DENITRIFICATRION IN EXTREME ENVIRONMENTS.

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1. Introduction

Denitrification is one of the metabolic pathways belonging to the nitrogen cycle (Figure 1). Thanks to this pathway nitrate is subsequently converted into nitrite, nitric oxide, nitrous oxide or nitrogen gas. The last three compounds are gases and they are not readily available for microbial growth; therefore, they are typically released to the atmosphere. Nitrogen gas makes up over 70% of atmospheric gases, thus the release of N_2 to the atmosphere is benign. However, nitric oxide and nitrous oxide have important implications in terms of climate change, the chemistry of the atmosphere, human health and the ecological functioning of natural ecosystems, especially aquatic systems and soils where nitrogen concentrations are increasing, causing eutrophication of lakes or rivers and oceanic dead zones through algal bloom-induced hypoxia (Howarth, 2004).

On the other hand, some nitrogen compounds resulting from human activities have great impact on denitrification causing several problems at environmental level (Martínez-Espinosa et al., 2011):

i) NO and N_2O emissions from fertilised soils due to denitrification. These gases are also produced through biomass burning, cattle and feedlots, fossil fuel combustion and other industrial sources. N_2O, carbon dioxide (CO_2) and methane (CH_4) are the three most important greenhouse gases. Consequently, recent strategies to mitigate climate change include the reduction of N_2O emissions. In addition, both N_2O and NO have deleterious effects on the stratosphere, where they are involved in the destruction of atmospheric ozone. Indeed, it has been reported that N_2O is currently the single most important ozone-depleting emission and it is expected to remain the largest throughout the 21st century (Figure 2).

ii) Excess NO_3^- and NO_2^- derived from fertilisers are leached from soils and enter the groundwater. At concentrations of <5 mM, NO_2^- is toxic for most microorganisms (Shen et al., 2003), and considerably lower concentrations represent a threat to aquatic invertebrates (Alonso and Camargo, 2006). High levels of nitrate in drinking water are a known risk factor for methaemoglobinaemia (a potential cause of the blue baby syndrome) and colon cancer.

Biological denitrification is an anaerobic respiration reaction in which nitrate (NO_3^-) is used as a terminal electron acceptor. Most denitrifying microorganisms are aerobic autotrophs or heterotrophs that can switch to anaerobic growth when nitrate is present in the media. Typical assays used to determine whether a new taxon is able to perform denitrification are usually based on the reduction of nitrate under anaerobic conditions. If this reaction takes place, the taxon is characterised as denitrifying. However, this assay is only demonstrating that nitrate reduction could take place anaerobically, but it is not demonstrating if complete or partial denitrification is performed by one specific taxon. Because of this, more effort should be put on the analysis of microbial denitrification capabilities in the next future.

From a biotechnological point of view, denitrification becomes such an important pathway because nitrogen may be completely removed from the system (soil, water, etc.) in gaseous form rather than simply recycled through the system in biomass. Denitrifying

microorganisms, and in particular extremophiles able to denitrify, have focused the research community attention on the design of new strategies for soil and wastewater bioremediation (Nájera et al., 2012; Martínez-Espinosa et al., 2015).

In the review presented here, main features about denitrification in extreme environments are highlighted. The biochemical characteristics of the denitrifying enzymes from extremophilic microorganisms are also described.

2. Extremophilic microbial communities in charge of denitrification.

Extreme environments are under conditions, which make the life of microorganisms difficult, like high or low values of temperature, pH, salinity, radiation, etc. These factors affect both the structure of microbial communities (in terms of composition and abundance) and the biochemical and genetic characteristics of denitrification.

2.1. Extremely low pH.

The pH range for denitrification is between 6 and 8 (Sánchez-Andrea et al. 2012), so an environment is considered to be acidic when pH is below 6. Other than natural causes, many of them have been generated because of the use of fertilisers during decades. The microbial biomass is lower in acidic soils than in neutral or alkaline soils (Čuhel et al. 2010) yet in extremely low pH habitats there is a broad range of denitrifying bacteria belonging to the genera *Paenibacillus, Bacillus, Sedimentibacter, Lysinibacillus, Delftia, Alcaligenes, Clostidrium* and *Desulfitobacterium*. An example of this kind of habitat is Tinto River in Huelva (Spain) where the oxidation of metal-sulphides results in waters at pHs around 2.3 (Sánchez-Andrea et al. 2012).

As a general rule, it can be stated that the denitrification rate decreases with low pH values; in contrast, the N_2O:N_2 ratio is negatively correlated with soil pH (Čuhel et al. 2010;

Huang et al. 2014; Liu et al. 2010; Sánchez-Andrea et al. 2012). However, it is clear that denitrification can occur in soil and water microbial communities at low pH values if they correspond to the optimum pH for optimum growth (Liu et al. 2010). Because of this, acid habitats have an important accumulation of nitrous oxide and they may contribute to climate change because it is a greenhouse gas that influences the ozone layer stability. In fact, 70% of nitrous oxide emissions to the atmosphere come from acid soils (Mosier 1998).

The accumulation of N_2O at low pHs is associated with the inhibition of the enzyme nitrous oxide reductase, which cannot catalyse the reduction of this gas to N_2 at these values of acidity. The transcription rate of *nosZ* (gene encoding N_2O reductase) compared to that of other denitrification genes like *nirS* is higher at low pHs than at high pHs (Liu et al. 2010), so the inhibition may occur at the post-transcriptional phase. This evidence suggests that there is a bug in the translation or assembly of nitrous oxide reductase, which causes the inhibition of the enzyme. Moreover, the N_2O reductase is the most sensitive enzyme to environmental conditions due to its localisation: in the periplasmic space in gram-negative bacteria (Šimek et al. 2002) and between the cell membrane and the S layer in archaea (some preliminary results obtained from the haloarchaea *Haloferax mediterranei* support this idea. Unpublished results).

2.2. Extremely high salinity.

Hypersaline environments have an extensive distribution on Earth like saline soils, alkaline salt lakes, saline ponds, etc. (Oren 2002). Extreme halophiles are generally defined as organisms with optimal growth in media with a concentration of 150 to 300 g L^{-1} (2.5 to 5.2 M NaCl) (Andrei et al. 2012).

Halophilic organisms are found in the three domains of life: Archaea, Bacteria and Eukarya, but at the highest concentrations of salt the dominant species are archaea, especially the family *Halobacteriaceae* (Andrei et al. 2012). Despite the fact that low concentrations of

NO_3^- are generally found in hypersaline environments (Andrei et al. 2012), some halophilic archaea can grow anaerobically using nitrate as an electron acceptor, forming N_2O or N_2, like *Haloarcula marismortui, Haloarcula hispanica, Haloferax mediterranei, Haloferax volcanii* or *Halogeometricum borinquense*. In saline and hypersaline environments, the denitrification process has some peculiarities focused on both nitrite reductases: NirK and NirS. No genome from a halophilic denitrifier has been found to date with both types present (Jones & Hallin 2010).

In general terms, there is a negative correlation between salt concentration and NirK richness (Santoro et al. 2006), which indicates some kind of specialisation of these enzymes. Studies in coastal ecosystems along salinity gradients conclude that with the increase of NaCl concentration (since 34,5 g L^{-1}), the predominant nitrite reductase in microbial communities is NirS (Jones & Hallin 2010; Santoro et al. 2006). However, in extreme hypersaline environments (since 300 g L^{-1}), the predominant nitrite reductase is NirK, which has been found in archaea species like *Haloarcula marismortui, Haloarcula hispanica, Haloferax mediterranei* or *Haloferax volcanii* (Inatomi and Hochstein, 1996; Hichiki, Tanaka na Mochizuki, 2001; Esclapez et al., 2013). This apparent contradiction may be explained by the more cosmopolitan nature of *nirS* sequences compared to *nirK* sequences along salinity gradients. A diversity of genes encoding NirS are found in places with high and low NaCl concentrations, while genes encoding NirK are restricted to low salinity points (Santoro et al. 2006) or really salty environments. Therefore, it is possible that *nirK* communities are more specialised in each habitat and are simply not detected using the currently available primer sets (Jones & Hallin 2010). The supposed advantage of NirK communities over NirS communities at the highest salt concentrations might lie in the different chemical structure of both enzymes. The first has copper centres which have higher reducing power than the iron sulphur centres of the NirS- cytochromes. These differences in cofactors could explain the prevalence of NirK in environments with low oxygen solubility and

high reducing power like hypersaline habitats. Nevertheless, at the time of writing this chapter there is no scientific evidence to verify this hypothesis.

