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ARTICLE

Materials and Corrosion

Influence of dissolved oxygen content on the bacteria-induced ennoblement of stainless steels in seawater and its consequence on the localized corrosion risk

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Abstract

The ennoblement of stainless steel (e.g., the increase of open circuit potential [OCP]) is associated with bacterial colonization. This increases the risk of localized corrosion as the critical pitting/crevice potential can be overcome, especially for lower grade stainless steel. In this study, we assessed the influence of dissolved oxygen content (DOC) on the crevice corrosion of duplex and super duplex stainless steels. In addition, we used DNA amplicon sequencing to identify the bacteria most likely associated with the ennoblement. Above approximately 100 parts per billion (ppb) of dissolved oxygen, the ennoblement of OCP was observed leading to an increased risk of localized corrosion. Below approximately 100 ppb of dissolved oxygen, no ennoblement occurred and the risk of localized corrosion was reduced. We identified certain hydrocarbon-degrading bacteria whose presence correlated with the ennoblement of super duplex stainless steel at saturated DOC. The role of these bacteria is not clear yet, but their distribution indicates a possible involvement in stainless steel ennoblement in seawater.

KEYWORDS

16S rRNA gene, electroactive bacteria, ennoblement, microbial ecology, natural seawater, stainless steel

1 | INTRODUCTION

The localized corrosion of stainless steels has received much attention in the last decades since it is the main limiting factor for the use of passive metallic alloys for seawater applications. One of the main environmental factors affecting the localized corrosion risk is microbially induced ennoblement. [1–13] The so-called ennoblement is a shift of the open circuit potential (OCP) of stainless steel to the noble direction, from below −100 mV/ScCE to approximately +300/+350 mV/saturated calomel electrode (SCE). This has been attributed to the development of bacteria on the passive oxide layer.[6–13] The other significant effect of bacteria on passive materials is an increase in the cathodic reduction efficiency (e.g., cathodic reduction of dissolved oxygen).[13,14] Both effects can significantly increase the risk of localized corrosion of stainless steels since the critical pitting/crevice potential

can be overcome during ennoblement, and once initiated, the localized corrosion will propagate much faster if the reduction of oxygen on cathodic surfaces is increased.

To better understand and prevent these localized corrosion risks, it is necessary to investigate the nature of the bacteria that are responsible for the ennoblement and the conditions in which they are promoted. This is particularly important to develop antibacterial strategies in, for instance, closed or semi-open cooling water circuits.[15]

The role of temperature on ennoblement has been investigated, showing that the incubation time for biofilm ennoblement is temperature-dependent and can vary from 1 to 2 days at tropical temperatures (i.e., around 30°C) to about a month at 5°C.[15] Also, the effect of bacteria vanishes above a critical temperature, which varies according to the geographical location. The critical temperature for bacteria-induced ennoblement is 32°C in the North Sea, around 37°C in the Bay of Brest (Atlantic Ocean) and above 40°C in the tropical sea of Singapore.[3,16–18] These results suggest that the microorganisms responsible for the ennoblement are probably not of the same nature worldwide, or that they adapt to the different local environments. The nature of the bacteria associated with the ennoblement was investigated in another study and suggests the involvement of electrotrophic bacteria, which are able to catalyze the oxygen reduction with the current provided by the cathode.[19]

The effect of dissolved oxygen content (DOC) in natural seawater was shown to have a significant influence on the ennoblement and consequently on the localized corrosion risks.[20] Stainless steels with a pitting resistant equivalent number ($PRENw = \%Cr + 3.3\ (\%Mo + 0.5\%W) + 16\%N$) lower than 40 are normally not recommended for seawater applications. However, if DOC concentrations are controlled (e.g., below 20 ppb), lower grade steels ($PREN < 40$) might be considered to reduce costs, for example, in treated seawater injection metallic wells where oxygen scavengers are generally used.

However, no data can be found in the literature on the influence of oxygen on bacteria associated with stainless steel. The identification and characterization of the bacteria that are present below and above the critical DOC for ennoblement could help to control the effective localized corrosion risk. The objective of the present study was to further investigate the critical DOC promoting bacteria-induced ennoblement and to perform microbiological analysis (marker gene sequencing) at low and saturated DOC. The conditions below and above the critical DOC for ennoblement were investigated and compared, in terms of both the extent of localized corrosion and on the composition of the bacterial community.

