Denitrifying haloarchaea within the genus *Haloferax* display divergent respiratory phenotypes, with implications for their release of nitrogenous gases

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Originality/significance statement

This is the first detailed study of respiratory physiology in denitrifying Archaea, specifically representatives of the haloarchaea. They thrive under high salt conditions and with the currently increasing aridity and salinity in temperate regions such organisms are becoming increasingly important participants in the global biogeochemical cycles. Until now, information about the potential release of nitrogenous intermediates from these organisms during anaerobic respiration is limited. This work is a contribution to rectifying this shortcoming as it describes the denitrification phenotypes of three of the most well-known Haloferax species with unsurpassed resolution. We find phenotypic variants similar to those reported in bacteria, suggesting comparable regulatory circuits. Moreover, we find that the presence of structural denitrification genes does not necessarily predict an organism's propensity for releasing gaseous intermediates, underscoring the importance of including physiological studies when evaluating potential candidates for application in bioremediation.

Summary

Haloarchaea are extremophiles, generally thriving at high temperatures and salt concentrations, thus with limited access to oxygen. As a strategy to maintain a respiratory metabolism, many halophilic archaea are capable of denitrification. Among them are members of the genus *Haloferax*, which are abundant in saline/hypersaline environments.

Three reported haloarchaeal denitrifiers, *Haloferax mediterranei*, *Haloferax denitrificans* and *Haloferax volcanii*, were characterized with respect to their denitrification phenotype. A semi-automatic incubation system was used to monitor the depletion of electron acceptors and

accumulation of gaseous intermediates in batch cultures under a range of conditions. Out of the species tested, only *H. mediterranei* was able to consistently reduce all available N-oxyanions to N_2, whilst the other two released significant amounts of NO and N_2O, which affect tropospheric and stratospheric chemistries, respectively.

The prevalence and magnitude of hypersaline ecosystems are on the rise due to climate change and anthropogenic activity. Thus, the biology of halophilic denitrifiers is inherently interesting, due to their contribution to the global nitrogen cycle, and potential application in bioremediation. This work is the first detailed physiological study of denitrification in haloarchaea, and as such a seed for our understanding of the drivers of nitrogen turnover in hypersaline systems.

Introduction

Saline and hypersaline environments are found worldwide. Among them are salty lakes and lagoons, salty ponds and saline soils (Oren, 2002; de la Haba *et al.*, 2011; Andrei *et al.,* 2012), and they include well-known ecosystems such as The Dead Sea (Israel-Palestine and Jordan), The Magadi Lake (Kenya) and The Great Salt Lake (USA). The biodiversity under hypersalinity is lower than in other, less extreme environments (Oren, 2002), but higher than one might expect. All three domains of Life are represented, but Haloarchaea have been found to dominate when the salt concentration exceeds 16% (Andrei *et al.,* 2012; Edbeib *et al.,* 2016; Torregrosa-Crespo *et al.,* 2016).

Haloarchaea live and proliferate in extreme environments, characterised by salt concentrations up to saturation, high temperatures (although some are cold adapted-

Dassarma *et al.*, 2013; Williams *et al.,* 2017), high solar radiation and high ionic strength (Oren, 2013[a]; Oren, 2015; Oren, 2016). Under these conditions, oxygen availability is low (Oren, 2013[a]). At 35^{o}C, the optimum temperature for many haloarchaea, the solubility of O_2 is 6.92 mg/l in distilled water (Sherwood *et al.,* 1991; Sherwood *et al.,* 1992), but drops approximately ten-fold in hypersaline environments (Rodríguez-Valera *et al.,* 1985). Hence, many indigenous organisms have made adaptations allowing them to maintain respiratory metabolisms when oxygen is scarce. One fascinating, but still poorly understood mechanism is the use of cytoplasmic gas vesicles for vertical migration to O_2 rich layers of the water body (Oren, 2013[b]; Pfeifer, 2015). Another common strategy is the use of alternative electron acceptors. Second only to aerobic respiration, denitrification, the sequential reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to dinitrogen (N_2) via nitric oxide (NO) and nitrous oxide (N_2O) is the most energy-yielding alternative (Zumft & Kroneck, 2006).

The ability to denitrify is widespread among prokaryotes and is one of the routes by which bioavailable nitrogen can be returned to the atmosphere as N_2. However, the process is often incomplete, leading to release of the gaseous intermediates NO and N_2O. Both of these gases affect the environment. NO is highly reactive and is involved in the formation of HONO and tropospheric ozone (Su *et al.,* 2011; Oswald *et al.,* 2013). N_2O is the most important destroyer of stratospheric ozone and a greenhouse gas 310 times more potent than CO_2 (Ravishankara *et al.,* 2009; Thomson *et al.,* 2012). Microbial denitrification is one of the largest sources and the only known biological sink of N_2O. This underscores its environmental significance in a time where anthropogenic sources have doubled the global nitrogen budget, accelerating the processes driving the nitrogen cycle (Schlesinger, 2009).

Saline and hypersaline areas are currently increasing in size and prevalence. This is due to desertification processes caused by climate change and intensive exploitation of water resources (Martínez-Espinosa *et al.,* 2011). Moreover, nitrate and nitrite concentrations are increasing annually in such systems due to the use of fertilizers in agriculture and the dumping of untreated urban and industrial wastewaters (Martínez-Espinosa *et al.,* 2007; Ochoa-Hueso *et al.,* 2014). Thus, it is likely that denitrifying haloarchaea contribute to the global nitrogen turnover and N-oxide emissions, although to what extent remains to be determined.

