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ORIGINAL RESEARCH PAPER

Marine bacterial community analysis on 316L stainless steel coupons by Illumina MiSeq sequencing

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Abstract In order to evaluate the corrosive action of microorganisms on 316L metal exposed directly to a marine environment, a system was designed to immerse coupons in seawater. After periods of 30, 60 and 90 days, the coupons were recovered, the corrosion rates evaluated and the biofilm samples on their surface were analyzed by 16S rRNA gene sequencing. The results of the corrosion rate showed an acceleration over the entire experimental period. Alpha diversity measurements showed higher rates after 60 days of the experiment, while abundance measurements showed higher rates after 90 days of exposure to the marine environment. The beta-diversity results showed a clear separation between the three conditions and proximity in the indices between replicates of the same experimental condition. The results of 16S rRNA gene sequencing showed that after 30 days of exposure to seawater, there was massive representativeness of the pioneer bacteria, *Gamma* and *Alphaproteobacteria*, with emphasis on the genera *Alcanivorax*, *Oceanospirillum* and *Shewanella*. At the 60-day analysis, the *Gammaproteobacteria* class remained dominant, followed by *Alphaproteobacteria* and *Flavobacteria*, and the main representatives were *Flexibacter* and *Pseudoalteromonas*. In the last analysis, after 90 days, a change in the described bacterial community profile was observed. The *Gammaproteobacteria* class was still the largest in diversity and OTUs. The most predominant genera in number of OTUs were *Alteromonas*, *Bacteriovorax* and, *Nautella*. Our results describe a change in the microbial community over coupons directly exposed to the marine environment, suggesting a redirection to the formation of a mature biofilm. The conditions created by the biofilm structure suggest said condition favor biocorrosion on the analyzed coupons.

Keywords Biocorrosion · Marine environment · Stainless 316L · Microbial community analysis · Bacteria · Microbiologically influenced corrosion (MIC)

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Introduction

The deterioration of metallic as well non-metallic materials by corrosion processes is a problem widely

present in many industry sectors, as well as having enormous impacts on public safety and the environment (Hamilton 2003; Muyzer and Stams 2008). The economic losses due to corrosion approximate US$2.5 trillion per annum, equivalent to roughly 3% of global Gross Domestic Product (GDP) (Koch et al. 2002). Although numerous efforts are made to prevent corrosion, for example by coating with paint or other metal covers, or even cathodic protection, the problem persists (Chandler 1985). Corrosion is an electrochemical process characterized by the dissolution of the zero-valence metal at an anode site, but, in turn, a secondary reaction is necessary, which consists of a cathodic site that acts as an external acceptor of electrons, to complement the reaction as a whole. The tendency of a material to act as an anodic or cathodic site, or a donor or electron acceptor, respectively, is dependent on the electrochemical potential of such material. The propensity of the anodic site undergoing corrosion will be dependent on the potential difference between the anode and the cathode sites. However, this difference in potential is necessary, but not sufficient for metals to corrode. In fact, for corrosion to occur, there must be a kinetic path available to facilitate the flow of electrons between the anode and the cathode, and the rate of that flux determines the rate of corrosion. This process is particularly accelerated in microbially stimulated corrosion when biofilms accumulate at the cathodic site (Hamilton 2003).

Microbiologically Influenced Corrosion (MIC) is a commonly related problem in damage to metal infrastructure, including in marine environments, with losses that can reach billions of USD worldwide (Koch et al. 2002). MIC over metallic surfaces is an interfacial process involving interactions between microbes and structures, by means of strong interactions of microbial communities, named biofilms (Bonifay et al. 2017). The adhered microbes can induce, facilitate or accelerate metallic damage as a result of the requirement for an energy source, electron donor or electron acceptor (Videla and Herrera 2005; Procópio 2019). The scientific literature describes corrosion as frequently linked to the activity of Sulfate-Reducing Bacteria (SRB) and Thiosulfate-Reducing Bacteria (TRB) in anaerobic systems (Boudaud et al. 2010). More recently, attention has been directed to corrosion processes, especially metal corrosion, by the presence of lithotrophic Fe-Oxidizing Bacteria (FeOB) (Lee and Newman 2003; Marty et al. 2014; McBeth and Emerson 2016). FeOB-marine members are commonly aerobic, lithotrophic and capable of oxidizing iron under neutral pH conditions (Emerson et al. 2010). This group has been more studied in recent years in different marine environments, such as near-shore and deep-marine environments (Edwards et al. 2003; Emerson and Moyer 2002; McBeth and Emerson 2016). FeOB participates in the biogeochemical cycle of environments with high Fe levels, through redox reactions, and have been widely described as participants in the early stages of acceleration of carbon steel corrosion. As a consequence of its colonization, surface rust is formed, which enables the subsequent growth of anaerobic bacteria in the formed microenvironment (McBeth et al. 2011). Microorganisms related to iron oxidation accelerate the process through the mechanisms of pH changing, acid productions, secretion of corrosive metabolites or extracellular enzymes (Kato 2016; Little et al. 2007). The basis of an aerobic corrosion reaction is characterized by electrochemical coupling between iron oxidation (anodic reaction) and oxygen reduction (cathodic reaction) (Lee and Newman 2003). The product of this reaction is ferrous iron Fe(II), which is further oxidized to ferric iron, Fe(III). Fe(III) serves as a final electron acceptor in the Electron Transport Chain (ETC), allowing the flow of electrons through the ATPase enzyme, thus generating electrochemical strength for the synthesis of ATP, coupling the corrosion reactions with Fe(III)-respiring bacteria (Kato 2016; Tremblay et al. 2017).

Metallic structures are materials commonly used in marine engineering and industrial facilities, as well as in the construction of piers, ships, and bridges near marine environments. As marine environments are very conducive to corrosive processes, such as chemical and microbiologically influenced corrosion, the safety and conservation of these structures are of great global concern (Dang et al. 2011). Submerged metallic surfaces in aquatic environments are rapidly colonized by microorganisms, which adhere strongly, forming biofilm structures (Procópio 2019). The bacterial growth and subsequent formation of biofilm structures over metallic surfaces modify the oxygen concentration gradient, essentially due to microbial respiration, which generates conditions that can induce and accelerate cathodic corrosion reactions (Lee and Newman 2003; Marty et al. 2014). Despite the fact that Fe(II) is their primary energy source and they are

frequently identified in corrosion biofilms, the role of the FeOB group in MIC is poorly described and the understanding of the role of FeOB in marine MIC is largely unvalued (Mumford et al. 2016). Studies have shown FeOB are able to colonize and grow either on metallic coupons introduced into the environment (Dang et al. 2011; McBeth et al. 2011), or are present on corroded steel associated with ALWC (Accelerated Low Water Corrosion) structures (Marty et al. 2014). Even structures made with metal alloys described as more resistant to corrosion are deteriorated by the action of microorganisms (Li et al. 2016; Sun et al. 2017). The 316L metal alloy is widely used in structures due to being more resistant to corrosion. Its application in structures close to marine environments is in the construction of deck components for boats and ships e.g. deck eyes, brackets for anchor ropes, housings for equipment, shackles, and handrails, among others.

