Electrochemical and molecular characterization of electroactive biofilms on stainless steel in marine environment

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LIST OF ABBREVIATIONS

ANI	Average Nucleotide Identity
ATP	Adenosine Tri-Phosphate
BES	Bioelectrochemical System
CBB	Calvin-Benson-Bassham
COG	Clusters of Orthologous Groups
DNA	Deoxyribonucleic Acid
DOC	Dissolved Oxygen Content
EET	Extracellular Electron Transfer
EPS	Extracellular Polymeric Substances
HMDS	Hexamethyldisilazane
ISO	International Organization for Standardization
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAG	Metagenome-Assembled Genome
MFC	Microbial Fuel Cell
MOB	Manganese Oxidizing Bacteria
$NADH/NAD^+$	Nicotinamide Adenine Dinucleotide
NGS	Next Generation Sequencing
NMDS	Non-metric Multidimensional Scaling
OCP	Open Circuit Potentiel

OHCB	Obligate Hydrocarbonoclastic Bacteria
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
Pfam	Protein Families
ppb	Parts-per billion
ppm	Parts-per million
PREN	Pitting Resistance Equivalent Number
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SCE	Standard Calomel Electrode
SCG	Single-copy Core Gene
SEM	Scanning Electron Microscopy
SHE	Standard Hydrogen Electrode
SS	Stainless Steel
TBS	Tris Buffered Saline
WGS	Whole Genome Sequencing

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INTRODUCTION

In this chapter, the aim is to introduce notions and the literature required to understand the work perform during this thesis. Consequently, some notion in electrochemistry and microbiology will be presented, reflecting the multidisciplinary approach. This introduction is composed of three main parts: ennoblement of stainless steel, electroactive bacteria and the new approaches in microbial eco-genomic.

1. ENNOBLEMENT

The main keyword of this thesis is 'ennoblement' and the simplest definition is: an increase of the open circuit potential of stainless steel in seawater. From this definition, I will elaborate on the nature of stainless steel, the electrochemical potential, the risk and reasons of this potential increase.

1.1. STAINLESS STEEL

Stainless steels are mainly used for their corrosion resistance property and are widely deployed in corrosive environments and commercially easily available. Example of applications are, but no limited to, pipelines, offshore structures, desalination plants, nuclear plants cooled down with seawater, bridges (Falkland et al., 2011).

1.1.1. COMPOSITION

The corrosion resistance of stainless steel is due to the addition of chromium in the alloy. A minimum composition of 10.5% (mass fraction) of chromium is required according to the International Standard ISO15510:2014.

Other elements such as molybdenum, nickel, carbon, nitrogen, manganese, tungsten can be added to the alloy to modify the material properties. Four different type of stainless steels can be described based on their microstructures: austenitic, ferritic, martensitic and austenitic-ferritic (Duplex).

Ferritic stainless steel are standard chromium alloys with no or little addition of nickel while austenitic stainless steels are obtained by addition of nickel, manganese and nitrogen. The former is magnetic and

the latter is non-magnetic (Streicher and Grubb, 2011). Martensitic are iron-chromium alloy that can be hardened and have good mechanical properties. They have a high carbon content and nitrogen can be added to improve their strengths (Grubb, 2011). Duplex stainless steel have a mixed ferritic and austenitic microstructure with combined benefits of high strength and high corrosion resistance (Falkland et al., 2011).

Stainless steels can be classified by their pitting resistance equivalent number (PREN) for which a higher value correspond to a better corrosion resistance. It is calculated with chromium, molybdenum, tungsten and nitrogen mass fraction. In this study, we were mostly interested in the stainless steel electrochemical potential, independently of the corrosion phenomenon. Hence, only super-duplex (high chromium content - PREN > 40) stainless steel has been used and its nominal composition is described in Table 1.

Table 1. Chemical composition (wt%) and pitting resistance equivalent number (PREN) of the tested stainless steel.

	UNS	Cr	Ni	Mo	Fe	Cu	N	PREN a)
Super duplex	S32750	25.1	7.0	3.8	Bal.	0.13	0.29	42.3

a) PREN = %Cr + 3,3%(Mo + 0.5%W) + 16%N

1.1.2. PASSIVATION

Some alloys are called passive alloy because of their ability to form a passive layer on the surface of the metal. This layer is made of oxide that remain stable and therefore prevent further corrosion to take place (Kruger, 2011). In the case of stainless steel, the presence of chromium is the most important element that will form the passive film, along with iron. Both iron and chromium oxide/hydroxide will form a passive layer of about a few nanometers. Despite the robustness and stability over time, there is a slow but continuous renewal of that passive layer. The very slow oxidation defines the passivation current that is several orders of magnitude lower than the oxidation current for pure iron (Kruger, 2011).

1.2. REDOX REACTIONS

Redox reactions are a central element of all the work presented in this thesis. It is therefore critical to introduce a general redox definition: an electron transfer from a reducer (electron donor) to an oxidant (electron acceptor). Redox reactions are defined by two half reactions: an oxidation for the loss of electron by the reducer, and a reduction for the gain of electron by the oxidant. In an electrochemical system, electrodes are named according to the redox reactions: the anode is where the oxidation happens and the cathode is the place of a reduction.

The redox reaction of an oxidant O with reducer R is given in half-reactions (A)(B) and complete reaction (C).

$$\text{(A) } R \rightleftharpoons R^{n+} + ne^{-}$$

$$\text{(B) } O + ne^{-} \rightleftharpoons O^{n-}$$

$$\text{(C) } O + \mathrm{R} \rightleftharpoons O^{n-} + R^{n+}$$

These reactions define a redox potential, which is the tendency of an element to accept (or donate) electron to get reduced (or oxidized). The standard redox potential for a half-reaction (A) and (B) is the

voltage—difference of potential—at equilibrium in standard condition with the oxidation of hydrogen (D), which is 0.0 V by convention.

$$\text{(D)}\ \frac{1}{2}H_2 \rightleftharpoons\ H^+ + e^-$$

The Nernst equation is used to get the redox potential of each half reaction with the standard redox potential, temperature and concentration of the reactants.

As for the total reaction :

$$\text{(E)}\ E^0_{total} \rightleftharpoons\ E^0_R - E^0_O$$

$$\text{(F)}\ E_{total} \rightleftharpoons E^0_{total} - \frac{RT}{nF} ln\ \frac{[R][O^{n+}]}{[R^{n+}][O]}$$

In many well oxygenated environments, the main reduction reaction is the oxygen reduction reaction as it is a strong oxidant that can undergo a four electron reaction to form water (G), or a two electron reduction to hydroxide peroxide (H), which can then be reduced to water with a second two electrons reaction (I).

$$\text{(G)}\ O_2 +\ 2H_2O + 4e^- \rightleftharpoons 4OH^- \qquad E^0 = +0.401\ V$$

$$\text{(H)}\ O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O_2 \qquad E^0 = +0.670\ V$$

$$\text{(I)}\ \ H_2O_2 + 2H^+ + 2e^- \rightleftharpoons 2H_2O \qquad E^0 = +1.77\ V$$

Another way to visualize redox reactions and redox potentials is with an Evan's diagram where the oxidation (anodic) and reduction (cathodic) reactions are plotted in a current vs. potential plot Figure 1. Since there is no accumulation of charge, the number of electrons generated by the oxidation must be consumed by the reduction reaction. Hence, the measured potential is at the crossing point of the anodic and cathodic curves.

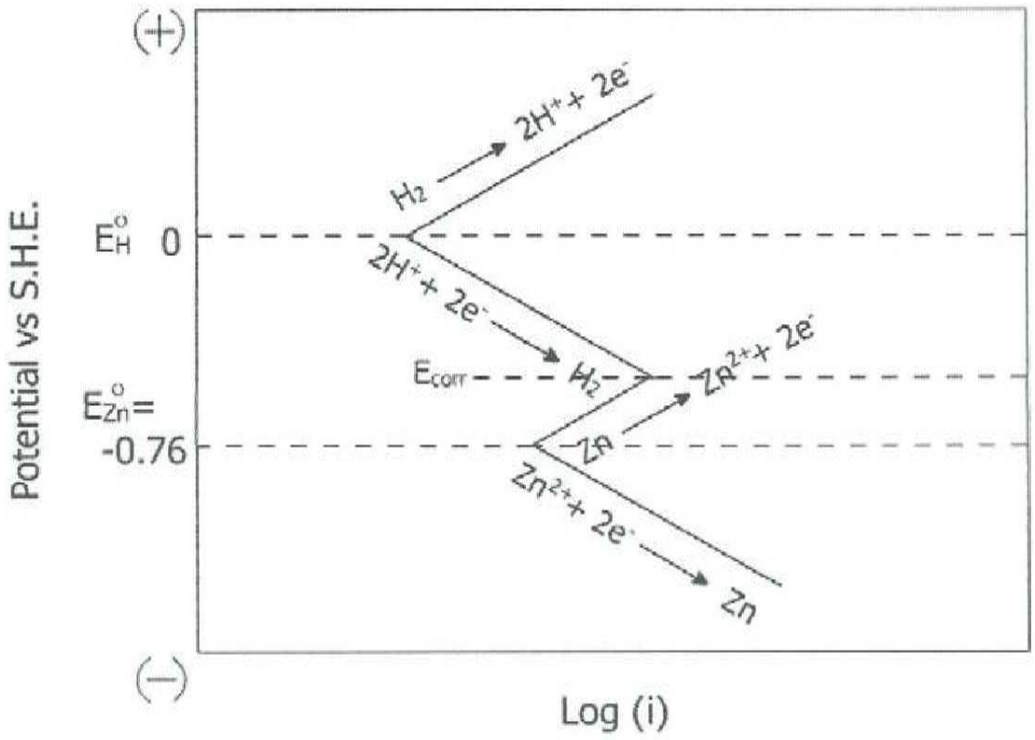

Figure 1. Example of an Evan's diagram for the oxidation of Zn and the reduction of H+. E_{corr} is the total redox potential. Adapted from (Ahmad, 2006).

1.3. INCREASE OF POTENTIAL

For practical reasons, we generally use Ag/AgCl reference electrodes to measure the potential of a material. These electrodes are regularly calibrated with Saturated Calomel Electrodes (SCE), itself around +244 mV vs. standard hydrogen electrode (SHE). When stainless steels are immersed in a solution like seawater, it is thus possible to measure the voltage difference between the stainless steel and saturated calomel electrode (SCE). If no current or potential are externally applied to the stainless steel, the measured potential is called the open-circuit potential, referred as OCP hereafter.

The OCP is defined by the chemical reactions at the interface of the stainless steel with its surrounding environment. The keys factors that will determine that potential are the concentration of the reactants, the half-cell reaction potential (E'^0) and the kinetics of each half cell reaction.

An increase of the OCP can be observed on stainless steel exposed in natural seawater and river water, *i.e.* naturally containing microorganisms (Figure 2). That spontaneous increase in OCP is called ennoblement and was first described by Mollica and Trevis, 1976. It is usually in the range of + 300 / + 500 mV. This phenomenon has been observed worldwide (Scotto and Lai, 1998), in seawater and freshwater systems. The ennoblement was observed on all types of stainless steel (Motoda et al., 1990), and also on glassy carbon electrode (Sridharan et al., 2011).

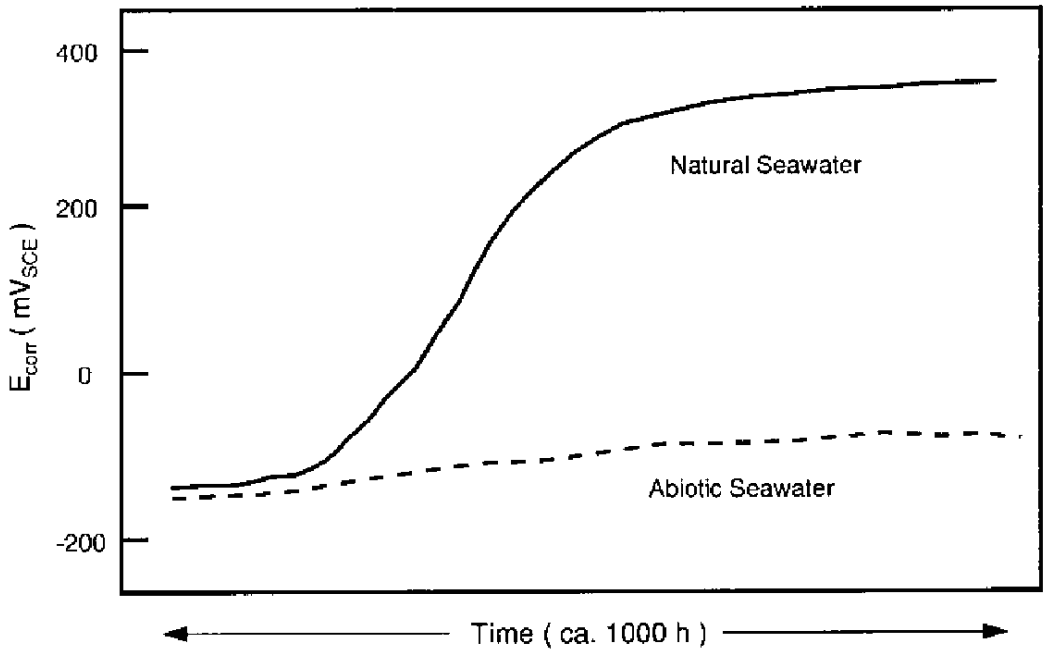

Figure 2. Schematic shift of potential with exposure time in natural and sterile sea water (Dickinson and Lewandowski, 1998).

