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On the bacterial communities associated with the corrosion product layer during the early stages of marine corrosion of carbon steel

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A B S T R A C T

Carbon steel coupons were immersed for 1–8 weeks at ~1 m deep in a French harbor of the Atlantic coast. The resulting corrosion product layers were characterized by μ-Raman spectroscopy. They consisted of an inner stratum of sulfated green rust covered by an outer stratum of lepidocrocite (predominantly), goethite and magnetite. Mackinawite FeS was detected after one month of immersion, but only locally. The bacterial characterization of these layers was coupled to this analysis. The diversity of the culturable bacteria grown in liquid media directly from biofilms or isolated on agar plates was evaluated by 16S rRNA gene sequencing. The microbial diversity was also estimated, without cultivation and after Temporal Temperature Gradient Electrophoresis (TTGE) separation. A moderate biodiversity was found in all cases and different bacteria associated with the redox cycles of Fe and S were identified. *Roseobacter*, *Erythrobacter* and *Bacillus* were dominant among culturable bacteria whereas *Sulfurimonas autotrophica*, a sulfur-oxidizing bacterium, was detected among the total bacterial community at all immersion times. After one month of immersion, sulfide-producing bacteria were detected, e.g. *Desulfovibrio profundus* in liquid mixed cultures and *Sulfurospirillum arcachonense* in the total bacterial community, in agreement with the local identification of FeS by μ-Raman spectroscopy.
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Introduction

According to the phenomenological model proposed by Melchers and Jeffrey (2005) and Melchers and Wells (2006), the corrosion process of carbon steel in seawater involves two main periods. During the first period, called the aerobic period, the corrosion rate is controlled by the reduction of dissolved O_2 (Melchers and Jeffrey, 2005). The thickness of the corrosion product layer increases and during the last phase of the aerobic period, the corrosion rate is controlled by the diffusion of O_2 through this layer. The consumption of O_2 by the aerobic micro-organisms leads to the second period, called the anaerobic period. The steel surface and the inner part of the corrosion product layer are then in anoxic conditions. The beginning of the anaerobic period is associated with an increase of the corrosion rate attributed to micro-organisms, notably sulfate-reducing bacteria (SRB). During the anaerobic period, the corrosion process is then basically a microbiologically influenced process and it was proposed that the corrosion rate could be controlled by the transport of nutrients (Melchers and Wells, 2006; Melchers and Jeffrey, 2012). For instance, during the last phase of the anaerobic period of the phenomenological model, the corrosion rate could be controlled by the transport of nutrients through the corrosion product layer.

Numerous studies were then devoted to the understanding of the mechanisms associated with the increase of the corrosion rate in the anaerobic period. The most recent studies demonstrated that electroactive SRB, able to use Fe° as the only source of electrons for sulfate reduction, could accelerate significantly the corrosion process in very specific conditions (Enning et al., 2012; Venzlaff et al.,

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2013; Yu et al., 2013). Besides, peculiar bacterial consortia are suspected to be responsible for accelerated localized corrosion phenomena. In particular, it was shown that the association between SRB and sulfur-oxidizing bacteria (SOB) contributed to the accelerated low water corrosion (ALWC) of carbon steel (Beech and Campbell, 2008). Iron reducing bacteria (IRB) could also be involved, but their influence is controversial. In some cases, corrosion rates were lowered when IRB were associated with SRB (Duan et al., 2008) while in other cases these micro-organisms enhanced corrosion in the absence or the presence of other bacteria (Herrera and Videla, 2009). This result however indicates that bacteria associated with the redox cycle of iron could play a role in the microbiologically influenced corrosion process. Other micro-organisms may influence the rate of cathodic and/or anodic reactions, for instance via the hydrogenase enzyme that catalyzes proton reduction (Bryant and Laishley, 1990; Mehanna et al., 2008) or by direct electron transfer with the steel surface (Mehanna et al., 2009).

According to the phenomenological model (Melchers and Jeffrey, 2005; Melchers and Wells, 2006), and in agreement with Malard et al. (2008), the micro-organisms may have only little influence on the corrosion mechanisms and kinetics during the aerobic period. However, the evolution with time of the bacterial communities forming a biofilm on the steel surface during this period may provide information on the initiation of the microbiologically influence corrosion processes (MIC). This evolution of the bacterial communities is necessarily associated with that of the corrosion product layer that develops rapidly on the steel surface (the initial corrosion rate can reach 5.7 mm per year according to Memet, 2000). So, it is likely that the evolution of this mineral layer influences that of the micro-organisms, and vice-versa. For instance, the combined study of both corrosion products and bacteria, performed at the beginning of the anaerobic period, that is ~12 months of immersion, revealed that SRB were associated with FeS and sulfated green rust ($GR(SO_4^{2-}) = Fe^{II}{}_4Fe^{III}{}_2(OH)_{12}SO_4 \cdot 8H_2O$) uniformly all over the steel surface (Pineau et al., 2008).