2 | EXPERIMENTAL PROCEDURE

2.1 | Materials

The materials used in this study were duplex stainless steel UNS S31803 and super duplex stainless steel UNS SS32750. Samples were tested as coupons of 100 mm × 50 mm × 10 mm. The nominal composition of the stainless steel is given in Table 1. Before exposure, the coupons were washed for 20 min in 20% nitric acid and sterilized by autoclaving for 20 min at 15 psi and 121°C (dry cycle). Metallographic investigations were performed on the two tested alloys. The results are given in Figure 1. The austenite/ferrite spatial distribution (50/50 with approximately 10 μm austenite spacing) and the microstructure of the inspected alloys showed no abnormal deviations from the expected microstructure features.[21]

2.2 | OCP and crevice corrosion testing

The stabilized OCP of the tested alloys was measured as a function of DOC in natural seawater. Titanium (grade 2) wires were used for electrical contact allowing OCP measurements. OCP was measured with high impedance ($>10^{11}\ \Omega$) data loggers connected to a gel reference electrode Ag/AgCl/KCl. The electrodes were calibrated weekly with a certified SCE. The criterion for stabilized potential was arbitrarily fixed to no potential evolution of more than ±5 mV over 48 hr (after a minimum exposure time of 15 days). Continuously renewed seawater (from the Bay of Brest, France) was used to allow a continuous supply of bacteria and nutrient from the natural seawater. The temperature was controlled at 30.0 ± 0.5°C (regulated by heating bands) and the renewal rate was about one complete renewal per day of the seawater in the cells (i.e., ~24 L/day). The

TABLE 1 Chemical composition of tested passive alloys (%wt – balanced Fe) and PREN

Elements	C	Mn	S	Ni	Cr	Mo	N	Cu	PREN[a]
UNS S31803	0.016	1.36	<0.01	5.73	22.5	3.0	0.17	0.23	34.9
UNS S32750	0.014	0.34	<0.01	7.0	25.1	3.8	0.29	0.13	42.3

Abbreviation: PREN, pitting resistant equivalent number.
[a]PREN = %Cr + 3.3 (%Mo + 0.5%W) + 16%N.

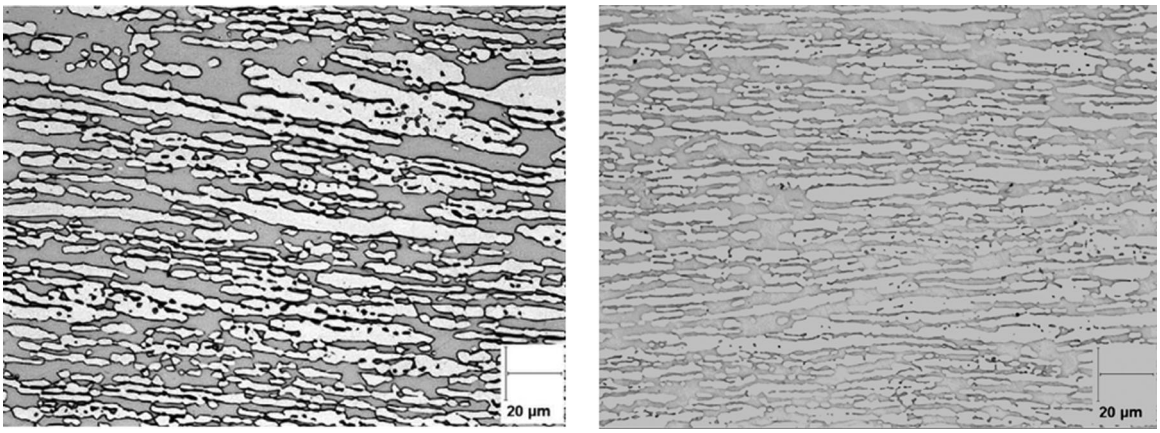

FIGURE 1 Metallographic cross-sections of (left) S31803 and (right) S32750

seawater we used showed the standard characteristics of the Atlantic Ocean, with a salinity of 34‰ ± 1 and pH 8.1 ± 0.1. Several dissolved oxygen levels were selected from <10 ppb to saturation (6 ppm) to plot OCP versus DOC and to define the critical DOC for bacterial-induced ennoblement. The DOC was adjusted with the use of controlled mass gas flow regulators connected to pure nitrogen and oxygen. The DOC was measured with light-dissolved oxygen probes allowing measurement of oxygen trace (<5 ppb). The principle of the set-up is given in Figure 2.

In parallel, crevice assemblies were used on the coupon samples to evaluate the risk of crevice corrosion at different DOC in natural seawater (from 10 ppb to saturated DOC of 6 ppm). The selected crevice formers for coupons were made of polyvinylidene difluoride (20 mm diameter gaskets), designed according to ISO18070:2015 recommendations. The gasket pressure of 20 N/mm^2 was applied on all systems, which is considered as a very severe crevice geometry for the used assembly.[5] A schematic description of the crevice former for coupon geometry is presented in Figure 3. The applied pressure on the gasket was controlled using a calibrated torque wrench and calibrated titanium disc springs. Titanium (Grade 2) fastenings, all insulated from the coupons, were used for the assembly. The OCP of the crevice samples was continuously monitored using a similar set-up as described above for non-crevice samples. The monitoring of the potential allowed detection of the eventual initiation of localized corrosion, that is, indicated by large potential drop(s). The exposures were stopped when corrosion was visually detected, with a maximum exposure duration of 3 months.

