

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Characterization of chlorate reduction in the haloarchaeon *Haloferax mediterranei*.

Rosa María Martínez-Espinosa*, David J. Richardson[#] and María José Bonete

División de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Alicante, Ap. 99, E-03080 Alicante, Spain

[#] School of Biological Sciences, Faculty of Science, University of East Anglia, Norwich, NR4 7TJ, UK

*Corresponding author. Tel.: 34 96 5901258. Fax: 34 96 5903880. E-mail: rosa.martinez@ua.es

Abbreviations: DT, dithionite; MV, methylviologen; NarGH, respiratory nitrate reductase; PCRB, perchlorate-respiring bacteria; CRB, chlorate-respiring bacteria.

1

2 **Abstract**

3 Background: *Haloferax mediterranei* is a denitrifying haloarchaeon using nitrate
4 as a respiratory electron acceptor under anaerobic conditions in a reaction
5 catalysed by pNarGH. Other ions such as bromate, perchlorate and chlorate can
6 also be reduced.

7 Methods: *Hfx. mediterranei* cells were grown anaerobically with nitrate as
8 electron acceptor and chlorate reductase activity measured in whole cells and
9 purified nitrate reductase.

10 Results: No genes encoding (per)chlorate reductases have been detected either
11 in the *Hfx. mediterranei* genome or in other haloarchaea. However, a gene
12 encoding a chlorite dismutase that is predicted to be exported across the
13 cytoplasmic membrane has been identified in *Hfx. mediterranei* genome. Cells
14 did not grow anaerobically in presence of chlorate as the unique electron
15 acceptor. However, cells anaerobically grown with nitrate and then transferred to
16 chlorate-containing growth medium can grow a few generations. Chlorate
17 reduction by the whole cells, as well as by pure pNarGH, has been characterised.
18 No clear chlorite dismutase activity could be detected.

19 Conclusions: *Hfx. mediterranei* pNarGH has its active site on the outer-face of
20 the cytoplasmic membrane and reacts with chlorate and perchlorate. Biochemical
21 characterisation of this enzymatic activity suggests that *Hfx. mediterranei* or its
22 pure pNarGH could be of great interest for wastewater treatments or to better
23 understand biological chlorate reduction in early Earth or Martian environments.

1 General significance: some archaea species reduce (per)chlorate. However,
2 results here presented as well as those recently reported by Liebensteiner and
3 co-workers [1] suggest that complete perchlorate reduction in archaea follows
4 different rules in terms of biological reactions.

5

6 *Keywords:* Halophile; Archaeon; respiratory nitrate reductase; chlorate reduction;
7 anaerobic respiration; chlorite dismutase.

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

1. Introduction

During the last 10 years, perchlorate (ClO_4^-) and chlorate (ClO_3^-) have been detected in several water supplies, ground waters, agricultural crops and even in soils as a result of human activities [2]. Perchlorate is used in the manufacture of propellants, explosives and pyrotechnic devices [3]. Perchlorate salts are extremely soluble, non-volatile, non-reactive and chemically very stable. The high water solubility and poor adsorption of perchlorate to soil and organic matter make its high mobility in the environment possible [4]. The concerns about perchlorate toxicity are its interference with iodide uptake by the thyroid gland, and the related potential carcinogenic effects [5]. Chlorate is present in several herbicides and defoliants, and it is released when chlorine dioxide (ClO_2) is used as a bleaching agent in the paper and pulp industry [6]. In humans, ClO_3^- may cause thyroid lesions and anaemia. Because of these health concerns, several organizations such as the World Health Organization or the Environmental Protection Agency have advised that (per)chlorate in water intended for human consumption should be minimized [7].

Perchlorate and chlorate are ideal electron acceptors for microorganisms due to their high redox potentials ($\text{ClO}_4^-/\text{Cl}^- E_0=1.287 \text{ V}$; $\text{ClO}_3^-/\text{Cl}^- E_0=1.03 \text{ V}$) [8]. It has been proposed that in perchlorate-respiring bacteria (PCRB) the (per)chlorate-reduction pathway consists of the (per)chlorate reductase, which sequentially reduces perchlorate to chlorate and in turn chlorate to chlorite (ClO_2^-), via sequential two-electron transfers [6,7]. Finally, chlorite dismutase

1 transforms chlorite into chloride and oxygen [9-13]. Perchlorate-respiring bacteria
2 (PCRB) are ubiquitous in the environment, and are mainly facultative anaerobes
3 and denitrifiers [14,15]. Perchlorate reductases isolated from PCRB react with
4 both perchlorate and chlorate [6], while chlorate reductases expressed by
5 chlorate-respiring bacteria (CRB) do not reduce perchlorate [16]. It has also been
6 demonstrated that perchlorate and chlorate reductases isolated from some
7 PCRB recognize nitrate as substrate [17].

8 Nitrate is also often present in environments where perchlorate or chlorate
9 are faced as contaminants [18]. In the denitrification pathway, nitrate is
10 sequentially reduced to dinitrogen gas: $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ [19].
11 Several nitrate reductases involved in anaerobic nitrate reduction also reduce
12 chlorate. However, these nitrate reductases have active sites facing the
13 membrane potential negative side (nNars) and the nitrate transporters that
14 deliver nitrate into the cytoplasm do not recognize chlorate, thus preventing the
15 potentially damaging intracellular reduction of chlorate to cytotoxic chlorite. In
16 previous studies on the respiratory nitrate reductase (NarGH) from *Haloferax*
17 *mediterranei*, a denitrifying halophilic archaeon able to use nitrate as nitrogen
18 source for growth or as electron acceptor under anaerobic conditions [20-22], it
19 was demonstrated that this enzyme has an active site facing the membrane
20 potential positive face (pNars) and is able to reduce chlorate [18]. The extra-
21 cytoplasmic active site could be accessible to chlorate and so, this reaction might
22 take place in the environment leading to the question of whether it could support
23 growth and also whether it could reduce perchlorate, a substrate for which the

1 role as electron acceptor in the reactions catalyzed by nitrate reductases has
2 been poorly described in haloarchaea.

3 Recent results reveal that (per)chlorate reductases establish a distinct
4 group with the archaeal p-type NarG nitrate reductases as the closest relatives
5 into dimethyl sulfoxide (DMSO) reductase family [23]. It has also been proposed
6 that chlorate reduction was built multiple times from type II dimethyl sulfoxide
7 (DMSO) reductases and chlorite dismutases [24, 25]. This work summarises the
8 biochemical characterisation of the NarGH chlorate reductase activity and
9 discusses different strategies that might be used by haloarchaea to deal with the
10 chlorite produced during chlorate reduction.

11

12 **2. Materials and Methods**

13

14 **2.1 Strains, media and growth conditions**

15 *Hfx. mediterranei* (ATCC 33500T) was grown anaerobically with nitrate
16 (100 mM) as electron acceptor as previously described [18], in a 25% (wt/vol)
17 mixture of salts (25% SW) [26] and 0.5% yeast extract (complex media). Cultures
18 with chlorate (100mM) as electron acceptor were prepared in the same way.
19 Growth was monitored by measuring the optical density at 600 nm. In some
20 experiments, *Hfx. mediterranei* cells were grown as already described to induce
21 the denitrification pathway (nitrate as electron acceptor). After that, cells were
22 harvested by centrifugation at 15000 x g for 20 min at 4 °C in a Beckman J2-21
23 centrifuge, washed with 25% SW, centrifuged again at 15000 x g for 20 min at 4

1 °C and transferred to fresh anaerobic complex medium (25% SW and 0.5% yeast
2 extract) containing chlorate at different concentrations ranging from 5 to 100 mM.

