{15}N_2$ production from each sample was calculated based on Holtappels et al. (2011) through the derivate of $^{29}N_2$ and $^{30}N_2$ based on the integrated peak area of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$. First of all, it is important to know that the measured peak area of a specific isotope, like $^{29}N_2$ is the sum of its background abundance in the sample and its amount produced in the course of the experiment (equation 9).

$$^{\delta}A_M = {}^{\delta}A_N + {}^{\delta}A_B \quad (9)$$

Where, δ can be the isotope 28, 29 or 30; *A* represents the peak area; *M* represents the measured peak area; *N* corresponds to the peak area due to $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ production; and *B* represents the background peak area.

In a simplified way, there are 3 steps to obtain the initial $^{29}N_2$ and $^{30}N_2$ concentration in the exetainer. The first step encompasses the calculation of the background peak in order to achieve the $^{\delta}N_2$ produced peak, according the following equation (10):

$$ {}^{\alpha}A_N = \left(\frac{{}^{\alpha}A_M}{{}^{28}A_M} - \frac{{}^{\alpha}A_{air}}{{}^{28}A_{air}} \right) . {}^{28}A_M \qquad (\mathbf{10}) $$

Where, α can be the isotope 29 or 30; $^{29}A_{air}$ /$^{28}A_{air}$ is the ratio of the areas in the air standard; $^{28}A_M$ is the $^{28}N_2$ measured peak area. The term inside the brackets is the excess ratio (Nielsen 1992). This way of calculating $^{29}A_N$ takes advantage of the high accuracy of sector field IRMS in measuring the relative abundance of stable isotopes.

From $^{\alpha}A_N$ determined (in unit peak area) we proceed to the second step where it is calculated the number of moles of $^{\alpha}N_2$ injected into the IRMS, using the air standard, of which we know the ratio of $^{28}N_2$ to peak area, according the following equation (11):

$$ {}^{\alpha}M_N = {}^{\alpha}A_N \ \frac{{}^{tot}M_{air}}{{}^{tot}A_{air}} \qquad (\mathbf{11}) $$

Where, α can be the isotope 29 or 30; *A* represents the peak area; *M* denotes the number of moles of N_2 injected into the MS; N represents the N_2 injected due to N_2 production; $^{tot}M_{Air}$ is calculated using the molar volume of air (at 20ºC and 1013 hPa) and the fraction of N_2 in the atmosphere; and $^{tot}M_{Air}$ is the sum of $^{28}A_M$, $^{29}A_M$ and $^{30}A_M$.

Lastly, from the injected moles of $^{\alpha}N_2$ (like $^{29}M_N$), the initial concentration in the exetainer is calculated according the following equation (12):

$$ {}^{\alpha}C_N = {}^{\alpha}M_N \ . \left(\frac{V_H + \left(\frac{V_W}{Fg/w} \right)}{V_I . V_W} \right) \qquad (\mathbf{12}) $$

Where, α can be the isotope 29 or 30; N represents the N_2 injected due to N_2 production; M represents the number of moles of N_2 injected into the IRMS; V_I represents the injected volume; V_H represents the headspace volume; V_W represents the water volume; and Fg/w fractionation factor of N_2 between gas and water phase.

6. Molecular analyses

6.1 Nucleic acid extraction and quantification

Total DNA was extracted from 0.5g wet weight of sediment using a PowerSoil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA). DNA extracted was later quantified using the Qubit Fluorometric Quantitation method (ThermoFisher Scientific) and used for amplification according to the protocol described below (PCR – amplification of *amoA* genes). Total RNA was extracted from 2g wet weight of sediment using the Power Soil RNA isolation kit (MoBio Laboratories Inc.). DNA was removed from RNA by treatment with 1 U μL^{-1} of DNase I (Sigma) using DNA- and RNA-free reagents, followed by PCR using general 16S rRNA gene bacteria primers to check for traces of genomic DNA contamination. Reverse transcription (RT) was performed using the Omniscript RT kit (Qiagen) by adding 13 μL of total RNA to an 18 μL RT mixture according to the manufacturer's instructions. Synthesized cDNA was further used for amplification according to the protocol described below (PCR – amplification of *amoA* genes). The DNA was quantified by the Qubit® dsDNA HS (High Sensitivity) assay according with the manufacturer's instructions. The assay is highly selective for double-stranded DNA (dsDNA) over RNA and is designed to be accurate for initial sample concentrations from 10 pg/μL to 100 ng/μL. The cDNA was quantified by using the Qubit® ssDNA Kit ideal for quantitating single-stranded DNA or oligonucleotides. In order to assess the RNA integrity determination, it was used the RNA StdSens analysis kit on the Experion automated electrophoresis station (Bio-Rad Laboratories, Inc.) which performs all of the steps of gel-based electrophoresis in one compact, durable unit. Therefore, it combines electrophoresis, staining, destaining, band detection, and imaging into a single, 30 min step.

6.2 Amplification of *amoA genes*

With the purpose to perform a DGGE analysis, bacterial and archaeal *amoA* genes were amplified in 25 µL reaction containing 0.75 µL of each primer at 10 µM and 12.5 µL of DreamTaq PCR Master Mix (Thermo Scientific).

In order to amplify the *amoA* genes of ammonia-oxidizing bacteria it was used the forward primer amoA-1F (5'-GGGGTTTCTACTGGTGGT) (Rotthauwe et al. 1997) with a GC clamp (CGCCCGCCGCGC CCCGCGCCCGGCCCGCCGCCCCCGCCCC) (Muyzer et al. 2004) and the reverse primer amoA-2R (5'-CCCCTCKGSAAAGCCTTCTTC [K = G or T; S = G or C]) (Rotthauwe et al. 1997) generating a specific PCR product of 491bp (Rotthauwe et al. 1997). Approximately ~6ng of DNA of each sample with the following cycling conditions: 95°C for 5 min; 35 cycles consisting of 94°C for 45 s, 58°C for 60 s, and 72°C for 60 s; and 72°C for 15min. Moreover, the PCR conditions for Crestuma cDNA was 35 cycles consisting of 94°C for 45 s, 58°C for 60 s, and 72°C for 60 s; and 72°C for 15min; and for Afurada cDNA was: 95°C for 3.5 min; 35 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 35 s; and 72°C for 5min.

In order to amplify the *amoA* genes of ammonia-oxidizing archaea, the forward primer CrenamoA23f (5'- ATGGTCTGGCTWAGACG) (Tourna et al. 2008) and the reverse primer CrenamoA616r (5'- GCCATCCATCTGTATGTCCA) (Tourna et al. 2008) were used generating a specific PCR product of 620 bp (Tourna et al. 2008)

On the DNA extracted from both Afurada and Crestuma composed samples,~10ng of DNA was used to amplify the *amoA* gene from ammonia-oxidizing archaea with the following cycling conditions: 95°C for 5 min; 35 cycles consisting of 94°C for 45 s, 58°C for 60 s, and 72°C for 60 s; and 72°C for 15min. Additionally, cDNA from both Afurada and Crestuma, was amplified following the PCR conditions: 95°C for 5 min for Crestuma cDNA and lack of initial denaturation for Afurada cDNA followed by 35 cycles consisting of 94°C for 45 s, 56°C for 60 s, and 72°C for 60 s; and 72°C for 15min.

Aliquots (4 mL) of each PCR products were electrophoresed and visualized in 1.5% agarose gels using standard electrophoresis procedures.

6.3 DGGE analysis of AOA and AOB *amoA* genes and its transcripts

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, Hertfordshire, UK) according to the manufacturer's instructions. Gels were prepared containing 8% (w/v) polyacrylamide (30 %, Carl Roth) with a linear gradient of 30% [9.6% (w/w) Urea (for electrophoresis, Aldrich), 2% (v/v) TAE (Life Technologies) 50x and 12% (v/v) Formamide (≥99.5%, Aldrich)] to 70% [29% (w/v) Urea, 2% (v/v) TAE 50x and 28% (v/v) Formamide] for *amoA* AOB assays, and a linear gradient of 20% [8.4% (w/w) Urea, 2% (v/v) TAE 50x and 8% (v/v) Formamide] to 60% [25.2% (w/w) Urea, 2% (v/v) TAE 50x and 24% (v/v) Formamide] for *amoA* AOA assays to create a vertical denaturing gradient using a gradient former (Fisher Scientific UK, Loughborough, United Kingdom). About 5 µL and 6 µL of the PCR products from *amoA* AOB and *amoA* AOA were loaded with 5 µL and 4 µL of buffer, respectively. Gels were electrophoresed in 7 L of 1% TAE buffer at a constant temperature of 60ºC during 480 minutes at 100 V for AOB, and during 570 minutes at 120 V for AOA.

Following electrophoresis, gels were silver-stained which consisted of covering the gels with a 150 mL fixation/staining solution [10% (v/v) Ethanol (absolute for analysis, Merck Millipore), 0.5% (v/v) Acetic acid (>99.7%, Aldrich), 0.2% (w/v) $AgNO_3$ (≥99,9 %, Carl Roth)] for 20 minutes. The fixation/staining solution was removed and briefly rinsed with dH_2O. The water was removed and the gels covered in a 150 mL developing solution [0.27% (v/v) Formaldeide (37%, Merck Millipore) and 50mL of 9% (w/v) NaOH (98%, Alpha Aesear)] in water bath at 55ºC until the gels were deemed sufficiently developed. Lastly, the developing solution was removed and it was added 500 mL of Stop solution [1.5% (w/v) $NaCO_3$ (ACS,Reag.Ph Eur, Merck Millipore)] for 15 minutes, before scanning the gel using a GS-800 Calibrated Densitometer (Bio-Rad, Hertfordshire, UK).

6.4 Next generation sequence analysis of the 16S rRNA gene

Amplification of 16S rRNA gene fragments was performed using the primer pair 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and the recently revised 926R (5′-CCGYCAATTYMTTTRAGTTT-3') (Parada et al., 2016) to increase detection of

SAR11, which were designed as part of the Earth Microbiome Project (Gilbert JA 2014). The complete reagent mixture contained PCR-grade water (13 µL) 5' hot master mix (10 µL), forward primer (10µM, 0.5 µL), reverse primer (10µM, 0.5 µL), and template DNA (1.0 µL) in a total reaction volume of 25 µL. The PCR amplification conditions (384-well-thermocycler) were: 94ºC for 3 min; 35 cycles of 94ºC for 45s, 50ºC for 60s, and 72ºC for 90s; 72ºC for 10 min, and then a 4ºC hold. Resulting amplicons were cleaned, pooled and quantified using Quant-iT picogreen double-stranded DNA assay kit following EMP benchmarked protocols. Pooled amplicons were then sequenced on a multiplexed 2- by 150bp Illumina MiSeq sequencing run at LGC Genomics of the German Helmholtz Association from Berlin (Germany).

6.4.1 SILVA NGS analysis

The raw sequence reads obtained were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3; http://www.arb-silva.de) (Quast et al. 2013), that follows the common process of analysing rDNA amplicon reads from next generation sequencing: alignment, quality management, de-replication, clustering at a user-defined threshold and classification of the OTUs/reads. And therefore, each read was aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008) against the SILVA SSU rRNA SEED and quality controlled. Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artefacts, reads with a low alignment quality (50% alignment identity, 40% alignment score reported by SINA), were identified and excluded from downstream analysis. After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered (OTUs), on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering was done using cd-hit-est (version 3.1.2) (Huang et al. 2010). The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 123.1; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho et al. 2009). The classification of each OTU reference read was mapped onto all

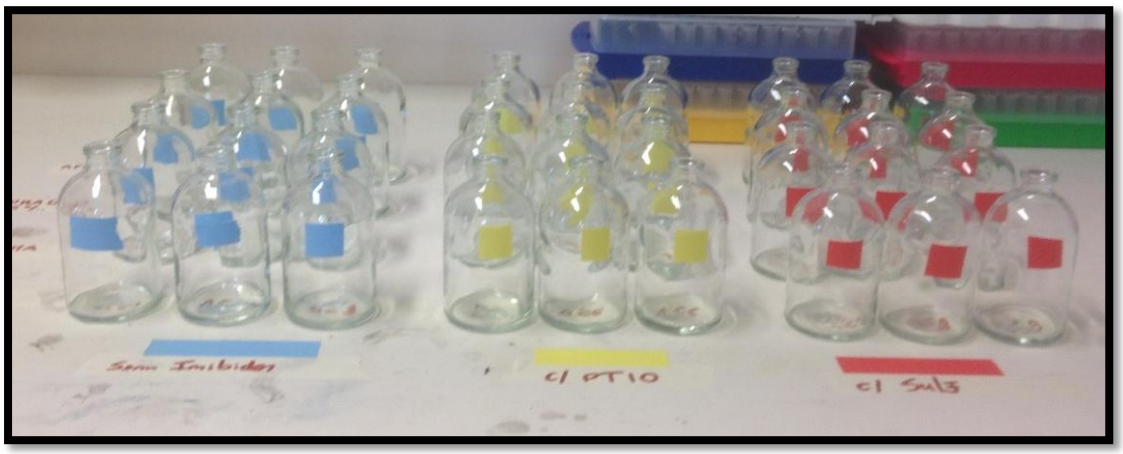
reads that were assigned to the respective OTU. Reads without any BLAST hits or reads with weak BLAST hits, where the function “(% sequence identity + % alignment coverage)/2" did not exceed the value of 93, remained unclassified. These reads were assigned to the meta group “No Relative" in the SILVAngs fingerprint and in Krona charts.

7. Data analysis

Data was analysed in triplicates for all relevant parameters. Data was tested for normality using the Kolmogorov-Smirnov test, and for homoscedasticity using Levene’s test. To compare differences between the means of each salinity and treatments, the parametric tests one-way ANOVA and two-way ANOVA (analysis of variance) were done. When significant differences were detected, a Tukey honestly significant difference (HSD) multiple comparison test was performed. Significant differences between the mean of two variables were assessed using t tests. All analysis were performed at the 95% confidence level. All statistical tests were done using the software STATISTICA, version 13, StatSoft, Inc.

The digitalized DGGE gels were analysed with the software package Quantity One (Bio-Rad) and band positions were processed in a spreadsheet. Using PRIMER 6 software (version 6.1.11) a data matrix of the gels was transformed based on the absence/presence of the bands. A distance matrix of each gel profile was generated using the Bray-Curtis Index and a dendrogram using the SIMPROF-test was generated.

Effect of a salinity gradient on AOA and AOB activity and *amoA* genes expression



Chapter I

Results

In order to test the effect of different salinities on the net fluxes of inorganic N and on the ammonia oxidation activity of archaea and bacteria, controlled slurries experiments with different salinities were set up for Afurada and Crestuma sediments according to what was described in the ′Material and Methods′ section.

1. Salinity effect on net inorganic nitrogen compounds

Results from net fluxes of NO_3^-, NO_2^- and NH_4^+ in Afurada and Crestuma sediment slurries subjected to different salinities (0‰, 15‰ and 30‰) are presented in Figure 13 and Figure 14, respectively. For further details on nutrients (NO_3^-, NO_2^- and NH_4^+) results see Appendix 1. Figure 1.

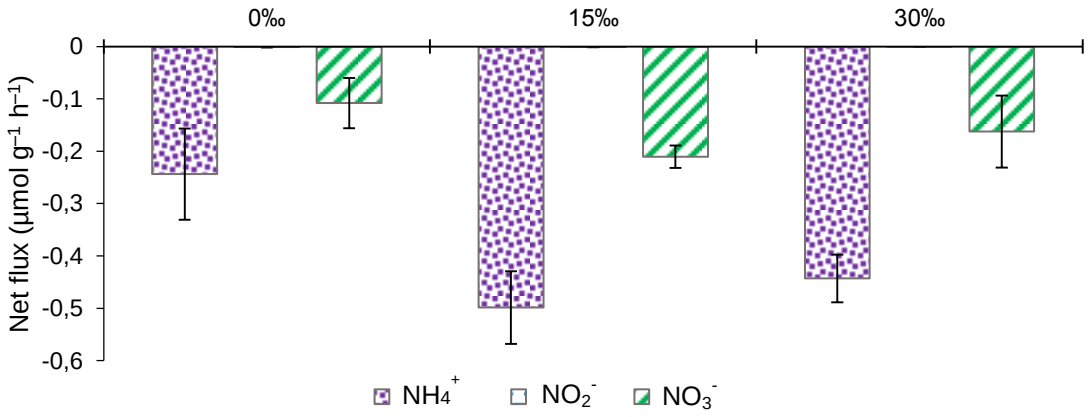


Figure 13. Mean ± SD of Afurada net fluxes of NH_4^+, NO_3^- and NO_2^- in sediment slurries subject to different salinities (0‰, 15‰ and 30‰).

Afurada net flux of NH_4^+ showed an uptake of NH_4^+ over time in all salinities (0‰, 15‰ and 30‰), suggesting a dominance of NH_4^+ uptake processes over NH_4^+ release processes. Nevertheless, according to the statistical analysis, the NH_4^+ net uptake from the intermediate and high salinity treatments (15‰ and 30‰, respectively) were significantly greater (one-way ANOVA, $P < 0.05$) compared to the uptake verified in low salinity treatment (0‰). Furthermore, the net flux of NO_2^- was almost null in all salinities (0‰, 15‰ and 30‰)

without significant (one-way ANOVA, $P > 0.05$) differences between treatments. Lastly, the net fluxes of NO_3^- were negative in all salinities (0‰, 15‰ and 30‰), and no significant (one-way ANOVA, $P > 0.05$) differences were found between treatments.

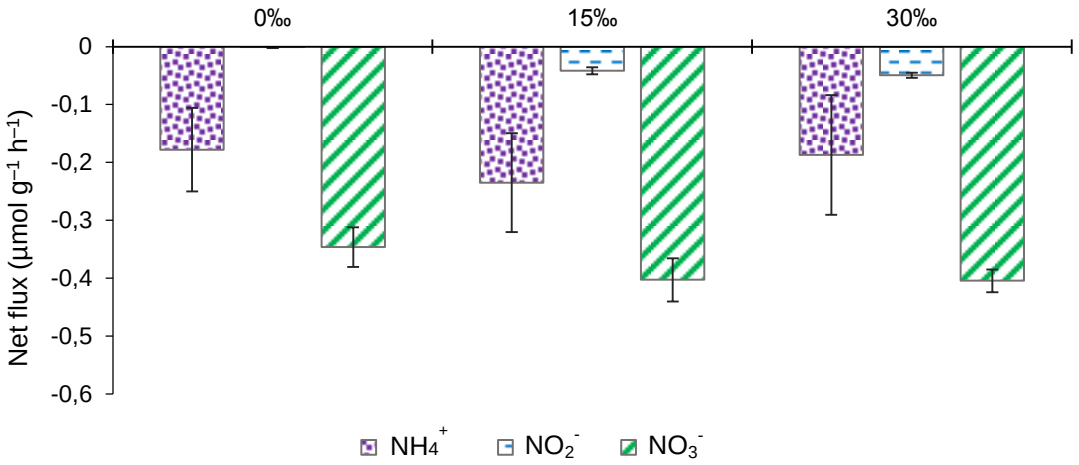


Figure 14. Mean ± SD of Crestuma net fluxes of NH_4^+, NO_3^- and NO_2^- in sediment slurries subject to different salinities (0‰, 15‰ and 30‰).

As it was observed in Afurada, Crestuma net fluxes of NH_4^+ were negative in all salinities (0‰, 15‰ and 30‰), suggesting a dominance of NH_4^+ uptake, however no statistical differences (one-way ANOVA, $P > 0.05$) were found between treatments. Crestuma's net fluxes of NO_2^- were measurable and a higher net uptake of this inorganic N compound was observed at the intermediate and high salinity treatments (15‰ and 30‰) compared to the low salinity treatment (0‰). On the other hand, in low salinity treatment (0‰), NO_2^- net flux result was approximately zero and significantly (one-way ANOVA, $P < 0.05$) different from higher salinity treatments (15‰ and 30‰). Last of all, net fluxes of NO_3^- presented the highest uptake rates, with no statistical differences (one-way ANOVA, $P > 0.05$) between the three salinity treatments.

2. Salinity gradient effect on Bacteria and Archaea nitrification activity

The rates of potential nitrification (based on the analyses of ^{15}N in the $^{15}N\text{-}NO_2^-$ and $^{15}N\text{-}NO_3^-$, after the addition of $^{15}NH_4^+$) on Afurada and Crestuma sediment slurries subjected to different salinities (0‰, 15‰ and 30‰) as well as to different inhibitory treatments are presented in Figures 15 and 16, respectively. For more detailed results, see Appendix 4, Table 1 and 2. In the treatment with no inhibitor, we expected to measure potential rates of nitrification of the total nitrifier microorganisms (TNM), the PTIO treatment (that inhibits ammonia-oxidizing archaea), gives rates of potential nitrification only for active ammonia-oxidizing bacteria (AOB), and the sulfathiazole treatment (that inhibits bacteria) gives rates of potential nitrification only for active ammonia-oxidizing archaea (AOA).