Establishing a correlation between the action of bacteria and the corrosive processes of metal alloys is a challenge in biocorrosion studies. Culture techniques usually do not reflect the actual composition present in the sample in studies, owing to the innumerable non-cultivated species or because they are limited to a single-species study. Today, large-scale sequencing techniques, such as Illumina MiSeq, enable the investigation of microbial communities in different environments, as well as tracking succession over a given period. Moreover, the analysis of environmental samples in situ, allow more realistic approaches, once the real composition of the bacterial community is identified (Procópio 2020a, b). One reflection of this is the growing number of metagenomic studies which became available in the past few years (An et al. 2016; Li et al. 2017a; Liang et al. 2016; McBeth and Emerson 2016; Ramírez et al. 2016).

Stainless steel is widely used in various structures close to marine environments. The reason for its use is that 316L steel has corrosion-resistant properties. However, despite its stainless property, corrosive deterioration processes, such as pitting, are commonly described in this metal exposed to corrosive environments (Marconnet et al. 2008). In addition, although microbial corrosion of metal structures has been widely studied in recent decades, most of these studies have been conducted under controlled laboratory conditions. This metagenomic study is the first attempt to assess corrosion influenced by microorganisms in an actual marine environment. Our results allowed us to evaluate the true conditions, with the numerous factors in the conduction of biocorrosion. The goal of our study was to evaluate the action of bacteria grown over a period of up to 90 days on 316L stainless steel coupons dipped in seawater and the resultant corrosion rates due to biofilm growth over them. For this, we analyzed, by means of 16S rRNA gene sequencing, the dynamics of the bacterial community during the time of exposure to the marine environment and verified the attendant corrosion of the metal coupon.

Materials and methods

Experiment design

The experiment was conducted in situ, in Guanabara Bay, Rio de Janeiro, RJ, Brazil, near the naval shipyard of the Brazilian Navy, at a depth of roughly 5 m. A 3-cubic meter (m^3) polypropylene box was designed, opened at the top. Fifteen stainless coupons of 3 cm^2 surface area and 0.1 cm thickness were placed in the box, suspended by nylon strings and separated from each other. The box was fixed to the bottom of the sea using ropes and weights to prevent it being turned or dragged by the tide. The experiment was conducted for 90 days, and at intervals of 30, 60 and 90 days the box was retrieved, and 5 coupons were removed, immediately transferred in an icebox to the Microbial Corrosion Laboratory, Estacio University (UNESA), Rio de Janeiro-Brazil, to evaluate the mass loss and analyze the presence of microbial communities over the coupon using 16S DNA gene sequencing.

Corrosion rate analysis

Stainless steel 316L coupons were used to evaluate microbial corrosion in a marine environment, with maximum chemical composition of 0.030% C, 2.00% Mn, 0.75% Si, 0.045% P, 0.030% S, from 16.00 to 18.00% Cr, from 10.00 to 14.00% Ni, from 2.00 to 3.00% Mo. The stainless steel was cut into coupons of approximately 3cm^2, with a small hole cut close to one of the edges, permitting them to be secured by nylon strings in the polypropylene box described above. Initially, the stainless steel coupons were sanded sequentially using sandpaper with decreasing grains. Then, they were then incubated for thirty minutes in

absolute ethanol to degrease the surface, washed twice with acetone to remove any organic matter and finally dried at 70 °C for thirty minutes and stored in a desiccator. Subsequently, all coupons were autoclaved, cooled and identified. The coupons were weighed twice employing precision scales with 0.00001 readability in order to evaluate any weight loss after exposure to seawater.

At each timepoint, five stainless coupons were carefully removed of the box, and each coupon was stored in a previously sterilized 200 ml glass filled with local seawater, then transported to the Microbial Corrosion Laboratory of Estácio University. Two of the coupons were separated to determine microbial community by 16S rRNA gene sequencing, and the other three coupons were used for mass loss analysis.

The biofilm formed over the coupons and corrosion debris were scraped and immediately immersed in acid pickling (15% HCl) to remove all surface corrosion products according to ASTM G1 (Standard Practice for Preparing, Cleaning, and Evaluating Corrosion Test Specimens) (ASTM G1-03 2017). The acid reaction was ended by the application of thiourea solution for 5 s, then the coupons were washed with distilled water, neutralized with 10% NaOH for 5 s, and finally immersed in acetone for the same period. Following this process, the coupons were dried and used to determine corrosion rates. Evaluation of corrosion progress was undertaken using the weight-loss method. The results of weight-loss measurement were employed to calculate the value of the corrosion rate (CR) following the equation:

$$CR = (W \times K)/(D \times A \times T),$$

where the W is the decrease in metal weight the during time period analyzed, K is the constant (8.76×10^4), D represents the metal density in g/cm^3, the coupon area (mm^2) and T the exposure time in days (ASTM G1-03 2017).

16S rRNA gene-based community analysis

The coupons immersed in the marine environment of each experimental condition were carefully removed and, rinsed with sterile water to remove residues loosely adhered to the coupons. Following this, the biofilm growth over the coupons surfaces were scraped with a swab, previously moistened to facilitate collection and, kept refrigerated in a thermal box at 0 °C. Sequentially, the replicates were immediately sent to Neoprospecta Microbiome Technologies (Florianópolis-SC, Brazil). The DNA total was extracted using the commercial DNeasy PowerSoil Kit (Qiagen), respecting the conditions suggested by the manufacturer. The sample preparation and sequencing were performed by Neoprospecta Microbiome Technologies. It consisted of the 16S rRNA V3/V4 region, which was amplified using 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers, employing Illumina adapters. Amplification consisted of 35 cycles at 50 °C annealing temperature of the primers. In each sample, the process was carried out in triplicate. Then, sequencing was performed by Illumina MiSeq using V2 kits, with a 300 nts run.

The bioinformatics analyses were performed by Neoprospecta Microbiome (Florianopolis-SC, Brazil). The primer and adapter sequences were trimmed from the reads and only sequences with 275nt or more were used in downstream analysis. Then, all reads with one or more indeterminate "N" bases and truncated sequences with two or more consecutive bases with quality scores below Q20 were eliminated. OTUs (Operational Taxonomic Units) were performed using BLASTN 2.2.28 against the GreenGenes 13.8 database (DeSantis et al. 2006). For the purpose of attributing taxonomy, only sequences with 99% identity hits and over 99% alignment coverage were considered. The phylogenetic tree was constructed initially using an alignment with high abundance OTUs by MUSCLE method (Edgar 2004a, b). Next, a tree was generated using the UPGMA method and MEGA6 software (Tamura et al. 2013), with 1000 bootstraps for branching, and the tree topology by 1000 re-samplings. The OTUs sequences are available in the NCBI Sequence Read Archive (SRA) database under accession no. PRJNA494887, in SAMN10184473-8 Biosample accession numbers.