Finally, with respect to the final stages of denitrification in haloarchaeas, there are not any detailed biochemical studies on nitric oxide reductases and nitrous oxide reductases, but there is evidence that some haloarchaea, such as *Haloferax mediterranei,* are complete denitrifiers because of their ability to reduce nitrous oxide to dinitrogen (Bonete et al. 2008). This evidence opens the doors to the use of these organisms in wastewater treatments with high salt concentrations, transforming large quantities of nitrates and nitrites into dinitrogen (Nájera-Fernández et al. 2012).

2.3. Extremely high temperature.

Hyperthermophile organisms grow at temperatures around 80 ^{0}C or higher (van Wolferen et al. 2013). They are found in hot terrestrial and marine environments: on land, some of the most common habitats are hot springs that come from volcanic emanations; in the sea, marine environments like hydrothermal systems, abyssal hot sediments or active seamounts are home to a broad variety of hyperthermophile communities (Stetter 1999). Until today, over 90 hyperthermophilic species have been discovered (van Wolferen et al. 2013), some of them are bacteria but the majority belong to the Archaea domain. However, there is little evidence of hyperthermophilic denitrifiers. *Aquifex pyrophilus* is the best characterised hyperthermophilic dentrifier bacteria , which grow optimally at oxygen concentrations below 5% (v/v) in the gas phase (Amo et al. 2002). Also, other bacteria belonging to the ε-proteobacteria (members of the genera *Sulfurimonas, Sulfurovum* and *Nitratifractor*) and γ-protetobacteria couple the oxidation of reduced sulphur species with NO_3^- reduction (Bourbonnais et al. 2012); examples in Archaea are *Pyrobaculum calidifontis* (Amo et al. 2002) and *Pyrobaculum aerophilum* (Volkl et al. 1993),

which are complete denitrifiers, growing because of the reduction of nitrate to dinitrogen as a final product.

The poor knowledge about the denitrification process in hyperthermophilic environments may be due to the difficulty to grow some of these organisms in culture media. However, a complete picture of the functional genes required for denitrification (nitrate, nitrite, nitric oxide and nitrous oxide reductases) have been detected in hydrothermal vent chimneys (Wang et al. 2009). Moreover, *nirK* seems to override *nirS* (Bourbonnais et al. 2012), which supports the idea that NirK is more resistant in environments with high reducing power. Studies of the genes involved in denitrification will be needed to increase the knowledge about it in these environments.

3. Extreme enzymes involved in denitrification.

Denitrification can be considered as the modular assemblage of four partly independent respiratory processes: nitrate, nitrite, nitric oxide and nitrous oxide reduction (Zumft 1997) (Figure 4). In the entire denitrification process, nitrate is reduced to N_2 by means of four reaction steps catalysed by the action of four metalloenzymes: respiratory nitrate reductase, respiratory nitrite reductase, nitric oxide reductase and nitrous oxide reductase. Physiological, biochemical and genetic evidence has provided a detailed process for this pathway in the *Bacteria* Domain (Zumft 1997). Nonetheless, the biochemical and genomic data related to the denitrification process in extremophiles is scarce, in fact, at the moment there is not a single archaeon whose denitrification pathway has been described not only at genetic but also at enzymatic level. Related to the genetic evidence, during the last years the high number of available genomes of *Archaea* has allowed scientists to identify denitrification genes by homology search with their bacterial counterparts. However, the few biochemical studies related to denitrification pathways in extremophiles are restricted to the purification and characterisation of respiratory

nitrate and nitrite reductases from halophilic microorganisms and the hyperthermophilic archaeon *Pyrobaculum aerophilum* (Table 1). No methanogenic archaeon has been described as denitrifying up to now. The existence of denitrification in the hyperthermophilic branches indicates an early origin and occurrence of this pathway before the branching of the archaeal and bacterial domains. That is why *Archaea* and *Bacteria* exhibit the same denitrification pathway with similar enzymes.

3.1. Respiratory nitrate reductases

Denitrifying microorganisms possess nitrate reductase as the terminal enzyme of the nitrate respiration (Zumft 1997). According to the structural and catalytic characteristics, dissimilatory nitrate reductases can be classified into two groups: periplasmic nitrate reductase (Nap) and membrane-bound nitrate reductase (Nar). The Nap enzyme is mainly found in Gram negative bacteria. Its function is related to different processes depending on the organism in which it is found, as, for example, the dissipation of excess reducing power for redox balancing, scavenging nitrate in nitrate-limited conditions, and aerobic or anaerobic denitrification (Potter et al. 2001; Gavira et al. 2002; Ellington 2003). Generally, Nap enzymes are heterodimers composed of a catalytic subunit (NapA) and a cytochrome *c* (NapB) which receives electrons from NapC, a membrane cytochrome *c* (Richardson et al. 2001). The Nar enzyme is more widely distributed in nitrate-respiring microorganisms and is involved in the generation of metabolic energy using nitrate as a terminal electron acceptor. It is negatively regulated by oxygen, induced by the presence of nitrate and unaffected by ammonium. In general, Nar complex is a heterotrimer composed of: a catalytic subunit (NarG) that binds a bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor for nitrate reduction, an electron-transfer subunit with four iron-sulphur centres (NarH), and a di- *b*-heme integral membrane quinol dehydrogenase subunit (NarI). The NarG and NarH are membrane-extrinsic domains while the NarI is a hydrophobic

membrane protein which attaches the NarGH complex to the membrane (Richardson et al. 2001; Cabello, Roldán, and Moreno-Vivián 2004).

At the time of writing, all nitrate reductases purified and characterised from extremophilic microorganisms are membrane-bound Nar enzymes (Table 2). In general, enzymatic and physicochemical analysis of these enzymes indicated a marked resemblance to the bacterial NarGH complex, although there was a relevant difference between the archaeal and bacterial enzymes, related to the subcellular localisation (Yoshimatsu, Iwasaki, and Fujiwara 2002; Martinez-Espinosa et al. 2007).

In the *Archaea* domain, the purification of respiratory Nar enzymes has been reported for several denitrifying halophilic microorganisms, including three *Haloferax* species and *Haloarcula marismortui*, and the hyperthermophilic *Pyrobaculum aerophilum.* The *Haloferax denitrificans* membrane-bound Nar was the first extremophilic respiratory nitrate reductase purified and characterised. This enzyme is a heterodimer (Table 2) with a K_m for nitrate of 0.2 mM. The enzyme is able to reduce both nitrate and chlorate using methyl viologen (MV) as an electron donor in vitro, and it is inhibited by azide and cyanide. Azide is a competitive inhibitor with respect to nitrate, it may act directly in the molybdenum-containing site of the Nar, probably by metal chelation. On the other hand, cyanide is a non-competitive inhibitor of nitrate reduction. Curiously, unlike other halophilic enzymes, this nitrate reductase is stable in the absence of salt and its activity decreases with increasing salt concentrations. Moreover, it was suggested that the enzyme contains molybdenum because tungstate represses nitrate reductase synthesis (Hochstein and Lang 1991). *Haloferax volcanii* contains a trimeric respiratory Nar with a K_m for nitrate of 0.36 mM and shows a remarkable grade of thermophilicity, similarly to other halophilic enzymes (Table 2). Like the *Hfx. denitrificans* Nar, the enzyme shows optimal activity in the absence of NaCl (Bickel-Sandkotter and Ufer 1995). The *Haloarcula marismortui* Nar was first described as a homotetramer of 63 kDa subunit