For all tested configurations (i.e., with and without crevice formers), five replicates were exposed to allow statistical evaluations. The material affected by corrosion was treated with 20% nitric acid before the evaluation. This treatment is used to remove the eventual ferrous

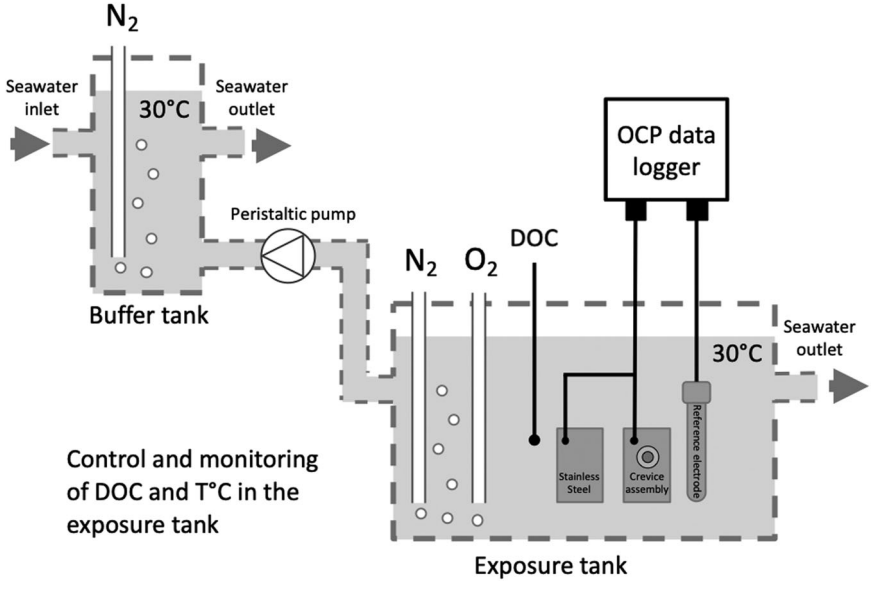

FIGURE 2 Principle of the set-up for dissolved oxygen control in electrochemical cells. DOC, dissolved oxygen content; OCP, open circuit potential

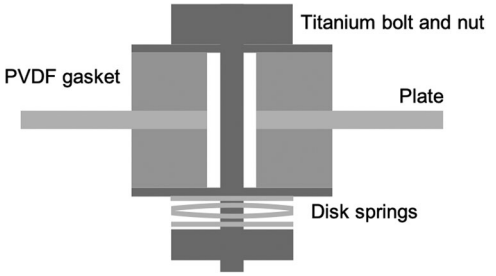

FIGURE 3 Schematic crevice former assembly based on ISO18070:2015. PVDF, polyvinylidene difluoride

pollution that could exist on samples after machining operations.

2.3 | Bacterial DNA collection and sequencing

Many bacteria can settle and form a biofilm on the surface of a stainless steel coupon, but biofilms can also develop on inert surfaces, like glass. It is crucial to identify the specific bacterial fraction of the community collected on stainless steel that is responsible for the ennoblement. We used glass coupons as nonconductive surface control to be compared with stainless steel bacterial communities.

A similar set-up as described in Figure 2 was used to expose super duplex stainless steel and to collect the bacteria at the surface of the material after 2 weeks of exposure. The two conditions used were fully aerated around 6 ppm and deaerated water under 10 ppb. The glass samples were exposed during the same time and in the same electrochemical cells.

Bacteria were collected immediately after coupon collection using a sterile cell lifter (Thermo Fisher Scientific) by gentle and uniform scratching into 100 mL of Tris-buffered saline (TBS) solution (50 mM Tris, 150 mM NaCl, pH 7.6) while an adjacent Bunsen burner kept the immediate area sterile. TBS solutions were then stored in ice for transport to the molecular laboratory. TBS solutions were filtered through 0.22 μm GTTP polycarbonate membranes (Merck Millipore) which were then transferred to PowerBiofilm® Bead Tubes from the PowerBiofilm DNA extraction kit (MoBio).