The physiology of denitrification has been studied in detail in some organisms, mostly bacteria, of which the majority belongs to the proteobacteria (Bakken *et al.,* 2012; Liu *et al.,* 2013; Bergaust *et al.,* 2014; Hassan *et al.,* 2014; Lycus *et al.,* 2017). In contrast, representatives of the Archaea domain have for the most part remained unexplored with respect to their ability to denitrify (Philippot, 2002; Yoshimatsu, 2002). This is particularly true for those that thrive in extreme environments, such as the haloarchaea, because only a few can grow under laboratory conditions and out of those, a minority is able to denitrify. Members of the genus *Haloferax* (family *Haloferacaceae*) have been used as models for the study of denitrification in hypersaline environments, and they are probably the best known haloarchaea in terms of biochemistry, molecular biology and microbial ecology (Lledó *et al.,* 2004; Martínez-Espinosa *et al.,* 2007; Nájera-Fernández *et al.,* 2012; Torregrosa-Crespo *et al.,* 2016).

As in their bacterial counterpart, full-fledged haloarchaeal denitrifiers carry the four redox enzymes nitrate-, nitrite-, nitric oxide- and nitrous oxide reductase (NAR, NIR, NOR and N_2OR, respectively). Out of these, only the first two have been characterized biochemically. The respiratory nitrate reductase, purified in species such as *Haloferax mediterranei* (Lledó *et al.,*

2004) and *Haloferax denitrificans* (Hochstein & Lang, 1991), is a pNarGH enzyme oriented towards the pseudo periplasm between the cell membrane and the S-layer (Martínez-Espinosa *et al.,* 2007). The native nitrite reductase, purified in *H. denitrificans*, is a copper-containing enzyme resembling the bacterial NirK (Inatomi & Hochstein, 1996). Neither of the remaining two reductases have been purified in haloarchaea, but the latest advances indicate that the nitric oxide reductase in this group of microorganisms is the qNOR type (Torregrosa-Crespo *et al.,* 2017).

Current knowledge in terms of the respiratory physiology of haloarchaeal denitrifiers is even slimmer. Studies have shown that these organisms produce nitrogenous gases when reducing nitrate under anaerobic conditions, e.g. N_2O accumulation by *H. mediterranei* using nitrate as electron acceptor (Bonete *et al.,* 2008) and production of N_2 and N_2O in *H. denitrificans* in ratios depending on the amount of nitrate available (Tindall *et al.,* 1989). In a third species, *H. volcanii*, the results appear conflicting, most likely because different strains have been used for denitrification studies. Even so, it is generally considered a denitrifying organism (Bickel-Sandkötter & Ufer, 1995; Hattori *et al.,* 2016).

The proportions of nitrogenous gases released from any system depend on the physiology of the organisms driving nitrogen turnover. Thus, our knowledge gaps in terms of the most relevant microbes in hypersaline environments leave us wanting in understanding the mechanisms controlling N-oxide emissions in these systems. Here we present the first comparative study of the physiology of denitrification in haloarchaea using three of the better-known species within the *Haloferax* genus: *H. mediterranei*, *H. denitrificans* and *H. volcanii*.

Results

The strains were tested with respect to release of intermediates and ability to make balanced transitions from aerobic respiration to denitrification under a range of conditions. Briefly, aerobically raised cells were transferred to sealed vials with Helium atmosphere and ~0, 1 or 7% initial O_2 in headspace and low mM concentrations of KNO_2 or KNO_3 in the medium. The subsequent depletion of O_2 and accumulation of NO, N_2O and N_2 were monitored by frequent automated sampling from headspace and NO_2^- in the liquid was quantified after manual sampling (See "Experimental Procedures" for details).

Denitrification phenotypes with 1% initial O_2 + 5 mM KNO_3 or 1 mM KNO_2.

In cultures with nitrate (5 mM) and 1% initial O_2 in headspace (Fig 1 and Table 1), *H. mediterranei* reduced all the available N oxyanion to N_2 with minimal and transient accumulation of intermediates. Onset of denitrification was apparently induced by low O_2 (O_2 at induction: 3.7 ± 0.6 µM in liquid) and was characterized by the concomitant increase of all intermediates, followed by exponential accumulation of N_2. The rapid and effective switch to denitrification was evident from the e^- flow (µmol $vial^{-1}$ h^{-1}), which showed a smooth transition from aerobic to anaerobic respiration with no apparent delay or decrease in total e- flow towards terminal e^- acceptors (Fig 1, panel B). NO and N_2O were kept at concentrations in the low nM range, but NO at transition to denitrification (= NO_{max}) was higher than during subsequent respiration; 39.8 ± 10.5 nM vs 12.0 ± 0.3 nM, respectively.