(Yoshimatsu, Iwasaki, and Fujiwara 2002), but the sequence of the gene as well as SDS-PAGE in the presence of reducing agent revealed that it is a heterodimer (Table 2). The present archaeal enzyme has a K_m for nitrate of 80 μM with 2.0 M NaCl. In relation to salt dependence, the *Har. marismortui* Nar is stable even in the absence of NaCl, however, salt-dependent enhancement of the enzymatic activity was observed. Besides, it was determined by electron paramagnetic resonance (EPR) measurements that the enzyme contains a Mo-molydobterin complex and iron-sulfur centres (Yoshimatsu, Sakurai, and Fujiwara 2000). Yoshimatsu, Iwasaki and Fujiwara 2002 proposed the *Har. marismortui* Nar as a new archaeal type of membrane-bound nitrate reductase based on two pieces of evidence: the loss of the NarI membrane-associated protein as well as the sequence and structure similarity of Nar to dissimilatory selenate reductases from *Thauera selenatis,* although the halophilic enzyme does not reduce selenate. Later, the similarity of Nar to selenite reductase was also supported, since both enzymes have an aspartic residue as ligand to the molybdenum atom (Jormakka et al. 2004). In *Haloferax mediterranei,* two different nitrate reductases involved in non-assimilatory processes have been described: a dissimilatory nitrate reductase described by Álvarez-Ossorio et al. 1992 and Nar characterised by Lledó et al. 2004. The first one is a salt requiring enzyme, with an optimal activity at 89 ºC in 3.2 M NaCl and K_m for nitrate between 2.5 and 6.7 mM depending on salt concentration (Álvarez-Ossorio et al., 1992). According to its molecular mass and enzymatic properties, Lledó et al. 2004 proposed that the enzyme purified by Álvarez allows the dissipation of reducing power for redox balancing. The *Hfx. mediterranei* Nar is a heterodimer (Table 2) and its K_m for nitrate is 0.82 mM, which is in the range of the values obtained from other nitrate reductases (Zumft 1997). Like other nitrate reductases, cyanide and azide are strong inhibitors of this enzyme. Other compounds as dithiothreitol and EDTA were also tested, but they are not effective inhibitors, since they only partially decrease the activity. The *Hfx. mediterranei* Nar does not exhibit a strong dependence on temperature at the different NaCl concentration assayed (0-3.8 M NaCl), showing the maximum activity at 70 ºC for all NaCl concentrations. Therefore, this halophilic

enzyme also exhibits a remarkable thermophilicity, although the Nar activity does not show a direct dependence on salt concentration, as described for *Hfx. denitrificans* Nar (Hochstein and Lang 1991) and *Hfx. volcanii* Nar (Bickel-Sandkotter and Ufer 1995). Not all nitrate reductase activities found in halophilic archaea exhibit a similar dependence (Álvarez-Ossorio et al., 1992; Yoshimatsu, Sakurai, and Fujiwara 2000). Even though most proteins for haloarchaea are stable and active at high salt concentrations, there are some, which are either active or stable in the absence of salt. The origin of haloarchaeal enzymes which do not require salt is unclear, but it has been proposed that extreme halophiles could obtain Nar from a eubacterial source (Hochstein and Lang 1991). The absorption spectrum of the *Hfx. mediterranei* Nar shows a broad band around 400 to 415 nm indicating that this enzyme has Fe-S clusters as other Nar purified from denitrifying microoganisms (Lledó et al. 2004). The respiratory nitrate reductase of *Pyrobaculum aerophilum,* a hyperthermophilic microorganism belonging to the *Archaea* domain, was also purified (Afshar et al. 2001). The hyperthermophilic enzyme is a heterotrimer (Table 2), and contains molybdenum, iron and cytochrome *b* as cofactors and its K_m for nitrate is 58 μM. Hyperthermophilic microorganisms, such as *P. aerophilum*, are naturally exposed to high concentrations of tungsten, a heavy metal which is abundant in high-temperature environments (Kletzin and Adams 1996). It has been demonstrated that tungstate inactivates molybdoenzymes as, for example, the nitrate reductase from *Escherichia coli,* whose function was abolished by the addition of this heavy metal to the medium. Nonetheless, the hyperthermophilic respiratory nitrate reductase remains active in *P. aerophylum* cultured in the presence of high tungstate concentrations (Afshar et al. 1998). Curiously, this nitrate reductase distinguishes itself from the nitrate reductases of mesophilic bacteria and archaea by its very high specific activity (about 7 to 40 times higher) using reduced benzyl viologen as the electron donor. This fact could be an adaptation of the thermophilic enzyme to counteract the inhibition carried out by the presence of tungsten under physiological growth conditions, since *P. aerophilum* needs to support growth by nitrate respiration even when the concentration of

tungsten in the environment is high. As a typical hyperthermophilic enzyme, the *P. aerophilum* nitrate reductase exhibits its maximum activity at or above 95 ºC. Under this condition, the enzyme could be stabilised by its membrane environment, since detergent extraction results in a 4-fold loss of the thermostability of the nitrate reductase activity. From an evolutionary point of view, the enzyme from this hyperthermophilic microorganism is the oldest nitrate reductase purified and characterised. Therefore, the nitrate reductase in the last common-ancestor group of microorganisms could be a heterotrimeric enzyme (Afshar et al. 2001).

Classically, it has been considered that NarG and NarH are located in the cytoplasm and associate with NarI at the membrane potential-negative cytoplasmic face of the cytoplasmic membrane, so the nitrate reduction takes place in the inside of this membrane. This arrangement is conserved in Gram-negative bacteria and indeed, for many years, it was assumed that this orientation would be conserved among prokaryotes in general. However, the presence of a typical twin-arginine signal in *Har. marismortui* and *Hfx. mediterranei* NarG suggests that nitrate reductases from *Archaea* are translocated across the membrane by TAT export pathway. Later, the analysis of the N-terminal region of the archaeal nitrate reductases revealed the conservation of a twin-arginine motif (Martinez-Espinosa et al. 2007). The data available suggests that the NarG protein is strongly attached to the membrane fraction and requires detergent solubilisation to release it (Yoshimatsu, Sakurai, and Fujiwara 2000; Afshar et al. 2001; Lledó et al. 2004). To answer the question of whether the subunit is located on the inside or the outside of the cytoplasmic membrane, different assays were carried out with intact cells of *Har. marismortui* (Yoshimatsu, Sakurai and Fujiwara 2000) and *Hfx. mediterranei* (Martinez-Espinosa et al. 2007). The results obtained revealed that the electron donation to the active site of an enzyme is on the outside, rather than the inside, of the cytoplasmic membrane. These experiments have not yet been reported for the other archaeal Nars with Tat sequences thus far identified. Nonetheless, the available evidence supports the fact that the active site of these

archaeal Nar systems is indeed on the outside of the cytoplasmic membrane (Martinez-Espinosa et al. 2007).

Hence, based on subunit composition and subcellular location, it can be suggested that archaeal Nars are a new type of enzyme with the active site facing the outside and attached to the membrane by the cytochrome *b* (as proposed for *Hfx. mediterranei*) or stabilised by the lipid environment in the membrane as described for *P. aerophylum*. This system could be an ancient respiratory nitrate reductase, although the nitrite formed may be introduced in the nitrogen assimilation pathway. The outside location of the catalytic site of archaeal NarG has important bioenergetic implications because being energy conserving requires coupling this process to a proton-motive complex, instead of the typical redox-loop mechanism, the NarI subunit described in bacteria. On the other hand, it appears that an active nitrate-uptake system would not be required for respiratory nitrate reduction in archaea, thus increasing the energetic yield of the nitrate reduction process (Bonete et al. 2008).