3

4

5 **2.2 Purification of respiratory nitrate reductase and characterization of the** 6 **NarGH chlorate reductase activity**

7 All the purification steps were carried out at 25 °C following the protocol
8 previously described [20]. The chlorate reductase activity of the NarGH was
9 measured using two different methods: i) colorimetric o-toluidine assay, which
10 allows chlorate quantification [27] and ii) methylviologen method (substrate-
11 dependent oxidation of reduced methylviologen) [28]. For the o-toluidine assay,
12 the reaction mixture contained, in a final volume of 1ml: 50 mM Tris-HCl pH (7-9),
13 0-2M NaCl or KCl, 5 mM MV (electron donor), 50 mM KClO₃ (substrate), 10 mM
14 Na₂S₂O₄ (freshly prepared in 0.1 M NaHCO₃) and 40 µl of pure enzyme (final
15 protein concentration around 0.03 mg protein per mL was constantly present in
16 the reaction mixture). The enzymatic activity was tested at temperatures between
17 25 and 70 °C, but most of the assays were developed at 35 °C. After 5 min of
18 incubation to allow enzymatic reaction, 0.25 ml of o-toluidine (0.4 g/l) and 1.25 ml
19 of concentrated HCl were added to reaction mixture. O-toluidine and HCl
20 additions destroy the protein and as a consequence, the enzymatic reaction is
21 stopped. The absorbance related to the yellow holoquinone finally produced in
22 the colorimetric reaction was checked at 490 nm. NarGH chlorate reductase
23 activity is expressed as micromoles of KClO₃ reduced per minute and chlorate
24 reductase specific activity is expressed as micromoles KClO₃ reduced per minute

1 per milligram of protein. All the assays were carried out in triplicate and against a
2 control assay without enzyme. The control without enzyme was used for two
3 different purposes: i) to check that there is no chlorate reduction when removing
4 the enzyme and ii) to quantify chlorate concentration within the reaction mixture
5 at zero time. This ensures that the kinetics of chlorate reduction take into account
6 the chlorate concentration within the reaction mixture at zero time. To determine
7 the optimal pH for chlorate reductase activity, 50 mM MES (pH 5.5.-6.7), 50 mM
8 MOPS (pH 6.5-7.9) and 50 mM carbonate (pH 9-11) buffers were also prepared
9 containing the aforementioned reaction mixtures. For the methylviologen assay,
10 chlorate reductase activity was measured spectrophotometrically in quartz
11 cuvettes equipped with rubber septa by monitoring the oxidation of reduced MV
12 ($\epsilon_{600 \text{ nm}} = 13700 \text{ M}^{-1} \text{ cm}^{-1}$) in presence of chlorate at different temperatures [28].
13 The reaction mixture contained, in a final volume of 800 μl , 50 mM Tris buffer pH
14 8, 0-2M NaCl or KCl, 5 mM MV and an appropriate amount of pure enzyme (final
15 protein concentration around 0.03 mg protein per mL was constantly present in
16 the reaction mixture). The assay mixture was flushed with nitrogen for 10 min,
17 and 5 mM dithionite solution (degassed and freshly prepared in 0.1 M NaHCO_3)
18 was added until an absorbance of 3.0 at 600 nm was obtained. The reactions,
19 incubated for 1 min at 40 °C, were initiated by the addition of KClO_3 (nitrogen
20 flushed) to a final concentration between 0-50 mM. Alternative electron acceptors
21 were tested in the same assay system, except that chlorate was replaced by
22 ClO_4^- , NO_3^- , IO_3^- , BrO_3^- and SeO_4^{2-} (potassium salts). All the assays were
23 carried out in triplicate and against controls without enzyme or without the
24 electron acceptors.

1 The MV assay method was also used to follow the chlorate reduction
2 using whole cells previously grown with nitrate or chlorate as electron acceptors.
3 In this case, harvested cells were resuspended in 50 mM Tris buffer containing
4 0.5 M NaCl up to a final O.D. around 0.2. The reaction mixture (1.2 ml final
5 volume) contained 1 ml resuspended cells, 5 mM MV, 5 mM dithionite solution
6 and 0-25 mM substrate. The assay was developed at room temperature. All the
7 assays were carried out in triplicate and against controls without cells or without
8 the electron acceptors. Data obtained by MV method were processed using the
9 Michaelis–Menten equation. The values of V_{max} and K_m were determined by
10 nonlinear regression analysis of the corresponding Michaelis–Menten curves
11 (rate vs. $[\text{ClO}_3^-]$) using the algorithm of Marquardt–Levenberg with the SigmaPlot
12 program (Jandel Scientific, version 1.02). The protein content was determined by
13 the Bradford method, with bovine serum albumin (fraction V) as a standard.

14 In order to check the effect of other anions (e.g. bromate and
15 (per)chlorate) on nitrate reduction catalyzed by *Hfx. mediterranei* pNarGH, the
16 nitrate reduction was also measured as previously described [20]. In that
17 instances, the standard reaction mixture contained 4mM MV (artificial electron
18 donor), 18 mM KNO_3 , and different chlorate, perchlorate and bromate
19 concentrations (from 0 up to 18 mM). We followed the nitrite production by pNar
20 using Griess method [29]. Nitrate reductase specific activity is expressed as
21 micromoles of NO_2^- appearing per minute per milligram of protein [20].

22
23
24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

3. Results

3.1 Chlorate reduction by *Hfx. mediterranei* cells

In order to analyze the capacity of *Hfx. mediterranei* cells to reduce chlorate, the cell growth was checked in anaerobic media using nitrate or chlorate as electron acceptors. When nitrate is present within the anaerobic media, denitrification is induced and as a consequence, *Hfx. mediterranei* is able to use nitrate as electron acceptor to support growth, as previously described [19-22, 30]. However, no growth was observed if chlorate was added as the unique electron acceptor to the anaerobic media. Specific growth rate (μ) and cell doubling time (t_d) for cultures under each of the assayed conditions are summarised in table 1. These results suggest that this haloarchaeon is unable to express chlorate inducible genes coding for a (per)chlorate reductase system. Recently, the *Hfx. mediterranei* genome draft has been obtained and no genes encoding (per)chlorate reductases have been detected (www.ncbi.nlm.nih.gov/genome/?term=haloferax+mediterranei).

However, when nitrate-respiring cells (anoxic conditions) were harvested at the beginning of the stationary phase of growth, washed and transferred to a fresh anaerobic chlorate media, the cells were able to use chlorate as electron acceptor. In this case, the optical density reached at stationary phase of growth is 0.8 (after 150 hours of batch culture), t_d is 27 hours and a μ of 0.019 h^{-1} is achieved. When the cells pre-grown with nitrate were transferred into chlorate-

1 containing medium more than twice, cells were not able to grow anymore. This
2 suggests that pre-induced respiratory nitrate reductase could be involved
3 physiologically in chlorate reduction supporting limited cell culture growth. In
4 earlier studies, it was stated that (per)chlorate and nitrate reduction were
5 catalyzed by the same enzyme (a nitrate reductase) in bacteria [31]. This implies
6 that the inability of several denitrifiers to grow using (per)chlorate is due to: (1)
7 failure to induced nitrate reductase in the presence of chlorate alone and (2) the
8 toxicity chlorite produced by nitrate reductase when chlorite dismutase is absent.
9 This explanation is supported by experiments where chlorate, perchlorate and
10 bromate reduction were measured using whole *Hfx. mediterranei* cells previously
11 grown with nitrate as electron acceptor (Fig. 1A), where the order of the reaction
12 velocities was $\text{ClO}_3^- > \text{NO}_3^- > \text{BrO}_3^- > \text{ClO}_4^-$, while no reduction activity was
13 detected in presence of IO_3^- or SeO_4^- , similarly to the purified enzyme (Fig. 1B).
14 This characteristic could be the explanation for the *Hfx. mediterranei* NarGH
15 chlorate reductase activity.

16

17 **3.2 Characterization of the NarGH chlorate reductase activity**

18 A preliminary report that pNarGH could reduce chlorate has previously
19 been made [32]. A more detailed study was undertaken of this reaction in the
20 present study to explore the use of this enzyme (and/or the whole cells) in waste
21 water treatments. The capacity of pure NarGH to reduce substrates such as
22 iodate, selenate, bromate and perchlorate has been checked. The results
23 obtained (Fig. 1B) have been compared with the nitrate reduction. Consistent
24 with the results from the whole cell experiments, no activity was detected when

1 iodate or selenate were used as substrate. The absence of NarGH reactivity
2 towards selenate was previously pointed out and it has been proposed that this
3 fact could be taken into account to distinguish pNars from nNars (the latter type
4 of Nars are highly reactive towards selenate) [22]. Nevertheless, pure *Hfx.*
5 *mediterranei* NarGH reduced chlorate, bromate and perchlorate with reaction
6 velocities in the order of $0.25 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $0.15 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and 0.11
7 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The order of effectiveness was $\text{ClO}_3^- > \text{NO}_3^- >$
8 $\text{BrO}_3^- > \text{ClO}_4^-$, which is the same pattern observed from the activity
9 measurements using whole cells (Fig. 1A). This pattern correlates with that
10 observed in whole cells suggesting it is due to the NarGH present in the
11 membranes of these cells.

