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# Structural and functional diversity of the diazotrophic community in xeric ecosystems: response to nitrogen availability

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# ABSTRACT

The effects of increased nitrogen (N) input in the ecosystems have brought concern for some time, however, the long-term consequences of this input to Mediterranean ecosystems are poorly studied. The Mediterranean ecosystem is considered a hotspot of biodiversity; the Mediterranean basin was considered one of the places in the world with most biodiversity. Taking in consideration that these ecosystems are regarded as environments under N limitation, changes in N availability will alter the relationships between the organisms. The research presented here was carried out on the Mediterranean basin and it is part of an ongoing study over the last 10 years, in which fields were fertilized with different quantities and forms of N. To understand the consequences of this fertilization, diazotrophic bacteria were isolated from rhizospheric soil of *Ulex densus* subjected to different N addition and characterized in terms of the structural and functional diversity of the communities, using this community as an ecological marker for comparing the different treatments.

The methodology applied to understand the ecological consequences was the isolation of the rhizospheric bacteria in N free media and characterization of this collection of isolates, in terms of diversity (phenotypic and genotypic), morphophysiology (distribution of the isolates by different morphotypes), structure (identification of the genera associated with the treatments), functionality (ability to perform ammonification, nitrification and/or denitrification) and metabolic plasticity (ability to use different carbon sources). The N fixation of these isolates was confirmed by their growth in solid and liquid N free media and by isotopic characterization of the ratio of $^{15}N$ over $^{14}N$; a search for the *nifH* gene was also performed.

For this community there were no differences in diversity nor at the morphophysiological level between the different treatments. However, a higher number of isolates was detected in the treatments with higher $NH_4^+$ inputs (40A, and 80AN). The ability to perform N fixation was confirmed through multiple passages in solid and liquid N-free media and *nifH* gene was detected through PCR and dot-blot hybridization, though this was not possible for every representative isolate. Differences with genera associated with the form and quantity of N added to the soil were observed, with some genera only appearing in certain treatments. Every isolate presented more than one function in the N cycle and a decrease in nitrification together with an increase of denitrification was observed along with the increase of bioavailable N. Isolates belonging to genera detected exclusively in treatment 80AN presented higher N content and higher denitrification rates. The variation of the metabolic plasticity between the treatments increased with N fertilization, since the control treatment had the more consistent response for metabolic plasticity.

The increase on the isolates number followed the same pattern as the proliferation of *U. densus*, showing that the potential for N fixation was not inhibited by $NH_4^+$ addition. The differences in genera associated with the treatments evidenced a change in the structure of the community. The results of multifunctionality showed a shift in the diazotrophic community, since nitrification and denitrification presented specificity towards different forms of fertilization. The higher variation of the metabolic plasticity associated with N fertilization can be related to changes in carbon source availability in the soil.

From the work presented here and future work can arise a platform to direct alterations on the agriculture and ecological guidelines. These alterations are essential to protect this kind of ecosystem, which is extremely important due to the unique biodiversity associated with it.

**Keywords:** N fixation, Mediterranean ecosystems, N fertilization, Multifunctionality in N cycle, Diazotrophic diversity

# RESUMO

Há algum tempo que os efeitos do aumento de nitrogénio (N) introduzido nos ecossistemas leva a preocupação, no entanto, as consequências a longo prazo desta introdução nos ecossistemas mediterrânicos estão pouco estudadas. O ecossistema mediterrânico é considerado um *hotspot* de biodiversidade; a bacia mediterrânica é um dos locais com mais biodiversidade no mundo. Tendo em consideração que estes ecossistemas são considerados ambientes com limitações ao nível do N, as mudanças na disponibilidade de N vão alterar as relações entre os organismos. O estudo aqui apresentado foi levado a cabo na bacia mediterrânica e faz parte de um estudo que já começou há 10 anos e continua a ser efetuado, em que os talhões foram fertilizados com diferentes formas e quantidades de N. De forma a entender as consequências desta fertilização, foi isolada a comunidade de bactérias diazotróficas a partir de solo rizosférico de *Ulex densus* sujeito a diferentes adições de N e foi caracterizada a diversidade estrutural e funcional da comunidade. A comunidade diazotrófica foi utilizada como um marcador ecológico para a comparação entre os diferentes tratamentos.

Os microrganismos que conseguem realizar a fixação de N, ou seja, a conversão de $N_2$ em $NH_3$, são chamados de diazotróficos. Esta conversão ocorre através da ação da enzima nitrogenase, e existem três tipos desta enzima que variam no cofator associado: Mo, Va ou Fe.

A estrutura da comunidade diazotrófica não apresentou diferenças entre os tratamentos em termos de diversidade, no entanto o índice de diversidade genotípico apresentou um poder de discriminação maior que o índice de diversidade fenotípico entre os isolados e, portanto, é essencial complementar os resultados fenotípicos com o perfil genotípico obtido por PCR *fingerprinting*. Foi detetado um número mais elevado de isolados diazotróficos heterotróficos nos tratamentos com maior adição de $NH_4^+$ (40A e 80AN), estes são os mesmos tratamentos em que existe proliferação de *Ulex densus,* mostrando assim que o potencial para a fixação de N não foi inibido pela adição desta forma de N. Foram observadas diferenças entre os géneros identificados dependendo da forma e quantidade de N adicionado ao solo, mostrando mudanças na estrutura da comunidade. Para esta identificação foram escolhidos 40 representantes, sendo 10 de cada tratamento, e foram considerados uma boa representação da coleção de isolados por não apresentarem valores de semelhança superiores a 55%.

A maioria dos géneros que foram identificados estão associados com a fixação de N ou pelo menos apresentam um espécie pertencente ao género que apresenta a capacidade de realizar fixação de N. A capacidade de realizar fixação de N foi confirmada através de múltiplas passagens por meio sem N quer na forma líquida quer na forma sólida e o gene *nifH* foi detetado através da técnica de PCR e por hibridação *dot-blot*. Apesar de os isolados representantes terem sido identificados como sendo pertencentes a géneros associados com a capacidade de realizar a fixação de N e os pares de *primers* escolhidos serem capazes de amplificar o gene *nifH* dos *clusters* onde estes géneros estão incluídos, não foi possível amplificar e/ou detetar o gene *nifH* da maioria dos isolados. Isto pode significar que o gene *nifH* destes isolados é diferente das sequências utilizadas para desenhar os *primers*.

Até há pouco tempo, era comum associar determinados grupos de microrganismos a uma biotransformação especifica do ciclo do N. Contudo ultimamente tem sido exposto que a maioria dos isolados tem potencial para realizar diversas biotransformações de N, nomeadamente combinações de fixação de N, amonificação, nitrificação e desnitrificação. Isto representa um fator extremamente importante, uma vez que a multifuncionalidade é essencial para a adaptação dos microrganismos ao meio ambiente. Os isolados representantes de cada um dos tratamentos foram testados para diferentes processos do ciclo do N: amonificação, nitrificação e desnitrificação. Todos os isolados apresentaram mais do que uma função no ciclo do N. Pode ser observada uma mudança na multifuncionalidade da comunidade diazotrófica devido ao aumento da biodisponibilidade de N. De facto, a capacidade de

realizar nitrificação foi encontrada mais frequentemente entre os isolados representantes da comunidade de tratamentos que recebem mais baixas quantidade de $NH_4^+$ (tratamento controlo e 40AN), o que está de concordo com o que foi reportado em estudos anteriores, em que o aumento da disponibilidade de $NH_4^+$ leva a uma inibição na nitrificação. Foi encontrada capacidade desnitrificante em todos os tratamentos, no entanto os isolados do tratamento 80AN (recebe mais nitrato) apresentaram taxas de desnitrificação mais elevadas. Taxas de desnitrificação mais elevadas podem ser relevantes como mecanismo de desintoxicação de nitrato, uma vez que elevadas concentrações de nitrato podem inibir a fixação de N.

A alteração da multifuncionalidade microbiana associada com biotransformações de N está associada a mudanças na composição da comunidade diazotrófica, sendo corroborado pelas diferenças nos géneros identificados nos diferentes tratamentos.

Com base nos resultados apresentados, é sugerido que o isolado identificado como pertencente ao género *Pedobacter* apresente uma sensibilidade para concentrações elevadas de N, e por isso pode ser possível que a ausência deste género possa estar associada a um aumento da disponibilidade de N. Por esta razão, este género pode ser um candidato a indicador biológico das alterações no ecossistema.

Não foi possível associar a multifuncionalidade dos isolados com a plasticidade metabólica. No entanto, quando foram analisados dois géneros comuns a diferentes tratamentos - *Pseudomonas* e *Stenotrophomonas* - foi possível observar diferenças na resposta à adição de N. Os isolados identificados como *Pseudomonas* apresentaram uma resposta uniforme ao longo dos diferentes tratamentos, correspondendo a uma elevada plasticidade metabólica. Contudo, no caso dos isolados identificados como *Stenotrophomonas* o mesmo já não foi observado, pois a plasticidade metabólica aumenta com o aumento da disponibilidade de N. Isto pode dever-se à adaptação destes microrganismos ao aumento da disponibilidade de N ou os isolados podem pertencer a espécies distintas com diferente plasticidade metabólica.

Apesar de ser possível identificar algumas diferenças entre os tratamentos em termos das propriedades funcionais da comunidade, estas diferenças não são tão evidentes como seria de esperar, de acordo com a literatura, e não corroboram completamente a teoria de que a comunidade diazotrófica devia ser afetada pela adição de N. Estas discrepâncias podem dever-se ao ecossistema que está a ser estudado, o ecossistema mediterrânico, que apresenta características distintas dos outros ecossistemas estudados em trabalhos semelhantes, e por isso os efeitos da adição de N podem não ser os mesmos.

Por último, a comunidade escolhida neste trabalho como indicador das respostas do ecossistema, a comunidade diazotrófica, pode não ter sido a escolha certa, uma vez que não se verificam grandes alterações estruturais com o aumento da biodisponibilidade de N. A comunidade estudada foi isolada a partir do solo rizosférico de *Ulex densus* que é uma espécie que prolifera nos tratamentos com maior disponibilidade de N. Desta forma a planta pode estar a contrabalançar os efeitos da adição de N levando a que não haja diferenças na comunidade diazotrófica entre os tratamentos. Pode ainda ser o facto de a comunidade rizosférica conseguir aguentar as pressões ecológicas e manter este grupo de microrganismos, uma vez que estes apresentam uma função importante que pode ser benéfica para a comunidade rizosférica quando em condições de N limitante.

O trabalho apresentado neste estudo, e trabalho futuro que possa advir dele, pode criar uma plataforma para alterações nas políticas agrícolas e ambientais, que são necessárias para a preservação deste tipo de ecossistemas que apresentam uma biodiversidade única.

**Palavras-chave:** Fixação de N, Ecossistemas mediterrânicos, Fertilização com N, Multifuncionalidade no ciclo do N, Diversidade diazotrófica

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# ABBREVIATIONS

**Anammox** - Anaerobic ammonium oxidation

**AOA** - Ammonia-oxidizing archaea

**AOB** - Ammonia-oxidizing bacteria

**ARA** - Acetylene reduction assay

**AUC** - Area under curve

**BCIP** – 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

**BNF** - Biological nitrogen fixation

**DIG** - Digoxigenin

**DNRA** - Dissimilatory nitrate reduction to ammonium

**EDTA** - Ethylenediamine tetraacetic acid

**GES** - Guanidium thiocyanate, EDTA, Sarkosyl

**GH** - Greenhouse effect

**IRMS** - Isotope-ratio mass spectrometry

**NAR** - Nitrate reductase

**NAUC** - Net area under curve

**NBT** – Nitro-blue tetrazolium

**NCBI** - National center for biotechnology information

**NFB** - Nitrogen free broth

**NIR** - Nitrite reductase

**NOB** - Nitrite-oxidizing bacteria

**NOR** - Nitric oxide reductase

**NOS** - Nitrous oxide reductase

**Nr** - Reactive nitrogen

**OM** - Organic matter

**PBS** - Phosphate buffer saline

**PC** - Principal component

**PCA** - Principal component analysis

**PCR** - Polymerase chain reaction

**PM** - Particulate matter

**RO-Water –** Reverse osmose purified water

**SDS** - sodium dodecyl sulfate

**SIIAF -** Stable isotopes and instrumental analysis facility

**SPW** - Sterile pure water

**SSC** - saline-sodium citrate

**TBE** - Tris-borate-EDTA

**TE** – Tris-EDTA

**TSA** - Tryptone soy agar

**UPGMA** - Unweighted pair group method with arithmetic average

# 1. INTRODUCTION

## 1.1. An overview of the nitrogen cycle

The nitrogen (N) cycle is one of the most important cycles in the ecosystems, given that sustainability of several environments is maintained by the accessibility of N. This element, in the form of dinitrogen ($N_2$), is the most abundant in Earth's atmosphere, however, in this form it is unavailable to most organisms. For N to become bioavailable, its conversion to reactive N is necessary. Reactive nitrogen (Nr) is a designation that includes inorganic reduced forms of N such as ammonia ($NH_3$) and ammonium ($NH_4^+$), inorganic oxidized forms that can be nitrogen oxides ($NO_x$), nitric acid ($HNO_3$), nitrous oxide ($N_2O$), nitrite ($NO_2^-$) and nitrate ($NO_3^-$) and organic compounds like urea, amines, proteins and nucleic acids (Galloway *et al.*, 2004; Thamdrup, 2012).

The bioavailability of N mostly depends on the action of microorganisms, either through the reaction for the conversion of biological unavailable N ($N_2$) into a bioavailable form ($NH_3$) or the conversion of a bioavailable form, like $NH_3$, into other kinds of bioavailable forms, such as $NO_3^-$ or $NO_2^-$ (Stein and Klotz, 2016).

The flux of N through the distinct ecosystem compartments, regularly called the biogeochemical N cycle, is mostly mediated through bioconversions which can be clustered in seven different processes (**Figure 1.1**): nitrogen fixation, nitrification, denitrification, anammox, assimilation, ammonification and dissimilatory nitrate reduction to ammonium (DNRA). The N cycle can be envisioned by starting with the transformation of a $N_2$ molecule into $NH_3$ through N fixation, the $NH_3$ produced will integrate the biomass of growing organisms and will be released after their death and/or decomposition of the organic matter produced through ammonification processes, followed by nitrification, with the transformation to $NO_2^-$ and of this to $NO_3^-$. The denitrification process will transform $NO_3^-$ to $NO_2^-$, $NO_2^-$ to nitric oxide (NO), NO to $N_2O$ and this to $N_2$. Each one of these processes is carried out by different groups of microorganisms (Kuypers, Marchant and Kartal, 2018).

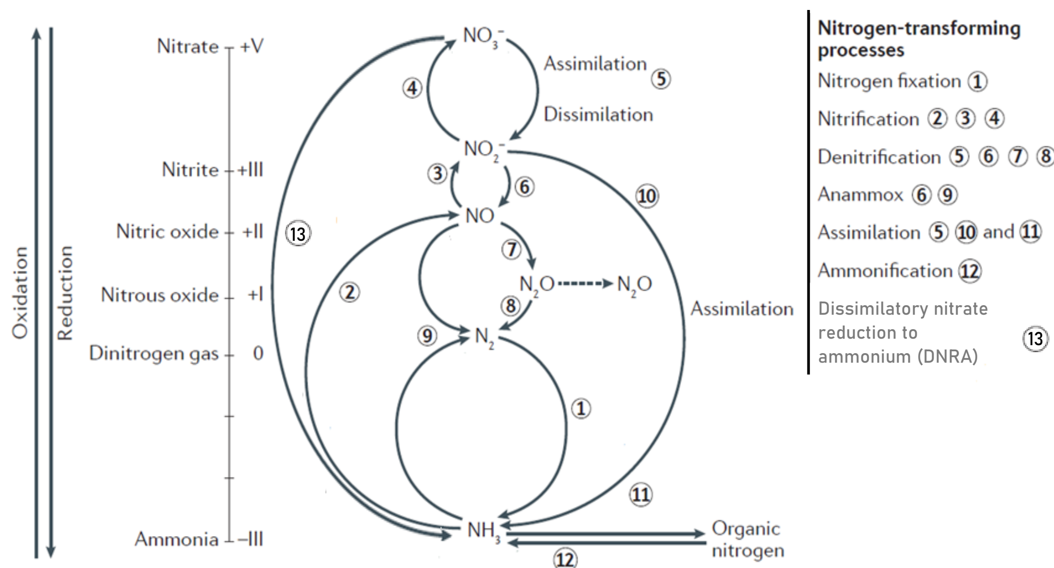


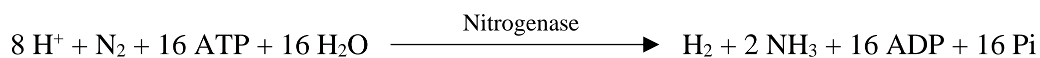

Figure 1.1: Nitrogen cycle. Adapted from Kuypers, Marchant and Kartal (2018)

The processes that make up the N cycle are not evenly distributed in space and in time. This is due to the different conditions necessary for each process to occur, as well as the microorganisms that are involved in each process. These microorganisms have specific requirements for pH, oxygen ($O_2$) concentration, and different N and carbon (C) source preferences. This, together with the anthropogenic input of N forms into the cycle, leads to an imbalance and to the accumulation of these compounds in the environment, given that the recycling of N, by its return to $N_2$ form, is only possible by the processes of denitrification and anammox (Smil, 2001; Vitousek *et al.*, 2002; Galloway *et al.*, 2004; Karl and Michaels, 2019).

Anthropogenic action has led to an increase in the quantity of N added to the environment, especially with leguminous cultures, production of fossil fuels and the addition of fertilizers to the soil. These fertilizers use Nr forms, usually $NH_4^+$, and this input of N in the soil has consequences (Watson, Valois and Waterbury, 1981).

## 1.2. Nitrogen fixation

The reaction where $N_2$ is reduced to $NH_3$ is designated by biological nitrogen fixation (BNF) and it is achieved by the enzyme nitrogenase, as presented in **Equation 1.1**. In this process, energy in the ATP form is necessary to compel $N_2$ to merge with Hydrogen (H) to create $NH_3$. This way, $NH_3$ can be used as an N source or it can be converted to $NO_3^-$ (Rascio and la Rocca, 2008; Lindström, Aserse and Mousavi, 2015).



$$8\ H^+ + N_2 + 16\ ATP + 16\ H_2O \xrightarrow{\text{Nitrogenase}} H_2 + 2\ NH_3 + 16\ ADP + 16\ Pi$$

Equation 1.1: Nitrogen fixation reaction.

BNF is considered one of the most primordial enzyme-catalyzed reactions and it is a vital component of the N cycle. The microorganisms that can perform this conversion are termed diazotrophs and are exclusive of the archaeal and bacterial domains. This ability is widely distributed and must have occurred early in prokaryotic evolution, to provide the N needed in the increased microbial biomass (Towe, 2002; Raymond *et al.*, 2004).

Diazotrophs can have different types of metabolic strategies, as well as different relationships with $O_2$ - they can be obligate or facultative anaerobes or aerobes. They can establish a symbiotic relationship, develop associative relationships with different organisms in the ecosystem or be free-living organisms in different environments as referred in Gaby and Buckley (2015).

Nitrogenase, being an enzyme deeply sensitive to $O_2$, requires fixation to occur in anaerobic conditions, which is not an obstacle for microorganisms that can grow in the absence of $O_2$. However, the obligate aerobes had to provide strategies to surpass this restriction. For example, *Azotobacter vinelandii* is a strict aerobe that erases the $O_2$ traces with an increased aerobic respiration rate, to guarantee an anoxic environment in the interior of the cell. Cyanobacteria, being autotrophic microorganisms had to develop other strategies: one of them is the formation of specialized cells called heterocysts, that have an anoxic environment, which allows nitrogen fixation to occur; another is the temporal and spatial separation of the photosynthesis process from the N fixation process

(Poole and Hill, 1997; Berman-Frank *et al.*, 2001; Peters and Boyd, 2015; Poza-Carrión, Echavarri-Erasun and Rubio, 2015).

Some diazotrophs, most of them from the alpha or betaproteobacteria class, designed a symbiotic association with plants. This relationship is profitable for both, since the plant provides a particular place with low $O_2$ concentration, the nodule, to allow N fixation, and provides C sources to bacteria. In return, microorganisms provide N, in the form of $NH_3$, to the plant, which is fundamental to its growth. There are also symbiotic relationships between members of the *Frankia* genus and actinorhizal plants, as well as between cyanobacteria and some plant genera, but these are less established and investigated (Osborne and Bergman, 2008; Franche, Lindström and Elmerich, 2009; Lindström and Mousavi, 2010).

Associative nitrogen fixation is a beneficial relationship, but not obligatory, between diazotrophs and some plants, where there is no colonization of the plant cytoplasm. This relation is common with Proteobacteria and can include Firmicutes members, like *Paenibacillus* species (van Dommelen and Vanderleyden, 2007).

### 1.2.1.Nitrogenase

Nitrogenase is the enzyme responsible for the transformation of $N_2$ towards $NH_3$, during which $H_2$ is released, reducing the efficiency of the reaction. There are three different types of this enzyme depending on the cofactor employed, which can be Molybdenum (Mo)-Iron (Fe), Vanadium (V)-Fe or Fe-Fe. This enzyme has two components: the first component has two subunits, denominated α, and β, and the second component is formed by a Fe-protein with two equal subunits, as presented in **Figure 1.2**. Microorganisms can have different combinations of the enzyme types or even have the three types, like the case of *Azotobacter vinelandii*, although every N-fixing microorganism has the MoFe-nitrogenase (Hausinger and Howard, 1983; Rees and Howard, 2000).

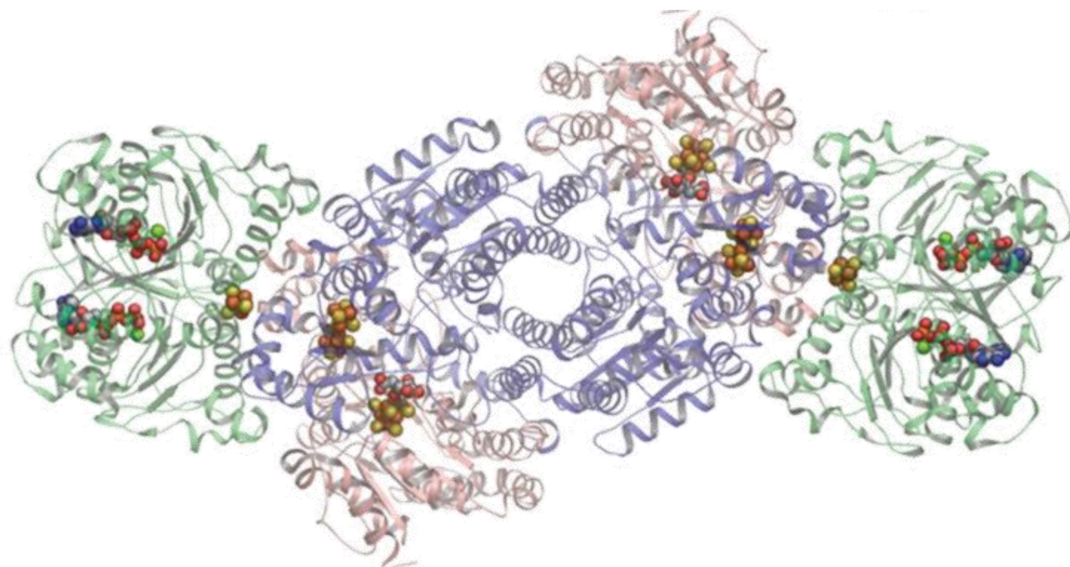


Figure 1.2: MoFe-nitrogenase complex. MoFe protein subunits in salmon (α subunits) and blue (β subunits) at the center with two Fe proteins (green) bound on each end (Danyal *et al.*, 2016).

The MoFe-nitrogenase has molybdenum (Mo) as its cofactor and the genes encoding for the architectural components are *nifD*, *nifK*, which form the catalytical part of the enzyme, and *nifH*,

encoding for the dinitrogenase reductase subunit (Fe-protein). This enzyme is the most efficient in reducing $N_2$ into $NH_3$ and with lower $H_2$ production, therefore microorganisms preferably express the genes for this enzyme (Simpson and Burris, 1984; Eady, 1996; Glazer, Kechris and Howard, 2015).

The MoFe protein contains two metal clusters: one is the MoFe cofactor which gives the active site for substrate attachment and reduction and the other one is the Phosphorus (P)-cluster which is involved in the transference of electrons from the Fe protein to the MoFe cofactor (Burgess, 1990; Kim and Rees, 1992; Peters *et al.*, 1995; Hoffman *et al.*, 2014).

Nitrogenase has the ability to reduce other triply bonded small molecules beside $N_2$ such as acetylene, azide, and cyanide (Berges and Mulholland, 2008).

Alternative nitrogenases can be VFe-nitrogenase or FeFe-nitrogenase, and the genes encoding for these enzymes are *vnfD, vnfK, vnfH,* and *anfD, anfK, anfH*, respectively, as shown in **Figure 1.3**. These enzymes require the hydrolysis of more ATP molecules per nitrogen fixed, between 24-48 ATP versus 16 ATP for MoFe-nitrogenase (Galloway *et al.*, 2004; Glazer, Kechris and Howard, 2015).

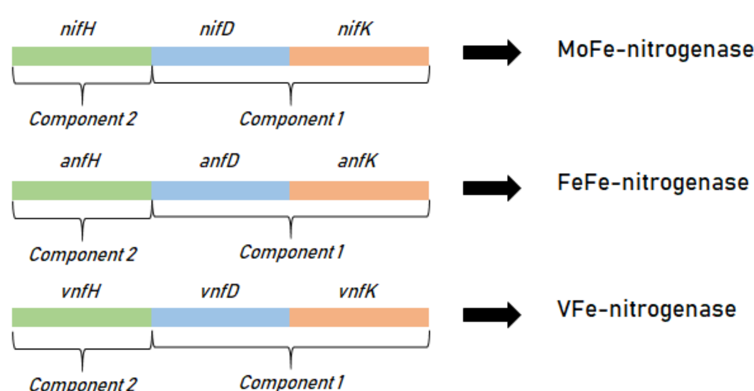



Figure 1.3: Scheme of the structural genes of the three types of nitrogenase.

### 1.2.2. The *nif*, *anf* and *vnf* gene cluster

For the synthesis of the MoFe-nitrogenase, there are quite a few genes implicated, which are crucial, not only for the creation of the nitrogenase complex but also for the catalytic metal cofactor biosynthesis. There are two clusters of *nif:* the major cluster comprises five transcriptional units which include *nifHDKTY* and *nifENX;* the minor cluster holds three operons, with *nifLA* being one of them, as presented in **Figure 1.4**. These genes are relevant because they form a complex, *nifA-nifL-GlnK*, which regulates *nif* gene expression (Jacobson *et al.*, 1989; Martinez-Argudo *et al.*, 2004).

The architectural genes for MoFe-nitrogenase *nifH, nifD* and *nifK* are in the major cluster and, although they are transcribed from the same promoter as *nifT* and *nifY*, the levels of transcription are up to 70-fold higher for the structural genes. Although all three genes, *nifH, nifD,* and *nifK*, are vital to nitrogenase arrangement, the transcript levels of *nifH* are higher than those of *nifDK*, which is related to the stoichiometry of nitrogenase component proteins (Hamilton *et al.*, 2011; Poza-Carrión, Echavarri-Erasun and Rubio, 2015).

Figure 1.4: Organization of Mo, V, and Fe nitrogenase gene clusters in *A. vinelandii*. Adapted from Mus *et al.* (2018).

The organization of the *vnf* gene cluster differs from the *nif* organization, seeing as the *vnfH* is in an operon distinct from the operon that contains *vnfDGK*. The *vnfG* encodes for the γ-subunit which does not exist in MoFe-nitrogenase. The *vnf* gene cluster contains the *vnfENX* operon homologous to *nifENX*. The *anf* gene cluster is organized as a single operon *anfHDGK* and *anfG* encodes for the same subunit as *vnfG.* The *vnfA* and *anfA* have regulatory properties similar to *nifA,* as shown in **Figure 1.4** (Joerger *et al.*, 1989; Waugh *et al.*, 1995; Mus *et al.*, 2018).

