

Microbiologically Influenced Corrosion by Halophilic (Salt-loving) Nitrate and Sulfate-reducing Microorganisms

Biwen Annie An
Bundesanstalt für Materialforschung und -
prüfung (BAM)
Unter den Eichen 87
Berlin, 12205
Germany

Andrea Koerd
Bundesanstalt für Materialforschung und -
prüfung (BAM)
Unter den Eichen 87
Berlin, 12205
Germany

Hans-Jörg Kunte
Bundesanstalt für Materialforschung und -
prüfung (BAM)
Unter den Eichen 87
Berlin, 12205
Germany

ABSTRACT

Microbiologically influenced corrosion (MIC) potential of halophilic (salt-loving) microorganisms have gained increased interests in recent years due to the expansion of industrial operations in saline environments i.e. shale. Survey of multiple shale reservoirs across the continent revealed a number of recurring taxa shared by many geological formations, including members of the genera *Halomonas*, *Halanaerobium*, *Methanohalophilus* and members of the order Desulfovibrionales.

In this paper, MIC potential of pure halophilic strains was evaluated and compared with mixed microbial communities at high salinity. Results showed that the MIC potential of the pure nitrate-utilizing strain *Halomonas halodenitrificans* was low (max: 0.144 mm/yr), but it formed a biofilm layer close to the steel surface. Whereas the highly corrosive sulfate-reducing bacteria *Desulfovibrio ferrophilus* formed a thick and compact corrosion layer at the same salinity.

Results of the mixed microbial community established using enrichments from a Canadian shale oil site revealed a close association between the activities of bacteria from the genus *Halanaerobium* and the other members of the halophilic community. The data indicate the interdependence between the halophiles will alter the overall MIC mechanism.

Key words: Halophile, halophilic microorganism, methanogenesis, high salinity, NaCl, shale, extremophile, sulfate-reducing bacteria, nitrate-reducing bacteria, microbiologically influenced corrosion, microbially induced corrosion, MIC, *Halanaerobium*

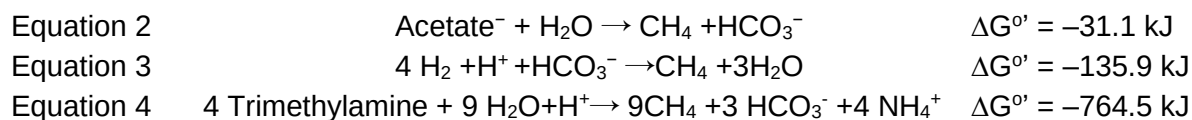
INTRODUCTION

Saline environments with greater than 10% NaCl (or approximately 1.5 M NaCl which is triple the seawater salinity) such as the Great-Salt Lake, salt marshes and salt caverns^{1,2}, are inhabited by a unique group of microorganisms, known as the halophiles or salt-loving microbes. An increased industrial exposure on the halophiles was brought upon by the exploratory activities of the energy industry, such as shale operations. Microbial community analyses of several deep surface shale reservoirs revealed high proportions of halophiles³⁻¹⁰, which raises many questions regarding the potential risks of the halophiles on the infrastructures.

Halophiles have evolved two basically different adaptation strategies to survive in environments with high level of salt stress^{2-4,11,12}, namely the “salt-in-cytoplasm” mechanism and the organic osmolyte mechanism. Organisms following the “salt-in-cytoplasm” mechanism adapt the interior protein chemistry of the cell to high salt concentration by employing highly acidic intracellular protein structures to accommodate the ionic stress. Organisms of the “salt in cytoplasm” strategy can be often found in environments with salt concentrations well above 15% NaCl¹².

In contrast, microorganisms applying the organic-osmolyte strategy accumulate uncharged, highly water-soluble organic compounds in order to maintain an osmotic equilibrium with the surrounding medium. These molecules do not disturb the cell's metabolism, and are thus aptly named *compatible solutes*². Compatible solute accumulation for osmoadaptation is widespread among Bacteria and also present in some methanogenic Archaea. The most prominent compatible solutes in Bacteria are the amino acid derivatives glycine betaine and ectoine¹³. Compatible solutes are beneficial for bacterial cells not only as osmoregulatory solutes, but also as protectants of proteins by mitigating detrimental effects of desiccation, freezing and high temperatures¹³.