The DNA extraction was performed according to the manufacturer's instructions of the PowerBiofilm DNA extraction kit (MoBio). The V4–V5 region of the 16S ribosomal RNA (rRNA) bacterial marker gene was amplified by a polymerase chain reaction (PCR) with the 518F and 926R primers fused with Illumina adapters and sample-specific sets of barcodes and indexes. PCR products were visualized on agarose gels and purified with AMPure XP (Agencourt) reagent. DNA concentrations were assessed with Quant-iT™ PicoGreen® double-stranded DNA (Invitrogen) before pooling the PCR products at equimolar concentration. Sequencing using Illumina MiSeq platform was performed at the Josephine Bay Paul Center (Woods Hole, MA). Sequences were deposited into the European Nucleotide Archive under the accession number PRJEB30977 (http://www.ebi.ac.uk/ena/data/view/PRJEB30977).

2.4 | Bioinformatic analysis of the DNA

The quality filtering of raw sequencing data was carried out following the recommendations of Minoche et al. (2011),[22] while merging paired-end reads with Illumina-Utils python scripts on demultiplexed raw reads.[23] We then performed operational taxonomic unit (OTU) delineation with the Swarm algorithm, which uses a local linking threshold (default, $d = 1$) for sequence clustering and internal abundance structure to break chained OTUs.[24] We used VSEARCH[25] to detect and remove chimeras, and the Silva NR 128[26] database for taxonomic assignment of Swarm representative sequences with mothur.[27]

Stacked bar plots were produced with ggplot2.[28] We used the two conditions "stainless steel with oxygen" and "glass with oxygen" to perform biomarker detection with LEfSe.[29]

A fully reproducible workflow is available at https://github.com/loimai/ennoblement_DOC_16S.

2.5 | Scanning electron microscope imaging

Some coupons were fixed for scanning electron microscopy with 2.5% glutaraldehyde seawater for 1 hr and then rinsed three times in seawater for 15 min. The dehydration process involved four washes of 15 min in increasing concentrations of ethanol (50%, 70%, 90%, and 100%) followed by similar washes in hexamethyldisilazane (HMDS) and ethanol solution (⅓ HMDS, ½ HMDS, ⅔ HMDS, and 100% HMDS). Observations were made with a Hitachi SU3500 microscope (Hitachi High-Technologies, Germany).

3 | RESULTS

3.1 | OCP and crevice corrosion testing

We first monitored the stabilized OCP versus DOC for the duplex UNS S31803 and the super duplex UNS S32750 in natural seawater at 30°C (Figure 4). Similar potentials were measured for both alloys without pitting corrosion. The OCP decreased from approximately −100 to

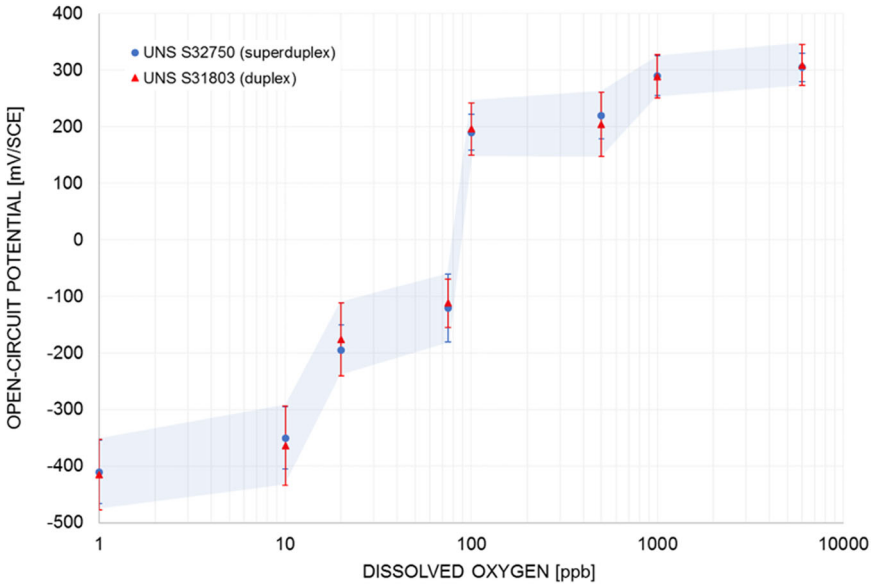

FIGURE 4 Stabilized OCP versus DOC curves in continuously renewed natural seawater at 30°C for stainless steels UNS S31803 and S32570. DOC, dissolved oxygen content; OCP, open circuit potential [Color figure can be viewed at wileyonlinelibrary.com]

−450 mV/SCE in deaerated water when DOC decreased from 100 ppb to less than 5 ppb. Under the tested conditions, the bacteria-induced ennoblement was observed in the range of 70–100 ppb, with an increase in OCP to approximately +200 mV/SCE. At higher DOC, the stabilized OCP after ennoblement ranged from +250 to +350 mV/SCE.