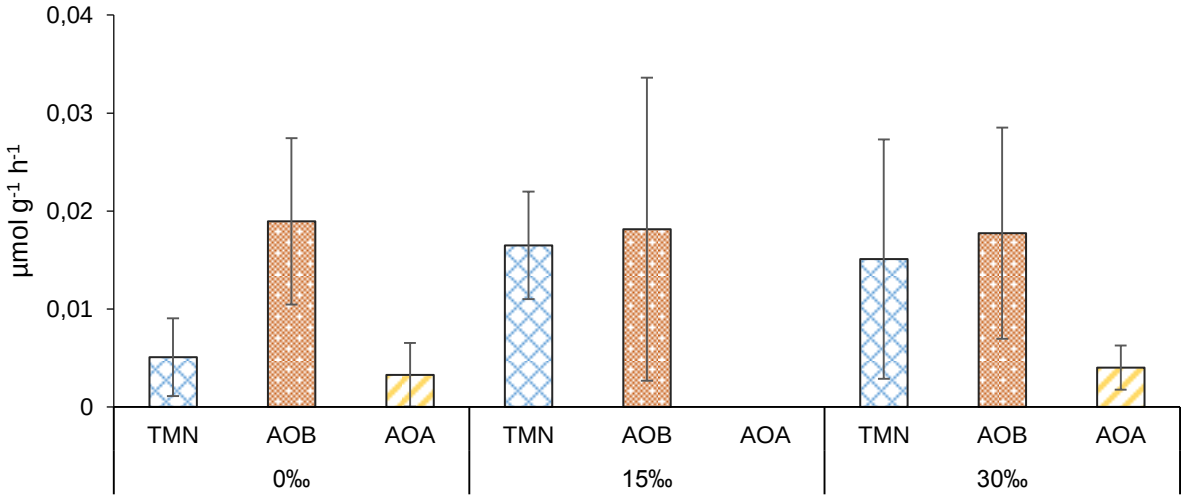


Figure 15 Mean ± SD of Afurada potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries subjected to different salinities (0‰, 15‰, and 30‰) and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and sulfathiazole (rates of AOA).

Afurada potential nitrification rates of TNM exhibited a tendency to be higher in the intermediate and higher salinity treatments, however with no significant (one-way ANOVA, *P* > 0.05) differences found. AOB potential nitrification rates in Afurada slurries were not significantly (one-way ANOVA, *P* > 0.05) affected by salinity variations. AOA potential nitrification rates were similar (one-way ANOVA, *P* > 0.05) among low and high salinity, with null nitrification rates in the intermediate salinity (15‰), and therefore significant (ANOVA, *P* < 0.05) lower rates than other salinities (0‰ and 30‰) tested. Nonetheless, AOB potential nitrification rates were higher (*t*-test, *P* < 0.05) than AOA potential nitrification rates under low and intermediate salinities.

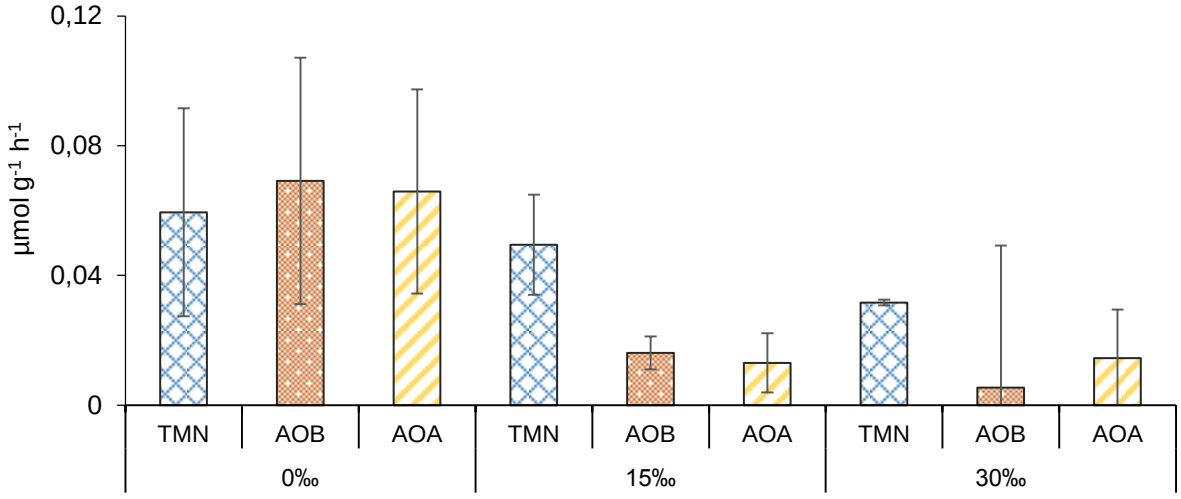


Figure 16 Mean ± SD of Crestuma potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries subjected to different salinities (0‰, 15‰, and 30‰) and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and sulfathiazole (rates of AOA).

TNM rates from Crestuma presented distinct tendencies from Afurada. Results suggest higher rates of potential nitrification at the lower salinity treatment followed by the intermediate and high salinity treatments. However, no significant (one-way ANOVA, *P* > 0.05) differences were found between TNM rates for the different salinities. AOA and AOB potential nitrification rates presented a similar pattern, decreasing with the increase of salinity but without statistically significant (one-way ANOVA, *P* > 0.05) differences between salinities for each group. Moreover, AOA and AOB potential nitrification rates under the different salinities presented similar results (one-way ANOVA, *P* > 0.05).

When comparing Afurada and Crestuma rates (Figure 15 and 16, respectively), the TNM results showed significant (*t*-test, $P < 0.05$) higher rates under low and intermediate salinities in Crestuma samples. The AOB group exhibited similar (*t*-test, $P > 0.05$) potential nitrification rates in all salinities tested, and, the AOA only presented higher (*t*-test, $P < 0.05$) PNR in Crestuma under 0‰.

3. Effect of salinity on archaeal and bacterial *amoA* gene and *amoA* expression

Fingerprinting DGGE analysis of AOA and AOB *amoA* genes fragments and transcripts (cDNA) was performed at the end of the salinity gradient experiment performed for Afurada and Crestuma sediments, in order to compare the community structure of ammonia oxidizers based on *amoA* gene and *amoA* transcripts.

3.1 Richness of archaeal *amoA* gene and its transcripts

DGGE analysis of archaeal *amoA* gene and *amoA* transcripts from Afurada sediments is presented in Figures 17A and 17B. Results from Afurada DNA sediment sample indicate a greater archaeal *amoA* gene richness with a total of DGGE 45-44 bands (Figures 17A, 17B) in comparison with *amoA* transcripts richness. As expected the active *amoA* community was substantially lower in the slurries experiments in comparison with archaeal *amoA* gene richness, and marked differences were observed between the level of AOA transcripts and the different salinity treatments (0‰, 15‰ and 30‰). Hierarchical cluster analysis based on the average linkage of Bray-Curtis similarities, using the presence/absence of AOA *amoA* transcripts and *amoA* gene from initial DNA (Afurada initial) revealed a high dissimilarity of the DGGE profile at 30‰ compared with the other two treatments with only 5 DGGE bands. A 50% of similarity was observed between salinity 0‰ and 15‰ treatments, with a total of 27 and 15 DGGE bands, respectively. These results suggested a low number of active AOA from Afurada site for the highest salinity tested (30‰), and a highly diverse activity of these communities under low salinity conditions, since a clear and progressive decrease on the

richness of the archaeal *amoA* transcripts were observed between the 0‰ to 30‰ salinity treatments.

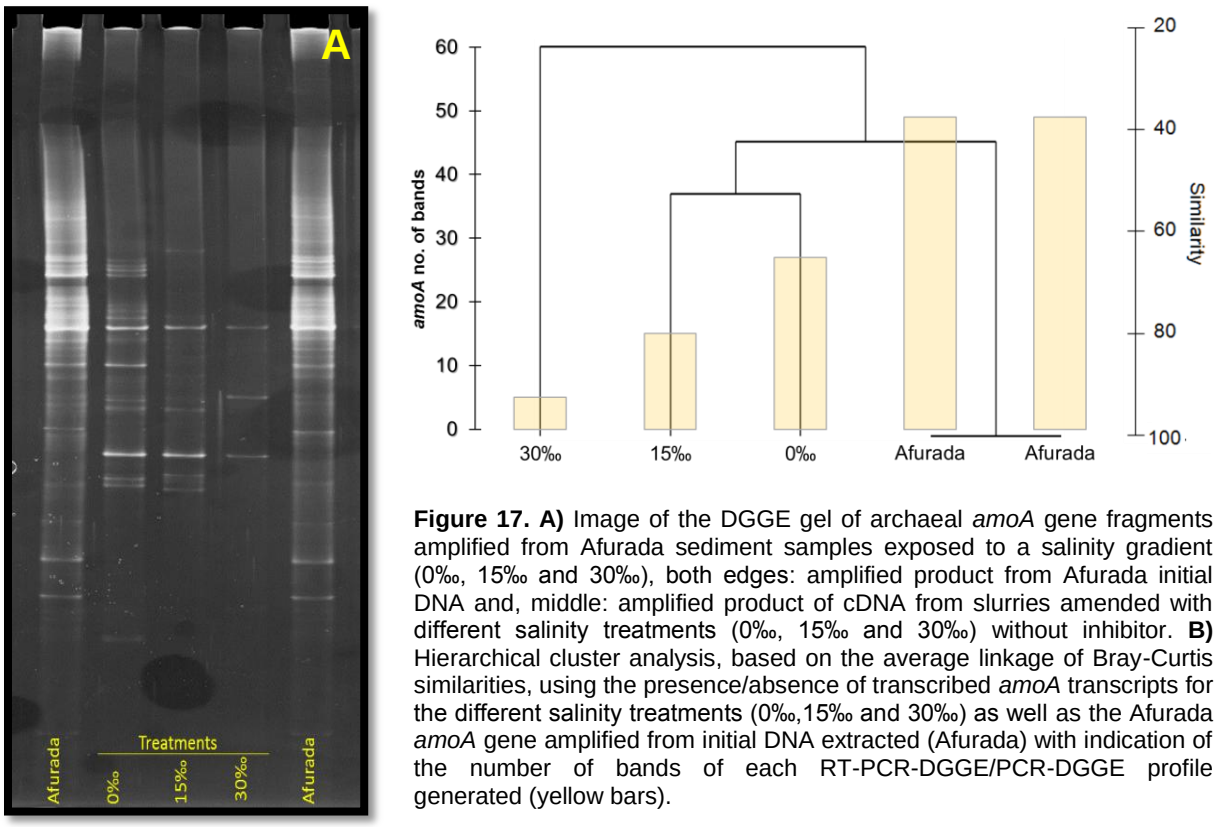


Figure 17. A) Image of the DGGE gel of archaeal *amoA* gene fragments amplified from Afurada sediment samples exposed to a salinity gradient (0‰, 15‰ and 30‰), both edges: amplified product from Afurada initial DNA and, middle: amplified product of cDNA from slurries amended with different salinity treatments (0‰, 15‰ and 30‰) without inhibitor. **B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of transcribed *amoA* transcripts for the different salinity treatments (0‰,15‰ and 30‰) as well as the Afurada *amoA* gene amplified from initial DNA extracted (Afurada) with indication of the number of bands of each RT-PCR-DGGE/PCR-DGGE profile generated (yellow bars).

Interestingly, similar DGGE band pattern was observed in Crestuma sediments. The treatment under lowest salinity (0‰) presented higher DGGE band pattern of *amoA* transcripts, compared to the higher salinity treatments (Figure 18A and 18B), with a total of 29 DGGE bands compared with 12 and 19 in the 15‰ and 30‰ salinity treatments, respectively.

Additionally, DGGE banding pattern from *amoA* gene derived from initial Crestuma total DNA indicate high AOA richness with a total of 56 bands. Furthermore, the AOA *amoA*

gene from Crestuma total DNA in comparison with Afurada total DNA *amoA* suggests a more diverse AOA community in the section of the estuary with lower salinity influence.

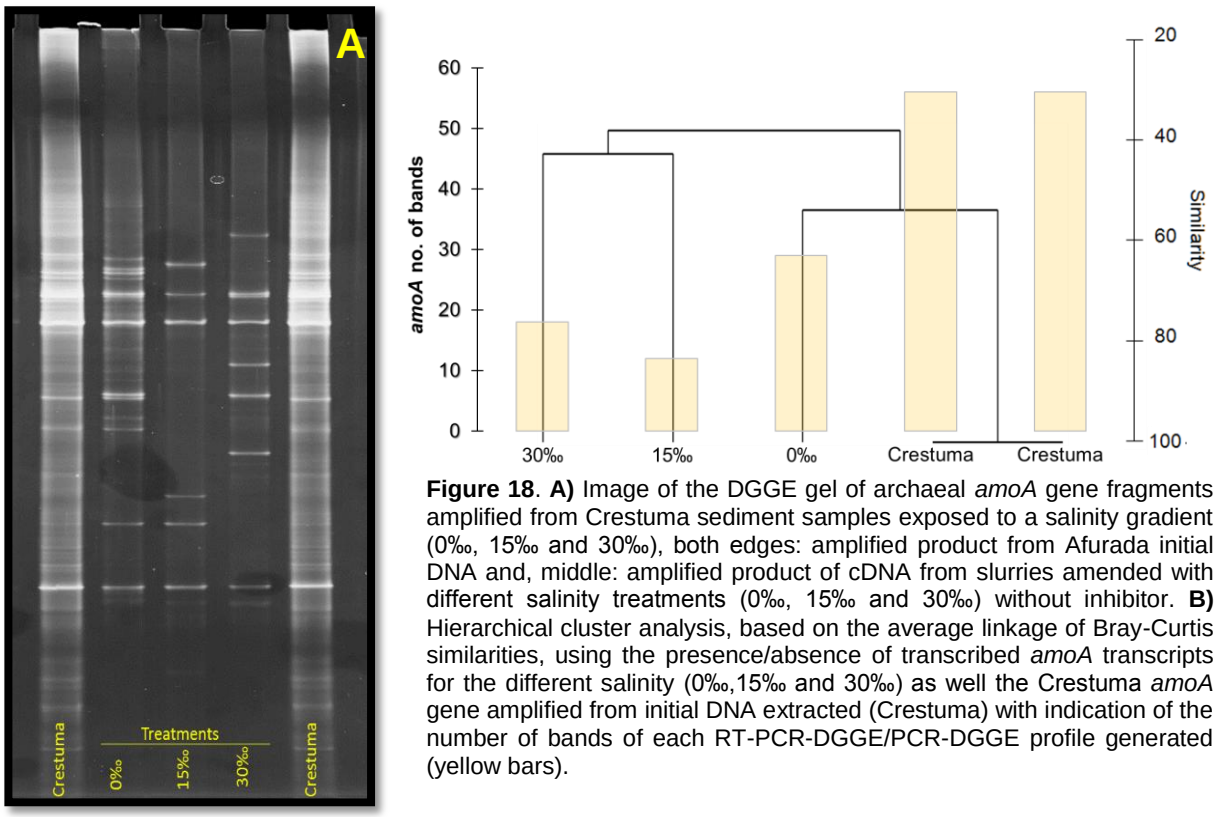


Figure 18. **A)** Image of the DGGE gel of archaeal *amoA* gene fragments amplified from Crestuma sediment samples exposed to a salinity gradient (0‰, 15‰ and 30‰), both edges: amplified product from Afurada initial DNA and, middle: amplified product of cDNA from slurries amended with different salinity treatments (0‰, 15‰ and 30‰) without inhibitor. **B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of transcribed *amoA* transcripts for the different salinity (0‰,15‰ and 30‰) as well the Crestuma *amoA* gene amplified from initial DNA extracted (Crestuma) with indication of the number of bands of each RT-PCR-DGGE/PCR-DGGE profile generated (yellow bars).

Based on the hierarchical cluster analysis of the DGGE results it is possible to assess that between salinity 30‰ and 15‰ there is a similarity of 40%. Moreover, the 0‰ is more similar to the initial Crestuma sediment with a similarity of 50%. These two clusters have a similarity of 35% (Figure 18B).

3.2 Richness of bacterial *amoA* gene and its transcripts

DGGE analysis of bacterial *amoA* gene and *amoA* transcripts from Afurada sediments is presented on Figures 19A and 19B. The results for Afurada DNA sediment indicate an elevated bacterial *amoA* gene richness with a total of 42-42 DGGE bands. The DGGE bands pattern for *amoA* transcripts of the different treatments differ considerably resulting in 25, 45

and 11 DGGE bands, respectively for 0‰, 15‰ and 30‰ treatments. The high DGGE band number of AOB *amoA* obtained for the 15‰ (Figure 19B), suggests that the original AOB community of Afurada station display highly diverse activity to operate at intermediate salinities. However, differences in DGGE band profiles between *amoA* transcripts from intermediate salinity and its gene is observed with a dissimilarity of 41%.

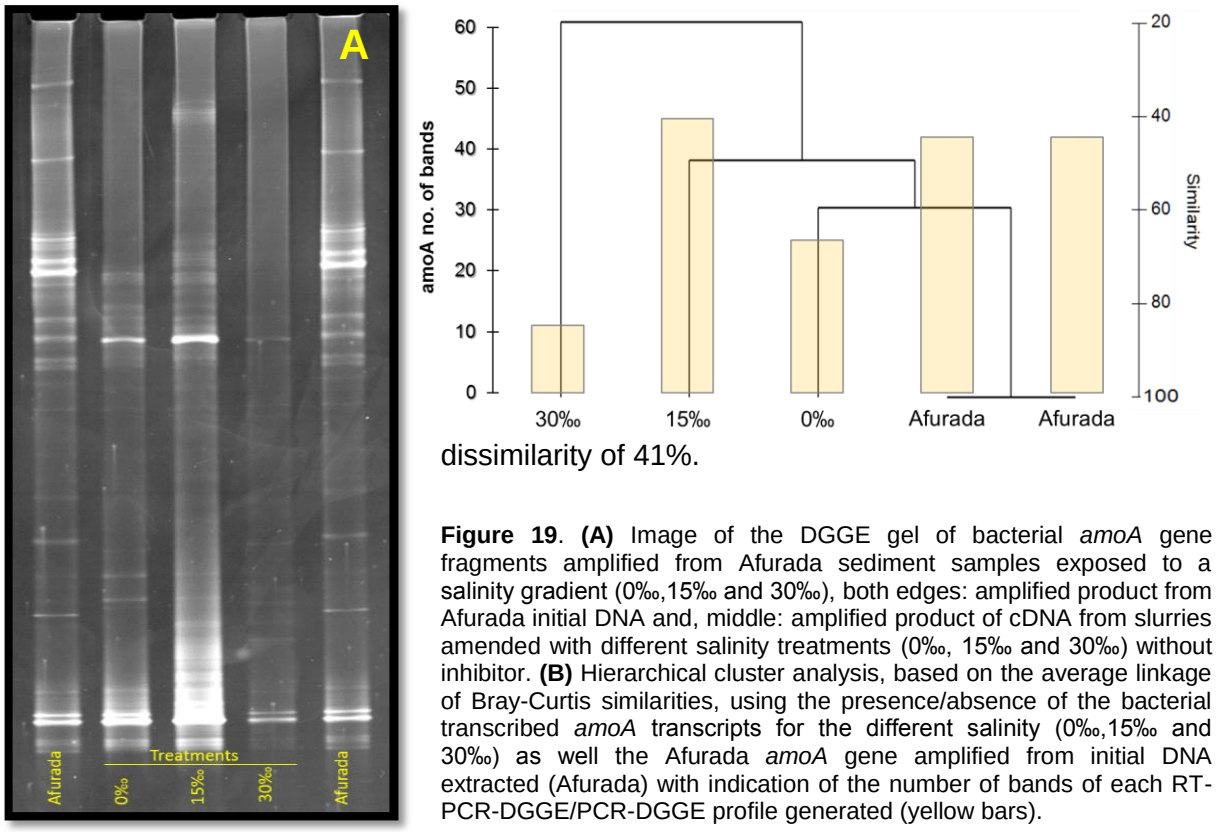


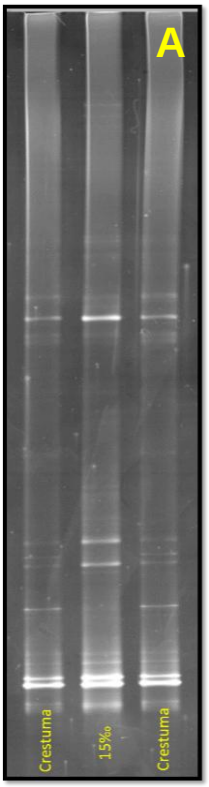
Figure 19. **(A)** Image of the DGGE gel of bacterial *amoA* gene fragments amplified from Afurada sediment samples exposed to a salinity gradient (0‰,15‰ and 30‰), both edges: amplified product from Afurada initial DNA and, middle: amplified product of cDNA from slurries amended with different salinity treatments (0‰, 15‰ and 30‰) without inhibitor. **(B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of the bacterial transcribed *amoA* transcripts for the different salinity (0‰,15‰ and 30‰) as well the Afurada *amoA* gene amplified from initial DNA extracted (Afurada) with indication of the number of bands of each RT-PCR-DGGE/PCR-DGGE profile generated (yellow bars).