In **Figure 1.5** is presented the 16S rRNA gene phylogenetic tree and the distribution of the diazotrophic microorganisms in the groups formed.

Figure 1.5: Neighbor-joining phylogenetic tree constructed from 16S rRNA gene sequences for the microbial genomes used in this analysis. Diazotrophic genomes, as determined by the presence of *nifHDKEN* operons, are indicated with bold lines (Raymond *et al.*, 2004).

### 1.2.3. *nifH* phylogeny

BNF is a process observed in bacteria and a limited group of archaea - the methanogenic archaea - but has not been detected in eukaryotes. This is a function that is broadly dispersed, both taxonomically and ecologically (Raymond *et al.*, 2004; Boyd *et al.*, 2011).

Of all the genes associated with this function, the most frequently sequenced one is *nifH* and it has been used as a marker gene to detect diazotrophic microorganisms, with applications in ecology and diversity comparisons. This choice is based on its structural function, which encodes for the Fe protein present in every type of nitrogenase and independent of the cofactor utilized. This characteristic makes it a conserved gene and one of the most ancestral genes in the history of gene evolution (Young, 1993; Ueda *et al.*, 1995; Zehr *et al.*, 2003).

The *nifH* gene was used on phylotype investigations where it was settled that *nifH* phylogeny can be divided into five clusters (Zehr *et al.*, 2003; Raymond *et al.*, 2004; Boyd *et al.*, 2011).

Cluster I essentially holds bacterial *nifH* and some *vnfH* sequences from aerobes and facultative anaerobes. Cluster II is composed of bacterial and methanogenic archaeal *anfH* sequences. Cluster III is comprised of *nifH* sequences of anaerobic bacteria plus archaea. Cluster IV includes *nifH* paralogs with no purpose in nitrogen fixation found. Cluster V holds paralogs of *nifH* implicated in the synthesis of bacteriochlorophyll. These groups are presented in **Figure 1.6** (Chien and Zinder, 1996; Glazer, Kechris and Howard, 2015).

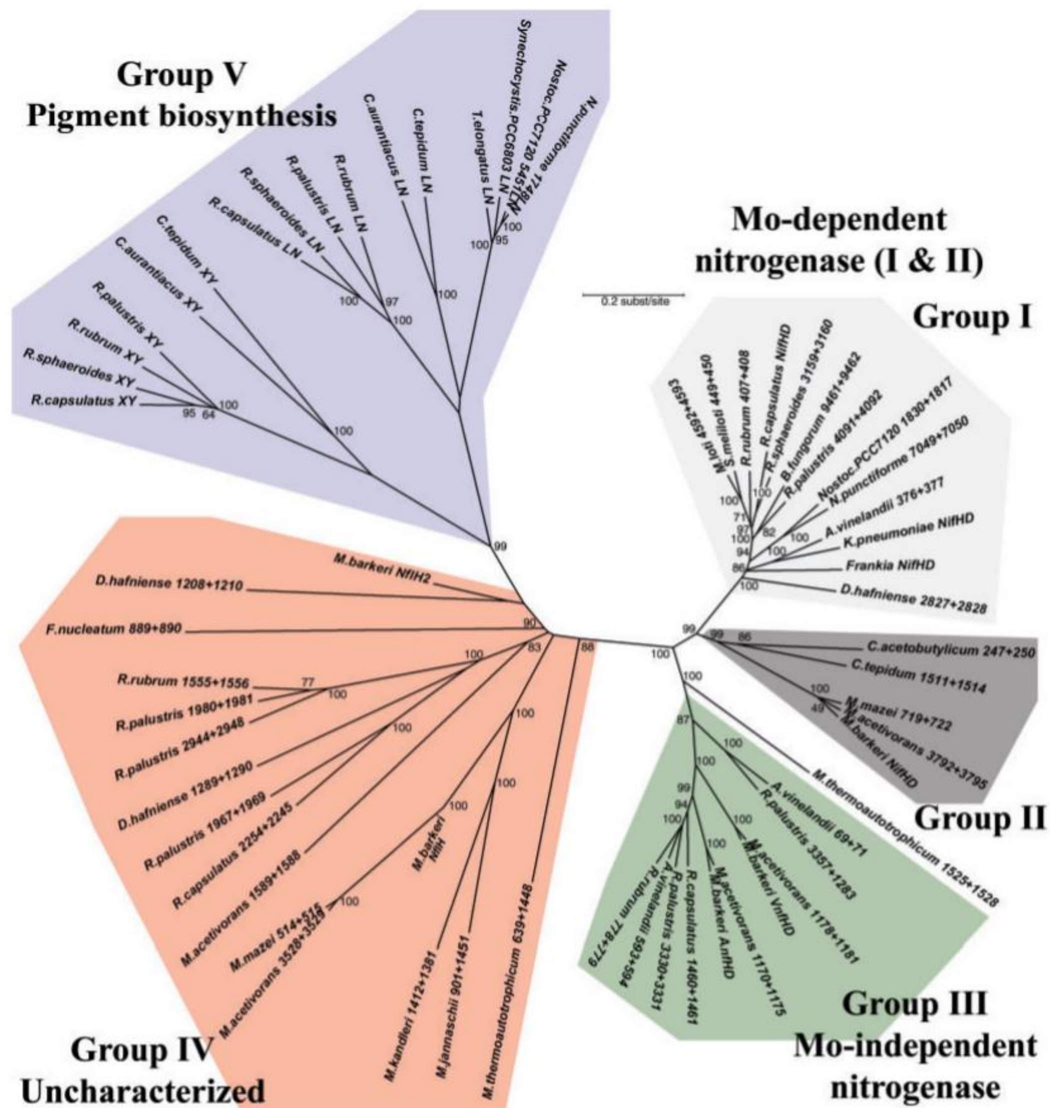



Figure 1.6: Overview of five phylogenetic groups shown on a concatenated phylogenetic tree composed of *nifH* and *nifD* homologs found in complete genomes (Raymond *et al.*, 2004).

## 1.3. Multifunctionality in the N cycle

Diazotrophic microorganisms can perform other functions of the N cycle, such as heterotrophic nitrification, denitrification, and ammonification. These functions are activated when the microorganisms are exposed to certain environmental conditions that induce changes in the normal metabolism, such as high $NO_3^-$ or $NH_4^+$ concentration, lower soil pH and high rates of C/N ratio (Thomas, Lloyd and Boddy, 1994; Princic *et al.*, 1998; Wang *et al.*, 2013; Svehla *et al.*, 2014).

### 1.3.1. Nitrification

Autotrophic nitrification is a chain of two biological oxidation processes, from $NH_3$ to $NO_2^-$ and $NO_2^-$ to $NO_3^-$, mediated by two groups of microorganisms as their energy acquiring process. The first process is realized by ammonia-oxidizing bacteria (AOB) or by ammonia-oxidizing archaea (AOA). The second process is the oxidation of $NO_2^-$ to $NO_3^-$ which includes another category of microorganisms, the nitrite-oxidizing bacteria (NOB) (Nunes-Alves, 2016; Cáceres, Malińska and Marfà, 2018).

Heterotrophic microorganisms, such as diazotrophs, can perform another type of nitrification, heterotrophic nitrification. Heterotrophic nitrification is the transformation of organic and inorganic N ($NH_3$) forms to generate $NO_3^-$ by some genera of bacteria. This form of nitrification is not coupled to energy conservation and the enzymes involved differ from the ones of autotrophic nitrification (Prosser, 2007; Shoda, 2017).

This process seems to be significant in environments with low pH values since it is a process less impacted by these conditions than autotrophic nitrification (Braker and Conrad, 2011; Pajares and Bohannan, 2016).

Nitrification utilizes $NH_3$ as the substrate for the process, so the accumulation of this form of N through the addition of fertilizers will promote the occurrence of nitrification. This leads to economic problems, because of the lower N fertilizer yield, requiring increases in N fertilizer inputs to compensate the yield and decreasing N use efficiency. Nitrification product, $NO_3^-$, has consequences in the ecosystem, such as its lixiviation, which occurs because of its negative charge, which does not favor the formation of aggregates with soil particles. This way it leaches away more easily than $NH_4^+$ and this accumulation of $NO_3^-$ in watercourses leads to their eutrophication (Menéndez *et al.*, 2012; Beeckman, Motte and Beeckman, 2018).

### 1.3.2. Denitrification

Denitrification is the sequential dissimilatory reduction of the ionic nitrogen oxides, $NO_3^-$ or $NO_2^-$, to gaseous oxides, such as NO and $N_2O$, and these forms can be additionally reduced to $N_2$, as shown in **Equation 1.2**. This process is an anaerobic respiratory pathway that can be performed by aerobic bacteria, in conditions of $O_2$ deficiency, or can be performed as a way of cellular detoxification, in environments with an excess of $NO_3^-$ or $NO_2^-$. This is a process with lower energy profit than oxygenic respiration, making denitrification only efficient in low $O_2$ concentrations (Knowles, 1982; Lu, Chandran and Stensel, 2014).

$$NO_3^- \xrightarrow{NAR} NO_2^- \xrightarrow{NIR} NO \xrightarrow{NOR} N_2O \xrightarrow{NOS} N_2$$

Equation 1.2: Denitrification process. NAR stands for nitrate reductase, NIR for nitrite reductase, NOR for nitric oxide reductase and NOS for nitrous oxide reductase.

Denitrification is a widely spread process, that can occur in terrestrial as well as aquatic systems (Skiba, 2008).

Microorganisms can have the ability to perform denitrification and N fixation (Kloos *et al.*, 1995). These two processes are usually temporally separated, however, their regulation is extremely important to maintain the N cycle fluxes balanced (Joye and Paerl, 1994; Fan, Bolhuis and Stal, 2015).

The widespread distribution of denitrifying genes can be the outcome of their cooccurrence with N fixation and can have arisen by divergence and lineage sorting and horizontal transfer events (Jones *et al.*, 2008; Alvarez *et al.*, 2011; Levy-Booth, Prescott and Grayston, 2014).

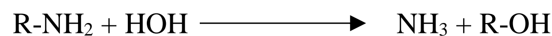
Microorganisms can use this process as a way to detoxify when under a high concentration of $NO_3^-$ or $NO_2^-$, including diazotrophs (Hopkinson and Giblin, 2008; Strock, 2008).

Denitrification can be important to diazotrophs because $NO_3^-$ is an inhibitor of nitrogenase, so it is essential to decrease inhibitory concentrations of this N form to allow N fixation to happen (Carroll and Gresshoff, 1983; Luciński, Polcyn and Ratajczak, 2002).

With the increase in $NO_3^-$ concentration by N deposition, there is an increase in the denitrification process. Denitrification can lead to different problems, such as loss of N fertilizers, when they are applied in this form, and atmospheric consequences by the production of NO and $N_2O$ (Hagman *et al.*, 2008; Portmann, Daniel and Ravishankara, 2012; Lu, Chandran and Stensel, 2014).

#### 1.3.3. Ammonification

Ammonification is the process by which organic N is converted into inorganic N in the forms of $NH_3$ or $NH_4^+$, as presented in **Equation 1.3**. Microorganisms derive energy from this metabolic process. Ammonification is a very widespread process in various environments (Hopkinson and Giblin, 2008; Strock, 2008) .



$$R\text{-}NH_2 + HOH \longrightarrow NH_3 + R\text{-}OH$$

Equation 1.3: Ammonification reaction.

### 1.4. Imbalances of N input in ecosystems

The natural processes through which Nr species are created in an ecosystem are via BNF or by lightning. Before the industrial revolution, N introduction in the soil occurred anthropogenically through food production - the legume production increases N input due to their association with N-fixing microorganisms - or through energy production, with NO emissions as a waste product of fossil fuel combustion (Vitousek *et al.*, 1997; Smil, 2001).

With the exponential growth of the population, the demand for food production substantially increased the need for a process that produced a bioavailable form of N. This method was possible in 1913, by producing $NH_3$ from $N_2$ and $H_2$, by the Haber-Bosh process (Aber, 1998).

The artificial synthesis of $NH_3$ drastically amplified the Nr added anthropogenically, and the increase of these compounds had several consequences, some of which are yet to be unraveled. The results of increased Nr concentration can increase ecosystem productivity in the short term, however, in the long term it can lower productivity. These changes can lead to a loss in ecosystem diversity, through acidification and eutrophication, which leads to imbalances in the biogeochemical cycles (Matson, Lohse and Hall, 2002; Vitousek *et al.*, 2002; Galloway *et al.*, 2003; Karl and Michaels, 2019).

The problems to the environment can occur in succession, referred to as nitrogen cascade, presented in **Figure 1.7**, which is described as “the sequential transfer of Nr through environmental systems, and which results in environmental changes, as Nr moves through or is temporarily stored within each system” (Galloway *et al.*, 2003). These problems arise from the accumulation of Nr in the environment because of the increase in the Nr input rate over the Nr removal rate, which is projected to continue to grow (Wang *et al.*, 2019).

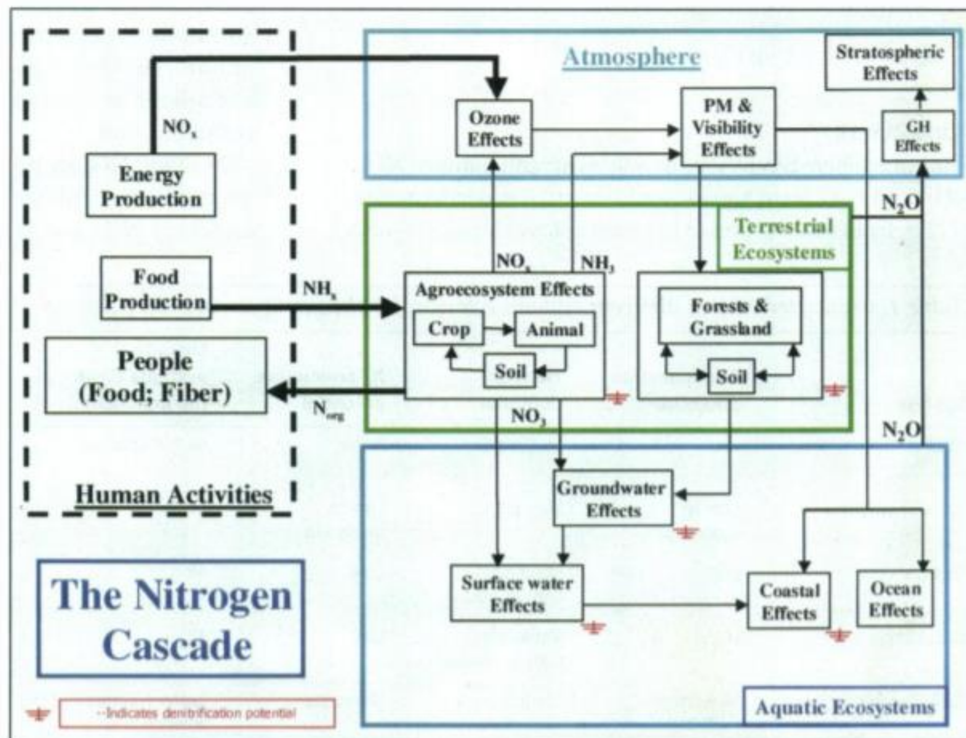



Figure 1.7: Illustration of the nitrogen (N) cascade showing the sequential effects that a single atom of N can have in various reservoirs after it has been converted from a nonreactive to a reactive form. Abbreviations: GH, greenhouse effect; PM, particulate matter (Galloway *et al.*, 2003).

The phenomenon of the atmospheric deposition of N can occur in two ways: by dry deposition, which is the uptake at surface level, such as soil, water or vegetation; or by wet deposition, which is the absorption of the gaseous forms into droplets, followed by the precipitation of these droplets (Zannetti and Zannetti, 1990; Pacyna, 2008).

The N use efficiency is a crucial factor in these ecosystems and consists of two parameters: the N uptake efficiency, which is the ability to take up N from the soil, and the use efficiency of the absorbed N, the efficiency with which organisms use the absorbed N to grow (Burns, 2006; Schenk, 2006).

As a result of the anthropogenic alterations to the N cycle, N deposition is increasing, leading to severe consequences to the ecosystems. N deposition has led plant communities in Europe and North America to shift their composition, which can result in a loss in plant diversity, as well as diversity loss of the associated microorganisms (Bobbink *et al.*, 2003; de Vries, Reinds and Vel, 2003; Kleijn *et al.*, 2008; Ochoa-Hueso *et al.*, 2011).

N deposition effects on plants depend on a series of factors, such as time, overall quantity and N form of the contribution, sensitivity of the plant species and abiotic circumstances of the ecosystem. These factors will promote different series of events, however, some effects are common such as: acidification of the soil, by the accumulation of sulfur compounds; decrease in plant resistance due to

stress; and toxic effects to susceptible plant species by N forms, such as $NH_3$ (Lee and Jose, 2003; LeBauer and Treseder, 2008; Yuan and Chen, 2012, 2015).

Previous studies on simulated N fertilization have shown that atmospheric N deposition can increase plant aerial and radicular growth. However, excessive N availability leads to soil acidification and decreased enzymatic activity, reducing soil organic matter decomposition, as well as reducing microbial respiration. N deposition can result in a reduction of the diversity of terrestrial plants, as well as alterations on C, N and P cycles (Lee and Jose, 2003; LeBauer and Treseder, 2008; Yuan and Chen, 2012, 2015).

The acidification of the soil can lead to the loss of base cations and Aluminum (Al) mobilization. This can result in alterations in soil chemistry, affecting soil food webs. The Al resulting from soil pH decreasing is toxic to soil microorganisms (Vitousek *et al.*, 1997; Matson *et al.*, 1999; Chen *et al.*, 2019).

The excessive amount of N added to the soil leads to changes in the prevalent microbial strategies, altering the microbial community, as well as the extracellular enzyme activity. A continuous N addition leads to ectomycorrhizal fungi suffering a decline and to the saprotrophs relative abundance increasing (Saiya-Cork, Sinsabaugh and Zak, 2002; Ramirez, Craine and Fierer, 2012; Morrison *et al.*, 2016).

The increase of the harmful effects of N addition on total microbial biomass, bacterial biomass, as well as microbial C, is more intense over time. A meta-analysis has shown an average decline of 15% on soil microbial biomass, being these results more pronounced in long term studies, as well as studies with elevated amounts of N addition to the soil (Pregitzer *et al.*, 2007; Treseder, 2008; Lu *et al.*, 2011; Ramirez, Craine and Fierer, 2012).

These effects are more notorious in microbial groups associated with N transformations, in particular diazotrophs, which, with N deposition, no longer have a competitive advantage in the community, since their input of Nr is no longer necessary.

N fixation is not energetically cost-effective so when conditions change and N availability increases, they switch off the process. However, this may not occur depending on the concentration of dissolved organic C, pH and the C/N ratio (Arp and Zumft, 1983; Cejudo and Paneque, 1988).

Although N deposition has inhibitory effects on nitrogenase action, studies have shown that when N availability decreases it is possible to reverse this process. However, it is unknown if that is still the case with chronic N addition to the soil (Reed, Cleveland and Townsend, 2011; Rousk, Jones and DeLuca, 2013).

For symbiotic diazotrophs, their association with plants is also affected by N deposition, since the plant has accessible N, so the association with the microorganisms is neglected. In short term, there is a reduction in nodule formation and in the case of $NO_3^-$-based fertilizer, nodule biomass, as well as its number, can be reduced along with nitrogenase activity (Bond and Mackintosh, 1975; Streeter and Wong, 1988; Wang *et al.*, 2018).

The negative effects of chronic N addition occur independently of the amount of N added to the soil, as well as the type of ecosystem, such as tundra, meadow, woodland, swamps and croplands, however, these effects on warmer environments are attenuated on a few levels (Zhang, Chen and Ruan, 2018).

## 1.5. Mediterranean ecosystems

Mediterranean-type ecosystems are the result of Mediterranean climate, described by hot dry summers and mild wet winters. Spring and autumn are considered the growing seasons and this climate is characterized by the presence of temperate natural and annual semi-natural grasslands, shrublands, dry woodlands, and forests (Cowling *et al.*, 1996; Myers *et al.*, 2000).

There are five zones on Earth where the Mediterranean ecosystem can be found: California, central Chile, Mediterranean Basin, southern Cape region and southwestern and southern Australia, as shown in **Figure 1.8**. There are some ecosystems exclusive of Mediterranean basins, like Portuguese *matos* and *montados*, Spanish *matorrales, garrigas* and *dehesas,* Italian *macchias* and Greek *phryganas* (Blondel, 2006).

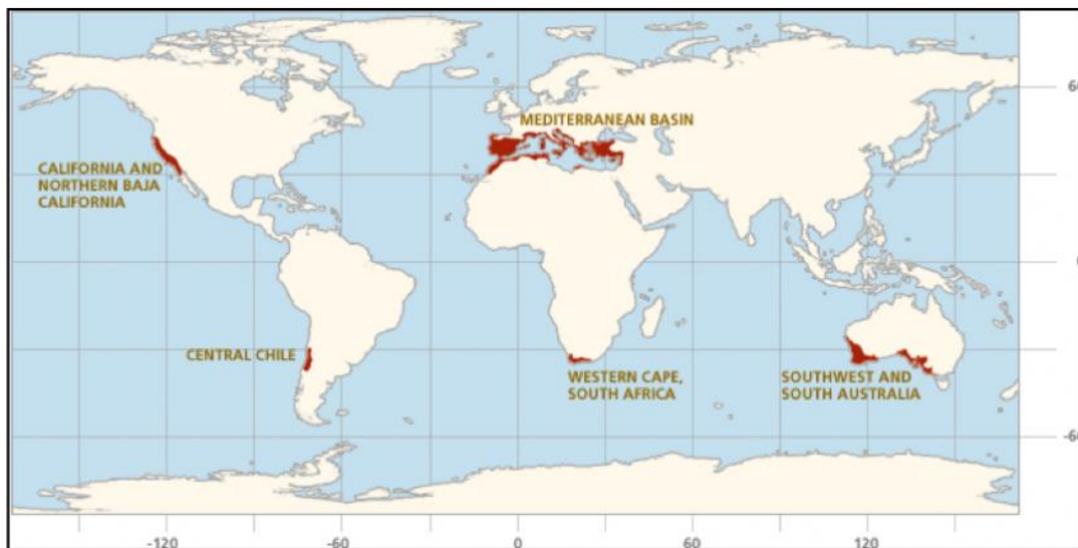



Figure 1.8: Representation of the location where mediterranean climate is present (https://www.iucn.org/commissions/commission-ecosystem-management/our-work/cems-specialist-groups/mediterranean-type-ecosystems)

The Mediterranean ecosystem is considered a hotspot of biodiversity; the Mediterranean basin was considered one of the places in the world with most biodiversity. Although its biodiversity is immense, the vegetation of these ecosystems can be categorized into two groups: summer semi-deciduous and evergreen sclerophylls (Pacyna, 2008).

These ecosystems are considered environments under N limitation, however, their primary structure and response to N input are specially conditioned by water restriction and P availability. Considering that these environments are used to having limited amounts of N, their communities have evolved to be adapted to these conditions, and as such, changes in N availability will alter the relations between the organisms (Chapin, 2003; Xia and Wan, 2008; Craine *et al.*, 2009).

Due to the distinctive features of the Mediterranean ecosystems, such as the climate, the soil (lower levels of nutrient and organic matter, although it has a high level of carbonate content), the increased N dry deposition, the discrepancy of N accessibility and biological action and even the spatial and chronological heterogeneity, it is predictable that this ecosystem will not have the same response as the north temperate ecosystems. This is important to establish since most of the studies done on N deposition were made in north Europe and America, which are north temperate ecosystems (Cruz *et al.*, 2008; Bobbink *et al.*, 2010; Ochoa-Hueso *et al.*, 2011).

Although there are studies on Mediterranean ecosystems, they come from California, which does not share the same environmental conditions, such as soil fertility and P availability (Cowling *et al.*, 1996; Fenn *et al.*, 2010; Ochoa-Hueso *et al.*, 2011).

As such, N deposition can have different impacts on Mediterranean basin ecosystems and given the impact these studies can have, the critical loads added to the soil can be determined and a change in agriculture practices and legislation involved can be encouraged. Moreover, the particularity of this being a long-term study can give an insight into a more profound background of the consequences of N deposition.

### 1.6. Study site

The research presented here was carried out on Serra da Arrábida in the Arrábida Natural Park (38° 27'34" N, 9° 0' 20" W), in a Natura 2000 site, positioned south of Lisbon, Portugal (PTCON0010Arrábida/Espichel). This study site is near urban and industrial areas, so the fertilization was made to mimic the inputs of agricultural sources (only N reduced added- $NH_4^+$), or the inputs of agricultural and industrial sources (added N reduced and oxidized- $NH_4^+$ and $NO_3^-$). This location is encompassed in a study since January of 2007 and the fertilization was performed 3 times a year: spring, summer, and middle autumn/winter. Before the present work, the field was fertilized on January 26$^{th}$ 2019 by the Plant & Soil Ecology group and the samples were collected on March 14$^{th}$ 2019.

This work is part of an ongoing study over the last 10 years, in which fields are fertilized with different quantities and forms of N. To characterize the soils from these fields, some soil characteristics were analyzed in different years of the study, namely 2007 (prior to fertilization), 2008 (one year fertilization), 2011 (five years fertilization) and 2018 (10 years fertilization). The characteristics evaluated were: pH, organic matter (OM), N%, C/N, soil inorganic N ($N_{inorg}$), $NH_4^+$, $NO_3^-$ (Dias, Malveiro, *et al.*, 2011; Dias *et al.*, 2014). The results of this characterization are shown in **Figure 1.9**.

The values of N% (Graphic A) have not changed in the 10 years of the study, presenting low values for all treatments every year (~0.1%). This indicates that in the fertilized treatments, the fertilization did not change the N content.

The concentration of $N_{inorg}$ and $NO_3^-$ (graphic B and C) exhibited more varied results. The concentration of $N_{inorg}$ has increased during the experiment, especially for the treatment 80AN which presents the highest values and differs from the other treatments in the years 2011 and 2018. The concentration of $NO_3^-$ follows the same pattern as the $N_{inorg}$ concentration, with an increase in the last two measurements especially the treatment 80AN.

The $N_{inorg}$ was measured to assess the N retention, since the lower the soil inorganic N, the higher the N retention. This way the increase of $N_{inorg}$ in treatment 80AN represents a decrease in the N retention and therefore alterations in the N cycle, possibly with $NO_3^-$ leaching (de Schrijver *et al.*, 2008; Dias *et al.*, 2014).