12 The pH dependence of enzyme activity in the range 5 to 11 revealed that
13 the optimal pH for pNarGH chlorate reductase activity was around pH 8 at 40 °C
14 (which is the average temperature detected in the *Hfx. mediterranei* natural
15 environment) (Fig. 2A). pNarGH nitrate reductase activity also showed maximum
16 activity at pH values around 8 [20]. Bacterial chlorate reductases recently
17 described had a temperature optimum between 40 and 70°C, which is perhaps
18 unexpected because most bacteria from which they were purified are mesophilic
19 [16].

20 NarGH chlorate reductase activity was also measured at different NaCl or
21 KCl concentrations at 40 °C (Fig. 2B). In those reaction mixtures containing NaCl
22 the enzymatic activity increased when the NaCl concentrations were increased
23 up to 1.4 M and at higher NaCl concentrations the activity remained stable.
24 However, in those assays carried out in presence of KCl, the enzymatic activity

1 increased when the KCl concentrations were increased up to 1M, but at higher
2 KCl concentrations, a decrease in the specific enzymatic activity could be
3 detected. This result could be related to the fact that in pNarGH the catalytic
4 subunit is oriented to the positive side of the membrane [22] and in natural salt-
5 marsh environments, NaCl is the predominant salt (instead of KCl which is
6 predominant in the cytoplasm).

7 In another set of experiments, NarGH chlorate reductase activity was
8 measured using an assay mixture with different NaCl concentrations at
9 temperatures from 25° to 70 °C (Fig. 3). The maximum specific chlorate
10 reductase activity was detected in the presence of low NaCl concentrations at 35
11 °C. At salt concentrations higher than 0.4 M, the maximum specific activity could
12 be detected at higher temperatures (between 40-45 °C). The data was subject to
13 an Arrhenius analysis that revealed that the lowest activation energy (around 2.2
14 $\pm 0.2 \text{ J mol}^{-1}$ in presence of 0.8-1.6 M NaCl) was observed at the highest NaCl
15 concentrations (versus $5 \pm 0.5 \text{ J mol}^{-1}$ in presence of 0-0.4 M NaCl. These results
16 strongly support the fact that activation energy of a halophilic enzyme is lower at
17 high salt concentrations. However, the higher the salt concentrations, the lower
18 NarGH chlorate reductase activity was observed. This is not the pattern expected
19 from halophilic enzymes, which are characterised by high enzymatic activity
20 values at high salt concentrations. The pattern here described for NarGH chlorate
21 reductase activity differs from the behaviour previously described for NarGH
22 nitrate reductase activity. In the latter, nitrate reduction was not strongly
23 dependent on temperature at different NaCl concentrations [20].

1 Like other halophilic nitrate reductases from the genus *Haloferax*, NarGH
2 from *Hfx. mediterranei* presented a remarkable thermophilicity and worked well
3 up to 70 °C with nitrate as substrate and this activity did not show a direct
4 dependence on salt concentration [20]. However, working with chlorate as
5 substrate, NarGH exhibited higher specific activity at low temperatures in
6 presence of low salt concentrations (Fig. 3). This may reflect the different
7 molecular geometry of the substrates (planar triangular for nitrate, triangular
8 pyramidal for chlorate or tetragonal pyramidal for perchlorate).

9 Kinetic parameters of NarGH were determined using different
10 concentrations of chlorate (as substrate), in the presence of 50 mM Tris buffer
11 (pH 8.0) containing 0.2 M NaCl. The halophilic enzyme followed Michaelis–
12 Menten kinetics. Apparent V_{max} and K_m values for chlorate were 0.280 ± 0.003
13 $\mu\text{mol min}^{-1} \text{ mg prot.}^{-1}$ and 2.41 ± 0.16 mM, respectively. These values are higher
14 than those described in several chlorate reductases isolated from bacteria such
15 as *Pseudomonas chloritidismutans* [16], which is in agreement with the fact that
16 although NarGH is able to reduce chlorate, it is not its natural substrate. The
17 turnover numbers as well as the specificity constant have been obtained taking
18 into account the molecular mass of the NarGH isolated from *Hfx. mediterranei*
19 [20] and the V_{max} obtained from reactions where chlorate or nitrate act as
20 substrates; results are summarized in table 2.

21 Finally, nitrate reduction activity has been measured in presence of
22 different anions such as perchlorate, chlorate and bromate using pNarGH from
23 *Hfx. mediterranei* or the whole cells. As previously mentioned, it is quite common
24 to find environmental samples or waste water samples containing nitrate and

1 (per)chlorate. We expected to see competitive inhibition of nitrate reduction due
2 to the presence of (per)chlorate ions in the reaction mixture. That's why, we
3 hypothesised that when the highest oxyanion concentration was added, the
4 lowest nitrite concentration would be produced. However, the opposite effect was
5 observed: the nitrite production was even more extensive when bromate, chlorate
6 and perchlorate were present within the reaction mixture containing nitrate as
7 substrate (Fig. 4). From these results, we conclude that in the presence of nitrate,
8 these oxyanions are neither inhibitors nor alternative substrates. We suspect that
9 the high redox potential defining each of those chemical compounds has an
10 important role in this enzymatic mechanism. Thus, they could act as electron
11 carriers in the reduction of nitrate to produce nitrite. Another interesting feature to
12 highlight from these results is that the reaction in presence of nitrate + other
13 oxyanion requires more time to take effect (nitrite is not detected prior to 2-3
14 minutes of incubation), but when nitrite production starts, the reaction is quicker
15 than that taking place in absence of bromate, chlorate and perchlorate. These
16 results are relevant when trying to explore the feasibility of bioremediation of
17 waste water samples containing more than one of those compounds.

18

19 **4. Discussion**

20 The results reported here indicate that *Hfx. mediterranei* is able to use
21 (per)chlorate as final electron acceptor in absence of oxygen using the nitrate
22 reductase (pNarGH) involved in denitrification process. Some recent reports
23 support the existence of separate pathways for (per)chlorate and nitrate
24 reduction under anaerobic conditions, although they have not completely

1 eliminated the potential of shared enzymes, being used for (per)chlorate and
2 nitrate reduction in some bacteria [33]. The physiological study previously
3 discussed suggests that chlorate respiratory enzymes are not inducible in *Hfx.*
4 *mediterranei* by chlorate under anaerobic conditions, which make sense taking
5 into account that genes coding for (per)chlorate reductases have not been
6 identify in *Hfx. mediterranei* genome.

7 One interesting aspect to be pointed out is what happens to the chlorite
8 produced by NarGH during chlorate reduction by *Hfx. mediterranei*. The analysis
9 of the *Hfx. mediterranei* genome draft shows that the gene encoding a putative
10 chlorite dismutase is present (Fig. 5), while no genes coding for (per)chlorate
11 reductases have been detected either in the *Hfx. mediterranei* genome nor in
12 other haloarchaea up to now. Database searches (HALOLEX:
13 www.halolex.mpg.de/public/) have pointed out that chlorite dismutase gene from
14 *Hfx. mediterranei* is a homolog to *pitA* from *Hfx. volcanii*, which is a fusion
15 between chlorite dismutase-like and antibiotic biosynthesis monooxygenase-like
16 domains within a single open reading frame. This fusion has been also described
17 from other haloarchaea and may represent a modification to limited oxygen
18 availability [34]. Preliminary studies to detect chlorite dismutase activity in *Hfx.*
19 *mediterranei* whole cells and extracts have been carried out in our laboratory
20 following oxygen production in presence of chlorite using Durham tubes and
21 oxygen electrodes. An activity toward chlorite could not clearly be identified either
22 in whole cells or in cell extracts. Some recent work from some bacteria suggest
23 that some chlorate reduction genes might constitute transposons flanked by
24 insertion sequences which show the potential to move horizontally [24]. The

1 phylogenetic analysis carried out using bacterial genomes reveals that chlorate
2 reduction was evolved multiple times from type II DMSO reductases and chlorite
3 dismutases [24]. It has also been suggested that chlorite dismutase has been
4 mobilised at least once from (per)chlorate reducers to build chlorate respiration
5 [24]. More studies focused on that subject should be done in archaea to
6 understand how chlorate respiration was built in this domain.

7 Other studies from hyperthermophilic archaea highlight that although
8 (per)chlorate can be used as electron acceptor using enzymes belonging to the
9 type II subgroup of DMSO reductase family, no chlorite dismutase activity has
10 been detected. In that case, the authors demonstrate that chlorite is eliminated
11 by interplay of abiotic and biotic redox reactions involving sulphur compounds
12 instead of being enzymatically split into chloride and oxygen [1]. This work has
13 been carried out using *Archaeoglobus fulgidus*, which is a hyperthermophilic
14 archaeon that thrive in environments resembling those of early Earth.