Statistical analysis and processing data

The abundances of phylum, class, genus and species levels, as well as their statistical differences in terms of the microbial community throughout the analyzed period were delineated in R language using Studio R software. Bar graphs were produced by the Studio R software, while a Heatmaps graph based on the relative abundance of OTUs at genetic levels was

constructed using the ggplot2 (Wickham 2016) packet R program. Statistical analyzes of corrosion rates were performed using the parametric t test, as well as the standard error of the means were calculated and using the program R.

The alpha-diversity analysis was calculated using Shannon and Simpson, whereas the non-parametric abundance Chao1. All these indices employed the EstimateS software. The rarefaction curves based on the six samples of the three conditions were constructed using the Past3 software. The stacked bars graphs, Venn diagram and Heatmap were made using the ggplot2 and Vegan packages available for the R Studio software. The beta-diversity index was used to verify the differences among the communities employing the Bray–Curtis index. The plots used to show these results were the principal component analysis (PCA) and the non-metric multidimensional scaling (NMDS) analysis, both using the Past3 software.

Results

Corrosion rate measurement

The coupons were submerged in seawater at a depth of about 5 m and recovered after 30, 60 and 90 days to evaluate corrosion rates. Deposits of rust on the surface were removed and then the coupon underwent the acid pickling process as described above. After the process, the coupons were weighed to compare the mass before and after the period of exposure to seawater. Even after the first collection and analysis at 30 days, biofouling was visible on the whole surface, and this characteristic deposit persisted, and gradually increased until the last analytical period. The results of the mass loss measures indicated a clear progression in the corrosion rate throughout the experiment. Figure 1 shows the corrosion rate over the assessed period of up to 90 days, showing a small loss of mass only after 90 days of exposure.

Richness of OTUs among the samples

The Shannon and Simpson indices were used to measure the alpha heterogeneity in the samples evaluated. Both index values indicated a great diversity in all the samples. The values presented in Table 1

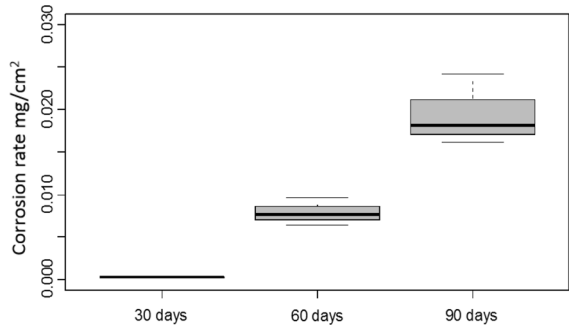

Fig. 1 Evaluation of the corrosion rate of 316L stainless steel coupons after 30, 60 and 90 days of exposure to seawater, at a depth of about 5 m. The x-axis represents the three timepoints of analysis, while the y-axis represents the corrosivity rate in mg/cm^2

show an increase in diversity between 30 and 60 days of exposure to the coupons in the marine environment. In fact, the condition where the greatest diversity occurred, according to the alpha diversity index was at 60 days. Although after 90 days there was a decrease in relation to the previous period of 60 days, a high diversity index was still maintained. The indices of abundance calculated by Chao1 also presented a profile where there is higher richness in the condition of 60 days of incubation in seawater, followed by 30 and 90 days (Table 1). The rarefaction curve analysis showing all replicates of the three analyzed conditions confirm the greatest richness after 60 days, while conditions at 30 and 90 days show similar indices (Fig. 2). The analysis of the structures of the three communities using Non-Metrric Multidimensional Scaling (NMDS) showed a distinct separation between them (Fig. 3).

The Illumina MiSeq sequencing generated a total of 307,183 high-quality OTUs sequences within the six sequenced samples of the three time points analyzed. In the first analysis, after 30 days of exposure in the marine environment, 163,356 OTUs were generated between the two sequenced coupons. At the time of the second analysis, there was a substantial decrease in the number of OTUs generated, 38,833 sequences in total for both coupons. And in the last two sequenced samples which refer to 90 days' exposure to seawater, there was an increase in the number of sequences, in which 104,994 OTUs were detected. The Venn diagram shows the OTU distribution and overlap for the different microbial communities throughout the study (Fig. 4a).

Table 1 Numbers of OTUs in each sample analyzed, diversity indices (Shannon and Simpson), abundance and incidence estimator (Chao1 and Chao2), rate numbers identified in each sample and Biosample access number

Sample name	OTUs	Shannon diversity index	Simpson (inverse) index	Chao1	Number of genera	Accession number
30 days A	65,908	1.58	0.66	60.6	56	316L30Daysa
30 days B	97,931	1.65	0.68	84.39	61	316L30Daysb
60 days A	21,975	3.34	0.93	96.97	25	316L60Daysa
60 days B	21,789	3.42	0.94	105.18	25	316L60Daysb
90 days A	46,990	2.00	0.71	108.0	69	316L90Daysa
90 days B	58,601	1.52	0.55	108.0	75	316L90Days