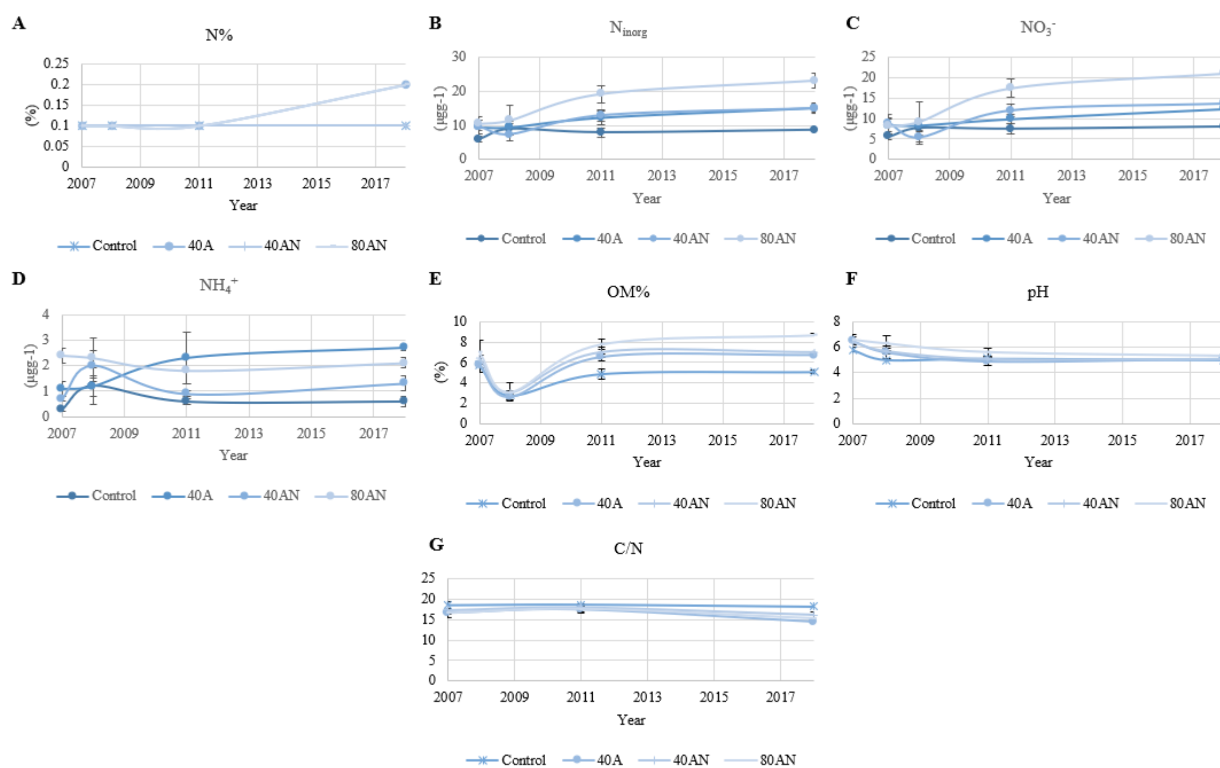



Figure 1.9: Graphic representation of the soil samples relative to the characteristics evaluated. A- N (%), B- $N_{inorg}$ ($\mu gg^{-1}$), C- $NO_3^-$ ($\mu gg^{-1}$), D- $NH_4^+$ ($\mu gg^{-1}$), E-OM (%), F- pH and G- C/N ratio. Error bars represent the mean (n = 3 experimental plots per treatment) ±SE

The concentration of $NH_4^+$ (graphic D) did not present differences during the study both among the treatments and throughout the years.

The OM% (graphic E) appears to have changed over the years - in the results from 2011 and 2018 the OM increases from the control treatment, to treatment 40A, followed by treatment 40 AN and treatment 80AN presents the highest values. These findings may have resulted from an increase in the decomposition, which has great relevance in soils of the mediterranean basin, since it has naturally low values of OM (Jones *et al.*, 2012; Dias *et al.*, 2013).

The pH (graphic F) does not present differences between the treatments in any year, however, throughout the years pH decreased in the treatments. Most studies report acidification with N addition; however, the soil high content of calcium carbonate may be the reason why it is not possible to observe a difference between the fertilized treatments and the control (Schultz, 2005; Bobbink *et al.*, 2010).

The same results can be observed for the C/N ratio (graphic G), throughout the years the ratio continues similar in the treatments, however, in the results of 2018 the control treatment appears to have a higher value then the N fertilized treatments. This way, the ratio of C/N remains within the range for the Mediterranean Basin (Sardans *et al.*, 2008).

## 1.7. Thesis purpose and goals

The effects of increased nitrogen (N) input in the ecosystems have brought concern for some time now, the long-term consequences of this input to Mediterranean ecosystems are poorly studied. These ecosystems are known for being hotspots of biodiversity and for this reason studies on the effects of N deposition on biodiversity in these places are necessary.

To understand these consequences, the diazotrophic community within the rhizosphere of *Ulex densus* was studied as a proxy to the ecosystem's response to different N addition to the soil, in the context of a long-term field study where various plots were continuously exposed to different forms and amounts of N-fertilizers.

For each soil treatment, selective isolation for diazotrophs was made and the isolates collection that resulted from that was characterized in order to evaluate the structural differences in the diazotrophic community on the distinct treatments. 40 representative isolates of this collection were selected and identified through 16S rRNA gene sequencing.

To evaluate the functional differences, other functions of the N cycle, such as denitrification, nitrification, and ammonification were evaluated in order to assess the multifunctionality of these isolates within the N cycle. For some of these isolates, the metabolic plasticity was studied to determine if the different fertilizations changed the metabolism of the microorganisms.

Under these circumstances, this thesis goals are:

- Characterize the effect of N availability on *Ulex densus* rhizospheric diazotrophic community, based on the structural and functional diversity of the respective isolates, in order to find an ecological marker of high N availability;
- Morphophysiological and genomic characterization of the rhizospheric diazotrophic community, obtained from isolation in N-free media;
- Evaluation of the structural differences of the community between the different treatments under distinct N addition;
- Evaluation of the multifunctionality of the community, within the N cycle, and comparison between the different treatments under distinct N addition;
- Evaluation of the differences in the metabolic plasticity of the community between the different treatments under distinct N addition.

## 1.8. Strategy

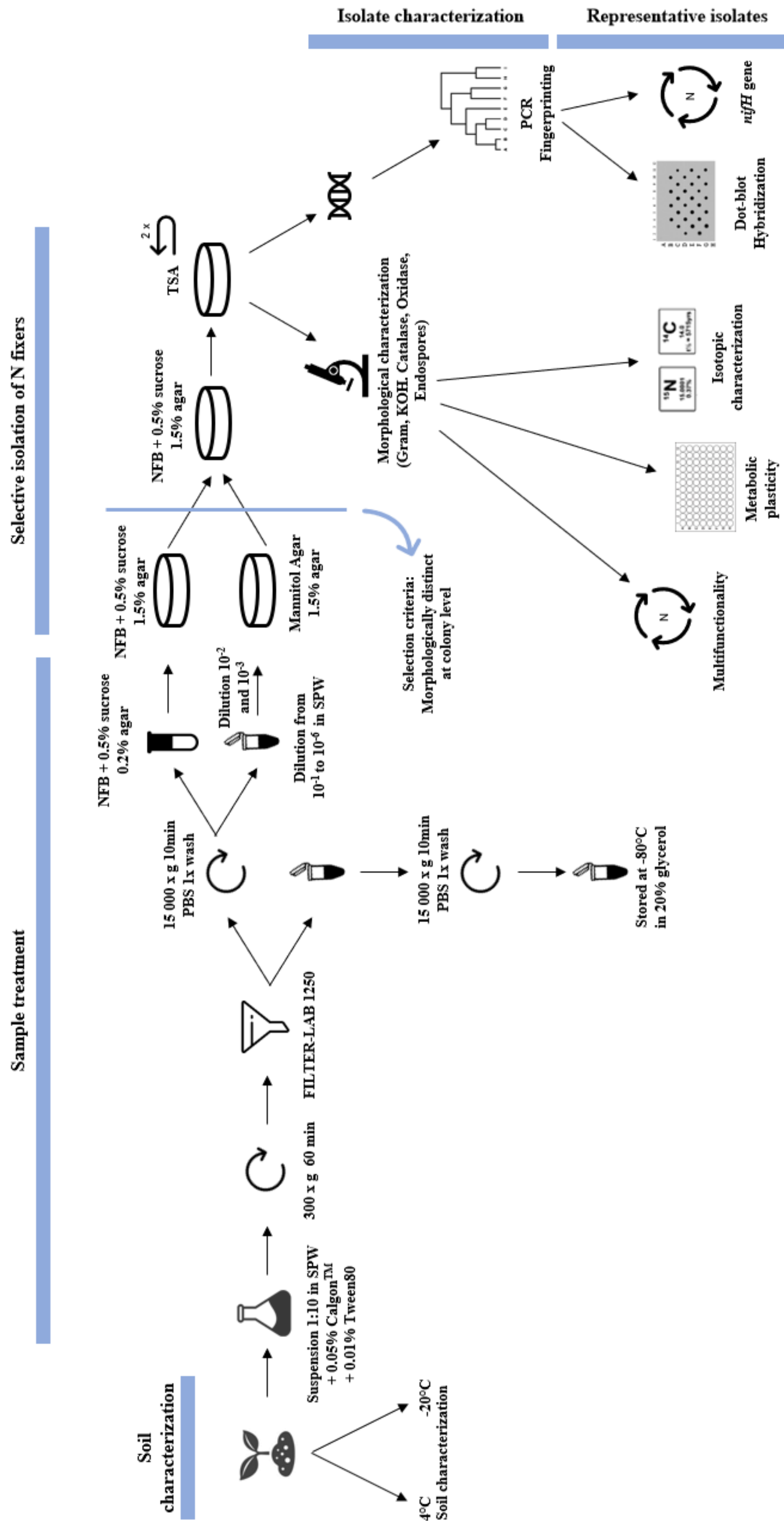



Figure 1.10 Scheme of the analytical workflow for the soil samples.

# 2. MATERIALS AND METHODS

## 2.1. Experimental design and soil sampling

The field experimental scheme consisted of 12 plots, of 400 $m^2$ each, as presented in **Figure 2.1**. All determinations and analyses were conducted inside an internal 100 $m^2$ square, to limit the boundary effect and dilution processes. Nitrogen availability was altered through the addition of 40 and 80 kg N $ha^{-1}$ $yr^{-1}$ in the form of $NH_4NO_3$ (doses designated 40 AN and 80 AN respectively) and 40 kg N $ha^{-1}$ $yr^{-1}$ as a 1:1 mix of N-$NH_4Cl$ and N-$(NH_4)_2SO_4$ (designated 40 A). Control plots were not fertilized. Each treatment had three replicates (3 plots). To counteract nitrogen 'pollution' flow-through from fertilized plots, the experimental plots were spread in three lines along the slope, with the controls being positioned in the top row. The principles of determining the nitrogen doses and forms applied to the soil are described in Dias, Neto, *et al.* (2011).

Soil samples were obtained from rhizospheric soil from four plants in each corner of the internal square and one plant in the center. For each plant, four samples were collected corresponding to the four cardinal points. This procedure was done in every plot as illustrated in **Figure 2.1**.

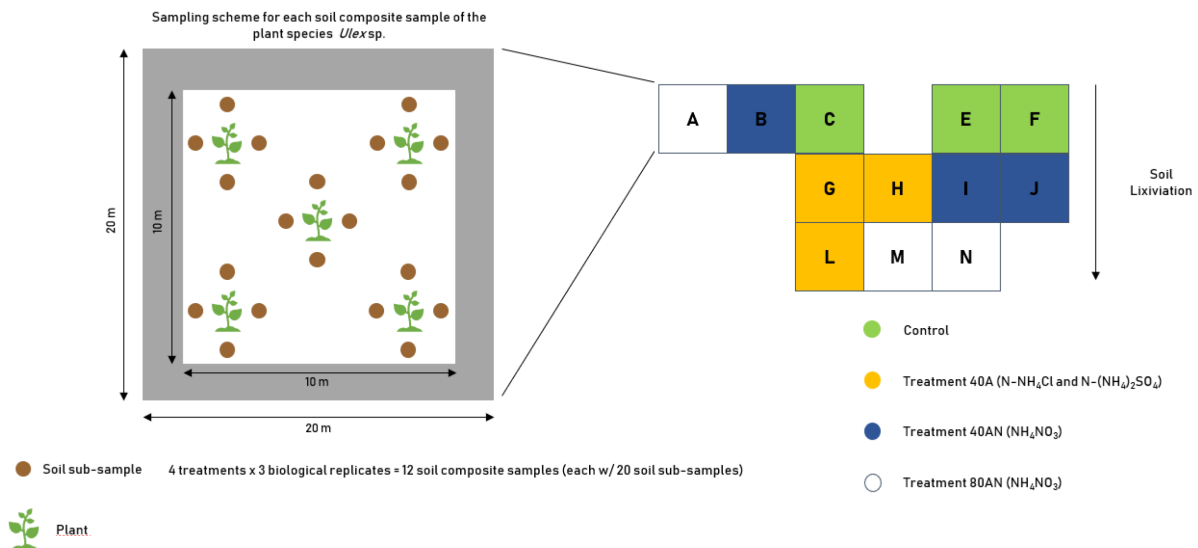



Figure 2.1: Experimental scheme of the plots in the field and the sampling scheme for the soil samples.

## 2.2. Sample treatment

Each sample was composed of soil collected from each cardinal point making a total of five samples per plot, one for the rhizospheric soil of each plant. For the processing of the samples, each of them was sieved through a 4 mm pore, to prevent debris passage. These samples were separated in three portions: two were stored at 4ºC (for the isolation of microorganisms) and one was placed at -20ºC (for posterior analysis, corresponding to the soil characterization in **Figure 1.10**).

As shown in the sample treatment section of **Figure 1.10**, the composite samples were constituted by 5 g of soil from each of the five individual samples described above, making a total of

25 g of soil per plot. These composite samples were placed in 500 mL Erlenmeyer flasks with 200 mL of Sterile pure water (SPW) with 0.05% (w/v) Calgon, acting as a chelating agent, and 0.01% (v/v) Tween 80, to release hydrophobic bacteria from the aggregates they form with soil particles. These suspensions were incubated for an hour at 28ºC at 160 rpm and then centrifuged at 300 *x g* in a Centrifuge 5810R (Eppendorf, Germany) for 60 min. The collected supernatants were filtered with FILTER-LAB1250 (10-13 µm).

For each suspension, 1 mL was collected in a microtube, centrifuged at 10000 *x g* in a Centrifuge 1-15P (Sigma, USA) for 10 min, washed in 1x Phosphate Buffer Saline (PBS) and stored in 20% (v/v) glycerol at -80ºC. The remaining volume was centrifuged at 10000 *x g* in a Centrifuge 1-15P (Sigma, USA) for 10 min and washed in 1x PBS. The obtained pellets were used for microorganism isolation in serial dilutions from $10^{-1}$ to $10^{-6}$.

### 2.3. Selective isolation of nitrogen fixers

For microorganism's isolation two different media were used: Nitrogen free broth (NFB) medium with sucrose addition and Mannitol agar. NFB is a more selective medium, since it uses malic acid as a carbon source, which can have inhibitory effects on some species (Rathnayaka, 2013). The medium was supplemented with sucrose, to allow the growth of microorganisms that cannot use malic acid as a carbon source. To ensure microorganisms incapable of growing on NFB would not be lost in the isolation process, Mannitol agar was used in parallel, since it is a more general medium that allows the growth of aerobic diazotrophic microorganisms. This medium usually contains calcium carbonate, but it was removed to facilitate colony visualization.

The Mannitol agar medium composition was: 15 g mannitol; 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4$, 0.1 g $CaSO_4$, 0.2 g NaCl, 15 g Agar, in 1 L of RO-Water. The medium was sterilized by autoclave, at 121ºC for 15 min.

The NFB medium with sucrose contained: 5 g malic acid, 5 g sucrose, 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2{\cdot}2H_2O$, 2 mL micronutrient solution (0.4 g $CuSO_4{\cdot}5H_20$, 0.12 g $ZnSO_4{\cdot}7H_20$, 1.4 g $H_3BO_3$, 1 g $Na_2MoO_4{\cdot}2H_2O$, 1.5 g $MnSO_4{\cdot}H_20$ in 1 L of RO-water), 2 mL bromothymol blue solution (0.5% (v/v) in 0.2 N KOH), 4 mL 1.64% (w/v) Fe(III) Ethylenediamine tetraacetic acid (EDTA), 1 mL vitamin solution (10 mg biotin and 20 mg pyridoxol HCl in 100 mL of SPW) in a final volume of 1 L RO-water, pH adjusted to 6.8 with 1 M NaOH, before sterilization by autoclave at 121ºC for 15 min. The vitamin solution was filtered (0.22 µm), and it was added after the medium was autoclaved. Both media were used with supplementation of 0.01% cycloheximide to prevent the growth of fungi.

NFB medium was first used in semi-solid form, 0.2% agar, as described by Baldani *et al.* (2014) and Chowdhury *et al.* (2007), as a means of enrichment for diazotrophic microorganisms. This occurs with the formation of a film within the medium, which provides a microaerophilic condition that favors the nitrogenase action. These enrichments were inoculated with $10^0$ and $10^{-1}$ dilutions in 3 mL of medium in transparent glass tubes, so it was possible to visualize film formation. The enrichments were incubated for a week at 28ºC, after which the films were collected and inoculated onto NFB with 1.5% agar plates. For Mannitol agar, $10^{-2}$ and $10^{-3}$ dilutions were inoculated. All plates were incubated at 28ºC until growth was visible.

All plates were observed under a stereomicroscope S4E (Leica, Germany) and the colonies with different morphology were picked off and streaked onto fresh NFB plates.

Colonies were then picked off onto Tryptone Soy Agar (TSA), because in NFB they were extremely mucous, not allowing isolation. Repeated passages were made to ensure isolation, prior to characterization and the creation of an isolate culture collection.

## 2.4. Isolate characterization

The isolates were characterized based on morphological features using classic phenotypic methods, namely Gram staining and KOH test combined with oxidase and catalase tests and endospore staining.

Genomic DNA was extracted from bacterial cells in a pure culture using an adapted Guanidium Thiocyanate method described by Pitcher, Saunders and Owen (1989). The modifications were made mainly in the first steps of the method. The bacterial cells were collected from the plate and were resuspended in 250 µL of lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0.3% SDS; pH 8.0) and 100 µL of microspheres were added. Posteriorly, the cells were homogenized in a vortex for 2 min, after which they were incubated at 65ºC for 30 min, followed by 2 min of homogenization. Then, the GES reagent was added (5 M guanidium thiocyanate; 10 mM EDTA; 0.5% Sarkosyl; pH 8,0) and the original method was followed, however, precipitation was achieved using 1 volume of isopropanol.

PCR-fingerprinting with primer M13 (5' – GAGGGTGGCGGTTCT – 3') (Meyer *et al.*, 1993) was performed in a total volume of 25 µL, with 1x PCR buffer (Invitrogen), 3 mM $MgCl_2$, 25 pmol of primer, 0.2 mM of dNTP's mix, 1 U of Taq Polymerase (Invitrogen) and 1 µL of template DNA. Amplification was performed in a UNO II Thermal Cycler (Biometra, Germany), with the following conditions: 5 min of initial denaturation at 95°C, followed by 40 cycles of 1 min of denaturation at 95°C, 2 min of annealing at 50°C and 2 min of extension at 72°C, followed by a final elongation step of 5 min at 72°C.

PCR products were visualized by gel electrophoresis, alongside with 1 kb plus DNA ladder (Invitrogen), in a 1.2% (w/v) agarose gel in 0.5x TBE buffer (40 mM Tris; 45 mM Boric acid; 1 mM EDTA; pH 8.3). Electrophoresis was run at 2.5 V/cm for 3 h, followed by gel staining in an Ethidium Bromide solution and shot in an Alliance 4.7 UV Transilluminator (UVItec, Cambridge), utilizing Alliance software version 15.15 (UVItec, Cambridge).

Gel images with the fingerprinting profiles obtained were uploaded and treated with Bionumerics software (version 6.6). Dendrograms were constructed for the isolates based on the profiles obtained for M13, using Pearson correlation coefficient to construct the similarity matrix and the Unweighted Pair Group Method with Arithmetic Average (UPGMA) as the clustering method. Reproducibility was established by randomly selecting 10% of the isolates to use as replicates in the amplification. A dendrogram constructed based on these duplicates was used to estimate the reproducibility cut-off and to calculate the optimization (0.5) and curve smoothing (1) parameters that better paired the repeats.

To assess the soils diazotrophic diversity Shannon (J') and Simpson's (D) diversity indices were used (Zar, 2010), establishing a cluster cut-off at 70% to define groups (Appendix A), which was the minimum similarity between the duplicates. The diversity indices were calculated based on **Equation 2.1**, **2.2**, **2.3** and **2.4**.

$$H' = -\sum_{i=1}^{k} p_i log p_i$$

Equation 2.1: Calculation of Shannon diversity index (H'), where 'k' is the number of categories and '$p_i$' is the number of observations in each category.

$$H'_{max} = log k$$

Equation 2.2: Calculation the $H'_{max}$ (maximum possible diversity for a set of data), where 'k' is the number of categories.

$$J' = \frac{H'}{H'_{max}}$$

Equation 2.3: Calculation of J' as a measure of the proportion between the observed and the maximum possible diversity.

$$D = 1 - \sum \frac{n(n-1)}{N(N-1)}$$

Equation 2.4: Calculation of Simpson's diversity index (D), where 'N' is the total number of individuals and 'n' is the number of individuals present in a specific group.

## 2.5. Representative isolates and sequencing

A dendrogram was constructed for each soil treatment and a cut-off line was elected for the selection of 10 clusters, making a total of 40 clusters for the four treatments. Given that the number of isolates obtained from each biological plot replicate was not the same, a proportion per biological replicate was considered for calculating its contribution to the total number of representative isolates for each cluster (Appendix A).

DNA of the representative isolates was used in molecular identification based on 16S rRNA gene partial sequence analysis. The amplification was made in a final volume of 50 µL with 1x PCR reaction buffer (Invitrogen), 2 mM $MgCl_2$, 50 pmol of each primer PA (5'–AGAGTTTGATCCTGGCTCAG–3') (Massol-Deya *et al.*, 1995) and 1392R (5'–ACGGGCGGTGTGTRC-3') (Baker, Smith and Cowan, 2003), 0.2 mM of dNTP's mix, 1 U of Taq polymerase (Invitrogen) and 1 µL of template DNA. Amplification was performed in a UNO II Thermal Cycler (Biometra, Germany), with the following conditions: 3 min of initial denaturation at 94°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 1 min of extension at 72°C, followed by a final elongation step of 3 min at 72°C.

PCR products were visualized by gel electrophoresis, alongside with 1 kb plus DNA ladder (Invitrogen), in a 1.2% (w/v) agarose gel in 0.5x TBE buffer (40 mM Tris; 45 mM Boric acid; 1 mM EDTA; pH 8.3). Electrophoresis was run at 2.5 V/cm for 1 h, followed by gel staining in an Ethidium Bromide solution and shot in an Alliance 4.7 UV Transilluminator (UVItec, Cambridge), utilizing Alliance software version 15.15 (UVItec, Cambridge).

PCR products were sequenced by Eurofins (Germany) using Sanger technology and the results were curated and BLASTed against NCBI database to obtain the most probable identification.

## 2.6. *nifH* gene detection

### 2.6.1. PCR approach

To assess the isolates that presented the *nifH* gene, a PCR strategy with search for the *nifH* gene was conducted. For this reason, a search of primer pairs for this gene was made and a compilation of data, for the microorganisms included in nitrogenase clusters for the cultivation conditions used, was elaborated to determine the sets of primers with the best results (Appendix D). **Table 2.1** includes the three sets of primers that presented the best results.

Table 2.1: Compilation of different primers for the *nifH* gene identification. The position presented is relative to *A. vinelandii nifH* gene (Genbank ACCN# M20568). 'I' stands for Iosine. The clusters are from the nitrogenase phylogeny. The references presented corresponds to the works in which these primers were used in the various clusters.

| Primers | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Results | | | | | | | | |
| | | | | | | Cluster 1 | | | | | | | Cluster 2 | |
| Name | Sequence (5' to 3') | Position | Degeneracy | Tm (°C) | Length | Subcluster 1J, 1K | Subcluster 1J, 1K,1P, 1U | Subcluster 1H,1K,1I, 1M, 1N, 1P,1T,1U | Subcluster 1F | Subcluster 1B | Subcluster 1D | Subcluster 1E, 2D | Subcluster 2E | Reference |
| | | | | | | α-Proteobacteria | β-Proteobacteria | γ-Proteobacteria | ε-Proteobacteria | Cyanobacteria | *Frankia* | Firmicutes (*Paenibacillus*) | δ-Proteobacteria | |
| IGK3 | GCI WTH TAY GGI AAR GGI GGI ATH GGI AA | 19-47 | 72 | 69.4-75.3 | 395 | Yes | Yes | Yes | | Yes | Yes | Yes | Yes | (Angel *et al.*, 2018; Chakraborty and Islam, 2018) |
| DVV | ATI GCR AAI CCI CCR CAI ACI ACR TC | 388-413 | 8 | 71.7-75.8 | | | | | | | | | | |
| F2 | TGY GAY CCI AAI GCI GA | 115–131 | 4 | 62.3-67.9 | 359 | Yes | Yes | Yes | | Yes | Yes | Yes | Yes | (Marusina *et al.*, 2001; Angel *et al.*, 2018) |
| R6 | GCC ATC ATY TCI CCI GA | 457–473 | 2 | 61.1-62.5 | | | | | | | | | | |
| IGK | AAR GGN GGN ATH GGN AA | 31-47 | 384 | 62.1-72.5 | 464 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | | (Gaby and Buckley, 2012) |
| nifh3 | ATR TTR TTN GCN GCR TA | 478-494 | 128 | 46.1-61.5 | | | | | | | | | | |

For each of the three sets of primers several attempts were made in order to optimize the amplification reaction, these attempts are exposed in **Table 2.2**, **2.3** and **2.4**.

Table 2.2: PCR conditions for the set of primers IGK and nifh3, with the different conditions tested.

| **Primers** | **IGK** | **nifh3** |
|---|---|---|
| **PCR conditions** | 10 min 94ºC; (45 s 94ºC; 30 s 45ºC-59ºC; 1 min 72ºC) x35; 10 min 72ºC | |
| | 10 min 94ºC; (45 s 94ºC; 30 s 49ºC-56ºC; 1 min 72ºC) x35; 10 min 72ºC | |
| **PCR reagents** | 1x PCR reaction buffer; 1 mM $MgCl_2$; 25 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA | |
| | 1x PCR reaction buffer; 1 mM $MgCl_2$; 50 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA | |
| | 1x PCR reaction buffer; 0.5 mM $MgCl_2$; 50 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA | |
| **Reference** | (Ohkuma *et al.*, 1996) | (Zani *et al.*, 2000) |
| **Negative control** | *Escherichia coli* K12 | |
| **Positive controls** | *Azospirillum brasilense* sp245 | |
| | *Azospirillum brasilence* ARG2 | |

Table 2.3: PCR conditions for the set of primers IGK3 and DVV, with the different conditions tested.

| **Primers** | **IGK3** | **DVV** |
|---|---|---|
| **PCR conditions** | 10 min 94ºC; (45 s 94ºC; 30 s 58ºC; 1 min 72ºC) x35; 10 min 72ºC | |
| | 10 min 94ºC; (45 s 94ºC; 30 s 50ºC-56ºC; 1 min 72ºC) x35; 10 min 72ºC | |
| **PCR reagents** | 1x PCR reaction buffer; 1 mM $MgCl_2$; 25 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA | |
| | 1x PCR reaction buffer; 1 mM $MgCl_2$; 50 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA | |
| **Reference** | (Ando *et al.*, 2005) | |
| **Negative control** | *Escherichia coli* K12 | |
| **Positive controls** | *Azospirillum brasilense* sp245 | |
| | *Azospirillum brasilence* ARG2 | |

Table 2.4: PCR conditions for the set of primers F2 and R6, with the different conditions tested.

<table>
<tr><th>Primers</th><th>F2</th><th>R6</th></tr>
<tr><td rowspan="2">PCR conditions</td><td colspan="2">3 min 94ºC; 3 min 50ºC; (3 min 72ºC; 30 s 94ºC; 2 min 50ºC; 30 s 72ºC) x5; (30 s 94ºC; 30 s 40ºC; 30 s 72ºC) x30; 7 min 72ºC</td></tr>
<tr><td colspan="2">3 min 94ºC; 3 min 45ºC; 3 min 72ºC; (30 s 94ºC; 2 min 45ºC; 30 s 72ºC) x5; (30 s 94ºC; 30 s 40ºC; 30 s 72ºC) x30; 7 min 72ºC</td></tr>
<tr><td rowspan="2">PCR reagents</td><td colspan="2">1x PCR reaction buffer; 1 mM $MgCl_2$; 25 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA</td></tr>
<tr><td colspan="2">1x PCR reaction buffer; 1 mM $MgCl_2$; 50 pmol each primer; 0.1 mM dNTP's mix; 0.5 U Taq polymerase; 1 µl of DNA</td></tr>
<tr><td>Reference</td><td colspan="2">(Marusina et al., 2001)</td></tr>
<tr><td>Negative control</td><td colspan="2">Escherichia coli K12</td></tr>
<tr><td rowspan="2">Positive controls</td><td colspan="2">Azospirillum brasilense sp245</td></tr>
<tr><td colspan="2">Azospirillum brasilence ARG2</td></tr>
</table>

PCR products were visualized by gel electrophoresis, alongside with 1 kb plus DNA ladder (Invitrogen), in a 1.2% (w/v) agarose gel in 0.5x TBE buffer (40 mM Tris; 45 mM Boric acid; 1 mM EDTA; pH 8.3). Electrophoresis was run at 2.5 V/cm for 1 h, followed by gel staining in an Ethidium Bromide solution and shot in an Alliance 4.7 UV Transilluminator (UVItec, Cambridge), utilizing Alliance software version 15.15 (UVItec, Cambridge).