The last advances related to the knowledge of respiratory Nar have been carried out in *Haloferax mediterranei* (Martínez-Espinosa, Richardson, and Bonete 2015), where the capacity of the whole cells and pure NarGH to reduce chlorate, perchlorate, bromate, iodate and selenate was tested. Not only whole *Hfx. mediterranei* but also pure NarGH are able to reduce chlorate, bromate and perchlorate, but no reduction activity is detected with iodate or selenate. Therefore, the same microorganism is able to reduce nitrate and chlorate thanks to the nitrate reductase under microaerobic or anaerobic conditions. These results are of great interest for wastewater bioremediation purposes since most of the wastewater samples containing nitrate also contain chlorate and other oxyanions. Although the removal process is not really fast (4.8 mM chlorate after 150 hours of incubation), the removed concentration using microorganisms is one of the highest described thus far (Bardiya and Bae 2005; van Ginkel, van Haperen, and van der Togt 2005). Moreover, one of the advantages of using *Hfx. mediterranei* cells or its NarGH is

that nitrate reduction is not inhibited by the presence of chlorate or perchlorate at high salt concentrations. These results make it possible to create new bioremediation process designs based on the use of haloarchaea, or even to improve the knowledge of biological chlorate reduction in early Earth or Martian environments (Martínez-Espinosa, Richardson, and Bonete 2015).

3.2. Respiratory nitrite reductases

The nitrite produced by the respiratory nitrate reductase is reduced to nitric oxide by the respiratory nitrite reductases (NiR), a key enzyme used to distinguish between denitrifiers and nitrate reducers. This reaction implies the return of nitrite to the gaseous state leading to a significant loss of fixed nitrogen from the terrestrial environment. Two types of different enzymes in terms of structure and the prosthetic metal have been reported in denitrifying bacteria: cytochrome cd_1-nitrite reductase (encoded by *nirS*) and Cu-containing dissimilatory nitrite reductase (encoded by *nirK*). The cd_1-nitrite reductase is homodimeric and contains hemes *c* and d_1 as prosthetic cofactors, whereas the Cu-nitrite reductase is homotrimeric and contains two Cu atoms per subunit molecule. Cu-NiR enzymes can be readily distinguished based on their spectra and their sensitivity to diethyldithiocarbamate (DDC) (Shapleigh and Payne 1985). The two NiR types are functionally and physiologically equivalent, but while the cd_1-nitrite reductase predominates in denitrifying bacteria, the Cu-nitrite reductase is present in a greater variety of physiological groups and bacteria from different habitats (Zumft 1997; Heylen et al. 2006).

The first evidence related to the activity of respiratory nitrite reductase was reported in *Har. marismortui* and *Hfx. denitrificans.* In 1978, the ability of *Har. marismortui* to reduce nitrite to nitric oxide in crude extracts using halophilic ferredoxin as an electron donor was identified (Werber and Mevarech 1978). Later, it was stated that the membranes from *Hfx. denitrificans*

reduce nitrite to nitric oxide by a reaction that is inhibited by DDC, which implies that the enzyme is a Cu-NiR (Tomlinson and Hochstein 1988). It was in 1996 when the first extremophilic respiratory nitrite reductase from *Hfx. denitrificans* was purified and characterised (Table 3) from soluble and membrane fractions (Inatomi and Hochstein 1996). The SDS-PAGE analysis of the purified protein resulted in the presence of two peptides of 64 and 51 kDa and the molecular mass of 127 kDa was determined by gel filtration. The authors suggested that the protein is a dimer and that the lower weight peptide was a degradation product of the larger subunit, although nowadays it is known that this data is inaccurate. Although the protein shows its maximum activity in the presence of 4 M NaCl (Table 3), there is no loss of activity when the enzyme is incubated in the absence of salt. Its absorption spectrum is characterised by maxima, located at 462, 594 and 682 nm, which disappeared after the addition of dithionite, concluding that this enzyme belongs to the green Cu-NiR. The assays carried out in the presence of DDC determined that this reagent inhibits the activity of NiR at relatively low concentrations, supporting the fact that this enzyme is a Cu-Nir. Although the membrane-bound Cu-NiR was not totally purified, its characteristics are similar to those of the enzyme purified from the soluble fraction (Inatomi and Hochstein 1996). The respiratory nitrite reductase was also purified from the halophilic archaea *Har. marismortui* (Table 3) (Ichiki, Tanaka, and Mochizuki 2001). The SDS-PAGE of the purified enzyme gave two protein bands, as in *Hfx. denitrificans*, whose molecular masses are 46 and 42 kDa. N-terminal amino acid sequences were determined obtaining that the sequence of the 46 kDa subunit after the 17^{th} amino acid is identical to the N-terminal sequence of the 42 kDa subunit, except for the 16 amino acid difference. The absorption spectrum of the purified Cu-NiR shows absorption maxima at 465 and 600 nm with a small shoulder around 820 nm in the visible region, suggesting that this halophilic enzyme is a blue Cu-NiR. EPR spectroscopy provided evidence that one molecule each of the type 1 and type 2 Cu centres is present in a subunit of this enzyme. The Cu-NiR is activated in the presence of high salt concentrations, reaching its maximum at NaCl concentrations higher than 2M while being

denaturated in the absence of salt, as most halophilic enzymes. The physiological electron donor remains unclear, although halocyanin could play this role in some Archaea. Analysis of the amino acid sequence of the *Har. marismortui* Cu-NiR suggests that the minimum functional unit of the archaeal enzyme is a trimer constituted by identical subunits. Furthermore, phylogenic analysis indicated that the halophilic enzyme is in quite a close relationship with the enzyme from the gonorrhoeal pathogen *Neisseria gonorrhoeae.* The structural similarities between these two enzymes suggest the lateral transfer of the *nirK* gene between halophilic archaea and the pathogenic proteobacteria (Ichiki, Tanaka, and Mochizuki 2001). The last studies related to respiratory nitrite reductases in extremophilic microorganisms have been carried out in *Hfx. mediterranei* (Esclapez et al. 2013). The respiratory nitrite reductase from *Hfx. mediterranei* was expressed in the halophilic host *Hfx. volcanii*. The enzymatic activity of the recombinant protein was detected in both cellular fractions (cytoplasmic fraction and membranes) and in the culture media. The enzyme isolated from the cytoplasmic fraction and the culture media were purified and characterised (Table 3). The cytoplasmic NirK is a trimeric protein which shows its maximum activity in the presence of 2 M of salt (NaCl or KCl) and at around 70 ºC. The sequence and structural analysis of this enzyme revealed the presence of four significant regions. The first of them involves the presence of a region similar to the distinctive Tat motif; therefore, it is probable that this region is acting as the Tat motif for the protein to be exported via the Tat system. The second conserved domain shows the presence of two possible cutting targets for proteases, located in positions 27 and 34 from the N-terminal end. The presence of this sequence is associated with the Tat signals, since the mature protein exportation through the cytoplasmic membrane requires the removal of the signal peptide. Finally, seven residues in copper binding were identified sited in a central position inside the chain. These residues may coordinate the type 1 and type 2 copper centres proposed for the Cu-NiR proteins. On the other hand, the UV-vis spectrum shows two different maxima absorption at 453 nm and 587 nm, suggesting that the enzyme belongs to the green Cu-NirK group. In order to elucidate the

composition of the native enzyme, an exhaustive study was carried out. A native PAGE of pure enzyme followed by activity NiR staining revealed that the intracellular Cu-NiR is composed of at least six different isoforms of the enzyme. The SDS-PAGE of each of the six bands showed that each one exhibits a different combination of two isoforms of 44.3 and 39.8 kDa, the smaller form being the predominant isoform protein in this cellular fraction. Taking into account the two cleavage sites present in the *Hfx. mediterranei* Cu-NiR sequence, it is possible to propose that the expression of recombinant proteins could conclude with the maturation of the initial polypeptide through a cut in one of the two targets present at its N-terminal extreme. Finally, the two possible isoforms could combine to form a pool of active trimers. This maturation mechanism could also explain why it is possible to observe two bands with slightly different masses to those NiR purifications carried out in *Har. marismortui* or *Hfx. denitrificans*. The extracellular pool of recombinant NiR was also purified and characterised. No significant biochemical differences are found between extracellular and intracellular NiR. However, the comparison of the isoform expression pattern of both samples reveals a remarkable difference. In the intracellular fraction, the 39.8 kDa isoform is predominant and the 44.3 kDa isoform appears slightly, while in the extracellular fractions the 44.3 kDa isoform is the predominant or even the only one. This data supports the fact that the halophilic NiR is involved in a maturation process and in exportation via the Tat system. In order to elucidate the maturation process of the protein and its exportation via the Tat system, the first eight amino acids of the two isoforms that appear in the SDS-PAGE were sequenced. The results show that the 44.3 kDa isoform is obtained as a consequence of the cleavage between the 33rd and 34th residues. Therefore, this isoform may be exported via the Tat system, being cleaved by the twin arginine signal sequence after its translocation to an extracellular medium. The sequence of the small isoform, 39.8 kDa, starts in the 52nd position. No cutting target is predicted in this location so , it seems more likely that this isoform could be obtained as a result of an alternative translation mechanism (Hering et al. 2009) or mRNA processing rather than as a cleavage process. Once the two possible