In contrast, *H. denitrificans* was inefficient in switching to denitrification. In most of the cultures, only a fraction of the nitrate supplied was recovered as gaseous N-oxides during the incubation. The transition to denitrification was typically slow and, as in the culture exemplified (Fig 1, panels C and D), with an apparent lag phase of approximately 12 hours. During this period, NO increased slowly, but stayed below 15 nM, while NO_2^-, N_2O and N_2 concentrations were negligible. From 60 to approximately 90 hours, there was a dramatic increase in denitrification rate (e-flow to N-oxides, μmol $vial^{-1}$ h^{-1}). As NO reached its maximum (63 nM), NIR and N_2OR appeared to stall, resulting in nitrite and N_2O accumulation. NIR activity resumed, and nitrite was reduced once NO decreased slightly, but at the end of the incubation, approximately 20% of the supplied nitrate was recovered as N_2O (Table 1 and Fig 1, panels C and D). A similar phenotype was also seen in cultures started under slightly different conditions (thus not included in Table 1 and Fig 1). When inspecting the length of the sub-oxic phase after first appearance of NO ($\Delta_t(NO_{inc}\text{-}O_{2depl})$h) vs time required for full reduction of nitrate to N_2O + N_2 ($\Delta_t(NO_{inc}\text{-}NO_3^-{}_{depl})$h) in cultures of *H. denitrificans* started with 1% O_2, there was a negative correlation between the two. Moreover, a similar correlation was evident when plotting ($\Delta_t(NO_{inc}\text{-}NO_3^-{}_{depl})$h) against NO (nM) at oxygen depletion. These correlations were not seen in cultures of *H. mediterranei* under the same conditions (Figs S7 and S8, Supplementary information).

H. volcanii was unable to reduce nitrate to nitrite. Thus, in cultures with nitrate supplemented medium, respiration stopped after depletion of O_2 (Fig 1, panels E and F). However, upon depletion of oxygen in the presence of 1 mM NO_2^-, there was some reduction to gaseous N-oxides. As O_2 reached 1.3 ± 0.5 μM, NO and N_2O increased to μM concentrations

within approximately 5 hours, after which respiration came to a halt (N_2O_{max} = 3.4 ± 0.13 µmol $vial^{-1}$, NO_{max} = 8.8 ± 0.48 µM in liquid). There was no detectable N_2 production, and the slight increase (Fig 2, panel E) was due to leakage during sampling.

[Insert Figure 1]

H. denitrificans resembled *H. volcanii* in its response to 1 mM NO_2^-. As O_2 approached depletion, there was a rapid increase in NO. When NO reached µM concentrations (NO_{max} = 8.20 ± 0.27 µM, Table 1), there was a general arrest in respiration, first in N_2O reduction, followed by upstream reduction steps (Figure 2, panels C and D).

Unlike the other two *Haloferax spp*, *H. mediterranei* successfully switched to denitrification and was able to reduce 1 mM NO_2^- to N_2. As O_2 approached depletion, the entire set of reductases was expressed, and N_2 accumulated exponentially until all the available nitrite had been consumed (Fig 2, panels A and B). NO was not as stringently regulated as seen in cultures with nitrate and it transiently reached µM concentrations during the anoxic phase (NO_{max} = 1.14 ± 0.08 µM in liquid; Table 1).

[Insert Figure 2]

[Insert Table 1]

Success of denitrification in a range of initial O_2 concentrations.

All three *Haloferax spp* were tested under a range of initial O_2 concentrations (~0, 1 or 7%) in combination with nitrite or nitrate as the alternative e^- acceptor. *H. mediterranei* consistently reduced the available N-oxides to N_2 at 1 or 7% initial O_2, but was more variable when challenged with sudden anoxia (transferred from oxic conditions to O_2 < 1 µM in liquid). In cultures with nitrate, only 50% of the replicates were approaching depletion of e^- acceptors (N_2 > 100 µmol $vial^{-1}$; Fig 3 and Fig S1, Supplementary information) by the end of the incubation, whereas in medium with nitrite, none of the cultures were able to denitrify within the timeframe of the experiment (Fig 3).

H. denitrificans responded differently to the addition of nitrite or nitrate as alternative electron acceptor. In cultures with nitrate, the probability of complete reduction to N_2 appeared to increase with initial oxygen concentration. In contrast, when challenged with nitrite and 0 or 1% initial O_2, excessive accumulation of NO led to respiratory arrest. *H. volcanii*, was unable to reduce nitrate under all conditions tested, and when supplied with 1 mM NO_2^- and 1 % initial O_2 it, like *H. denitrificans* accumulated µM concentrations of NO (Figs 2 and 3). However, in cultures experiencing sudden anoxia, there was a prolonged period of slow and gradual accumulation of NO and N_2O. Injection of O_2 led these *H. volcanii* cultures to promptly switch to aerobic respiration and growth (Fig S9, C and D, Supplementary information), whereas cultures exposed to µM concentrations of NO were unable to reduce O_2 (Figure S9 A and B, Supplementary information).

[Insert Figure 3]

Growth rates

Apparent specific growth rates (μ h^{-1}) were derived from observed gas kinetics under oxic and anoxic conditions (Table 2). Estimates for aerobic growth rates (μ_{ae}) were based on regression of log-linear e-flow rates to O_2 (µmol $vial^{-1}$ h^{-1}) in cultures with 7% initial oxygen (n=4). Likewise, anaerobic growth rates (μ_{an}) were derived from e-flow rates to electron acceptors during exponential anaerobic growth in nitrate amended (5 mM) cultures with 0% initial oxygen (n=3). Growth yields (Y, cells mol^{-1} e^- to e^- acceptors) were estimated based on OD_{600} and total mol e^- acceptor reduced at the end of incubations. Aerobic growth yields (Y_{ae}) were derived from cultures with 7% initial oxygen and no added N oxyanions (n=3) and anaerobic growth yields were based on cultures with 0% initial oxygen and 5 mM NO_3^-.