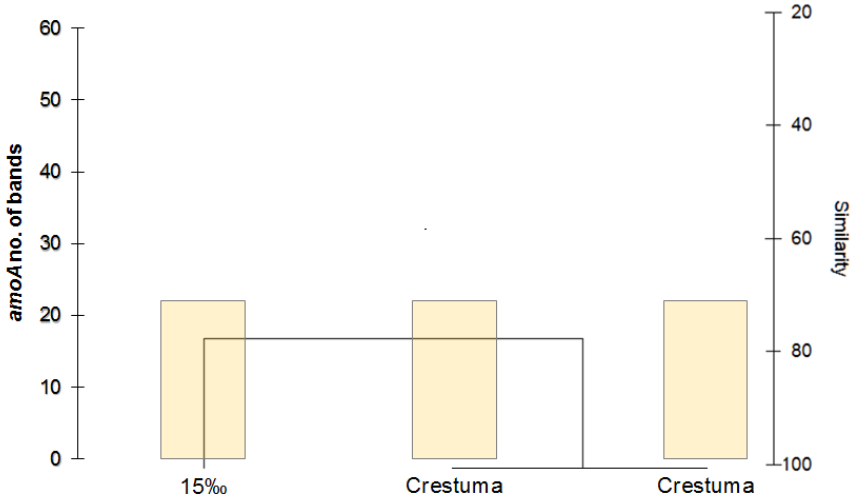
Based on the hierarchical cluster analysis of the DGGE profiles, the results showed a similarity of 60% between the active AOB community structure subjected to 0‰ and the bacterial community structure from initial DNA. Richness of AOB *amoA* transcripts in higher salinity treatments (15‰ and 30‰) presented a decrease in the level of similarity.

Bacterial *amoA* gene and *amoA* transcripts analysis for Crestuma sediments are present on Figure 20A and 20B. AOB *amoA* transcripts were only amplified in the 15‰. While

null PCR results of cDNA samples from 0‰ and 30‰ are not conclusive of an absence of *amoA* (as seen on potential nitrification rates), they are indicative of lower levels of mRNA transcription in these treatments compared with 15‰. Indeed, the lower richness of AOB *amoA* gene (22 DGGE bands) observed for Crestuma initial DNA sample compared with AOA *amoA* gene richness (56 DGGE bands), suggest that the structure of Crestuma ammonia oxidizers communities might be highly dominated by AOA, contrary to what was observed in Afurada site.

Figure 20. **(A)** Image of the DGGE gel of bacterial *amoA* gene fragments amplified from Crestuma sediment samples exposed to a salinity gradient (15‰), both edges: amplified product from Crestuma initial DNA and, middle: amplified product of cDNA from slurries amended with intermediate salinity (15‰) without inhibitor. **(B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of the bacterial transcribed *amoA* transcripts for 15‰ as well the Crestuma bacterial *amoA* gene amplified from initial DNA extracted (Crestuma) with indication of the number of bands of each RT-PCR-DGGE/PCR-DGGE profile generated (yellow bars).





Additionally, hierarchical cluster analysis of the DGGE profile from the AOB *amoA* gene and *amoA* transcripts indicate considerable high similarity (76%) between the bacterial *amoA* transcription community structure subjected to 15‰ and the bacterial community structure from initial DNA.

Discussion

Several studies focusing on ammonia-oxidizing communities have evaluated the *in situ* distribution, abundance and activity of these communities across various ecosystems, such as lakes (Zhao et al. , Hou et al. 2013), estuaries (Magalhães et al. 2009), extreme environments (Alves et al. 2013), coastal areas (Heiss and Fulweiler 2016), oceans (Zehr and Kudela 2011), rivers (Sun et al. 2014) and soils (Zhalnina et al. 2012). However, there is an issue when performing studies *in situ*, due to the fact that it is impossible to independently

study all processes, energy trades as well as factors occurring during the *in situ* experiments (Magalhães et al. 2005a, Liu et al. 2013). Therefore, the evaluation of the effect of one or more variables in *in situ* experiments turn to be more difficult as well as inaccurate. In a parallel/complementary way, a common used approach is the microcosm experiment, that despite not reflecting the actual environment, it allows us to evaluate a direct impact of a variable or the interaction of two or more variables (Vidican and Sandor 2015).

In this study, we used microcosm incubation experiments to test the effect of salinity on net inorganic nitrogen compounds as well on Bacteria and Archaea nitrification activity (through ^{15}N tracer technique) in two different estuarine sediments (Afurada and Crestuma). We also used bacterial and archaeal *amoA* transcripts as an indicator of the microbial community responsible for ammonia oxidation (Santoro 2009). Additionally, *amoA* gene (amplified from DNA) was studied in the initial sediment as indicator of the community structure present in both locations.

1. Salinity gradient effect on net inorganic nitrogen compounds

The inorganic nitrogen compounds (NO_3^-, NO_2^-, NH_4^+) net fluxes were measured for both sites. The results obtained might explain differences on net fluxes due to the salinity gradient impact on microorganisms involved on the N-pathway. However, it is important to have in account that these net fluxes reflect the result of several N recycling processes that occurred simultaneously in the slurries and therefore, it is only possible to assume and/or predict the occurrence of individual N processes.

Results from Afurada sediment slurries presented higher net fluxes of NH_4^+ on intermediate and high salinity treatments (15‰ and 30‰) compared with the lowest salinity treatment (0‰). These results suggest that microorganisms responsible for the NH_4^+ uptake might tolerate higher salinities. Unlikely Afurada, Crestuma sediment slurries presented similar net fluxes of NH_4^+ between different salinities. However, the net fluxes of NO_2^- under the lowest salinity (0‰) was very low when compared to higher salinities (15‰ and 30‰) where uptake of NO_2^- was observed. Furthermore, the net fluxes of NO_3^- on both sediments were always negative, suggesting that denitrification might be the dominant process for NO_3^- net uptake. These results are in agreement with Magalhães et al. (2005a) that found similar

denitrification rates at Afurada site when sediments were exposed to different salinities (0‰, 15‰ and 30‰). However, the differences reported between salinities and sites might be due to the occurrence of other N-pathways, like dissimilatory nitrate reduction to ammonium (DNRA). In fact Magalhães et al. (2005b) previously suggested the possibility of the occurrence of both denitrification and DNRA in those sediments.

2. Salinity effect on bacteria and archaea nitrification activity

Afurada potential nitrification rates by total nitrifier microorganisms (TNM) tended to be higher in the intermediate salinity (15‰), followed by the higher salinity (30‰), which is in line to what was previously observed in other estuarine systems (Magalhães et al. 2005a).

Additionally, nitrification rates by AOB exhibited higher rates than AOA and almost no fluctuations between salinities were registered in Afurada, suggesting that ammonia-oxidizing bacteria communities from Afurada are adapted to a wide range of salinities. However, it is important to notice that this similarity between salinities might be due to different ammonia oxidation rates produced between replicates (elevated SD), and therefore the existence of core variability (Santoro et al. 2008). Nonetheless, nitrification rates by AOA were much lower than the magnitudes registered for AOB, suggesting that AOB are the most active nitrifier community in Afurada site.

In general, at Afurada sediment slurries incubation within the different salinities, the nitrification rates were higher by AOB over AOA communities, suggesting that AOB are the principal responsible for nitrification at Afurada site along the daily salinity fluctuations. Our findings are in agreement with a study in Douro River estuary by Magalhães et al. (2009) where it was reported that AOB were more abundant than AOA. Additionally, other studies in estuarine systems have shown that β-AOB *amoA* genes were more abundant then AOA *amoA* genes specially with the proximity of the ocean and higher salinity (Mosier and Francis 2008, Santoro et al. 2008, Wankel et al. 2011, Li et al. 2015). Furthermore, Li et al. (2015) with the use of allylthiourea, an inhibitor of bacteria (Wang and Gu 2014), reported a significant decrease on nitrification rates, suggesting that AOB communities are the principal responsible for nitrification at Colne estuary.

The potential nitrification rates from Crestuma supported some of the previous results from nutrient net fluxes analysis where it was suggested higher nitrification rates under low salinity. TNM potential nitrification rates decrease with the increase of salinity. These results are in agreement with Rysgaard et al. (1999) who reported that nitrification was lower at higher salinities, however with minor differences after 10% of increment. Moreover, the tendencies of nitrification rates are also in line with a study performed by Zhang et al. (2015) that through a microcosm experiment of 56 days with estuarine sediments (salinity site = 5‰, similar to Crestuma) amended with three different salinities (5‰, 15‰, 30‰) found a significant higher nitrification from the lowest salinity in comparison with the higher salinities. Nonetheless, the nitrification rates from AOB displayed higher rates on low salinity, with the decrease with the increase of salinity. Although the decrease was significant between low salinity and intermediate salinity, at higher salinity due to the elevated SD there was no significant differences. Once again, these results suggest variability between replicate sediment slurries (Santoro et al. 2008) and therefore, making it difficult to obtain statistical support. However, the tendencies observed indicate a decrease of AOA and AOB nitrification rates with the increase of salinity.

The overall of AOA and AOB nitrification in Crestuma were similar, suggesting that both AOA and AOB communities share similar role on the nitrification process at Crestuma lower salinity site. According to Magalhäes et al. (2005b), Crestuma site is highly influenced by freshwater NO_3^- inputs, and at the same time, it shows low levels of NH_4^+. Inversely, Afurada site has high NH_4^+ concentrations with an annual range between 21 – 168 µM (Magalhães et al. 2009). These NH_4^+ levels might derive from sources and/or internal inputs by the human-induced contamination in these areas of the estuary as previously described (Azevedo et al. 2008, Magalhães et al. 2008). As a result, the AOB nitrification rates dominance over AOA presented at the Afurada site might be explained by the fact that thaumarchaeal have extremely low half saturation constant K_m values (in the nM range) for NH_4^+ (Martens-Habbena et al. 2009) and therefore, higher NH_4^+ concentrations of Afurada site may inhibit AOA activity. However, in advantage, low K_m values may well turn AOA highly competitive at low NH_4^+ concentration (Fukushima et al. 2012, Hatzenpichler 2012, Li et al. 2015). In fact, there has been reports about AOA dominance in soils (Leininger et al. 2006, Chen et al. 2015), open ocean (Wuchter et al. 2006, Mincer et al. 2007) and estuaries

(Caffrey et al. 2007, Bernhard et al. 2010b). And therefore, this results are in agreement with previous findings on the detection of high abundances of AOB in environments with higher NH_4^+ input, while AOA are more abundant in low-NH_4^+ environments (He et al. 2007, Jia and Conrad 2009, Verhamme et al. 2011). Although, a conjugation of factors such as salinity, C/N ratio and also the NH_4^+ concentration in water and sediments will certainly play a role on the magnitudes of the activity by AOA and AOB (Hatzenpichler 2012), which could explain why both AOA and AOB communities presented similar role on the nitrification process in Crestuma sediments.

Lastly, the results presented higher nitrification rates in Crestuma site in comparison with Afurada. These results might be explained not only by the fact that these locals are characterized by different AO organisms with different tolerance to salinity, but also due to sediment features such as large grain sizes allowing easy oxygen diffusion within the sediments creating conditions to enhance nitrification process (Caffrey et al. 2007), and therefore this report is in agreement with the different results obtained between Afurada and Crestuma (large grain size). Moreover, high percentages of organic matter in the sediments might also stimulate H_2S production which inhibits nitrification (Magalhães et al. 2002), which might be occurring at Afurada site.

3. Effect of a salinity gradient on AOA and AOB *amoA* transcripts richness

The Afurada DGGE result from archaeal *amoA* transcripts exhibited a decrease in AOA *amoA* richness expression with the increase of salinity, i.e. the amount of possible different AOA species transcribing the enzyme responsible for ammonia oxidation. These results suggest that AOA community present at Afurada sediments is composed by more ecotypes that are active by lower salinities over high salinities, though the presence of AOA ecotypes with different salinity affinities seems to coexist. In fact, a study performed by Beman and Francis (2006) showed that archaeal *amoA* sequences from Bahía del Tóbari were included in freshwater (or soil) and marine cluster. In addition, Park et al. (2008) found that CG I.1a-related *amoA* was the dominant group of AOA in marine sediments. These results showed that AOA from soil cluster could subsist under high salinities. In a study

performed on Afurada site, Magalhães et al. (2009) showed that the phylogenetic relationship of archaeal *amoA* sequences retrieved from Afurada were considerably diverse, with representatives in previously defined cluster such as the 'water column', 'sediments', and 'soil/sediments', supporting our hypothesis of a wide range of different AOA ecotypes. Furthermore, an ecophysiology study revealed the preference for low-salinity habitat of an enriched AOA, *Candidatus Nitrosoarchaeum limnia* strain SFB1, which is included in group I.1a, *Nitrosopumilus* cluster (Mosier et al. (2012b), showing the occurrence under low salinity environments of species from this cluster. Additionally, this strain was capable of growing at 75 % of seawater salinity at a slower growth rate, and so, supporting the presence of identical transcribed AOA *amoA* fragments under different salinities on Afurada and Crestuma. Moreover, it might clarify the differences on nitrification rates between the different Crestuma treatments.

Our results suggest that most of AOA richness present at Afurada operate under lower salinities. One possible reason is the fact that NH_4^+ levels might comprise the establishment of AOA from marine group that prefer low NH_4^+ levels (as previous mentioned). Actually, most of AOA had a preference for low NH_4^+ content and can survive in extreme low NH_4^+ concentrations (Martens-Habbena and Stahl 2010, Zhou et al. 2015) with some exceptions as previously reported by Fukushima et al. (2012) that found a few AOA ecotypes under conditions of high NH_4^+ concentrations. This hypothesis is reinforced by a study at Afurada site performed by Magalhães et al. (2009) where it was found that archaeal *amoA* sequences in Douro estuary were included on the soil cluster and were similar to sequences retrieved from agricultural soils (Leininger et al. 2006), hypernutrified estuarine sediments (Beman and Francis 2006) and pristine pasture soil (Leininger et al. 2006), suggesting that NH_4^+ levels play a role on AOA niche specialisation and differentiation. Additionally, our hypothesis is also supported by Baolan et al. (2012) who found that *Nitrososphaera viennensis* could grow well in the media containing NH_4^+ concentration as high as 15 mM, indicating a low affinity to NH_4^+ of this strain of AOA. Contrary, *Nitrosopumilus maritimus* showed extremely high affinity for NH_4^+ and the NH_4^+ inhibition concentration was as low as 2 mM to 3 mM (Baolan et al. 2012).

The Crestuma DGGE profile from archaeal *amoA* transcripts presented higher AOA *amoA* transcripts richness under low salinity (0‰), followed by high salinity (30‰) and lastly

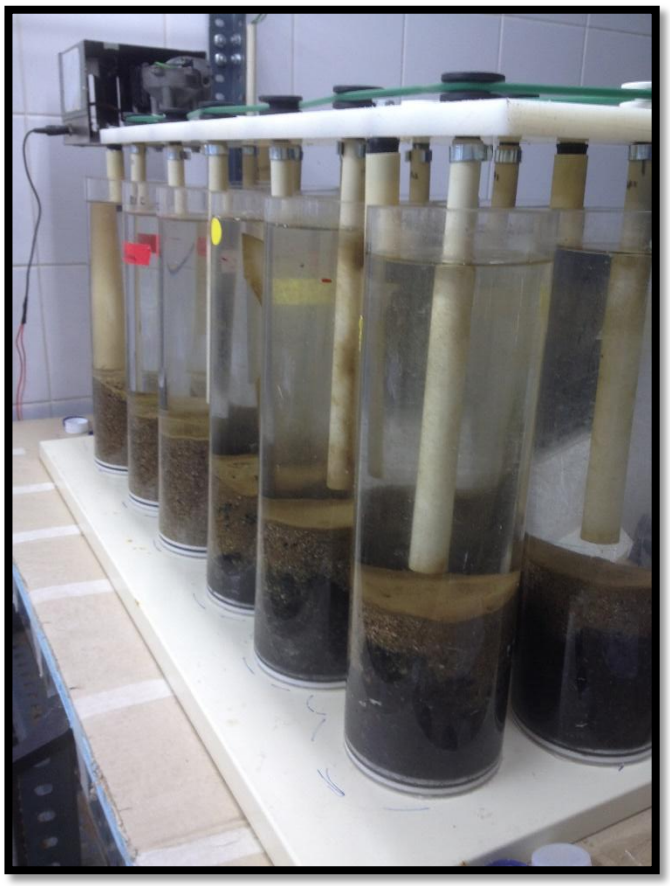
intermediate salinity (15‰). These results showed a similarity of 45% between intermediate and high salinity, and a similarity of 40% between the previous salinities and the low salinity treatment (0‰), suggesting that even in Crestuma sediments (characterized by low salinities) there are AOA ecotypes that are active in a wide range of salinities. Moreover, it seems that some AOA ecotypes operate under more restricted salinities. Similar results have been reported by Zhang et al. (2015) throughout the analyses of the distribution of phylogenetic AOA groups under a range of salinities (5‰, 15‰ and 30‰).

The Afurada DGGE results from bacterial *amoA* transcripts showed that 50% of the active AOB under 15‰ were the same that were also active at 0‰, suggesting that these AOB ecotypes operate under these range of salinities, while a few species are only active at 0‰. Additionally, lower AOB *amoA* transcription richness was registered for 30‰ with a similarity of 20% with the bands from lower salinities (0‰ and 15‰), suggesting the existence of some AOB ecotypes able to perform NH_4^+ oxidation in all salinities. In fact, some AOB species have been reported as salt tolerant and moderately halophilic like *Nitrosomonas marina*, *Nitrosomonas Europea*), *and Nitrosococcus mobilis* within Betaproteobacteria (Ventosa et al. 1998, Koops and Pommerening-Röser 2001). Furthermore, these differences might be explained with the presence of riverine, marine and native estuarine phylotypes, as previously demonstrated in other studies (Herlemann et al. 2011, Xie et al. 2014), and consequently are dependent to cope with osmotic stress (Bernhard et al. 2005, Koops et al. 2006). However, it is interesting to note, that changes in AOB *amoA* transcripts richness did not reflected into different magnitudes of AOB nitrification rates.

In Crestuma, AOB *amoA* transcripts amplification was only possible for the intermediate salinity. These results might suggest that under the lower and the highest salinity, the transcribed *amoA* gene abundances was too low to be detected by PCR. This issue was also reported by Zhang et al. (2015) which recover relative low AOB *amoA* gene abundance in cDNA samples, with not sufficient mRNA for pyrosequencing analysis of AOB *amoA* transcripts in the salinity amplitudes previously referred.

Furthermore, the number of bands from the transcribed bacterial *amoA* gene was equal to the number of bands from the bacterial *amoA* gene fragments, suggesting that the entire AOB community from Crestuma site are triggered by intermediate salinities.

The effect of salinity fluctuations on nitrification activity and communities



Chapter II

Results

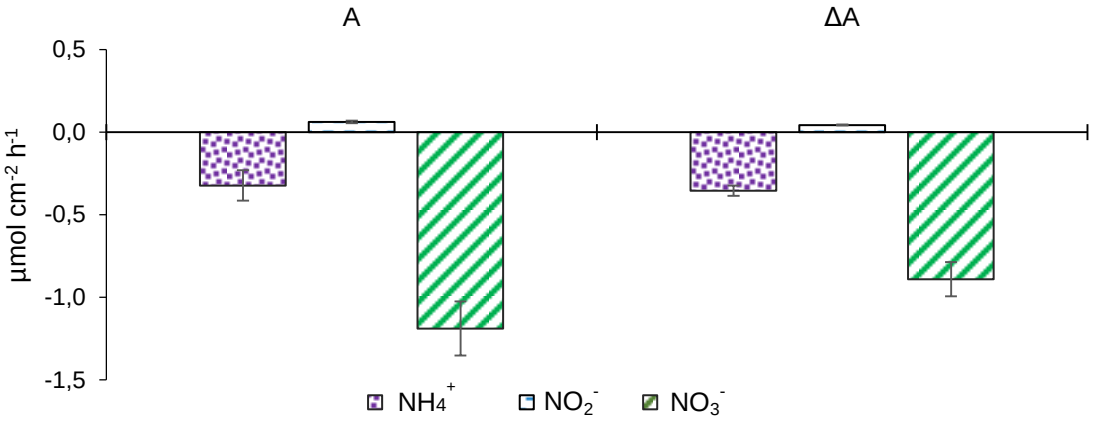
In order to evaluate the response of ammonia-oxidizing communities in terms of their activity and structure to salinity fluctuations, an experiment of 36 days was performed with sediment cores from Afurada and Crestuma, exposed to two salinity regimes (constant vs fluctuations). Salinity and temperature were monitored according to Table 1.