Afterwards another strategy was used to determine which of the isolates had the *nifH* gene present in their genome.

### 2.6.2. Dot-blot hybridization

The first step of the dot-blot hybridization was the construction of the probe by PCR, using *Azospirillum brasilense* sp245 DNA as template and the set of primers IGK3 and DVV, resulting in a 395 bp fragment of the *nifH* gene. The system used for probe labeling was the Digoxigenin-labeled DNA.

The PCR approach is based on the amplification with the kit PCR DIG Labeling mix (Roche, Germany) and was made in a final volume of 50 µL with 1x PCR reaction buffer (Invitrogen), 1 mM $MgCl_2$, 50 pmol of each primer IGK3 and DVV, 0.5 mM of dNTP's Kit PCR DIG labeling mix (2 mM dATP, dCTP, dGTP each, 1.9 mM dTTP, 0.1 mM digoxigenin-11-dUTP (DIG-11-dUTP)), 0.5 U of Taq polymerase and 1 µL of template DNA. The amplification was performed in a UNO II Thermal Cycler (Biometra, Germany) and the PCR conditions were: 10 min of initial denaturation at 94°C, followed by 35 cycles of 45 s of denaturation at 95°C, 30 s of annealing at 58ºC and 1 min of extension at 72°C, followed by a final elongation step of 10 min at 72°C.

This technique was chosen because it is possible to verify the labeling of the probe by electrophoresis, seeing as the labelled probe has a higher molecular weight than the amplicon.

After this, it was necessary to know the labeling reaction yield, by comparing a control labelled DNA in a known concentration with the probe constructed. This comparison was made with the protocol provided in DIG High Prime DNA Labeling and Detection Starter Kit I, following manufacturer instructions, by a colorimetric reaction. This reaction showed that the probe labelling had low yield and so another approach was performed.

The other approach was the High prime kit (Roche, Germany) where 1 µg of template DNA was added to PCR water to a final volume of 16 µl in a microtube. DNA was denatured by heating in a boiling water bath for 10 min and quickly chilled in an ice/water bath, 4 µL of mix DIG-High prime was added to the DNA, mixed and spinned. The mixture was incubated for 3 h at 37ºC, and the reaction was stopped by adding 2 µL of 0.2 M EDTA (pH 8.0). The yield of the probe labelling was assessed as described above.

For the genera identified through 16S rRNA gene sequencing, sequences of *nifH* gene were searched in the NCBI database. These sequences were BLASTed against the sequence of the probe and the homology values obtained are shown in **Table 2.5**.

Table 2.5: Homology values (percentage of identity) of the probe with the different genera obtained of the representative isolates.

| | Max score | Total score | Query cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| ***Chryseobacterium* sp.** | 355 | 355 | 96% | 2.00E-102 | 82.20% | Query_202929 |
| ***Ensifer* sp.** | 474 | 474 | 53% | 7.00E-138 | 88.38% | Query_181963 |
| ***Microbacterium* sp.** | 271 | 271 | 73% | 2.00E-77 | 84.00% | Query_150083 |
| ***Novosphingobium* sp.** | 380 | 380 | 99% | 5.00E-110 | 86.48% | Query_118509 |
| ***Pantoea* sp.** | 375 | 375 | 96% | 2.00E-108 | 92.55% | Query_224697 |
| ***Pseudomonas* sp. D5-2** | 402 | 402 | 52% | 4.00E-116 | 82.53% | Query_12693 |
| ***Pseudomonas stutzeri*** | 359 | 359 | 97% | 2.00E-103 | 83.00% | Query_1559 |
| ***Rhizobium* sp.** | 475 | 475 | 58% | 2.00E-138 | 85.17% | Query_244305 |
| ***Serratia* sp.** | 52,7 | 52,7 | 38% | 4.00E-11 | 67.76% | Query_8457 |
| ***Stenotrophomonas* sp.** | 250 | 250 | 97% | 7.00E-71 | 75.71% | Query_112687 |
| ***Variovorax* sp.** | 529 | 529 | 47% | 1.00E-154 | 88.04% | Query_227783 |

The average sequence homology obtained, 83.26%, was used to calculate the optimal hybridization temperature based on **Equation 2.5** and **2.6**, resulting in an optimal hybridization temperature of 50ºC.

$$Tm = 49.82 + 0.41\ (\%\ G + C) - (600/l)$$

Equation 2.5: Calculation of probe optimal hybridization temperature according to GC content and percent homology of probe to target. l corresponds to length of hybrid in base pairs.

$$T_{opt.} = Tm - 20 \text{ to } 25°\ C$$

Equation 2.6: Calculation of probe optimal hybridization temperature with DIG Easy Hyb (Roche).

The hybridization reaction was performed using the 40 isolates, a negative control (*E. coli* K12) and two positive controls (*A. brasilense* sp245 and *A. brasilense* ARG2).

Each isolate DNA was denatured at 100ºC for 10 min, followed by quick chilling on ice to obtain single-stranded DNA, and 1 µL of this DNA was spotted onto the dry positive charged membrane.

The membrane was baked for 2 h at 80ºC to fixate the DNA, after which the membrane was placed in a tube with 20 mL of prehybridization solution (hybridization buffer DIG Easy Hyb (Roche)) and incubated at 50ºC for 30 min. This prehybridization solution has the same composition as the hybridization solution except for the probe, and its purpose is to prepare the membrane for hybridization.

The probe was denatured at 100ºC for 5 min and rapidly cooled in ice-water. Afterwards, the probe was added to 4 mL of pre-heated hybridization buffer and mixed by inversion. The prehybridization solution was poured off and the hybridization solution was added. The membrane was incubated with gentle agitation at 50ºC overnight.

Subsequently, the membrane was washed two times at room temperature for 5 min with 50 mL of 2x saline-sodium citrate (SSC); 0.1% (v/v) sodium dodecyl sulfate (SDS), to clean the membrane from hybridization solution remains. Next was the low stringency wash, in which the membrane was washed with 50 mL of 0.2x SSC; 0.1% (v/v) SDS at room temperature for 10 min with rotation.

For the immunological detection, the membrane was washed with 100 mL washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween 20, pH 7.5) for 1 min at room temperature, to clean the low stringency wash remains. After that, the membrane was incubated with 100 mL of blocking solution (dilution of 10x blocking solution with maleic acid buffer - 0.1M maleic acid, 0.15 M NaCl, pH 7.5) for 30 min at room temperature, in order to block every spot which does not contain the DNA from the isolates.

Afterwards, the membrane was incubated with 20 mL of antibody solution (dilution of Anti-DIG 1:5000 in 1x blocking solution) for 30 min at room temperature.

To remove the unbound antibody conjugate, the membrane was washed two times with 100 mL of washing buffer at room temperature, after which the pH of the membrane was equilibrated to pH 9.5, to allow the colorimetric detection, with 20 mL of detection buffer (0.1M Tris-HCl, 0,1M NaCl, pH 9.5) for 2 min at room temperature.

For the revelation, the membrane was incubated with 10 mL of color-substrate solution (40 µL of Nitro-blue tetrazolium (NBT)/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) stock solution to 2 mL of detection buffer) for 16 h in the dark. The reaction was stopped with 50 mL of TE-buffer (10 mM Tris-HCl, 1mM EDTA, pH 8) for 5 min at room temperature. The scheme for this procedure is presented in **Figure 2.2**.

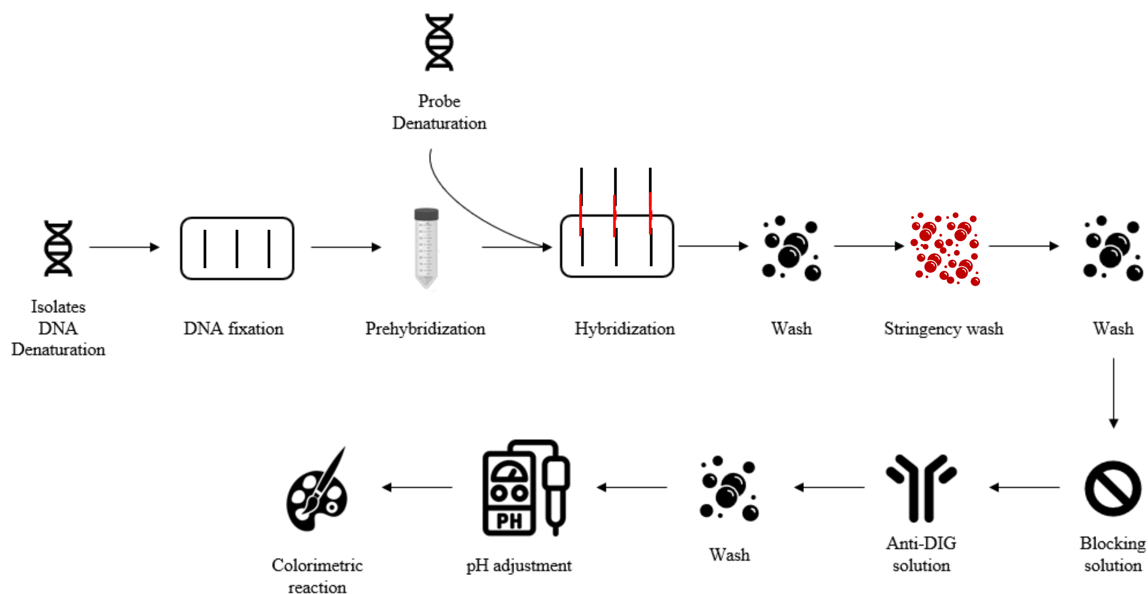



Figure 2.2: Schematic representation of the hybridization procedure.

### 2.7. C and N isotopic analysis

For this analysis, the microorganisms were grown in NFB medium and the lyophilized cultures of the 40 representative isolates were prepared.

The technique used for this analysis was isotope-ratio mass spectrometry (IRMS) and it was performed to determine the ratio between $^{15}N$ and $^{14}N$ ($\delta^{15}N$), the percentage of N and C (%N and %C) to calculate the ratio of C and N (C/N ratio).

This technique was a contract service performed by the Stable Isotopes and Instrumental Analysis Facility (SIIAF) - Plant Biology Department (Faculty of Sciences, University of Lisbon).

### 2.8. Multifunctionality assay

For the multifunctionality assay, three processes were studied: ammonification, nitrification, and denitrification. Urea, $NH_4Cl$ and $NaNO_3$, respectively, were used as substrates for these reactions and measurements of $NH_4^+$ were taken to assess ammonification while measurements of $NO_2^-$ were taken to assess both nitrification and denitrification.

The media used in these assays were:

- Ammonification - NFB supplemented with Urea at 2% (w/v) (Christensen, 1946);
- Nitrification - NFB supplemented with $NH_4Cl$ at 40 mM (Hartmann, Prabhu and Galinski, 1991);
- Denitrification - NFB supplemented with $NaNO_3$ at 0.008% (w/v) (Willis, Chuang and Burford, 2016).

A pre-inoculum for each of the 40 isolates grown in N free medium was performed in falcon tubes with 11 mL of each of the media described above and incubated for a week at 28ºC.

The falcon tubes were centrifuged at 4000 *x g* for 15 min in a centrifuge 5810R (Eppendorf, Germany), the medium was renewed and the cultures were incubated a secound time in the same conditions; 1 mL aliquotes of each assay were collected in microtubes, for the 18, 24 and 48 h timepoints.

In the case of denitrification, anaerobiosis flasks with 5 mL of NFB supplemented with $NaNO_3$ were prepared and the medium was degassed and autoclaved. After this, the pre-inocula resuspended in 1 mL of 1x PBS were used to inoculate the flasks with 200 µL. After 72 h of growth at 28ºC, an aliquot of 1 mL was collected in a microtube.

The collected aliquots were centrifuged at 10 000 *x g* for 10 min in a Centrifuge 1-15P (Sigma, USA) and the supernatant was collected and stored at 4ºC.

$NH_4^+$ and $NO_2^-$ measurements were performed in 96-well microplates using triplicates for all measurements.

Calibration curves were done in triplicates, using standard solutions of $NaNO_2$ and $NH_4Cl$, with dilutions up to 10 mg/L for $NaNO_2$; and up to 100 mg/L for $NH_4Cl$. Each curve was tested for linearity and slope significance of the regression (Appendix E).

In each microplate, a sample of known concentration from the standard solutions was added to the microplate, to ensure that its estimated concentration was within the boundaries of the confidence interval determined for the inverse prediction, calculated using the calibration curve.

Ammonium quantification followed the Berthelot reaction (Hood-Nowotny *et al.*, 2010): 50 µL of 5% sodium citrate solution (pH 7) were added to 50 µL of each sample and incubated for 1 min at room temperature; after that 50 µL of a freshly prepared solution of PPS-nitroprusside (2-phenylphenol nitroprusside) were added, followed by the addition of 25 µL of hypochlorite buffer (pH 13) and 100 µL of RO-water. The microplate was incubated in the dark at room temperature for 45 min, after which the absorbance was read at 665 nm in a spectrophotometric microplate reader (Tecan SPECTRA Rainbow, Switzerland).

For the nitrite quantification, the Griess method (Hood-Nowotny *et al.*, 2010) was used, where 100 µL of each sample were added to the microplate followed by 50 µL of sulphanilamide solution (Griess Reagent I), 50 µL of N-(1-naphthyl)-ethylenediamine solution (Griess Reagent II) and 100 µL of RO-water. The microplate absorbance was read at 520 nm in a spectrophotometric microplate reader (Zenyth 3100, Anthos).

## 2.9. Metabolic profiling

The metabolic profiling of these isolates was performed with the utilization of EcoPlates™ from Biolog. These plates contain 31 different substrates and their distribution in the plate is shown in **Figure 2.3**.

| A1 Water | A2 β-Methyl-D-Glucoside | A3 D-Galactonic Acid γ-Lactone | A4 L-Arginine | A5 Water | A6 β-Methyl-D-Glucoside | A7 D-Galactonic Acid γ-Lactone | A8 L-Arginine | A9 Water | A10 β-Methyl-D-Glucoside | A11 D-Galactonic Acid γ-Lactone | A12 L-Arginine |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B1 Pyruvic Acid Methyl Ester | B2 D-Xylose | B3 D-Galacturonic Acid | B4 L-Asparagine | B5 Pyruvic Acid Methyl Ester | B6 D-Xylose | B7 D-Galacturonic Acid | B8 L-Asparagine | B9 Pyruvic Acid Methyl Ester | B10 D-Xylose | B11 D-Galacturonic Acid | B12 L-Asparagine |
| C1 Tween 40 | C2 i-Erythritol | C3 2-Hydroxy Benzoic Acid | C4 L-Phenylalanine | C5 Tween 40 | C6 i-Erythritol | C7 2-Hydroxy Benzoic Acid | C8 L-Phenylalanine | C9 Tween 40 | C10 i-Erythritol | C11 2-Hydroxy Benzoic Acid | C12 L-Phenylalanine |
| D1 Tween 80 | D2 D-Mannitol | D3 4-Hydroxy Benzoic Acid | D4 L-Serine | D5 Tween 80 | D6 D-Mannitol | D7 4-Hydroxy Benzoic Acid | D8 L-Serine | D9 Tween 80 | D10 D-Mannitol | D11 4-Hydroxy Benzoic Acid | D12 L-Serine |
| E1 α-Cyclodextrin | E2 N-Acetyl-D-Glucosamine | E3 γ-Amino Butyric Acid | E4 L-Threonine | E5 α-Cyclodextrin | E6 N-Acetyl-D-Glucosamine | E7 γ-Amino Butyric Acid | E8 L-Threonine | E9 α-Cyclodextrin | E10 N-Acetyl-D-Glucosamine | E11 γ-Amino Butyric Acid | E12 L-Threonine |
| F1 Glycogen | F2 D-Glucosaminic Acid | F3 Itaconic Acid | F4 Glycyl-L-Glutamic Acid | F5 Glycogen | F6 D-Glucosaminic Acid | F7 Itaconic Acid | F8 Glycyl-L-Glutamic Acid | F9 Glycogen | F10 D-Glucosaminic Acid | F11 Itaconic Acid | F12 Glycyl-L-Glutamic Acid |
| G1 D-Cellobiose | G2 Glucose-1-Phosphate | G3 α-Keto Butyric Acid | G4 Phenylethyl-amine | G5 D-Cellobiose | G6 Glucose-1-Phosphate | G7 α-Keto Butyric Acid | G8 Phenylethyl-amine | G9 D-Cellobiose | G10 Glucose-1-Phosphate | G11 α-Keto Butyric Acid | G12 Phenylethyl-amine |
| H1 α-D-Lactose | H2 D,L-α-Glycerol Phosphate | H3 D-Malic Acid | H4 Putrescine | H5 α-D-Lactose | H6 D,L-α-Glycerol Phosphate | H7 D-Malic Acid | H8 Putrescine | H9 α-D-Lactose | H10 D,L-α-Glycerol Phosphate | H11 D-Malic Acid | H12 Putrescine |

Figure 2.3: Scheme of the carbon sources in the Ecoplates from the Ecoplates manual (https://www.biolog.com/products-portfolio-overview/microbial-community-analysis-with-ecoplates/)

Suspensions equivalent to 5 McFarland Standard were prepared for each of the 20 isolates, grown in NFB, in 1x PBS. Each plate was inoculated with 100 µl of these suspensions, incubated at

25ºC in the dark and read at 595 nm at the 0, 3, 19, 24, 43, 48, 67, 72, 91, 96, 120, 144, 168, 192, 216 and 240 h timepoints in a spectrophotometric microplate reader (Zenyth 3100, Anthos).

In order to analyze the isolates growth with different carbon sources, the net area under curve (NAUC) was calculated for each growth curve. For this analysis, each curve is transformed in a value that corresponds to the area between the curve and the x-axis - area under curve (AUC) - with the subtraction of the value corresponding to AUC for the negative control.

### 2.10. Integrative data analysis

For the results obtained from the multifunctionality, the isotope characterization and the metabolic plasticity assays, a Principal Component Analysis (PCA) was performed. The data matrix, characteristics vs OTUs, was imported to the NTSYSpc software (version 2.20d; Exeter Software, Setauket, NY, USA).

# 3. RESULTS AND DISCUSSION

The present work's aim was to isolate diazotrophic bacteria, from rhizospheric soil subjected to four different N treatments, and characterize them in terms of the structural and functional diversity of the communities, using this as an ecological marker for comparing different treatments.

The enrichment and isolation strategy selected, using semi-solid medium and N-free media, worked as seen by the formation of the film in the medium and the subsequent growth on agar plates. Every colony picked off from mannitol agar grew in NFB which demonstrates that inhibition of growth by malic acid did not occur for these microorganisms. The microorganisms obtained from these isolations made up a collection with 232 isolates from the four soil treatments. These isolates were characterized as shown below.

## 3.1. Morphophysiological and genotypic analysis

With the results obtained from the morphophysiological characterization of the isolates, such as microscopic observation of cell morphology, Gram staining, catalase and oxidase tests, and endospore staining, the isolates were grouped into morphophysiological types according to the flowchart presented in **Figure 3.1**.

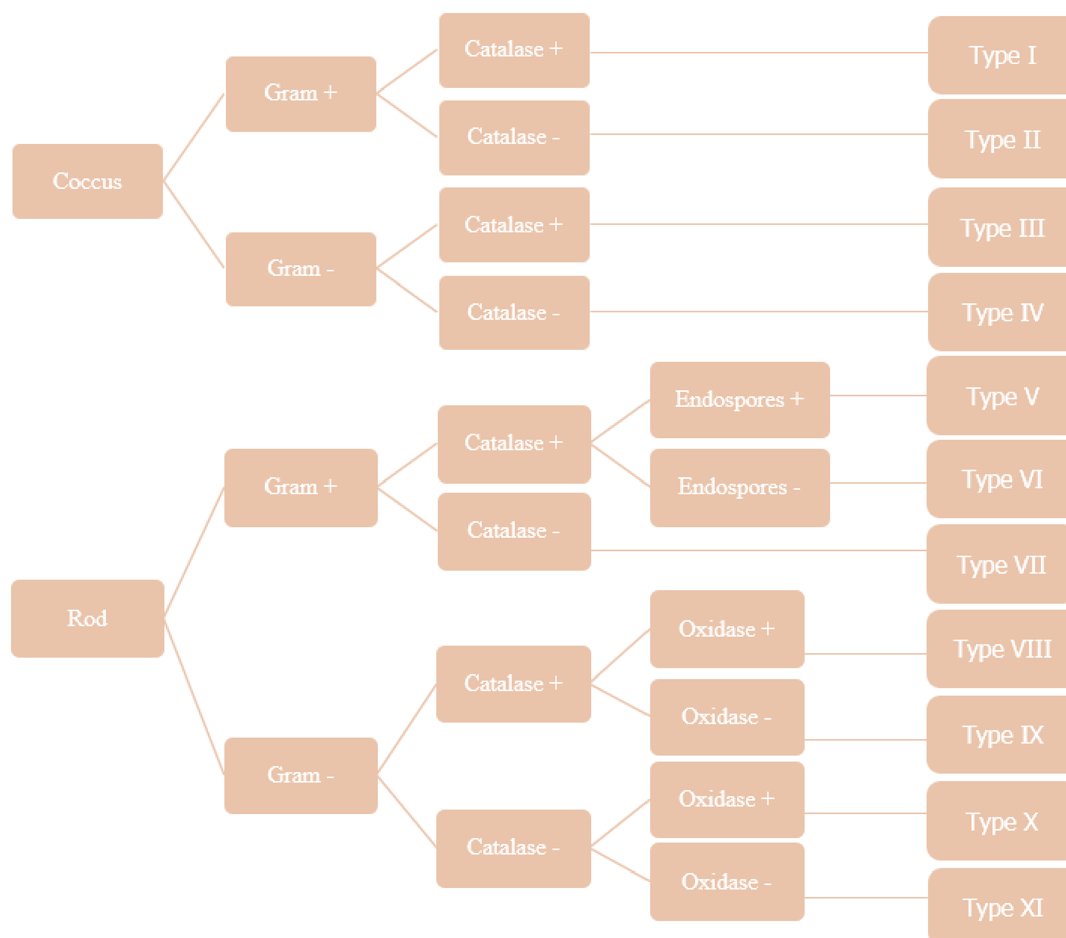



Figure 3.1: Flowchart used for differentiation of the isolates into different morphophysiological types using cell morphology, Gram staining and catalase and oxidase tests and endospore-forming ability.

The distribution of these morphotypes for each treatment was observed, as shown in **Figure 3.2**.

The diversity of the isolates was evaluated with the dendrograms presented in Appendix A, one dendrogram for the isolates of each treatment. The maximum similarity found between the isolates in each dendrogram was 17.2% for the control treatment, 16.3% for treatment 40A, 19.7% for treatment 40AN and 22.3% for treatment 80AN. These similarities show the high diversity of the isolates which was expected due to the nature of the matrix of isolation - rhizospheric soil - and the group of isolates for which the isolation was directed - diazotrophs.

Figure 3.2: Graphic representation of the total number of isolates per treatment, the distribution of these isolates in the morphophysiological groups and the phenotypic and genotypic diversity indices - Shannon-Wiener and Simpson - of each treatment.

In **Figure 3.2**, the total number of isolates for each treatment is represented, as well as the distribution of these isolates among the types defined. It is possible to observe that the total number of isolates is higher in the treatment 40A followed by 80AN, Control and at last the 40AN treatment.

The diversity indices for these isolates, presented in this figure, were calculated by two methods, Shannon-Wiener and Simpson. The phenotypic indices were calculated based on the morphophysiological groups and the genotypic indices were calculated based on PCR fingerprinting using only one primer (M13).

The majority of characterized isolates had a rod-shaped morphology, and mainly a Gram-negative type wall. This goes according to what was expected, since, despite their wide distribution, the most common groups of diazotrophs found in soils are members of the phylum Proteobacteria, which share this morphology. Gram positive rod-shaped diazotrophs found in soil are usually associated with *Bacillus* spp. and *Frankia* spp.

In control treatment, the most relevant types are VI, VII, VIII, IX, and X. In treatment 40A, the most relevant types are VI, VIII, and X. In treatment 40AN, the most relevant types are V, VII and VIII. In treatment 80AN, the most relevant types are VI, VIII, IX, and X. All the relevant groups are formed by Gram-negative bacteria.

In type V, the genus of interest *Bacillus,* which is a spore-forming genus, can be found. Some isolates belonging to this type could have been misplaced in type VI, if it was not possible to observe the endospores (Thwaite and Atkins, 2012).

In type VI genera of interest like *Frankia* and *Streptomyces,* among others, can be found (Rosenberg *et al.*, 2014b) .

Type VII is characteristic of lactic acid bacteria and one of the genera that can be found is *Lactobacillus* (Rosenberg *et al.*, 2014a).

Type VIII, which is the most represented one in all the plots, contains most of the phylum Proteobacteria as well as some of the phylum Cyanobacteria. In these phyla, there are many genera known as diazotrophic, such as *Azotobacter, Azospirillum, Herbaspirillum, Rhizobium, Nostoc,* and *Anabaena* (Kersters *et al.*, 2006; Whitton and Potts, 2012).

Type IX is characteristic of the family Enterobacteriaceae, which contains genera of interest, such as *Enterobacter* and *Citrobacter* (Line, 1990).

In this research, the diversity studied is limited to the soil from *Ulex densus* rhizosphere and to diazotrophic bacteria that can be cultivated under aerobic conditions on media without N, so only a small percentage of the actual soil diversity is represented here. To assess the total diversity associated with these plots, a different approach should be attempted, such as microbial profiling or metagenomics studies to complement the cultivation approach.

The soil samples from which the isolates were obtained, were collected from the rhizospheric soil of *Ulex densus* which is in the category of evergreen sclerophylls species. The evergreen sclerophylls species have a high water use efficiency and a higher tolerance to certain stress conditions; this may provide advantages to these species to proliferate in soil with increased N fertilization (Correia and Catarino, 1994; Canadell *et al.*, 1996; Dias, Neto, *et al.*, 2011).

In previous studies in these soils, it was shown that the proportion of evergreen sclerophylls to summer semi-deciduous - another prevalent group of plants in these soils - increased under higher N dose (Dias *et al.*, 2013).

Since the microorganisms isolated in this work were from the rhizosphere of *Ulex densus,* the results obtained for the total number of isolates per treatment are concordant to results for the plant richness for each treatment (Dias *et al.*, 2014).

The data suggest that the microbial community is driven by the amount of $NH_4^+$, since the treatments with more $NH_4^+$ (40A and 80AN) present a higher total number of isolates. This N form is of special importance in Mediterranean ecosystems due to the extreme limitation of N. Similar results were obtained for the plants of this study as presented in Dias *et al.* (2014).

The results between the types of diversity indices, genotypic and phenotypic, are quite distinct. The phenotypic indices present lower values than the genotypic indices, which can be due to isolates that present similar results in morphologic charcterization and for that are placed in the same type, however, the genotypic profiles of these isolates are different.

In the phenotypic indices, the treatments present differences: treatment 40A shows a lower diversity index value than the other treatments due to the number of isolates that are presented in the type VIII of this treatment.