transcripts are translated, a random trimerisation occurs between these two possible isoforms. This process originates the pool of possible isoforms found both inside and outside the cell. Finally, the Tat system of *Hfx. volcanii* facilitates the exportation of recombinant Cu-NiR active trimers whenever any of the three contain the signal peptide. In the process of exportation through the membrane, the signal peptides of the large isoform are cleaved. Thus, outside the cell it can find a mixture of the cleaved and signal-avoided NiR, prevailing over the large isoform. In contrast, only the trimers remain inside the cell exclusively composed by untargeted peptides that not are able to cross the membrane and go outside the cell. This discrimination between targeted and non-targeted peptides looks like a mechanism for regulating the system and final NiR location. The location of recombinant NiR outside the cell agrees with the results related to the extracellular location of membrane-associated NarGH from *Hfx. mediterranei* detailed above. For this reason, there is increasing evidence that the complete reduction of NO_3^- to N_2 could take place through an extracellular enzymatic complex, which is part of the machinery associated with the outer face of the cytoplasmic membrane, while the rest of soluble enzymes and metabolites are embedded in the porous S-layer. This atypical respiratory complex orientation offers advantages to these microorganisms in oxygen-poor environments such as hypersaline ecosystems. With this modification, the presence of NO_3^- transporters is not required and the electron acceptor can be reduced directly in the growth media, increasing the efficiency of the process. Finally, the mobilisation of the proteins involved in NO_3^- respiration appears to be regulated by the Tat system so that they are folded and loaded with metallic cofactors inside the cell before being exported out of the cell, where they will take part in their physiological role.

Regarding the cd_1-nitrite reductase, a *nirS* homologous gene has been identified in the genome of the hyperthermophile *P. aerophilum* (Cabello, Roldán, and Moreno-Vivián 2004), although the enzyme has not been purified or characterised at the time of writing this review. However, polarographic studies carried out with purified membranes revealed that this nitrite

reductase uses menaquinol as an electron donor (de Vries and Schröder 2001). This data could suggest the existence of cd_1-nitrite reductase in thermophilic microorganisms, while halophilic microorganisms possess Cu-containing nitrite reductase.

3.3. Nitric oxide reductases.

Nitric oxide is the product of the respiratory nitrite reductase. This compound is toxic to cells and for that reason, it is immediately reduced to N_2O by nitric oxide reductases (Nor). Several enzymes with Nor activities have been described. In fungal denitrification, Nor enzymes are soluble monomeric proteins belonging to the cytochrome P-450 family (Nakahara et al 1993). In most denitrifying bacteria, Nor is a heterodimer membrane complex of a cytochrome *c* (encoded by *norC*) and a cytochrome *b* with 12 transmembrane regions (encoded by *norB*). This enzyme is known as cNor. On the other hand, a monomeric Nor with 14 transmembrane regions has been described in other bacteria. This enzyme is called qNor due to its quinol-oxidising activity. The qNor enzyme is similar to the NorB subunit, although it contains an N-terminal extension, with a quinone-binding site, absent in NorB (Hendriks et al 2000; Cabello, Roldán, and Moreno-Vivián 2004; Bonete et al. 2008).

Despite the fact that there is only one study related to the characterisation of Nor in extremophilic microorganisms thus far, gas formation from nitrite has been reported for a number of archaeal microorganisms as, for example, *P. aerophilum*, *Hfx. denitrificans, Hfx. mediterranei, Haloarcula hispanica* and *Har. marismortui* (Zumft and Kroneck 2006) and for the halophilic bacteria *Halomonas halodenitrificans* (Sakurai et al. 2005). The first studies of Nor in *P. aerophilum* demonstrated the formation of N_2O using menaquinol as an electron donor and the presence of Nor bound to its membrane (de Vries and Schröder 2001). Later, in 2003, the nitric oxide reductase from the hyperthermophilic microorganism was purified and characterised (de Vries et al. 2003). The enzyme is a monomeric protein of 78.8 kDa and contains

heme and nonheme iron in a 2:1 ratio. The EPR, resonance Raman and UV-vis spectroscopy analyses show that one of the hemes is a bis-His-coordinated low spin, whereas the other heme adopts a high spin configuration. In comparison with other thermophilic enzymes, the thermostability of the isolated Nor from *P. aerophilum* is very low, while the enzyme bound to the membrane is in the average. It is possible that the removal of the membrane lipids by detergent contributes to the lower thermostability, although it is unclear how this would occur (de Vries et al 2003).

Regarding the genetic analysis, *nor* genes have been identified in few genomes of halophilic and hyperthermophilic microorganisms. *Har. hispanica, Har. marismortui, Hfx. mediterranei, Hfx. volcanii* and *P. aerophilum* contain in their genomes a copy of a *norB* gene, and up to now, there is only one example of *norZ* gene in the halophile *Halogeometricum borinquense*, suggesting a possible case of horizontal gene transfer between *Bacteria* and *Archaea*.

3.4. Nitrous oxide reductases.

The reduction of N_2O to N_2 is the last step of denitrification, which is catalysed by nitrous oxide reductases (Nos). This reaction is of great environmental importance because it closes the N-cycle. N_2O is less toxic than NO or nitrite and the vast majority of microorganisms could manage without converting N_2O to N_2. Nonetheless, there are many bacteria, which contain nitrous oxide reductases encoded by the *nosZ* gene. These bacterial enzymes are located in the periplasm and they are multicopper homodimers whose electron donor is the cytochrome *c* or pesudoazurin (Zumft 1997). Each monomer contains two copper centres, a di-copper cluster CuA resembling that of cytochrome oxidase, and a CuZ cluster which consists of 4 Cu atoms ligated by 7 His residues (Rasmussen et al 2000). The putative *nosZ* gene has been identified in the halophilic archaea *Har. marismortui*, *Hfx. mediterranei, Haloarcula hispanica* and *Hfx.*

denitrificans. The gene which encodes the nitrous oxide reductase has been also identified in other halophilic archaea such as *Halopiger xanaduensis, Halogeometricum borinquense* and *Halorubrum lacusprofundii.* These genes have not been classified as *nosZ*. *P. aerophilum* and neither has the *nosZ* gene, but, recently, a thermophilic multicopper oxidase which shows nitrous oxide reductase activity has been purified (Fernandes et al. 2010). This multicopper oxidase is a thermoactive and thermostable metallo-oxidase, it follows a ping-pong mechanism, its sequence contains a putative TAT-dependent signal peptide and it shows a 3-fold higher catalytic efficiency when it uses N_2O as an electron acceptor compared to when it uses dioxygen, the typical oxidising substrate of muticopper oxidases. This fact represents a completely new function among multicopper oxidases, and it could be a novel archaeal nitrous oxide reductase which is probably involved in the final step of the denitrification pathway of *P. aerophilum* (Fernandes et al. 2010).

4. Future prospects.

Denitrification is the major biological pathway for N loss from ecosystems, and the gaseous intermediates, nitric oxide and nitrous oxide, have implications in global warming (Prather et al. 2012). Nitrous oxide has become the third most important anthropogenic greenhouse gas (IPCC 2014), and it is today's single most important ozone-depleting emission (Ravishankara et al., 2009). When aiming to mitigate N_2O emissions, an accurate understanding of the biochemical processes responsible for N_2O production is crucial (Richardson et al. 2009).