The ennoblement is associated with the presence of microorganisms that colonize the surface of stainless steel. The removal of the surface attached microorganisms triggers a loss of ennoblement (Motoda et al., 1990), and the exponential increase of bacterial colonization on stainless steel has been correlated with the increase of potential, which suggests that the number of bacteria is linked to the potential. To further relate the presence of microorganisms and potential increase, Gümpel et al., 2006, have added ethanol during the bacterial colonization and the potential decreased for a short time as the ethanol has a reversible negative effect on bacterial growth. The addition of potassium cyanide had the same effect but for a longer period of time. Since the cyanide is toxic and prevents cell respiration, it means that microorganisms have to be metabolically active.

1.4. RISKS

The presence of the passive film on stainless steel prevents localized corrosion. However, there is a critical potential above which localized corrosion can occur for stainless steel. That potential is called the pitting potential (Marcus, 2011) (Figure 3). This critical potential is depending on the composition of stainless steel and in general, the higher the PREN value is, the higher this critical potential is.

The ennoblement, or the increase of potential, triggered by the microorganisms increases the likelihood to reach a potential higher than the critical pitting corrosion for a given alloy, especially for lower alloys stainless steel (less chromium content) (Geiser et al., 2002; Hakkarainen, 2003; Liao et al., 2010; Shi et al., 2003).

As stainless steel applications rely on the corrosion resistance, the ennoblement is a real concern. As a consequence, higher grades and more costly stainless steels like super-duplex materials with high PREN have to be used to prevent the occurrence of localized corrosion.

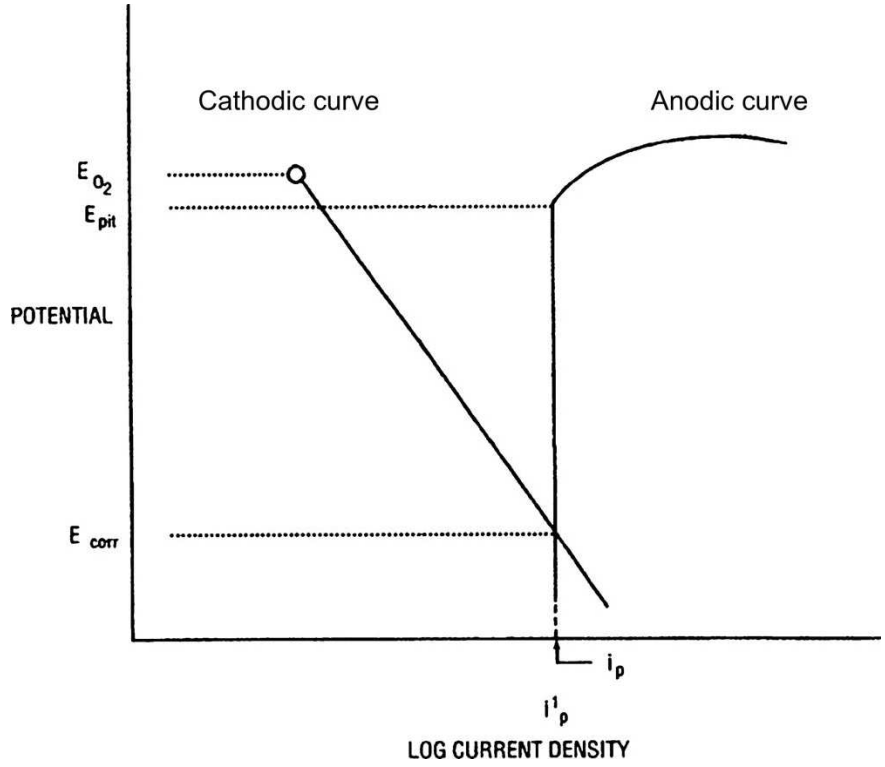

Figure 3. Schematic polarization curve for stainless steel. A polarization curve represents the evolution of the current (in log scale) with the potential, *i.e.* it describes the current obtained when increasing or decreasing the potential. This figure is a schematic representation of the anodic curve (the oxidation reaction) of stainless steel, and the cathodic curve (reduction reaction) of oxygen. E_{pit} is the pitting corrosion, above which a small increase of potential strongly increase the current. The measurable potential (E_{corr}) is the potential where both curves cross, *i.e.* at the same current density (i_p) since all electron produced by the oxidation are used in the reduction reaction. Adapted from (Little et al., 2008).

1.5. CAUSES

Despite the description of ennoblement more than 40 years ago, the microbial mechanism(s) that can explain how microorganisms can increase the potential of stainless steel immersed in seawater is still debated. Nevertheless, several hypotheses have been proposed in the literature, both theoretical and empirical, but none could explain the ennoblement for all reported conditions.

The theoretical ways to increase the OCP have been summarized (Little et al., 2008, 2012) and consists of three possibilities: thermodynamics, kinetics and alteration of the reduction reaction.

1.5.1. THERMODYNAMIC

Given the Nernst equation, there are multiple ways to change the potential and a first parameter is the concentration of the reactants involved in the redox reaction. As we posit that the cathodic reaction is the oxygen reduction, the two critical elements are the pH as protons are involved in the reactions and oxygen partial pressure.

A decrease of the pH at the stainless steel surface can change the redox potential of the cathodic half reaction (Figure 4). Theoretically, a change of pH from 8 to 3 would account for a +300mV increase (Little et al., 2008). However, Dexter and Chandrasekaran, 2000, showed that pH measured on 24 month old biofilms was highly variable, ranging from 6 to 2. Hence, the pH contribution to the ennoblement is possibly relevant but cannot be entirely responsible of the observed ennoblement. It also requires a fully developed biofilm covering the metal in order to counter proton diffusion and maintain a pH gradient with the surrounding medium.

An increased in the partial pressure of oxygen can also increase the OCP (**Figure 4. Schematic polarization curve for stainless steel.** Change of either pH or O2 partial pressure would change the reversible potential $E_{O_2}^{0.1}$ to $E_{O_2}^{0.2}$. As a result, the measured potential (also referred as Ecorr) would raise from E_{corr}^{1} to E_{corr}^{2}. Adapted from (Little et al., 2008).Figure 4). Phototrophic microorganisms can

increase this partial pressure of oxygen with photosynthesis and therefore can play a role in ennoblement (Landoulsi et al., 2011). But the ennoblement is also observed in dark conditions.

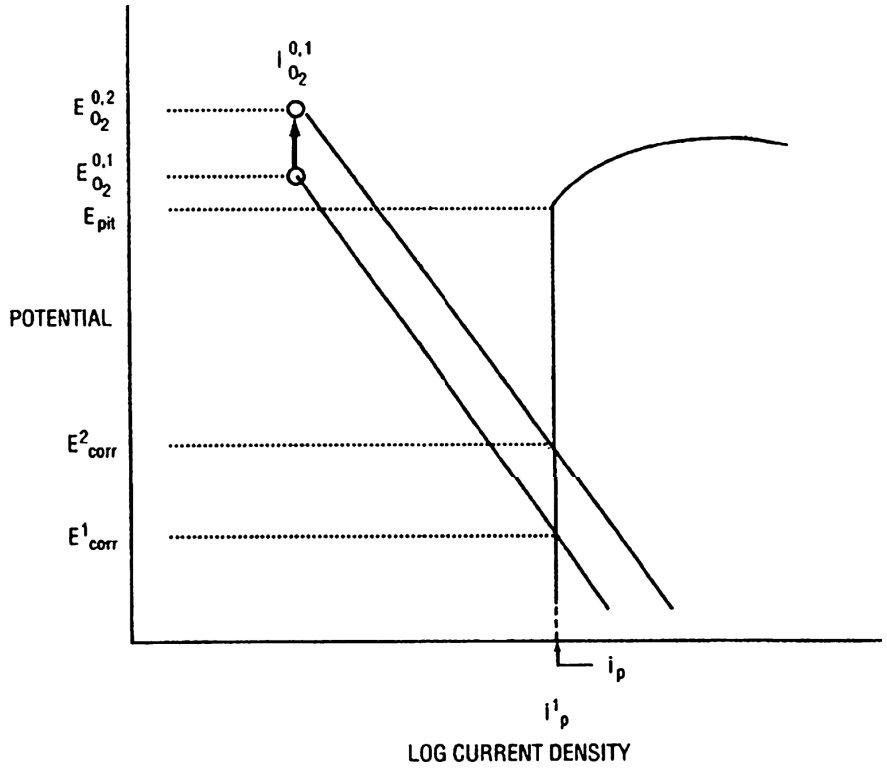

Figure 4. Schematic polarization curve for stainless steel. Change of either pH or O_2 partial pressure would change the reversible potential $E_{O_2}^{0.1}$ to $E_{O_2}^{0.2}$. As a result, the measured potential (also referred as E_{corr}) would raise from E_{corr}^1 to E_{corr}^2. Adapted from (Little et al., 2008).

1.5.2. KINETICS

The potential can be modulated by the concentration of the reactant involved in the redox reaction, and therefore also by the kinetics of the associated reaction. As two half reactions are involved here, the reduction at the cathode and the oxidation at the anode, either an increase of the cathodic reduction or a decrease of the anodic oxidation can account for an overall increase in potential.

1.5.2.1. CATHODIC DEPOLARIZATION

An increased rate of oxygen reduction can theoretically increase the OCP as shown in the Figure 5.

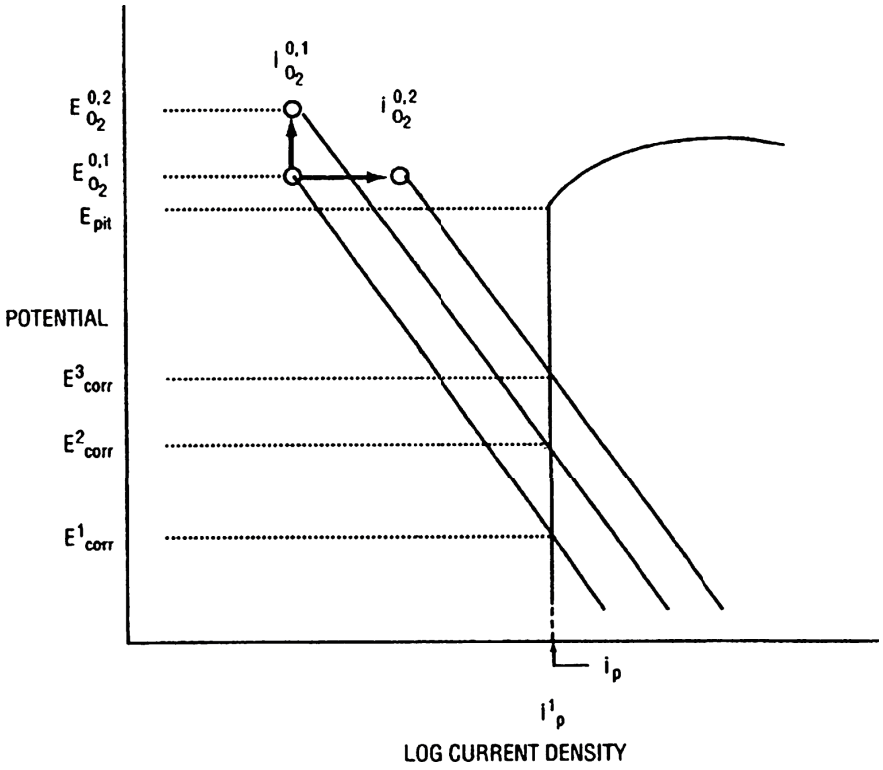

Figure 5. Schematic polarization curve for stainless steel. An increase rate of oxygen reduction would change $i_{O_2}^{0.1}$ to $i_{O_2}^{0.2}$. As a result, the measured potential (also referred as E_{corr}) would raise from E_{corr}^{1} to E_{corr}^{3}. Adapted from (Little et al., 2008).