Table 1. Salinity and temperature from all cores during the salinity fluctuations experiment with total number of water exchanges, mean, minimum, maximum and standard deviation for constant salinity treatment in Afurada (A_1, A_2 and A_3) and Crestuma (C_1, C_2 and C_3) and for salinity fluctuations treatment in Afurada (ΔA_1, ΔA_2 and ΔA_3) and Crestuma (ΔC_1, ΔC_2 and ΔC_3).

Cores	Total exchanges	Salinity (‰)				Temperature (^{0} C)			
		Mean	Min	Max	SD	Mean	Min	Max	SD
A1	69	15,30	15,00	16,10	0,23	18,8	15,3	25	2,15
A2	69	15,34	15,00	16,10	0,20	18,8	15,3	25	2,15
A3	69	15,32	15,00	16,10	0,21	18,8	15,3	25	2,15
∆A1	69	15,52	1,50	29,20	9,42	18,8	15,3	25	2,15
∆A2	69	15,46	1,50	29,30	9,49	18,8	15,3	25	2,15
∆A3	69	15,49	1,50	29,10	9,37	18,8	15,3	25	2,15
C1	69	0,15	0,10	1,70	0,19	18,8	15,3	25	2,15
C2	69	0,16	0,10	1,40	0,21	18,8	15,3	25	2,15
C3	69	0,14	0,10	1,30	0,15	18,8	15,3	25	2,15
∆C1	69	15,49	1,40	29,30	9,50	18,8	15,3	25	2,15
∆C2	69	15,40	1,30	29,30	9,54	18,8	15,3	25	2,15
∆C3	69	15,45	1,20	29,30	9,56	18,8	15,3	25	2,15

1. Effect of salinity fluctuations on net inorganic nitrogen compounds

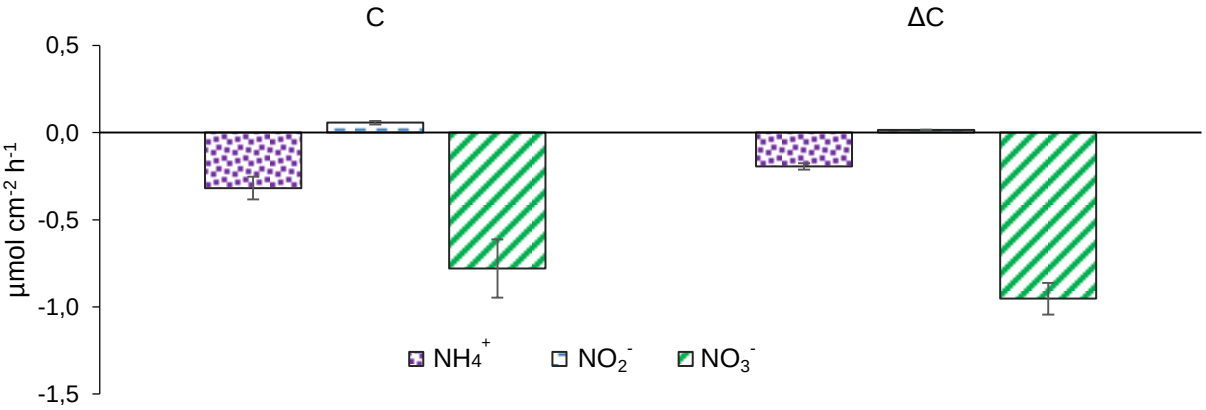
Results from net fluxes of NO_3^-, NO_2^- and NH_4^+ in Afurada and Crestuma sediment cores exposed to constant (A, C) and to salinity fluctuations (ΔA, ΔC) are presented in Figure



21 and 22, respectively. For further details on nutrients (NO_3^-, NO_2^- and NH_4^+) results see Appendix 1, Figure 2.

Figure 21. Mean ± SD of Afurada net fluxes of NH_4^+, NO_3^- and NO_2^- in sediment cores subject to constant (A) and salinity fluctuations (ΔA).

Net flux of NH_4^+ in Afurada sediment cores subject to constant (A) and fluctuations (ΔA) treatments showed similar uptake rates (*t*-Test, $P > 0.05$). The net flux results of NO_2^- showed a consistent release over time with significantly (*t*-test, $P < 0.05$) higher efluxes at constant salinity regime (A) in comparison with ΔA. Lastly, the net flux of NO_3^- presented high uptake rates over time in both treatments, with higher (*t*-Test, $P < 0.05$) uptakes in A



treatment in comparison with ΔA treatment.

Figure 22. Mean ± SD of Crestuma net fluxes of NH_4^+, NO_3^- and NO_2^- in sediment cores subject to constant (C) and salinity fluctuations (ΔC).

Net fluxes of NH_4^+ in Crestuma sediment cores (C and ΔC) presented considerably higher uptakes in C cores in comparison with ΔC cores, which is statistically significant (*t*-Test, $P < 0.05$). Furthermore, C cores also presented higher (*t*-Test, $P \leq 0.05$) NO_2^- release in comparison with ΔC. Lastly, the ΔC cores presented higher tendencies of NO_{3-} uptake in comparison with C cores, however it was not statistical (*t*-Test, $P > 0.05$) supported.

2. Salinity fluctuations effect on nitrification activity

Results of nitrification rates in Afurada (A and ΔA) and Crestuma (C and ΔC) sediment cores are presented in Figure 23.

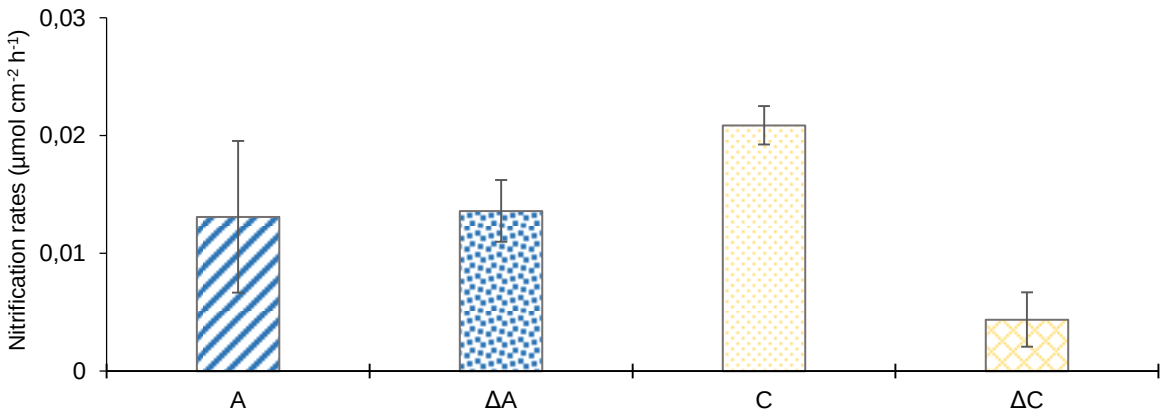


Figure 23. Mean ± SD of Afurada and Crestuma potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediment cores subjected to different salinity treatment (constant (A and C) and salinity fluctuations (ΔA and ΔC).

The potential nitrification rates in Afurada sediments in both treatments (A and ΔA) were identical without significant differences (*t*-Test, $P > 0.05$). On the contrary, results of Crestuma sediments subjected to the different salinity treatments (C and ΔC) presented significantly (*t*-Test, $P < 0.05$) higher rates of nitrification in the sediment cores subjected to constant salinity than the ones under salinity fluctuations.

3. Salinity fluctuations effect on bacteria and archaea nitrification activity

Potential nitrification rates measured in Afurada and Crestuma sediment slurries subjected to the two salinity regimes (constant and fluctuations) were measured with and without selective inhibitors for AOA and AOB. The treatment without inhibitor measured the nitrification rates from all active nitrifier microorganisms (TNM), the PTIO treatment (that inhibits ammonia-oxidizing archaea) measured the activity from ammonia-oxidizing bacteria (AOB), and the sulfathiazole treatment (that inhibits ammonia-oxidizing bacteria) measured the nitrification activity of ammonia-oxidizing archaea (AOA). Results of these selective measurements are presented in Figures 24 and 25, respectively. For more detailed results, see Appendix 4, Tables 3 and 4.

In Afurada, the potential nitrification rates pattern observed in A and ΔA treatments (Figure 24) were similar for all selective treatments (constant and salinity fluctuations treatments), showing a similar (one-way ANOVA, $P > 0.05$) response of TNM, AOB and AOA to the different salinity regimes. However, it is possible to observe that nitrification rates by AOB are significantly higher (two-way ANOVA, $P < 0.05$) than AOA nitrification rates.

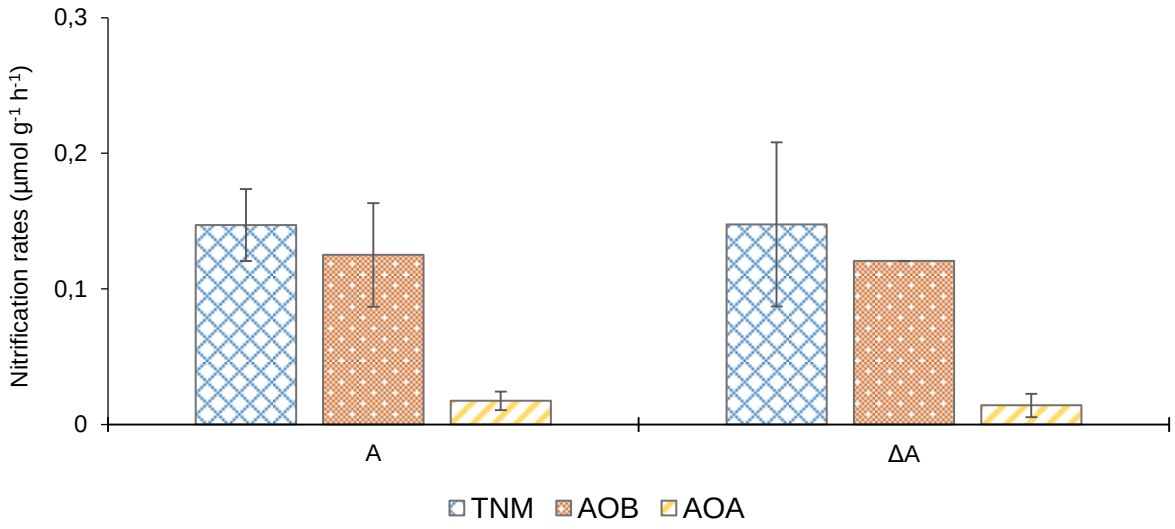


Figure 24. Mean ± SD of Afurada potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries from the two salinity regimes (constant (A) and fluctuation (ΔA)) amended with a salinity of 15‰ and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and sulfathiazole (rates of AOA).

In Crestuma, the total potential nitrification rates by TNM (Figure 25) were higher (one-way ANOVA, $P < 0.05$) at the C treatment slurries in comparison with salinity fluctuations treatment (ΔC). The AOB potential nitrification rates presented also a significant (one-way ANOVA, $P < 0.05$) decrease in the salinity fluctuations treatment. On the other hand, AOA did not show significant differences in their nitrification activity between C and ΔC treatments (one-way ANOVA, $P > 0.05$).

Lastly, potential nitrification rates by AOB were significantly (two-way ANOVA, $P < 0.05$) higher in comparison with AOA in C treatment. However, no significant differences (two-way ANOVA, $P > 0.05$) were found between AOB and AOA nitrification activity in ΔC treatment.

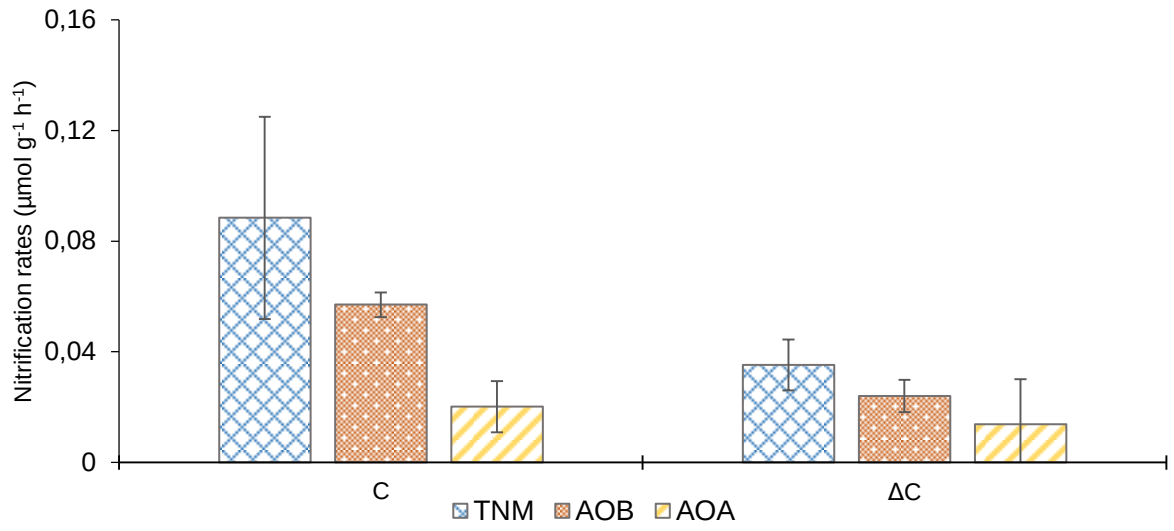


Figure 25. Mean ± SD of Crestuma potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries from the two salinity regimes (constant (C) and fluctuation (ΔC)) amended with a salinity of 15‰ and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and sulfathiazole (rates of AOA).

4. Salinity fluctuations effect on archaeal and bacterial *amoA* richness and *amoA* expression

4.1 Richness of archaeal *amoA* gene and its transcripts

DGGE analysis of archaeal *amoA* gene and *amoA* transcripts from Afurada sediments is presented in Figures 26A and 26B. Results showed a high number of DGGE bands for archaea *amoA* gene with, 39, 40 and 40 DGGE bands in constant salinity sediment cores, and 43, 43 and 44 DGGE bands in salinity fluctuations sediment cores. Therefore, few differences were observed between *amoA* gene richness (Figures 26A, 26B) as exhibited by the hierarchical cluster analysis that revealed a high similarity between the DGGE profiles (90%; Figure 26A).

As expected *amoA* transcripts richness representing the active *amoA* community was lower than the *amoA* gene richness, and noticeable differences were observed between the levels of AOA transcripts among the different salinity treatments (constant and salinity fluctuations). Hierarchical cluster analysis based on the average linkage of Bray-Curtis similarities, using the presence/absence of AOA *amoA* transcripts revealed substantial similarity of 70% and 65% between salinity fluctuations replicates (ΔA_1, ΔA_2 and ΔA_3) with 28, 31 and 25 DGGE bands respectively, and the constant salinity treatment core replicate A_1 (32 DGGE bands), and replicates A_2 and A_3 (19 and 15 DGGE bands), respectively. These results suggest a higher tolerance or adaptation of AOA that inhabit Afurada site to salinity fluctuations (0‰ – 15‰ – 30‰).

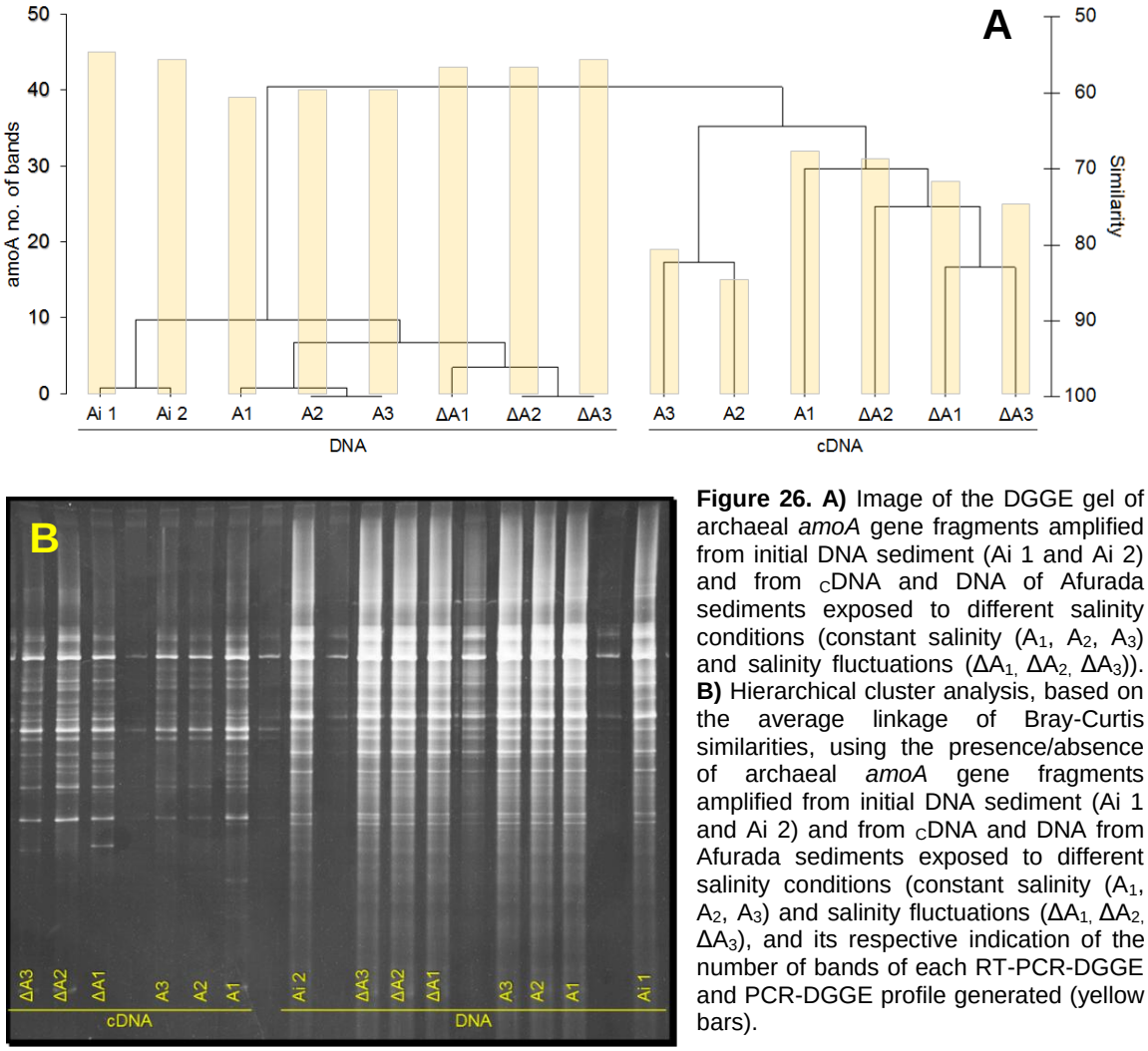


Figure 26. A) Image of the DGGE gel of archaeal *amoA* gene fragments amplified from initial DNA sediment (Ai 1 and Ai 2) and from $_{C}$DNA and DNA of Afurada sediments exposed to different salinity conditions (constant salinity (A_1, A_2, A_3) and salinity fluctuations (ΔA_1, ΔA_2, ΔA_3)). **B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of archaeal *amoA* gene fragments amplified from initial DNA sediment (Ai 1 and Ai 2) and from $_{C}$DNA and DNA from Afurada sediments exposed to different salinity conditions (constant salinity (A_1, A_2, A_3) and salinity fluctuations (ΔA_1, ΔA_2, ΔA_3), and its respective indication of the number of bands of each RT-PCR-DGGE and PCR-DGGE profile generated (yellow bars).

Interestingly, a different pattern was observed in Crestuma sediment cores, where AOA *amoA* genes retrieved from DNA collected from the beginning of the incubation presented less DGGE bands (31 and 35) in comparison with *amoA* gene richness from constant salinity (where it showed 49, 49 and 49 DGGE bands) and salinity fluctuations (45, 42 and 38 DGGE bands) after 36 days of incubation (Figures 27A, 27B). Furthermore, hierarchical cluster analysis based on the average linkage of Bray-Curtis similarities, using the presence/absence revealed only 62% of similarity between *amoA* gene from initial DNA

(Crestuma) and final DNA of constant and salinity fluctuations sediments (Figures 27A and 27B).

The salinity fluctuations treatment presented lower level of AOA *amoA* transcripts richness compared with constant salinity treatment, with a total of 9 DGGE bands compared with 33 and 22 DGGE bands, respectively. Additionally, hierarchical cluster analysis based on the average linkage of Bray-Curtis similarities, using the presence/absence revealed a high AOA *amoA* transcripts dissimilarity between constant and salinity fluctuations treatments of 72%. These results suggest that salinity fluctuations affected negatively AOA activity at Crestuma site.