We then exposed specimens with crevice formers for a maximum of 3 months at different DOC and stopped the exposures when corrosion was visually detected. The crevice corrosion results are given in Table 2. The super duplex UNS S32750 only showed crevice corrosion at DOC of 600 ppb. At this level, the OCP range of uncreviced specimens was between +250 and +320 mV/SCE. For the duplex UNS S31803, crevice corrosion was observed at 100 ppb and above, while no corrosion was observed at 60 ppb and below.

3.2 | Microbiological analyses

In parallel, we recorded the potential of stainless steel coupons used for the microbiological analysis during the 2 weeks of exposure (Figure 5). A typical ennoblement potential up to +300 mV/SCE mediated by bacteria was measured after 3 days in aerated conditions. In contrast, the potential decreased below −400 mV/SCE at the end of the exposure under 10 ppb of DOC.

To characterize the surface bacterial communities for each condition, we constructed and sequenced 12 amplicon libraries and obtained 974,457 raw sequences of the bacterial 16S rRNA marker gene V4–V5 region. After quality filtering and paired-end read merging, 754,082 sequences were retained, resulting in 50,342 OTUs, and 24,695 chimera-free OTUs finally comprised of 641,550 sequences.

In general, the bacterial composition was different with or without oxygen, with only one shared OTU above a 1% of relative abundance cutoff (affiliated with *Muricauda*; Figure 6). Under anaerobic conditions, the bacterial communities were similar between stainless steel and glass coupons, with only one OTU (affiliated with *Rhodothermaceae*; Figure 6) exclusively present on glass coupons.

The bacterial communities in aerobic conditions were also very similar between the stainless steel and glass coupons, but we observed additional OTUs on the

TABLE 2 Corrosion results for stainless steels UNS S31803 and S32570 as a function of DOC in continuously renewed natural seawater at 30°C—crevice formers according to ISO18070:2015 with gasket pressure of 20 N/mm^2

DO (ppb)	OCP range without crevice (mV/SCE)	Electroactive ennoblement	Duplex S31803	Super duplex S32750
10	−420/−300	No	No corrosion	No corrosion
60	−200/−80		No corrosion	No corrosion
100	+150/+240	Yes	Crevice corrosion	No corrosion
200	+150/+240		Crevice corrosion	No corrosion
600	+250/+320		Crevice corrosion	Crevice corrosion

Abbreviations: DO, dissolved oxygen; DOC, dissolved oxygen content; OCP, open circuit potential; SCE, saturated calomel electrode.