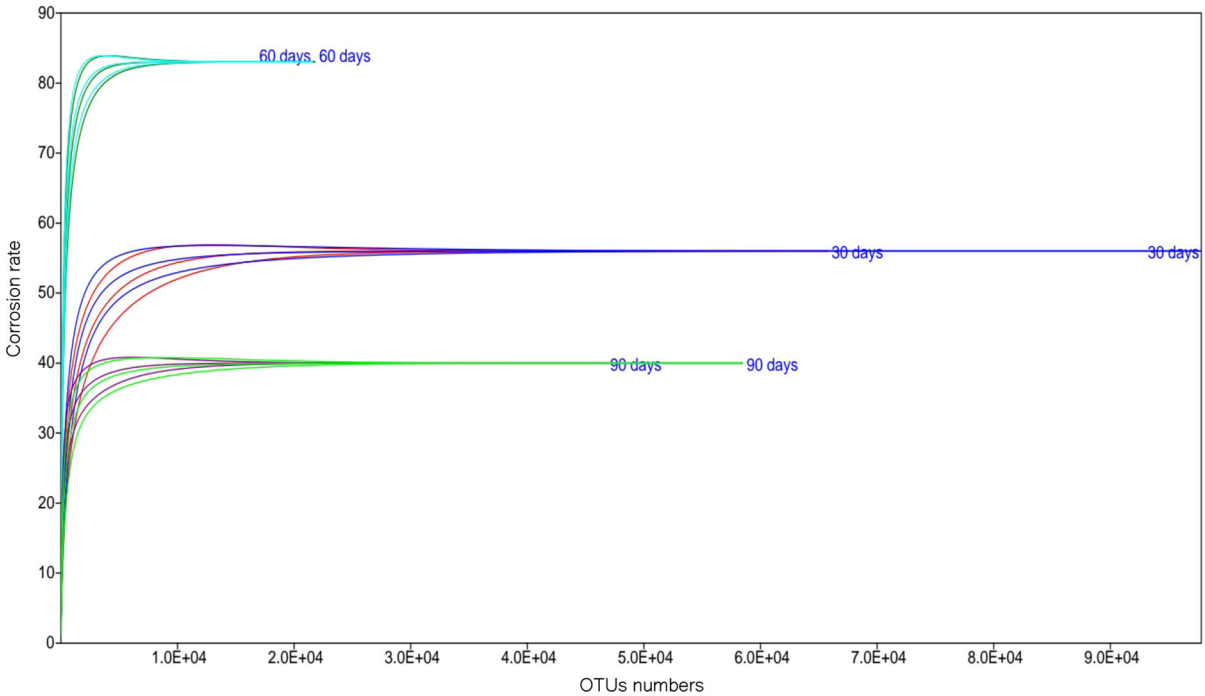

Fig. 2 Venn diagram describing OTU richness and overlap in microbial communities analyzed after 30, 60 and 90 days in a marine environment

The results of the OTUs similarity analysis enabled the identification of 229 different bacterial species (Fig. 4b). When comparing microbial diversity during the period in which the coupons were exposed in the marine environment, it was noted that between the first 30 and 60 days, there was a small increase in the number of species detected, from 127 to 135 bacteria, although the number of OTUs was lower at the second time point. The analysis of the species detected throughout the experiment shows that the largest number was identified at 60 days, followed by 30 days. Despite the fact that number of OTUs present exclusively after 90 days was the highest when compared to those at 30 and 60 days, the number of bacteria species present only at day 90 was the lowest (Fig. 4b).

Annotated diversity analysis

The results showed that, in general, there were consistent patterns of taxonomic distributions between replications of sequences in coupons, as can be seen in taxonomic analyses of the phylum and class groups. At the phylum level, *Proteobacteria*, *Bacteroidetes*,

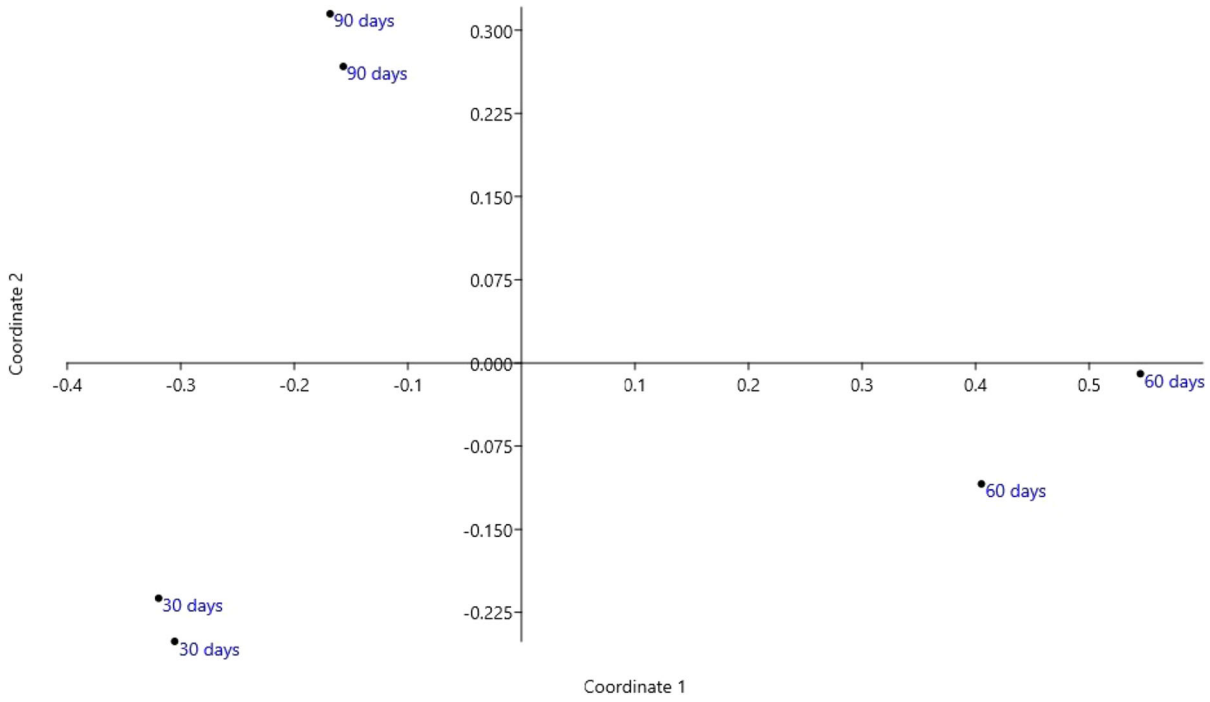

Fig. 3 Rarefaction curve analysis of OTUs referent to three conditions, with receptively replicates, showing the relationship between OTU numbers (x-axis) and taxonomic diversity (y-axis)

Firmicutes, and *Verrucomicrobia,* were represent (Table 2). Members of *Proteobacteria* phyla were dominant in all samples sequenced. In this group alone, 288,856 OTUs were detected, constituting a total of 94.03% of the total detected sequences. The next most abundant group was *Bacteroidetes*, which comprised about 5.62% of the sequences, with 17,252 OTUs. *Firmicutes* (0.34%/1035 OTUs) and *Verrucomicrobia* (0.01%/40 OTUs) were detected only at 30 and 60 days of incubation, respectively (Fig. 4). In general, when analyzing the core of class-level sequencing, community composition was overwhelmingly dominated by *Gammaproteobacteria* (86.8%), *Alphaproteobacteria* came second with 6.3%, and *Flavobacteriia* with 3,6% was the third most predominant class. *Saprospiria* (1.2%), *Oligoflexia* (1%), *Bacilli* (0.4%), *Epsilonproteobacteria* (0.05%) and *Cytophagia* (0.9%) were detected in lesser proportions.