Bacterial populations thriving in a marine environment (3% NaCl) will remain stable and unchanged up to a salinity of 10%. Above 10% NaCl, marine (or halotolerant) bacteria struggle and the composition of the microbial community changes. Based on this environmental observation, Oren (2008)² defines halophilic microorganisms as organisms that can grow in salt containing environments well above 10% NaCl even if their optimum for growth is below that threshold. However, even with this plain definition it is not always simple to separate halotolerant from halophilic microorganisms. Resistance to salt stress is interconnected to the metabolic efficiency of a microorganism¹⁴ and the thermodynamic constraints imposed by the environment. Halophiles using the salt-in-cytoplasm mechanisms can grow at extreme salinity ranges, due to the low cost of their osmoregulation process¹⁴. Microorganisms employing the organic osmolyte mechanism can also tolerate saturating concentrations (> 300 g/L) of NaCl if they can generate high yields of ATP by photosynthesis and aerobic respiration. For anaerobic microorganisms, such as acetoclastic and hydrogenotrophic methanogens, the low energy yield from their methanogenic reactions (Equation 2 and Equation 3)¹⁵ prohibits their growth at salinities above 120 g/L of NaCl. Whereas, methylotrophic methanogenic reactions yield much higher energy output, allowing the microbes to grow well up to 250 g/L of NaCl (Equation 1)¹⁵.



Industrial applications of halophile focusing on the production of compatible solutes such as ectoine, for pharmaceutical, cosmetic and other biotechnological purposes are well known¹³. However, the involvement of halophiles on structural integrity and material biodeterioration, i.e. microbiologically influenced corrosion (MIC), remain mostly unknown.

Halophiles and MIC

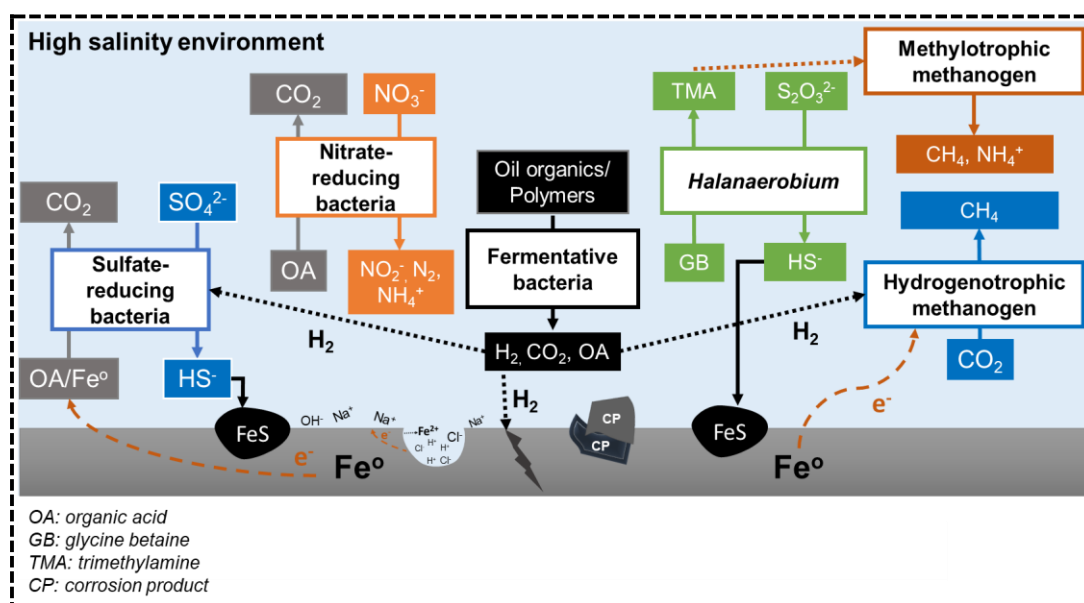


Figure 1 Anaerobic metabolic pathways and corrosion mechanisms at high salt by different groups of microorganisms

Shale exploration through hydraulic fracturing has offered a glimpse into the subsurface microbial community structure¹⁶. Microbial community analyses of various shale reservoirs across the North American continent exhibited high percentages of halophiles including members of *Halanaerobium*, *Halomonas*, *Marinobacter*, *Desulfovermiculus* and *Methanohalophilus*^{3-7,10,17-24}. Notably, *Halanaerobium* was shown to be the dominant taxa of several reservoirs^{4,10,22}. Several members of the halophilic community are considered to be corrosive, including the fermentative, thiosulfate reducer *Halanaerobium* (HA), sulfide-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB).

HA uses KCl for osmoregulation and thrive up to 5.2 M NaCl (saturation)²⁵. HA can breakdown complex oil organics, polymers (i.e. guar gum)²⁶ and even osmotic solutes produced by other members of the community into metabolic end products^{6,22}. It was proposed that HA and methylotrophic methanogens (MM) i.e. *Methanohalophilus*, exhibit a synergistic relationship^{4,7} (Figure 1). MM produces glycine betaine (GB) for osmoregulation, which HA breaks down into trimethylamine (TMA) that is further used by MM for methanogenesis⁷ (Figure 1). Additionally, HA contributes to increased corrosion risks by reducing thiosulfate into sulfide (Figure 1).