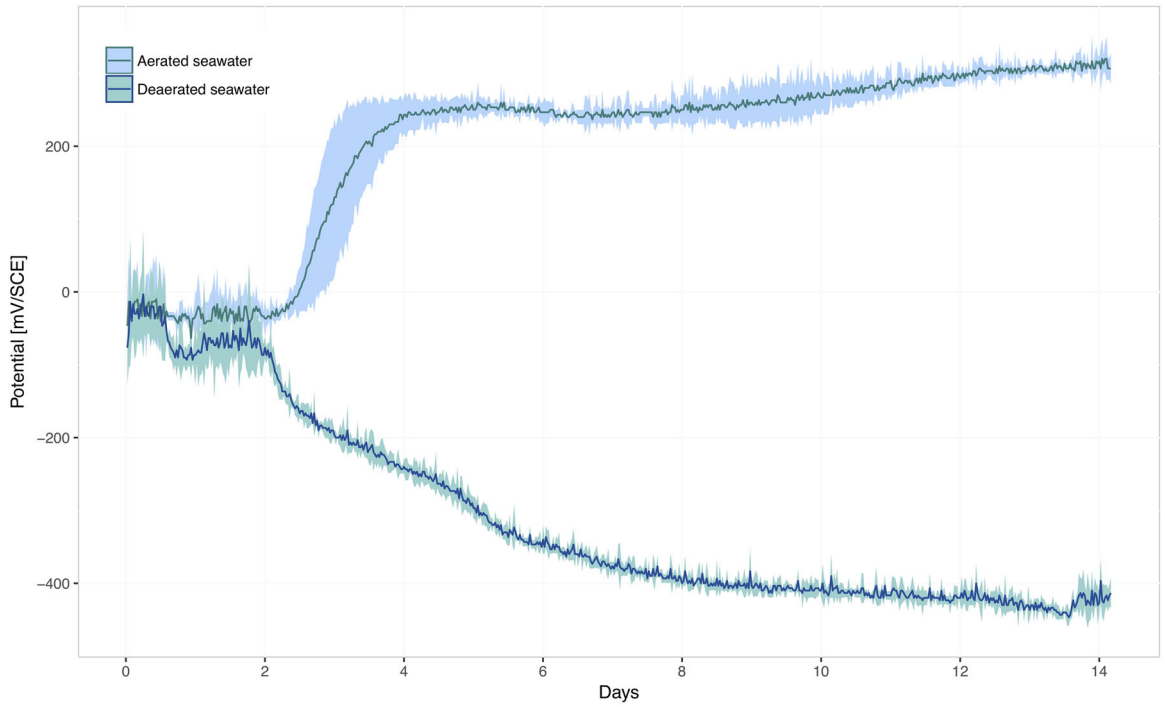

FIGURE 5 Open circuit potential of UNS S32570 in aerated (approximately 6 ppm) and deaerated (<10 ppb) seawater [Color figure can be viewed at wileyonlinelibrary.com]

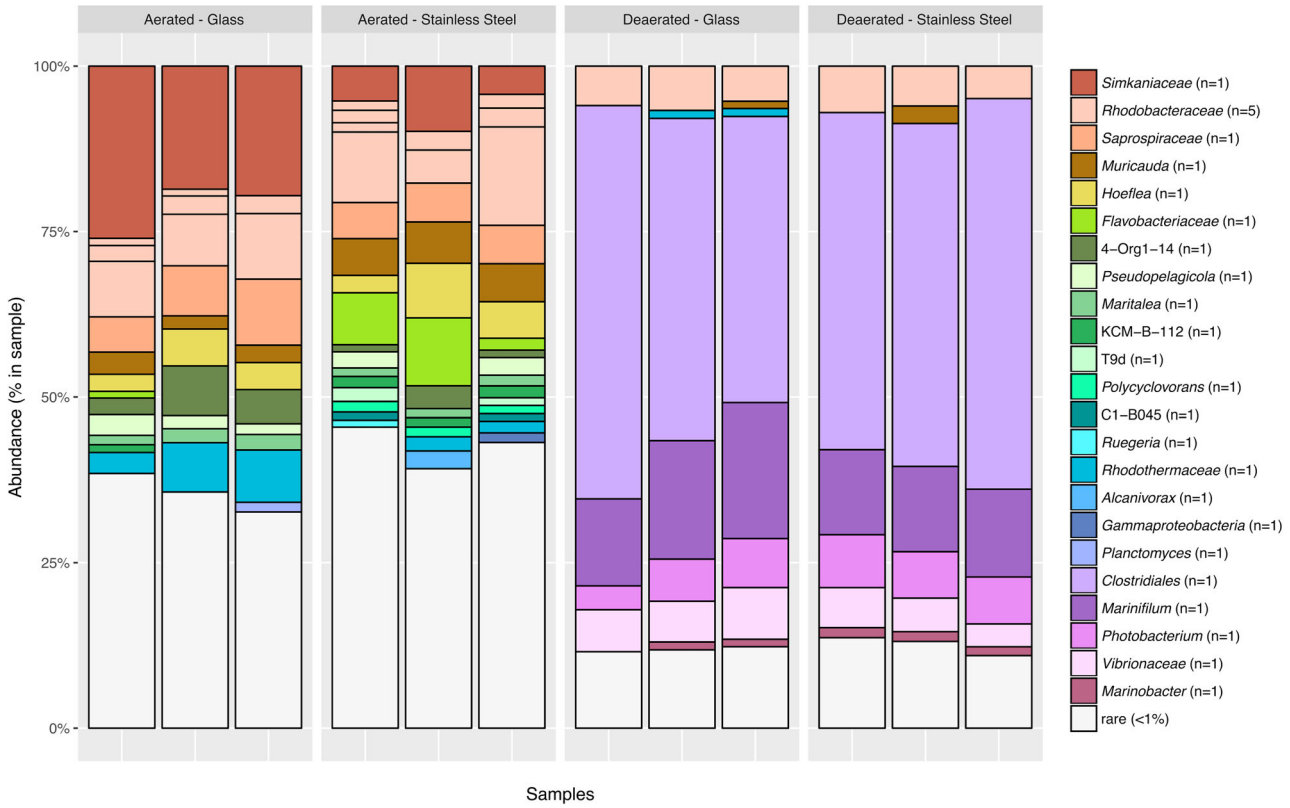

FIGURE 6 Relative abundance distribution of phylotypes in the different experimental conditions. Only OTU with relative abundance above 1% are displayed and grouped according to their taxonomic affiliation (phylotypes). Results are shown for three replicates per conditions (aerated or deaerated seawater, glass or stainless steel coupons). The taxonomic assignment is represented by the different colors and *n* indicates the number of OTUs within each phylotype. OTU, operational taxonomic unit [Color figure can be viewed at wileyonlinelibrary.com]