15 In the study presented here, we have not added specific sulphur
16 compounds to check the mentioned interplay of abiotic and biotic redox reactions
17 and no evidence on that subject has been reported so far from haloarchaea. So,
18 it would be worth exploring these abiotic and biotic redox reactions involving
19 sulphur in the future within in member of the Halobacteriaceae family. Regarding
20 this,it is interesting to draw attention to: i) the natural salted water where these
21 microorganisms live contains sulphur salts, ii) when growing the haloarchaea in
22 the lab, salted water also contains sulphur salts (ammonium sulphate,
23 magnesium sulphate, etc.) and iii) some of the protocols used to purify proteins
24 from haloarchaeal involve buffers containing sulphur compounds (i.e ammonium

1 sulphate). Therefore, we cannot dismiss the possibility that abiotic and biotic
2 redox reactions involving sulphur compounds take place in haloarchaea. Some
3 works related to bacterial communities in marine sediments have also stated
4 recently that there are (per)chlorate effects on metabolic pathways related to
5 sulphur [35].

6 This research as well as other recent published reports suggest that
7 haloarchaea can reduce perchlorate and chlorate anaerobically. This fact has
8 recently been connected to studies of the anaerobic Martian environment where
9 perchlorate among the salts as detected by the Phoenix Lander on Mars, may
10 support halophilic life in a similar way to the halophilic environments on Earth
11 [36].

12 The results presented show that (per)chlorate anions can be efficiently
13 removed from the media where the *Hfx. mediterranei* cells are growing.
14 Perchlorate and chlorate reduction take place efficiently thanks to *Hfx.*
15 *mediterranei* cells, even in presence of low salt concentrations. This fact could
16 suggest that changes in the structure of the enzyme, and therefore the chlorate
17 reductase activity, could be caused by salt.

18 If this is the case for other denitrifying halophilic archaea, then those type
19 of microorganisms could be excellent models for the bioremediation of brines
20 [37]. Most of the ground waters or waste waters contaminated with chlorate also
21 contain nitrate [16] and several previous studies have shown that (per)chlorate
22 and nitrate are simultaneously degraded by several bacteria [38]. A better
23 understanding of the factors that regulate the expression of the enzymes involved
24 in (per)chlorate, chlorate, and nitrate reduction are important as several

1 microorganisms could play a key role in water bioremediation. The processes
2 based on bioremediation could replace or even improve those protocols where
3 perchlorate and nitrate are removed from brines by ion exchange techniques [5].
4 Several studies have highlighted that (per)chlorate-reducing bacteria removed
5 (per)chlorate at such slow rates to make them impractical for application in
6 treatment systems. Moreover, the enzymes involved in this pathway are only
7 active at low salt concentrations [5]. Other previous studies reported rapid
8 perchlorate and nitrate removal using mixed cultures in presence of 10% NaCl
9 [39]. Other systems use permeable barriers containing vegetable oil that would
10 promote the degradation of (per)chlorate [7]. However, each process has a
11 limitation, namely salt tolerance.

12

13 **5. Conclusions**

14 Respiratory nitrate reductases, mainly those belonging to the pNarGH
15 group [20] may have a relevant role in (per)chlorate reduction in those halophilic
16 archaea lacking genes coding for (per)chlorate reductases. Biological
17 per(chlorate) reduction by ancient archaea might have taken place during pre-
18 anthropogenic times thanks to (per)chlorate reductases or even nitrate
19 reductases. As a consequence of these enzymatic activities members of the
20 archaea domain may have prevented perchlorate accumulation in early Earth
21 giving rise to the environmental conditions characterising the Earth [1]. Taking
22 into account the biochemical parameters defining NarGH chlorate reductase
23 (reported here) and NarGH nitrate reductase activities in *Hfx. mediterranei*
24 [20,22], waste water treatment approaches could consider the relevance of

1 halophilic denitrifiers to explore a role in bioremediation in the near future.
2 Preliminary results obtained in our lab reveal that chlorate removal by *Hfx.*
3 *mediterranei* cells is more efficient than per(chlorate) removal. In those media
4 containing 5 mM chlorate, the final chlorate concentration quantified after 150
5 hours of incubation was 0.2 mM. On the other hand, nitrate reduction is not
6 inhibited in presence of either (per)chlorate or bromate, it can even be concluded
7 that bromate is able to slightly stimulate nitrate reduction (see results summarised
8 in figure 4). So, the same microorganism could reduce nitrate and chlorate in
9 presence of other ions thanks to the nitrate reductase under microaerobic or
10 anaerobic conditions. These results are quite interesting in terms of waste water
11 bioremediation purposes because most of the waste water samples containing
12 nitrate also contain chlorate and other oxyanions. The removal ratio for chlorate
13 estimated in our study is around 4.8 mM chlorate in approximately 6 days.
14 Although the removal process is not really fast, the removed concentration is one
15 of the highest described at the time of writing when using microorganism for
16 waste water bioremediation [40,41]. Furthermore, one of the advantages of using
17 *Hfx. mediterranei* cells or its pNarGH is that nitrate reduction is not inhibited in
18 presence of (per)chlorate (and vice versa) at high salt concentrations. These
19 results open a new way to explore bioremediation processes making
20 haloarchaeon-based remediation of brines and waste waters feasible.

21
22

23 **Acknowledgements**

1 This work was funded by research grant from the MINECO Spain
2 (CTM2013-43147-R), MEC Spain (BIO2008-00082), Generalitat Valenciana
3 (GV/2011/038) and University of Alicante (GRE09-25).

4

5

6

7

8

9 **References**

- 10 [1] M.G. Liebensteiner, M.W.H. Pinkse, P.J. Schaap, A.J.M Stams, B.P. Lomans,
11 Archaeal (per)chlorate reduction at high temperature: an interplay of biotic
12 and abiotic reactions, *Science* 350 (2013) 85-87.
- 13 [2] C. Hogue, Rocket-fueled river, *Chem. Eng. News* 81 (2003) 37-46.
- 14 [3] E.T. Urbansky, M.R. Schock, Issues in managing the risks associated with
15 perchlorate in drinking water, *J. Environ. Manag.* 56 (1999) 79-95.
- 16 [4] B.E. Logan, Assessing the outlook for perchlorate remediation, *Environ. Sci.*
17 *Technol.* 23 (2001) 482A-487A.
- 18 [5] S.G. Lehman, M. Badruzzaman, S. Adham, D.J. Roberts, D.A. Clifford,
19 Perchlorate and nitrate treatment by ion exchange integrated with biological
20 brine treatment, *Water Res.* 42 (2008) 969-976.
- 21 [6] S.W.M. Kengen, G.B. Rikken, W.R. Hagen, C.G. van Ginkel, A.J.M. Stams,
22 Purification and characterization of(per)chlorate reductase from the
23 chlorate-respiring strain GR-1, *J. Bacteriol.* 181 (1999) 6706-6711.

- 1 [7] W.J. Hunter, Bioremediation of Chlorate or Perchlorate contaminated water
2 using permeable barriers containing vegetable oil, *Curr. Microbiol.* 45
3 (2002) 287-292.
- 4 [8] S.A.B. Weelink, N.C.G. Tan, H. ten Broeke, C. van den Kieboom, W. van
5 Doesburg, A.A.M. Langenhoff, J. Gerritse, H. Junca, A.J.M. Stams, Isolation
6 and characterization of *Alicyclophilus denitrificans* Strain BC, which grows
7 on benzene with chlorate as the electron acceptor, *Appl. Environ.* 74 (2008)
8 6672-81.
- 9
- 10 [9] C.G. van Ginkel, G.B. Rikken, A.G.M. Kroon, S.W.M. Kengen, Purification and
11 characterization of chlorite dismutase: a novel oxygen-generating enzyme,
12 *Arch. Microbiol.* 166 (1996) 321-326.
- 13 [10] T. Giblin, W.T. Frankenberger, Perchlorate and nitrate reductase activity in
14 the perchlorate-respiring bacterium *perclace*, *Microbiol. Res.* 156 (2001)
15 311-315.
- 16 [11] K.S. Bender, S.M. O'Connor, R. Chakraborty, J.D. Coates, L.A. Achenbach,
17 Sequencing and transcriptional analysis of the chlorite dismutase gene of
18 *Dechloromonas agitata*, and its use as metabolic probe, *Appl. Environ.*
19 *Microbiol.* 68 (2002) 4820-4826.
- 20 [12] B.R. Streit, J.L. DuBois, Chemical and steady-state kinetic analyses of a
21 heterologously expressed heme dependent chlorite dismutase,
22 *Biochemistry* 47 (2008) 5271-5280.