The genotypic diversity indices presented high diversity values as well, yet no differences between the treatments were found. It is necessary to consider that these indices were calculated based on PCR fingerprinting using only one primer (M13), which may not have sufficient discriminatory power between distinct isolates. For a more accurate estimation of the diversity found, further analysis using complementary PCR fingerprinting technique should be performed.

Diversity index results show that even though the genotypic characterization perfomed did not allow accurate distinction between all isolates, it is still more discriminatory than phenotypic characterization and so, it is essential to complement phenotypic results with the genotypic profile from PCR fingerprinting.

## 3.2. Genera associated with the diazotrophic community

With the dendrograms constructed for each treatment based on the fingerprinting profiles of the isolates (Appendix A), 10 representatives of each treatment were selected to be identified at the genus level through 16S rRNA gene partial sequencing (Appendix B).

The dendrograms with the representative isolates per treatment (Appendix B) were considered a good representation of the isolates diversity, since the similarity between isolates from the same treatment was not higher than 59.3%.

For the identification of isolates through 16S rRNA partial gene sequencing the results obtained are presented in the Appendix C. A query cover of at least 90% as well as a percentage of identity of at least 95% were used as minimum parameters for genus determination. Isolate 3 was an exception due to the reduced size of the nucleotidic sequence recovered and the low query cover percentage. The values for this isolate are lower and only identification at order level was possible. The results of these identifications are presented in **Figure 3.3**.

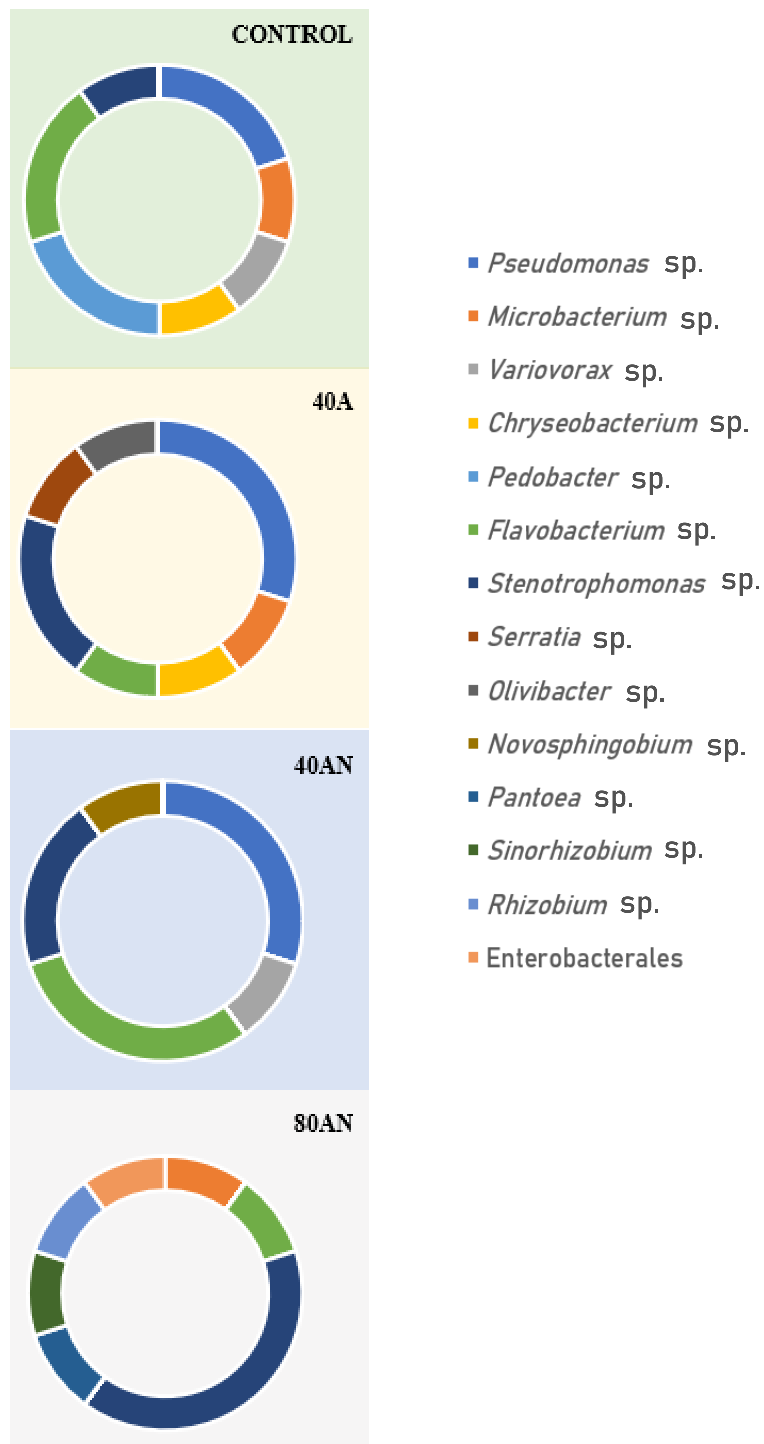



Figure 3.3: Graphic representation of genus level isolate identification by 16S rRNA gene sequencing, and the distribution of these genera per treatment.

Some of the genera identified are common to all treatments, such as *Flavobacterium* and *Stenotrophomonas*, however, there are some genera that were only identified in certain treatments. The control treatment is the only treatment where the genus *Pedobacter* is present. The *Serratia* and *Olivibacter* genera only appear in treatment 40A, the genus *Novosphigobium* was only detected in treatment 40AN and the *Pantoea, Sinorhizobium*, and *Rhizobium* genera are only present in treatment 80AN.

The treatments differ in diversity at the genus level; treatment 40AN shows a lower number of different genera associated with it. This was also the treatment with the lower total number of isolates and this treatment presents the lowest value of $NH_4^+$ input of all fertilized plots (Dias *et al.*, 2014).

Most of the genera that were found are associated with N fixation or at least one species in the genus has the ability to perform biological N fixation (Ruppel, 1991; Loiret *et al.*, 2004; Shabayev, 2010; Galardini *et al.*, 2011; Han *et al.*, 2011; Lin *et al.*, 2012; Smit *et al.*, 2012; Cerezer *et al.*, 2014; Glaeser *et al.*, 2015; Harsha Shelat and Panpatte, 2017; Poole, Ramachandran and Terpolilli, 2018).

All the genera are commonly found in soils and are aerobic or facultative anaerobic bacteria (Spain, Krumholz and Elshahed, 2009; Schrempf, 2013; Wolińska *et al.*, 2017). The phyla that include the found genera are not only commonly found in soil, but are actually the most prevalent phyla in the soil microbial community (Fierer, Bradford and Jackson, 2007; Hillel, 2008; Siles *et al.*, 2014).

## 3.3. Nitrogen fixation ability

All the isolates grew in N free media which indicates that they are able to uptake N from the atmosphere and use it in their metabolism.

### 3.3.1. *nifH* gene search

Since the *nifH* gene is present in every diazotroph, a search for this gene was performed. The choice of primer sets used for this purpose was based on the broad range for nitrogenase clusters of interest. The nitrogenase clusters that were important to be targeted by the primers were the clusters of aerobic diazotrophs and of great importance in the soil, such as Proteobacteria, Cyanobacteria, *Frankia* and *Paenibacillus* as presented in **Table 2.1**.

The first approach was the search for the *nifH* gene by PCR. For this approach, three sets of primers were used, and optimization attempts were made. This approach did produce a single band with the right molecular weight for the positive control and no bands in the negative control, however, none of the isolates presented the band of interest or any other bands.

The stringency conditions were lowered, by the decreasing the annealing temperature, until the negative control presented an unspecific band. In all these attempts it was not possible to detect the gene of interest, which does not mean the isolates are not diazotrophs. This may be due to high diversity of the isolates which has been evidentiated in the dendrograms of the Appendix A, and sequence may be too distinct from the other *nifH* sequences used to design the primers.

To surpass this issue, the dot-blot hybridization technique was used. This technique has the advantage that the probe used, in this case a 395 bp probe, is longer than the primer and, even if the probe only has partial homology it still can attach and be detected.

With the dot-blot hybridization it was possible to identify seven isolates as diazotrophs, one isolated from the control treatment, two from the 40A treatment, two from the 40AN treatment and the last two from the 80AN treatment. These isolates pertain to the genera *Variovorax* (two isolates), *Microbacterium, Serratia, Novosphingobium, Stenotrophomonas,* and *Rhizobium,* based on 16S rRNA gene sequencing (**Figure 3.3**), these are genera described as presenting species with the ability to perform N fixation.

For the remaining isolates, it was not possible to confirm this, which does not permit to infer that they are not diazotrophs. One of the reasons for this is the fact that *nifH* is a single copy gene and for those, the proportion of DIG-11-dUTP and dTTP in the reaction must be higher so that there can be more incorporation of DIG label into the probe. A low proportion can cause low efficiency of the colorimetric reaction and the visualization of the hybridization may not be achieved.

Although the representative isolates were identified as belonging to genera associated with the ability to perform N fixation and the sets of primers chosen are able to amplify the *nifH* gene of the clusters were these genera are present, it was not possible to amplify and/or detect the *nifH* gene for the majority of the isolates. This could mean that the *nifH* gene of these isolates is different from the sequences used in the primer design.

### 3.3.2. N isotopic analysis

For this assay the isolates were grown in liquid N free medium and were able to accumulate biomass, which corroborates the isolation strategy. However, two isolates out of the 40 representative isolates - isolates 271 and 285 - did not create enough biomass to perform the analysis. This could be due to a slower metabolism or the liquid conditions may not be propitious to their growth.

In this analysis, the ratio of $^{15}N$ to $^{14}N$ ($\delta^{15}N$) was determined for the representative isolates. In enzymatic processes, the two isotopes can be incorporated; some enzymes prefer $^{14}N$ because it is the lightest form, others do not have a preference. This way, this ratio can be used to follow the transformation of N through the different processes of N conversion, since different enzymes have different affinities for $^{15}N$.

N fixation is the only process associated with N transformations in which the $\delta^{15}N$ is null, since nitrogenase is an enzyme that does not discriminate between the isotopes *in vivo*, which is due to the high energetic cost of N fixation for the microorganisms. Studies of the symbiotic relationship of diazotrophs with plants have shown values of $\delta^{15}N$ are approximately null.

Although N fixation does not discriminate between isotopes, this is not the only process involved in nitrogen metabolism. The uptake of $NO_3^-$ can discriminate slightly, however the most discriminatory processes are the conversion of $NO_3^-$ to $NO_2^-$ and the conversion of $NH_4^+$ into organic N. The enzymes involved in these processes, nitrate reductase and glutamine synthetase, can present values of $\delta^{15}N$ of 15‰ and 17‰, respectively (Robinson, 2001; Needoba, Sigman and Harrison, 2004; Craine *et al.*, 2015).

The results from the IRMS for the $\delta^{15}N$ of the isolates per treatment are presented in **Figure 3.4**. The values for $\delta^{15}N$ are quite variable within and between treatments, however, all the isolates present positive values for $\delta^{15}N$, which indicates that discrimination has occurred.

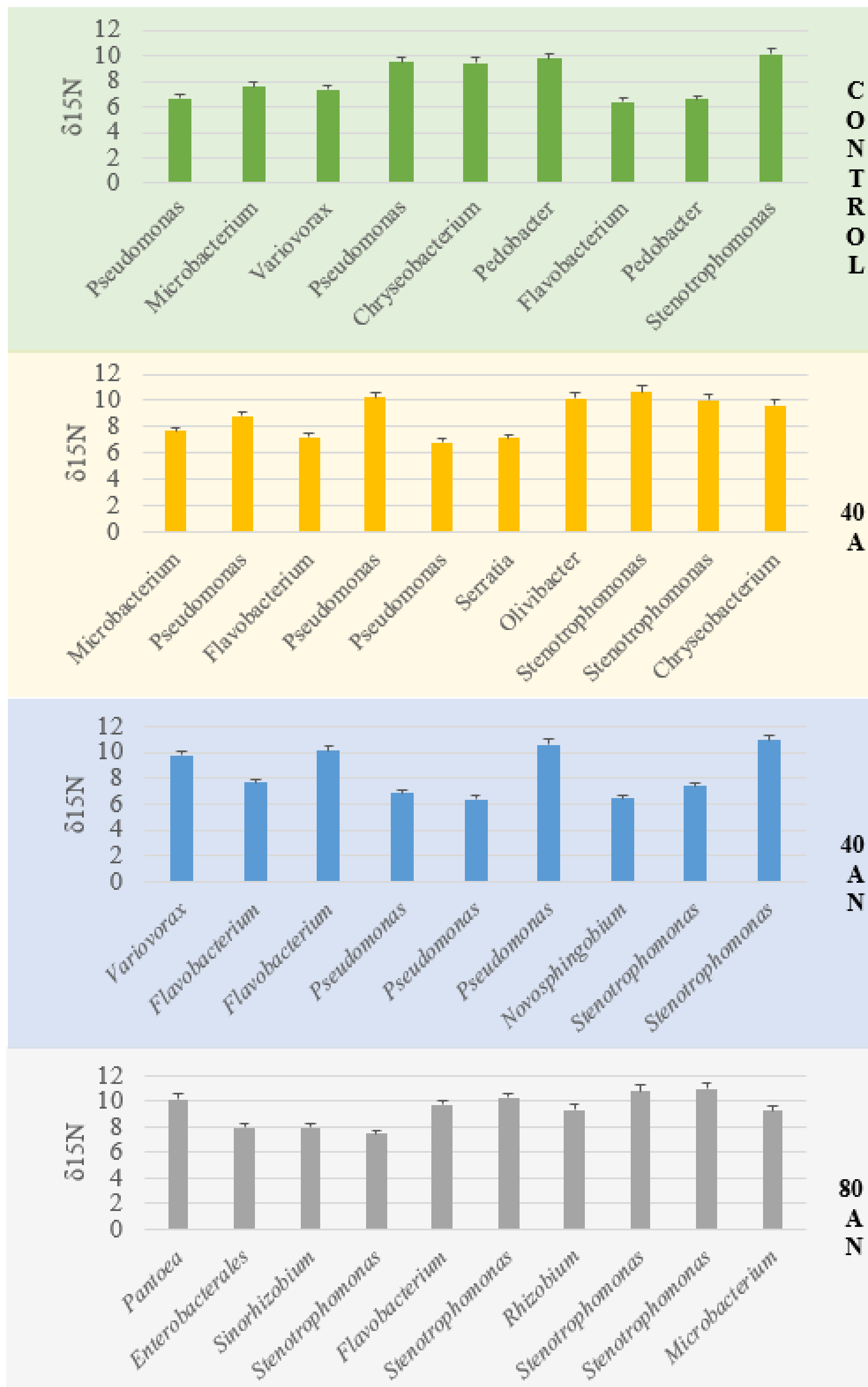



Figure 3.4: Representation of the results from IRMS for the $\delta^{15}N$ for the representative isolates per treatment. The error bars represent the methodological coefficient of variation. The numbers presented in the graphic are the isolates identification number.

The results presented in **Figure 3.4** support the previous information that these isolates are able to perform N fixation, since a positive value of $\delta^{15}N$ is shown. A positive value for this ratio means that an enrichment in $^{15}N$ has occurred in the interior of the cell. Since these isolates were grown in N free medium, a release of N to the medium must have occurred. In this process the lightest form, $^{14}N$, was the one to be released to the medium, because the membrane transporters do discriminate between the two forms and have a preference for $^{14}N$. For N to be released to the medium, the concentration of N in the cell must increase and in this circumstances N fixation must have happened (Coleman, 1991; Mary, Mariotti and Morel, 1992).

The differences between the isolates for the $\delta^{15}N$ values, may be due to distinct metabolism; some may have produced more N than others, which led to higher release rates, increasing the $\delta^{15}N$, while others may have higher N needs for various biosynthesis purposes (e.g.: cell wall, proteins or nucleic acid synthesis).

Acetylene reduction assays (ARA) were considered for detection of nitrogenase activity, however, due to the bias and the lack of reliability in the results that are presented by this assay, this technique was not used (Witty and Minchin, 1988).

## 3.4. The ratio of C and N (C/N)

The results from the IRMS for the ratio C/N of the isolates per treatment are presented in **Figure 3.5**.

The use of elemental ratios is fundamental for the prediction of the nutrient retention and biomass production from cellular level to ecosystem scale. Microorganisms can be described in terms of elemental composition such as C and N (Sinsabaugh, Hill and Follstad Shah, 2009).

The control treatment and treatment 40A appear to have a lower variation for the ratio C/N than the treatments 40AN and 80AN. The higher values obtained are from isolates in the treatments with addition of N in the form of NO3-, 40AN and 80AN.

A compilation of data from various studies concluded that the mean C/N ratio of microbial biomass was 5.3 (Bleam, 2017).

For the control treatment the mean value of C/N ratio is 3.438, being the minimum value 3.116 and the maximum 3.779. The treatment 40A presents a mean value of 3.495, with minimum value of 3.233 and maximum of 3.726. The treatment 40AN has a mean value of 3.554, being the minimum value 3.202 and the maximum 4.503. For last, the treatment 80AN presents a minimum value of 3.573 with minimum value of 3.157 and maximum of 4.515.

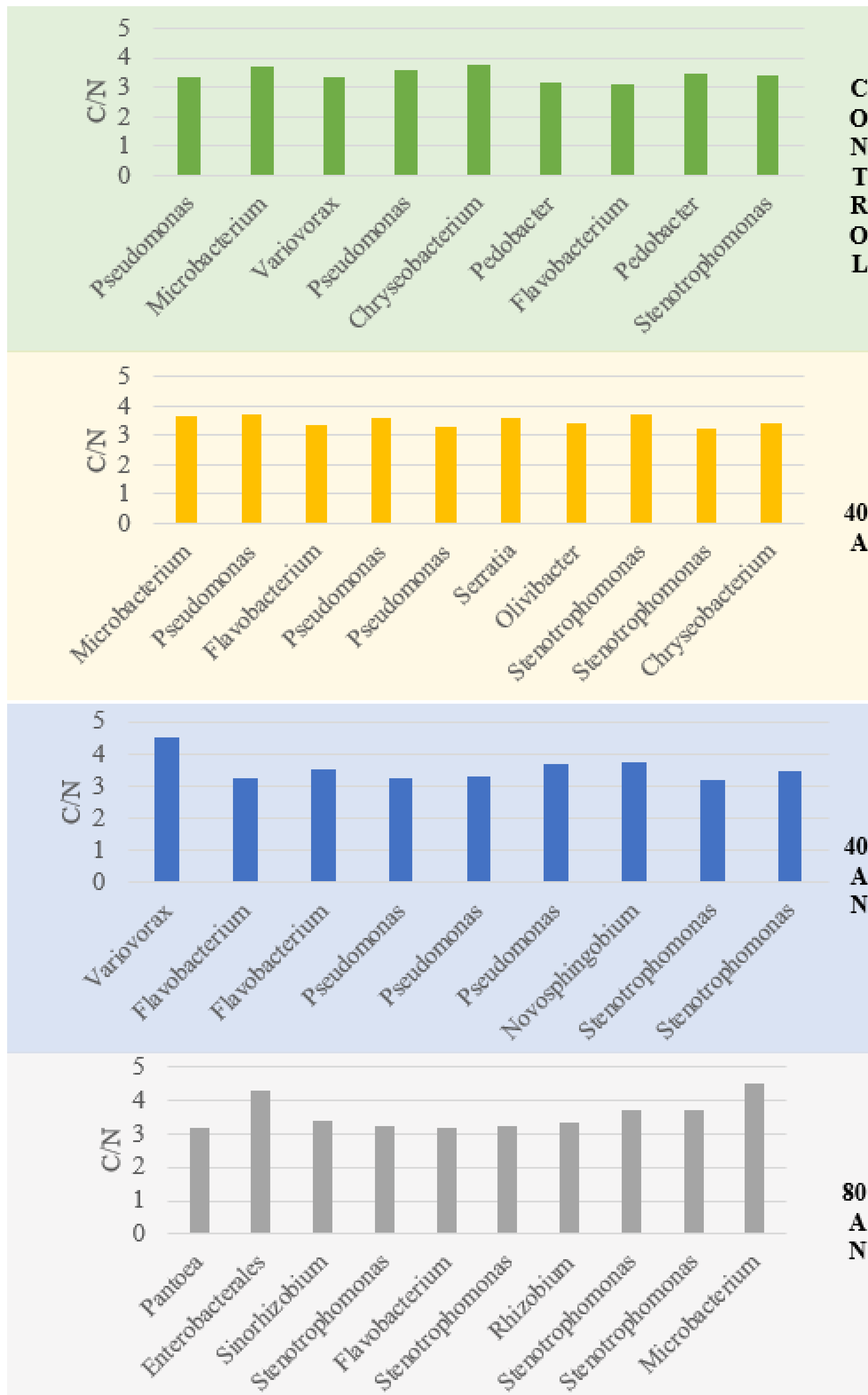



Figure 3.5: Representation of the results from IRMS for the C/N or the representative isolates for each treatment.

The mean value of C/N ratio is similar in all treatments, although it increases from the control to the 80AN treatment. Previous studies demonstrate that the N fertilization can reduce the C/N ratio of total microbial biomass (Aanderud *et al*., 2018).

The major difference is in the maximum values, which increase in the treatments 40AN and 80AN, associated with the genera *Variovorax* and *Microbacterium*. Although the maximum values are different, further studies with biological replicates to allow a statistical analysis are necessary to determine if these differences are significant.

Based on the results from this analysis, a correlation between the treatments and the differences in the C/N ratio does not appear to exist.

### 3.5. Multifunctionality in the N cycle

Multifunctionality is extremely important for microorganisms' adaptation to the environment. The diazotrophic isolates were tested for different processes of the N cycle: ammonification, nitrification and denitrification (**Figure 3.6**).

In the multifunctionality assays the positive results were verified by the use of internal controls, corresponding to aliquots of the media, used in each assay, incubated in the same conditions and taken at the same timepoints to control the abiotic degradation of the substrates as well as the abiotic production of the products.

It must be considered that the concentration of the substrates used in the denitrification and nitrification, $NH_4^+$ and $NO_3^-$, was the same for all isolates so this concentration may not be enough for some isolates or even be toxic to others, which could result in false negatives.

As only heterotrophic N fixers were isolated, only heterotrophic nitrification could be detected. From the 40 representative isolates tested only three presented potential heterotrophic nitrification. It is possible that some of the negative responses are false negatives because heterotrophic nitrification is a slow process and the reaction may not have been detected within the timeframe of the assay. As some of the N fixers that have the potential for heterotrophic nitrification are also able to perform denitrification, the detection of the nitrification potential will only be detected if the denitrification is not occurring simultaneously, or it is occurring at much smaller rates.

In the denitrification assay, false negatives can be present, since only the production of nitrite was measured, and if complete denitrification was underway the process would not be detected. Given that $NO_3^-$ was the only substrate used, in the case of the bacteria that were not capable of undergoing the first step in the process, denitrification could not be detected, because it would be necessary to provide $NO_2^-$ as a substrate. For the ammonification assay, false negatives can result from the fact that $NH_4^+$ produced may be uptaken by the cells and used in a biological process and as such cannot be measured.

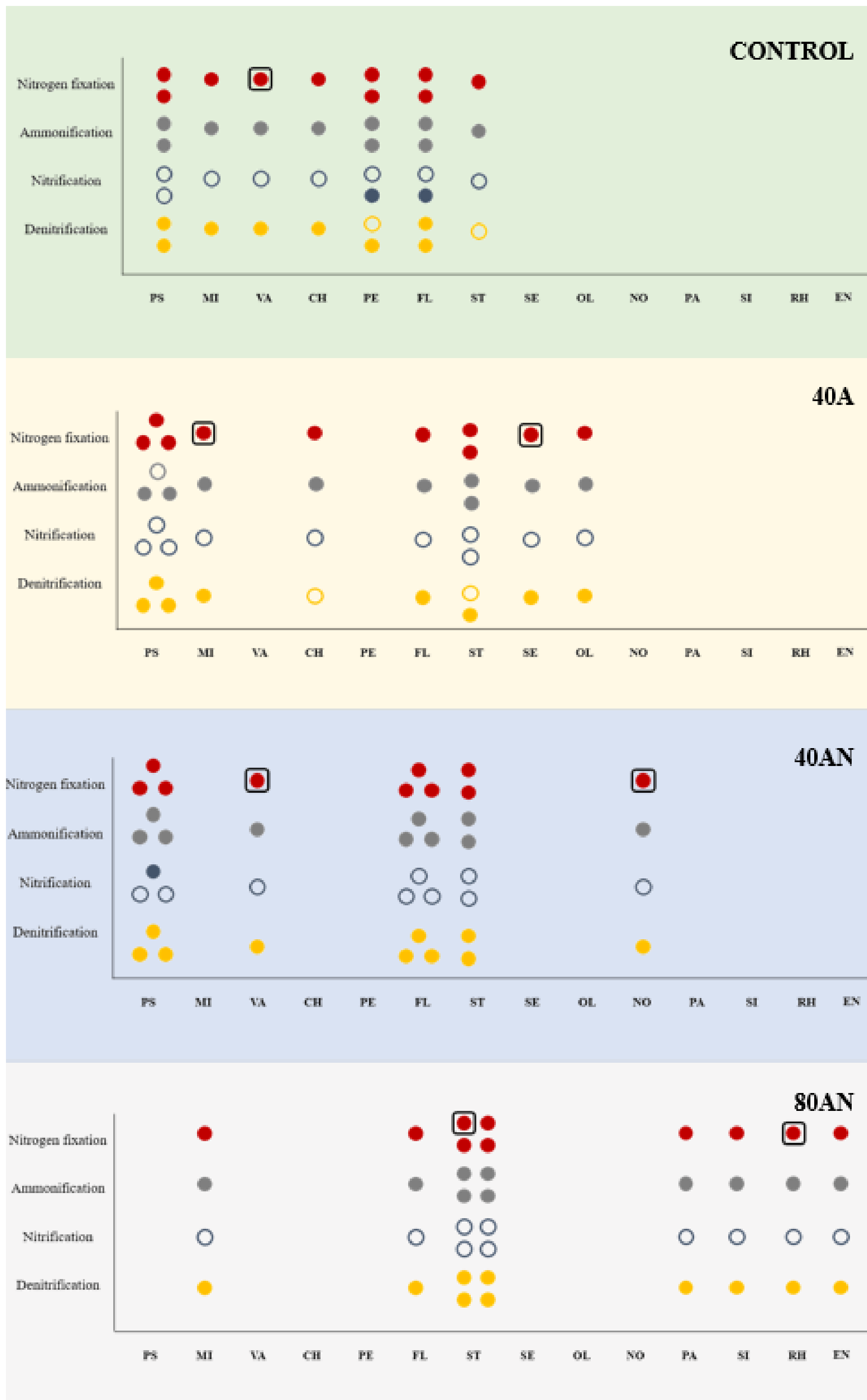



Figure 3.6: Graphic summarizing the multifunctionality data. Filled circles illustrate positive results and unfilled circles refer to negative results. In the N fixation the circles with the square frame are the isolates for which nifH detection was possible. The number of isolates per treatment can be seen per line. PS - *Pseudomonas*, MI - *Microbacterium*, VA - *Variovorax*, CH - *Chryseobacterium*, PE - *Pedobacter*, FL - *Flavobacterium*, ST - *Stenotrophomonas*, SE - *Serratia*, OL - *Olivibacter*, NO - *Novosphingobium*, PA - *Pantoea*, SI - *Sinorhizobium*, RH - *Rhizobium*, EN - Enterobacterales.

The three isolates (two from the control and one from the 40AN treatment) that presented the ability to convert $NH_4^+$ into $NO_2^-$, based on the 16S rRNA gene sequencing (**Figure 3.3**) belong to the *Pseudomonas, Pedobacter, and Flavobacterium* genera. For these isolates heterotrophic nitrification potential has been described (Prosser, 2005; Stein, 2011; Trung Tran *et al.*, 2019). Heterotrophic nitrification is a process described as being associated with stress conditions, such as low soil pH and high C/N ratio. In the case of this assay, soil pH and C/N ratio were very similar in all treatments (**Figure 1.9**), so they cannot be used to explain differential potential of heterotrophic nitrification among treatments. But it is interesting to find isolates with potential for heterotrophic nitrification in well aerated soils with pH above 5, such as those of Arrábida. Based on the obtained results it is possible to argue that the function of heterotrophic nitrification may be associated with low $NH_4^+$ availability conditions, which is in agreement with the high $NH_4^+$ susceptibility found in some heterotrophic nitrifiers (Princic *et al.*, 1998; Svehla *et al.*, 2014).