Under the conditions tested*, H. mediterranei* had the highest average aerobic and anaerobic growth rates and the highest aerobic cell yield compared to the other *Haloferax* species. However, μ_{an} in *H. denitrificans* and *H. mediterranei* were not significantly different (0.089 ± 0.024 and 0.092 ± 0.001, respectively). *H. denitrificans* had the highest anaerobic growth yield, which was comparable to and even slightly exceeding its aerobic cell yield. *H. volcanii* did not denitrify and thus anaerobic growth rate could not be estimated. Moreover, under oxic conditions it had the longest apparent generation time and lowest growth yield out of the three species (Table 2).

[Insert Table 2]

Discussion

Denitrification is a major source of nitrogenous gases from a range of environments and the only known sink of N_2O. In line with this, there has been a longstanding interest in the biochemistry and physiology of denitrifying bacteria. Meanwhile, the knowledge of archaea and extremophiles in this context has remained limited. We selected three *Haloferax spp* based on reports of their ability to denitrify, and conducted a set of incubation experiments where cultures faced transitions from oxic to anoxic conditions in nitrate or nitrite amended medium. Each organism displayed a distinct denitrification phenotype.

H. volcanii is genetically tractable and has been used as a model for studies of haloarchaea. It has been identified as a denitrifier capable of anaerobic growth under microoxic conditions using nitrate as alternative electron acceptor (Hattori *et al.,* 2016). In contrast to findings by Hattori *et al*., *H. volcanii* did not reduce nitrate to nitrite in our hands. The experimental approach differed between the studies, e.g. with respect to nitrate concentration and stirring conditions, but the phenotypic discrepancy is more likely due to inter-strain differences.

Although unable to reduce nitrate to nitrite, *H. volcanii* did denitrify *sensu stricto* as it reduced nitrite to gaseous products. The respiratory arrest due to high NO seen in nitrite-amended cultures (Fig 2 and 3) can be seen as an artefact of the experimental set-up, where cultures were confined in closed systems. Moreover, if cultivated with less intensive stirring, allowing the formation of aggregates, *H. volcanii* would perhaps have been able to switch to denitrification, reducing nitrite to N_2O while accumulating less NO. Nevertheless, as N_2O is the

end product, *H. volcanii* DS2 is intuitively not a good candidate for controlling N-oxide emissions.

The remaining two organisms, *H. denitrificans* and *H. mediterranei*, were both capable of reducing nitrate to N_2. Their individual denitrification phenotypes where distinct, but resembled variants seen in bacterial counterparts. This indicates that similar regulatory circuits govern the respiratory physiology across kingdoms. *H. mediterranei* showed the most reproducible phenotype. When transferred to suboxic conditions (1 or 7% initial O_2) in nitrate or nitrite amended media, it consistently reduced all available N-oxyanions to N_2 with low and transient accumulation of intermediates. During the transition to denitrification, electron flow (µmol e^- $vial^{-1}$ h^{-1}) to terminal acceptors was maintained at levels comparable to the initial aerobic phase, followed by apparent balanced growth. Apparent specific growth rate (µ h^{-1}) was approximately halved during denitrification as compared to aerobic respiration and the Y_{an}:Y_{ae} ratio was ~0.7 (Table 2). These ratios are comparable to those found in the model organism *Paracoccus denitrificans* (Bergaust *et al.,* 2010) and in efficient denitrifiers newly isolated from soil (Roco *et al.,* 2017). Put together, the phenotypic data are indicative of a well-orchestrated, full onset of the denitrification apparatus in the vast majority of the population. As such, *H. mediterranei* shows promise both as a model for denitrifying extremophiles and as a candidate for future application in bioremediation.

Unlike *H. mediterranei, H. denitrificans* was unable to make a successful transition to anaerobic respiration in the presence of 1 mM NO_2^-. In nitrate-amended medium, it was notoriously unreproducible with respect to timing of the apparent induction of N-oxide reductases. Whereas cultures of *H. mediterranei* originating from the same aerobic inoculum

would display highly similar phenotypes, replicates of *H. denitrificans* spanned from failure in switching to denitrification to full reduction of nitrate to N_2. As cultures faced anoxia, there was an apparent delay in the expression of N-oxide reductases, seen as a dramatic decrease in e^--flow towards terminal electron acceptors and slow accumulation of gaseous N-oxides (Fig 1, panel D). In other denitrifiers, similar gas kinetics have been taken to suggest that only a small fraction of cells synthesize the core denitrification enzymes and then grow exponentially. This phenomenon has been explored by modelling and experiments in the α-proteobacterium *P. denitrificans*, where a low probability of *nirS* expression and high probability of *nar* expression results in nitrite accumulation, followed by its reduction by a minority of NIR positive cells (Hassan *et al.,* 2014; Hassan *et al.,* 2016; Lycus *et al*., 2018). In *H. denitrificans*, there is no significant accumulation of nitrite, thus the gatekeeper appears to be NAR. However, instead of full expression in a fraction of the population, there is more likely a slow, gradual onset of NAR expression in the entire population. In the event of cell diversification as seen in *P. denitrificans*, the drop in e-flow should be followed by balanced, exponential growth by the active minority of cells throughout the anoxic phase. Instead, there is a late, sudden and dramatic increase in e^--flow to N-oxides. In denitrifying bacteria, NAR is often regulated negatively by O_2 and positively by nitrate/nitrite/NO via O_2- and NO_x-sensing transcription factors (Spiro, 2012). A possible underlying mechanism for this phenotype is thus an initial weak induction of the denitrification apparatus in all cells through oxygen sensing factors, followed by a stronger N-oxide (e.g. NO) mediated induction across the population once N-oxides accumulate to sufficient concentrations.

