Corrosion of carbon steel influenced by anaerobic biofilm in natural seawater

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1. Introduction

Corrosion can cause great damages to marine steel infrastructures such as bridges, wharfs, platforms and pipeline systems. It has been estimated that some 20% of the corrosion cost is due to microbial corrosion and degradation [1], of which a significant part is due to anaerobic corrosion influenced by sulfate-reducing bacteria (SRB) [2]. Their activity often causes the formation of biofilms on iron and steel and tends to promote the formation of cavity on them.

The corrosion behaviors of single SRB strain have been investigated extensively and many corrosion mechanisms have been suggested [3–7]. Some SRB strains can accelerate corrosion by consuming cathodic hydrogen [3] and inducing the formation of ferrous sulfides [4], and hydrogenase-catalyzed mechanisms were suggested [5,6]. Some SRB-like bacteria could acquire energy using iron as electron supplier through a putative pathway involving a membrane-associated cytochrome and an intracellular hydrogenase-mediated electron transfer system [7].

However, bacterial communities in the biofilms on the surface of steel in natural seawater environment are usually heterogeneous; therefore the anaerobic corrosion should be affected by the whole communities. Marine anaerobic biofilms on steel are also thought to contain dissimilatory iron-reducing bacteria (DIRB), because solid ferric oxides are good electron acceptors for DIRB [8]. The single DIRB usually decreases the rate of corrosion, partly due to the high concentrations of soluble Fe(II) that scavenges O2 [9]. These findings have led to the active researches on “dual-species” in order to understand the role of both SRB and DIRB in biofilm-mediated corrosion [10].

Rusting is also an important phenomenon accompanied with the corrosion of carbon steel. It was generally thought that the formation of corrosion products such as iron oxides is an abiotic process of chemical reactions. However, considering the bacterial activities in the rust layer and their important influences on the corrosion process, microbiologically influenced biomineralization or transformation of corrosion products has been suggested [11,12]. Although the presence of iron sulfide such as pyrite FeS2 in seawater is often associated with the SRB activities [11], there are reports indicating that the Fe(II–III) hydroxysulfate, i.e. the sulfated green rust GR(SO42−), are the important component in the rust layer in marine environment [13–15]. Green rusts can be produced by the slow reduction of various ferric compounds in the absence of bacteria [13]. In recent studies, sulfate- and iron-reducing bacteria are thought to participate in their formation, and attempts were made to elucidate the mechanisms [16–18].

The knowledge of bacterial diversity in the anaerobic biofilm of rust layer is helpful to understand how the anaerobic bacteria influence the corrosion and rusting processes. Several studies based on culture-independent phylogenetic technologies have been performed to investigate the composition of microbial communities...
in corrosive biofilms formed under laboratory conditions using samples collected from marine biofilms and marine sediments [19–21]. However, few studies are carried out directly using corrosive biofilm in the rust layer.

The aim of this study was to analyze the bacterial communities in the rust layer biofilm on steel in natural seawater with culture and molecular biology technologies. The characteristics of corrosion products, especially the existence of green rust with the connection to bacterial activity were analyzed. Further studies were also carried out to investigate the corrosion and rusting process by single and mixed bacterial species in laboratory conditions to confirm the field data and to reveal the corrosion mechanisms of anaerobic biofilms.

2. Experimental

2.1. Collection of rust layer biofilms

Fresh rust layers, ~10–15 mm thick, were scraped using a sterile scalpel from Q235 carbon steel specimens (100 mm × 200 mm × 5 mm, chemical composition: C, 0.16%; Mn, 0.53%; Si, 0.30%; P < 0.045; S < 0.055; balance, Fe) that had been immersed in seawater for about 1 and 2 years in the Qingdao marine corrosion experimental station (NL 36° 03′, EL 120° 25′; average seawater temperature 13.7 °C, dissolved oxygen 8.4 mg L−1, salinity 32, pH 8.3, flow velocity 0.1 m s−1). The rust layer samples were roughly divided into three parts, outer, middle and inner rust layers, which were put into a special media for the enrichment and isolation of corrosive anaerobic bacteria, such as SRB, and also for the analysis of the morphology and compositions of the corrosion products.