Denitrification is a largely dispersed process and it is common among bacteria - the results show that out of the 40 representative isolates only four did not present this ability. Two of these isolates belong to the control treatment and the remaining two belong to the 40A treatment. The genera associated with these were *Pedobacter* sp.*, Stenotrophomonas* sp. (two isolates) and *Chryseobacterium* sp.*,* however, other isolates from the same genera presented this ability (Verstraete and Focht, 1977; Lu, Chandran and Stensel, 2014). Thus, the lack of capacity of these four isolates is not genera related, it is probably species related, for which 16S rRNA gene sequencing does not have enough discriminatory power to identify, in some cases.

In the treatments with $NO_3^-$-N added to the soil, 40AN and 80AN, all isolates demonstrate the ability to perform denitrification. Since $NO_3^-$ is a substrate for denitrification, a high concentration of this compound may promote this process, as a way to remove the excessive amounts that can be toxic (Thomas, Lloyd and Boddy, 1994; Wang *et al.*, 2013).

Ammonification is a process commonly found in bacteria, corresponding to the conversion of organic N into $NH_4^+$, which can be used in biological processes. In the ammonification assay, only one isolate did not demonstrate the ability to convert urea into $NH_4^+$. This isolate belongs to the 40A treatment and was identified as *Pseudomonas* sp. (Strock, 2008).

As presented in **Figure 3.6**, the diazotrophs in which it was possible to detect the *nifH* gene are present in all treatments in a similar number and associated with different genera. Most of the isolates presented the ability to perform denitrification as well as ammonification and only a few isolates were able to perform nitrification. The potential for N fixation was not affected by the N fertilization input to the soil, having even increased the number of diazotrophic isolates in the treatments with higher input. In the literature it is suggested that N input negatively affects N fixation and should thus result in lower diversity of diazotrophs (Kox *et al.*, 2016; Zheng *et al.*, 2019).

The *Flavobacterium* and *Stenotrophomonas* genera are present in every treatment which implies that they are not sensitive to N addition, as opposed to the genus *Pedobacter,* which only appears in the control treatment. Based on the results presented, it is suggested that *Pedobacter* presents a sensitivity for high concentration of N, so it may be possible that the absence of this genus is related to the N availability increase. For this reason, this genus may function as a candidate to indicator of this alteration in the ecosystem (Wolińska *et al.*, 2017).

The genus *Chryseobacterium* is only present in the treatments without $NO_3^-$ addition - the control and 40A. The addition of this N form can behave as stress for this genus and inhibit metabolism, since one way to alleviate this stress is denitrification and the isolates of these genus either do not present this ability or present it in very low rates.

Isolates identified as *Pseudomonas* sp. are present in every treatment with the exception of 80AN treatment, which poses the hypothesis that this genus cannot compete in this environment, since low denitrification rates are presented these isolates cannot be able to detoxify efficiently the quantities of $NO_3^-$ that are applied in treatment 80AN.

With the results obtained for the multifunctionality in the N cycle, a schematic representation of the isolates abilities to convert N forms was elaborated and is presented in **Figure 3.7**.

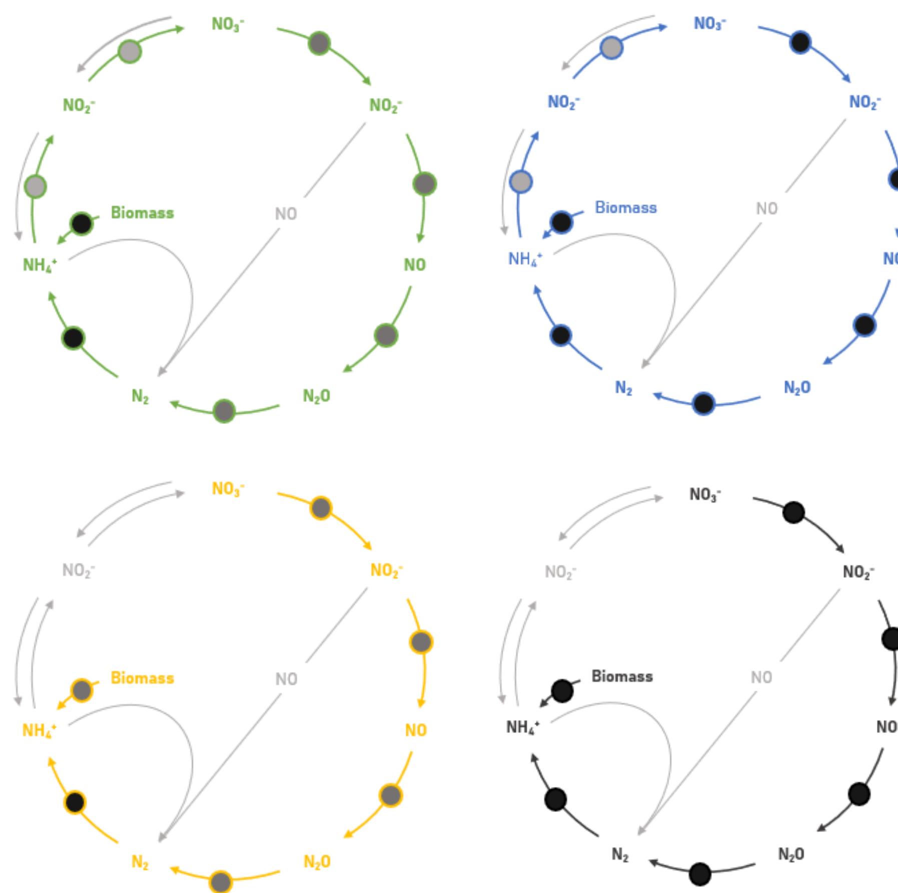



Figure 3.7: Graphic representation of the N cycle and the transformations within. The graphic A represents the process of the N cycle that the isolates of the control treatment are able to perform. The graphics B, C, D are the treatments 40A, 40AN and 80AN, respectively. The darkness of the color inside the circle is related to the number of isolates able to perform the corresponding process.

In an overview of the results presented for the multifunctionality assays, a shift in the diazotrophic community can be observed with the increase of bioavailable N. The genera identified in the control treatment differ from the ones found in the treatment 80AN, with the absence of some genera, such as *Pseudomonas* and the detection of other genera, namely *Rhizobium, Sinorhizobium* and *Pantoea.* For the isotopic characterization results, C/N ratio and %N, some differences were present - the isolates from the treatment 80AN present a higher %N than the ones from the control. These results suggest a higher demand for N from the isolates that only appear in the treatment 80AN, which may explain why it was not possible for them to grow in the other treatments.

Although the results of denitrification are presented as positive or negative to facilitate the representation, the denitrification rate was measured. The rates of denitrification of the isolates from the control treatment and the treatment 80AN were compared and the highest rates for this ability were found in treatment 80AN. Their presence in the treatment with the highest N addition corroborates their ability of detoxifying the system from toxic elements such as $NO_3^-$. The isolates from the control treatment, which do not present high rates of denitrification, may not be able to subsist in this environment.

## 3.6. Metabolic plasticity

Combining the results obtained from the identification of the isolates at genus level and the results from multifunctionality assays, a total of 20 isolates were chosen from different genera, with different multifunctionality patterns (Appendix F).

The substrates present in the Ecoplates were placed in categories to facilitate the visualization of the results and are shown in Appendix H, in the same order by which they are presented in the graphics.

To determine the metabolic plasticity of the selected isolates, the metabolization of 31 different substrates was followed using Ecoplates (Biolog). The values of NAUC calculated for each substrate are shown in **Figure 3.8**. In these figures the pattern of each graphic can be observed, demonstrating the metabolic plasticity of the isolates.

Plasticity of bacteria is linked with morphological and physiological properties and, in different environmental conditions, shifts in these properties can occur, in order to increase the tolerance to environmental stresses. The individuals in a community with high plasticity can accommodate changes in the environment without changes at the community level (Comte and del Giorgio, 2011).

Metabolic plasticity appears to play a part in terms of the pathways by which the microbial communities respond to environmental changes. This property establishes whether the community response involves changes in the community composition or not (Comte and del Giorgio, 2011; Comte, Fauteux and Giorgio, 2013).

Metabolic plasticity appears to be an intrinsic property of the community defined by the composition of the collective genomes, and as such, some communities are more plastic than others. This suggests that different ecosystems with distinct environments may promote the growth of different taxa that can be more specialized in a determined function or opt for a group of microorganisms with wider functions associated (Comte and del Giorgio, 2011).

The pattern presented in the control treatment has less variation between the isolates than the other treatments. This could be because in this treatment the community does not have genera specialized in specific functions, but a homogeneous group with similar functions within the community.

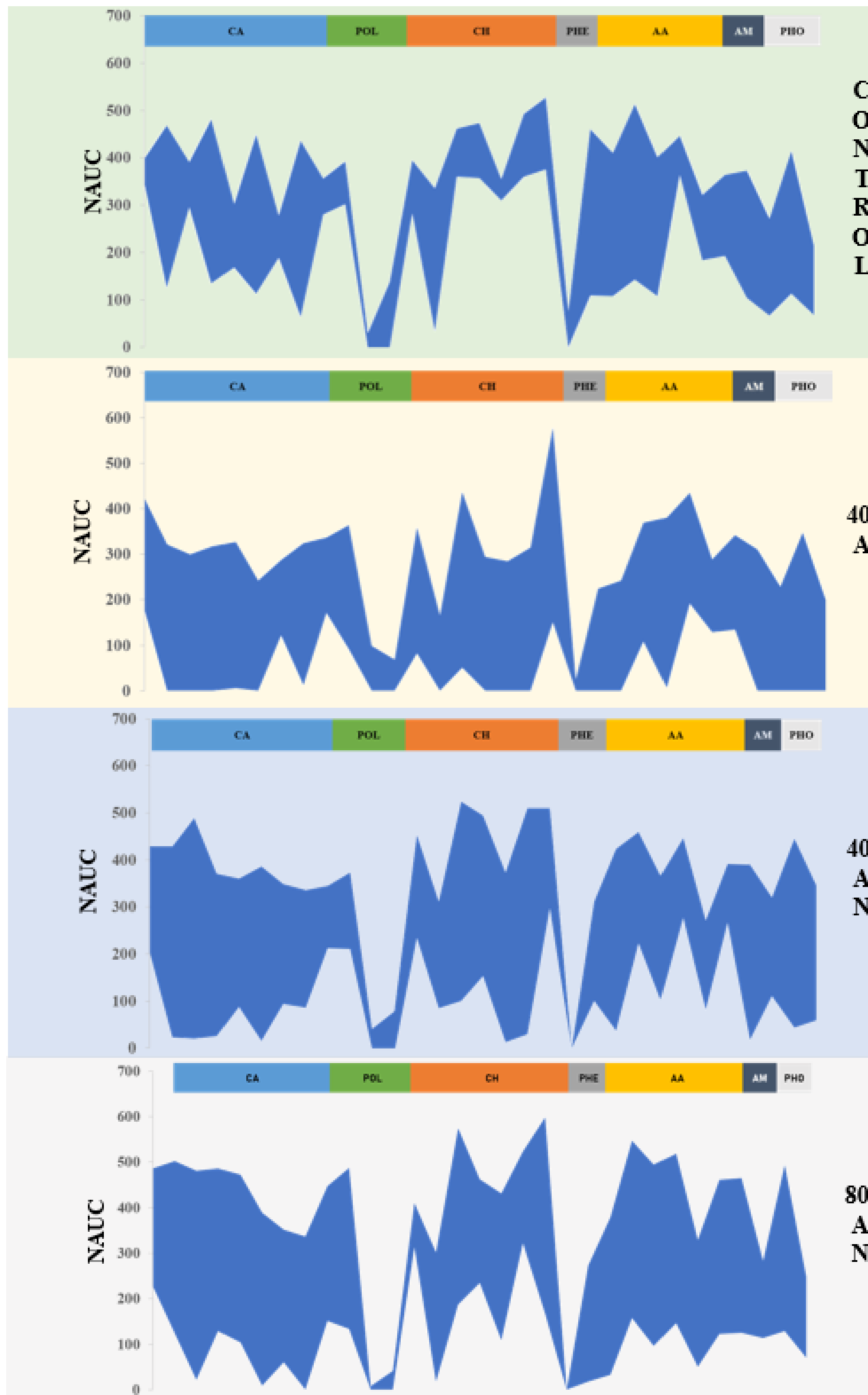



Figure 3.8: NAUC values of the Ecoplate substrates for each treatment. The blue area represents the average ± standard deviation. The colors in the top bar of each graphic correspond to the colors of the substrate category: blue are carboxylic acids, green are polymers, orange are carbohydrates, light grey are phenolic compounds, yellow are amino acids, dark grey are amines and brown are phosphorylated chemicals.

Treatment 40A presents the pattern with lower values which means that the isolates of this treatment have lower metabolic plasticity, however, some isolates present high metabolization for some of the carbon sources. The community from this treatment may be exposed to some stress conditions that lead to a specialization of the individuals in the community, and the isolates with lower values may be involved in other functions that were not considered in this assay.

The isolates from the treatments 40 AN and 80AN present a wide range of values for the carbon sources studied, which suggests that the community in these treatments had the need to specialize in different functions to allow the survival of the group.

The metabolic plasticity of the *Pseudomonas* and *Stenotrophomonas* genera are presented individually because these two genera are represented in different treatments, and a comparison between the same genera in different treatments can be performed. These results are shown in **Figure 3.9**.

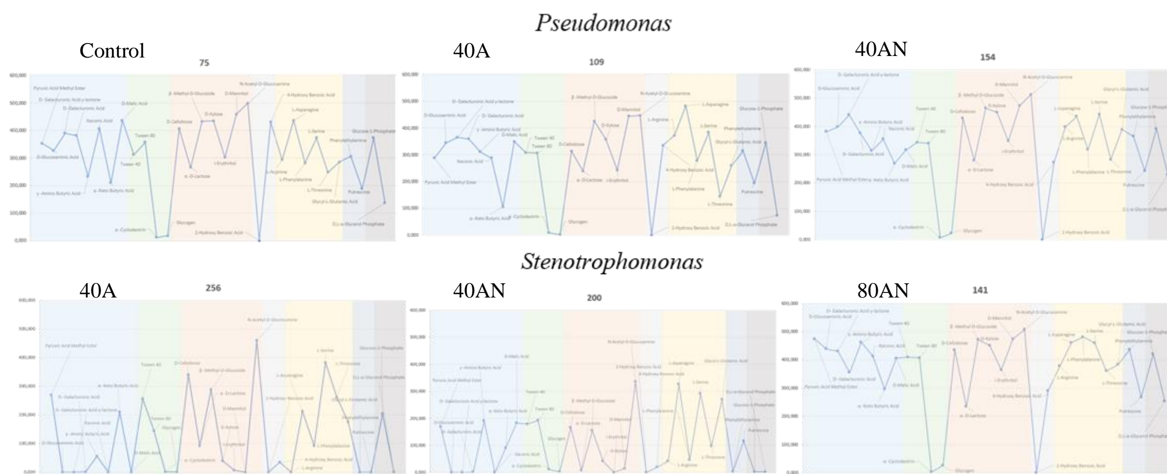



Figure 3.9: NAUC values of the Ecoplate substrates for each isolate from the *Pseudomonas* and *Stenotrophomonas* genera.

In the graphics of the isolates that belong to the genus *Pseudomonas*, it is possible to observe that the pattern of the isolates from three different treatments is quite similar. These isolates, although belonging to the same genus, are present in different communities. These communities are under different environmental stresses and these isolates may be involved in the community response to these stresses (Comte and del Giorgio, 2011; Comte, Fauteux and Giorgio, 2013).

In the graphics of the isolates that belong to genus *Stenotrophomonas*, it can be observed that the first two isolates, from the treatments 40A (isolate 256) and 40N (isolate 200), have a similar pattern. However, isolate 141, that belongs to treatment 80AN, shows a different pattern with higher values. These isolates, although belonging to the same genus, are present in different communities, under different environmental stresses, and the conditions in treatment 80AN may have caused a shift in metabolic plasticity in order to overcome this adversity. It is also possible that isolate 141, although belonging to the same genus, may be from a different species, that proliferates in an environment with higher N availability.

## 3.7. Integrative analysis

To better understand the response of the diazotrophic community to N availability, in terms of structure and functional diversity, a PCA of the results from the multifunctionality assays for denitrification, nitrification and ammonification, the isotopes analysis and the metabolic plasticity, was performed and it is shown in **Figure 3.10**.

The analysis of the variables in the PCs space showed that PC1 represents a gradient toward higher metabolic plasticity, PC2 represents a gradient from denitrification to nitrification and PC3 represents a gradient toward a lower ratio of C/N and glycogen metabolization. These 3 PCs explain 68% of the variance.

In Appendix G the analysis of the explicative variables for PC1, PC2 and PC3 is presented.

The integrative analysis of the results allows an observation of the pattern of the isolates in each treatment and the comparison between the different treatments.

The results from the control treatment show a stricter response to the different variables than the other treatments. In this treatment, all isolates present high metabolic plasticity and different isolates are associated with different functions in the N cycle.

These results may indicate a community not driven by environmental stresses, with a homogenous response to the carbon sources available and different responses to the N availability. In treatment 40A, the N added to the soil in $NH_4^+$ form has led to a community with microorganisms presenting lower metabolic plasticity, which could be due to the environmental stress. In this case the community may have shifted the metabolic plasticity of carbon sources to other functions, maybe linked to the removal of $NH_4^+$. Other hypothesis is that the community is comfortable with the input of N and there is no reason to maintain a high metabolic plasticity.

Treatment 40AN has an input of N in two different ways, $NO_3^-$ and $NH_4^+$, and presents isolates with high values of metabolic plasticity and others with low values. This shows a community with a distribution of functions, which is also corroborated by the presence of an isolate with the ability to perform nitrification.

The pattern presented by the isolates of treatment 80AN shows a community with high metabolic plasticity and a stricter response than other fertilized treatments. This is the treatment with higher input of N which probably places the community under stress, which could lead to a community with individuals geared towards a more comprehensive metabolism, rather than individuals specialized in certain functions.

In this treatment, 80AN, there is a shift to a community with lower C/N ratio, which means a higher content in N. This way, the isolates of this treatment have a high demand for N and for this reason are present in the treatment with the higher N available. These isolates may not be present in other treatments because of this high demand for N.

Treatments 40A and 80AN do not present isolates with the ability to perform nitrification. This may be due to the inhibitory character of $NH_4^+$ in nitrification, since these are the treatments with higher input of N in this form.

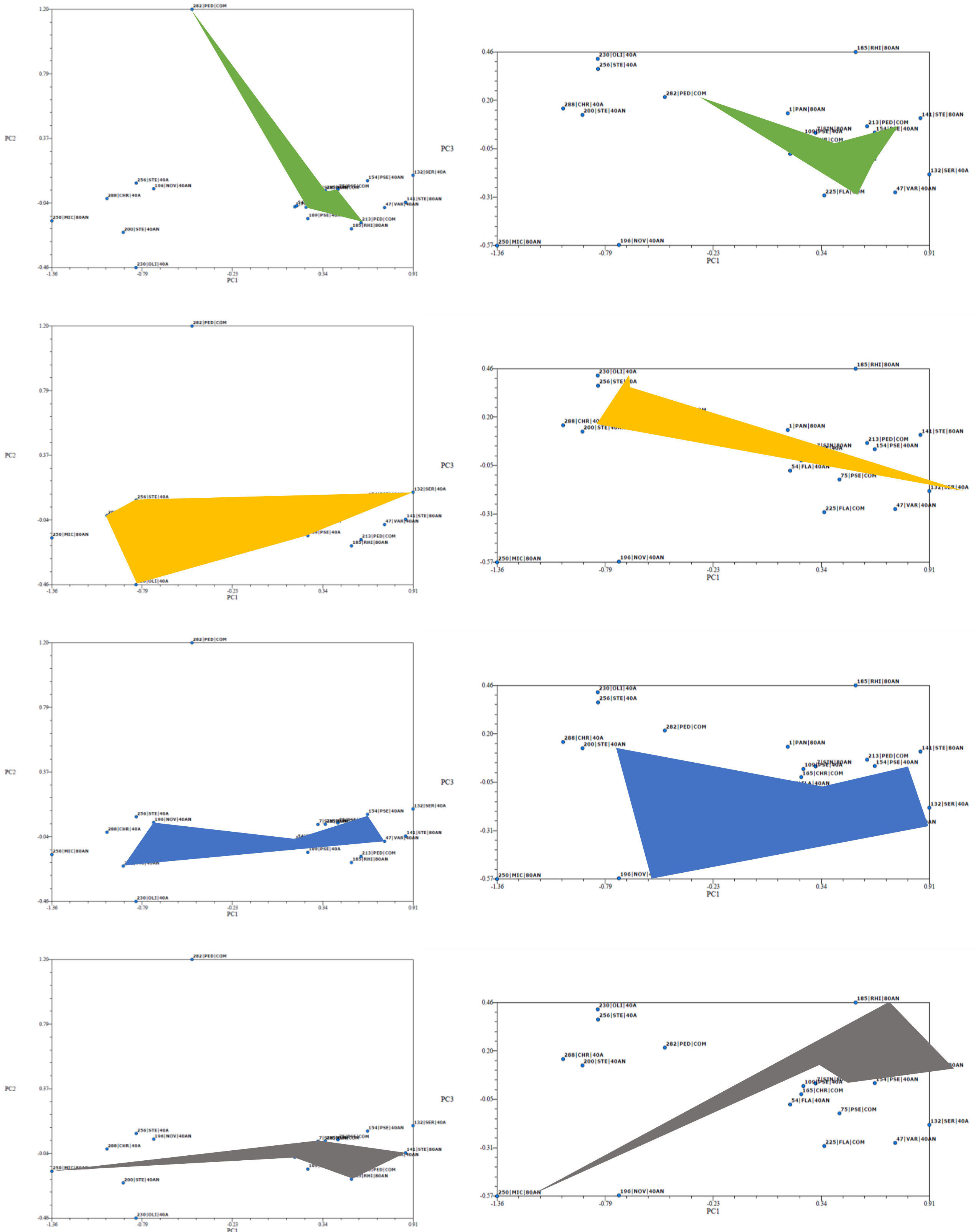


Figure 3.10: Principal Component Analysis of the results from the multifunctionality assays for denitrification, nitrification and ammonification, the isotopes analysis and the metabolic plasticity. The left graphics are for PC1 and PC2, the right graphics are for PC1 and PC3. The figure represents the projection of each isolate (OTUs) in the space defined by the first three PCs. The green color unites the isolates from the control treatment, yellow unites the isolates from treatment 40A, blue unites the isolates from the treatment 40AN, and the grey unites the isolates from the treatment 80AN.

# 4. CONCLUSIONS AND FUTURE PERSPECTIVES

Mediterranean ecosystems present unique characteristics, being hotspots of diversity as well as very responsive to N additions (Dias *et al.*, 2011, 2013, 2014). A long-term field study is being conducted in Arrábida, where plots have been subjected to different N inputs for more than 10 years. *Ulex densus* is a legume plant that, surprisingly, profits from the increased N availability along this period. In this work we aimed at understanding how the structure and functionality of the diazotrophic community associated with the rhizosphere of this plant was affected by the increased N availability.

The N added throughout the 10 years of this study was incorporated in the biotic compartment without significantly increasing the soil $NO_3^-$ or $NH_4^+$ concentrations (Dias *et al.*, 2011; Dias *et al.*, 2014). So, it is reasonable to think that the incorporation of the inorganic N into the biotic compartment may be mediated, at least in part, by the soil microbial community and that one of the most affected functional groups is that of the N fixing bacteria.

However, the highest numbers of diazotrophic isolates were detected in the treatments with higher $NH_4^+$ inputs (40A, and 80AN), the same treatments were *U. densus* proliferates (Dias *et al.*, 2011) showing that the potential for N fixation was not inhibited by $NH_4^+$ addition. In ecosystems with low soil fertility, root exudates and root debris are the main carbon sources supporting the microbial community. The proliferation of diazotrophs with N addition may result from a stress alleviation associated with better plant nutrition, resulting in increased root exudates that can support the heterotrophy of these diazotrophs. Apart from N fixation other functions performed by diazotrophs such as phosphate solubilization may contribute to their fitness at higher $NH_4^+$ availability (Marra *et al.*, 2012; Wang, Chen and Fu, 2019).

While data show an increase in the number of diazotrophs with increased $NH_4^+$ availability, a change in the structure of the community was observed at the genus level. This may suggest that diazotrophs differ in their tolerance and/or fitness towards fixed N.

Until recently, it was common to associate certain groups of microorganisms to specific biotransformations of the N cycle. However, lately it has been shown that most of the N cycle related microorganisms have the potential to perform several of the N biotransformations, namely combinations of N fixation, ammonification, nitrification or denitrification (Delgado-Baquerizo *et al.*, 2017; Blesh, 2018). It is then crucial to understand if the N addition selects for microorganisms with distinct multifunctionalities. In the present work, it was found that the ability to perform nitrification was more frequent among the representative isolates of the diazotrophic community from treatments receiving lower $NH_4^+$ inputs (control and the 40AN treatments), which agrees with the reported inhibition of nitrification by $NH_4^+$ availability (Princic *et al.*, 1998; Svehla *et al.*, 2014). Denitrification was found in all treatments, however, the isolates from the treatment 80AN (receiving more $NO_3^-$) presented higher denitrification rates, which may be relevant as $NO_3^-$ detoxification mechanisms, since high $NO_3^-$ concentrations may inhibit N fixation. The proof that the microbial community was involved in the N incorporation in the biotic compartment lies in the highest C/N ratio of the isolates exclusive of the treatment with the higher N availability (80 AN). This ratio indicates a higher incorporation of N by the microorganisms, which in turn increases the N present in the ecosystem's biotic compartment.

These changes in the microbial multifunctionality associated with the N biotransformations were not reflected in a loss of diversity, but in a change in composition of the community inferred by the distinct taxonomic identification of the representative isolates of the community associated with each treatment.

The variation of the metabolic plasticity between treatments changed along with N fertilization, since the control treatment had the more consistent response for metabolic plasticity. The higher variation of this property is associated with N fertilization treatments and can be related to changes in carbon source availability in the soil.

Although it was possible to identify some differences between the treatments in terms of the functional properties of the community, these differences were not as evident as expected, according to the literature, and do not completely corroborate the theory that the diazotrophic community should be affected by the N additions. These discrepancies may be occurring since the ecosystem that is under study - the Mediterranean ecosystem - presents different characteristics from other ecosystems in similar studies, and so the effects of N addition may not be the same for the Mediterranean ecosystem. Lastly, the community chosen to work as a proxy for the ecosystem's response, the diazotrophic community, may not be the most appropriate choice. This can be due to the wide phylogenetic distribution of genera associated with N fixation ability; additionally, the rhizospheric community may support the ecological stresses and may strive towards maintenance of this functional group, given their importance in the rhizospheric community under N limitation.

As to better understand the ecological responses of the Mediterranean ecosystems under different N inputs, further studies are necessary. Strategies for this purpose can be the characterization of the diazotrophic community in the rhizosphere of another plant species, presenting a different response to N addition, preferably one which suffered a detrimental effect due to N addition.

The study of the total microbial community of the rhizospheric soil can elucidate the effect of N input, since studying the total community would allow to perceive which microorganisms beneficiate from an increase of N availability and which ones are negatively affected by this increase, as well as whether the proportions of the different groups of microorganisms is affected. Moreover, studying the total microbial community may reveal a specific community within, other than the diazotrophic community, that could be used as an indicator of the detrimental effects of N input for the global community in Mediterranean ecosystems.