Interestingly, among the *H. denitrificans* cultures that successfully reduced the available nitrate to gaseous N-oxides, approximately half accumulated a mixture of N_2O and N_2 as end product (Figs 1 and 3; Figs S2, S3 and S5, Supplementary information). The shutdown of N_2O reductase appeared stochastic but was likely due to the accumulation of some substance with inhibitory effects, either on the expression or more directly on the function of the enzyme. It appeared that this inhibition transiently affected NIR as well, resulting in a rapid increase in nitrite and a plateau in NO accumulation. NIR eventually resumed its activity, but N_2O reductase did not recover within the time-span of the incubation. Denitrification is a play with fire in terms of the toxic intermediate NO. The fact that NO is a major controller of gene expression, in bacteria commonly inducing the transcription of its own enzymatic origin nitrite reductase (Spiro, 2012), underscores the importance of a finely tuned regulation. Bacterial N_2O reductase has been shown to be inhibited by NO (Frunzke & Zumft, 1986), and it is not unlikely that the N_2O reductase in *H. denitrificans* is more sensitive to NO than the other denitrification enzymes.

Under the present conditions, *H. denitrificans* was frequently unable to carry out a balanced transition from aerobic respiration to denitrification. This may be a disadvantage in competition with more aggressive denitrifiers, displaying rapid and full onset of denitrification enzymes in response to oxygen depletion. Moreover, NO released in the open environment quickly disperses. Thus, if it is in fact the major positive controller of NAR in *H. denitrificans*, the organism may not see NO at sufficient concentrations for induction of denitrification enzymes. On the other hand, NO is intuitively not likely to reach local concentrations high enough to

inhibit N_2O reductase in free-living cells. Thus, if at all able to synthesize the N-oxide reductases under natural conditions, *H. denitrificans* would most likely release N_2 during denitrification.

The strains used in this work have all been fully sequenced, the genes encoding the central denitrification apparatus have been identified and the biochemistry of some of the core enzymes have been explored (Bickel-Sandkotter & Ufer, 1995; Inatomi & Hochstein, 1996; Lledó *et al.,* 2004; Torregrosa-Crespo *et al.,* 2016). Apart from the lack of *nos* genes in *H. volcanii*, the relatively subtle differences in the respective functional genes and amino acid sequences of the main enzymes of the pathway (Fig S10, Table S1, Supplementary information) offer little explanation to the dramatic differences in denitrification phenotypes. This strengthens the theory that gene regulation is the key. Currently, little is known about the regulation of denitrification in *Haloferax* species. However, Hattori *et al.* (2016) recently identified a putative O_2 sensor, NarO, regulating *nar* and *nir* transcription in *H. volcanii*. A similar sequence is found in *H. denitrificans*, but in *H. mediterranei*, the corresponding position is occupied by an AcrR-like transcriptional regulator (Fig S11 and Table S2, Supplementary information). This may account for some of the observed phenotypic differences.

Our results fall in line with observations made in bacteria: Genetic make-up and phylogeny are poor predictors of a denitrifying organism's propensity to release N-oxides (Liu *et al.,* 2013; Lycus *et al.,* 2017). This underscores the importance of including physiological studies when assessing denitrifiers and their potential in nitrogen removal. At this point, there is limited knowledge of denitrification in halophilic prokaryotes in general. With modern days' increasing N-input and salinization of water systems, these organisms are gaining importance as possible tools for mitigation of N-oxide emissions. Hence, this study can be regarded as a

starting point towards a deeper understanding of the respiratory physiology of the organisms driving the reduction of nitrate and nitrite under high salinity.

Experimental Procedures

Archaeal strains

The strains used in this study were *H. mediterranei* (R-4)*, H. denitrificans* (S1) and *H. volcanii* (DS2) provided by DSMZ (Leibniz Institute DSMZ-German collection of microorganism and cell cultures).

The correlation between OD_{600} and cell number for *H. mediterranei* (Cells ml^{-1}= OD_{600} * 9.69E8) was estimated using a cell staining protocol with SYBR green (Noble & Fuhrman, 1998).

Media, incubation conditions and experimental design.

For the comparative phenotype analyses all the strains were grown on their respective optimal media: 20% (w/v) mixture of salts (20% SW) (Rodríguez-Valera *et al.,* 1980; DasSarma *et al.,* 1995) and 0.5% (w/v) yeast extract. The pH was adjusted to 7.3, 6.5 and 7.0 for *H. mediterranei, H. denitrificans* and *H. volcanii*, respectively.

Inocula were raised aerobically at 35°C in 120 ml serum vials containing 50 ml medium. The cultures were vigorously stirred (700 rpm, triangular magnetic stirring bar; Cowie 25 x 8 mm, VWR International) to ensure full dispersal of cells and homogenous conditions. Upon reaching OD_{600} ~ 0.2, cells were transferred to new vials for monitoring of gas kinetics (see below). The volume transferred was adjusted according to the measured OD_{600} of the aerobic pre-culture ($V = 0.2/OD_{600}$) to secure similar initial cell density for all strains and experiments (~ 1.94×10^8 cells ml^{-1}).