The cathodic depolarization, or the enhanced oxygen reduction reaction is thought to be conducted by the microorganisms that quickly settled and developed on the surface of a stainless steel in seawater. Their presence and activity has been investigated with the use of bacterial inhibitor such as sodium azide and glutaraldehyde by Scotto et al., 1985, 1986; Scotto and Lai, 1998).

Sodium azide is a general enzyme inhibitor that is reversible and does not cause cell death. Therefore, all enzymatic activities of the cells are reversibly turned off including oxygen reduction. Sodium azide was added into the medium after several days of incubations on stainless steel showing potential ennoblement in seawater. This caused a sharp decrease of the OCP. Removal of sodium azide led to the recovery of ennoblement. This suggests that the ennoblement could be caused by an enzymatic activity of the microorganisms that developed on the stainless steel.

Glutaraldehyde is usually used to fix cells, and therefore kills microorganisms. But the addition of glutaraldehyde did not induce a decrease in OCP, which remained at a high value. Addition of sodium azide irreversibly decreased the OCP. The authors concluded that only exoenzyme (enzyme outside the cells) could remain functional after the addition of glutaraldehyde as the cells were dead. These exoenzymes would then be inhibited by the sodium azide, irreversibly because the microorganisms were unable to regenerate these exoenzymes.

An enzymatic explanation of the cathodic depolarization is really interesting but since all bacterial activities were inhibited in this experiment, it is hard to conclude on the exact mechanisms through which the bacteria can increase the OCP. Nevertheless, it shows that an enzymatic activity is continuously required to keep the OCP at high potential. In contradiction, (Dupont et al., 1998; Marconnet et al., 2008) have shown that the addition of catalase enzyme decreased the ennoblement with the removal of hydrogen peroxide. Hence if that enzyme was affected by sodium azide, the ennoblement should raise or at least remain the same.

While the biofilm can affect the oxygen reduction reaction, it is also interesting to better understand the changes in the stainless steel passive film composition. It has been investigated according to the oxygen reduction by Le Bozec et al., 2001 and the highest current density generated by oxygen reduction was partly connected with the Fe^{2+} content of the passive film.

1.5.2.2. ANODIC DEPOLARIZATION

Theoretically, it is also possible that a decrease in the passivation current density could increase the OCP as shown in Figure 6.

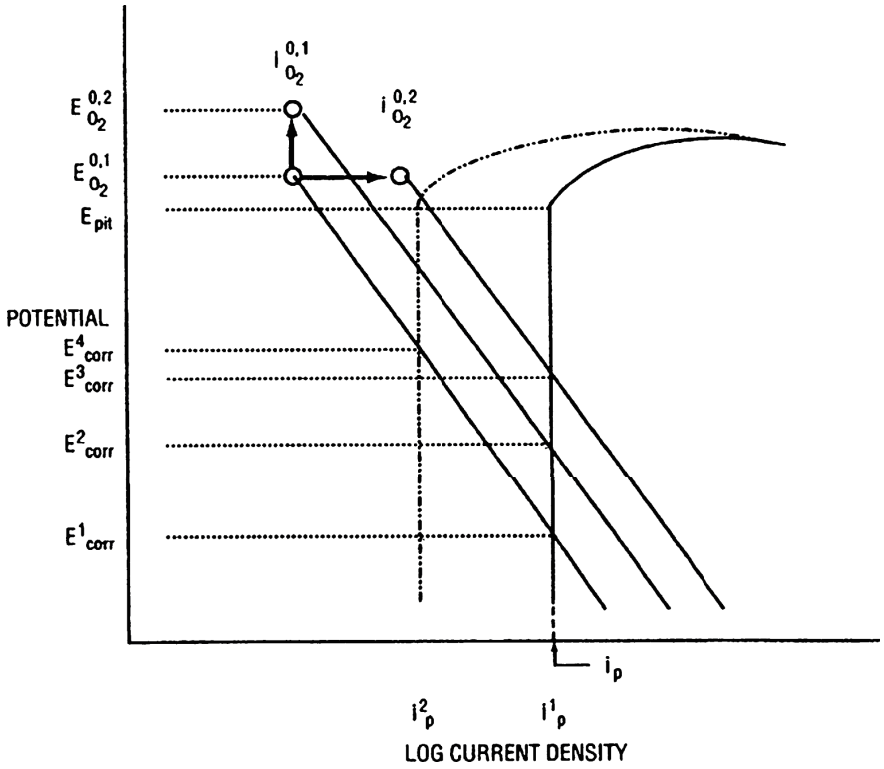

Figure 6. Schematic polarization curve for stainless steel. A decrease of passivation current density would change i_p^1 to i_p^2. As a result, the measured potential (also referred as E_{corr}) would raise from E_{corr}^1 to E_{corr}^4. Adapted from (Little et al., 2008).

Maruthamuthu et al., 1996, proposed a model based on the decrease of the passivation current by an increase in the passive film thickness. They argue that the production of anions by the microbial metabolism was responsible for the decrease in electron donors present in the passive film by neutralization of excess cations. The increasing film thickness by anions continuously produced by the biofilm is a process that can strengthen the oxide film and make it more stable. That stability may explain why some stainless steel can resist pitting corrosion while the ennoblement has shifted the potential

value above their critical pitting corrosion potential. They discussed the role of siderophores, which are iron chelators produced under neutral pH condition. Siderophores act as corrosion inhibitor, while strengthening passive film on passive alloys (Eashwar and Maruthamuthu, 1995).

These authors also proposed a neutral pH model of the titanium ennoblement (Eashwar et al., 1995b; Eashwar and Maruthamuthu, 1995), where bacteria are responsible of an acidification of the biofilm under dark conditions, while diatoms counter the acid effect under light expositions. The neutral pH is not responsible for the ennoblement, but rather a decrease in the passive current density as observed by the authors. Their conclusion is that the biofilm enhances passivity, increasing the titanium potential. The enhance passivity may explain why the ennobled potential, which is often above the critical pitting potential, remains stable without causing any pitting corrosion. The same authors demonstrated that sunlight radiation inhibited the ennoblement (Eashwar et al., 1995a).

Other experiments carried out by Xiangbo et al., 2007, have described an anodic depolarization as the main reason for the ennoblement. They observed a decreased passive current density and an increased pitting potential once the potential had reached its highest value.

1.5.3. ALTERATION OF THE REDUCTION REACTION

The concentration of the redox reactants as well as the kinetic of the two half-rea=ction can impact the potential. But there is a third way to theoretically increase the OCP, and that is to involve other elements in the reduction reaction.

The addition of another oxidant with a high standard potential would increase the observed OCP. The two elements generally considered are hydrogen peroxide and manganese oxide as they can become present in the environment.

1.5.3.1. HYDROGEN PEROXIDE

The oxygen reduction by aerobic bacteria involves the formation of reactive oxygen species as the four electrons reduction of oxygen can be decomposed into two steps. One involves the formation of

hydrogen peroxide by the superoxide dismutase enzyme, which is then degraded by catalase or peroxidase (Madigan et al., 2014).

Amaya and Miyuki, 1994, proposed the presence of substances of higher redox potential in mature biofilms. Le Bozec, 2000, have measured hydrogen peroxide content in the range of 1 mM in a biofilm after 20 days in seawater and Marconnet et al., 2008, have measured 0.29 mM of hydrogen peroxide in naturally grown biofilm in freshwater with ennoblement. Washizu et al., 2004, showed a seasonal change of OCP with higher value during summer (+ 0.350 V vs SHE) compared to winter (+ 0.050 V vs SHE). Hydrogen peroxide fluctuates between higher value in summer and lower in winter. The addition of catalase enzymes, which decompose hydrogen peroxide to water and oxygen, decreased the OCP by 0.300 V (Dupont et al., 1998; Washizu et al., 2004).

Landoulsi et al., 2008b, 2008a, 2009, investigated the enzymatic activity responsible of the ennoblement. They chose to mimic the oxygen consumption and hydrogen peroxide production with the use glucose oxidase which oxidize glucose to gluconolactone and reduce oxygen to hydrogen peroxide. They exposed stainless steel plates directly to hydrogen peroxide and to glucose and glucose oxidase in aerated conditions. Hydrogen peroxide concentrations were around 2 mM, which is approximately 7 times more that natural values (Marconnet et al., 2008). The OCP increased quickly with hydrogen peroxide and a similar increase was measured in presence of glucose oxidase with a slight delay due to the enzymatic activity in the production of hydrogen peroxide (Figure 7). They analyzed the ratio of iron and chromium oxide of the passive layer and conclude that hydrogen peroxide does not have an influence on the chemical nature of the passive film, but rather directly influence the OCP, either added or produced with glucose oxidase.

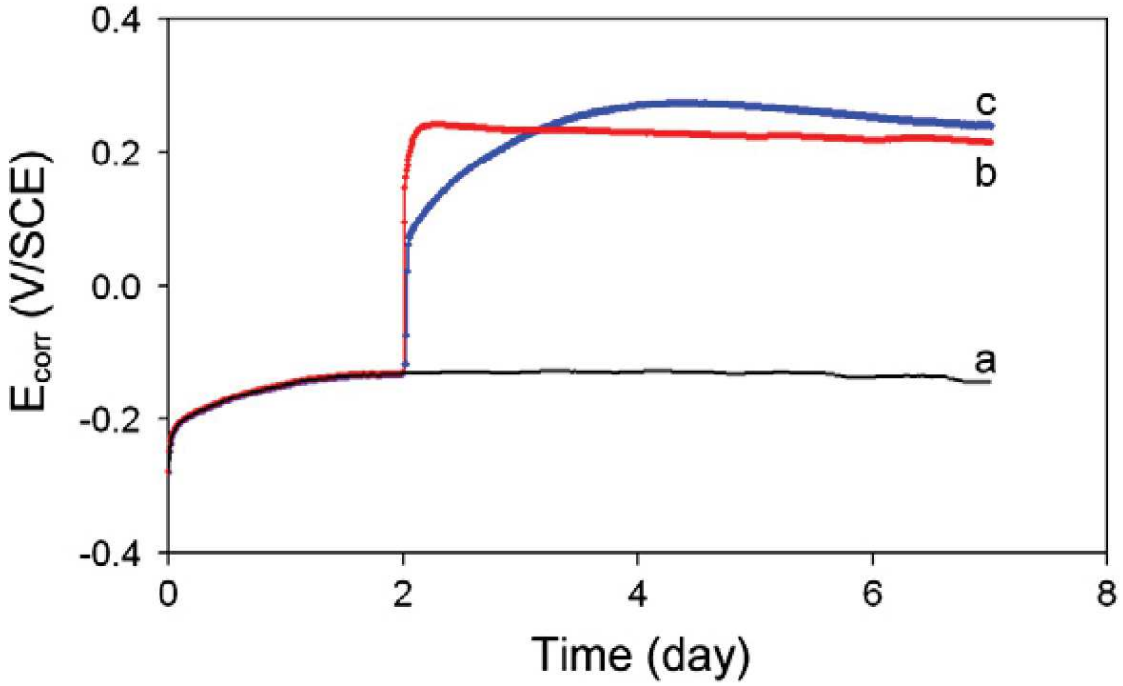

Figure 7. Evolution of stainless steel potential as function of immersion time. (a) in synthetic fresh water, (b) after addition of hydrogen peroxide and (c) after introduction of glucose oxidase and glucose (Landoulsi et al., 2008b).

Zhang et al., 2009, have developed a device called wire beam electrode for potential and current measurements on a surface. It highlights potential/current patterns and distribution in an artificial biofilm and stainless steel interface. They later used this system to study the effect of glucose oxidase and the production of hydrogen peroxide (Wang et al., 2009b). The potential was not evenly distributed as the glucose oxidase capsules. Cathodic current zones were surrounded by anodic zones. Such a tool does not use a uniform stainless steel surface but many small stainless steel wires entrapped in insulating epoxy resin. Wang et al., 2009a, have shown that the addition of hydrogen peroxide leads to the ennoblement as well as an increased risk of crevice corrosion.

The presence of hydrogen peroxide may be enough to trigger an increase of OCP, but its importance in real conditions is not yet defined as experiments were carried out in laboratory conditions and with high concentration of hydrogen peroxide. This is all the more relevant in the presence of oxygen respiring

organisms, since hydrogen peroxide is generally decomposed by catalase and peroxidase to prevent damages due to its biological toxicity.