Another MIC contributor is halophilic sulfate-reducing bacteria (SRB), i.e. *Desulfovermiculus*⁴ (Figure 1). Generally, SRB-induced MIC is through two pathways: direct electron uptake and production of sulfide²⁷. Energy yield of sulfate-reduction with iron as the electron donor at high salinity is currently undefined¹⁴. But the growth of halophilic SRB with incomplete oxidation of lactate to acetate can tolerate up to 230 g/L NaCl¹⁴. Regardless of the electron donor, production of hydrogen sulfide and deposition of ferrous sulfide are causative agents of MIC.

The addition of nitrate to stimulate the activities of nitrate-reducing bacteria (NRB) is often suggested to inhibit the activities of SRB^{4,28,29} (Figure 2). The souring-inhibitory agent, nitrite, is fully reduced to N_2 or ammonium under standard conditions (low salinity, mesophilic)^{4,28-30} (Figure 2). Interestingly, nitrite retains under extreme conditions, i.e. high salinity or high temperature^{4,28}, which continuously inhibit the sulfate-reduction pathway (Figure 2). NRB are also considered to stimulate MIC by producing nitrite that reacts abiotically with sulfide to produce corrosive sulfur³¹.

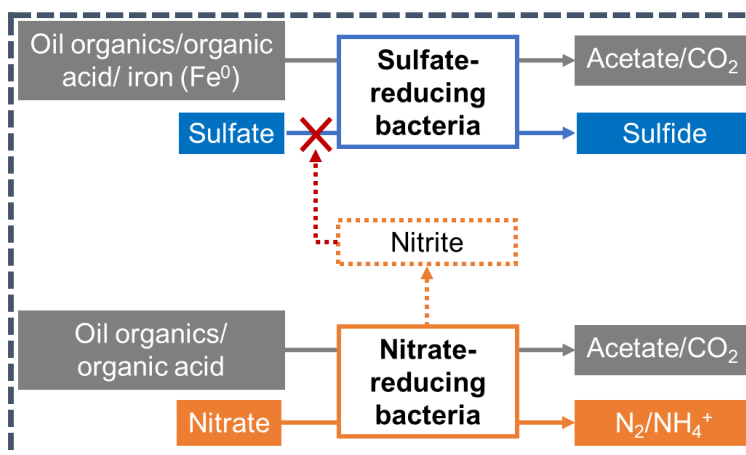


Figure 2 Inhibition of sulfate-reducing bacteria (SRB) by nitrate-reducing bacteria (NRB)

Another group of MIC microbes, hydrogenotrophic methanogens, i.e. *Methanobacterium*-affiliated IM1, *Methanococcus maripaludis* KA1, OS7 and Mic1c10, were isolated from marine environments (up to 70% v/v NaCl_{eq} or 1.2 M NaCl)³²⁻³⁴ at mesophilic conditions. The absence of produced corrosive by-products suggest direct electron uptake through electroactive proteins i.e. hydrogenases, might be their primary corrosion route³³. However, the effect of high salinity environments on the corrosiveness of hydrogenotrophic methanogens needs further investigation.

In this paper, the corrosion potential of different groups of halophilic sulfate-reducing bacteria, nitrate-reducing bacteria and methanogenic archaea are presented. Corrosion tests using both laboratory strains and environmental enrichments were conducted. The corrosion potential of *Halanaerobium* as part of a halophilic microbial community was also assessed. This paper also explores the MIC potential of NRB capable of complete nitrate reduction (no nitrite accumulation) in comparison to incomplete nitrate reduction. Overall, the goal of the study is to expand the understanding of MIC under high salinity and to aid the operators with the development of effective mitigation strategies suitable for environments with elevated osmotic stress.

EXPERIMENTAL PROCEDURE

Pure strain corrosion tests

Type strains *Halomonas halodenitrificans* DSM 735 and *Desulfovibrio ferrophilus* IS5 or DSM 15579 were enriched anaerobically in artificial seawater medium (ASW) with salinity ranging between 0.45-1 M NaCl. ASW was prepared as previously described³⁵ with N₂-CO₂ (80-20, v/v) headspace. Nitrate (10 mM) and lactate (20 mM) served as the electron acceptor and donor, respectively, for *H. halodenitrificans*. Sulfate (10 mM) was supplemented to *D. ferrophilus* as the electron acceptor, and lactate (20 mM) or elemental iron (Fe⁰) served as the electron donor.