The incubation experiments were conducted in 120 ml serum vials with 50 ml medium and a triangular magnetic stirring bar. After autoclaving, KNO_3 or KNO_2 was added to final concentrations of 5 mM or 1 mM, respectively. Prior to inoculation, vials were crimp sealed with rubber septa (Matriks AS, Norway) and aluminium caps and conditions were made anoxic through cycles of evacuation and He-filling (Molstad *et al.,* 2007) with constant stirring (700 rpm) to ensure sufficient gas exchange between liquid and headspace. The vials were then either inoculated directly (0% initial oxygen) or pure O_2 was added to initial headspace concentrations of 1 or 7% (v/v) before inoculation. Calibration gases were filled into evacuated, crimp-sealed serum vials without medium.

The N-oxyanion concentrations used in this set of experiments were, at least for nitrite, high compared to what would normally be expected in relevant environments. The rationale for using mM concentrations of nitrate and nitrite was that it facilitates sufficiently long aerobic and anaerobic growth phases for generation of high-resolution data using the incubation system. This is necessary for the estimation of parameters such as apparent growth rates and

for the assessment of e^--flow towards terminal acceptors during the oxic-anoxic transition. Such data sets also aid the generation of hypotheses with regards to the regulation of the respective reductases.

The cultures were incubated at a constant temperature of 35^{o}C in a robotized incubation system designed for measuring gas kinetics (Molstad *et al.,* 2007; Bergaust *et al.,* 2008). Samples from the headspace were taken in intervals by an auto-sampler unit connected to a peristaltic pump and led to a gas chromatograph (CP-4700 Micro-GC or Varian; 7890A GC, Agilent) for analysis of O_2, CO_2, N_2O and N_2. NO was measured by a chemiluminescence NO_x analyser (M200A or M200E, Teledyne). Each headspace sample was replaced by an equal amount of He to maintain ~1 atm pressure.

For NO_2^- and OD_{600} measurements, liquid samples were taken using sterile syringes. For NO_2^- quantification, 10 µL sample was injected into a purge vessel containing 3 ml reducing agent (NaI, 1% w/v in glacial acid) connected to a chemiluminescence NO-analyser (Sievers 280i, GE Analytical Instruments). N_2 was continuously bubbled through the reducing agent to maintain an anoxic environment in the system and to transport the NO through the NO analyser (Walters *et al.,* 1987).

Analysis of gas kinetics data and apparent specific growth rates

Sampling dilution of headspace gases and leakage of O_2 and N_2 as well as solubility of the respective gases in the medium, and transport rates between liquid and headspace were considered when analysing the gas data (Molstad *et al.* 2007). Briefly, under the current

conditions (35°C), the solubility of the relevant gasses were 0.01858 (N_2O), 0.00169 (NO), 0.00058 (N_2), 0.00024 (O_2) and 0.15636 (CO_2, pH dependent) mol/L*atm (derived from Wilhelm *et al*., 1977). The concentration of O_2 in the bulk liquid was estimated taking aerobic respiration rate into account, while the concentration of NO and N_2O were calculated by assuming equilibrium between headspace and liquid. This enabled the estimation of total amount $vial^{-1}$ and liquid- and headspace concentrations of each gas at each time point or mid-increment.

Apparent specific aerobic (μ_{ae}) and anaerobic (μ_{an}) growth rates were estimated based on the observed kinetics of O_2 consumption and denitrification, respectively, through the regression of log (ln) transformed respiration rates versus time during log-linear increases in electron transport rates.

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Figure and table legends

Figure 1: Gas kinetics and e^--flow to terminal e^--acceptors during transition to anoxia in nitrate-supplemented medium. Top panels: consumption of O_2 and subsequent accumulation of N-oxides in *H. mediterranei* (A)*, H. denitrificans* (C) and *H. volcanii* (E) with 1% initial O_2 in headspace and 5 mM KNO_3 in the medium. The experiment was conducted at 35°C and in triplicate batch cultures. However, for *H. denitrificans*, only one vial is shown. The bottom panels (B, D, F) show e- flow (µmol $vial^{-1}$ h^{-1}) to O_2 and N-oxides for the respective strains based on nitrite and gas measurements.