1.5.3.2. MANGANESE OXIDE

In manganese rich environments like freshwater rivers, manganese oxides (MnO_2) can be reduced to manganese II (Mn^{+2}) and contribute to the stainless steel ennoblement (Dickinson et al., 1996). Manganese oxidizing bacteria (MOB) are able to oxidize soluble Mn^{2+} to insoluble MnO_2 coupled with the reduction of oxygen. These bacteria would be able to influence ennoblement by recycling the insoluble MnO_2 which is then reduced in the cathodic reaction happening on the surface of the stainless steel (Linhardt, 2006; Olesen et al., 2000).

The contribution of MOB has been shown in freshwater systems, or estuarine area rich in manganese (Braughton et al., 2001; Dickinson and Lewandowski, 1998; Linhardt, 2004, 2006; Marconnet et al., 2008).

To understand the role of the manganese oxide in the ennoblement, Dickinson et al., 1996, have used sulfite to remove manganese oxide from the surface of an ennoblement coupon with a biofilm. As a result, the OCP dropped to low values close to the control coupons without biofilms. They also observed that a manganese oxide coating was able to increase the potential in a similar way than a biofilm. Gümpel et al., 2006, have shown that the addition of a high concentration of manganese in the freshwater inoculum led to a quicker OCP increase but failed to reach natural ennoblement values. The authors argued that excess amount of manganese oxide could have reduced the surface availability of stainless steel for the bacteria.

The genus *Leptothrix discophora*, which is a known MOB, was used in pure culture conditions to assess the effect on the OCP. It was able to trigger ennoblement on stainless steel (Shi et al., 2002, 2003) and glassy carbon (Nguyen et al., 2007). *Leptothrix discophora* in pure culture was responsible for pitting corrosion of stainless steel once the OCP was increased and with addition of sodium chloride (Geiser et al., 2002).

1.6. TEMPERATURE EFFECT

Several works have reported that the ennoblement was inhibited at temperature above 39°C for heated seawater in Brest (Thierry et al., 2015) (Figure 8). Scotto et al., 1986, have shown that the OCP did not increased at 40°C in heated Mediterranean seawater (Italy). Gümpel et al., 2006, have shown that the ennoblement was no longer observed above 40°C in the freshwater of the river Rhine. Both teams assumed that a loss of bacterial activity was responsible for the absence of ennoblement. Dupont et al., 1997, observed the same critical temperature effect in seawater with identical amount of extracellular polymeric substance but different bacteria at different temperatures. They conclude that different bacterial metabolisms could lead to either ennoblement or non-ennoblement of stainless steel and explain the critical temperature effect.

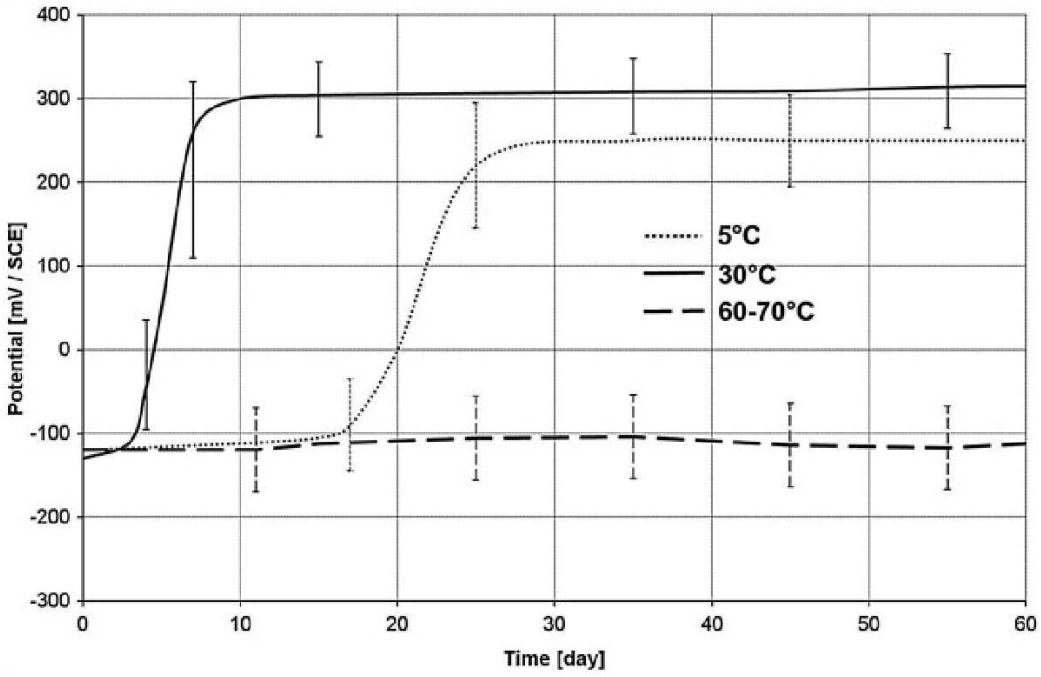

Figure 8. Influence of the seawater temperature on the open circuit potential of stainless steel (Thierry et al., 2015).

In the tropical climate of the Key West Island, Florida, Martin et al., 2003, have also observed a lack of OCP increase above 40°C. But in lower average temperature seas like the Baltic Sea (Mattila et al., 2000) and the North Sea (Bardal et al., 1993), the critical temperature was around 32°C. Hence, both the critical temperature and the average seawater temperature were lower for these two geographical locations.

1.7. OXYGEN EFFECT

An essential environmental factor is the presence of oxygen. Indeed, under anaerobic conditions, the shift of potential is no longer observed (Dupont et al., 1997; Moos and Gümpel, 2008; Thierry et al., 2015).

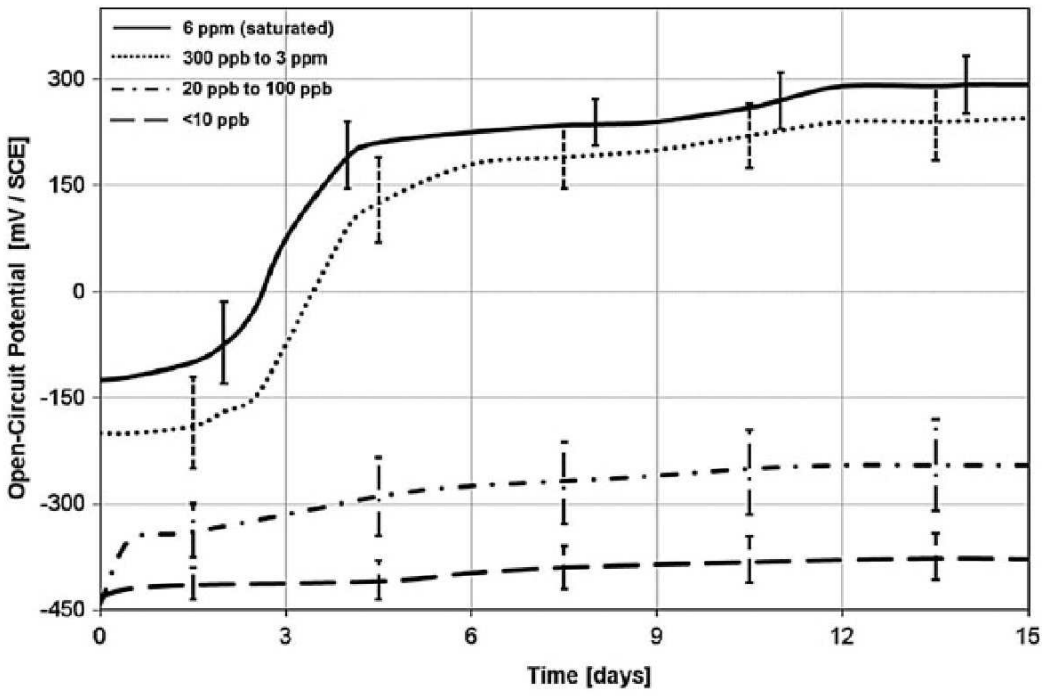

Figure 9. Oxygen influence on the open circuit potential of stainless steel in seawater (Thierry et al., 2015).

Oxygen removal induce a decrease in potential which is quickly reversible (Moos and Gümpel, 2008). Thierry et al., 2015, have shown that intermediate oxygen (300 ppb to 6 ppm) content did not change the kinetic of the OCP shift. The absence of ennoblement was only observed under 100 ppb of oxygen (Figure 9).

The need for oxygen to observe OCP ennoblement is an additional contribution in favor of the cathodic depolarization hypothesis.

2. ELECTROACTIVE BACTERIA

One of the possible reasons the OCP can be increased by the microorganisms is by an enhanced oxygen reduction happening on the surface of the stainless steel. Enhanced stainless steel surface oxygen reduction by microorganisms is a possible reason for OCP increase. Interestingly, electromicrobiology or the study of microbial electron exchange with conductive surfaces (Lovley, 2012) is a field in microbiology seeking to understand how bacteria reduce oxygen on the surface of an electrode.

2.1. ELECTROMICROBIOLOGY

Microorganisms rely on the oxidation of an electron donor coupled with the reduction of electron acceptor to conserve energy for growth. Most of them use soluble electron donors and acceptors but in some environments where there is a lack of either soluble electron donors or acceptors, microorganisms were found to be able to use insoluble/solid-state compounds. These bacteria require extracellular electron transfer mechanisms to transport electron to an electron acceptor or from an electron donor. The following chapter will discuss the bacteria involved in this process, their electron transfers mechanisms, their application and relations with stainless steel ennoblement.

Electroactive bacteria are used and studied in bioelectrochemical system (BES) which consists of two compartments with two electrically connected electrodes. The anode is the electrode that receives the electron via the oxidation reaction in one part of the cell, and the cathode delivers electron for a reduction reaction in the other part of the cell. Microbial fuel cell (MFC) are a prime example of BES in which microorganisms are used to harvest an electrical current. Bacteria able to reduce insoluble electron acceptor can also use the anode. These bacteria that provide electron to the anode have been called Anode-reducing bacteria (Logan and Regan, 2006), Anode-respiring bacteria (Torres et al., 2007), Anodophile (Park and Zeikus, 2003), Electricigen (Lovley, 2006) or Electrogen (Oh and Logan, 2007).

On the other side of the MFC, bacteria able to use the cathode as an electron donor were called Cathode oxidizing bacteria (Martin et al., 2011), Cathodophile (Rinaldi et al., 2008), or Electrotroph (Lovley, 2011).

To maintain consistency, the terms electrogen and electrotroph will be used in this thesis to describe these two extracellular electron transfer metabolisms.

Bacteria have developed means to achieved extracellular electron transfers which can be divided into three categories: direct electron transfer, indirect electron transfer and electron transfer between microorganisms. In the first condition, they use membrane bound complexes that are reduced or oxidized on direct contact with the extracellular electron donor or acceptor.

The second category, indirect electron transfer or mediated electron transfer, required the bacteria to secrete extracellular molecules that will act as electron shuttles between the extracellular electron donor/acceptor prior to reabsorption by the bacteria.

The third mechanism is electron transfer between microorganisms which is involved in syntrophic relationships when more than one species are required to gain energy from a reaction. For instance, hydrogen or formate can be used as electron shuttles from fermenting bacteria to methane producing archaea (Stams and Plugge, 2009). Cells to cell electron transfer has also been proposed for 'cable bacteria' that can couple sulfide oxidation in marine sediments with oxygen reduction with electron transfer over centimeter scale in marine sediment (Pfeffer et al., 2012).

2.2. ELECTROGENIC BACTERIA

There is a knowledge gap between electrogenic bacteria and electrotrophic bacteria, the first being extensively studied compared to the second one. In the present work, we are more interested in electrotrophic bacteria since our system involves cathodes, but a short review of the work done on electrogenic bacteria is required as the extracellular electron transfer mechanisms were first described on electrogenic bacteria, and also because similar processes could be reversible and involved in electrotrophic bacteria.