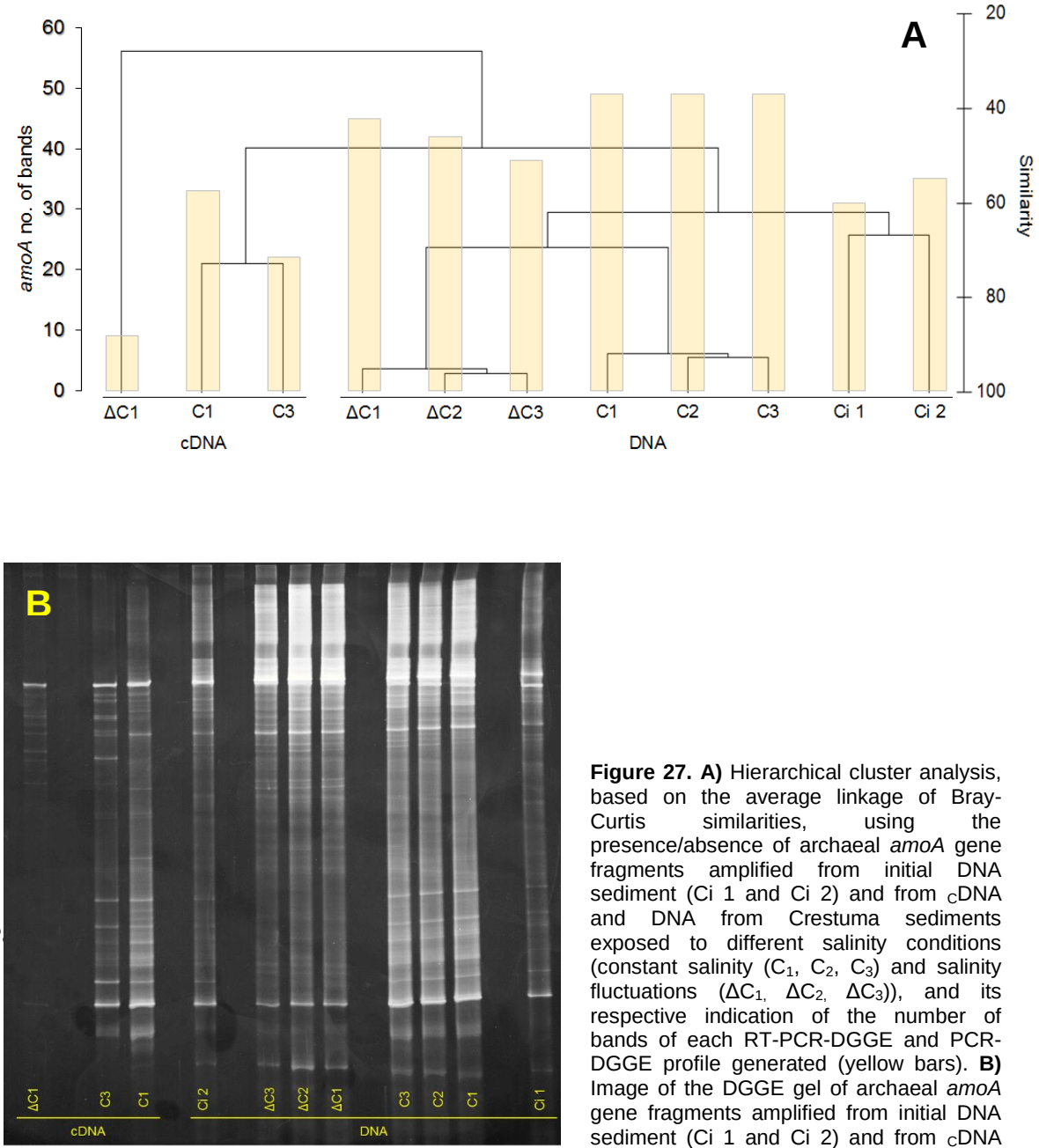


Figure 27. A) Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of archaeal *amoA* gene fragments amplified from initial DNA sediment (Ci 1 and Ci 2) and from $_C$DNA and DNA from Crestuma sediments exposed to different salinity conditions (constant salinity (C_1, C_2, C_3) and salinity fluctuations (ΔC_1, ΔC_2, ΔC_3)), and its respective indication of the number of bands of each RT-PCR-DGGE and PCR-DGGE profile generated (yellow bars). **B)** Image of the DGGE gel of archaeal *amoA* gene fragments amplified from initial DNA sediment (Ci 1 and Ci 2) and from $_C$DNA

P

4.2 Richness of bacterial *amoA* gene and its transcripts

DGGE analysis of bacterial *amoA* gene and *amoA* transcripts in Afurada sediments are present on Figures 28A and 28B.

In Afurada sediments the number of DGGE bands for *amoA* genes retrieved from the beginning of the incubation (initial DNA) and after 36 days in constant and salinity fluctuations treatments were similar (with 26 and 28 DGGE bands for initial DNA; 25, 25 and 26 DGGE bands for constant salinity treatment; and 29, 27 and 28 DGGE bands for the fluctuations treatment). According to the hierarchical cluster analysis, there was a similarity of 82% between all samples analysed, suggesting that the AOB community structure did not changed at the DNA level.

Afurada AOB *amoA* transcripts showed higher mean transcription richness in the salinity fluctuations treatment (19, 21 and 19 DGGE bands) compared with constant salinity treatment (21, 12 and 11 DGGE bands), however there was an exception of sample A_1 that showed higher levels of AOB *amoA* transcripts at the constant treatment. Hierarchical cluster analysis based on *amoA* transcripts, showed considerable low similarity of 59% between constant treatment replicates (A_2 and A_3) and salinity fluctuations replicates (Figure 28B).

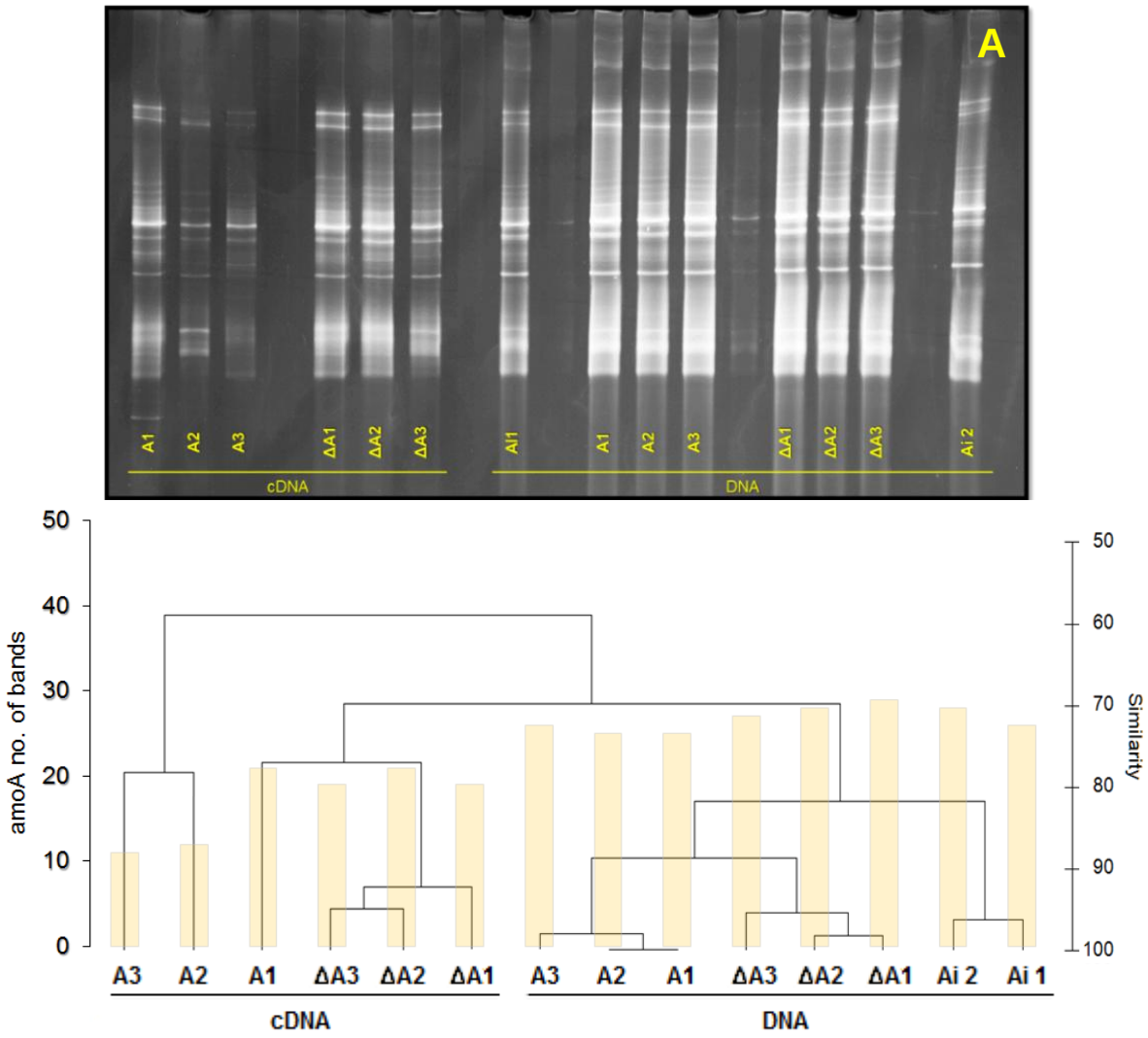


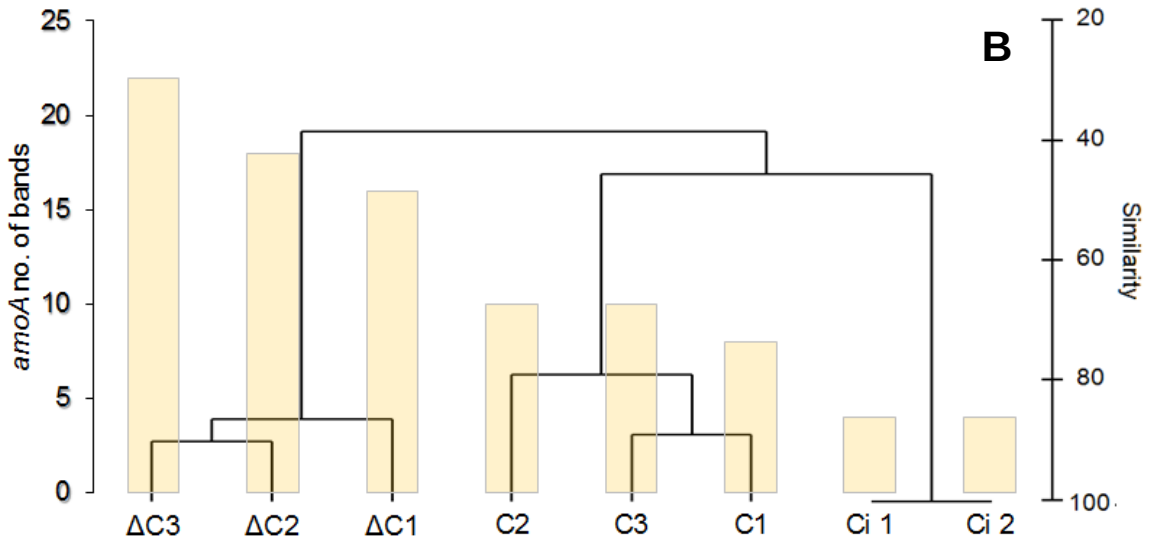
Figure 28 A) Image of the DGGE gel of bacterial *amoA* gene fragments amplified from initial DNA sediment (Ai 1 and Ai 2) and from cDNA and DNA of Afurada sediments exposed to different salinity conditions (constant salinity (A_1, A_2, A_3) and salinity fluctuations (ΔA_1, ΔA_2, ΔA_3)). **B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of bacterial *amoA* gene fragments amplified from initial DNA sediment (Ai 1 and Ai 2) and from cDNA and DNA from Afurada sediments exposed to different salinity conditions (constant salinity (A_1, A_2, A_3) and salinity fluctuations (ΔA_1, ΔA_2, ΔA_3), and its respective indication of the number of bands of each RT-PCR-DGGE and PCR-DGGE profile generated (yellow bars).

On the other hand, in Crestuma sediments the number of DGGE bands for *amoA* genes retrieved from the beginning of the incubation (initial DNA), and after 36 days in constant and salinity fluctuations treatments were considerably different (with 4 and 4 DGGE bands for initial DNA; 8, 10 and 10 DGGE bands for constant salinity treatment; and 16, 18

and 22 DGGE bands for the fluctuations treatment), suggesting that both treatments promoted the increase of AOB *amoA* richness (Figures 29A and 29B). According to the hierarchical cluster analysis (Figure 29A), there was a dissimilarity of 58% between all samples analysed, suggesting that the AOB community structure changed at the DNA level.

The null PCR results for cDNA samples from both treatments are not conclusive on the total absence of *amoA* transcripts, however they are indicative of lower levels of transcription in these treatments.

Finally, the lower richness of AOB *amoA* gene (4 DGGE bands) observed for Crestuma initial DNA sample compared with AOA *amoA* gene richness (26 and 28), indicate that the structure of Crestuma ammonia oxidizers communities are highly dominated by AOA.



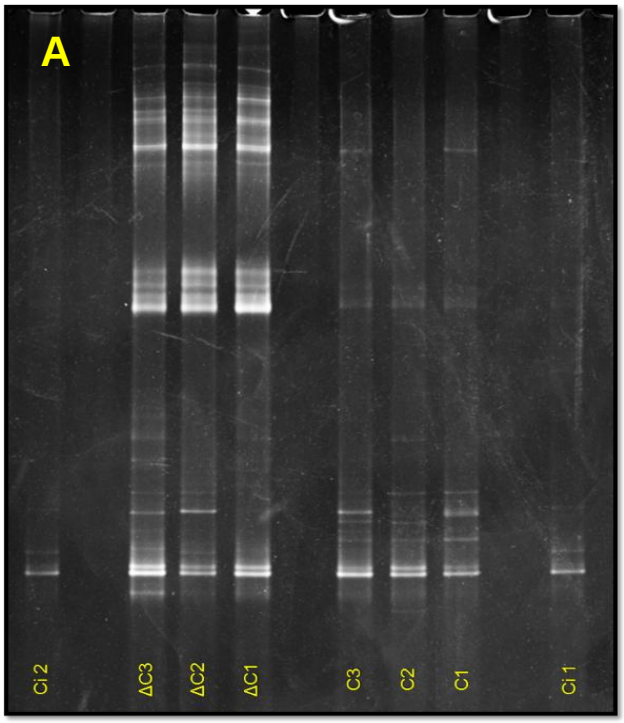


Figure 29 A) Image of the DGGE gel of bacterial *amoA* gene fragments amplified from initial DNA sediment (Ci 1 and Ci 2) and from DNA of Crestuma sediments exposed to different salinity conditions (constant salinity (C_1, C_2, C_3) and salinity fluctuations (ΔC_1, ΔC_2, ΔC_3)). **B)** Hierarchical cluster analysis, based on the average linkage of Bray-Curtis similarities, using the presence/absence of bacterial *amoA* gene fragments amplified from initial DNA sediment (Ci 1 and Ci 2) and from DNA of Crestuma sediments exposed to different salinity conditions (constant salinity (C_1, C_2, C_3) and salinity fluctuations (ΔC_1, ΔC_2, ΔC_3), and its respective indication of the number of bands of each PCR-DGGE profile generated (yellow bars).

5. Shifts on the diversity of bacterial and archaeal nitrifying organisms based on 16s rRNA next generation sequencing analysis

The 16S rRNA gene of 8 samples from Afurada and Crestuma sediments at the end of the incubation experiment (36 days), including two replicates of each salinity treatment (constant, fluctuations), were sequenced using the IIlumina NGS amplicon workflow (see "Material and Methods" section). The result reads were assembled, filtered for prokaryotic sequences, and annotated using SILVAngs pipeline. Sequences details from each sample are presented on Table 2.

Sample	Sample ID	Raw total reads	Raw read pairs	Adapter clipped total reads	Adapter clipped read pairs	Primer clipped total reads	Primer clipped read pairs	Combined reads
A1	A1	113,764	56,882	113,756	56,878	106,978	53,489	51,696
A2	A2	79,828	39,914	79,824	39,912	75,416	37,708	36,597
C2	C2	111,342	55,671	111,324	55,662	100,436	50,218	48,368
C3	C3	30,402	15,201	30,398	15,199	27,132	13,566	13,077
ΔA1	DA1	31,046	15,523	31,046	15,523	29,828	14,914	14,417
ΔA2	DA2	123,470	61,735	123,462	61,731	117,628	58,814	57,206
ΔC1	DC1	19,206	9,603	19,204	9,602	17,218	8,609	8,188

Table 2. Sequences output information from silva ngs analysis for each sample.

ΔC2	DC2	126,634	63,317	126,628	63,314	120,224	60,112	58,144

Bacteria kingdom abundance dominated all samples. Results at both constant and salinity fluctuations treatments showed that on average 96% and 97% of the total relative sequences from Afurada belong to bacteria phylotypes, respectively. While in Crestuma sediment cores, Bacteria presented an average relative abundance of 99% for all samples and treatments. Within the Bacteria kingdom, the dominant phyla were Proteobacteria with relative sequences abundance between 45%-48% and 49%-52% in the constant salinity treatments of Crestuma and Afurada sediments, respectively. Nevertheless, Proteobacteria was a better represented phylum in the salinity fluctuation treatments, with a relative percentage of 61% to 62% and 53% to 51%, respectively for Crestuma and Afurada.

In the present work, we are interested in evaluating shifts on relative abundance of nitrifier OTU's, and therefore on Fig. (30 - 34) it is displayed the relative abundance and taxonomy report of AOB, AOA and NOB from all treatments and sites studied.

In Figure 30, it is represented the taxonomy report of the Nitrosomonadales order of which the β-AOB *Nitrosomonas* and *Nitrosospira* genus belong. The results exhibited the presence by *Nitrosomonas* ecotypes in all samples, however the *Nitrosospira* ecotypes only appeared on A_1, A_2, C_2 and DA_2 samples. Furthermore, it is also possible to observe the occurrence of *Candidatus Nitrotoga*, a nitrite-oxidizing bacteria that was retrieved from one of each duplicated treatment.