Weight-loss corrosion tests were conducted using carbon steel coupon and beads. The coupons (99.5 % Fe⁰, 0.3 % Mn, 0.08 % C, SA=1.6 centimeters², Goodfellow, Germany) and beads (99.18-99.62 % Fe⁰, 0.3-0.6 % Mn, 0.08-0.13 % C, 55.22 ± 0.026 mg, SA=0.178 cm², Simply bearings, England) were sterilized using the NACE protocol SP0775-2013³⁶. For the NRB enrichments, ASW with various salinities were supplemented with nitrate (10 mM) and with or without lactate (20 mM). Each enrichment contained one carbon steel coupon used for surface analyses and ten pre-weighted sterile beads. SRB enrichments were treated in the same way. Pre-cultures (10%) were transferred into sterile, metal-containing anaerobic medium. Enrichments were incubated at 28°C for 30 days. Biological replicates were duplicated. Post experiment, metal coupons were extracted and fixated using glutaraldehyde (2.5 %) followed by ethanol dehydration³⁵. Carbon steel beads were cleaned³⁶ then weighed to calculate the corrosion rate.

Field enrichment corrosion tests

Halophilic SRB, NRB and methanogenic enrichments were established using water samples from a Canadian shale oil field^{4,5}. The SRB and NRB enrichments were established as chemostats, which were discussed elsewhere⁴. The chemostats had a running salinity of 2.5 M NaCl and supplemented with volatile fatty acid (3 mM each of acetate, propionate, and butyrate) as the electron donor and sulfate or nitrate (10 mM) as the electron acceptor. The halophilic methanogenic enrichment was established using the produced water of the same shale oil field⁵. The methanogenic culture was established using 20 mM trimethylamine (TMA) to stimulate the growth of methylothrophic methanogens⁵.

To test their corrosion potential, anaerobic minimal medium (2.5 M NaCl)⁴ were supplemented with one sterile pre-weighed carbon steel coupon (SA=4.6 cm²) in each testing vial. Each testing vial was amended with 10 mM of sulfate (SRB) or nitrate (NRB). Abiotic control consists of sterile medium, iron coupon and substrates only. Three types of corrosion tests were established for NRB and SRB: VFA amended, GB amended or iron only. Glycine betaine (10 mM) was supplemented (SRB_GB) to stimulate the growth of *Halanaerobium*. VFA was supplemented to stimulate the growth of SRB or NRB (SRB_VS or NRB_VN). To verify lithotrophic growth, no additional electron donor was supplemented (SRB_Fe⁰ or NRB_Fe⁰). To test the corrosion activities of methanogens, 20 mM trimethylamine (MA_TMA) or 10 mM glycine betaine (MA_GB) was supplemented. All biological replicates were duplicated and incubated for 30 days at 30°C. At the end of the incubation, coupons were extracted, cleaned³⁶ and weighed.

Corrosion rate calculation

Corrosion rates were calculated using the weight-loss method. Prior to incubation, carbon steel coupons or beads were polished using grit size 320 sandpaper, weighed and then sterilized³⁷. For the post corrosion experiments the testing specimen were treated with the same protocol then weighed using the same analytical scale. The general corrosion rate was calculated using the standard weight-loss formula (Equation 5):

$$\text{Equation 5 CR} = 87600 \times \Delta W / (D \times A \times T)$$

The Corrosion rate is calculated in relations to the change in weight (ΔW) over the incubation time (T, hr.), density of the material (D, 7.85 g/cm³ for carbon steel) and the surface area of the material (A, cm²). To convert from the unit cm/hr to mm/yr, the multiplication factor of 87600 is applied³⁸.

Surface characterization

After the corrosion experiment, the carbon steel coupons incubated with pure strains of NRB and SRB were fixated using 2.5 % glutaraldehyde followed by ethanol dehydration³⁵. The fixated coupons were imaged with a scanning electron microscope equipped with a focused ion beam (FIB-SEM). A layer of platinum (~5 nm) was first deposited on to the gold-coated (15 nm) coupon surface to aid the slicing process. Then a cross-section was made inside the FIB (FEI 200 xP, Thermo-Fischer Scientific, Germany) and imaged by the SEM. Energy dispersive X-ray spectroscopy (EDS) measurements were also conducted on the fixated coupons (Pathfinder Basecamp EDS, Thermo-Fischer scientific, Germany). Coupons incubated with the mixed community was cleaned using the same NACE protocol³⁷ and imaged with the SEM (FEI Quanta 250 FEG variable pressure/environmental field emission SEM) only to visualize the pit formations.

RESULTS

Halophilic NRB and SRB corrosion characterization—pure strains

Corrosion rates of *Halomonas halodenitrificans* and *Desulfovibrio ferrophilus*