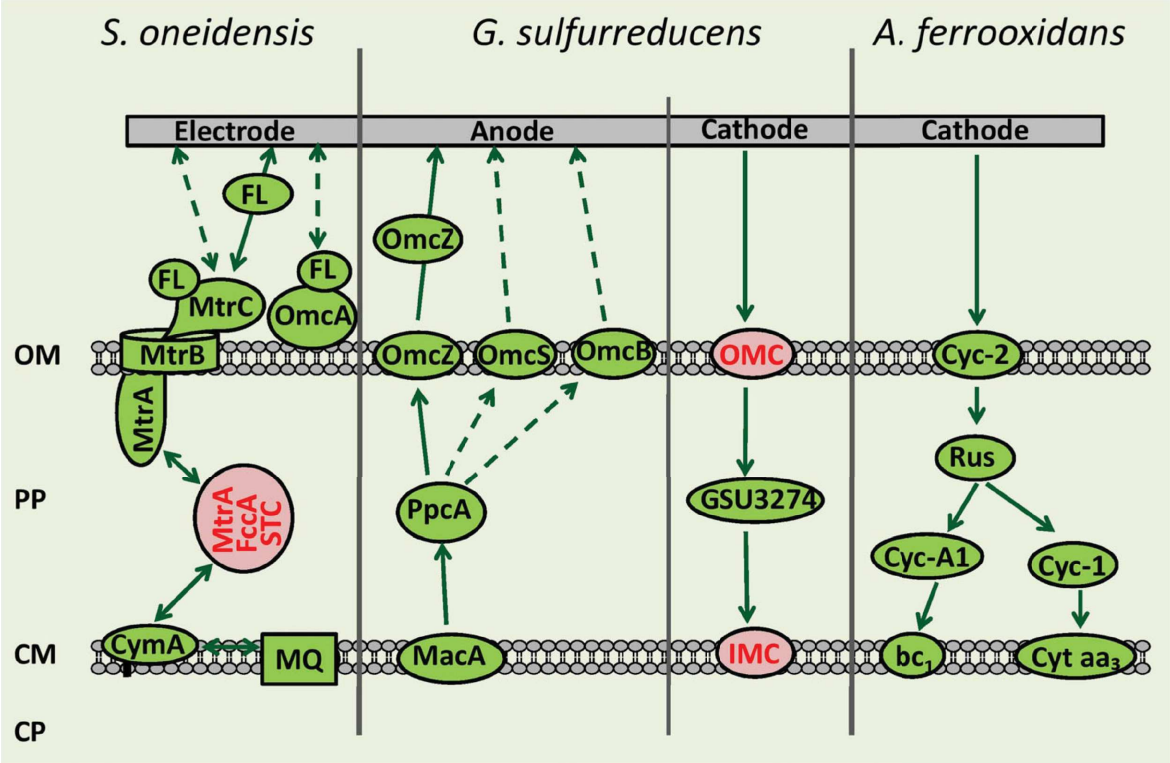

Figure 10. Main extracellular electron transfer pathways in *Geobacter sulfurreducens*, *Shewanella oneidensis* and *Acidithiobacillus ferrooxidans*. MQ menaquinone, FL flavins, CP cytoplasm, PP periplasm, CM cellular membrane, OM outer membrane. (Sydow et al., 2014).

Many Fe(III) reducer are very good at delivering electron to an anode, mainly because in natural environments they have to donate electron to solid-state Fe(III) oxide (Lovley et al., 2011). *Geobacter sulfurreducens* and *Shewanella oneidensis* are two model bacteria that are known Fe(III) reducer and were later described as able to transfer electron to an anode (Bond and Lovley, 2003; Kim et al., 1999). *G. sulfurreducens* is better known for its direct electron transfer mechanisms and *S. oneidensis* for the use of mediated electron transfer.

Regarding the direct electron transfer mechanisms, the key molecule are the cytochromes produced by the bacteria, mainly multiheme and membrane bound c-type cytochrome (Shi et al., 2007). Around 100 of putative c-type cytochromes have been identified in *G. sulfurreducens* (Mehta et al., 2005). While

OmcS is the essential cytochrome for Fe(III) reduction in *G. sulfurreducens*, a different one is involved in electron transfer to the anode: OmcZ (Nevin et al., 2009) (Figure 10). *G. sulfurreducens* is able to grow thick biofilm on the surface of an anode, which questions the long range ability of the cell to perform direct electron transfer. Electrically conductive type-IV pili, also called nanowire, have been identified in *G. sulfurreducens* (Reguera et al., 2005). The nanowire is covered in c-type cytochrome that provides the conductive property and micrometer-long distances electron transfer and thicker biofilm on an anode. *S. oneidensis* is better known for its mediated electron transfer mechanisms but nonetheless has multiple c-type cytochromes (Shi et al., 2007). The key proteins are MtrC and OmcA on the outer membrane of the cell (Figure 10). While these cytochromes can perform direct electron transfer to an anode, they are also essential to reduce the flavin mediator involved in mediated electron transfer. *S. oneidensis* does not have nanowire-like structure as for *G. sulfurreducens*, but rather extracellular extension at the micrometer-scale (Gorby et al., 2006; Pirbadian et al., 2014).

Mediated electron transfer relies on electron shuttles: organic compounds that can be reversibly reduced to transfer electron to the anode. They can be artificially added to the medium to help electroactive (Sund et al., 2007) and non-electroactive (Pham et al., 2008) to interact with an anode. Electron shuttles can also be secreted by bacteria, such as *S. oneidensis*, which produced flavin molecules like riboflavin and flavin mononucleotide (Canstein et al., 2008; Marsili et al., 2008) (Figure 10). Some membrane bound cytochromes (MtrC, OmcA) are used to reduce and recycle the extracellular flavin molecules (Brutinel and Gralnick, 2012). Other compounds involve phenazines, like the one produced by *Pseudomonas sp*. (Pham et al., 2008).

2.3. ELECTROTROPHIC BACTERIA

Conversely, some bacteria are able to use a cathode as an electron donor (Sydow et al., 2014). The associated reduction can be oxygen (Rabaey et al., 2008), nitrate (Gregory et al., 2004), nitrite (Clauwaert et al., 2007), proton (Rozendal et al., 2008), CO_2 (Cheng et al., 2009; Nevin et al., 2011) or organic contaminants (Strycharz et al., 2008). Most electrotrophic bacteria have an autotrophic metabolism, *i.e.* they use CO_2 as sole carbon source (Philips et al., 2015).

Some bacterial communities grown on an anode were able to draw electrons from a cathode as well (Cheng et al., 2010; Rimboud et al., 2017; Rozendal et al., 2008). This suggests that the extracellular electron transfer used at the anode could be reversed. Biofilms with *S. oneidensis* were described as able to use a cathode, hence using the same mediated electron transfer Mtr pathway but reversed (Ross et al., 2011). *G. sulfurreducens* is also able to switch from an electrogenic metabolism to an electrotrophic while still using a direct electron transfer mechanism (Gregory et al., 2004). However, the cytochromes and pili involved at the anode are not essential to draw electron from the cathode, but another mono-heme cytochrome (GSU3274 - Figure 10) is required suggesting a different electron uptake pathway (Strycharz et al., 2011).

2.3.1. OXYGEN REDUCING ELECTROTROPH

In seawater, the oxidant with the highest potential is oxygen, and some bacteria are able to coupled oxygen reduction with electron drawn from a cathode. The following part is about the identified bacteria associated with electrotrophic metabolism.

Rabaey et al., 2008, have used a carbon cathode open to air in a microbial fuel cell system with measured current density up to 220 µA/cm^2. They identified three major lineages, *Sphingobacterium* (*Bacteroidetes*), *Acinetobacter* (*Gamaproteobacteria*), *Acidovorax* (*Betaproteobacteria*). They successfully isolated a *Sphingobacterium* and an *Acinetobacter* with an enrichment using H_2/O_2 medium, which is quite far from normal seawater conditions.

Erable et al., 2010, used electrode polarized at -245 mV (vs SCE) to enriched biofilm with electroactive bacteria and then use it as inoculum for electrochemical reactors. They only reach the best current density value (60 µA/cm^2) with continuously renewed filtered seawater suggesting the need for nutrient renewal. Some bacteria were isolated from the mixed community enrichment: *Actinobacteria*, *Gammaproteobacteria*, *Flavobacteriaceae* and *Firmicutes*. Only *Winogradskyella poriferorum* and *Acinetobacter johsonii* were able to generate a current density when used as pure culture in the electrochemical bioreactors. The current density produced was 7% and 3%, respectively, of the

maximum current density reached with the wild biofilm, which suggest a strong synergetic effect in mixed communities.

Rimboud et al., 2017, designed three methods to obtain oxygen reducing biofilm on biocathode: open circuit conditions, aerobic polarization to -200 mV (vs SCE), and reversion of an anaerobic bioanode. Aerobic polarization lead to the best current density and the bacterial community was driven by *Sporosarcina* (*Firmicutes*) and *Brevundimonas* (*Alphaproteobacteria*). With cyclic voltammetry, they proposed that direct electron transfer was used in all conditions, while the mediated electron transfer mechanism was only used in the aerobic polarization conditions.

Parot et al., 2009, have used grass compost to assess the possible bacteria able to grow on a cathode. They found *Enterobacter* and *Pseudomonas* as able to catalyze the reduction of oxygen.

Milner et al., 2016, have used activated sludge from a wastewater treatment to increase the oxygen reduction on biocathodes. The power output were similar to the use of titanium cathode and bacterial community analysis revealed a dominant unknown *Gammaproteobacteria*. This bacterium was close to other *Gammaproteobacteria* found on biocathodes (Rothballer et al., 2015).

Debuy et al., 2015 used seawater as inoculum for the biofilm on the cathode. The bacterial communities were able to generate current density up to 80 µA/cm2 at -600 mV (vs SCE) and was mainly composed of *Gammaproteobacteria*. They isolated four bacteria (*Pseudoalteromonas sp.*, *Marinobacter sp.*, *Roseobacter sp*. and *Bacillus sp.*) that were all able to produce current in pure culture conditions.

As Fe(III) reducers were used at the anode, Fe(II) oxidizing bacteria are also able to use a cathode as an electron donor like *Acidithiobacillus ferrooxidans* which was described as able to switch from a chemolithoautotrophic to an electroautotrophic lifestyle (Ishii et al., 2015). It generated 2.2 µA/m^2 at +150 mV (vs SCE) in low pH condition (pH = 1.8). They have shown that the electron pathway involves an outer membrane cytochrome cyc2, cytochrome aa3 complex and a cytochrome bc1 complex to eventually reduce oxygen (Figure 10).

Another Fe(II) oxidizer, *Mariprofundus ferrooxidans*, is able to draw electron from a cathode polarized at -243 mV (vs. SCE) at neutral pH (Summers et al., 2013). Electron transfer pathway may involve molybdopterin oxidoreductase and alternative complex III respiratory system (Singer et al., 2011).

Rhodopseudomonas palustris is also a neutrophilic Fe(II) oxidizer able to use a cathode polarized at -243 mV (vs. SCE) (Bose et al., 2014).

While most of these studies used cathodes set at low potentials, some other have used high cathodic potentials and recovered better current density despite a lower energy available from the reduction of oxygen. Desmond-Le Quéméner et al., 2016, compared the effect of cathode potential on the current production and bacterial composition. At low potential (-400 mV vs SCE), the current density was around 10 µA/cm^2, while at higher potential (+100 mv vs SCE) the current generated was around 60 µA/cm^2. The most abundant bacteria recovered at low potential were assigned to the order *Bacillales* while the high potential biofilm were dominated by unidentified *Gammaproteobacteria* close to the *Ectothiorhodospiraceae* in the *Chromatiales* order. The metagenome of the unidentified *Gammaproteobacteria* biofilm revealed a putative CO_2 fixation metabolism.

Rothballer et al., 2015, used surface modification (electrochemical reduction of aryl diazonium salts) and assess the performance and composition of microbial communities across these surface modifications. The best performing cathode was the unmodified electrode with current density up to 90 µA/cm^2 at +105 mV (vs SCE). They found a correlation with the relative abundance of an unidentified *Gammaproteobacteria*.

2.3.2. "*CANDIDATUS* TENDERIA ELECTROPHAGA"

One of the key results of this thesis is the bacteria '*Candidatus* Tenderia electrophaga' which was identified in a biocathode community. Here is a summary of the work that led to its identification and description of its putative extracellular electron transfer mechanisms.

Strycharz-Glaven et al., 2013, have used microbial solar cell which enable continuous power generation with the fuel being renewed by the photosynthetic activity of the biofilm. They performed biocathode enrichment with and without illumination to identify the contribution of photosynthetic versus non-photosynthetic bacteria for the oxygen reduction at a high cathodic potential (+66 mV vs SCE). The highest current density was achieved with the dark system suggesting that light independent bacteria were principally responsible for the oxygen reduction. 16S rRNA gene sequencing reveals that *Gammaproteobacteria* were dominant in dark biocathode, with the noted presence of *Marinobacter spp*.