The potential environmental importance of denitrification has led to numerous measurements of the process in a range of habitats. To know the extent of this process in extreme environments will be essential to understand the contribution of the denitrifying microorgansims to the greenhouse effect. Unfortunately, denitrification is very difficult to measure, mainly in extreme environments or extreme microcosms. So, the existing methods are problematic and the methodology still needs development. Although one review on methods for measuring denitrification is available (Groffman et al. 2006), the development of molecular approaches is necessary. In the pre-genomic era, establishing whether a microorganism was a denitrifier entailed testing its ability to grow under O_2-limiting conditions with nitrate most frequently provided as a terminal oxidant (Payne 1981). Therefore, nearly all denitrifiers

characterised were complete denitrifiers that showed robust growth under denitrifying conditions. However, with genome analysis supplanting phenotypic assignment as the principal means of identifying denitrifiers, both complete and partial denitrifiers can be identified (Shapleigh 2013).

The application of molecular methods to study denitrification can lead to understanding how the composition and physiology of the microbial community affects N transformations in the environment. Bacteria, fungi and archaea are capable of denitrification and it can be considered to be a community process, as many denitrifying organisms do not produce the complete suite of enzymes and could work together to complete the process (Wallenstein 2006). Understanding the responses of microbial communities to environmental factors and the impact of the community composition on the rate of denitrification is essential to know this process and its impact on gas emissions, even more so in extreme environments, where the denitrification community has been less studied.

Approaches based on the direct extraction of DNA from the natural environment and PCR amplifications can overcome limitations due to archaea and bacteria cultivation and isolation (Demanèche et al. 2009). It must also be taken into account that denitrification is, nearly exclusively, a facultative respiratory pathway and, in some environments, genes for denitrification are often detected where there is no measurable denitrification activity (Groffman 2006). A few studies have attempted to extract mRNA from environmental samples and use reverse transcriptase PCR to measure the active denitrified community (Nogales et al. 2002). This could be a potentially powerful approach.

The most fundamental need for molecular studies of denitrifier communities is an improved database of functional genes. Until now, most of the molecular tools used for studying denitrifier community composition begin by selectively amplifying the target functional genes using PCR. The problem was the degree to which the selective primers target all variants of these genes. The inventory of genes involved in denitrification and the extent of their diversity in extremophilic environments are yet to be explored, and the characterisation of whole or partial denitrification pathways with gene sequences becomes necessary (Wallenstein 2006). Previous analysis of genome organisation and comparative genomics in bacterial and archaeal genomes indicated complex genetic bases of the process and allowed the identification of new putative denitrifying genes (Philippot, 2002). A metagenomic approach has been carried out in order to identify and characterise gene clusters involved in the denitrification process in soil bacteria and the analysis led to the identification and the subsequent characterisation of nine denitrification

gene clusters (Demanèche et al. 2009). In archaea, there is not an extensive analysis on the gene cluster organisation involved in denitrification but, taking into account the previous works (Cabello et al, 2004; Philipot, 2002), the variability in archaeal genomes will also be important, and their analysis will shed light on the denitrification processes carried out by extremophiles.

The predictive genomic data must be confirmed by experimental data. Isolation and characterisation of the proteins and complexes involved in the denitrification process are compulsory to understand it. As it has been mentioned, there are some works focused on archaeal nitrate and nitrite reductases but much less is known about nitric oxide and nitrous oxide reductases. The characterisation of these enzymes and the identification of the electron transport intermediates are necessary to understand the extent of the final step of denitrification in extremophiles and the conditions in which the process is more active.

Other important consideration is the regulation of denitrification. In most denitrifier bacteria, the expression of genes encoding these proteins depends on the presence of nitrogen oxides. In general, Nir and Nor respond to NO stimuli and Nos responds to N_2O. Studies with model organisms have found that reduction of nitrate and nitrite to gaseous products occurs at low O_2 (Shapleigh, 2011; Zumft, 1997). In particular, the regulation of *nir* and *nor* genes is especially sensitive to O_2. A review of transcriptional regulation in bacteria is available (Shapleigh 2013) but there is no data on transcriptional regulation in archaea.

The knowledge on the regulation of denitrification in archaea would be very important, not only to understand the physiological conditions in which denitrification becomes important to the organisms, but also to improve potential biotechnological applications of the pathway in bioremediation or to understand the contribution of halophilic archaea to N-gas emissions (Najera et al. 2012; Martinez-Espinosa et al. 2015).

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REFERENCES

Afshar, S, C Kim, HG Monbouquette, and I Schroder. 1998. “Effect of Tungstate on Nitrate Reduction by the Hyperthermophilic Archaeon Pyrobaculum Aerophilum.” *Applied and*

Environmental Microbiology 64 (8): 3004–8. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=106806&tool=pmcentrez&rendertype=abstract.

Afshar, S, E Johnson, S de Vries, and I Schroeder. 2001. “Properties of a Thermostable Nitrate Reductase from the Hyperthermophilic Archaeon Pyrobaculum Aerophilum Properties of a Thermostable Nitrate Reductase from the Hyperthermophilic Archaeon Pyrobaculum Aerophilum.” *Journal of Bacteriology* 183 (19): 5491–95. doi:10.1128/JB.183.19.5491.

Alonso, A. and Camargo, J.A. (2006) Toxicity of nitrite to three species of freshwater invertebrates. Environmental Toxicology, 21, pp.90–94

Alvarez-Ossorio, MC, FJG Muriana, FF de la Rosa, and AV Relimpio. 1992. "Purification and chareacterization of nitrate reductase from the halophile archaebacterium Haloferax mediterranei." *Z Naturforsch* 47C: 670-676.

Amo, T. et al., 2002. Pyrobaculum calidifontis sp. nov., a novel hyperthermophilic archaeon that grows in atmospheric air. *Archaea (Vancouver, B.C.)*, 1(2), pp.113–121

Andrei, A.Ş., Banciu, H.L. & Oren, A., 2012. Living with salt: Metabolic and phylogenetic diversity of archaea inhabiting saline ecosystems. FEMS Microbiology Letters, 330(1), pp.1–9.

Bardiya, N, and JH Bae. 2005. “Bioremediation Potential of a Perchlorate-Enriched Sewage Sludge Consortium.” *Chemosphere* 58 (1): 83–90. doi:10.1016/j.chemosphere.2004.09.001.

Bickel-Sandkotter, S, and M Ufer. 1995. “Properties of a Dissimilatory Nitrate Reductase from the Halophilic Archaeon Haloferax Volcanii.” *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences* 50: 365–72.

Bonete, MJ, RM Martínez-Espinosa, C Pire, B Zafrilla, and DJ Richardson. 2008. “Nitrogen Metabolism in Haloarchaea.” *Saline Systems* 4: 9. doi:10.1186/1746-1448-4-9.

Bourbonnais, a. et al., 2012. Activity and abundance of denitrifying bacteria in the subsurface biosphere of diffuse hydrothermal vents of the Juan de Fuca Ridge. *Biogeosciences*, 9(11), pp.4661–4678.

Cabello, P, MD Roldán, and C Moreno-Vivián. 2004. “Nitrate Reduction and the Nitrogen Cycle in Archaea.” *Microbiology* 150: 3527–46. doi:10.1099/mic.0.27303-0.

Čuhel, J. et al., 2010. Insights into the effect of soil pH on N2O and N2 emissions and denitrifier community size and activity. *Applied and Environmental Microbiology*, 76(6), pp.1870–1878.

Demanèche, S., Philippot, L., David, M. M., Navarro, E., Vogel, T. M., and Simonet, P. 2009. Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. *Applied and Environmental Microbiology*, *75*(2), 534–7.

de Vries, S, and I Schroder. 2001. “Comparison between the Nitric Oxide Reductase Family and Its Aerobic Relatives, the Cytochrome Oxidases,” *Biochemical Society Transactions* 30:662-667. http://www.biochemsoctrans.org/bst/030/0662/bst0300662.htm.

de Vries, S, MJF Strampraad, S Lu, P Moënne-Loccoz, and I Schröder. 2003. “Purification and Characterization of the MQH2:NO Oxidoreductase from the Hyperthermophilic Archaeon Pyrobaculum Aerophilum.” *The Journal of Biological Chemistry* 278 (38): 35861–68. doi:10.1074/jbc.M300857200.