- 1 [13] B. Goblirsch, R.C. Kurker, B.R. Streit, C.M. Wilmot, J.L. DuBois, Chlorite
2 dismutases, DyPs, and EfeB: 3 microbial heme enzyme families comprise
3 the CDE structural superfamily, *J. Mol. Biol.* 408 (2011) 379-398.
- 4 [14] J.D. Coates, L.A. Achenbach, Microbial perchlorate reduction: rocket-fuelled
5 metabolism, *Nat. Rev. Microbiol.* 2 (2004) 569-580.
- 6 [15] J. Cameron Thrash, J. Pollock, T. Torok, J.D. Coates, Description of the
7 novel perchlorate-reducing bacteria *Dechlorobacter hydrogenophilus* gen.
8 nov., sp. nov. and *Propionivibrio militaris*, sp. nov., *Appl. Microbiol.*
9 *Biotechnol.* 86 (2010) 335-343.
- 10 [16] A.F.W.M. Wolterink, E. Schiltz, P.L. Hagedoorn, W.R. Hagen, S.W.M.
11 Dengen, A.J.M. Stams, Characterization of the chlorate reductase from
12 *Pseudomonas chloritidismutans*, *J. Bacteriol.* 185 (2003) 3210-3213.
- 13 [17] N. Bardiya, J.H. Bae, Dissimilatory perchlorate reduction: a review, *Microbial*
14 *Res.* 166 (2011) 237-254.
- 15 [18] D.C. Herman, W.T. Frankenberger, Bacterial reduction of perchlorate and
16 nitrate water, *J. Environ. Qual.* 28 (1999) 1018-1024.
- 17 [19] M.J. Bonete, R.M. Martínez-Espinosa, C. Pire, B. Zafrilla, D.J. Richardson,
18 Nitrogen metabolism in haloarchaea, *Saline Systems*, 4 (2008) 9.
- 19 [20] B. Lledó, R.M. Martínez-Espinosa, F.C. Marhuenda-Egea, M.J. Bonete,
20 Respiratory nitrate reductase from haloarchaeon *Haloferax mediterranei*:
21 biochemical and genetic analysis, *Biochim. Biophys. Acta* 1674 (2004) 50-
22 59.

- 1 [21] R.M. Martínez-Espinosa, D.J. Richardson, J.N. Butt, M.J. Bonete,
2 Respiratory nitrate and nitrite pathway in the denitrifier haloarchaeon
3 *Haloferax mediterranei*, Biochem. Soc. Transac. 34 (2006) 115-117.
- 4 [22] R.M. Martínez-Espinosa, E.J. Dridge, M.J. Bonete, J.N. Butt, C.S. Butler, F.
5 Sargent, D.J. Richardson, Look on the positive side! The orientation,
6 identification and bioenergetics of “Archaeal” membrane-bound nitrate
7 reductases, FEMS Microbiol. Lett, 276 (2007) 129-139.
- 8 [23] T. Nilsson, M. Rova, A. Smedja Bäcklund, Microbial metabolism of
9 oxochlorates: a bioenergetic perspective, Biochim. Biophys. Acta 182
10 (2013) 189-197.
- 11 [24] I.C. Clark, R.A. Melnyk, A. Engelbrektsen, J.D. Coates, Structure and
12 evolution of chlorate reduction composite transposons, mBio 4 (2013) 1-
13 11, e00379-13.
- 14 [25] I.C. Clark, R.A. Melnyk, A.T. Iavarone, P.S. Novichkov, J.D. Coates,
15 Chlorate reduction in *Shewanella* algae ACDC is a recently acquired
16 metabolism characterized by gene loss, suboptimal regulation and oxidative
17 stress, Mol. Microbiol. 94 (2014) 107-125.
- 18 [26] F. Rodríguez-Valera, F. Ruiz Berraquero, A. Ramos Cormenzana, Behaviour
19 of mixed populations of halophilic bacteria in continuous cultures, Can. J.
20 Microbiol. 26 (1980) 1259-1263.
- 21 [27] P. Urone, E. Bonde, Colorimetric determination of chlorates in well waters,
22 Anal. Chem. 12 (1960) 1666-1668.
- 23 [28] V.A. Bamford, H.C. Angove, H.E. Seward, A.J. Thomson, J.A. Cole, J.N.
24 Butt, A.M. Hemmings, D.J. Richardson, Structure and spectroscopy of the

- 1 periplasmic cytochrome c nitrite reductase from *Escherichia coli*,
2 Biochemistry 41 (2002) 2921-2931.
- 3 [29] C.D. Snell, C.T. Snell, Colorimetric Methods of Analysis, Vol. 2, (1949) pp.
4 802-807. Van Nostrand, New York.
- 5 [30] R.M. Martínez-Espinosa, B. Zafrilla, M. Camacho, M.J. Bonete, Nitrate and
6 nitrite removing from salted water by *Haloferax mediterranei*, Biocatal.
7 Biotrans. 25 (2007) 295-300.
- 8 [31] M.D. Roldán, F. Reyes, C. Moreno-Vivián, F. Castillo, Chlorate and nitrate
9 reduction in the phototrophic bacteria *Rhodobacter capsulatus* and
10 *Rhodobacter sphaeroides*, Curr. Microbiol. 29 (1994) 241-245.
- 11 [32] R.M. Martínez-Espinosa, M.J. Bonete, Bioremediation of chlorate and
12 perchlorate salted water using *Haloferax mediterranei*, J. Biotechnol. (2007)
13 S227. DOI:10.1016/j.jbiotec.2007.07.411
- 14 [33] J. Xu, J.T. Trimble, L. Steinberg, B.E. Logan, Chlorate and nitrate reduction
15 pathways are separately induced in the perchlorate-respiring bacterium
16 *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas*
17 sp. PDA, Water Res. 38 (2004) 673-680.
- 18 [34] E. Bab-Dinitz, H. Shmueli, J. Maupin-Furlow, J. Eichler, B. Shaanan,
19 *Haloferax volcanii* PitA: an example of functional interaction between the
20 Pfam chlorite dismutase and antibiotic biosynthesis monooxygenase
21 families?, Bioinformatics 22 (2006) 671-676.
- 22 [35] A. Engelbrekton, C.G. Hubbard, L.M. Tom, A. Boussina, Y.T. Jin, H. Wong,
23 Y.M. Piceno, H.K. Carlons, M.E. Conrad, G. Anderson, J.D. Coates,

- 1 Inhibition of microbial sulphate reduction in a flow-through column system
2 by (perchlorate treatment, *Front. Microbiol.* 26 (2014) 5:315.
- 3 [36] A. Oren, R. Elevi Bardavid, L. Mana, Perchlorate and halophilic prokaryotes:
4 implications for possible halophilic life on Mars, *Extremophiles* 18 (2014)
5 75-80.
- 6 [37] C. Nájera-Fernández, B. Zafrilla, M.J. Bonete, R.M. Martínez-Espinosa, Role
7 of the denitrifying Haloarchaea in the treatment of nitrite-brines, *Int.*
8 *Microbiol.* 15 (2012) 111-119.
- 9 [38] M.J. Oosterkamp, F. Mehboob, G. Schraa, C.M. Plugge, A.J.M. Stams,
10 Nitrate and (per)chlorate reduction pathways in (per)chlorate-reducing
11 bacteria, *Biochem. Soc. Trans.* 39 (2011) 230-235.
- 12 [39] B.C. Okeke, T. Giblin, W.T. Frankenberger, Reduction of perchlorate and
13 nitrate by salt tolerant bacteria, *Environ. Pollut.* 118 (2002) 357-363.
- 14 [40] C.G. van Ginkel, A.M. van Haperen, B. Van der Togt, Reduction of bromated
15 to bromide coupled to acetate oxidation by anaerobic mixed microbial
16 cultures, *Water Res.* 39 (2005) 59-64.
- 17 [41] N. Bardiya, J.H. Bae, Bioremediation potential of a perchlorate-enriched
18 sewage sludge consortium, *Chemosphere* 58 (2005) 83-90.

19
20
21
22
23
24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Legend to figures

Figure1. Reduction of different substrates by whole *Hfx. mediterranei* cells (A). Cells were grown under anaerobic conditions with nitrate as electron acceptor as cited in materials and methods. Harvested cells were resuspended in fresh complex culture medium containing 5 mM MV, 5 mM DT. Substrates final concentration was 20 mM. The reaction mixture was incubated at 40 °C for 15 minutes. 100 % Activity = 0.76 U.