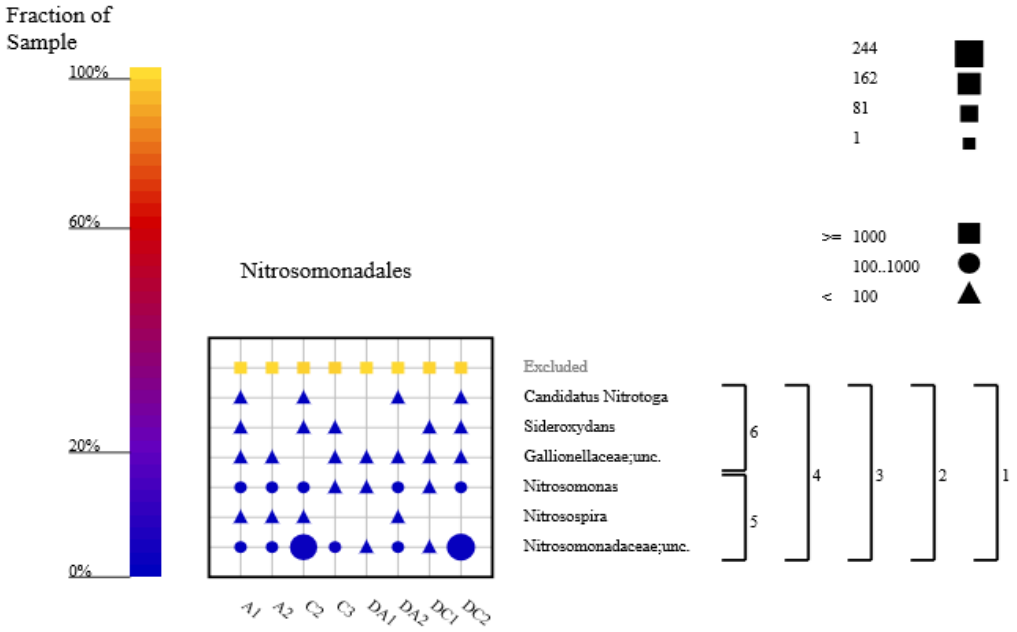
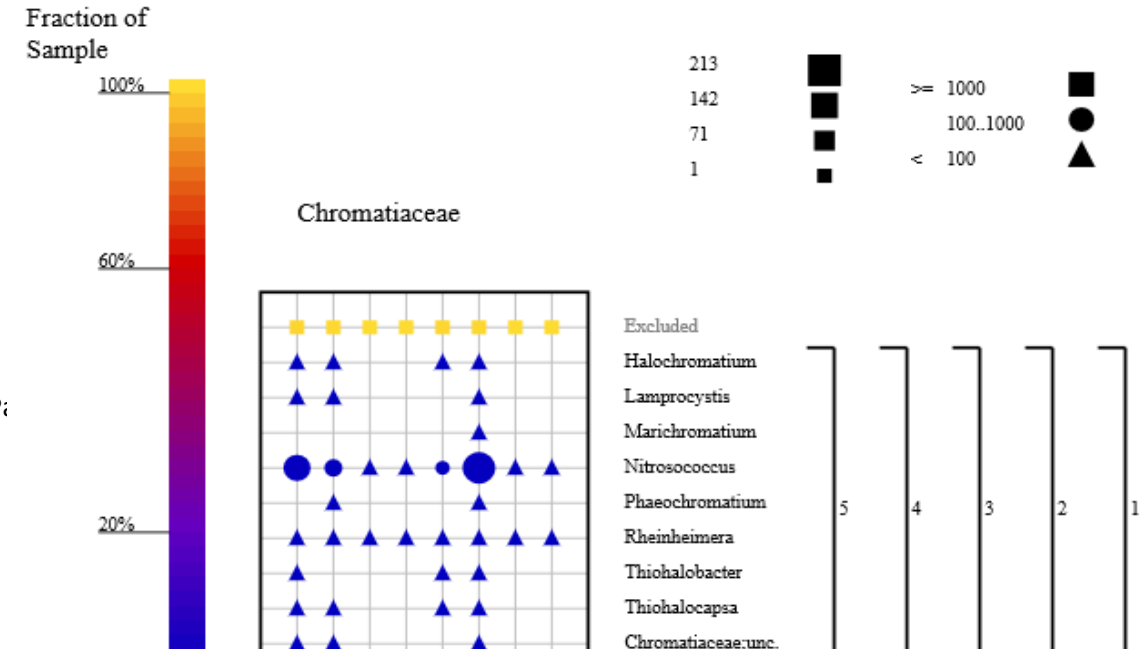


Figure 30. Taxonomy report of the Nitrosomonadales order where AOB *Nitrosomonas* and *Nitrosospira* genus are included as well the newly proposed NOB, the '*Candidatus Nitrotoga'*, which belong to the Betaproteobacteria class. Samples from Afurada constant salinity A_1 and A_2), Crestuma constant salinity C_2 and C_3), Afurada salinity fluctuation (DA_1 and DA_2) and Crestuma salinity fluctuation (DC_1 and DC_2) are presented. The number of sequences is given by the different shape, whereas the number of OTUs is given by the size of the different shape.

The γ-Proteobacteria comprises also ammonia-oxidizing bacteria from the *Nitrosococcus* genus. The *Nitrosococcus* genus was present in all samples however, higher abundance and diversity (nº of OTUs) were found on Afurada samples treatments (Figure 31).

Concerning the other groups of nitrite-oxidizing bacteria, on Figure 32 it is displayed the taxonomic abundance of Bradyrhizobiaceae family of which *Nitrobacter* genus belongs. Nevertheless, it was only observed the presence of this group of organisms on DC_2 (ΔC_2) sample. Moreover, on Figure 33 is represented the relative abundance and taxonomy report for Nitrospirales order of which *Nitrospira* genus is included. This group of NOB is present in all samples; however, it was more abundant and diverse in Crestuma sediments. Lastly, NOB *Nitrospina* genus which is included in Nitrospinaceae order, was highly represented in Afurada sediments (Figure 34), and it was observed on A_1, A_2, DA_2 (ΔA2) and DC2 (ΔC2) samples.



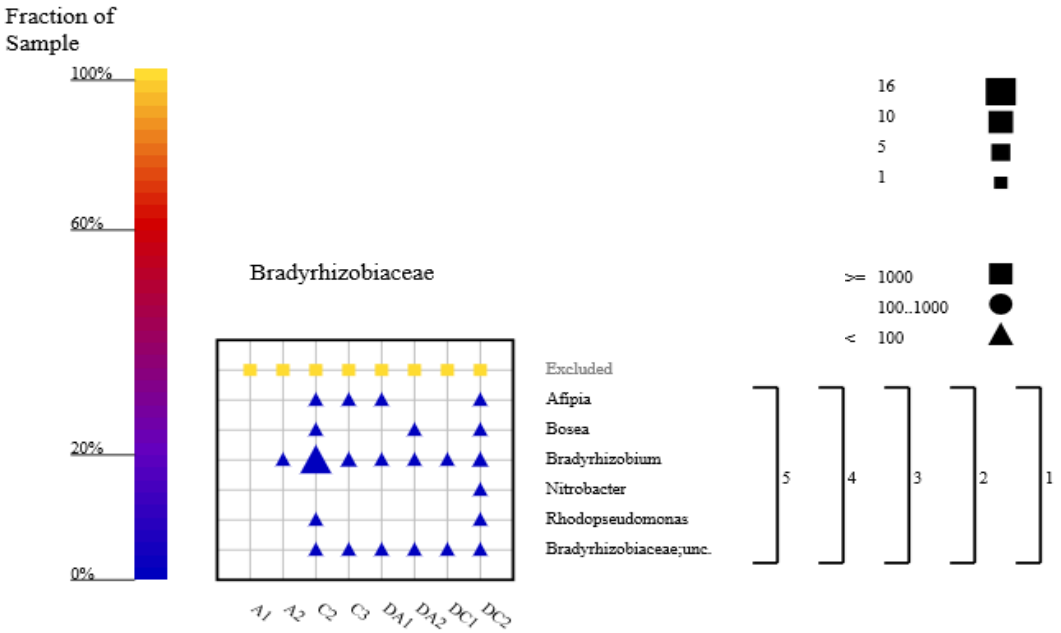


Figure 32. Taxonomy report of the Bradyrhizobiaceae family where NOB *Nitrobacter* genus are included which belong to the Alphaproteobacteria class. Samples from Afurada constant salinity A_1 and A_2), Crestuma constant salinity C_2 and C_3), Afurada salinity fluctuation (DA_1 and DA_2) and Crestuma salinity fluctuation (DC_1 and DC_2) are presented. The number of sequences is given by the different shape, whereas the number of OTUs is given by the size of the different shape.

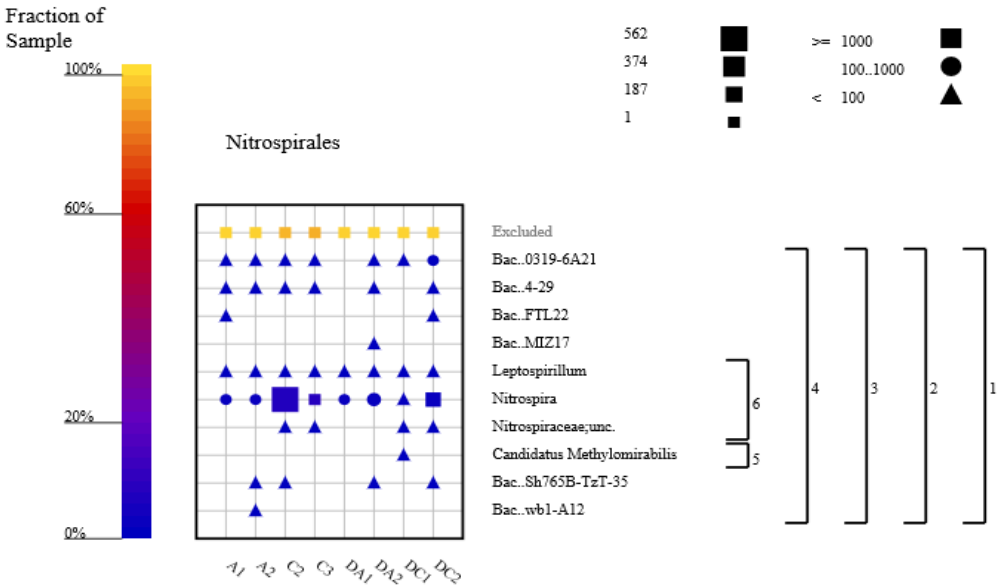
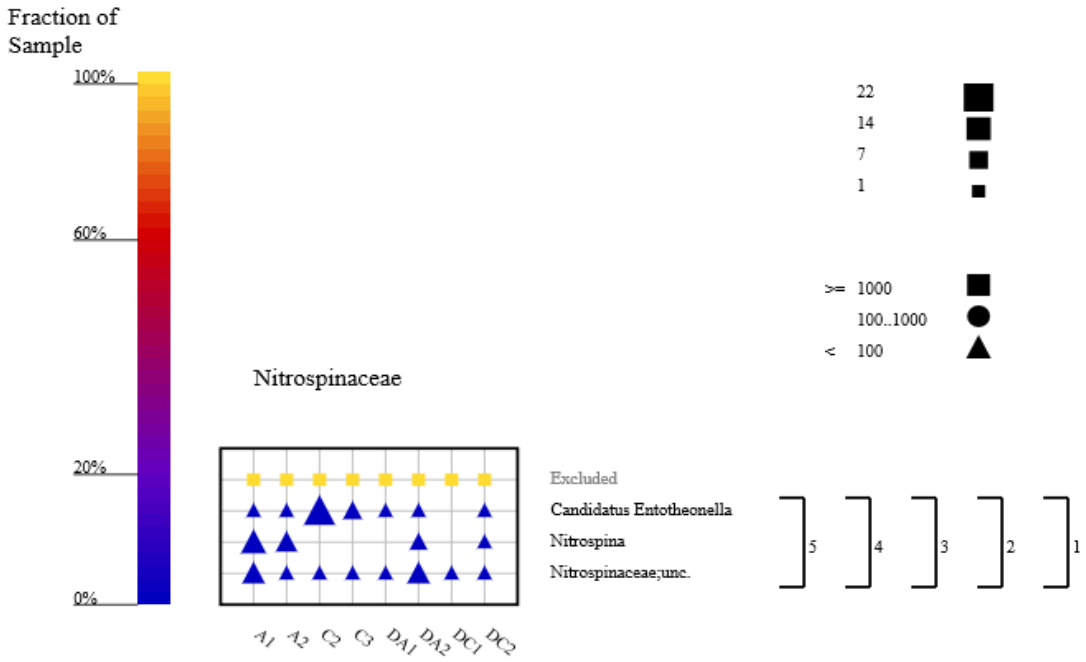


Figure 33. Taxonomy report of the Nitrospirales order where NOB *Nitrospira* genus are included which belong to the Nitrospirae phylum. Samples from Afurada constant salinity A_1 and A_2), Crestuma constant salinity C_2 and C_3), Afurada salinity fluctuations (DA_1 and DA_2) and Crestuma salinity fluctuations (DC_1 and DC_2) are presented. The number of sequences is given by the different shape, whereas the number of OTUs is given by the size of the different shape.



The Archaea group was present in all treatments however in different frequencies. On Afurada samples it represented on average between 2%-3% of the relative abundance of all sequences in both salinity treatments. On the other hand, at Crestuma samples it presented considerable lower abundance within constant and salinity fluctuations treatments (0.4%-0.3% and 0.5%-0.3%, respectively). When we look deeper on Archaea kingdom, it is possible to observed that Thaumarchaetoa phylum dominate Archaea abundances on these samples (Figure 35), exhibiting >98% and 94% of the total Archaea sequences, in Crestuma and Afurada samples, respectively.

Furthermore, it is possible to observe (Figure 35) a differentiation of some AOA ecotypes by sediment sites like the case of *Candidatus Nitrosospharae* and Arc. South African Gold Mine Group I OTUs that were only found on Crestuma site, as well the Arc.

Figure 34. Taxonomy report of the Nitrospinaceae order where NOB *Nitrospina* genus are included. Samples from Afurada constant salinity A_1 and A_2), Crestuma constant salinity C_2 and C_3), Afurada salinity fluctuation (DA_1 and DA_2) and Crestuma salinity fluctuation (DC_1 and DC_2) are presented. The number of sequences is given by the different shape, whereas the number of OTUs is given by the size of the different shape.

suggesting a wide distribution by these organisms such as *Candidatus Nitrosoarchaeum*, Arc.

Marine Group I and Arc. Sc-EA05. Lastly, there were two AOA ecotypes that appeared on both sediments but not in all treatments like the case of *Candidatus Nitrosopumilus* highly represented in Afurada site and Arc. Soil Crenarchaeotic Group (SCG), that did not appear on constant salinity treatment in Crestuma and on salinity fluctuations treatment in Afurada, respectively.

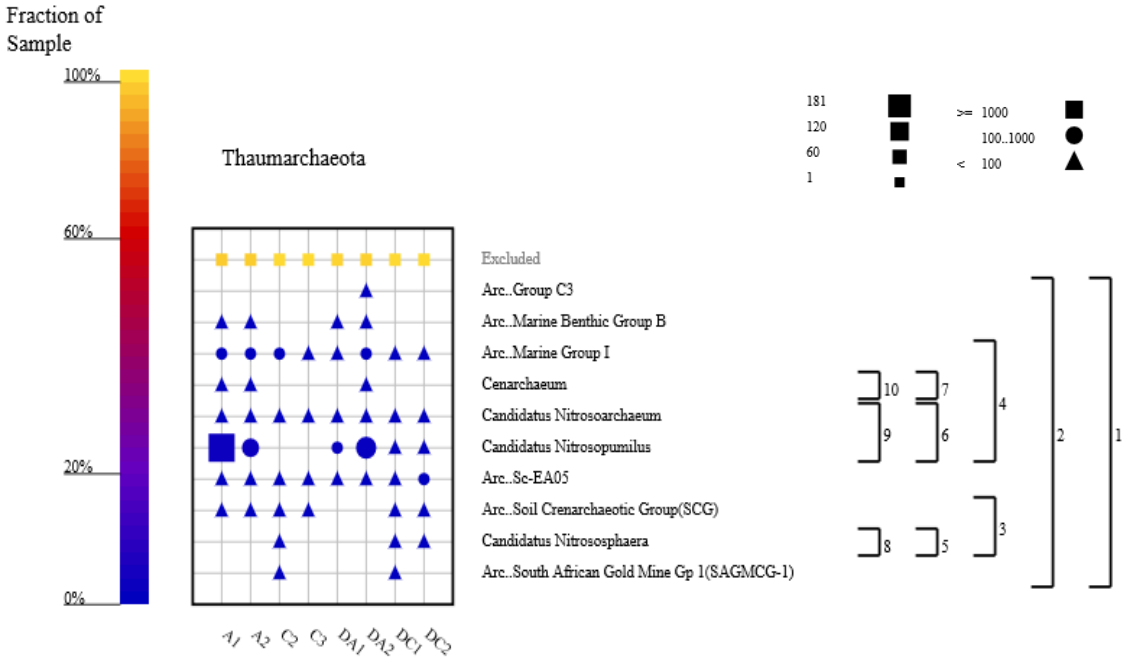


Figure 35. Taxonomy report of the Thaumarchaeota phylum which is represented by ammonia oxidizing archaea. Samples from Afurada constant salinity A_1 and A_2), Crestuma constant salinity C_2 and C_3), Afurada salinity fluctuation (DA_1 and DA_2) and Crestuma salinity fluctuation (DC_1 and DC_2) are presented. The number of sequences is given by the different shape, whereas the number of OTUs is given by the size of the different shape.

Discussion

Salinity is an important factor in regulating the functionality and distribution of the nitrifier microorganisms (Bernhard and Bollmann 2010a), where salt ions exert an osmotic pressure on microorganisms and, depending on the presence of mechanisms for the adaptation to different salt concentrations, microorganisms are able or not to survive to osmotic pressure conditions, or even become dormant (Bassin et al. 2011). In fact, previous studies have shown that salt concentration >10‰ causes plasmolysis and loss of microbial activity in cells when organisms are not adapted to saline environments (Kargi and Dincer 1996, Aslan and Simsek 2012).

In Chapter I, we evaluated the effect of a salinity gradient (short/direct term effect) on two type of sediments collected upstream and downstream Douro River estuary (Crestuma and Afurada, respectively). The analysis of these results showed differences between AOA and AOB ammonia oxidation rates from both sites according with the salinity treatments applied (0‰, 15‰, 30‰), suggesting the occurrence of different AOM ecotypes among sites. Moreover, the analysis of the *amoA* transcripts (cDNA-derived pattern) from both sites suggests a preference for lower salinities by the AOA ecotypes, and a preference for intermediate salinities by AOB ecotypes at both sites.

However, in estuarine systems, salinity can shift from salty to fresh during river inflows, or from fresh to salty during storm events, and consequently the occurrence of daily salinity fluctuations (Giblin et al. 2010) is an important factor to be considered, which vary with the estuary location (e.g. upper estuary has low salinity variations compared with lower estuarine sites). Moreover, the range of salinity fluctuations might promote the presence of different dominant halotolerant species (Vreeland 1987) and even the occurrence of non-tolerant species that might be inactivated under high salinities (Moussa et al. 2006).

So far, the impact of salinity fluctuations on nitrifier communities have not been investigated. Previous studies focused on evaluating AOM communities' activity, abundance and distribution in estuarine environments (Magalhães et al. 2005a, Giblin et al. 2010). Other controlled experiments have investigated the long-term effect on nitrification by increasing salinity gradually (Moussa et al. 2006, Bassin et al. 2011, Aslan and Simsek 2012, Gonzalez-

Silva et al. 2016), and studies to assess the salinity shock effect on nitrifiers cultures have been performed (Wu et al. 2008, Bassin et al. 2012, Jonassen 2013).

In Chapter II, we designed an experiment to test the effect of salinity fluctuations by developing a core incubation system that allowed us to simulate daily salinity fluctuations vs a constant salinity regime in Afurada and Crestuma sediments. This laboratory experiment, allowed us to study a long-term effect (36 days) of salinity fluctuations on AOM by monitoring inorganic nitrogen net fluxes, nitrification rates (^{15}N labelled technique), *amoA* gene and its transcripts richness, as well as shifts on 16S rRNA sequences of both archaea and bacteria nitrifiers diversity and abundance.

1. Salinity fluctuations effect on net inorganic nitrogen compounds and nitrification rates

The net fluxes of inorganic nitrogen compounds (NO_3^-, NO_2^-, NH_4^+) were measured in the different salinity treatments (constant and salinity fluctuations). As previously stated, the inorganic N net fluxes reflect the sum of the complex nitrogen compound processes occurring at the same time, and consequently, the results obtained can only assist as a guide of which process are more likely to be occurring and/or to be affected.

Afurada presented similar net uptake of NH_4^+ in constant salinity (A) treatment and salinity fluctuations (ΔA) treatment, suggesting that N-pathways might not be affected by salinity fluctuations as well as that part of these fluxes might be due to ammonia oxidation. On the other hand, net fluxes of NO_2^- exhibited significant differences between treatments, with the A treatment presenting higher levels of nitrite efluxes, suggesting that salinity fluctuations stimulated nitrogen processes responsible for the uptake of NO_2^- (nitrite oxidation through nitrification, denitrification and DNRA). In fact, nitrite-oxidizing bacteria (NOB), have been reported to be sensitive to high salinities (Bassin et al. 2011), which eventually would lead to an accumulation of nitrite. Nevertheless, net influx of NO_3^- was significantly higher in A treatment compared with ΔA treatment, suggesting higher activity of denitrification and/or DNRA processes at constant salinity.

Furthermore, nitrification rates measured in the undisturbed Afurada cores revealed an absent of significant differences between A and ΔA treatments, which is supported by the similar uptake rates of NH_4^+, suggesting that TNM activity from Afurada site is not affected by

salinity fluctuations. Additionally, these results are in agreement with the salinity gradient experiment (Chapter I), where no significant differences were found on potential nitrification rates in Afurada sediment slurries at the different salinities tested (0‰, 15‰ and 30‰).

On the other hand, divergent results were found in Crestuma sediments subjected to constant and salinity fluctuations treatments. Net fluxes of NH_4^+ from constant salinity treatment presented higher uptake rates compared to salinity fluctuations treatment, suggesting that salinity fluctuations exerted a negative effect on the processes responsible for NH_4^+ uptake (like nitrification and anammox) and/or stimulates ammonification rates. Similarly, the net flux of NO_2^- also presented significant higher uptakes of NO_2^- in the C treatment over the ΔC treatment, which might suggest that salinity fluctuations affected nitrogen processes responsible for the uptake of NO_2^- (nitrite oxidation through nitrification, denitrification and DNRA). Lastly, no differences were found between net uptake of NO_3^-, suggesting no distinct effect by the treatments on denitrification and DNRA.