After 30 days of seawater incubation, 132 different species were indentified growing over coupons. The main group was *Gammaproteobacteria,* with 153,353 OTUs (93.9%) distributed among 88 different representatives. The second most prevalent group was *Alphaproteobacteria*, with 4620 OTUs (2.8%), and 24 different members, with a value well below the first group. Next, *Flavobacteria* class presented 4210 OTUs (2.6%) and 18 different species (Fig. 5). The *Bacilli* (1035 OTUs) and *Epsilonproteobacteria* (138 OTUs) classes were detected only at this point of analysis, *Bacillus subtilis* and *Arcobacter bivalviorum* (138 OTUs) being the only representatives of each class, respectively (Fig. 6). The dominant genus in this analysis was *Vibrio*, with 38 different species, followed by *Pseudoalteromonas*, with 13 species, both representing 42.4% and 38.8%, respectively. However, when observing the number of sequences between the two groups, *Pseudoalteromonas* had 63,308 sequences, in close proximity to *Vibrio*, which despite having almost double the number of representatives, accounted for 69,284 sequences detected. Moreover, the bacterium *Pseudoalteromonas mariniglutinosa* was present in 37,736 sequences, which alone accounted for 23% of all sequences. The *Alcanivorax borkumensis* was represented by 5644 OTUs, with 3.5% of the detected sequences, while other more representative genera were *Shewanella,* with 3.7% of OTUs, *Oceanospirillum* was present in

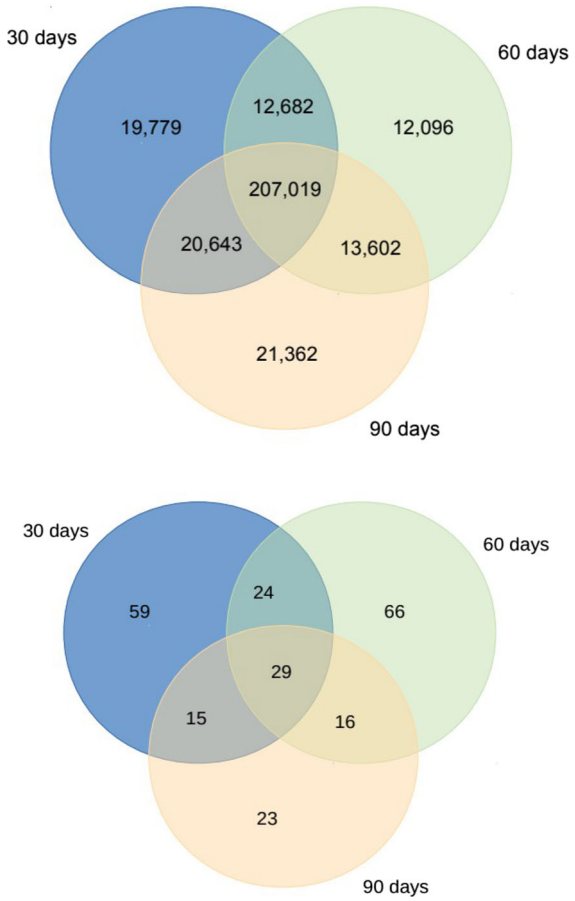

Fig. 4 Non-metric multidimensional scaling (nMDS) of metagenomic 16S DNA showing distances of the communities in different incubation periods. The ellipses show a significant separation of the communities present in the samples

Table 2 Distribution of OTUs among the Phylum detected in the six sequenced samples

Phylum	OTUs	Representativeness (%)
Bacteriodetes	17,252	5.62
Firmicutes	1035	0.34
Proteobacteria	288,856	94.03
Verrucomicrobia	40	0.01

1.3% and *Alteromonas* in 1.2%, and all other genera had values below 1% (Fig. 6).

With 60 days of exposure to seawater 135 different species was identified. The most representative group, in contrast to the first time point, was the class *Alphaproteobacteria* with 54 different species. The second group was members from class *Gammaproteobacteria,* with 45 members, followed by the *Flavobacteria* class represented in the 31 different genera. Members of the *Cytophagia, Sapropiria* and *Verrucomicrobiae* classes were identified for the first time at this timepoint. Although class *Alphaproteobacteria* was the most diverse, *Gammaproteobacteria* still accounted for the largest number of OTUs, 21,761 versus 9951 OTUs detected for *Alphaproteobacteria* (Fig. 5). The *Flexibacter echinicida* of the *Flavobacteria* class was one of the bacteria that showed the greater number of OTUs, 3504. The *Vibrio* genus, despite still being the most abundant genus, had a clear reduction in the number of OTUs and species, from 36 representatives detected at 30 days to 15 at this timepoint. Other *Gammaproteobacteria* members detected were represented by *Aestuariibacter halophilus* (5.1%), *A. borkumensis* (4.9%) and *Alteromonas macleodii* (4.1%). *Pseudoalteromonas* and *Shewanella* were present in five representatives each, being the most notable *Pseudoalteromonas spongiae* (3.5%) and *Shewanella waksmanii* (3.1%). Also, the oxyhydroxide-reducing *Ferrimonas* (0.7%), and the hydrocarbon degrader *Marinobacter hydrocarbonoclasticus* (0.2%) were detected. Along with *M. hydrocarbonoclasticus*, two other species of this genus were also identified, *Marinobacter coastalis* and *Marinobacter xestospongiae,* both wit 0.2% of representativeness. Among the *Alphaproteobacteria,* the *Roseovarius* genus was represented with seven species, followed by *Erythrobacter* and *Ruegeria* genera, with five and four representatives, respectively. The *Sulfitobacter* genus was represented by three different members, *Sulfitobacter donghicola* (0.15%), *Sulfitobacter mediterraneus* (0.1%), and *Sulfitobacter pontiacus* (0.9%). 16S DNAs from the *Donghicola*, *Hyphomonas*, *Marivita*, *Pseudoruegeria*, *Shimia*, *Thalassobius,* and *Tropicibacter* genera were also represented. Finally, the extremophilic bacterium *Antarctobacter heliothermus* was detected in this analysis with 0.2% representativity (Fig. 7).

The last time point analyzed was after 90 days of exposure to the marine environment. At this point, despite the fact the number of OTUs was the largest in this study (104,994), there was a decrease in the diversity of bacteria, 83 different species. The dominant group was class *Gammaproteobacteria*, with 55 members identified, and 87.4% representativeness, followed by *Alphaproteobacteria* (4.4%) and