The same group then used metagenomics and metaproteomic to further characterize the bacterial composition of the biocathode community and identify the extracellular electron transfer mechanism, as well as the inorganic carbon fixation pathway since the biofilm was able to grow with only CO_2 as carbon source (Wang et al., 2015b). The most represented bacteria had their genome sequenced and were identified as *Marinobacter* (Wang et al., 2015a), an unknown member of the family *Chromatiaceae* (Eddie et al., 2016), and *Labrenzia* (Wang et al., 2016). Eventually, the unknown member of the family *Chromatiaceae* was described but could not be placed in either the *Chromatiales* or *Thiotrichales* order. It was named '*Candidatus* Tenderia electrophaga', a member of the *Gammaproteobacteria* (Eddie et al., 2016). Putative extracellular electron transfer mechanisms were found for '*Candidatus* Tenderia electrophaga' and involved the MopB-containing alternative complex III (Figure 11), a putative iron oxidation pathway in *Zetaproteobacteria Mariprofundus ferrooxydans*, the bacterium able to oxidize solid-state Fe(II) at neutral pH. A c-type monoheme cytochrome Cyc2 from *Acidithiobacillus ferrooxidans* was also found. The latter is involved in Fe(II) oxidation at low pH (Figure 11). In addition to the extracellular electron transfer, '*Candidatus* Tenderia electrophaga' was the only genome with the Calvin-Benson-Bassham cycle for CO_2 fixation, including the presence of form I RubisCO. It was therefore the only bacteria able to fix carbon for the non-autotrophic member of the community.

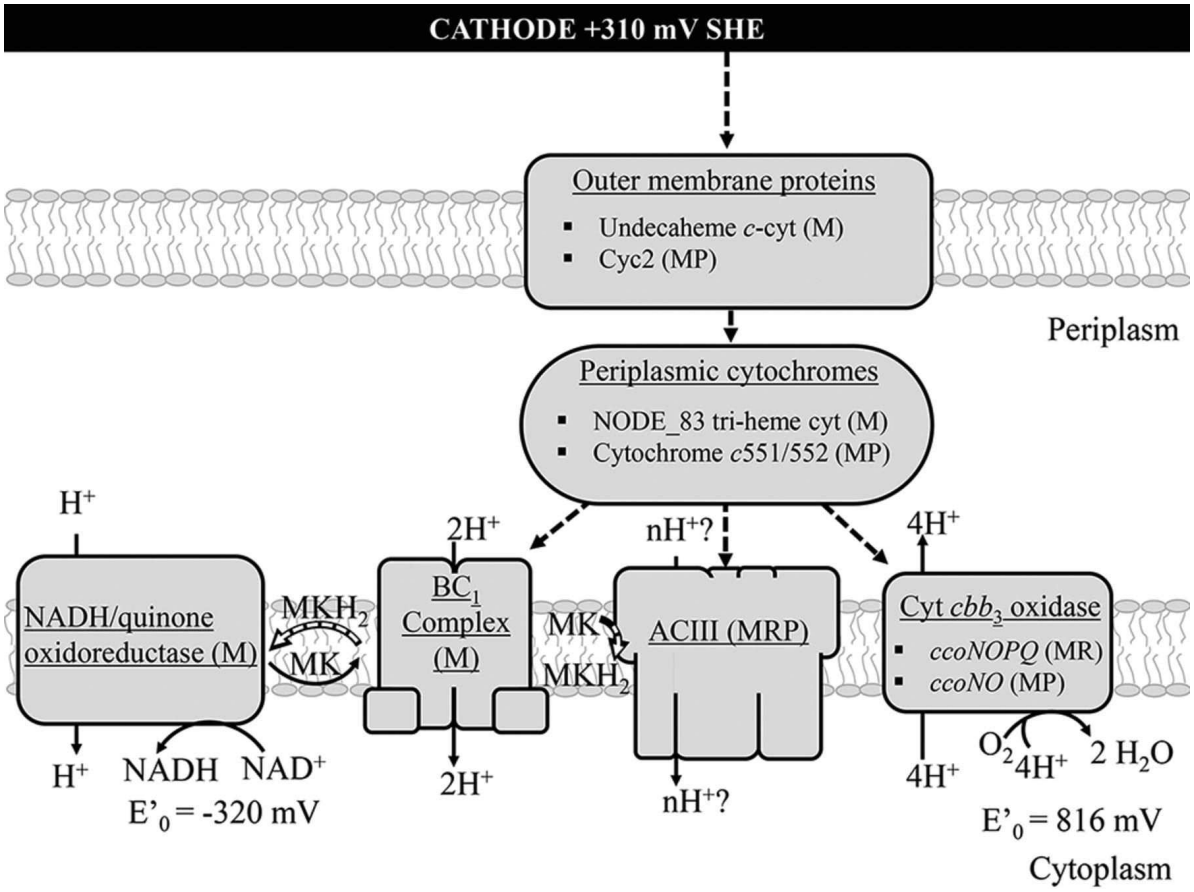

Figure 11. Putative extracellular electron transfer system in '*Candidatus* Tenderia electrophaga' (Wang et al., 2015b).

Metaproteomic was used again to compare the effect of the set cathodic potential on the bacterial composition and protein expression (Leary et al., 2015). The two potentials used were an optimal of +66 mV (vs SCE) and a suboptimal potential of +226 mV (vs SCE). The majority of the proteins expressed at the suboptimal potential were in protein turnover/synthesis, motility, and membrane transport. Five proteins were associated with the optimal potential and electron transfer: c-type cytochrome, two flavoproteins, quinoprotein alcohol dehydrogenase and Methionine sulfoxide reductase. Despites changes in protein expression, community's composition was not different from the optimal to suboptimal potential. It was verified in a second similar experiment where Eddie et al., 2017, used a

metatranscriptomic approach to assess the effect of the potential change from optimal to suboptimal. '*Candidatus* Tenderia electrophaga' activity was correlated with the current density which support the extracellular electron transfer model. The Cyc2 gene, predicted to have a role in extracellular electron transfer, was more highly expressed at the suboptimal potential, when more electrons are needed to generate the same amount of energy. The genes involved in CO_2 fixation were also more expressed reflecting the need to increase the efficiency of CO_2 fixation.

An additional study used metagenomics and reconstructed bin affiliated with '*Candidatus* Tenderia electrophaga', *Labrenzia*, *Marinobacter*, *Kordiimonas*, *Alcanivorax*, and *Muricauda*. The current density generated on these biocathode was correlated with the relative abundance of '*Candidatus* Tenderia electrophaga' (Malanoski et al., 2018).

3. ECO-GENOMIC APPROACH

Eco-genomic is a combination of ecological and DNA sequencing based approaches to study the microbial diversity. The ecology is the study of organism interactions with other organisms and their environment. In this work, we wanted to use this approach to better identify the fraction of the microbial community that could be responsible for the ennoblement. The genomic, or DNA based approach refers to the use of culture-independent techniques to access the broad diversity of microorganisms as only a fraction of the microorganisms can be identified using classical culture methods.

Previous studies on stainless steel ennoblement used a variety of approaches to identify the microbial community, such as denaturing gradient gel electrophoresis (Baker et al., 2003; Faimali et al., 2010; Machuca et al., 2014), microscopy (Dickinson et al., 1996; Kolari et al., 1998; Mattila et al., 1997; Wang et al., 2004), bacterial isolation and culture identification (Dickinson et al., 1996), lipid composition and EPS quantification (Acuña et al., 2006). High throughput sequencing technology was only used recently to assess the biofilm composition in brackish water on stainless steel (Huttunen-Saarivirta et al., 2018).

In this study, we intend to use the latest sequencing technology to assess the microbial population associated with stainless steel in seawater. The different technologies and methods to study a microbial community will be discussed in this chapter for the reader to better understand our approach.

3.1. SEQUENCING IN MICROBIOLOGY

In marine environment, the presence of microorganisms has been shown since the beginning of microbiology at the end of the 17th century by Antonie van Leeuwenhoek, a Dutch trader. Since then, the microbiology field was defined by the use of culture media to grow and characterize microorganisms. Microbiologists later realized that traditional culture techniques could only be used for a very small fraction of the bacteria in a given environment. This was defined as “Great Plate Count Anomaly” because the bacterial abundances observed on microscopes were much higher than those obtained by culture methods. It is estimated that less than 1% of microorganisms in the environment are potentially “cultivable” (Staley and Konopka, 1985), although this concept is debated as new isolation methods

permitted to increase the number of isolated strains (Rappé et al., 2002). Counting the number of bacteria contained in a seawater sample was only possible in the late 1970s with the emergence of epifluorescence microscopy techniques and DNA staining using fluorescent markers (Hobbie et al., 1977) as well as the development of flow cytometry and its use on seawater samples (Yentsch et al., 1983). Many of the major discoveries in microbiology in recent decades strongly relied on key technological advances capable of overcoming the difficulties inherent in the study of these organisms invisible to the naked eye, and mostly uncultivated.

As DNA store all the evolutionary and metabolic properties of living organisms, it became obvious that knowing the order of nucleic acid in genomes would contribute to understand living matter. A key technological advance in the sequencing of DNA was brought by Fred Sanger (Sanger et al., 1965, 1977) with the so-called Sanger sequencing technique, which led to the complete sequencing of first organism genome: the bacteriophage MS2 (Fiers et al., 1976). Later, the automation of the principle led to the first sequencing machines (Hunkapiller et al., 1991) and ultimately to the sequencing of the human genome (International Human Genome Sequencing Consortium, 2001).

The “genomic revolution” really happened with the second generation of sequencer called next-generation sequencing (NGS) with sequencing capacity growing at a faster rate than the Moore’s law for the computing revolution: between 2005 and 2010 sequencing capabilities doubled every five months (Stein, 2010). The two main technologies developed during this period were “pyrosequencing” by Roche 454 and “bridge amplification” by Solexa/Illumina, and both relied on sequencing by synthesis, providing high sequencing depth and accuracy. They were limited by the homopolymer (repetition of the same nucleic acid) for pyrosequencing, and size for both technologies (max 300bp, Illumina MiSeq). Beyond the very high sequencing throughput of the latest Illumina machines (around 4 billion paired-end per run for the NovaSeq), the sequencing error rate, *i.e.* the probability of a sequencer to call the wrong base in a given position, is relatively low (0.24 ± 0.06% per base and the percentage of mutated sequences was found to be 6.4 ± 1.24%) (Pfeiffer et al., 2018).

A third generation of sequencer was defined by the ability to sequence single molecule of DNA and therefore avoid the amplification process inherent to previous methods. Pacbio and Oxford Nanopore represent the main manufacturers of single molecule and real time sequencer. Reads quality and sequencing depth is often poor, around 10-15% of raw error rate before correction (Weirather et al., 2017), but sequence length can be very high and can be associated with NGS sequences to reconstruct long and accurate sequences of DNA.

3.2. AMPLICON BASED SEQUENCING

3.2.1. HISTORY OF MARKER GENES

Traditionally, organisms were classified according to phenotypic features (prokaryotes, eukaryotes) but Carl Woese and other used the small subunit ribosomal RNA gene—also called 16S rRNA (Figure 12)—as a phylogenetic marker to compare and reconstruct the tree of life and eventually define the three domains of life that are Bacteria, Archaea and Eukarya (Woese et al., 1990; Woese and Fox, 1977). The 16S rRNA gene has the advantage of being present in every single living organism (18S rRNA for Eukaria), and around 1500 bp long which was in the range of the Sanger sequencing technology. Since then, this marker gene has been extensively used to compare microorganisms, describe microbial communities and estimate biodiversity with PCR amplification of the gene, cloning and sequencing. With this fastidious approach, it was possible to obtain around 100 sequences for one sample and observe mainly the dominant species. This method has dominated the field of microbiology for 20 years before the introduction of NGS. Today, Illumina sequencers can produce 10^7 to 10^8 reads and therefore assess the nearly exhaustive microbial diversity of an environmental sample. A marker gene approach like the sequencing of 16S rRNA is also called amplicon sequencing because PCR amplification prior to the sequencing. It may also be referred as amplicon-based metagenomic. NGS and amplicon sequencing enabled microbial ecologist to describe many environments like the human microbiome (Turnbaugh et al., 2007), oceanic microbiome (Sunagawa et al., 2015), or soil microbiome (Fierer and Jackson, 2006).