Reduction of different substrates by pure NarGH (B). Assays were carried out in 50 mM Tris buffer pH 8 at 40 °C for 15 minutes. Substrates final concentration was 30 mM. 100 % Activity = 0.28 $\mu\text{moles MV oxidized min}^{-1} \text{ mg prot.}^{-1}$ MV assay was used in this experiment.

1 **Figure 2.** Optimum pH (A) and effect of salts concentration on chlorate reductase
2 activity (B) Reactions mixtures were incubated at 40 °C for 10 minutes. In panel
3 A, 100 % Activity correspond to 0.13 $\mu\text{moles KClO}_3$ reduced $\text{min}^{-1} \text{mg prot.}^{-1}$. O-
4 toluidine assay was used in this experiment.

5

6 **Figure 3.** Effect of temperature and salt concentration on chlorate reductase
7 activity. Assays carried out in 50 mM Tris buffer pH 8, at 40 °C, 10 minutes. O-
8 toluidine assay was used in this experiment.

9

10

11

12 **Figure 4.** Nitrate reductase activity in presence of different oxyanions. (triangle:
13 18 mM nitrate + bromate; rhombus: 18 mM nitrate + perchlorate; square: 18 mM
14 nitrate + chlorate). MV was used in this assay as electron donor. Nitrite
15 production by pNarGH was follow using Griess method.

16

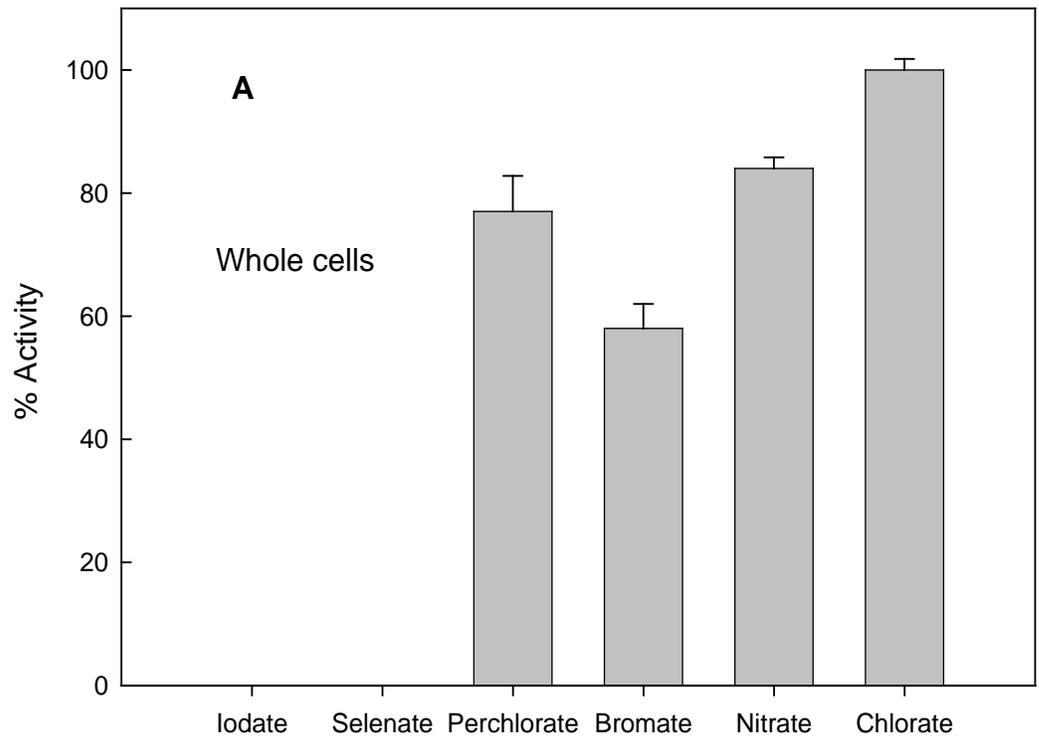
17 **Figure 5.** Protein sequence alignment *Hfx. mediterranei* chlorite dismutase-like
18 protein with *Hfx. volcanii* PitA and *Halorubrum lacusprofundi* B9LRB6,
19 *Haloterrigena turkmenica* D2RQG0 and *Natrialba magadii* D3STR5 using Clustal
20 2.0.12. Histidine residues lying in the region linking chlorite dismutase-like and
21 monooxygenase-like domains are in bold and underlined.