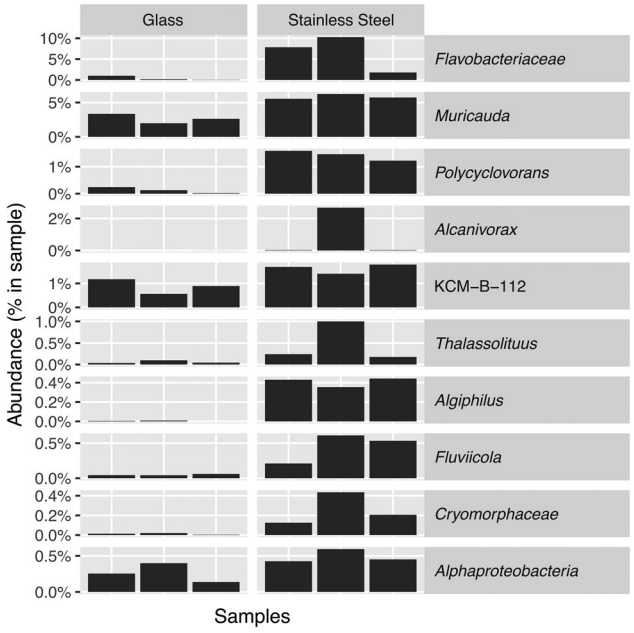

FIGURE 7 Relative abundance and distribution of the most significant stainless steel biomarker OTUs under aerobic conditions. OTU, operational taxonomic unit

stainless steel coupons affiliated with the family Flavobacteriaceae and the genus *Polycyclovorans*.

To identify bacteria that were differentially represented on stainless steel and not on the glass coupons, we performed a biomarker detection and retained the 10 OTUs with the highest linear discriminant analysis score from LEfSe analysis (Figure 7). These bacteria were all affiliated with microorganisms described as able to degrade hydrocarbon compounds.

The bacterial colonization of the UNS S32750 under both conditions is shown in Figure 8. Various bacterial morphologies can be observed under the two distinct oxygen content conditions: *Bacillus*, *Coccus*, segmented- and nonsegmented-filamentous bacteria. The bacterial morphology and coverage of the surface were relatively similar between the two conditions.

4 | DISCUSSION

Low DOC below 70–100 ppb prevents the bacteria-induced ennoblement of stainless steel, limiting the risk