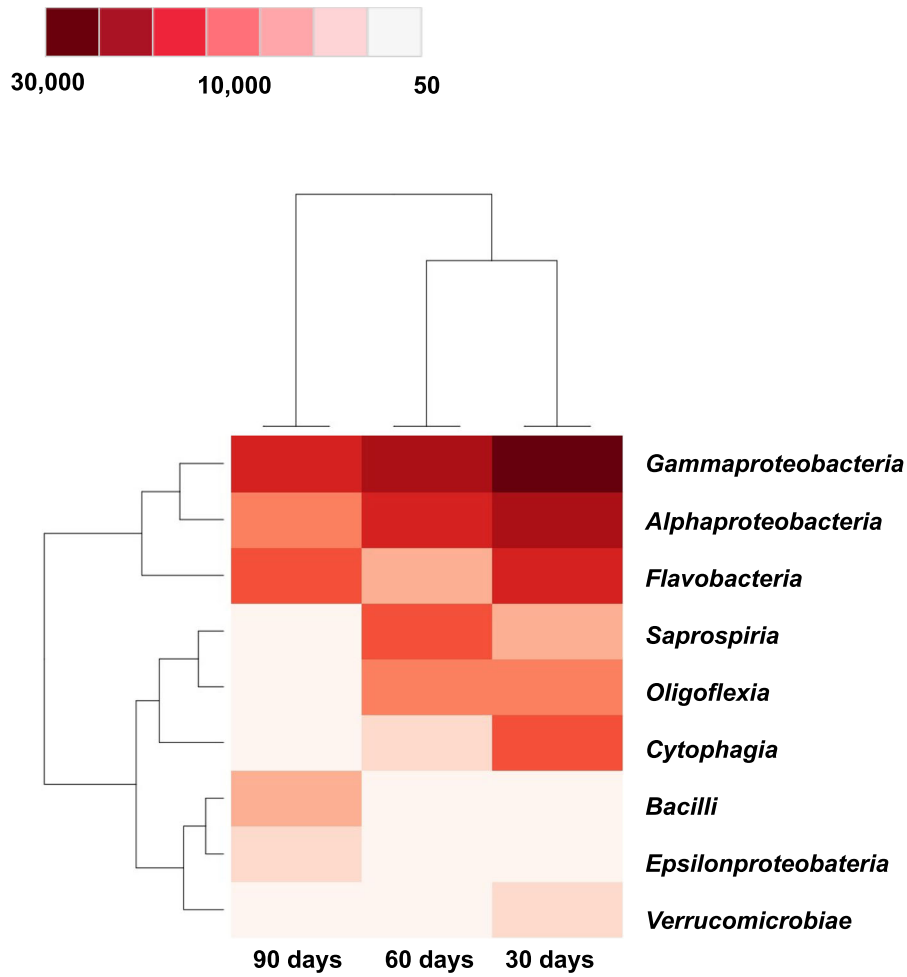

Fig. 5 Heatmap showing the distribution of the bacterial community throughout the experiment. On the x-axis the replicates of the sequenced samples are described, in the right column the classes are presented

Flavobacteria (2.4%) (Fig. 5). The *Vibrio* genus was again the most prevalent on the analyzed coupons. Even though, the number of species was 16, similar to 60 days, there was an increase in the number of OTUs detected in the group, with 58.3% representativeness. Seven members of the *Pseudoalteromonas* genus were detected with 14,551 sequences (13.9%), and three species of *Pseudomonas*, including the petroleum-degrading *Pseudomonas putida*. Additionally, the genus *Ferrimonas*, previously described, was detected again, with the *Ferrimonas futtsuensis* (0.02%), and *Ferrimonas kyonanensis* (0.2%) species. Other *Gammaproteobacteria* members, previously described in other analyses, such as *Aestuariibacter* (2.0%), *Alcanivorax* (0.3%), *Alteromonas* (2.0%), *Shewanella* (1.3%), *Thalassolituus* (0.3%), persisted over metallic coupons until the end of the experiment. The *Ruegeria* genus belonging to the class *Alphaproteobacteria* continued to be detected, with four members and 1065 OTUs (1%). Futhermore, other genera that persisted as shown by 16S rRNAs gene sequencing were *Celeribacter* and *Thalassobius* genera. *Cytophagia* class contained two identified members, *Algoriphagus vanfongensis*, *Algoriphagus zhangzhouensis,* and the *Saprospiria* class with a single representative, *Saprospira grandis* (3.3%/3485 OTUs). Among the members of the *Flavobacteria* class, *Bizionia palithoae*, *Corallibacter vietnamensis* and *Meridianimaribacter flavus* predominated in our analyses. Finally, the two representatives of the *Tenacibaculum* genus, detected in the 60-day analysis, persisted to this point with a representativeness of 1.1% of the sequences (Fig. 8).

Fig. 6 Phylogenetic tree based on the 16S rRNA gene sequences obtained after 30 days of exposure in seawater. Parenthesis indicate the total number of individual sequences within the OTUs. The bootstraps values were calculated from 1000 re-samplings

Discussion

After each period analyzed, the corrosion rate measurements indicated an acceleration of the deterioration of the metal exposed to the marine environment. Initially, the low corrosion rate in the first analysis was supplanted by the later results. Due to the resistance characteristics inherent in 316L stainless steel, because of the protective layer of Cr-oxides, the corrosive processes are normally slower (Landolt 2007). However, studies have shown that the presence of bacterial biofilms accelerates its corrosion by reducing oxygen in the cathodic zone (Videla 1994). As a consequence, pitting damage is identified in these situations. In a recent study on the effects of tidal cycles on the corrosion of 316L stainless steel, the acceleration of the corrosion rate influenced by the biofilm present on the surface was demonstrated (Daille et al. 2020). Furthermore, in this study three coupons were used to measure corrosion rates after 1 day, 5 and 15 weeks. Similarly, in our analysis, three coupons were also evaluated for each measure.

Fig. 7 Phylogenetic tree based on the 16S rRNA genes sequence obtained after 60 days of exposure in seawater. Parenthesis indicate the total number of individual sequences within the OTUs. The bootstraps values were calculated from 1000 re-samplings

However, the choice to start corrosion assay after 30 days was due to the prior knowledge that mass loss is insignificant in the first weeks of exposure.

One of the major challenges in understanding how the corrosion process occurs in a natural environment is to establish a relationship between the microorganisms associated with the process, the intrinsic environmental factors and the characteristics of the deteriorated material. The pivot for this understanding lies in deciphering the role of the microbial community throughout this process. Experiments carried out in controlled laboratory environments greatly aid in this task, but in situ experiments shed light on the evolution of the microbial taxa involved in the corrosive process, despite bringing more factors to be considered. Taxonomic information provides valuable insights into the microbial structure formed on the coupon by establishing functional relationships of the