So, the main aim of our study was to characterize thoroughly both bacterial communities and solid phases present in the corrosion product layers during the early stages of marine corrosion of carbon steel. For this purpose, carbon steel coupons were permanently immersed at a constant depth (~1 m) in a French Atlantic harbor (i.e. in natural seawater) for 1 week to 2 months. At the end of each immersion period, the layers covering the steel surface were studied via a combination of physico-chemical investigations with microbiological and molecular biology techniques. The corrosion products were characterized by µ-Raman spectroscopy while the associated bacteria were studied simultaneously. A combination of culture-dependent (traditional culture techniques) and culture-independent approaches (16S rDNA PCR amplification and TTGE) was used. The culture-independent approach was required because of the limitations of culture-based profiling of microbial communities. The more traditional culture-based methods were used in the attempt to obtain bacteria representative of biocorrosion phenomena that can be grown in culture and thus used for further experiments.

Materials and methods

Sampling of steel coupons immersed in seawater

Carbon steel coupons (70 × 70 × 6 mm) were immersed in one of the harbor of La Rochelle (Atlantic coast, France) for 1 week to 2 months at a constant depth of 1 m. The approximate steel composition (in weight %) was: 98.2% Fe, 0.122% C, 0.206% Si, 0.641% Mn, 0.016% P, 0.031% S, 0.118% Cr, 0.02% Mo, 0.105% Ni and 0.451% Cu. The surface was shot blasted (Sa 2.5, angular shot) to obtain a roughness value of 50–70 µm, degreased with acetone and dried. The coupons were disposed in a Teflon holder and set in the immersion site. At the end of the experiment, the corroded coupons were removed, carried to the laboratory in sealed bags filled with seawater and immediately processed. For each immersion time, one coupon was used for microbiological analysis in aerobic conditions, a second was used for coupled µ-Raman analysis and microbiological analysis in anaerobic conditions and two other coupons were stored at −20 °C for DNA extraction. The experimental procedure is summarized in Fig. 1.

Preparation of corrosion product layers for microbiological analysis

The corrosion product layer was scraped from the corroded coupons with a scalpel, weighed and then resuspended in 2 ml of synthetic sterile seawater (NaCl 23.4 g l^{-1}; KCl 1.5 g l^{-1}; $MgSO_4 \cdot 7H_2O$ 1.2 g l^{-1}; $CaCl_2 \cdot 2H_2O$ 0.15 g l^{-1}; $CaCl_2$ 0.15 g l^{-1}). Some samples were then subjected to mechanical grinding with glass beads for 1 min to dissociate adherent bacteria from the particles of corrosion products. This step allowed us to increase the number of bacteria counted by microscopy as well as by culture on solid media (data not shown). These samples were used to enumerate total and culturable bacteria and to inoculate the different media (solid and liquid).

In order to determine the amount of water in each sample, approximately 3–4 g of each sample were dried at 80 °C and weighed. All bacterial concentrations were expressed as the number of bacteria per gram of dry sample.

Numeration and isolation of culturable bacteria (Fig. 1, "path 1")

Isolates were obtained from the corrosion product layers by inoculation of 100 µl of serial dilutions (10^{-1} to 10^{-6}) on solid culture media (with 1.2% agar). Marine Agar (Difco) was used in aerobic and anaerobic conditions and Baar modified solid medium for sulfate reducers (Atlas, 2005) was used in anaerobic conditions. Baar modified solid medium contained: $MgSO_4$ 2 g l^{-1}; sodium citrate 5 g l^{-1}; $CaSO_4 \cdot 2H_2O$ 1 g l^{-1}; NH_4Cl 1 g l^{-1}; NaCl 25 g l^{-1}; K_2HPO_4 0.5 g l^{-1}; sodium lactate 3.5 g l^{-1}; yeast extract 1 g l^{-1}; agar 12 g l^{-1} (pH 7.5). After sterilization (115 °C, 20 min), Baar modified medium was supplemented with $Fe(NH_4)_2(SO_4)_2$ 1 g l^{-1}. After 3 weeks at 30 °C in an anaerobic incubator (Don Whithey Scientific limited MAC500, atmosphere $N_2/H_2/CO_2$, 80/10/10) or in aerobic conditions, bacterial growth was evaluated by counting the number of colony forming units (CFU) (two replicates). The results are expressed in CFU g^{-1} of dry sample. Purification of the isolates was achieved by selecting colonies with different morphologies. The colonies formed on plates were picked up and further purified by re-streaking on the corresponding medium and by incubation in the same conditions as described above.

Culture of mixed bacteria in liquid medium (Fig. 1, "path 2")

For each immersion time, 5 ml of liquid medium (Marine Broth and Baar modified medium) were inoculated with 2% of the corrosion product sample and incubated for 7 days at 30 °C under aerobic and anaerobic atmospheres. The cultures were then transferred to 100 ml of fresh medium and incubated under the same conditions for 3 additional weeks.

Determination of total bacterial abundance (Fig. 1, "path 3")

The cells from the corrosion product layers were fixed in formaldehyde (final concentration 1%). The mixture was then

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