2.2. Enrichment and isolation of anaerobic microorganism

Fresh rust layers including outer, middle and inner rust layers were inoculated respectively into sterile modified Postgate’s C seawater (PCS) medium to enrich anaerobic microbes in 30 mL glass culture bottles with 20 mL culture medium. The bottles were sealed with butyl rubber and aluminum lid and kept at 28 °C constant temperature. After 2 weeks, 100 μL liquid medium containing bacterial species was inoculated to an agar plate with PCS medium. The plate was put into an anaerobic box to cultivate anaerobic microbes under the carbon dioxide atmosphere. Colonies formed in the plates were selected and inoculated again into PCS medium. The procedure was repeated until the colonies of single bacterial strain were formed on the solid PCS medium. A modified PCS medium containing 0.5 g KH₂PO₄, 1 g NH₄Cl, 0.06 g CaCl₂·2H₂O, 0.06 g MgSO₄·7H₂O, 6 mL 70% sodium lactate, 1 g yeast extract, and 0.3 g sodium citrate in 1 L aged seawater from a Qingdao offshore area was prepared. A small quantity of scrap iron was used to substitute ferrous ion in mixed and isolated bacteria incubation. To prepare solid PCS medium, 0.06 g (NH₄)₂Fe(SO₄)₂·6H₂O and 1.5% mass agar were added into 1 L PCS medium.

2.3. Phylogenetic diversity analysis of the bacteria in the rust layer

Fifty millilitre cultures of mixed species and single colony were centrifuged at 15,000 × g for 5 min. The precipitate was added with 700 μL CTAB extraction buffer (100 mmol/L Tris–Cl, 20 mmol/L EDTA, 1.4 M NaCl, 2% CTAB, 0.1% β-mercaptoethanol, 0.15 mg/mL protease K, 0.05 mg/mL RNase A, pH 8.0) and incubated at 65 °C for about 30 min. An equal volume of PCI (phenol 25:chloroform 24:isoamyl 1), mixed well, and centrifuged at 15,000 × g for 10 min. The upper layer was transferred into a new tube containing an equal volume of CI (chloroform 23

2.4. Characteristics of the rust layers in natural seawater

Scanning electron microscopy (SEM) was used to characterize the rust layers. Energy dispersive X-ray spectrometry (EDS) coupled with field-emission SEM was used to determine the chemical composition of the rust layers. The rust layers, classified roughly as outer, middle and inner layers, were scraped from the Q235 carbon steel immersed in seawater for 12 and 24 months.

For SEM and EDS analysis, the fresh rust layer samples were immersed in sterile seawater and brought to the laboratory for further treatment. Considering the existence of possible biofilm on the rust layers, before SEM observation, the rust samples were cut into small pieces no more than 1 cm and pretreated by dehydration in a graded ethanol series, followed by fixation with phosphate buffer containing 2.5% glutaraldehyde. The samples were then subjected to carbon dioxide critical temperature desiccation, treated and coated with gold to allow morphological and mineralogical analysis with SEM (JEOL JSM–840) and EDS–SEM (JSM–6700F, Philips XL30, Phoenix EDAX).

2.5. Bacteria morphology by TEM

The morphologies of mixed bacteria were examined by transmission electron microscopy (TEM, JEM–1200EX). Several drops of mixed bacteria culture incubated for 7 days were placed on the glass slide. Copper meshes were put into the culture, which was fixed in 2.5% glutaraldehyde for 15 min and postfixed for 15 min with 0.1% phosphotungstic acid. Then the copper meshes were dried and observed under TEM.