Ellington, MJK. 2003. “Rhodobacter Capsulatus Gains a Competitive Advantage from Respiratory Nitrate Reduction during Light-Dark Transitions.” *Microbiology* 149 (4): 941–48. doi:10.1099/mic.0.26090-0.

Esclapez, J, B Zafrilla, RM Martínez-Espinosa, and MJ Bonete. 2013. “Cu-NirK from Haloferax Mediterranei as an Example of Metalloprotein Maturation and Exportation via Tat System.” *Biochimica et Biophysica Acta - Proteins and Proteomics* 1834 (6). Elsevier B.V.: 1003–9. doi:10.1016/j.bbapap.2013.03.002.

Fernandes, AT, JM Damas, S Todorovic, R Huber, MC Baratto, R Pogni, CM Soares, and LO Martins. 2010. “The Multicopper Oxidase from the Archaeon Pyrobaculum Aerophilum Shows Nitrous Oxide Reductase Activity.” *FEBS Journal* 277: 3176–89. doi:10.1111/j.1742-4658.2010.07725.x.

Gavira, M, MD Roldan, F Castillo, and C Moreno-Vivian. 2002. “Regulation of Nap Gene Expression and Periplasmic Nitrate Reductase Activity in the Phototrophic Bacterium Rhodobacter Sphaeroides DSM158.” *Journal of Bacteriology* 184 (6): 1693–1702. doi:10.1128/JB.184.6.1693-1702.2002.

Groffman, P. M., Altabet, M. A., Böhlke, J. K., Butterbach-Bahl, K., David, M. B., Firestone, M. K., … Voytek, M. A. 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. Ecological Applications, 16(6), 2091-2122.

Hering, O, M Brenneis, J Beer, B Suess, and J Soppa. 2009. “A Novel Mechanism for Translation Initiation Operates in Haloarchaea.” *Molecular Microbiology* 71 (6): 1451–63. doi:10.1111/j.1365-2958.2009.06615.x.

Heylen, K, D Gevers, B Vanparys, L Wittebolle, J Geets, N Boon, and P de Vos. 2006. “The Incidence of nirS and nirK and Their Genetic Heterogeneity in Cultivated Denitrifiers.” *Environmental Microbiology* 8 (11): 2012–21. doi:10.1111/j.1462-2920.2006.01081.x.

Hochstein, LI, and F Lang. 1991. “Purification and Properties of a Dissimilatory Nitrate Reductase from Haloferax Denitrificans.” *Archives of Biochemistry and Biophysics* 288 (2): 380–85. http://www.ncbi.nlm.nih.gov/pubmed/1654831.

Howarth, R.W. (2004) Human acceleration of the nitrogen cycle: drivers, consequences, and steps toward solutions. Water Sci. Technol. 49, 7–13

Huang, Y. et al., 2014. Acidophilic denitrifiers dominate the N2O production in a 100-year-old tea orchard soil. *Environmental Science and Pollution Research*, 22(6), pp.4173–4182. Available at: http://link.springer.com/10.1007/s11356-014-3653-6.

Ichiki, H, Y Tanaka, and K Mochizuki. 2001. "Analysis of Cu-Containing Dissimilatory Nitrite Reductase from a Denitrifying Halophilic Archaeon , Haloarcula Purification , Characterization , and Genetic Analysis of Cu-Containing Dissimilatory Nitrite Reductase from a Denitrifying Halophilic Archaeon" *Journal of Bacteriology* 183 (14): 4149–56. doi:10.1128/JB.183.14.4149.

Inatomi, K, and L I. Hochstein. 1996. "The Purification and Properties of a Copper Nitrite Reductase from Haloferax denitrificans." *Current Microbiology* 32 (2): 72–76. doi:10.1007/s002849900013.

IPCC 2014, IPCC web page http://www.ipcc.ch/

Jones, C.M. & Hallin, S., 2010. Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. *The ISME journal*, 4(5), pp.633–641. Available at: http://dx.doi.org/10.1038/ismej.2009.152.

Jormakka, M, D Richardson, B Byrne, and S Iwata. 2004. "Architecture of NarGH Reveals a Structural Classification of Mo-bisMGD Enzymes." *Structure* 12 (1): 95–104. doi:10.1016/j.str.2003.11.020.

Kletzin, A, and MWW Adams. 1996. "Tungsten in Biological Systems." *FEMS Microbiology Reviews* 18 (1). The Oxford University Press: 5–63. doi:10.1111/j.1574-6976.1996.tb00226.x.

Liu, B. et al., 2010. Denitrification gene pools, transcription and kinetics of NO, N 2O and N2 production as affected by soil pH. *FEMS Microbiology Ecology*, 72(3), pp.407–417.

Lledó, B, RM Martínez-Espinosa, FC Marhuenda-Egea, and MJ Bonete. 2004. "Respiratory Nitrate Reductase from Haloarchaeon Haloferax Mediterranei: Biochemical and Genetic Analysis." *Biochimica et Biophysica Acta - General Subjects* 1674: 50–59. doi:10.1016/j.bbagen.2004.05.007.

Martinez-Espinosa, RM, EJ Dridge, MJ Bonete, JN Butt, CS Butler, FSargent, and DJ Richardson. 2007. "Look on the Positive Side! The Orientation, Identification and Bioenergetics of 'Archaeal' Membrane-Bound Nitrate Reductases." *FEMS Microbiology Letters* 276: 129–39. doi:10.1111/j.1574-6968.2007.00887.x.

Martínez-Espinosa, RM, DJ Richardson, and MJ Bonete. 2015. "Characterisation of Chlorate Reduction in the Haloarchaeon Haloferax Mediterranei." *Biochimica et Biophysica Acta (BBA) - General Subjects* 1850 (4). Elsevier B.V.: 587–94. doi:10.1016/j.bbagen.2014.12.011.

Mosier, a. R., 1998. Soil processes and global change. *Biology and Fertility of Soils*, 27(3), pp.221–229.

Nájera-Fernández, C., Zafrilla, B., Bonete, M. J., and Martínez-Espinosa, R. M. 2012. Role of the denitrifying *Haloarchaea* in the treatment of nitrite-brines. *International Microbiology*. doi:10.2436/im.v15i3.64744

Nakara, K, T Tanimoto, K Hatano, K Usuda, and H Shoun. 1993. "Cytochrome P-450 55A1 (P450dNIR) acts as nitric oxide reductase employing NADH as direct electron donor." *Jouranl of Biological Chemistry* 268:8350-8355.

Nogales, B., Timmis, K. N., Nedwell, D. B., and Osborn, A. M. 2002. Detection and Diversity of Expressed Denitrification Genes in Estuarine Sediments after Reverse Transcription-PCR Amplification from mRNA. *Applied and Environmental Microbiology*, *68*(10), 5017–5025.

Oren, a, 2002. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *Journal of industrial microbiology & biotechnology*, 28(1), pp.56–63.

Payne, W. J. 1981. Denitrification. John Wiley & Sons Inc.

Philippot, L. 2002. Denitrifying genes in bacterial and archaeal genomes. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, *1577*(3), 355–376.

Potter, L, H Angove, DJ Richardson, and JA Cole. 2001. "Nitrate reduction in the periplasm of Gram negative bacteria." *Advances in Microbial Physiology* 45: 51-112.

Prather, M. J., Holmes, C. D., and Hsu, J. 2012. Reactive greenhouse gas scenarios: Systematic exploration of uncertainties and the role of atmospheric chemistry. *Geophysical Research Letters*, *39*(9), doi:10.1029/2012GL051440

Rasmussen, T, BC Berks, J Sanders-Loehr, DM Dooley, WG Zumft, and AJ Thomson. 2000. "The catalytic center in nitrous oxide reductase, CuZ, is a copper-sulfide cluster." *Biochemistry* 39:12753-12756.

Ravishankara, A. R., Daniel, J. S., and Portmann, R. W. 2009. Nitrous oxide (N_2O): the dominant ozone-depleting substance emitted in the 21st century. *Science 326*(5949), 123–5.