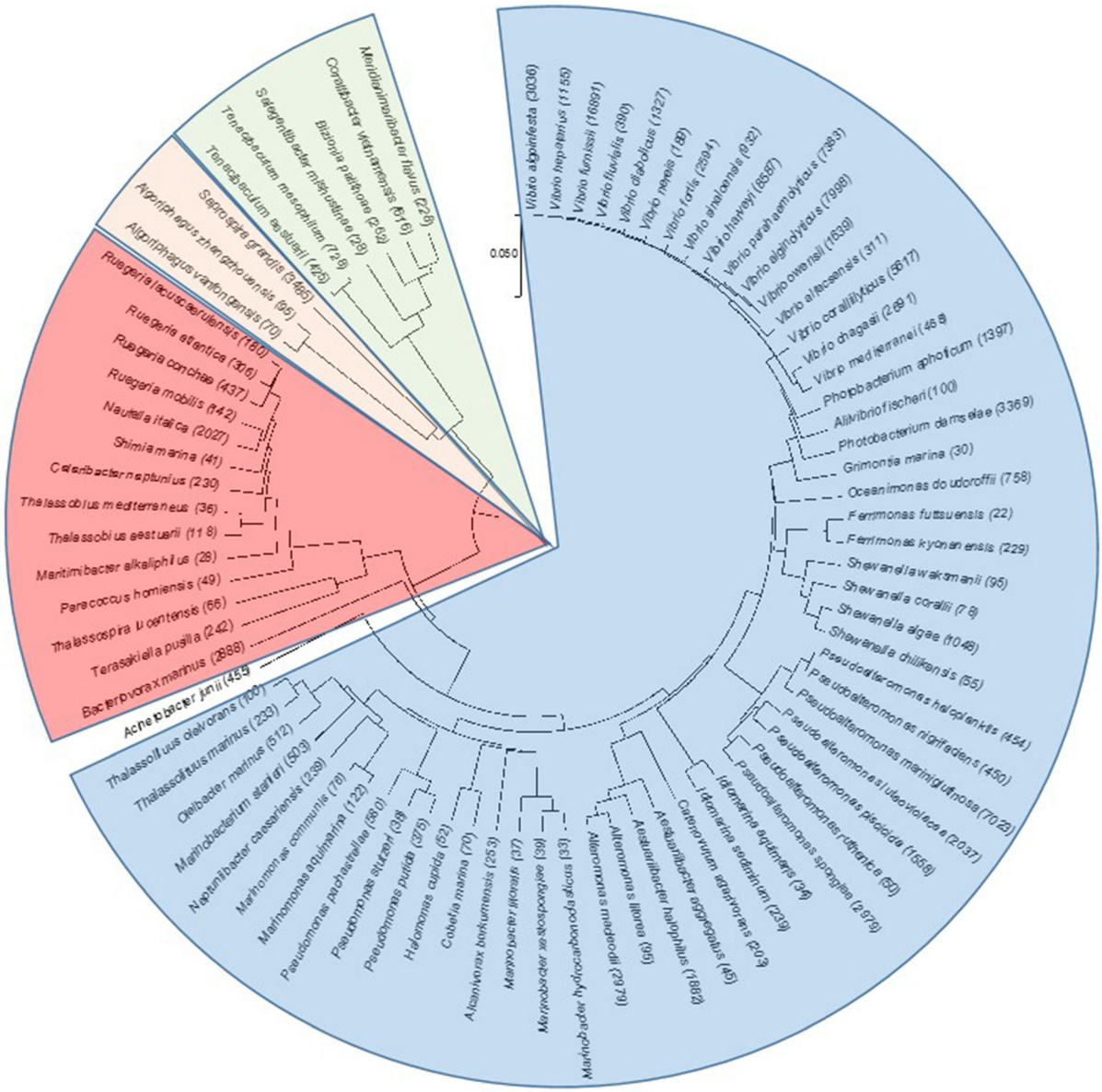

Fig. 8 Phylogenetic tree based on the 16S rRNA gene sequences obtained after 90 days of exposure in seawater. Parenthesis indicate the total number of individual sequences within the OTUs. The bootstraps values were calculated from 1000 re-samplings

biofilm structure grown on the structure. Thus, our experiment was conducted in situ deep-water over a 90-day period in a marine environment. During this period, coupons immersed in seawater were analyzed at predetermined intervals by sequencing the 16S rRNA gene in order to determine the microbial taxonomy grown, evaluating for bacterial succession over time. This approach was made possible by Next-generation sequencing techniques, which allows for an easy and inexpensive analysis of microbial communities present in different environments (Parada et al. 2016). A key factor for the success of this analysis is the choice of primers for the amplification of the 16S genes. Criteria for choosing the most satisfactory primers include depth of sequencing, high coverage of taxa, including Bacteria and Archaea, ability to compare the results obtained and, clear data that permit inferring phylogenetic relationships (Parada

et al. 2016). The 16S rRNA gene contains nine hypervariable regions (V1–V9) commonly used in taxonomic studies (Yang et al. 2016). The choice of the V3–V4 region of the 16S gene was due to its high sensitivity and accuracy. The amplification products of this region were 283 bp, and the following steps of the pipeline allowed confidence in sequenced reads, not considering sequencing errors, chimeras or PCR artifacts in the bacterial taxonomic evaluation.

In analyzing the sequencing results, the predominance of certain groups over the 90 days of exposure to seawater was clear. *Proteobacteria* phyla was widely distributed in all metagenomic analyses of replicate samples. Several other similar studies describe *Proteobacteria* as dominant at the core, based on 16S rRNA gene analyses (Bermont-Bouis et al. 2007; Dang et al. 2011; Garcia and Procópio 2020; Jones et al. 2007; Li et al. 2017b; McBeth and Emerson 2016; Moura et al. 2018; Vigneron et al. 2017). Although most of these studies have been directed to metagenomic analysis on offshore oil production facilities, a relationship between the microbial community structure present in both conditions is possible, especially when we analyze the initial periods in the microbial colonization on the rusting metallic surfaces. One of the possible reasons for this similar ecological behavior is suggested by Li et al. (2017b), who describe the members of the phylum *Proteobacteria* as pioneers in the colonization of surfaces, including metal surfaces. This step is central to the emergence of new bacterial species and the development and stability of the biofilm structure formation (Slightom and Buchan 2009).