The differences between salinity treatments were also seen on nitrification rates measured at Crestuma undisturbed cores, which exhibited higher rates on C treatment compared with ΔC treatment. These results together suggest that TNM activity of Crestuma were negatively affected by salinity fluctuations treatment. In fact, Crestuma site undergoes low salinity fluctuations (Azevedo et al. 2010), and therefore the Crestuma nitrifier community when faced against an induced high salinity fluctuations regime (our study) can suffer a shock effect, causing plasmolysis and loss of microbial activity, as previously described Kargi and Uygur (1997). Moreover, the non-tolerant species of AOM from Crestuma might survive when faced to high salt concentration through inactivation mechanism, as previously reported in a study by Moussa et al. (2006). This authors found that the nitrifier communities from a domestic wastewater treatment plant in operation during four years on a reference reactor (no salt added), after being exposed to high salinity gradient (118 days), some of the species that disappeared, started to recover after 2 days, indicating a recovering of the organisms to salt stress. And so, our results suggest that AOM from Crestuma sediments are adapted to low salinities, which is in agreement with the results presented in Chapter I, where significant lower potential nitrification rates were found for the highest salinity treatments (15‰ and 30‰).

In addition, selective measurements of potential nitrification rates (PNR) indicate that the activity of AOB were always higher than AOA in Afurada sediments. These results suggest that AOB are the main responsible organisms for ammonia oxidation in both Afurada treatments. In fact, the dominance of AOB communities over AOA on high salinity regions of estuarine systems have been reported on Weeks Bay (Caffrey et al. 2007), Douro River estuary (Magalhães et al. 2009), Cochin estuary (Veettil et al. 2015) and Colne estuary (Li et al. 2015). Furthermore, our results are in agreement with previous findings presented in Chapter I, where it was shown that Afurada sediment slurries presented higher nitrification rates by AOB.

Nevertheless, the potential nitrification rates in Crestuma for AOA and AOB activity were identical on salinity fluctuations treatment but different on constant salinity treatment, where AOB presented higher PNR. In fact, the results on Chapter I presented similar potential nitrification rates between AOA and AOB under different salinities suggesting that both AOM group display identical roles on the nitrification process in Crestuma. And so, our results suggest that the *in situ* daily low salinity fluctuations in Crestuma site might have contributed for bacteria and archaea nitrifiers identical roles, and consequently the increase to higher salinity fluctuations (salinity fluctuations treatment) have altered AOM community structure (as seen on DGGE fingerprint) but did not change nitrification performance (Bassin et al. 2012).

2. Salinity fluctuations effect on bacteria and archaea nitrifiers communities and on their transcripts

Ammonia-oxidizing bacteria communities robustness in higher salinities towards lower salinities have been described by Jonassen (2013) where he showed that these communities under higher salinities displayed a certain plasticity that can be advantageous on environments subjected to salinity amplitudes. Furthermore, Gonzalez-Silva et al. (2016) reported that a culture from a brackish environment (like Afurada site) when amended to different salinities (0‰, 13‰, 22‰ and 33‰) in a period of 54 days presented 28% higher NH_4^+ oxidation in lower salinities compared to the tested *in situ* salinity (22‰) and higher

salinity (33‰), suggesting a wide plasticity by AOM (from a brackish zone) to different salinities. In fact, our results from Afurada bacterial *amoA* gene DGGE analysis indicate high similarities between treatments, suggesting that AOM assemblages in these sediments are most likely acclimated to salinity fluctuations regimes. Indeed, salinity fluctuations are typically accompanied by changes in a suite of physico-chemical properties, demanding physiological plasticity of the resident microbes, and therefore, enabling a community of microbes with different tolerances to these conditions (Bernhard and Bollmann 2010a). However, despite the few differences of AOB community structure between treatments, DGGE profiles of bacterial *amoA* transcripts presented considerable differences between salinity treatments (constant and salinity fluctuations), suggesting that despite the maintenance of the AOB structure and the similar potential nitrification rates, shifts in the active AOB strains occurred. Indeed, *amoA* transcripts richness is considerable different between treatments, suggesting that the nitrification process is carried by different groups of AOB that are enhanced by the specific salinity treatments. And so, it seems that salinity fluctuations favoured bacterial *amoA* transcripts in Afurada sediments since it presented considerable higher expressed *amoA* richness compared with constant salinity treatment. However, it is important to have into account that higher *amoA* transcripts richness does not reflect necessarily higher nitrification performance (Prosser and Nicol 2012, Berg et al. 2015).

Additionally, NGS sequencing analysis at the DNA level showed that β-AOB presented similar relative abundances of the *Nitrosomonas* genus between Afurada treatments cores. Additionally, *Nitrosospira* genus were present with similar abundances on Afurada constant treatment cores (A_1 and A_2), one duplicate of Crestuma constant treatment (C_2) and Afurada salinity fluctuations treatment (DA_2) with the remain samples (C_3, DA_1, DC_1 and DC_2) not indicating the presence of *Nitrosospira* genus.

However, differences between duplicate cores can be attributed to the low number of sequences retrieved from MiSeq sequencing analysis of samples C_3, DA_1 and DC_1 (Table 2). The presence of Nitrosomonadales (*Nitrosonomas* and *Nitrosospiras*) have been shown on different cultures from seawater, brackish areas and freshwater (Gonzalez-Silva et al. 2016) with a percentage average under the different habitats of 6%, 34.7% and 14.7% of the total reads, respectively.

In Afurada, the γ-AOB group presented in general similar abundances of *Nitrosococcus* genus, which is expected since this group of AOB are obligatory halophilic (Koops and Pommerening-Röser 2001). Moreover, the *Nitrospira* genus from which belong the recently discovered species able to perform both steps of nitrification (Daims et al. 2015) also presented similar abundances. These results support the previous assumption of high similarity between treatments, suggesting an elevated tolerance by these group of AOB.

On the contrary, AOB communities from Crestuma presented higher PNR on constant salinity treatment, suggesting that ammonia-oxidizing bacteria from Crestuma are more sensitive to salinity fluctuations. The higher sensitivity toward the salinity fluctuations might be explained by the fact that AOB from Afurada are well adapted to daily high salinity fluctuations and, on the contrary, AOB communities from Crestuma are probably composed by species adapted to low salinity fluctuations, which may have been affected by osmotic pressure when exposed to a high salinity. Likewise, Cortés-Lorenzo et al. (2015) showed that concentrations of 24.1 g NaCl/L in a freshwater water influent resulted in a remarkable decrease of the NH_4^+ oxidation capacity of the system and a shift in AOB species present in the biofilm, as well as the inhibition of the nitrification process.

Moreover, if we take into account the bacterial *amoA* richness given by the DGGE analysis on Crestuma treatments, results suggested that both treatments favoured the appearance of new AOB ecotypes that were initially under low number. Even though an increase of AOB richness was registered in both treatments, the salinity fluctuations treatment presented a considerable higher bacterial *amoA* richness, suggesting high plasticity of these organisms towards salinity fluctuations. Consequently, lower potential nitrification rates in salinity fluctuations treatment in comparison with constant salinities in Afurada treatment might be due to the fact that AOB ecotypes are adapted to a wide range of salinity (Bernhard and Bollmann 2010a), with optimal activity under higher salinities. In fact, the MiSeq sequencing analysis showed for Crestuma sediments that the salinity fluctuations treatment in comparison with constant salinity treatment, favoured a small increase of operational taxonomic unit's (OTU) from the *Nitrosococcus* genus which are known to be obligate halophilic species (Grommen et al. 2005, Cui et al. 2016).

However, the *amoA* cDNA amplification for both treatments were null suggesting either a low expression by this group of organisms, or the non-capacity of our primers (which amplify β-AOB) to target the active AOB (such as the γ-AOB), and/or a low RNA quality as seen in Appendix 3. And therefore, the results obtained from AOB nitrification rates in Crestuma treatments might not be entirely explained by the action of β-AOB communities. Another possible explanation is the occurrence of *Nitrospira* species able to perform the complete nitrification process - commamox (Daims et al. 2015). In fact, studies have reported that NOB are more sensitive to salinity than AOB (Bassin et al. 2011) and consequently, the differences between AOB potential nitrification rates might be in part due to the activity of NOB. When we look at the MiSeq sequencing analysis, it is possible to see that *Nitrospira* group are greatly abundant on constant salinity treatment cores (C_2 and C_3) and decrease considerable on the salinity fluctuations cores (DC_1 and DC_2). And therefore, the *Nitrospira* species able to perform commamox might explain the contradictory differences between PNR and DGGE results.

Regarding ammonia-oxidizing archaea, in Afurada sediments, similar potential nitrification rates were observed in both salinity treatments. This results suggest an elevated plasticity by these group of organisms toward salinity fluctuations, as it was observed for AOB. Therefore, AOM from Afurada are likely to be adapted to salinity fluctuations as a consequence of a daily salinity fluctuations (Giblin et al., 2010).

The NGS analysis support these results by showing high similarity between salinity treatments with the exception of Arc. Group C3 OTU's that only appeared in a sample subjected to salinity fluctuations (DA_2). Likewise, DGGE analysis found high similarities in *amoA* gene analysis, but with DGGE profiles of archaeal *amoA* transcripts showing considerable differences between salinity treatments, with salinity fluctuations presenting higher number of transcripts and therefore, suggesting that salinity fluctuations favoured a highly diverse active AOA community. Even though these differences were found, the potential nitrification rates were similar between treatments which might be explained by the fact that AOA phylotypes might have different physiologies and tolerances that affect nitrification rates in ways that are not reflected by total *amoA* gene abundance (Puthiya Veettil et al., 2015).

On the other hand, despite Crestuma sediments presented similar PNR between salinity treatments, the AOA *amoA* gene DGGE analysis exhibited considerable dissimilarity between treatments, suggesting that salinity fluctuations affected AOA composition. In fact, the results suggest that both treatments altered AOA composition with an increase of their richness compared with the initial sample. However, AOA *amoA* transcripts showed a dissimilarity between constant and salinity fluctuations treatments of 72%, suggesting that salinity fluctuations affected AOA *amoA* expression differently at Crestuma sediments. These considerable differences between AOA composition, was not reflected on nitrification performance, most probably since different phylotypes might have different physiologies and tolerances to salinity that affect nitrification rates in ways that are not reflected by *amoA* gene abundance/diversity/transcription (Bassin *et al.*, 2012, Puthiya Veettil et al., 2015). The most outstanding difference weas registered on the NGS analysis with the appearance of the *Candidatus Nitrosopumilus* in the salinity fluctuations treatment which did not appear in the constant salinity treatment, suggesting that salinity fluctuations favoured *Nitrosopumilus* genus on Crestuma sediment. In fact, the *Nitrosopumilus maritimus*-like dominance has been reported in a laboratory-scale bioreactor treating saline wastewater under 10‰ (Jin et al. 2010). Moreover, Wu et al. (2013b) hypothesized that a high salinity condition may promote the growth of the marine group representative, *Nitrosopumilus maritimus*, while a low salinity condition may promote the growth of the soil group representative, *Nitrososphaera gargensis*, and therefore supporting our findings.

In general, the results obtained from inorganic net fluxes, potential nitrification of TNM and the potential nitrification rates from AOA and AOB presented concordant results where salinity fluctuations treatment seems to affect Crestuma ammonia-oxidizing organisms, more precisely, ammonia-oxidizing bacteria. On the contrary, at Afurada site ammonia-oxidizing bacteria seem to display an elevated plasticity toward salinity fluctuations, as expected due to site characteristics. Similar result was reported in a study by Magalhães et al. (2009) in the sediments of Douro River estuary, where the abundance of AOB *amoA* gene was found to be always greater than that of AOA *amoA* gene with a salinity variation of 1.5‰–26.8‰ in a whole year. These results are also in agreement with Gonzalez-Silva et al. (2016) that

showed high nitrification rates from a brackish culture under different salinities, and a decrease on nitrification rates by the freshwater environment.

Conclusion and future perspectives

Estuarine systems are characterized as highly dynamic zones due to their continuously mixing of freshwater and saltwater creating a physical-chemical gradient that shape the resident microbial communities. The microbial communities' functionality and distribution is affected by the estuarine salinity gradient, and therefore understand how these group of organisms respond to different salinity regimes under the estuarine systems is desired.

The salinity gradient experiment (Chapter I) aims to analyze both AOA and AOB response to different fixed salinity regimes in two distinct sites of the Douro River estuary. The combined inorganic net fluxes, potential nitrification rates and fingerprinting analysis of *amoA* transcripts brought new insights on how salinity might select AOA and AOB groups along an estuarine salinity gradient. Our findings suggest that different salinity regimes have shaped the occurrence of different nitrifier communities at both estuarine downstream and upstream locations. Moreover, downstream site with daily strong salinity regimes (Afurada) promoted the occurrence of more tolerant AOM (AOA and AOB) to wide range of salinities (0‰ – 15‰ – 30‰), which was not observed in the upstream stations (Crestuma), where AOM activity was clearly affected by high salinities. Comparative DGGE profiles of bacterial and archaeal *amoA* transcripts from both sites indicated a higher preference by AOA ecotypes for lower salinities, and AOB preference for intermediate salinities, which in part explain why AOB is the main responsible for nitrification activity at Afurada.

The salinity fluctuations experiment (Chapter II) represents the first study to analyze the plasticity of both AOA and AOB communities to daily changes of salinity regimes in laboratory. Our combined inorganic net fluxes, potential nitrification rates, fingerprinting analysis of *amoA* transcripts and 16S rRNA sequencing add interesting findings on the

response of both AOA and AOB groups to salinity fluctuations in two distinct sites of Douro River estuary. Our findings suggested that AOM (AOA and AOB) from Afurada sediments are well adapted to daily salinity fluctuations, and AOB are the main responsible for nitrification activity. On the other hand, at Crestuma sediments our findings suggest that salinity fluctuations negatively affected nitrification rates. Furthermore, salinity fluctuations treatment in Crestuma favoured the increase of high tolerant AOA and AOB species from *Nitrosopumilus* genus and *Nitrosococcus* genus, respectively. Lastly, the absence of AOB *amoA* transcripts in Crestuma station and the highly relative abundance of *Nitrosospira* species in constant salinity treatments based on 16S rRNA NGS analysis leads us to hypothesized that strains able to perform both steps of nitrification represent a main role on Crestuma nitrification process being negatively affected by salinity fluctuations.

The data generated from this study represents an important background to help us understand the distribution of the nitrifier microbial communities along an estuarine salinity gradient, and to understand their response to daily salinity fluctuations. However, future studies are required focusing on quantitative studies of *amoA* gene abundances from both AOA and AOB groups as well as a detailed analysis on the role of the recently discovered commamox species (*Nitrospiras* species) in environments subjected to salinity gradients and strong daily salinity fluctuations.

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Supplementary information

Appendix 1. Inorganic nitrogen compounds quantification

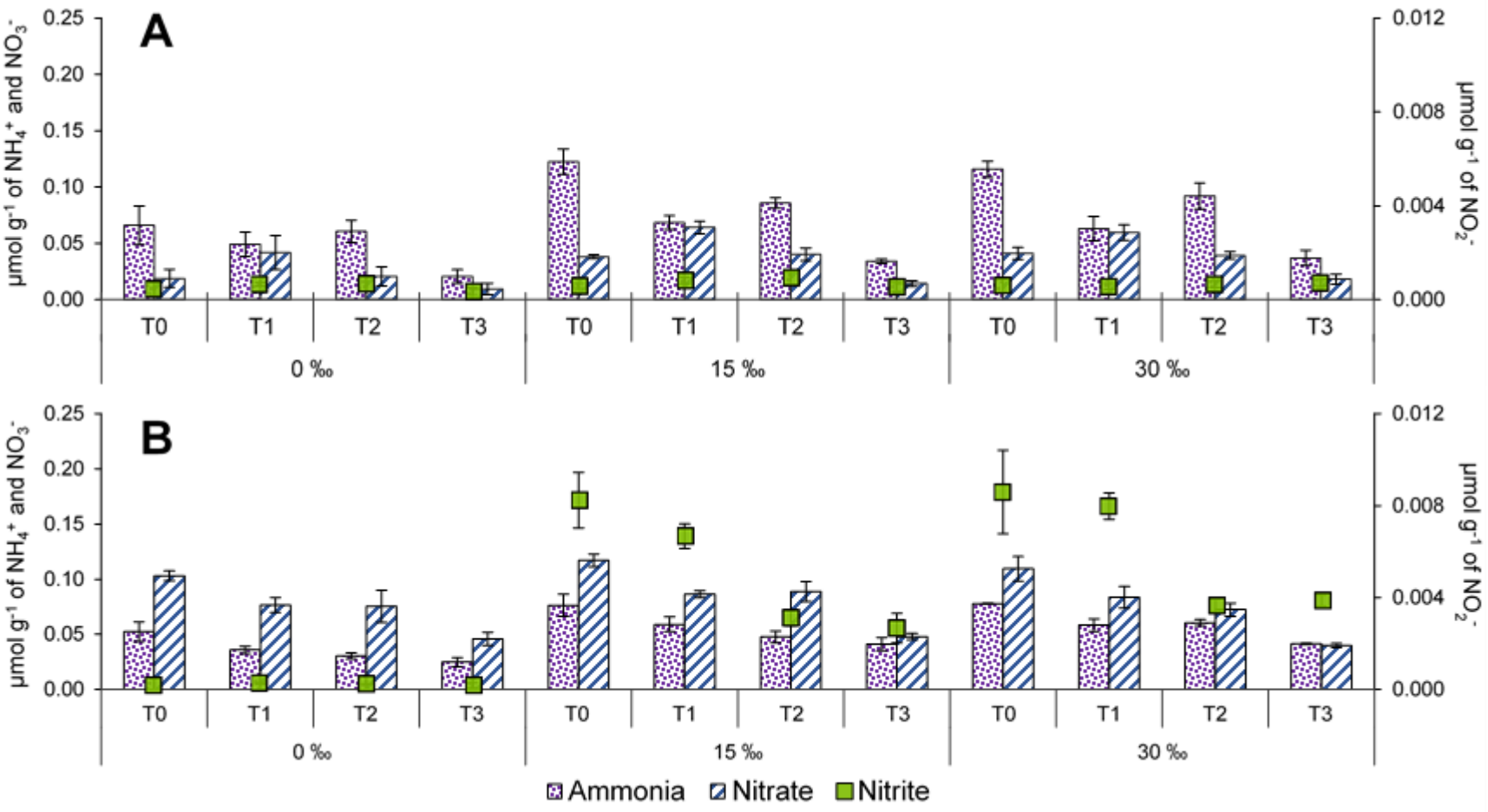

Figure 1. The concentration of ammonia (purple bars), nitrate (blue strips bars) and nitrite (green plot) with its respective standard deviation (n=3) from Afurada (A) and Crestuma (B) sediments subjected to a salinity gradient experiment (0‰, 15‰ and 30‰) and collected during 3 hours (time 0h, 1h, 2h and 3h).

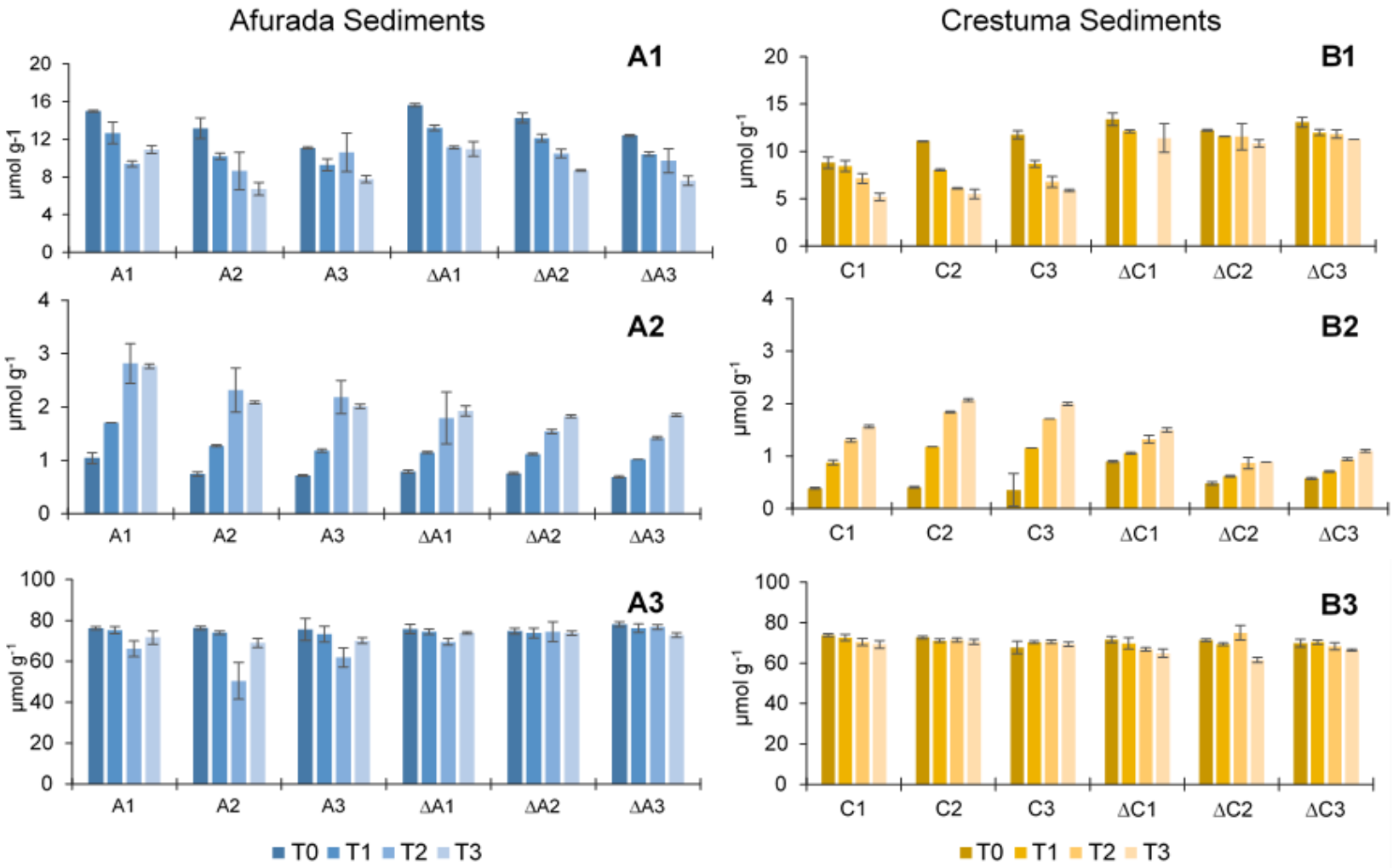

Figure 2. The concentration of ammonia (A1 and B1), nitrate (A3 and B3) and nitrite (A2 and A3) with its respective standard deviation (n=3) from Afurada (A) and Crestuma (B) sediments subjected to salinity fluctuations (ΔA and ΔC) as well to a constant salinity treatment (A and C) and collected during 3 hours (time 0h, 1h, 2h and 3h).