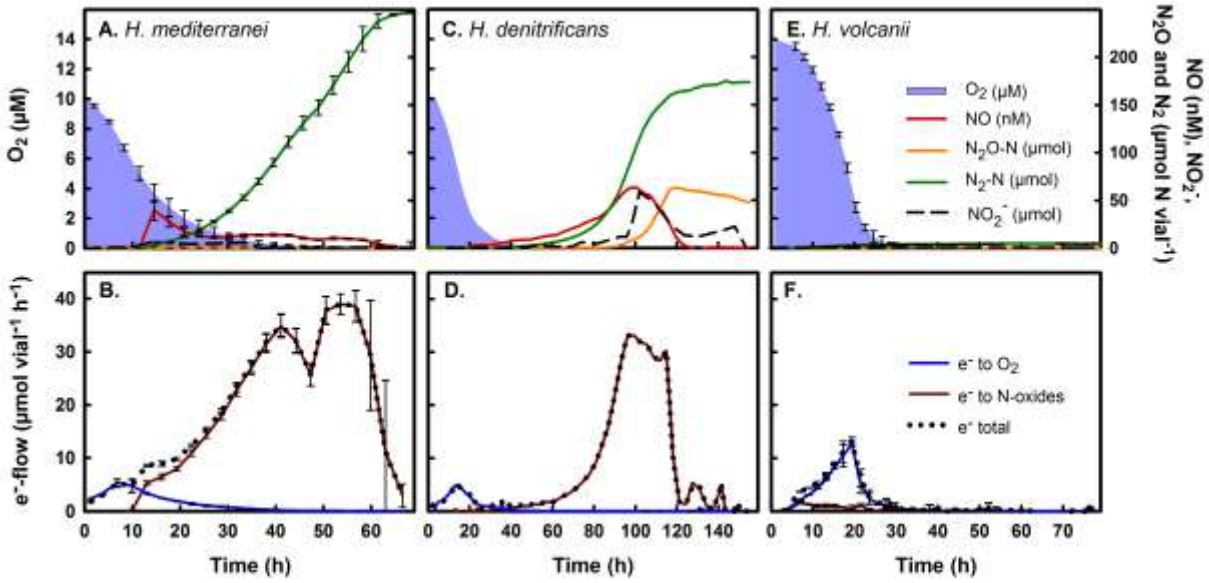
Figure 2: Gas kinetics and e^--flow to terminal e^--acceptors during transition to anoxia in nitrite-supplemented medium. Top panels A, C, E: consumption of O_2 and subsequent accumulation of N-oxides in *Haloferax spp* with 1% initial O_2 in headspace and 1 mM KNO_2 in the medium (n=3). The bottom panels B, D and F, show e- flow (µmol $vial^{-1}$ h^{-1}) to O_2 and N-oxides based on gas measurements.

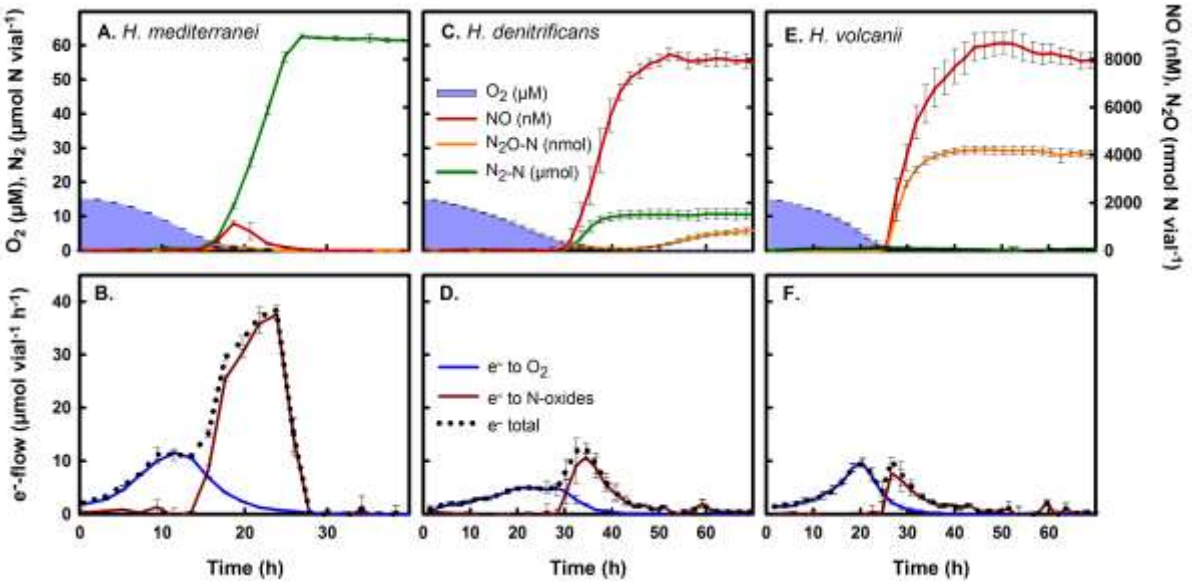
Figure 3: Denitrification phenotypes at a glance. Recovery of added N-oxyanions as gaseous N-oxides in batch cultures of *Haloferax spp* with 0, 1 or 7% initial O_2 in the headspace and 1 mM KNO_2 or 5 mM KNO_3 (2 mM for *H. volcanii*) in the medium (see Supplementary information, Fig S1-6 for underlying gas data). The figure illustrates the robust phenotype of *H. mediterranei*, which under all but one of the conditions tested reduced the available N-oxides to N_2 in all replicates (green). The exception was cultures in nitrite-supplemented medium starting at near anoxia, where all replicates were slow (grey), leading to only partial recovery of nitrite as N_2 within the time-frame of the experiment. *H. denitrificans* was unable to make a successful transition to denitrification in nitrite supplemented medium. Instead, toxic levels of NO accumulated, leading to full arrest in respiration (red). In nitrate-supplemented medium, the phenotype was somewhat erratic. Cultures with ~0% initial oxygen reduced nitrate either to $N_2O + N_2$ (yellow; n = 3) or to pure N_2 (green; n = 1) as end-product. This was also the case with 7% initial oxygen, but the success rate was higher; three out of four cultures reduced the available nitrate to N_2. At 1% initial oxygen, two out of three replicates did not complete denitrification within the timeframe of the experiment (grey) and the one replicate that did, reduced nitrate to $N_2O + N_2$ as end products (yellow). *H. volcanii* did not reduce nitrate under any of the conditions tested (black colour coding) and oxygen depletion in the presence of 1 mM NO_2^- resulted in accumulation of NO to crippling concentrations, leading to arrest in respiration (red).