2.6. Electrochemical impedance spectroscopy (EIS) measurements

To investigate the effect of anaerobic biofilms in seawater on the corrosion of steel, microbial corrosion tests were carried out in laboratory conditions. To compare the influence of different anaerobic biofilms on corrosion, four types of corrosion media were selected: (1) sterile PCS medium, a de-aerated sterile PCS medium; (2) SRB culture, SRB strain, enriched and purified from rust layer biofilms,
was grown for 1 week in anaerobic bottle and inoculated into sterile PCS medium as the SRB culture (5%, v/v); (3) SRB broth, SRB incubated for 7 days, SRB was removed by filtration through a 0.22-μm polycarbonate membrane to prepare the SRB broth; (4) mixed bacteria culture, fresh rust layer was inoculated into sterile PCS medium.

The growth curve of isolated SRB was obtained by the most probable number (MPN) method. The pH values and the sulfide concentrations were monitored using the pH electrode and the Ag/Ag2S sulfide ion-selective electrode, respectively.

Carbon steel poles of ϕ 10 mm or piece samples of 10 mm × 10 mm were cut from Q235 carbon steel. The steel pole electrodes sealed with epoxy were polished with wet SiC sandpaper to 600#. The counter and reference electrodes were 10 mm × 20 mm platinum pieces and saturated calomel electrodes (SCEs).

To study the corrosion of steel in seawater containing active anaerobic bacteria, EIS measurements were conducted at the corrosion potential by applying a 5 mV sinusoidal voltage in the frequency range of 0.0015–10 kHz with five data points. EIS measurements were made with a PARSTAT 2273 electrochemistry apparatus, and the data were analyzed by the software supplied by the PARSTAT 2273.

2.7. Observation and analysis of the morphology of coupons in laboratory condition

Rusted steel samples immersed in different media were also analyzed by SEM and EDS–SEM with the same procedure mentioned above. In addition, some steel sample surfaces were stripped of corrosion products by adding Clark acid cleaning solution (1 L 36% HCl, 20 g Sb2O3 and 50 g SnCl2) for 10–15 s. The exposed sample surface was finally rinsed with distilled water, cleaned in absolute ethanol, and dried under nitrogen flow. The surface was analyzed by SEM.

3. Results

3.1. Characterization of the bacteria on rusted steel immersed in natural seawater

Bacteria were enriched and isolated by inoculating culture medium with fresh rust, which were then transferred onto anaerobic solid agar culture media. In the enriched liquid culture, a black pigment was observed for the first 1–2 days after inoculation. It indicated the formation of iron sulfide by the reaction of iron oxide in the rust and hydrogen sulfide produced by SRB. At longer incubation times, the color of the culture shifted from black to laurel-green, possibly because the Fe(III) oxides in the rust layer were reduced to Fe(II) ions. On the solid agar plates, round and elliptical black colonies were observed, indicating SRB proliferation in the medium. Yellow elliptical colonies were also observed, often together with black colonies, which may be a characteristic of Fe(III) reducers [24]. Cultures inoculated with the inner rust layer showed only black colonies. However, in enriched mixed culture inoculated with the whole rust layer, two kinds of bacteria were found (Fig. 1), indicating that SRB existed in the inner rust layer, and iron-reducers existed in the middle or outer layer.

The evolutionary relationship between our isolated bacteria and other bacteria based on the phylogenetic tree of 16S DNA was analyzed (Fig. 2). The bacteria S–12 is most closely related to the strain Desulfovibrio, particularly similar to Desulfovibrio caledoniensis (Fig. 2a), which can use acetate as the electron donor and carbon source to reduce polychlorinated biphenyls [25]. Another strain (I–5) identified in this study is most similar to Clostridium sp. uncultured (Fig. 2b). Indeed, some Clostridiaceae strains, such as Clostridium butyricum, have been shown to be DIRB [24] and reduce Fe(III) oxides by metabolic organic carbon. Some strains of archaea may also exist in the rust layer. Although archaea DNA was successfully extracted from the cultures, subsequent analysis did not yield useful results. Further analysis is underway to identify these strains. Phylogenetic analysis of enriched bacterial cultures confirmed the existence of sulfate-reducing bacteria and iron-reducing bacteria in the microbial communities on the marine rust layer.