Towards a better understanding of the structural and functional properties of the community, the incorporation of a metagenomic analysis to complement the cultured-based approach would be important. This way, not only the culturable portion of the microbial community would be evaluated, but also the unculturable portion, which could bring an important insight to the consequences of the N input. A community that should also be considered is the anaerobic community, that was not evaluated in this work, but represents an essential part of the soil microbial composition.

The work presented in this study, and the future work that can arise from it, could create a platform that leads to changes in agricultural and environmental policies, which are necessary to preserve this type of ecosystem that is important due to its unique biodiversity.

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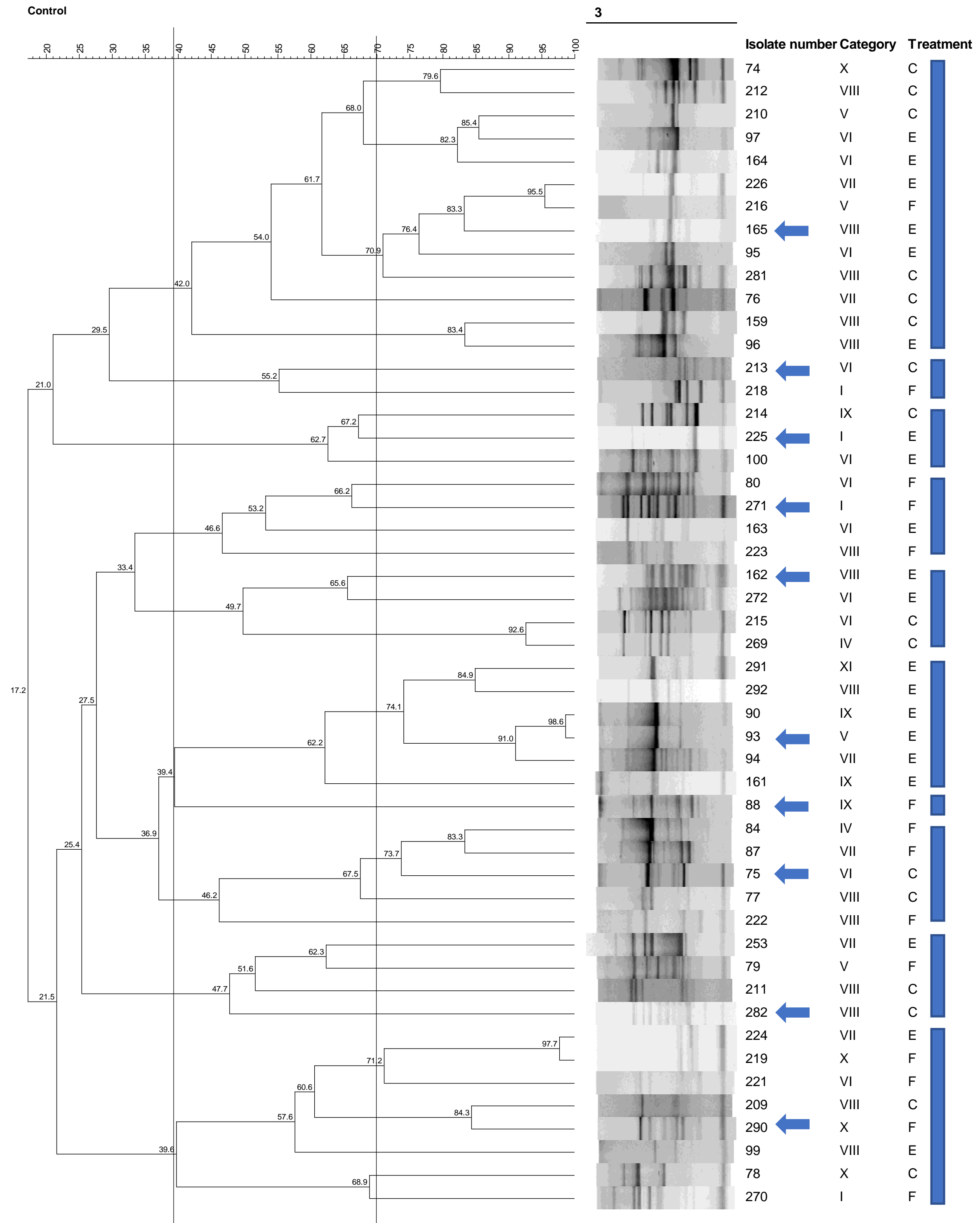
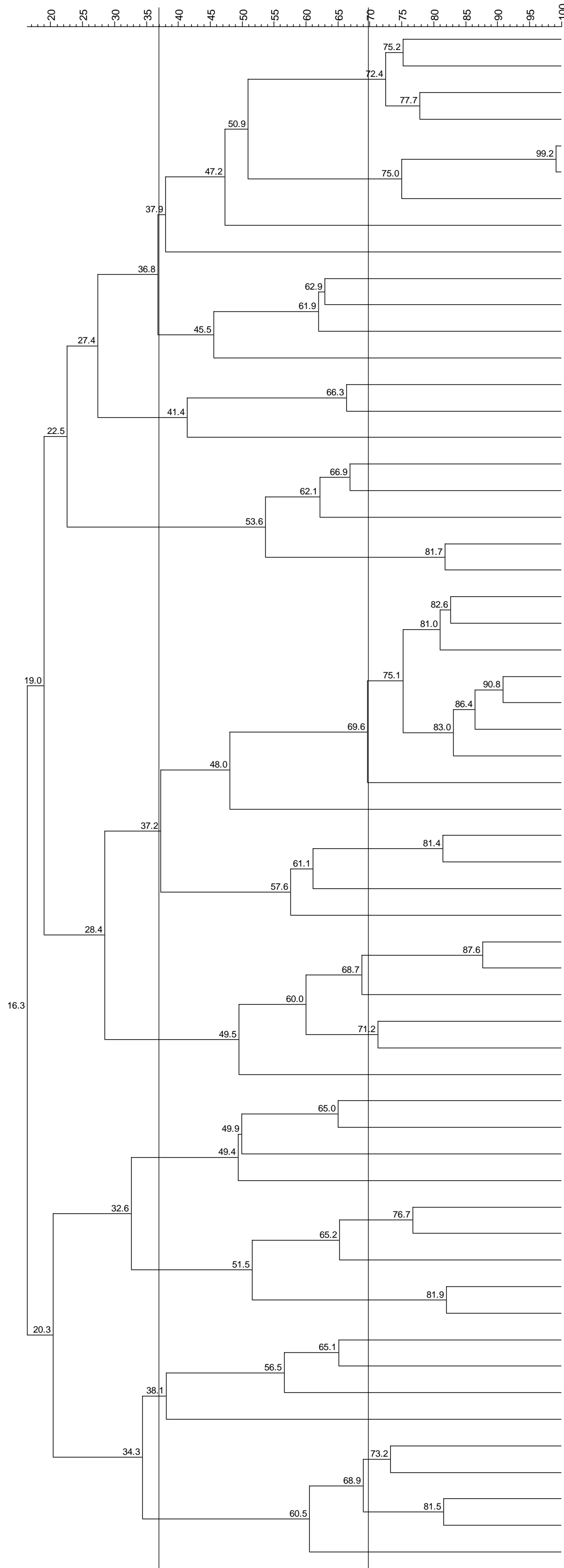
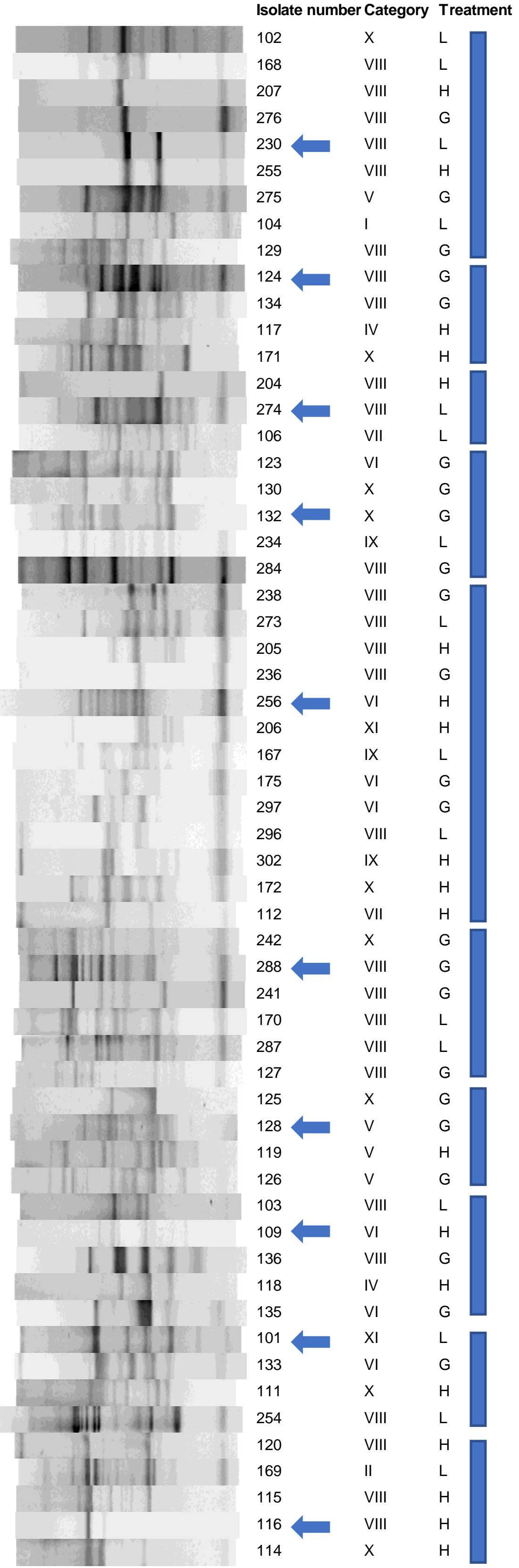
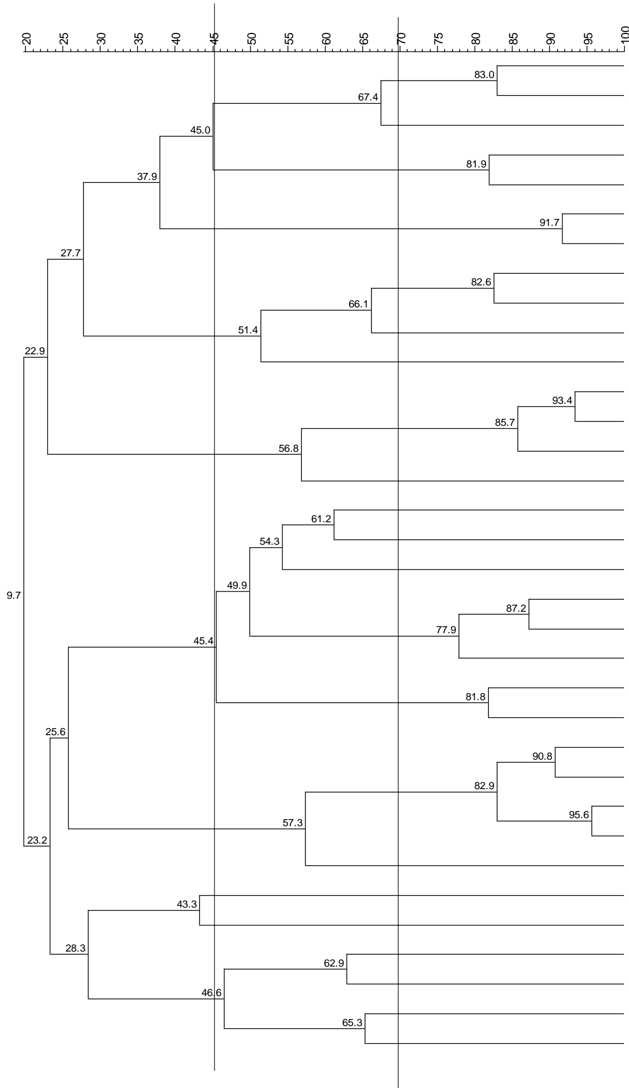
# Appendix

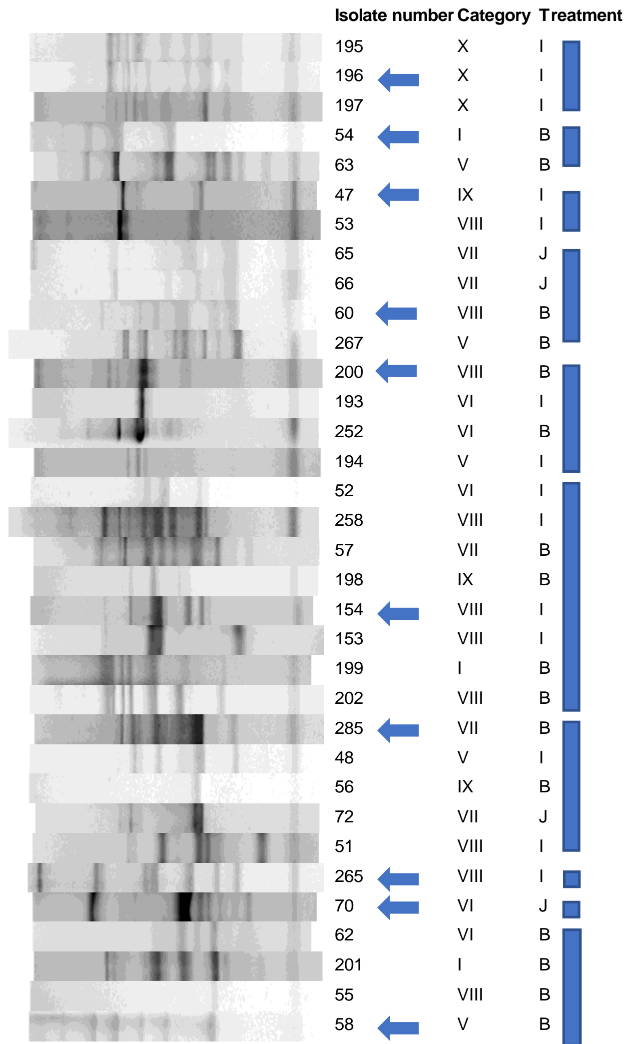
## Appendix A: Dendrograms of the isolates with representative isolates selection




40A

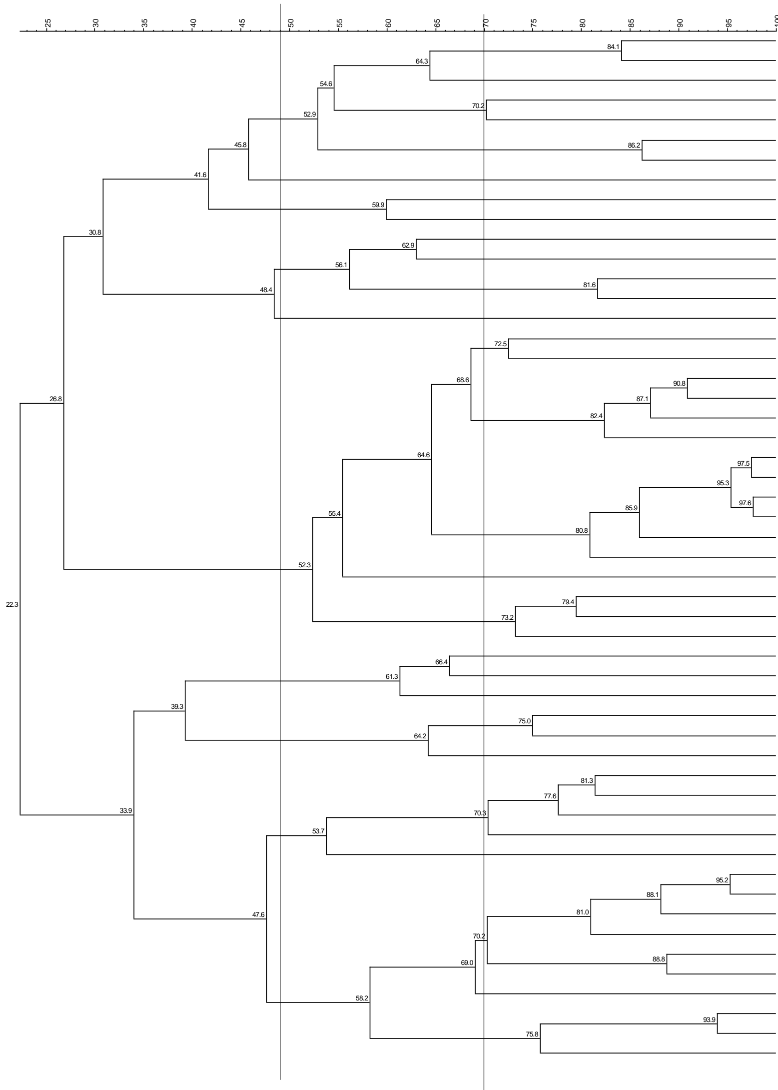

3
Isolate number Category Treatment
102 X L
168 VIII L
207 VIII H
276 VIII G
230 VIII L
255 VIII H
275 V G
104 I L
129 VIII G
124 VIII G
134 VIII G
117 IV H
171 X H
204 VIII H
274 VIII L
106 VII L
123 VI G
130 X G
132 X G
234 IX L
284 VIII G
238 VIII G
273 VIII L
205 VIII H
236 VIII G
256 VI H
206 XI H
167 IX L
175 VI G
297 VI G
296 VIII L
302 IX H
172 X H
112 VII H
242 X G
288 VIII G
241 VIII G
170 VIII L
287 VIII L
127 VIII G
125 X G
128 V G
119 V H
126 V G
103 VIII L
109 VI H
136 VIII G
118 IV H
135 VI G
101 XI L
133 VI G
111 X H
254 VIII L
120 VIII H
169 II L
115 VIII H
116 VIII H
114 X H

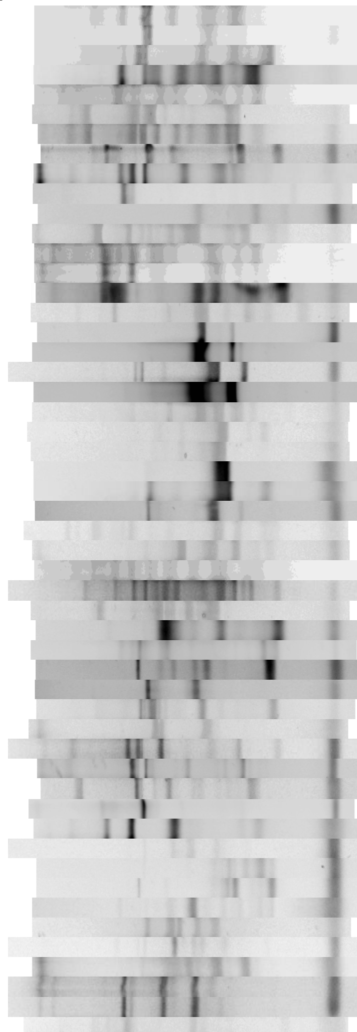

40AN
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
83.0
67.4
45.0
81.9
37.9
91.7
27.7
82.6
66.1
51.4
22.9
93.4
85.7
56.8
61.2
54.3
9.7
49.9
87.2
77.9
45.4
81.8
25.6
90.8
82.9
95.6
57.3
23.2
43.3
28.3
62.9
46.6
65.3


3
Isolate number Category Treatment
195 X I
196 X I
197 X I
54 I B
63 V B
47 IX I
53 VIII I
65 VII J
66 VII J
60 VIII B
267 V B
200 VIII B
193 VI I
252 VI B
194 V I
52 VI I
258 VIII I
57 VII B
198 IX B
154 VIII I
153 VIII I
199 I B
202 VIII B
285 VII B
48 V I
56 IX B
72 VII J
51 VIII I
265 VIII I
70 VI J
62 VI B
201 I B
55 VIII B
58 V B

80AN

3

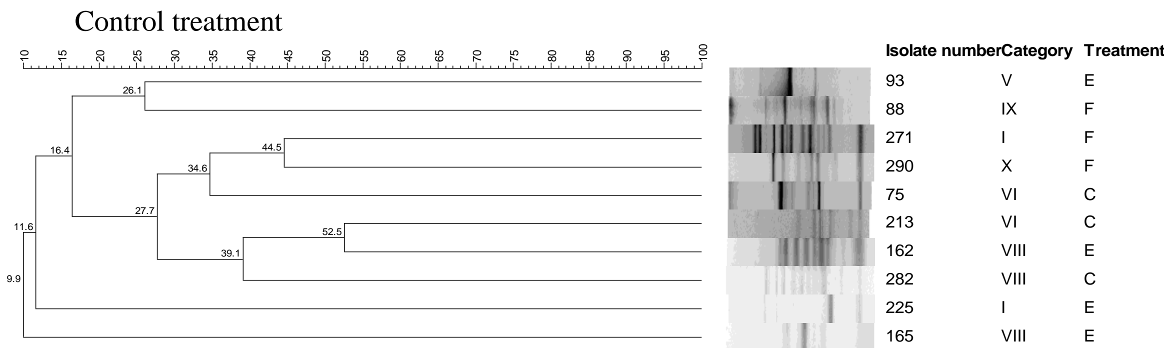


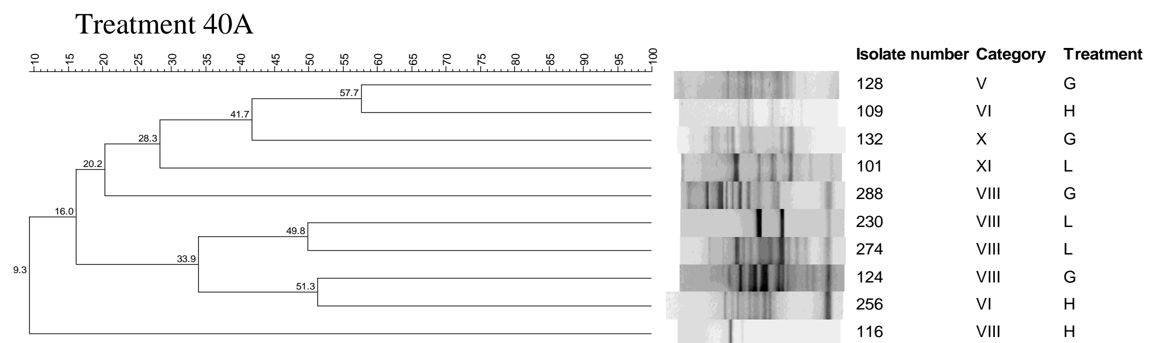
| Isolate number | Category | Treatment |
|---|---|---|
| 188 | VI | A |
| 191 | I | A |
| 186 | VIII | M |
| 149 | VI | A |
| 185 | VIII | M |
| 16 | VIII | M |
| 293 | VI | M |
| 250 | X | A |
| 141 | VI | M |
| 137 | IV | N |
| 8 | VII | N |
| 10 | II | N |
| 182 | IX | M |
| 183 | X | M |
| 1 | V | N |
| 19 | VIII | M |
| 243 | X | N |
| 29 | III | A |
| 263 | V | M |
| 42 | VIII | A |
| 13 | VIII | M |
| 27 | VIII | A |
| 20 | IX | M |
| 146 | VIII | A |
| 151 | V | A |
| 184 | VIII | M |
| 40 | VIII | A |
| 15 | VII | M |
| 189 | VI | A |
| 261 | VIII | N |
| 18 | VI | M |
| 279 | VIII | M |
| 3 | II | N |
| 23 | VIII | A |
| 187 | IX | M |
| 176 | IV | N |
| 21 | VI | M |
| 249 | VIII | A |
| 260 | V | N |
| 26 | IX | A |
| 2 | VII | N |
| 150 | VIII | A |
| 247 | VIII | M |
| 6 | VIII | N |
| 5 | VIII | N |
| 181 | VIII | M |
| 7 | VI | N |
| 244 | VIII | N |
| 299 | X | A |
| 264 | X | M |
| 262 | X | N |
| 41 | IX | A |

## Appendix B: Dendrograms of the representative isolates.

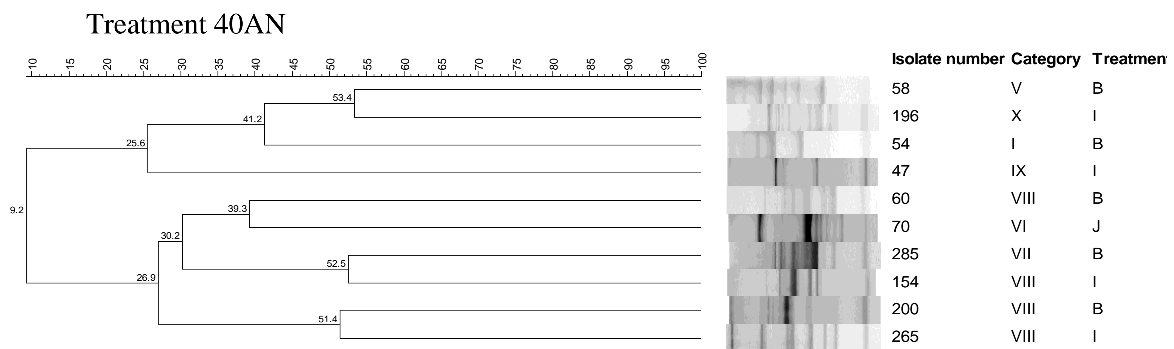
Control treatment



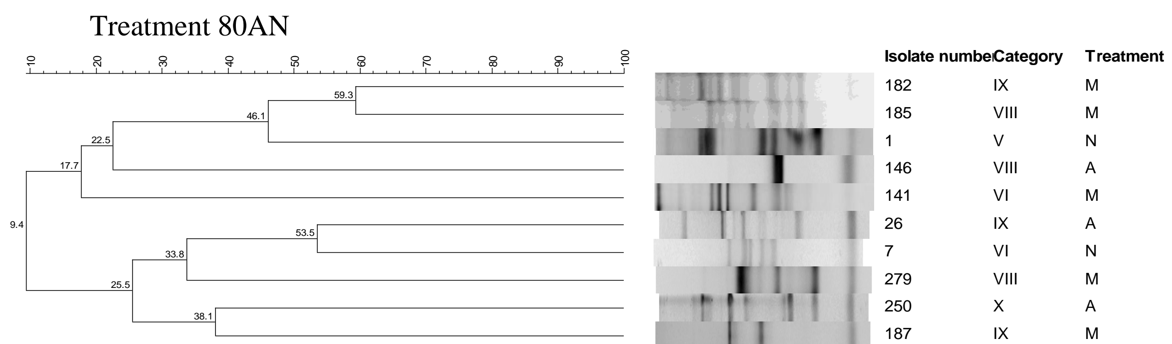

Treatment 40A




Treatment 40AN




Treatment 80AN



## Appendix C: 16S rRNA gene sequencing results.

| Isolate number | Closest hits | Sequence length (bp) | Query cover | Identity (%) | Genera |
|---|---|---|---|---|---|
| 1 | *Pantoea conspicua* LMG 24534 [NR_116247.1] | 931 | 98% | 98.80% | *Pantoea* |
| | *Pantoea allii* BD 390 [NR_115258.1] | | 98% | 98.69% | |
| | *Pantoea ananatis* 1846 [NR_026045.1] | | 98% | 98.69% | |
| 3 | *Enterobacter cloacae* isolate Ecl_IIT-BT08 [DQ347838.1] | 247 | 90% | 82.46% | order: Enterobacterales |
| | Uncultured *Serratia* sp. clone LSVP09 [MG016536.1] | | 91% | 81.74% | |
| | *Arsenophonus endosymbiont* [LN830038.1] | | 87% | 79.64% | |
| 7 | *Ensifer adhaerens* NBRC 100388 [NR_113893.1] | 1140 | 99% | 98.24% | *Sinorhizobium* |
| | *Ensifer adhaerens* LMG 20216 [NR_042482.1] | | 99% | 98.24% | |
| | *Ensifer sesbaniae* CCBAU 65729 [NR_133053.1] | | 99% | 98.15% | |
| 47 | *Variovorax boronicumulans* NBRC 103145 [NR_133053.1] | 1000 | 100% | 99.20% | *Variovorax* |
| | *Variovorax boronicumulans* NBRC 103145 [NR_041588.1] | | 100% | 99.20% | |
| | *Variovorax paradoxus* NBRC 15149 [NR_113736.1] | | 100% | 98.90% | |
| 54 | *Flavobacterium branchiicola* 59B-3-09 [NR_145953.1] | 1208 | 95% | 98.95% | *Flavobacterium* |
| | *Flavobacterium tructae* 435-08 [NR_133749.1] | | 94% | 98.95% | |
| | *Flavobacterium spartansii* T16 [NR_133748.1] | | 94% | 98.87% | |
| 58 | *Flavobacterium aquidurense* WB 1.1-56 [NR_042470.1] | 1151 | 99% | 98.26% | *Flavobacterium* |
| | *Flavobacterium collinsii* 983-08 [NR_145952.1] | | 99% | 98.17% | |
| | *Flavobacterium aquidurense* DSM 18293 [NR_118475.1] | | 99% | 98.17% | |
| 60 | *Pseudomonas* sp. PNS4 [MK602397.1] | 1090 | 95% | 96.09% | *Pseudomonas* |
| | *Pseudomonas* sp. YL24 [MK574810.1] | | 95% | 96.09% | |
| | *Pseudomonas* sp. YL20 [MK574805.1] | | 95% | 96.09% | |
| 70 | *Pseudomonas brassicacearum* subsp. *neoaurantiaca* CIP 109457 [NR_116299.1] | 865 | 99% | 99.65% | *Pseudomonas* |
| | *Pseudomonas thivervalensis* SBK26 [NR_024951.1] | | 99% | 99.54% | |
| | *Pseudomonas frederiksbergensis* DSM 13022 [NR_117177.1] | | 99% | 99.42% | |
| 75 | *Pseudomonas weihenstephanensis* DSM 29166 [NR_148764.1] | 827 | 99% | 98.91% | *Pseudomonas* |
| | *Pseudomonas helleri* DSM 29165 [NR_148763.1] | | 99% | 98.91% | |
| | *Pseudomonas endophytica* BSTT44 [NR_136473.1] | | 99% | 98.91% | |
| 88 | *Microbacterium tumbae* strain T7528-3-6b [NR_156954.1] | 888 | 99% | 99.21% | *Microbacterium* |
| | *Microbacterium diaminobutyricum* RZ63 [NR_152648.1] | | 99% | 98.76% | |
| | *Microbacterium kyungheense* THG-C26 [NR_134086.1] | | 99% | 98.76% | |
| 93 | *Variovorax boronicumulans* NBRC 103145 [NR_114214.1] | 1015 | 100% | 99.21% | *Variovorax* |