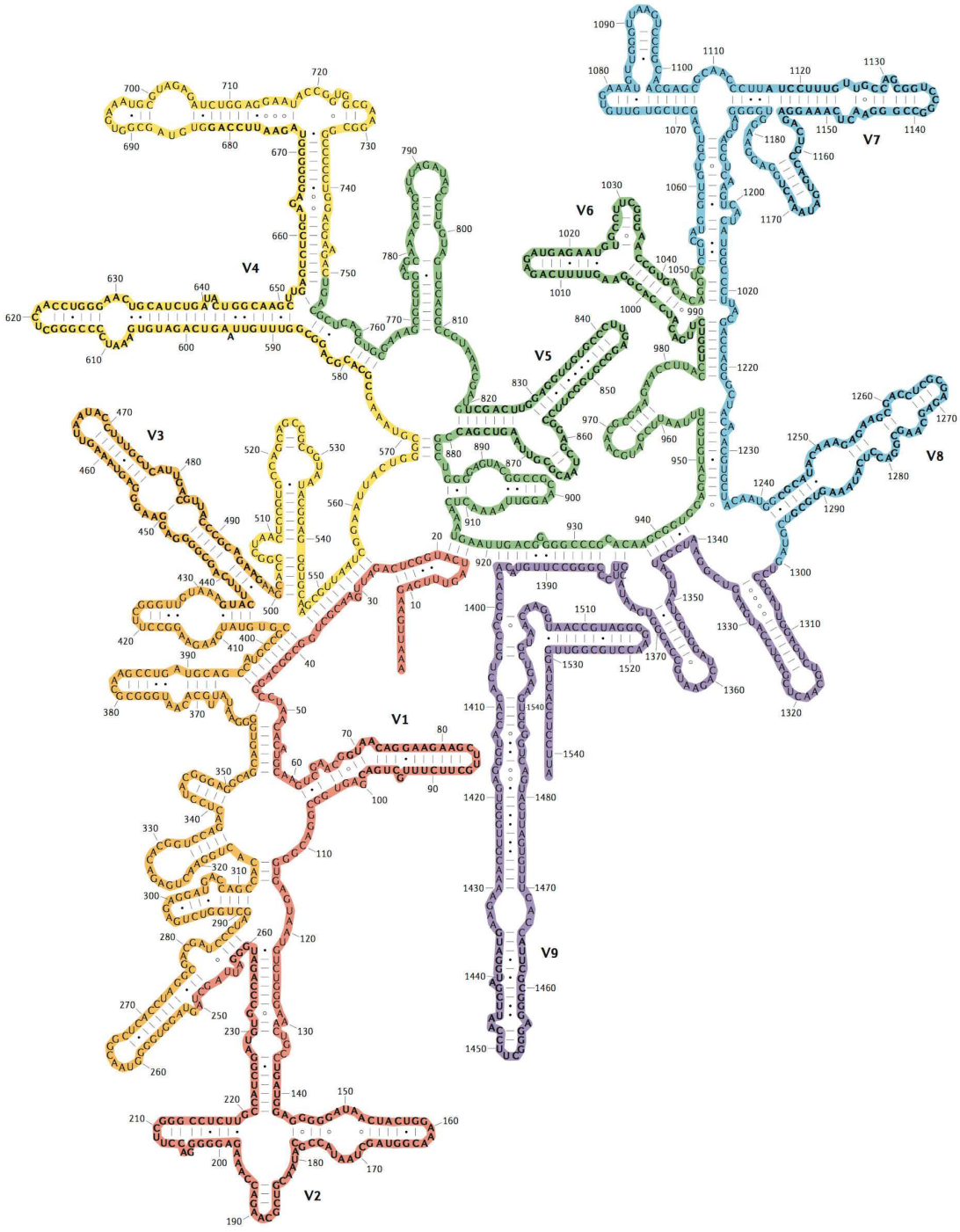

Figure 12. Secondary structure of the small subunit ribosomal RNA with the hypervariable regions (V1 to V9). In this thesis, we used the V4-V5 region.

3.2.2. THE CLUSTERING CHALLENGE

The gold standard to define bacterial species was defined by DNA-DNA hybridization with a recommended similarity threshold of 70%, under which the compared organisms belong to different species (Wayne et al., 1987). Marker gene similarity can also be used to define species boundaries, and a minimum similarity of 97% was required to consider two 16S rRNA sequences to belong to the same microbial species (Stackebrandt and Goebel, 1994). This threshold is still used nowadays to cluster sequence of amplicon based study, but has been re-evaluated to value closer to 98.65 % (Kim et al., 2014; Stackebrandt and Ebers, 2006). 16S rRNA amplicons are clustered into operational taxonomic units (OTUs) to account for that intra-species variability, but also the sequencing errors that are inherent to the NGS technology.

3.2.3. HIGH RESOLUTION METHODS FOR AMPLICON ANALYSIS

The use of an a priori genetic distance threshold to delineate fundamental units of diversity has however some strong limitations because it is not always applicable or relevant (Brown et al., 2015; Nebel et al., 2011) and limits the resolution of microbial diversity analysis by masking important patterns occurring between closely related organisms (Eren et al., 2013a). Hence, the recent development of new high resolution methods leveraging sequencing error analysis (DADA2, Callahan et al., 2016), information entropy analysis (oligotyping and MED, Eren et al., 2013a, 2014) or error distribution profiles (SWARM, Mahé et al., 2015) constitutes an important advance in microbial diversity analysis.

For instance, the Swarm algorithm agglomerates sequences based on pairwise similarity with maximum one nucleotide deference (default) and delineate OTUs based on abundance values—*i.e.* break in abundance “valley” linking two otherwise separate OTUs (Mahé et al., 2015).

3.2.4. LIMITATIONS AND BIASES

3.2.4.1. PARTIAL GENE SEQUENCING

Because NGS technology only generate small length sequence, smaller than the 16S rRNA gene itself, some region of the gene were selected based on their variability (hypervariable regions) which are found around conserve regions (Figure 12). The sixth hypervariable region (V6) was used because of its small size (60pb) fitting the length capacity of the Illumina HiSeq. With the MiSeq technology, longer region can be sequenced like the region V4-V5 (around 450pb). As a consequence, some studies are not comparable as the regions sequenced are not the same.

3.2.4.2. CHIMERA FORMATION

Other biases are inherent with the use of 16S rRNA amplicon sequencing, such as the creation of chimeric sequences during the PCR amplification. These artificial sequences are made with the fusion of two (or more) sequences, but are—by definition—lower in abundance than the "parent" sequences and can be identified by software such as VSEARCH (Rognes et al., 2016).

3.2.4.3. LACK OF UNIFYING TAXONOMIC FRAMEWORK

16S rRNA reference databases of known microorganisms can be used to assign taxonomy to OTUs. There are three 16S rRNA databases: Greengenes (McDonald et al., 2012), the Ribosomal database project (RDP) (Wang et al., 2007) and Silva (Quast et al., 2013). They differ in size and resolution, Silva being the largest with manually curated taxonomic rank assignment (Balvočiūtė and Huson, 2017). This taxonomic information can be used to infer hypothetical metabolisms of OTUs, but maker gene does not reflect the whole genome content, let alone the actual gene expression.

3.3. WHOLE GENOME SEQUENCING

3.3.1. PRINCIPLE AND RELEVANCE

The cost-effective high-throughput sequencing allowed for great improvement over amplicon: instead of sequencing a marker gene, all genomes present in a sample are fragmented and sequenced—hence the name of whole genome sequencing (WGS). The genetic potential of community can therefore be assessed, and genome populations can be reconstructed into metagenome-assembled genome (MAG) (Nielsen et al., 2014).

The clear advantage of this approach is to access the functional genes, which help to understand the potential metabolism occurring in a given sample. MAG can also be compared to known microbial genome using comparative genomics and metrics such as in silico DNA-DNA hybridization or the average nucleotide identity (ANI), which has become a new gold standard method to compare genomes, replacing the DNA-DNA hybridization (Goris et al., 2007; Richter and Rosselló-Móra, 2009). The accepted boundary for species delineation is 96% ANI (Kim et al., 2014).

Messenger RNA can be sequenced in addition to the DNA to assess the gene expression in particular conditions and associate MAGs genetic potential to actual activity.

3.3.2. ASSEMBLY AND BINNING CHALLENGE

New generation sequencers produce short reads of DNA (around 150pb, Illumina HiSeq/NextSeq). As a consequence, the original genomes need to be re-assembled in a process called metagenomic assembly. The products of that assembly are longer fragment of DNA called contigs. The challenge with assembly is that conserved part of genome among different organisms will break the assembly, as well as variations within a species population. In addition, low abundance organisms may not have enough short read sequenced to be re-assembled. To overcome this issue, co-assembly can be performed : DNA sequences from different samples are put together to increase the chance of recovering low abundance microorganism genome.

The binning process is the classification of contigs into discrete clusters in which a bin represent a genome. It can be done automatically using computational tools such as CONCOCT (Alneberg et al., 2014), MetaBAT (Kang et al., 2015), BinSanity (Graham et al., 2017) or Maxbin2 (Wu et al., 2016). The method is based on the sequence composition of contigs and their differential coverage across samples. But automatic methods are not perfect and can result in conflation errors (more than one genome in a bin) or fragmentation errors (one genome in multiple bins), which can be solved by manual curation of the binning results with visualization software such as Anvi'o (Eren et al., 2015). The single-copy core genes (SCG), which are expected to be in all microorganisms and only once, are used to assess the completion and redundancy of a bin—and therefore the quality of the binning. Because some microorganisms can lack some of these genes and/or have multiple copy, there is an accepted margin of 90% completion and 5% redundancy to characterize a MAG as a high quality MAG (Bowers et al., 2017).

3.3.3. LIMITATIONS AND BIASES

The first bias of this approach is the same as for the amplicon sequencing technique: the need for DNA amplification which can result in chimera sequences and overestimation of abundant sequences.

Microbial population with high intra-species variability would be poorly assembled because these variations are likely to break the assembly step. A prime example is the SAR11 clade, the major marine microbial clade (identified with amplicon sequencing) often poorly recovered in metagenomics studies (Venter et al., 2004). Longer DNA sequences would help overcome this issue.

For an equal number of DNA sequenced, the rare biosphere—microorganisms of very low abundance—can be more easily accessed with marker genes than with metagenomic because in the first case, one DNA sequence correspond to one microorganism (prior to the DNA amplification), and in the second case many DNA sequences belong to the same microorganism.

OVERVIEWS AND OBJECTIVES

The objective of the thesis is to address an unsolved microbial phenomenon : the ennoblement of stainless steel in seawater and to propose a mechanism reconciling previous observations. We aim to combine electrochemistry, molecular biology and bioinformatics in a collaboration between the French Corrosion Institute and the Laboratory of Microbiology from Extreme Environments.

The stainless steel ennoblement is impacted by environmental factor, such as the seawater temperature and the oxygen content. We used these conditions to described microbial communities that colonized stainless steel and infer the fraction of these microbial communities associated with ennoblement.

In the Chapter I, we used a temperature gradient and biomarker detection to identify the bacterial OTUs only present at temperatures with the ennoblement observed. We found an OTU affiliated with '*Candidatus* Tenderia electrophaga', and electrotrophic bacterial able to use a cathode as an extracellular electron donor to reduce oxygen and we proposed a model in which electrotrophic bacteria are able to use the stainless steel passivation current to reduce oxygen, hence increasing the potential. We also identified hydrocarbon degrading bacteria associated with ennoblement with no explanation so far.

The second environmental factor influencing the ennoblement is the oxygen content, which was assessed in the Chapter II. In addition, we compared the microbial communities on non-conductive surface like glass. Then again, we found OTUs affiliated to hydrocarbon degrading bacteria but interestingly, no known electroactive bacteria, suggesting that diverse, yet unidentified bacteria are able to trigger ennoblement

In the Chapter III, to confirm the putative role of electrotrophic bacteria, we used potentiostatic condition to identify the electrotrophic bacteria naturally occurring in open circuit condition. This time, we used metagenomics instead of 16S rRNA amplicons and identified a metagenome-assembled genome (MAG) very close to the genome of '*Candidatus* Tenderia electrophaga'. This MAG was only occurring under open circuit condition which provides more evidence for the model—proposed in the Chapter I—in which electrotrophic bacteria would influence the open circuit potential and consequently mediate the ennoblement

L'objectif de cette thèse est d'étudier un phénomène microbiologique non-élucidé à ce jour : l'anoblissement des aciers inoxydables en eau de mer, et de proposer un mécanisme microbien en accord avec les observations rapportées dans la littérature. Pour cela, nous avons associé l'électrochimie, la biologie moléculaire et la bioinformatique dans une collaboration entre l'Institut de la Corrosion et le Laboratoire de Microbiologie des Environnements Extrêmes.

L'anoblissement des aciers inoxydables est modulé par des facteurs environnementaux comme la température de l'eau de mer ou la teneur en oxygène. Nous avons utilisé ces conditions pour décrire les communautés microbiennes qui colonisent les aciers inoxydables et identifier la fraction microbienne associée à l'anoblissement.