3.2. Characterization of the corrosion products on carbon steel immersed in natural seawater

In this study, we intended to explore the possible connection between the formation of corrosion products and bacterial activity. The rust layer sampled from carbon steel immersed in seawater can be divided roughly into three layers: outer (red-brown), middle (yellow), and inner (black) layers. Downy globular cluster is the main morphology of the middle rust layer. Many studies by SEM and XRD analysis of the outer and middle rust layers confirmed the principal components to be goethite (α-FeOOH) and lepidocrocite.

![Fig. 1. TEM images showing the different bacteria morphologies in the culture inoculated by natural rust in modified Postgate’s C seawater medium.](image-url)
(γ-FeOOH) iron oxides with globular and acicular morphologies [26]. Our result is consistent with the previous observations.

Fig. 3 are the morphologies of inner rust layer of carbon steel immersed 1 and 2 years in seawater. For the inner rust layer of 1 year, it has a composition of C 0.59, O 39.54, Na 5.16, Mg 1.42, S 9.18, Fe 33.63, Au 10.48, and it is a classical hydroxysulfate green rust component. For the inner rust layer of 2 years, the main composition of the inner rust layer is a hexagonal platelet structure together with globular particles. The platelet structure has a composition of O 74.31, P 2.72, S 0.30, and Fe 22.67, and it could be hydroxysulfate green rust. The globular particles have a composition of C 38.66, O 36.75, S 5.92, Fe 18.67, and they may include siderite (FeCO3). Small platelet clusters (O 26.05, S 13.77, Cl 1.99, Fe 32.86) with attached bacteria, perhaps SRB or DIRB, could be clearly observed on the hexagonal platelets (Fig. 3d). The present results indicate that microbial activities influence the formation of green rust.

3.3. Carbon steel corrosion influenced by anaerobic biofilms in culture media

Through EIS measurement and surface observation, we investigated the effects of isolated SRB and mixed bacteria on the corrosion of carbon steel. EIS measurements were used to evaluate how the anaerobic biofilms influenced the corrosion rate of steel in different corrosion media.

Fig. 4 are the Nyquist graphs in different corrosion conditions. The impedance graphs obtained for the Q235 carbon steel exposed to various experimental conditions were fitted via the different equivalent circuits (Fig. 5) to: (a) single-layer circuit R(RC) for sterile medium condition; (b) single-layer Warburg circuit R(C(RW)) used for broth; (c) a double-layer equivalent circuit R(C(RC))) for the cultures of SRB and mixed bacteria. These respective fits were based on a minimum systematic deviation between the data measured and the fit results. Based on the equivalent circuits, the EIS data were fitted well. Fig. 6 shows the Nyquist plots after 2 days of exposure in different corrosion media. The electric parameters of $R_{ct}$ and $C_p$ were obtained by simulating experimental impedance diagrams using equivalent circuits (Fig. 7).

As shown in the changes of $R_{ct}$ over time, we observed different corrosion behaviors in different media. In the case of the sterile PCS medium, the $R_{ct}$ increased with time, indicating a decreased corrosion rate, possibly due to the effect of phosphate salt in the buffer. For the SRB culture, however, the $R_{ct}$ decreased continuously, indicating that isolated SRB accelerated corrosion. In contrast, in the SRB culture broth not containing SRB, the $R_{ct}$ of steel decreased slightly at first, then increased with exposure time. These results indicated that the metabolic products in the broth alone did not
accelerate corrosion in the longer term. Acceleration of the corrosion rate may depend on SRB acting directly on steel, or it may depend on the continued supply of metabolic products. Our results indicated that iron sulfide films by themselves did not promote corrosion; the continuous existence of SRB was necessary for accelerated corrosion.

In contrast to this effect of accelerated corrosion, the mixed anaerobic bacteria generally inhibited the corrosion of carbon steel. During the first 3 days, the $R_{ct}$ increased rapidly, after which it fluctuated and decreased, possibly due to the proliferation of SRB. However, the $R_{ct}$ was generally higher than that in the culture of isolated SRB (Fig. 7b).