| | | | | | |
|---|---|---|---|---|---|
| | *Variovorax boronicumulans* NBRC 103145 BAM-48 [NR_041588.1] | | 100% | 99.21% | |
| | *Variovorax paradoxus* NBRC 15149 [NR_113736.1] | | 100% | 98.92% | |
| **101** | *Microbacterium tumbae* T7528-3-6b [NR_156954.1] | 883 | 99% | 98.64% | *Microbacterium* |
| | *Microbacterium diaminobutyricum* RZ63 [NR_152648.1] | | 99% | 98.41% | |
| | *Microbacterium lacus* A5E-52 [NR_041563.1] | | 99% | 98.30% | |
| **109** | *Pseudomonas chlororaphis* NBRC 3904 [NR_113581.1] | 850 | 99% | 98.82% | *Pseudomonas* |
| | *Pseudomonas chlororaphis* DSM 50083 [NR_116723.1] | | 99% | 98.82% | |
| | *Pseudomonas chlororaphis* ATCC 9446 [NR_116763.1] | | 99% | 98.82% | |
| **116** | *Flavobacterium collinsii* 983-08 [NR_145952.1] | 1148 | 99% | 99.13% | *Flavobacterium* |
| | *Flavobacterium saccharophilum* NBRC 15944 [NR_112839.1] | | 99% | 98.87% | |
| | *Flavobacterium aquidurense* WB 1.1-56 [NR_042470.1] | | 99% | 98.87% | |
| **124** | *Pseudomonas synxantha* NBRC 3913 [NR_113583.1] | 416 | 100% | 99.76% | *Pseudomonas* |
| | *Pseudomonas extremaustralis* [NR_114911.1] | | 100% | 99.76% | |
| | *Pseudomonas grimontii* CFML 97-514 [NR_025102.1] | | 100% | 99.76% | |
| **128** | *Pseudomonas weihenstephanensis* DSM 29166 [NR_148764.1] | 1138 | 99% | 97.45% | *Pseudomonas* |
| | *Pseudomonas deceptionensis* M1 [NR_117552.1] | | 99% | 97.45% | |
| | *Pseudomonas fragi* ATCC 4973 [NR_024946.1] | | 99% | 97.45% | |
| **132** | *Serratia fonticola DSM 22080* C1 [NR_116808.1] | 1092 | 99% | 98.17% | *Serratia* |
| | *Serratia fonticola* DSM 4576 [NR_025339.1] | | 99% | 98.07% | |
| | *Serratia fonticola strain* LMG 7882 [NR_114577.1] | | 99% | 97.90% | |
| **141** | *Stenotrophomonas rhizophila* e-p10 [NR_121739.1] | 1182 | 99% | 99.06% | *Stenotrophomonas* |
| | *Stenotrophomonas rhizophila* e-p10 [NR_028930.1] | | 99% | 98.81% | |
| | *Stenotrophomonas bentonitica* BII-R7 [NR_157765.1] | | 99% | 98.55% | |
| **146** | *Flavobacterium hydatis* DSM 2063 [NR_118476.1] | 1069 | 100% | 98.60% | *Flavobacterium* |
| | *Flavobacterium hydatis* NBRC 14958 [NR_113710.1] | | 100% | 98.60% | |
| | *Flavobacterium hydatis* DSM 2063 [NR_114993.1] | | 100% | 98.60% | |
| **154** | *Pseudomonas synxantha* NBRC 3913 [NR_113583.1] | 683 | 100% | 98.98% | *Pseudomonas* |
| | *Pseudomonas extremaustralis* [NR_114911.1] | | 100% | 98.98% | |
| | *Pseudomonas grimontii* CFML 97-514 [NR_025102.1] | | 100% | 98.98% | |
| **162** | *Pseudomonas reinekei* MT1 [NR_042541.1] | 984 | 99% | 97.35% | *Pseudomonas* |
| | *Pseudomonas vancouverensis* DhA-51 [NR_041953.1] | | 99% | 97.25% | |
| | *Pseudomonas koreensis* Ps 9-14 [NR_025228.1] | | 99% | 97.25% | |
| **165** | *Chryseobacterium oleae* CT348 [NR_134002.1] | 1108 | 99% | 98.82% | *Chryseobacterium* |

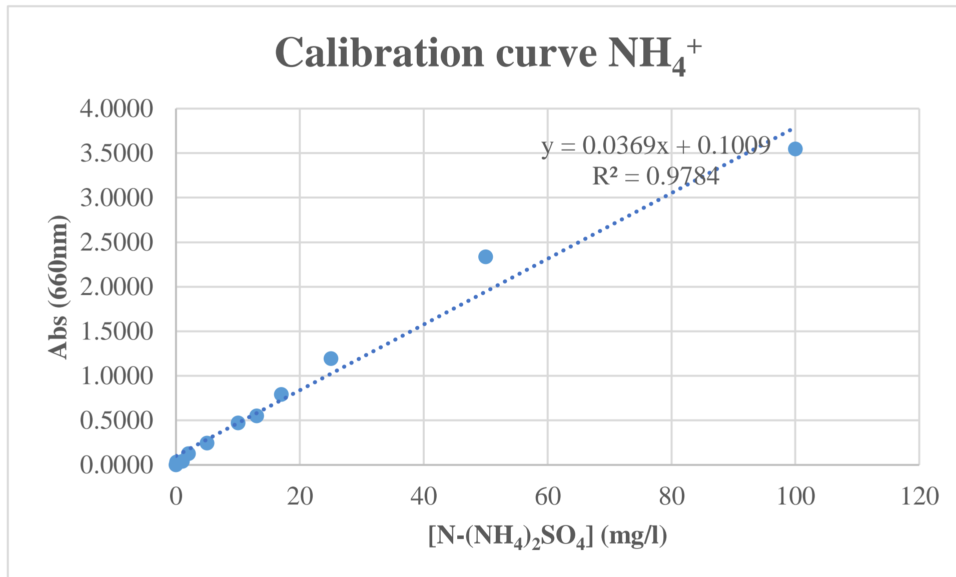
| | | | | | |
|---|---|---|---|---|---|
| | *Chryseobacterium vrystaatense* R-23566 [NR_042370.1] | | 99% | 97.81% | |
| | *Chryseobacterium joostei* LMG 18212 [NR_025387.1] | | 99% | 97.81% | |
| **182** | *Stenotrophomonas maltophilia* [LC379132.1] | 1063 | 96% | 95.53% | *Stenotrophomonas* |
| | *Stenotrophomonas terrae* Ff7b [MF801331.1] | | 96% | 95.53% | |
| | *Stenotrophomonas terrae* R-32768 [NR_042569.1] | | 96% | 95.53% | |
| **185** | *Rhizobium kunmingense* LXD30 [NR_132597.1] | 994 | 99% | 98.59% | *Rhizobium* |
| | *Neorhizobium huautlense* SO2 [NR_024863.1] | | 99% | 97.78% | |
| | *Rhizobium wenxiniae* 166 [NR_157780.1] | | 99% | 97.58% | |
| **187** | *Stenotrophomonas rhizophila* e-p10 [NR_121739.1] | 1010 | 99% | 97.43% | *Stenotrophomonas* |
| | *Stenotrophomonas rhizophila* e-p10 [NR_028930.1] | | 99% | 97.13% | |
| | *Stenotrophomonas bentonitica* BII-R7 [NR_157765.1] | | 99% | 96.83% | |
| **196** | *Novosphingobium lindaniclasticum* LE124 [NR_118312.1] | 1144 | 100% | 98.16% | *Novosphingobium* |
| | *Novosphingobium gossypii* JM-1396 [NR_137206.1] | | 100% | 97.73% | |
| | *Novosphingobium barchaimii* LL02 [NR_118314.1] | | 100% | 97.64% | |
| **200** | *Stenotrophomonas rhizophila* e-p10 [NR_121739.1] | 1146 | 98% | 98.32% | *Stenotrophomonas* |
| | *Stenotrophomonas rhizophila* e-p10 [NR_028930.1] | | 98% | 98.06% | |
| | *Stenotrophomonas bentonitica* BII-R7 [NR_157765.1] | | 98% | 97.97% | |
| **213** | *Pedobacter roseus* CL-GP80 [NR_043555.1] | 1052 | 99% | 99.14% | *Pedobacter* |
| | *Pedobacter kyonggii* K-4-11-1 [NR_159165.1] | | 99% | 98.47% | |
| | *Pedobacter ginsenosidimutans* THG-45 [NR_108685.1] | | 99% | 98.47% | |
| **225** | *Flavobacterium tyrosinilyticum* THG-DN8.8 [NR_149794.1] | 1127 | 99% | 98.75% | *Flavobacterium* |
| | *Flavobacterium banpakuense* 15F3 [NR_117276.1] | | 99% | 98.40% | |
| | *Flavobacterium chungbukense* CS100 [NR_109016.1] | | 99% | 97.77% | |
| **230** | *Olivibacter soli* Gsoil 034 [NR_041503.1] | 1049 | 99% | 99.23% | *Olivibacter* |
| | *Pseudosphingobacterium domesticum* DC-186 [NR_042571.2] | | 99% | 98.28% | |
| | *Olivibacter ginsengisoli* Gsoil 060 [NR_041504.1] | | 99% | 94.92% | |
| **249** | *Stenotrophomonas tumulicola* T5916-2-1b [NR_148818.1] | 845 | 100% | 98.46% | *Stenotrophomonas* |
| | *Stenotrophomonas chelatiphaga* LPM-5 [NR_116366.1] | | 100% | 98.35% | |
| | *Stenotrophomonas maltophilia* ATCC 13637 [NR_112030.1] | | 100% | 97.99% | |
| **250** | *Microbacterium saperdae* IFO 15038 [NR_024637.1] | 696 | 100% | 98.71% | *Microbacterium* |
| | *Microbacterium koreense* JS53-2 [NR_043304.1] | | 100% | 98.71% | |
| | *Microbacterium maritypicum* DSM 12512 [NR_114986.1] | | 100% | 98.71% | |
| **256** | *Stenotrophomonas maltophilia* ATCC 13637 [NR_112030.1] | 1029 | 99% | 98.24% | *Stenotrophomonas* |

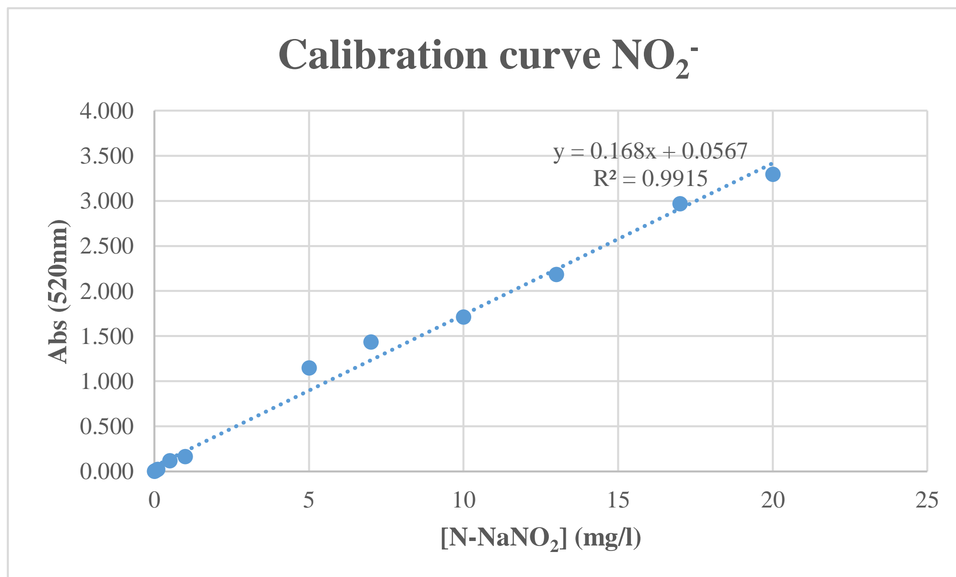
| | | | | | |
|---|---|---|---|---|---|
| | *Stenotrophomonas maltophilia* NBRC 14161 [NR_113648.1] | | 99% | 98.14% | |
| | *Stenotrophomonas pavanii* LMG 25348 [NR_118008.1] | | 99% | 98.14% | |
| **265** | *Stenotrophomonas rhizophila* e-p10 [NR_121739.1] | 698 | 100% | 99.00% | *Stenotrophomonas* |
| | *Stenotrophomonas rhizophila* e-p10 [NR_028930.1] | | 100% | 98.57% | |
| | *Stenotrophomonas bentonitica* BII-R7 [NR_157765.1] | | 100% | 98.28% | |
| **271** | *Flavobacterium chilense* LM-09-Fp [NR_108512.1] | 1151 | 99% | 98.26% | *Flavobacterium* |
| | *Flavobacterium spartansii* T16 [NR_133748.1] | | 99% | 98.09% | |
| | *Flavobacterium aquidurense* WB 1.1-56 [NR_042470.1] | | 99% | 98.09% | |
| **274** | *Stenotrophomonas terrae* R-32768 [NR_042569.1] | 1047 | 99% | 99.23% | *Stenotrophomonas* |
| | *Stenotrophomonas pictorum* JCM 9942 LMG 981 [NR_041957.1] | | 99% | 98.95% | |
| | *Stenotrophomonas humi* R-32729 [NR_042568.1] | | 99% | 98.76% | |
| **282** | *Pedobacter zeae* 22 [NR_156064.1] | 1043 | 98% | 98.16% | *Pedobacter* |
| | *Pedobacter vanadiisoli* XNV015 [NR_153693.1] | | 98% | 98.16% | |
| | *Pedobacter roseus* CL-GP80 [NR_043555.1] | | 98% | 98.06% | |
| **285** | *Flavobacterium hydatis* DSM 2063 [NR_118476.1] | 1076 | 99% | 98.88% | *Flavobacterium* |
| | *Flavobacterium hydatis* NBRC 14958 [NR_113710.1] | | 99% | 98.88% | |
| | *Flavobacterium hydatis* DSM 2063 [NR_114993.1] | | 99% | 98.88% | |
| **288** | *Chryseobacterium piperi* CTM [NR_108294.1] | 1102 | 99% | 98.81% | *Chryseobacterium* |
| | *Chryseobacterium soli* JS6-6 [NR_044299.1] | | 99% | 97.99% | |
| | *Chryseobacterium soldanellicola* NBRC 100864 [NR_113952.1] | | 99% | 97.90% | |
| **290** | *Stenotrophomonas chelatiphaga* LPM-5 [NR_116366.1] | 1121 | 99% | 97.68% | *Stenotrophomonas* |
| | *Stenotrophomonas tumulicola* T5916-2-1b [NR_148818.1] | | 99% | 97.32% | |
| | *Stenotrophomonas pavanii* ICB 89 [NR_116793.1] | | 99% | 96.51% | |

**Appendix D: Table with the compilation of data for the primers most used for the PCR search of *nifH* gene.**

| Primers | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Name | Sequence (5' to 3') | Position | Degeneracy | Tm (°C) | Length | Results | | | | | | | | Reference |
| | | | | | | Cluster 1 | | | | | | | Cluster 2 | |
| | | | | | | Subcluster 1J, 1K | Subcluster 1J, 1K,1P, 1U | Subcluster 1H,1K,1I, 1M, 1N, 1P,1T,1U | Subcluster 1F | Subcluster 1B | Subcluster 1D | Subcluster 1E, 2D | Subcluster 2E | |
| | | | | | | α-Proteobacteria | β-Proteobacteria | γ-Proteobacteria | ε-Proteobacteria | Cyanobacteria | *Frankia* | Firmicutes (*Paenibacillus*) | δ-Proteobacteria | |
| Ueda19 F | GCI WTY TAY GGI AAR GGI GG | 19–38 | 16 | 62.4-67.9 | 455 | Yes | Yes | Yes | | Yes | | | Yes | (Angel *et al.*, 2018) |
| R6 | GCC ATC ATY TCI CCI GA | 457–473 | 2 | 61.1-62.5 | | | | | | | | | | |
| IGK3 | GCI WTH TAY GGI AAR GGI GGI ATH GGI AA | 19-47 | 72 | 69.4-75.3 | 395 | Yes | Yes | Yes | | Yes | Yes | Yes | Yes | (Chakraborty and Islam, 2017; Angel *et al.*, 2018) |
| DVV | ATI GCR AAI CCI CCR CAI ACI ACR TC | 388-413 | 8 | 71.7-75.8 | | | | | | | | | | |
| F2 | TGY GAY CCI AAI GCI GA | 115–131 | 4 | 62.3-67.9 | 359 | Yes | Yes | Yes | | Yes | Yes | Yes | Yes | (Marusina *et al.*, 2000; Angel *et al.*, 2018) |
| R6 | GCC ATC ATY TCI CCI GA | 457–473 | 2 | 61.1-62.5 | | | | | | | | | | |
| IGK | AAR GGN GGN ATH GGN AA | 31-47 | 384 | 62.1-72.5 | 464 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | | (Gaby and Buckley, 2012) |
| nifh3 | ATR TTR TTN GCN GCR TA | 478-494 | 128 | 46.1-61.5 | | | | | | | | | | |
| F1 | TAY GGI AAR GGI GGI ATI GGI AAR TC | 25–50 | 8 | 60.4-74.5 | 467 | Yes | Yes | Yes | | | | | Yes | (Fedorov *et al.*, 2008) |
| nifH-3r | TTG TTG GCI GCR TAS AKI GCC AT | 469–491 | 8 | 68.5-72.1 | | | | | | | | | | |
| nifHfor | TAY GGN AAR GGN GGH ATY GGY ATC | 136-159 | 768 | 51.4 | 467 | Yes | Yes | Yes | | | | | Yes | (Sarita *et al.*, 2008) |
| nifHrev | ATR TTR TTN GCN GCR TAV ABB GCC ATC AT | 427-452 | 3496 | 54.3 | | | | | | | | | | |
| nifH2 | TGY GAY CCN AAR GCN GA | 115–131 | 128 | 54.0-68.1 | 362 | Yes | Yes | Yes | | Yes | | | | (Zehr and McReynolds, 1989; Zehr, Mellon and Zani, 1998) |
| nifH1 | ADN GCC ATC ATY TCN CC | 460-476 | 96 | 52.5-63.9 | | | | | | | | | | |
| PolF | TGC GAY CCS AAR GCB GAC TC | 115-134 | 24 | 63.8-70.1 | 362 | Yes | Yes | Yes | | | Yes | Yes | | (Poly, Monrozier and Bally, 2000; Beneduzi *et al.*, 2008) |
| PolR | ATS GCC ATC ATY TCR CCG GA | 457-476 | 8 | 63.7-67.5 | | | | | | | | | | |

**Appendix E: Graphic representation of the calibration curves for calculation of $NH_4^+$ and $NO_2^-$ concentration.**



**Appendix F: Table with the representative isolates and the results of denitrification (yellow), nitrification (dark blue) and ammonification (dark grey) for selection of the 20 isolates for the assay of metabolic plasticity. The numbers in red are the chosen for the group of 20.**

| *Pseudomonas* | | | | | | | | *Rhizobium* |
|---|---|---|---|---|---|---|---|---|
| CONTROL | | 40A | | | 40AN | | | 80AN |
| **75** | 162 | **109** | 124 | 128 | 60 | 70 | **154** | **185** |

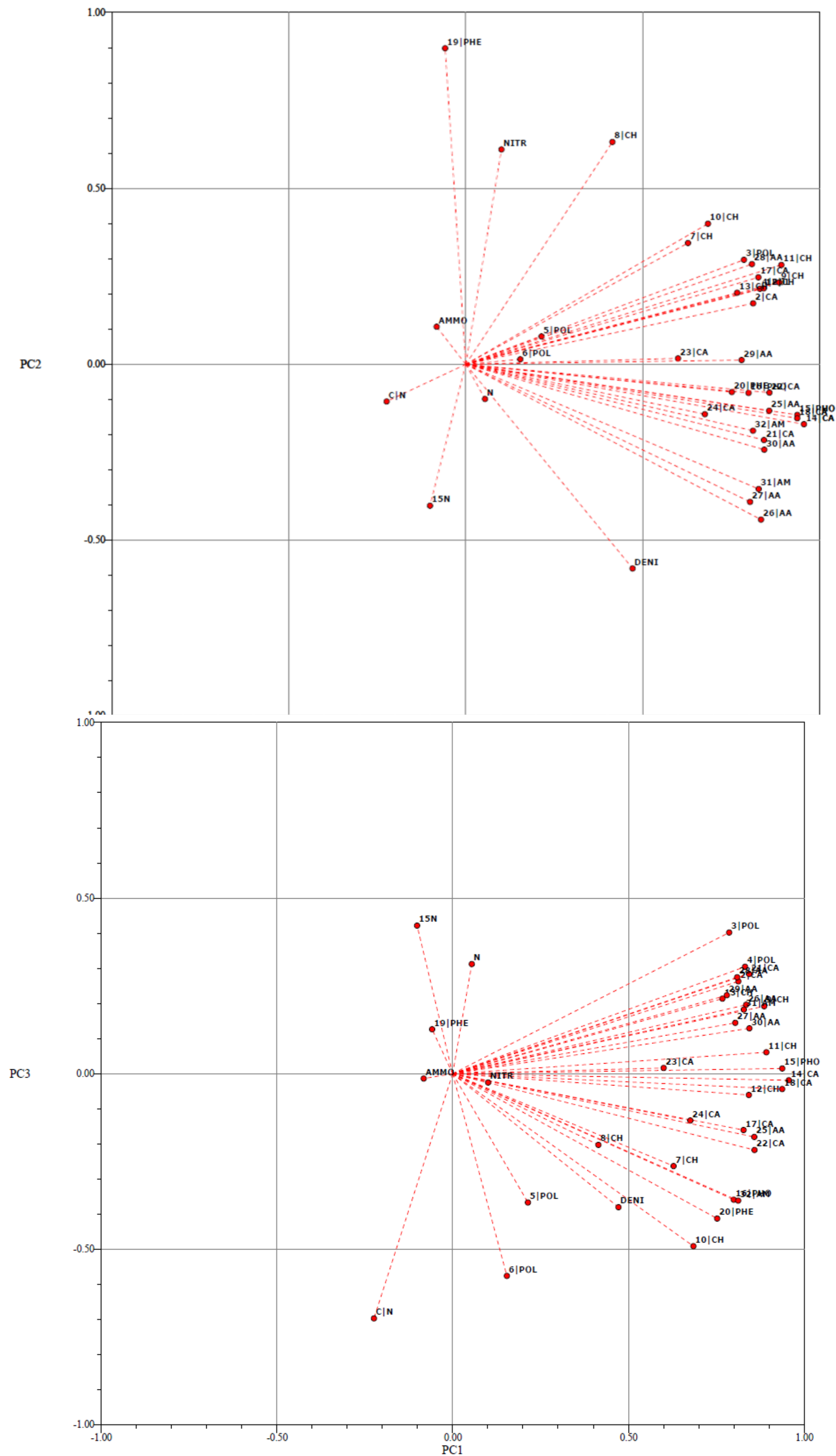
| *Stenotrophomonas* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CONTROL | 40A | | 40AN | | 80AN | | | |
| 290 | **256** | 274 | **200** | 265 | **141** | 182 | 187 | 249 |

| *Flavobacterium* | | | | | | | | *Variovorax* |
|---|---|---|---|---|---|---|---|---|
| CONTROL | | 40A | 40AN | | | 80AN | CONTROL | 40AN |
| **225** | 271 | 116 | **54** | 58 | 285 | 146 | 93 | **47** |

| *Chryseobacterium* | | *Pedobacter* | | *Serratia* | *Olivibacter* | *Novosphingobium* | *Pantoea* | *Sinorhizobium* |
|---|---|---|---|---|---|---|---|---|
| CONTROL | 40A | CONTROL | | 40A | 40A | 40AN | 80AN | 80AN |
| **165** | **288** | **213** | **282** | **132** | **230** | **196** | **1** | **7** |

| *Microbacterium* | | |
|---|---|---|
| CONTROL | 40A | 80AN |
| 88 | 101 | **250** |

## Appendix G: Analysis of the explanatory variables for PC1, PC2 and PC3



**Appendix H: Table with Ecoplate substrates in categories according to respective characteristics.**

| Ecoplate substrate | Substrate category |
|---|---|
| Pyruvic Acid Methyl Ester | Carboxylic acid (CA) |
| D-Glucosaminic Acid | |
| D- Galacturonic Acid γ-lactone | |
| D- Galacturonic Acid | |
| γ -Amino Butyric Acid | |
| Itaconic Acid | |
| α -Keto Butyric Acid | |
| D-Malic Acid | |
| Tween 40 | Polymer (POL) |
| Tween 80 | |
| α -Cyclodextrin | |
| Glycogen | |
| D-Cellobiose | Carbohydrate (CH) |
| α -D-Lactose | |
| β -Methyl-D-Glucoside | |
| D-Xylose | |
| i-Erythritol | |
| D-Mannitol | |
| N-Acetyl-D-Glucosamine | |

| Ecoplate substrate | Substrate category |
|---|---|
| 2-Hydroxy Benzoic Acid | Phenolic compound (PHE) |
| 4-Hydroxy Benzoic Acid | |
| L-Arginine | Amino acid (AA) |
| L-Asparagine | |
| L-Phenylalanine | |
| L-Serine | |
| L-Threonine | |
| Glycyl-L-Glutamic Acid | |
| Phenylethylamine | Amine (AM) |
| Putrescine | |
| Glucose-1-Phosphate | Phosphorylated chemical (PHO) |
| D,L-α-Glycerol Phosphate | |