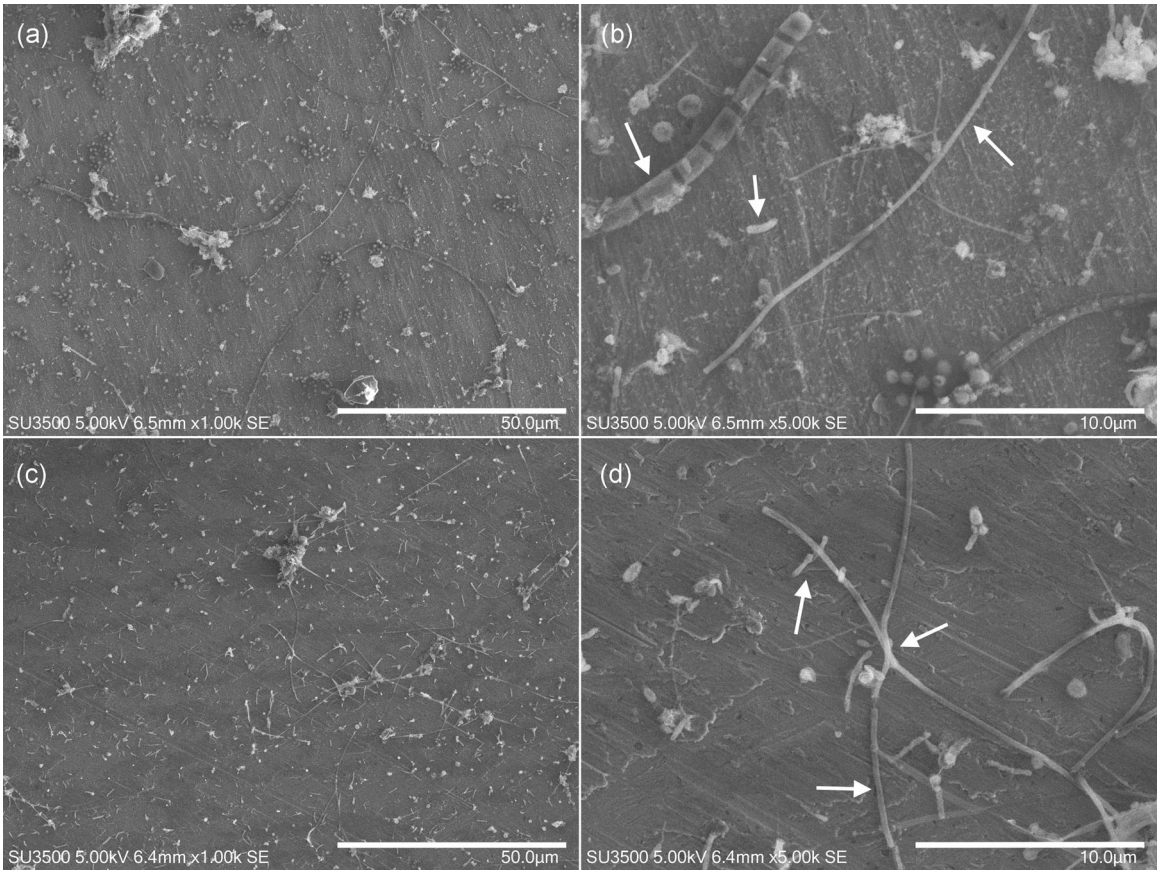

FIGURE 8 Scanning electron microscopy micrographs at 1 K (a,c) and 5 K (b,d) magnification. In aerobic (a,b) and anaerobic (c,d) conditions. UNS S32750 after 2 weeks of immersion. Some cells are indicated by arrows

of initiation of crevice corrosion for both duplex (UNS S31803) and super duplex (UNS S32750) stainless steels. Under such conditions, duplex stainless steels can then be considered for applications in seawater. For higher DOC (100 ppb–6 ppm), the ennoblement of OCP increased the risk of crevice corrosion and a higher grade of stainless steel is recommended.

Bacteria use redox reactions for energy conservation and growth and a large diversity of bacteria are able to reduce oxygen in the four-electron reduction to water. On the other hand, bacteria can also live without oxygen and reduce other elements such as nitrate or sulfate. Overall, it is not a surprising result to have different bacterial communities on the super duplex coupons with and without oxygen, and therefore these two conditions cannot be compared to infer which bacterial fraction is responsible for the ennoblement. With the DOC under 10 ppb, the bacterial communities were similar for both UNS S32750 and glass coupons, which is consistent with the ability of bacteria to settle on different surfaces. In fully aerated conditions, the bacterial communities were also globally similar between the two surfaces but some bacteria were only present on stainless steel. These biomarkers of the stainless steel communities can then be considered as the most interesting candidate bacteria involved in ennoblement.

Among the biomarkers, some are related to the known hydrocarbon-degrading bacteria like *Alcanivorax*, *Polycyclovorans*, *Algiphilus*, and *Thalassolituus*. It should be noticed that this type of bacteria was also identified in a study about the temperature effect on stainless steel ennoblement.[19] A member of the Alcanivoraceae family was also found in a biocathode experiment where bacteria are fed by a cathode as a sole electron donor source.[30] Oil-derived compounds in the tubing and pumping seawater system could be a source of contamination leading to the presence of bacteria with that particular type of metabolism. However, their recurrence in our studies and their presence in another study which involves electroactive bacteria[30] indicate that they might have a role in the ennoblement. Another biomarker of our study, *Muricauda*, was also identified by Wang et al.[30] in a biocathode biofilm.

In this study, we propose a list of bacteria associated with ennoblement (Figure 7) which is a new step to better understand the bacterial mechanisms responsible for the change of potential and the increased risk of localized corrosion. Interpretations about the role of biomarkers bacteria are limited to either poor taxonomic resolution (Flavobacteriaceae, KCM-B-112, Cryomorphaceae, *Alphaproteobacteria*) or the role of hydrocarbon-degrading bacteria in ennoblement which is not identified. Nonetheless, their association with the ennoblement question their role as hydrocarbon-degrading bacteria and future work should consider the possibility that these bacteria could have an electroactive metabolism capable of modulating stainless steel OCP.

5 | CONCLUSIONS

DOC impacts OCP and bacterial community structure. We found that critical DOC exists between 70 and 100 ppb, below which no bacteria-induced ennoblement is observed. Under these conditions in seawater at 30°C (i.e., below 70–100 ppb of DOC), no crevice corrosion initiated on duplex stainless steel. Above 100 ppb of DOC, biofilm-induced ennoblement was detected, increasing the risk of localized corrosion, and therefore promoting the use of more resistant alloys. The bacteria correlated with the ennoblement were related to hydrocarbon-degrading bacteria, which were also found in another study about the role of temperature on the bacteria-induced ennoblement,[19] and associated with electroactive bacteria by other authors.[30] Future studies should consider and further investigate the role of hydrocarbon-degrading bacteria to understand their relation with the ennoblement of stainless steel.

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