Richardson, DJ, BC Berks, D Russell, S Spiro, and C J Taylor. 2001. "Functional, Biochemical and Genetic Diversity of Prokaryotic Nitrate Reductases." *Cellular and Molecular Life Sciences : CMLS* 58: 165–78. doi:10.1007/PL00000845.

Richardson, D., Felgate, H., Watmough, N., Thomson, A., and Baggs, E. 2009. Mitigating release of the potent greenhouse gas N(2)O from the nitrogen cycle - could enzymic regulation hold the key? *Trends in Biotechnology*, *27*(7), 388–97.

Sánchez-Andrea, I. et al., 2012. Screening of anaerobic activities in sediments of an acidic environment: Tinto River. *Extremophiles*, 16(6), pp.829–839.

Sakurai, T, S Nakashima, K Kataoka, D Seo, and N Sakurai. 2005. "Diverse NO Reduction by Halomonas Halodenitrificans Nitric Oxide Reductase." *Biochemical and Biophysical Research Communications* 333: 483–87. doi:10.1016/j.bbrc.2005.05.149.

Santoro, A.E. et al., 2006. Denitrifier Community Composition along a Nitrate and Salinity Gradient in a Coastal Aquifer Denitrifier Community Composition along a Nitrate and Salinity Gradient in a Coastal Aquifer. *Applied and Environmental Microbiology*, 72(3), pp.2102–2109.

Shapleigh, WJ, and WJ Payne. 1985. "Differentiation of cd_1 cytochrome and copper nitrite reductase production in denitrifiers." *FEMS Letter* 26:275-279.

Shapleigh, J. P. 2013. Denitrifying prokaryotes. In The prokaryotes (pp. 405-425). Springer Berlin Heidelberg.

Shen, Q.R., Ran, W. and Cao, Z.H. (2003) Mechanisms of nitrite accumulation occurring in soil nitrification. Chemosphere, 50, pp.747-753.

Šimek, M., Jíšová, L. & Hopkins, D.W., 2002. What is the so-called optimum pH for denitrification in soil? *Soil Biology and Biochemistry*, 34(9), pp.1227–1234.

Stetter, K.O., 1999. Extremophiles and their adaptation to hot environments. *FEBS Letters*, 452(1-2), pp.22–25.

Tomilson, GA, and LI Hochstein. 1988. Abstracts of the Annual Meeting of the American Society for Microbiology. 1988:209.

Van Ginkel, CG, AM van Haperen, and B van der Togt. 2005. “Reduction of Bromate to Bromide Coupled to Acetate Oxidation by Anaerobic Mixed Microbial Cultures.” *Water Research* 39 (1): 59–64. doi:10.1016/j.watres.2004.09.005.

Van Wolferen, M. et al., 2013. How hyperthermophiles adapt to change their lives: DNA exchange in extreme conditions. *Extremophiles*, 17(4), pp.545–563.

Volkl, P. et al., 1993. Pyrobaculum aerophilum sp. nov, a Novel Nitrate- Reducing Hyperthermophilic Archaeum. *Applied and Environmental Microbiology*, 59(9)(9), pp.2918–2926.

Wang, F. et al., 2009. GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent. *Proceedings of the National Academy of Sciences of the United States of America*, 106(12), pp.4840–4845.

Wallenstein, M. D., Myrold, D. D., Firestone, M., and Voytek, M. 2006. Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecological applications, 16(6), 2143-2152.

Werber, MM, and M Mevarech. 1978. "Induction of a dissimilatory reduction pathway of nitrate in *Halobacterium* of the Dead Sea." *Archives Biochemistry Biophysics* 186:60-65.

Yoshimatsu, K. 2000. “Purification and Characterization of Dissimilatory Nitrate Reductase from a Denitrifying Halophilic Archaeon, Haloarcula Marismortui.” *FEBS Letters* 470: 216–20. doi:10.1016/S0014-5793(00)01321-1.

Yoshimatsu, K, T Iwasaki, and T Fujiwara. 2002. “Sequence and Electron Paramagnetic Resonance Analyses of Nitrate Reductase NarGH from a Denitrifying Halophilic Euryarchaeote Haloarcula Marismortui.” *FEBS Letters* 516: 145–50. doi:10.1016/S0014-5793(02)02524-3.

Yoshimatsu, K, T Sakurai, and T Fujiwara. 2000. “Purification and Characterization of Dissimilatory Nitrate Reductase from a Denitrifying Halophilic Archaeon, Haloarcula Marismortui.” *FEBS Letters* 470 (2): 216–20. doi:10.1016/S0014-5793(00)01321-1.

Zumft, W G. 1997. “Cell Biology and Molecular Basis of Denitrification.” *Microbiology and Molecular Biology Reviews : MMBR* 61 (4): 533–616.

Zumft, WG., and PMH Kroneck. 2006. *Respiratory Transformation of Nitrous Oxide (N2O) to Dinitrogen by Bacteria and Archaea*. *Advances in Microbial Physiology*. Vol. 52. doi:10.1016/S0065-2911(06)52003-X.

Table 1. Summary of the purified and characterised denitrifying enzymes from extremophiles.

MICROORGANISMS	**DOMAIN**	**PURIFIED AND CHARACTERISED ENZYMES**	**REFERENCE**
Haloferax mediterranei	*Archaea*	Respiratory nitrate reductase Respiratory nitrite reductase	Lledó et al. 2004 Martinez-Espinosa et al. 2007 Esclapez et al. 2013
Haloarcula marismortui	*Archaea*	Respiratory nitrate reductase Respiratory nitrite reductase	Yoshimatsu, Sakurai, and Fujiwara 2000 Yoshimatsu, Iwasaki, and Fujiwara 2002 Ichiki, Tanaka, and Mochizuki 2001
Haloarcula denitrificans	*Archaea*	Respiratory nitrate reductase Respiratory nitrite reductase	Hochstein and Lang 1991 Inatomi and Hochstein 1996
Haloferax volcanii	*Archaea*	Respiratory nitrate reductase	Bickel-Sandkotter and Ufer 1995
Pyrobaculum aerophilum	*Archaea*	Respiratory nitrate reductase	Afshar et al. 2001

Table 2. Characteristics of respiratory nitrate reductases from extremophiles.

MICROORGANISM	**STRUCTURE FEATURES**	**OPTIMAL ACTIVITY CONDITIONS**	**SUBSTRATES**	**INHIBITORS**
Haloferax denitrificans	Heterodimer: 116 and 60 kDa	Absence of salt	Nitrate Chlorate	Azide Cyanide
Haloferax volcanii	Heterotrimer: 100, 61 and 31 kDa	Absence of salt Temperature 80 ºC pH 7.5	Nitrate	Azide Cyanide Thiocyanate
Haloarcula marismortui	Heterodimer: 117 and 47 kDa	2 M NaCl pH 7.0	Nitrate Chlorate	Non determined
Haloferax mediterranei	Heterodimer: 112 and 61.5 kDa	Absence of salt pH 7.9 at 40 ºC pH 8.2 at 60 ºC	Nitrate Chlorate Perchlorate Bromate	Dithiothreitol Azide Cyanide EDTA
Pyrobaculum aerophilum	Heterotrimer: 130, 52 and 32 kDa	pH 6.5 Temperature 95 ºC	Nitrate Chlorate	Azide Cyanide

Table 3. Characteristics of respiratory Cu-nitrite reductases from extremophiles.

MICROORGANISM	**STRUCTURE FEATURES**	**OPTIMAL ACTIVITY CONDITIONS**	**K_m nitrite**	**INHIBITORS**	**ABSORPTION PEAKS (nm)**
Haloferax denitrificans	Homotrimer	4 M NaCl pH 4.8 -5.0	4.6 mM	DDC	462, 594, 682
Haloarcula marismortui	Homotrimer	2 M NaCl pH 8.0	*	DDC EDTA	465, 600
Haloferax mediterranei	Homotrimer	2 M NaCl pH 5.5	4 mM	*	453, 587

(*) Not determined