Capacitance increased in the SRB culture and the broth without the bacteria. In contrast, it decreased in the sterile medium and the culture of mixed anaerobic bacteria. There were higher capacitance values in the isolated SRB culture than in the mixed bacteria culture. High capacitance value indicates that the corrosion products are high electric conductor and usually indicates porous FeS formation in the presence of SRB [27].

It is very interesting to observe that there exists a distinct difference between the capacitance values in isolated SRB and mixed bacteria culture. Many studies have also observed that there are increasing interfacial capacitance and decreasing polarization resistance values in SRB culture [10,27,28]. However, no reason has been provided on why this phenomenon occurs.

![Fig. 4. Nyquist plots of carbon steel in different corrosion conditions with time: (a) sterile medium; (b) SRB culture; (c) SRB culture broth; (d) mixed bacteria culture.](image)

![Fig. 5. The diagrams of equivalent circuits used for the modeling of spectra of impedance for Q235 carbon steel immersed in different corrosive media: (a) single-layer model for sterile medium; (b) single-layer Warburg model for SRB broth; (c) double-layer model for the media containing SRB and mixed bacteria. $R_{sol}$ is the solution resistance; $C_d$ is the capacitance of the biofilm; $R_f$ is the resistance of the biofilm; $C_f$ is the capacitance of the corrosion product film; $R_{ct}$ is charge transfer resistance.](image)

![Fig. 6. Nyquist plots of the measured (symbols) and fitted data (lines) on day 2 of exposure in sterile medium (▲), SRB culture (●), culture broth (■), and mixed bacteria culture (○).](image)
Fig. 7. Time-dependent changes of charge transfer resistance (a) and capacitance (b) of steel immersed in sterile medium (▲), SRB culture (●), culture broth (■), and mixed bacteria culture (○).

Fig. 8. SEM and FT-SEM micrographs of carbon steel or its corrosion products after immersion in different corrosion media: (a) corrosion products of steel immersed in culture broth for 24 h; (b) corrosion products of steel immersed in enriched mixed anaerobic bacteria culture for 1 week; (c) the morphology of steel after removal of corrosion products using Clark solution; (d) magnification of (c) to show the bacterial biofilm attached to the steel.

We used SEM and EDS to examine the surface of steel immersed in different corrosion media (Fig. 8). For steel immersed in SRB culture broth, representative FeS\(_{1-x}\) structures were observed (S 5.23, Fe 94.77). Fig. 8b and c shows the morphologies of steel immersed in mixed anaerobic bacteria culture before and after Clark acid treatment. The complex chemical composition (C 49.83, O 25.40, Mg 1.48, P 7.92, S 2.19, Fe 13.19) may indicate the formation of iron (hydro)oxides, hydroxysulfate green rust, and vivianite [Fe\(_3\)(PO\(_4\))\(_2\)·8H\(_2\)O]. Removal of the corrosion products revealed bacteria biofilm attached to the steel surface (Fig. 8d). The findings indicated that iron sulfide did not form on steel in the presence of mixed anaerobic bacteria. The iron-reducing bacteria are likely to play a key role in inhibiting the corrosion of carbon steel under these conditions.

4. Discussion

4.1. Implication of rust layer anaerobic biofilm on steel

Studies have suggested that SRB is the most important corrosive bacteria in the anaerobic biofilm on the steel in marine environment. Our study indicated that a special SRB strain, most similar to \textit{D. caledoniensis}, existed in the rust layer. Our results also indicated that a kind of DIRB, \textit{Clostridium} sp. uncultured, also existed in the rust layer. Based on the initial observation, \textit{Desulfovibrio} species mainly existed in the inner rust layer; however, \textit{Clostridium} sp. uncultured more likely existed in the middle rust layer. At the steel/rust layer interface, there exists a redox zone, where the bacteria and possibly the anaerobic archaean form a special microbial micro-ecosystem. It is interesting that we identified two bacteria in the rust layer. Their presence may be important to the corrosion of steel. For the \textit{Clostridium} species (\textit{Clostridium} sp. uncultured), which is anaerobic, it may supply the carbon source for SRB. \textit{D. caledoniensis} is capable of reducing sulfate, sulfite, thiosulfate, and nitrate that are used as electron acceptors for growth. The isolated bacteria also used lactate as electron donors for growth coupled with reductive dechlorination [25]. It is interesting to note that the rust layer and the steel may play some roles in the bacteria growth. As an electron source, iron may supply the hydrogen indirectly or electron directly. However, further studies are needed.