Among the representatives of *Proteobacteria,* the predominant class consisted of *Gammaproteobacteria* members. As in other similar studies, this group was present in much higher numbers than others described in all the replicates analyzed, especially members of the *Alteromonadales*, *Oceanospirillales*, and *Vibrionales* families (Dang et al. 2011; McBeth and Emerson 2016; Moura et al. 2018). The scientific literature describes these bacteria as having important roles in the initial formation of the structure of biofilms on surfaces, being detected soon after the metal surface is exposed to the experimental conditions, such as coastal and marine environments (Dang and Lovell 2015; Dang et al. 2008, 2011; Moura et al. 2018; Slightom and Buchan 2009). The genus *Pseudoalteromonas,* described in the literature as potential marine FeOB (Ramírez et al. 2016), was extensively detected in all 16S rRNA sequencing of our samples. In a study evaluating the participation of *P. lipolytica* and *B. subtillis* demonstrated the corrosive action of *P. lipolytica*, especially in the formation of pitting, as a result of the inhibitory action on steel corrosion (Guo et al. 2017). Members of the *Shewanella* genus were detected in all samples collected. These bacteria are widely associated with microbial corrosion is different conditions and metal (Miller et al. 2018; Philips et al. 2018). In our study, the species *Shewanella loihica* was identified after 60 days of exposure to seawater. The halophilic *S. loihica* has been described as facultative anerobic and iron-reducing, having an active action on the corrosion of the metallic surface (Kooli et al. 2018). *Shewanella algae* were only detected in the last (60 days) analysis. Although *S. algae* are not directly related to the corrosion of metal surfaces, they have been described as an iron-reducing bacteria, suggesting their corrosive role in this study (Gandhi et al. 2002). The high number of specimens was also accompanied by a high number of OTUs in all replicates. Three different species of the *Marinobacter* genus were detected in this study, with greater prevalence after 90 days of exposure to seawater. *Marinobacter* species are commonly identified in similar studies and are associated with metal corrosion processes, mainly in offshore oil structures (Cluff et al. 2014; Brauer et al. 2015; Vigneron et al. 2016). The *Ruegeria* genus was present consistently in all three conditions analyzed in this experiment. This genus was also identified in a study on the colonization by bacteria of different metals and rust (Zhang et al. 2019). Some of the representatives detected belonging to the class *Gammaproteobacteria* are described as hydrocarbon-degrading, such as *A. borkumensis* (Konieczna et al. 2018), *Pseudomonas* (Brown et al. 2017; Gomila et al. 2017; Li et al. 2016; Maia et al. 2019; Rajasekar et al. 2010; Xia et al. 2015; Xu et al. 2017), *Acinetobacter venetianus* and *Thalassolituus* (Choi and Cho 2013, Golyshin et al. 2013). The presence of these bacteria can be explained by proximity to the location where the Brazilian naval ships are maintained, and the attendant oil leakage into the water.

The second group with the highest number of Bacteria identified and OTUs in the analysis was *Alphaproteobacteria*. Members of this group are commonly reported in marine biocorrosion studies

(Dang and Lovell 2015; Lenhart et al. 2014; McBeth and Emerson 2016; Vigneron et al. 2016). In a microcosm experiment simulating the marine environment, *Alphaproteobacteria* were also extensively identified, especially *Rhodobacteriales* (Moura et al. 2018). In homology analysis, 90 different specimens were detected in all the replicates analyzed, and were present in the first analysis at 30 days, suggesting a preponderant role in the colonization and formation of the biofilm structure over the coupons exposed to seawater. Similar to *Alphaproteobacteria* described in marine corrosion studies, *Alphaproteobacteria* are suggested as polysaccharide producers used in the formation of biofilms, thus creating a microenvironment between the metallic surfaces and the bacterial community present (Elifantz et al. 2013; Kip and van Veen 2015; Videla and Herrera 2005). Additionally, among the *Alphaproteobacteria*, the bacteria *Shimia marina* was identified, which are reported in the literature as a hydrocarbon-degrading strain, including aromatic compounds (Rodrigo-Torres et al. 2016). Another sequence identified was *Roseobacter denitrificans*, whose genome sequencing indicates the possibility of being related to sulphur oxidation under oxic and anoxic conditions (Lenk et al. 2012). Two different species of *Hyphomonas* were detected in the 60-day experimental period. The *Hyphomonas* genus is not related to the corrosion of metals, although it is found in corrosive biofilms, probably due to its ability to manufacture a polysaccharide capsule that allows it to become adhesive in relation to mineral oxides (Bhosle et al. 1998). A single *Epsilonproteobacteria* were detected in the 16S rRNA gene sequencing of the 316L submerged coupons. The *A. bivalviorum* was identified in the samples at 30 days, but was no longer present at other timepoints. Although the genus *Arcobacter* is commonly detected in environments of greater marine depth, being related to hydrothermal sediments (Alain et al. 2004; An et al. 2016; López-García et al. 2003), the *A. bivalviorum* is related to colonization of seafood (Levican et al. 2012).

Flavobacteria represented a considerable community in analyzed sequences, both in the number of representatives and in OTU sequences. *Flavobacteria* were detected in all samples with special representativity after 60 days. This group has already been described in a previous experiment under controlled laboratory conditions, although in a lower number of representativeness (Moura et al. 2018). However, in several other studies on metallic corrosion in marine environments *Bacteroidetes* phylum are always highly represented (Li et al. 2017a, b). Microbiological succession analysis of mild steel in marine and estuary environments showed high percentages of representative indices, reaching up to 75% of the sequences when analyzed within a period of up to 24 days (McBeth and Emerson 2016). *Bacteroidetes* have an important influence on the other bacteria and the maintenance of the formed structure on surfaces. Predator specimens of *Bacteroidetes* act on the degradation of polymers present in biofilms, thus contributing to the maintenance of aerobic microenvironments within biofilms (Kirchman 2002).

In the analysis of 16S rRNA gene sequences in this study, only one representative of the *B. subtilis* was detected, though the number of OTUs was high. *Firmicutes* are constantly described in corrosion studies of metals in marine environments (Guo et al. 2017; Lenhart et al. 2014; Li et al. 2017a, b; Rajasekar et al. 2010). Normally, these studies demonstrate the role of *Bacillus* genus acting as pioneers in bacterial adhesion on metallic surfaces and thus acting in the initial processes of formation of bacterial biofilms (Karn et al. 2017; Wan et al. 2018). Moreover, the role of species of the *Bacillus* genus in the corrosion of metals is widely described in scientific literature, whether acting on corrosion actively or as a process inhibitor (Guo et al. 2019; Kang et al. 2019; Suma et al. 2019; Wang et al. 2018). Recently, the corrosive action by marine *B. subtilis* was demonstrated in a study with 10MnNiCrCu steel (Wang et al. 2018). Metagenomic survey on the bacterial community involved in the corrosion of petroleum pipelines also detected the presence of species of the genus Bacillus, including *B. subtillis* in the corrosive biofilm (Rajasekar et al. 2010). Finally, the genus *Bacillus* was also identified in a study concerning bacterial community participating in the corrosion of carbon steel under microcosm conditions (Procópio 2020a, b).

Conclusions

Based on the analyses of the sample sequencing, it was possible to describe in detail all the dynamics of the microbiological succession that occurred on the coupons throughout the entire period of the experiment. Initially, pioneer specimens that formed the

bacterial biofilm structure were identified. However, over the course of the experiment, there was a change in the profile of the community present, characterized by microorganisms related to the maintenance of the biofilm and the appearance of specimens related to corrosive processes. Although it is not possible to relate the loss of mass of the coupons with the presence of the growing bacterial community, changing the bacterial profile over time seems to exert an influence on the microenvironment formed, which favors the conditions necessary to initiate or accelerate a corrosive process of the analyzed metal.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The current research work did not involve either of human or animal studies.

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