22

23

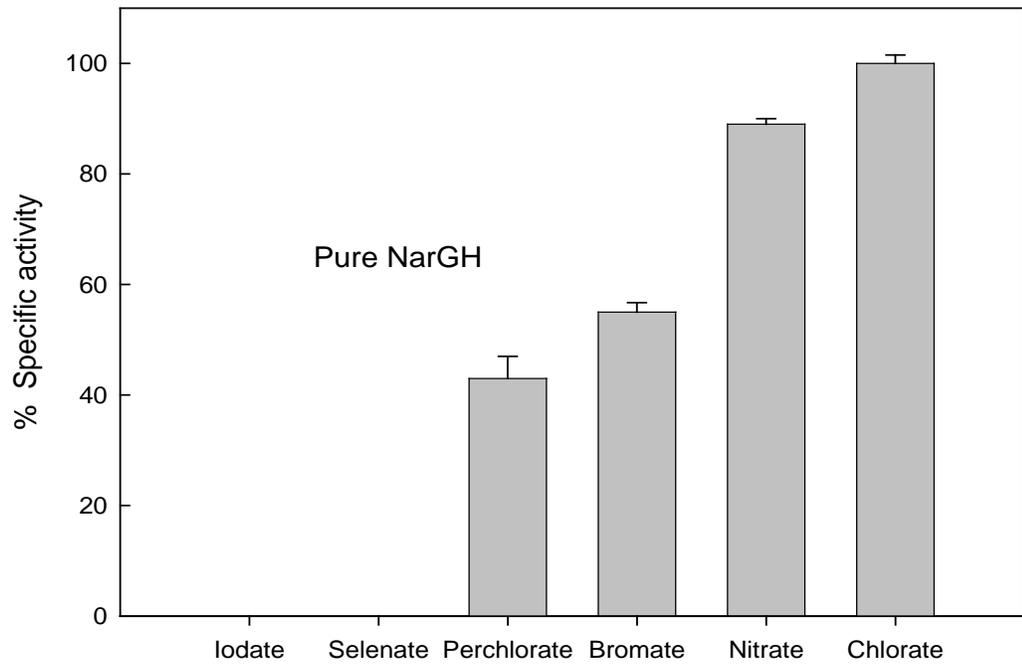
24

1
2
3
4
5
6
7
8
9
10
11
12 **Figure 1**



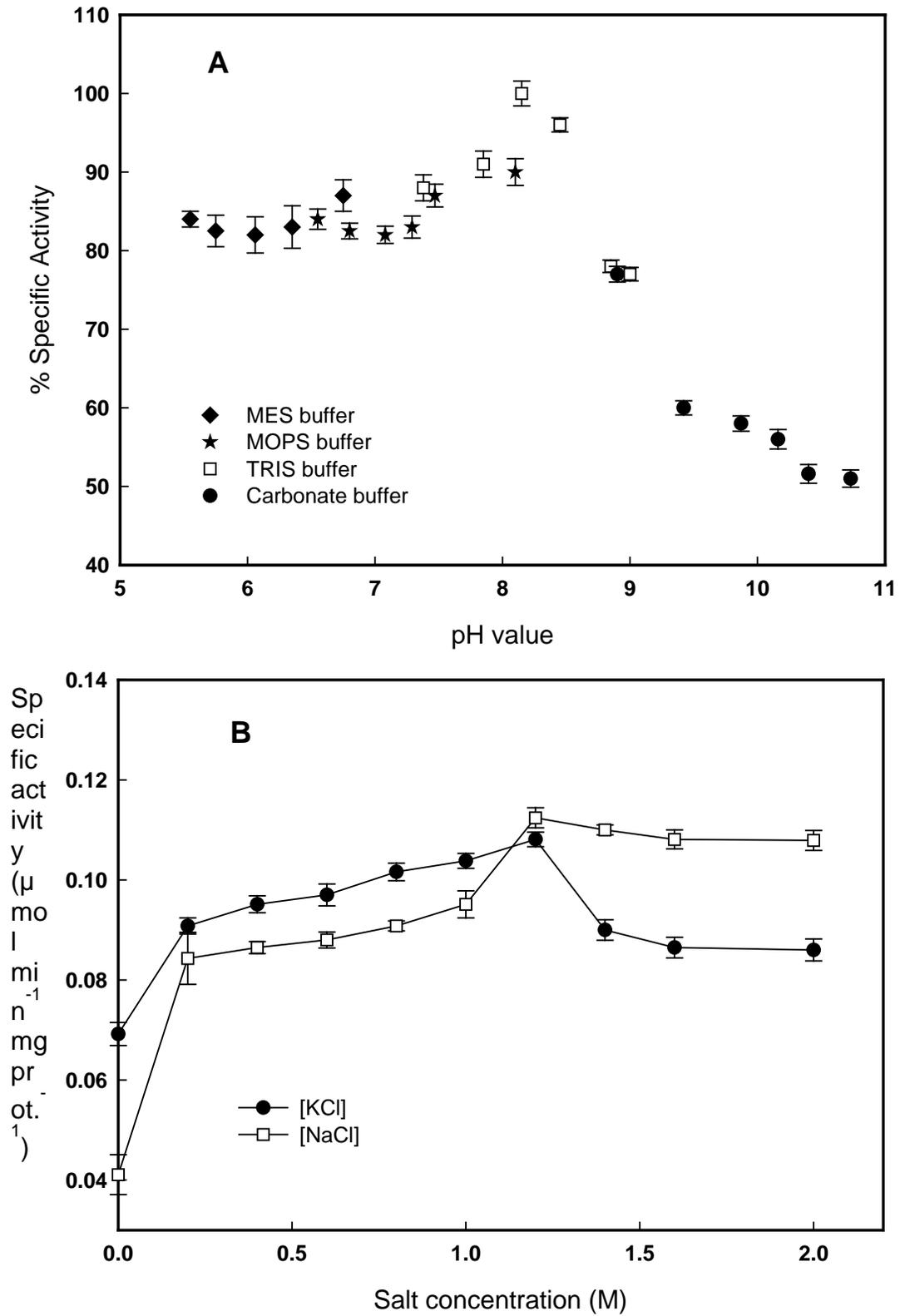
13

B



- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14

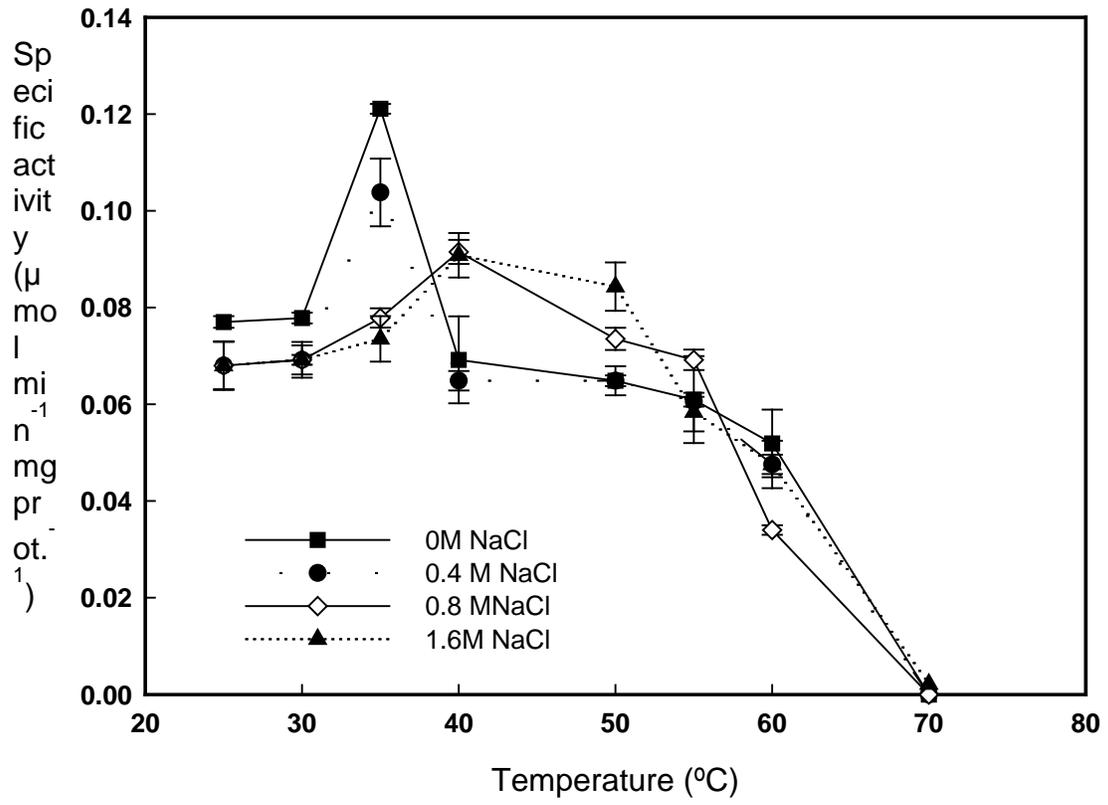
1 **Figure 2**



1 **Figure 3**

2

3



4

5

6

7

8

9

10

11

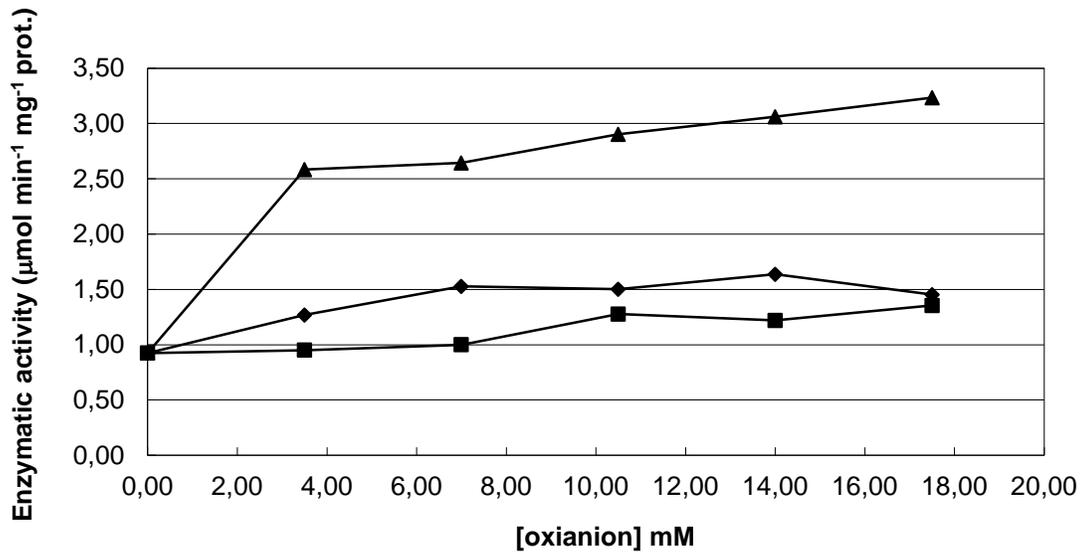
12

1

2 **Figure 4**

3

4



5

6

7

8

9

10

11

12

13

14

15

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68

Figure 5

<i>H mediterranei</i>	--MVEAPQTDEGWALHDFRTVDWDAWRDAPEHERRRAIEEGVAYLNSHE	48
<i>H volcanii</i>	--MVEAPQTDEGWALHDFRTVDWDAWRDAPDRERRRAIEEGVAYLDAHE	48
<i>H lacusprofundi</i>	--MVEAPQTEEGWFALHDFRSIDWDAWRDAPERERKRAIEEGKAFKHKRE	48
<i>H turkmenica</i>	MERRQPPQTEEGWVVLHDFRSIDWDAWRDAPERRRSRAIEEGIEYLSAAE	50
<i>N magadii</i>	MERRQPPQTEEGWVVLHDFRSIDWDAWRDAPEHRRSRALEEGIDYLTAAN	50
<i>H mediterranei</i>	AVEDAAEGTSAIFSVLGHKADFMVVHFRPTLDDISRAERQFEQTALAEFT	98
<i>H volcanii</i>	AVEDAAEGASAVFVSVLGHKADFVVHFRPTLDDISRAERQFERTALAAFT	98
<i>H lacusprofundi</i>	LVADADEGDSGLFVSVLGHKADLLFVHFRPTLDDLSIERRFEDTALANFT	98
<i>H turkmenica</i>	SVADAEEGDSATFVAVLGHKADLLVHLRPTLADLDALERRFEGTALAEFT	100
<i>N magadii</i>	DVADAEEGDSAVFVSVLGHKADLLVHLRPTLADLDALERRFEGTALAEFT	100
<i>H mediterranei</i>	EQPTSYSVSVTEVSGYVSDDYFEGNKEDIDTGLLRYIEGKLPDIPDDTYM	148
<i>H volcanii</i>	EQPTSYSVSVTEVSGYVSDDYFEGNEEDIDAGLLRYIEGKLPDIPEDTYM	148
<i>H lacusprofundi</i>	ERTTSYSVSVTEVSGYVSDDEFFE-DPESVDTGLKRYIEGKMTPEIPDDEYV	147
<i>H turkmenica</i>	ERADSYLSVTEVSGYMSQDYFDEDAEVEDTGMARYIETRLKPEIPDSEFL	150
<i>N magadii</i>	ERADSYLSVTEVSGYMSQDYFEEDGEIEDTGTKRYIESRLKPTIPDSEFV	150
<i>H mediterranei</i>	SFYPMKRRRGEKHNWYDLPFDERRELMSVHGDTGRKYAGKIKQVIASSVG	198
<i>H volcanii</i>	SFYPMKRRRGEHNWYDLPSFDERDLMSVHGDTGRQYAGKIKQVIASSVG	198
<i>H lacusprofundi</i>	CFYPMKRRRGEYHNWYDLPSFEDRADLMADHGEVGGKEYAGKIKQVIASSVG	197
<i>H turkmenica</i>	SFYPMKRRRGPEDNWYDLPFDERAEHLSSHGDI GKDYAGRVTQIIISGSIG	200
<i>N magadii</i>	SFYPMKRRRGPENWYDLPFDERADHLSSHGELGRNYAGRVTQIIISGSVG	200
<i>H mediterranei</i>	FEFEWGVTLFGDDPTDIKDIVYEMRFDEVS AKYGEFGEFYVGRFPFPPSD	248
<i>H volcanii</i>	FDDYEWGVTLFGDDPTDIKDIVYEMRFDEVS SKYGEFGEFYVGRFPFPPSD	248
<i>H lacusprofundi</i>	FDSHEWGVTLFGSDPTDIKDIVYEMRFDPASSRYGEFGEFYIGRRFPFPPD	247
<i>H turkmenica</i>	LDDFEWGVTLFGDDPTDVKELLYEMRFDPSSSRFAEFGRFLSARRFPFPPD	250
<i>N magadii</i>	LDDFEWGVTLFADDDPTDVKELLTEMRFDPSSSAFAEFGRFLSARRFPFPPAN	250
<i>H mediterranei</i>	LGAFFLAGDAVPT----SEFGDES <u>HHHAHAHG</u> -EGG <u>HHHGEGGHAHG</u> EDGH	293
<i>H volcanii</i>	LGAFFLAGDGVPT----SEFGDESHHGAHAHG-EGG-HHGEGG-----DGH	287
<i>H lacusprofundi</i>	LGAYFAGETVPTPAGDGDGTGDTEDGHGHAHG-EGHDHAGSGGGS--AHGD	294
<i>H turkmenica</i>	LGAFLAGERLIPR-----EGEESHGHEPHAGGESGGHHHGEGSGHHHEGSGD	295
<i>N magadii</i>	LGAFLAGEEVP-----QEQTSGHQHAGG--HGHNNGHGHGSDSSG	290
<i>H mediterranei</i>	<u>HHG</u> ESG <u>HGHG</u> EGG <u>HHG</u> -----GDSDEA----DETDIRGQLDDLNIY	331