4.2. Microbial influence on the formation of green rust

The present study suggested that the sulfate-reducing bacteria and possibly also the iron-reducing bacteria in anaerobic biofilms participate in the corrosion and rust mineralization of steel
in seawater environments. Under laboratory conditions designed to simulate seawater, electrochemical measurements indicated that the isolated SRB accelerated corrosion, while enriched mixed anaerobic bacteria inhibited corrosion.

Previous studies and our current study indicate that the green rust is the main component of the inner rust layer, not the ferrous sulfides. The middle and outer rust layers are mainly made of iron oxides. In many studies with a single strain, it has been observed that the ferrous sulfides are the main corrosion products, suggesting that the iron sulfides is converted to green rust.

The EIS results provided strong evidences for the transformation of corrosion products. A smaller capacitance value was observed in the mixed bacteria culture, and a larger capacitance value was observed in the single SRB culture. Clostridium sp. uncultured likely participates in the formation of green rust. Fe(III) oxide may be reduced by the bacteria to form ferrous ion, which in high concentration could form sulfated green rust because sulfate can be adsorbed easily than chloride ion. In addition, some researches indicate that green rust may supply sulfate, an electron acceptor, for the SRB [16]. An explanation may be that Fe(II) is produced when the iron-reducing bacteria reduce iron oxides, which are present in higher concentration than ferrous ions, causing the mackinawite to convert to green rust 2, which usually reduces the corrosion rate [11]. In culture medium, the high concentrations of phosphate and ferrous ion may produce vivianite, which is known to form films that inhibit corrosion [29], and this phenomenon may be one of the reasons for the decrease of the initial corrosion rate.

4.3. Mechanism of microbial corrosion of steel and its environmental significance

Steel, with its negative redox potential value ($E^0 = -0.44$ V), may act as a potential electron donor. In the inner rust layer of steel in seawater, it is possible that bacteria acquire energy for growth by taking up cathodic hydrogen from steel or by obtaining electrons through direct or indirect contact with steel. Corrosion due to Desulfovibrio species is generally thought to follow the cathodic depolarization theory, according to which iron acts as a hydrogen supplier to accelerate corrosion [3,7]. The results of the present study cannot confirm this theory. Nevertheless, they do support the idea that iron sulfide can enhance hydrogen production. We found that the culture broth containing biogenic sulfide accelerated corrosion of the carbon steel, but only temporarly; the continued existence of SRB was the key to the accelerated corrosion, implying that steel and bacteria should make direct or indirect contact through conducting FeS or possibly through electron shuttles. Steel is generally an electron supplier in microbiologically influenced corrosion. In light of our results, it is likely that in microbial corrosion, microorganisms mediate electron transfer from steel to different electron acceptors. There may exist complex electron acceptors, such as organic chloride and nitrate, especially in polluted marine environment. Iron may act as a special direct or indirect electron donor to accelerate the biodegradation and promote the bacteria growth.

5. Conclusions

Under laboratory conditions, sulfate-reducing bacteria D. caledoniensis and iron-reducing bacteria Clostridium sp. uncultured were found in the anaerobic biofilm under the rust layer on carbon steel immersed in natural seawater. SRB existed in the inner rust layer, and IRB existed in middle and outer layer. The study of the microbial community is important to understand microbial corrosion.

We found that the green rust was the main component in the inner rust layer, and both SRB and IRB have affected the formation of green rust.

The isolated sulfate-reducing bacteria accelerated corrosion and the mixed anaerobic bacteria inhibited corrosion. The main mechanism of corrosion inhibition is the biofilm-induced formation of green rust. The change of capacitance in EIS may be used as a marker for the change of corrosion products.

The anaerobic microbial corrosion of steel may have potential effect on the bioremediation in polluted marine environment.

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References

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