Table 1: Phenotypic parameters in *Haloferax spp.* Cultures were monitored during the transition from aerobic growth (initial O_2: 1% in headspace) to denitrification in nitrate or nitrite

supplemented medium. Initial KNO_3 was 5 mM for *H. mediterranei* and *H. denitrificans*, and 2 mM for *H. volcanii*; initial KNO_2 was 1 mM for all species.

Table 2: Specific growth rates (μ h^{-1}) and cell yield (Y) under oxic and anoxic conditions in *Haloferax spp*. Growth yields were estimated assuming the same conversion factor for all three strains: cells ml^{-1} = OD_{600} * 9.69E8. We further assumed that the cell number to OD_{600} ratio remained constant under all the conditions tested.





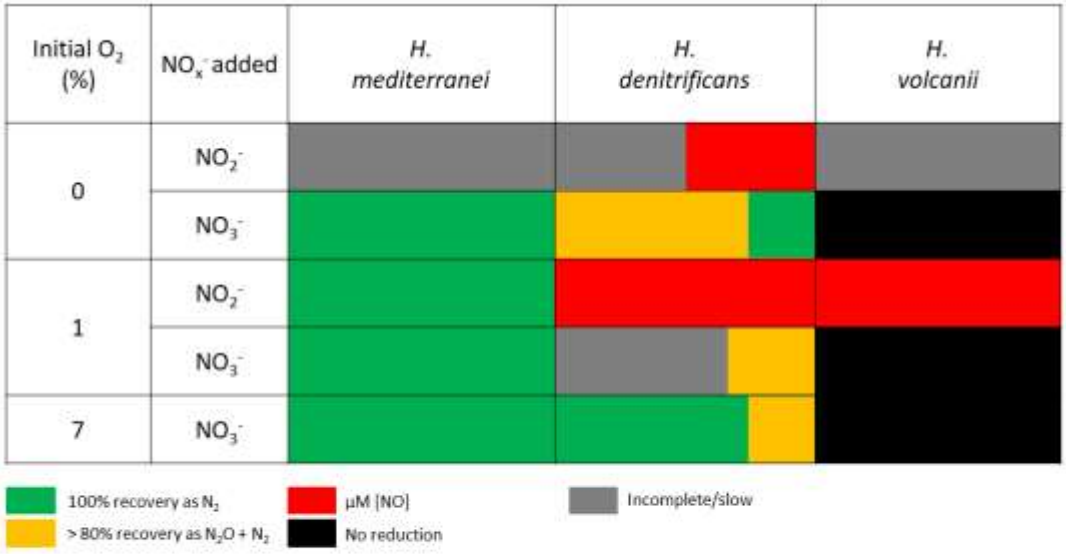


Table 1: Phenotypic parameters in *Haloferax* spp. monitored during the transition from aerobic growth (initial O_2: 1% in headspace) to denitrification in nitrate or nitrite supplemented medium. Initial KNO_3 was 5 mM for *H. mediterranei* and *H. denitrificans*, and 2 mM for *H. volcanii*; initial KNO_2 was 1 mM for all species.

	H. mediterranei	*H. denitrificans*	*H. volcanii*

	NO_3^- Average (SD)	NO_2^- Average (SD)	NO_3^- [c]	NO_2^- Average (SD)	NO_3^- Average (SD)	NO_2^- Average (SD)
O_{2Ind} (µM in liquid)[a]	3.7 (0.6)	2.3 (0.1)	2.8	1.8 (0.4)	n. a.	1.3 (0.5)
$NO_2^-{}_{max}$ (µmol $vial^{-1}$)	6.6 (0.2)	n. a.	58.4	n. a.	n. a.	n. a.
NO_{max} (µM in liquid)	0.04 (0.01)	1.14 (0.08)	0.06	8.20 (0.27)	n. a.	8.75 (0.48)
N_2O_{max} (µmol N $vial^{-1}$)	0.06 (0.00)	0.15 (0.01)	63.48	0.98 (0.14)	n. a.	3.36 (0.13)
NO_x^- recovery (%)[b]	100 (0)	100 (0)	100	57.9 (6.8)	< 1	31.8 (1.1)[d]

[a] $O_{2Induction}$: O_2 concentration at the time of NO appearance (NO > 2 nM in liquid)

[b] Reduction of added NO_x^--N to gaseous N-oxide at end of incubation

[c] Data from one successful culture

[d] Gaseous end products were NO and N_2O

n.a. not applicable

Table 2: Specific growth rates (µ h^{-1}) and cell yield (Y) under oxic and anoxic conditions in *Haloferax* spp. Growth yields were estimated assuming the same conversion factor for all three strains: cells ml^{-1} = OD_{600} * 9.69E8. We further assumed that the cell number to OD_{600} ratio remained constant under all the conditions tested.

	H. mediterranei Average (SD)	*H. denitrificans* Average (SD)	*H. volcanii* Average (SD)
μ_{ae} (h^{-1})	0.197 (0.007)	0.163 (0.009)	0.134 (0.022)
μ_{an} (h^{-1})	0.092 (0.001)	0.089 (0.024)	n.a.

Y_{ae} (cells mol^{-1} e^- to O_2)	4.18E+13 (1.01E+12)	3.18E+13(1.04E+12)	2.63E+13(6.12E+11)
Y_{an} (cells mol^{-1} e^- to NO_x)	2.93E+13 (2.95E+12)	3.22E+13 (4.99E+12)	n.a

n.a. not applicable