<i>H volcanii</i>	HH----HDDGDGDHPH-----GDDGDEA----ADEDIRGQLEDLNIY	321
<i>H lacusprofundi</i>	HPHGEEETSGEGDHPHSGEEGGGHGEDGDDP----SDADIRGELADLNIY	340
<i>H turkmenica</i>	HHHGDSSSSRGDDHGGS-----GGPHGD-----DDEDLRSELEDMGVY	333
<i>N magadii</i>	HHHGDSG-HGHGHGHS-----GDPHDDAGADEDDSVRSELEELGVY	332
<i>H mediterranei</i>	AGKPHGEDVYATVLYSEADAEVFEVEEGLRGNFDHYPTHVKTAVYEAND	381
<i>H volcanii</i>	AGKPHGEDVYATVLYSEADAEVFEVEEGLRGNFDHYPTHVKTAVYEANE	371
<i>H lacusprofundi</i>	AGKPSGEDVYATVLYSEADVDELFEVEEGLRGNFDHYGTHVKTAVYEGRV	390
<i>H turkmenica</i>	AGQPHGEDVHAVVLYSAADAEEVFEVDGLRGNFDHYDTHVKTAVYEPQD	383
<i>N magadii</i>	AGQPHGEDVHAVVLYSAADAGELFEVEEGLRTNFDHYDTHVKTAVYEPQN	382
<i>H mediterranei</i>	RD---RNAVVISWETASAAETAAGFLSELPGIVERAGEESG--FGTMGMF	426
<i>H volcanii</i>	RG---RVAVVISWETASAAETAAGFLSELPGIVERAGEESG--FGTMGMF	416
<i>H lacusprofundi</i>	TD---RAAVVISWETASAAETAAGFLSELPEVVARAGEESG--FGTMGMF	435
<i>H turkmenica</i>	GGDDSETAVVSLWETERAASTAAGFLADLPDIVRQAGDDEGDSWGTMGMF	433
<i>N magadii</i>	DDSDAETA VVSLWETDRAANTAAGFLADLPDIVRQAGDDEDDSWGTMGMF	432
<i>H mediterranei</i>	YTVKPEHREDVFEKFGVVGGLDDMDGHFDTDLMVNLEDENDMFIASQWR	476
<i>H volcanii</i>	YTVKSEHRGDFVEKFGTVVGLLEEMDGHFDTDLMVNVEDEDDMFIASQWR	466
<i>H lacusprofundi</i>	YTVKPEHQEDFTDFFDDVGEILAEMDGHVETDLMNVNVEDENDMFIASQWH	485
<i>H turkmenica</i>	YSVKPEHRGDFLGTFFEEAGELLAEMDGHKTDLLINREDENDMFIASRWD	483
<i>N magadii</i>	YTVKPEHRGDFIGVFDDAASILAEMDGHKSDLLVNREDENDMFIASRWD	482
<i>H mediterranei</i>	SQEDAMGFFRSDEFRTVQWGRDVLADRPRHVFLA	511
<i>H volcanii</i>	SQDDAMEFFRSDAFRDVTQWGRDVLADRPRHVFLA	501
<i>H lacusprofundi</i>	AKEDAMAFFGSDEFRETVQWGREVLADRPRHVFLA	520
<i>H turkmenica</i>	SREDAMQFFRSDAFSEAVEFGRDVLDRPRHVFLA	518
<i>N magadii</i>	SREDAMQFFRSDEFAETVEFGRDVLADRPRHVFLA	517

1
2
3
4
5
6

Table 1: Specific growth rate (μ) and cell doubling time (t_d) calculated from *Hfx. mediterranei* cultures anaerobically grown in complex media [20] in presence or absence of electron acceptor.

Electron acceptor	Optical density (600nm) at stationary phase of growth	Batch culture time (hours)	μ (h^{-1})	t_d (hours)
None	0.15	150	0.0058	79
Nitrate	2	150	0.11	6.5
Chlorate	0.15	150	0.0060	75
Nitrate growing cells transferred to chlorate media	0.8	150	0.019	27

7
8
9
10
11
12
13
14
15

1

2 **Table 2:** Turnover numbers (K_{cat}) and kinetic constants for NarGH. Kinetic
3 parameters related to nitrate were obtained at pH 8 in presence of 3.6 M NaCl
4 and MV as electron donor. Regarding to chlorate, kinetic parameters were
5 obtained in presence of 0.5 M NaCl and MV as electron donor. Activity is
6 expressed as $\mu\text{moles MV oxidized min}^{-1} \text{ mg prot.}^{-1}$

7

Substrate	K_m (mM)	V_{max} ($\mu\text{moles MV oxidized}$ $\text{min}^{-1} \text{ mg prot.}^{-1}$)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{mM}^{-1}\text{s}^{-1}$)	Reference
Nitrate	0.8	0.25	0.042	0.052	[20, 22]
Chlorate	2.4	0.28	46	19	This paper

8

9
10
11
12
13