Appendix 2. Solution and reagents used for the inorganic nutrient quantification

Ammonia Reagents

Magnesium Reagent

- ✓ Dissolve 45 g of Sodium Chloride (NaCl) and 20 g de Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in 200 mL of dH_2O.
- ✓ Add few drops of NaOH at 1M until start to form a precipitate.
- ✓ Add few pieces of glass to the solution and put the solution boiling (to remove ammonia) until reach a quantity of less than 200 mL.
- ✓ Allow the solution to cooldown and fulfill to 200mL with dH_2O.

Phenol and nitroprusside reagent

- ✓ Dissolve 3.8 g of phenol (C_6H_5OH) in 100 mL of dH_2O.
- ✓ Dissolve 0.04 g of nitroprusside ($Na_2Fe(CN)_5NO.2H_2O$) in the previous solution.

Hypochlorite reagent

- ✓ Dilute 0.25 g of Trione (corresponding to 750 mg of Cl-) in 100 mL of NaOH at 0.5 N.

Standard primary solution (100 mM)

- ✓ Dry the ammonium chloride (NH_4Cl) at 50°C overnight (to constant weight).
- ✓ Dissolve 0.5349 g of the above reagent in 100 mL of dH_2O.
- ✓ Preserve the solution by adding a few drops of chloroform.

Nitrate and Nitrite reagents

Colored reagent

- ✓ Dilute 50 mL of Phosphoric acid (H3PO4, 80%) in 400 mL of dH_2O.
- ✓ Add to the above solution 5 g of sulfanilamide ($4\text{-}NH_2C_6H_4SO_2NH_2$) and dissolve.

- ✓ Add to the above solution 0.5 g of NNED (N-1-naphthylethylenediamine dihydrochloride) and dissolve.
- ✓ Make up to 250 mL with dH_2O.

Solution of Ammonium Chloride Buffer at 0.7 M

- ✓ Dissolve 37.4 g of NH_4Cl in 800 mL of dH_2O.
- ✓ Add approximately 40 mL of NaOH at 1N until reach pH of 8.5.
- ✓ Fulfil up 1 L with dH_2O.

Cadmium Sulfate Solution

- ✓ Dissolve 200 g of $CuSO_4$ or $3CdSO_4.8H_2O$ into 1 L of dH_2O.

Hydrochloric acid solution at 6 N

- ✓ Dilute 492 mL of concentrated HCl (37%) in 1L of dH_2O.

Primary standard solution of Nitrate (100 mM)

- ✓ Dry KNO_3 for several hours at 60°C.
- ✓ Dissolve 1.011 g of the above reagent in 100 mL of dH_2O.

Primary standard solution of Nitrite (50 mM)

- ✓ Dry Sodium nitrite ($NaNO_2$) for about 1h at 100°C.
- ✓ Dissolve 0.345 g in 100 mL of dH_2O.
- ✓ Add a few drops of chloroform to retain the reagent (1 mL per L of solution).

Appendix 3. RNA integrity determination by Experion Automated Electrophoresis System

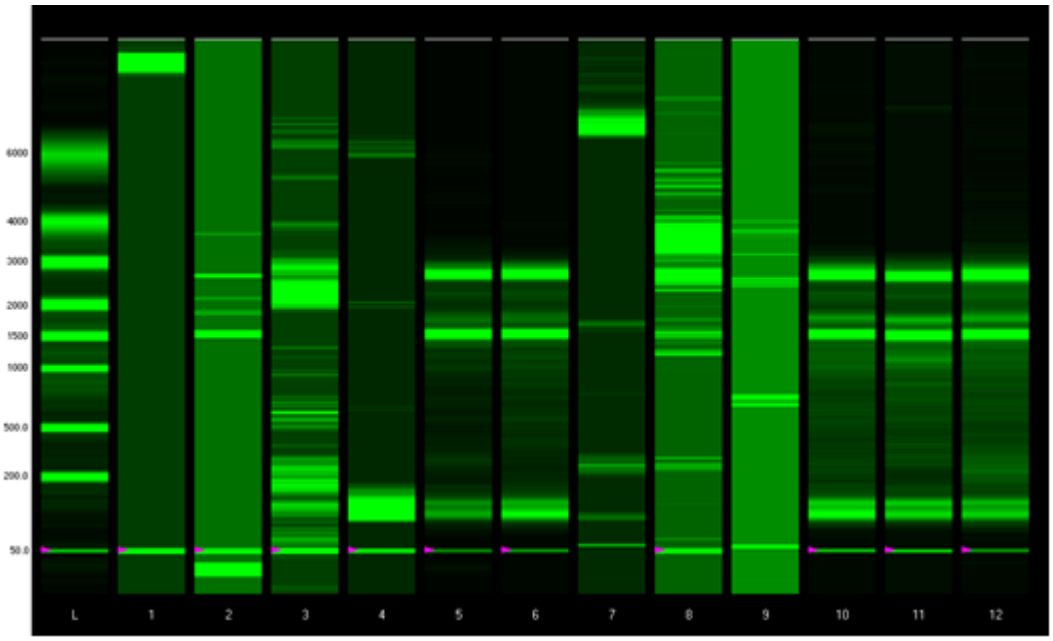

Figure 1. Image of Experion Automated Electrophoresis analysis

Table 1. Descriptive results from Experion analysis on each sample with Sample ID, RNA Area, RNA Concentration (ng/µl) and Ratio [23S/16S].

ID	Sample ID	RNA Area	RNA Concentration (ng/µl)	Ratio [23S/16S]
L		91.92	160.00	
1	C1	.24	.42	1.33
2	C2	2.34	4.07	.65
3	C3	8.21	14.30	2.55
4	ΔA1	15.81	27.52	1.36
5	ΔA2	52.34	91.10	.95
6	ΔA3	51.93	90.38	1.05
7	ΔC1	0	0	0
8	ΔC2	3.44	5.99	.44
9	ΔC3	0	0	0
10	A1	38.04	66.21	1.08
11	A2	27.81	48.41	.85
12	A3	45.29	78.84	1.15

Appendix 4. Potential nitrification rates of sediments slurries

Table 1 Results of each triplicate ± SD of Afurada potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries subjected to different salinities (0‰, 15‰, and 30‰) and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and Sulfathiazole (rates of AOA).

Salinity	Treatment	ID	µmol/L	
			Time 0	Time 2
0‰	TNM	4A	0.410 ± 0.005	0.412 ± 0.005
		5A	0.439 ± 0.005	0.457 ± 0.003
		6A	0.435 ± 0	0.452 ± 0.005
	AOB	7A	0.407 ± 0	0.427 ± 0.023
		8A	0.437 ± 0	0.480 ± 0

		9A	0.439 ± 0	0.478 ± 0.004
	AOA	10A	0.487 ± 0.004	0.498 ± 0.030
		11A	0.494 ± 0.002	0.501 ± 0.005
		12A	0.508 ± 0.019	0.507 ± 0.005
	TNM	16A	0.382 ± 0.007	0.410 ± 0.009
15‰		17A	0.410 ± 0.006	0.451 ± 0.006
		18A	0.436 ± 0.006	0.459 ± 0.017
	AOB	19A	0.436 ± 0	0.442 ± 0.001
		20A	0.382 ± 0.006	0.444 ± 0.003
		21A	0.406 ± 0.001	0.437 ± 0.007
	AOA	22A	0.467 ± 0.019	0.443 ± 0.004
		23A	0.488 ± 0	0.470 ± 0.001
		24A	0.471 ± 0.003	0.464 ± 0.01
	TNM	28A	0.473 ± 0.001	0.480 ± 0
30‰		29A	0.500 ± 0.025	0.525 ± 0
		30A	0.469 ± 0.003	0.522 ± 0.016
	AOB	31A	0.495 ± 0.003	0.514 ± 0
		32A	0.498 ± 0	0.545 ± 0.002
		33A	0.538 ± 0.004	0.500 ± 0.02
	AOA	34A	0.493 ± 0.018	0.504 ± 0.02
		35A	0.512 ± 0.005	0.515 ± 0.033
		36A	0.486 ± 0.007	0.495 ± 0.007

Salinity	Treatment	ID	µmol/L	
			Time 0	Time 2
0‰	TNM	4C	0.532 ± 0.001	0.590 ± NR
		5C	0.504 ± 0	0.693 ± 0.008
		6C	0.445 ± 0.003	0.555 ± 0.021
	AOB	7C	0.532 ± 0.026	0.665 ± 0.006
		8C	0.512 ± 0.015	0.709 ± 0.012
		9C	0.607 ± 0.008	0.660 ± 0.003
	AOA	10C	0.554 ± 0.006	0.663 ± 0

		11C	0.616 ± 0.031	0.686 ± 0.034
		12C	0.564 ± 0.024	0.761 ± 0
15‰	TNM	16C	0.536 ± 0.01	0.652 ± 0.003
		17C	0.526 ± 0.031	0.624 ± 0.006
		18C	0.551 ± 0.005	0.676 ± 0.006
	AOB	19C	0.605 ± NR	0.647 ± 0.035
		20C	0.607 ± 0.024	0.629 ± 0.110
		21C	0.590 ± 0.028	0.620 ± 0.009
	AOA	22C	0.642 ± 0.007	0.668 ± 0.023
		23C	0.649 ± 0.005	0.691 ± 0.004
		24C	0.617 ± 0.082	0.624 ± 0.02
30‰	TNM	28C	0.574 ± NR	0.637 ± NR
		29C	0.563 ± 0.020	0.626 ± 0.011
		30C	0.660 ± 0.01	
	AOB	31C	0.688 ± 0.077	0.751 ± 0.023
		32C	0.485 ± 0.005	0.537 ± 0.071
		33C	0.540 ± 0.012	0.453 ± 0
	AOA	34C	0.390 ± 0.015	0.434 ± 0.002
		35C	0.428 ± 0.003	0.423 ± 0.009
		36C	0.417 ± 0.012	0.464 ± 0.004

Table 2. Results of each triplicate ± SD of Crestuma potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries subjected to different salinities (0‰, 15‰, and 30‰) and different inhibitory treatments such as no inhibitor (rates of TNM), PTIO (rates of AOB) and Sulfathiazole (rates of AOA).

Figure 3. Results of each triplicate ± SD of Crestuma potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries from the two salinity regimes (constant (C) and salinity fluctuations (ΔC)) amended with a salinity of 0‰ and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and Sulfathiazole (rates of AOA).

Cores	Treatment	ID	µmol/L	
			Time 0	Time 2
	TNM	C1	0.51 ± 0.01	0.66 ± 0.02

C		C2	0.53 ± 0.01	0.61 ± 0.01
		C3	0.52 ± 0.01	0.74 ± 0.001
	AOB	C4	0.55 ± 0.03	0.64 ± 0.01
		C5	0.49 ± 0.03	0.59 ± 0.01
		C6	0.49 ± 0.02	NR
	AOA	C7	0.49 ± 0.03	0.52 ± 0.02
		C8	0.42 ± 0.02	0.48 ± 0.02
		C9	0.52 ± 0.004	0.54 ± 0.01
ΔC	TNM	ΔC1	0.52 ± 0.03	0.58 ± 0.03
		ΔC2	0.51 ± 0.01	0.59 ± 0.004
		ΔC3	0.53 ± 0.01	0.59 ± 0.03
	AOB	ΔC4	0.40 ± 0.02	0.46 ± 0.01
		ΔC5	0.42 ± 0.01	0.47 ± 0.02
		ΔC6	0.44 ± 0.03	0.47 ± 0.03
	AOA	ΔC7	0.35 ± 0.004	0.37 ± 0.02
		ΔC8	0.38 ± 0.01	0.44 ± 0.004
		ΔC9	0.42 ± 0.01	0.42 ± 0.01

Figure 4. Results of each triplicate ± SD of Afurada potential nitrification rates ($^{15}NO_2^-$ + $^{15}NO_3^-$ produced) in sediments slurries from the two salinity regimes (constant (A) and salinity fluctuations (ΔA)) amended with a salinity of 0‰ and different inhibitory treatment such as no inhibitor (rates of TNM), PTIO (rates of AOB) and Sulfathiazole (rates of AOA).

Cores	Treatment	ID	µmol/L	
			Time 0	Time 2
A	TNM	A1	0.47 ± 0.01	0.79 ± 0.003
		A2	0.51 ± 0.005	0.74 ± 0.02
		A3	0.49 ± 0.02	0.70 ± 0.01
	AOB	A4	0.51 ± 0.02	0.65 ± 0.02
		A5	0.52 ± 0.03	0.72 ± 0.03
		A6	0.53 ± 0.002	0.79 ± 0.002
	AOA	A7	0.50 ± 0.01	0.54 ± 0.03
		A8	0.49 ± 0.01	0.51 ± 0.02
		A9	0.51 ± 0.002	0.53 ± 0.01
ΔA	TNM	ΔA1	0.56 ± 0.02	0.79 ± 0.01
		ΔA2	0.55 ± 0.01	0.96 ± 0.07
		ΔA3	0.55 ± 0.02	0.75 ± 0.01
	AOB	ΔA4	0.54 ± 0.02	NR
		ΔA5	0.51 ± 0.01	0.73 ± 0.04
		ΔA6	0.51 ± 0.01	NR
	AOA	ΔA7	NR	0.41 ± 0.01
		ΔA8	0.44 ± 0.02	0.47 ± 0.02
		ΔA9	0.47 ± 0.002	0.48 ± 0.01

Appendix 5. Nitrification rates measurement protocol

1. Reduction of NO_3^- to NO_2^- for N_2 analysis

I. Prepare 5M NaCl solution;

II. Prepare sponge cadmium by adding HCl 6M during 3 minutes following by several washes with water until reach neutral pH.

III. Unfreeze the samples for analysis and vortex in order to homogenise them.

IV. Prepare tubes (e.g. 15mL Falcon) and label them properly;

V. Add 0.5 g of sponge cadmium to each tube;

VI. Add 2.25 mL of samples to each respective tube;

VII. Add 2.25 mL of 5M NaCl solution;

VIII. Store the tubes overnight in a dark place with constant agitation;

IX. After incubation, centrifuge at 4000 rpm during 8 minutes to deposit cadmium.

X. Took 4 mL of sample for the Extainer of 6 mL previously labelled (Labco Ltd)

2. Conversion of NO_2^- to N_2

I. Extainers were opened and filled with 4 mL of sample for analysis;

II. Before the conversion of NO_2^- to N_2 the Extainers were thoroughly closed with extreme care in order to avoid possible air contamination afterwards;

III. Few drops of water were place over the septum to ensure that air did not enter through the punctured septum;

IV. The extainers were then flushed with Argon for 10 minutes (Figure 1).

V. During the flushing, the gas stream exited the flasks through a second needle in the septum (Figure 1).

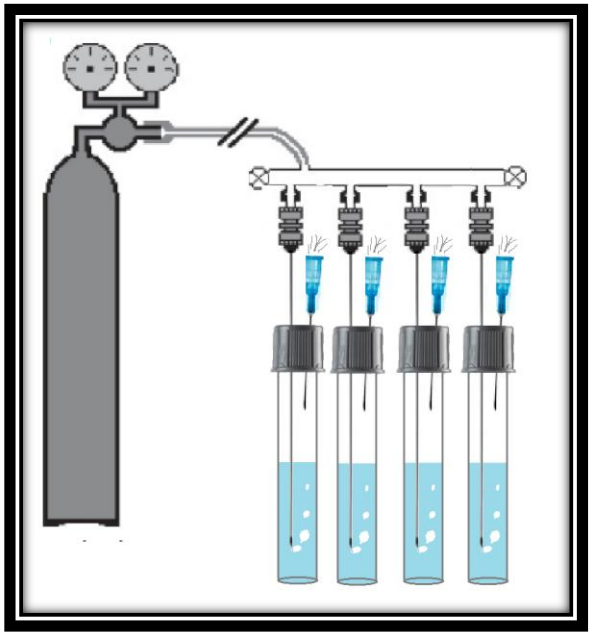

Figure 1. Scheme of extainers being purged with argon

VI. After being purged the Extainers were put upside down to prevent any air contamination;

VII. Subsequently, 50 µL ml of Sulfamic acid is injected into the Exetainer. The Exetainer is vigorously shaken to mix the acid and to transfer the dissolved N^2 into the headspace (Figure 2).

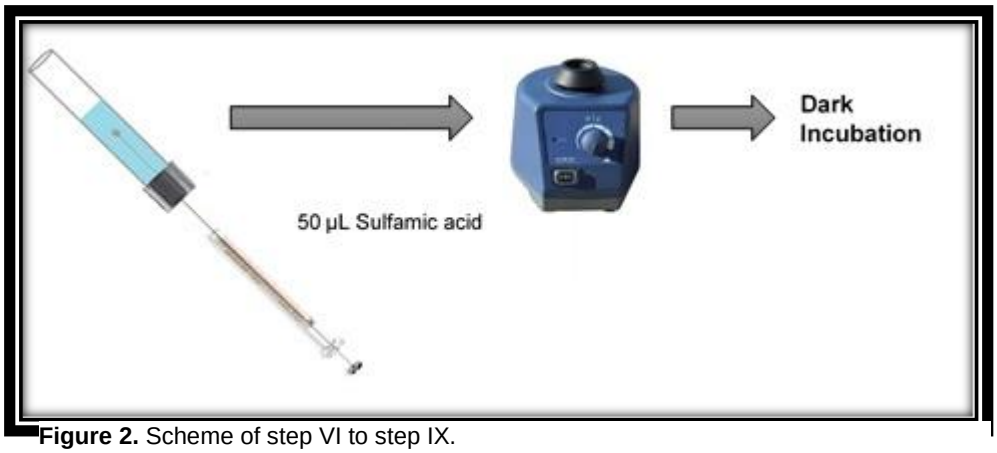

Figure 2. Scheme of step VI to step IX.

VIII. Finally, the Exetainer is stored upside down so that the headspace is not in contact with the cap and its perforated septum.

IX. The Exetainers are stored in the dark at stable temperatures;

3. Measurement of the N_2 gas in the IRMS

I. Aliquots of atmospheric air are prepared in an Extainer and measured prior to samples and blanks as a test of accuracy and precision.

II. Every five samples one air standard is measured. Carefully Vortex the Extainer in order to release N_2 formed on the wall of the tube.

III. Clean the needle to be used to insert the gas into de IRMS with Argon previously purged during 10 minutes into an Extainer.

IV. Insert the needle and took 250 µL of air;

V. At the same time insert a needle with water to equilibrate the atmosphere inside the vials

VI. Push back and forward around 100/150 µL of air (3/5 times) to be certain that air was mixed and wait 1 minute to equilibrate the pressure in the needle.

VII. When the gas syringe is pulled out of the Exetainer, it is important to avoid a vacuum in the syringe because atmospheric gas would enter the syringe immediately and contaminate the sample. The sample is quickly injected into the linear flow injector.

VIII. All gas tight syringes can easily clog and should, therefore, be tested after every injection by filling the syringes with air (or helium) and slowly pushing the gas into some water. If a bubble stream appears immediately, the needle is not clogged.

IX. After the injection on the IRMS repeat again the steps III, IV, V, VI, VII and VIII.

X. Switch the argon vials every 2/3 samples;