Dans le Chapitre I, nous avons utilisé un gradient de température et une détection de biomarqueurs pour identifier les OTUs présents uniquement lors de l'anoblissement. Nous avons trouvé un OTU affilié à '*Candidatus* Tenderia electrophaga', une bactérie électrotrophe capable d'utiliser une cathode comme donneur d'électrons pour réduire de l'oxygène. Nous avons proposé un modèle dans lequel les bactéries électrotrophes utilisent le courant du film passif de l'acier inoxydable pour réduire de l'oxygène, et ainsi augmenter le potentiel. Nous avons aussi identifié des bactéries, dégradant des hydrocarbures, associées avec l'anoblissement, sans connaître leur rôle à ce jour.

Le second facteur environnemental qui influence l'anoblissement est la teneur en oxygène, étudié dans le Chapitre II. Nous avons aussi comparé les communautés microbiennes avec des surfaces ne conduisant pas l'électricité comme le verre. Encore une fois, nous avons identifié des OTUs affiliés à des bactéries dégradant des hydrocarbures mais pas de bactéries électrotrophes ce qui suggère qu'une diversité de bactéries, non-identifiées, sont capable de produire l'anoblissement.

Dans le Chapitre III, nous avons utilisé une condition potentiostatique pour identifier les bactéries électrotrophes naturellement présentes en condition de potentiel libre pour confirmer leur rôle supposé dans l'anoblissement. Nous avons utilisé une approche de métagénomique et identifié un génome issu de l'assemblage métagénomique (MAG) très proche du génome de '*Ca.* Tenderia electrophaga'. Ce MAG n'était présent qu'en condition de circuit ouvert ce qui renforce notre hypothèse proposée dans le Chapitre I.

CHAPTER I:
INFLUENCE OF SEAWATER TEMPERATURE

Electroactive bacteria associated with stainless steel ennoblement in seawater.

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1. RÉSUMÉ

Les microorganismes peuvent augmenter le potentiel libre des aciers inoxydables immergés en eau de mer par plusieurs centaines de millivolts dans un phénomène appelé anoblissement. Cela augmente les risques de corrosion car le potentiel libre peut alors dépasser le potentiel critique de corrosion localisé. Malgré l'impact de l'anoblissement, aucun mécanisme n'a été décrit pour expliquer le phénomène. Dans ce travail, nous montrons que la bactérie électrotrophe '*Candidatus* Tenderia electrophaga' est identifiée comme biomarqueur de l'anoblissement et n'est retrouvée qu'à des températures d'eau de mer où l'anoblissement est observé. Lors d'une autre étude, cette bactérie a été enrichie sur une biocathode. Nos résultats suggèrent que '*Candidatus* Tenderia electrophaga', et son métabolisme de transfert d'électron extracellulaire couplé à la réduction de l'oxygène, pourrait avoir un rôle important dans la modulation du potentiel libre des aciers inoxydables et par conséquent induire l'anoblissement.

2. ABSTRACT

Microorganisms can increase the open-circuit potential of stainless steel immersed in seawater of several hundred millivolts in a phenomenon called ennoblement. It raises the chance of corrosion as the open-circuit potential may go over the pitting corrosion potential. Despite the large impact of the ennoblement, no unifying mechanisms have been described as responsible for the phenomenon. Here we show that the strict electrotroph bacterium '*Candidatus* Tenderia electrophaga' is detected as an ennoblement biomarker and is only present at temperatures at which we observe ennoblement. This bacterium was previously enriched in biocathode systems. Our results suggest that '*Candidatus* Tenderia electrophaga', and its previously described extracellular electron transfer metabolism coupled to oxygen reduction activity, could play a central role in modulating stainless steel open-circuit potential and consequently mediating ennoblement.

3. INTRODUCTION

When immersed in oxic seawater, metals and alloys can form an electrochemical cell with metal oxidation as an anode reaction and oxygen reduction at the cathode. In the case of stainless steel, metal (Cr, Ni, Mo, …) oxides form a so called passive layer largely preventing electron flow between these two electrodes. As a consequence, stainless steel exhibit a measurable electrochemical potential between these two half cells, called Open Circuit Potential (OCP) since no current is drawn from the system. Stainless steel OCP results from the concentration of the reactants, formal half-cell reaction potential (E°') and the kinetic parameters associated with each half-cell reaction. It can be measured in situ using a reference electrode of a known potential. Stainless steel ennoblement is a well-known phenomenon corresponding to an increase of the OCP, typically by 400-500 mV, when these alloys are immersed in seawater (Mollica and Trevis, 1976). As the OCP value gets closer to the pitting corrosion potential, the probability of stainless steel pitting and crevice corrosion initiation increases, hence the central problem raised by ennoblement (Mollica, 1992; Zhang and Dexter, 1995). Ennoblement is a biotic process as it is dependent on microbial colonization and development on the stainless steel surface (Gümpel et al., 2006; Le Bozec et al., 2001; Motoda et al., 1990; Scotto and Lai, 1998; Wei et al., 2005).

Stainless steels are commonly used building material in seawater systems and despite the large industrial impact of the ennoblement, relatively little is known about the diversity and the actual activity of marine microorganisms that colonize stainless steel. There are various hypotheses regarding their possible contributions to ennoblement, as summarized in Little et al., 2008. There are three ways of increasing the OCP: (1) thermodynamics, (2) kinetics and (3) alteration of the nature of the reduction reaction. A decrease of the surface pH could thermodynamically increase the observed OCP. However, as shown by Dexter and Chandrasekaran, 2000, the pH changes in a heterogeneous biofilm are highly variable and also challenging to measure. Kinetically, an increase of the cathodic reaction rate can also result in an increase of the OCP. Previous works have demonstrated that surface microorganisms increase cathodic reduction efficiency (Audouard et al., 1994; Holthe et al., 1989; Johnsen and Bardal, 1985; Larché et al., 2011; Le Bozec et al., 2001; Mollica and Scotto, 1996; Rogne and Steinsmo, 1996; Thierry et al., 2015; Zhang and Dexter, 1995). Other reactions such as manganese oxide reduction in freshwater (Dickinson

et al., 1996; Gümpel et al., 2006) and the formation of hydrogen peroxide could also contribute to the increase of the OCP as it is a stronger oxidant than oxygen with a higher redox potential (Landoulsi et al., 2008c).

While the mechanisms of ennoblement are still discussed, the seawater temperature has been identified as a critical parameter. Ennoblement is a temperature dependent process, undergoing a complete inhibition above a critical temperature around 40°C in temperate seawater and freshwater (Dupont et al., 1997; Gümpel et al., 2006; Martin et al., 2003; Scotto et al., 1986; Thierry et al., 2015) This critical temperature seems to vary with geography as it is 32°C in the Norwegian and Baltic sea (Bardal et al., 1993; Mattila et al., 2000). Despite sustained research on this important potential modulation, no mechanisms have been described that can explain the potential ennoblement, nor could the primary source of electrons for these cathodic reactions be identified.

Some microorganisms can perform direct extracellular electron transfer to and from electrodes. This was demonstrated in controlled systems such as microbial fuel cells. A wide diversity of microorganisms can channel electrons resulting from soluble substrate oxidation toward an anode, thus creating a measurable current in these bioelectrochemical systems (Philips et al., 2015). Far fewer microorganisms were identified as cathodic electron acceptor (Rabaey et al., 2008). Electroactive microorganisms such as *Geobacter sulfurreducens* or *Shewanella oneidensis* have been used as models to understand the electrogenic metabolism or the electron pathway from a soluble electron donor to an anode. However, they were also described as electrotroph, performing the reverse reaction using an electrode as electron donor. *Shewanella oneidensis* is able to switch from an electrogenic metabolism to an electrotrophic by reversing its electron transport pathway (Ross et al., 2011). Similarly, *Geobacter* species are also able to act as electrotrophs to reduce fumarate to succinate or nitrate to nitrite with electrons provided from a cathode through a direct electron uptake mechanism (Gregory et al., 2004; Strycharz et al., 2011). *Geobacter sulfurreducens* can increase the open-circuit potential by several hundred mV on stainless steel under anoxic condition via an electrotrophic metabolism to reduce fumarate to succinate (Mehanna et al., 2009b, 2010). Despite the absence of a model electrotrophic bacterium cultivated under aerobic conditions, some pure cultures were able to catalyze the electrochemical reduction of

oxygen (Erable et al., 2010; Rabaey et al., 2008), as well as some environmentally enriched communities (Milner et al., 2016; Rimboud et al., 2017; Rothballer et al., 2015; Strycharz-Glaven et al., 2013). Recently, the study of a bacterial community developed on a biocathode led to the identification of '*Candidatus* Tenderia electrophaga': a strict electroautotrophic bacterium able to use a cathode as an electron donor to reduce oxygen and able to fix carbon dioxide (Eddie et al., 2016). The presence of electrotrophic bacteria under natural conditions with aerated seawater has not yet been proposed as a possible reason for potential ennoblement despite their apparent ability to change the OCP.

As the rationale for this study, we hypothesize that electrotrophic bacteria could be involved in ennoblement by drawing electrons from immersed stainless steel in open-circuit condition (without additional current provided). However, to our knowledge, no study of stainless steel surface microbial community structure with high throughput sequencing methods has been carried out yet, even less so in relation to modulation of the electrochemical potential of this material. To test our hypothesis, we thus used the temperature dependence property of ennoblement and examine distinctive taxa in ennoblement vs. non ennoblement conditions.

4. MATERIAL AND METHODS

4.1. MATERIALS

The material used in all experiments was super duplex stainless steel (S32750) plates of 100 mm x 50 mm x 10 mm (French Corrosion Institute, France). The nominal composition of the stainless steel is 25.1% Cr, 7.0% Ni, 3.8% Mo, 0.13% Cu, 0.29% N, completed with Fe. The pitting resistance equivalent numbers is 42.3 (%Cr + 3,3%(Mo + 0.5%W) + 16%N). Prior to exposure, the plates were washed for 20 min in 20 % nitric acid and sterilized by autoclaving for 20 min at 121°C (dry cycle).

4.2. EXPERIMENTAL SET-UP

We exposed all coupons in 300 L seawater tanks renewed at an approximated rate of 12 L/h with an incoming seawater from the bay of Brest (France) (48°21'32.1"N 4°33'07.4"W). Seawater tanks were heated at 30°C, 33°C, 36°C, 38°C, and 40°C (+/-0.5°C). Since only three tanks were available, the experiment was run twice: 30°C, 33°C and 36°C; and 30°C, 38°C, and 40°C one week after the first series. Samples were collected after 7 days of exposure during March 2015. We also used two natural seawater samples (5 L, n = 3) collected from a Bay of Brest coastal microbial observatory close to the tanks' seawater pump intake. The two samplings took place on the 2015-03-02 and 2015-03-26, before and after the coupon exposure. Seawater was pre-filtered on a 3 µm filter and bacteria collected on a 0.22 µm sterivex filter.

4.3. OCP MEASUREMENTS AND CATHODIC POLARIZATION CURVES

Stainless steel samples were held by a titanium wire to measure the open-circuit potential with an Ag/AgCl reference electrode. The electrodes were calibrated with saturated calomel electrode (SCE) REF421 (Radiometer, France). The use of titanium wires has been documented in previous works and inhibits galvanic corrosion at the point of contact with stainless steel (Espelid, 2003). Measurements of OCP and temperature were recorded every 30 min. Five replicates were used per condition.

The cathodic polarization curves were drawn on samples exposed for two weeks under the two OCP conditions of interest: with ennoblement at 36°C and without the shift of potential at 40°C. We used a Gamry Reference 600 (Gamry Instruments, United States) from 20 mV over the open-circuit potential to -1.2 V vs Ag/AgCl electrode with a scan rate of 0.167 mV/s. The dynamic polarization curves started at +20 mV in order to get first points of the anodic branch without perturbing the oxide layer before cathodic scan. All potential values were corrected based on the reference electrode calibration with SCE. We obtained an estimation of the passivation current by drawing the intersection of the tangent of the anodic and cathodic branches close to OCP value.

4.4. SEM IMAGING

Dedicated coupons (20 mm x 20 mm x 1.5 mm) were fixed for scanning electronic microscopy (SEM) with 2.5% glutaraldehyde seawater for one hour, 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). We observed surface communities with SEM on samples exposed in seawater at 36°C and 40°C using a Hitachi SU3500 machine (Hitachi High-Technologies, Germany). To perform cell counting, we imaged ten random areas for each condition at x1000 magnification using backscattered-electron imaging. Pictures were processed with the ImageJ software for automatic cell detection. After the background removal, images were converted into binary black and white with the default threshold of the software. The particle analysis was used with a minimum area of 0.5 μm^2 up to 4 μm^2 for cell detection (SI Figure 3).

4.5. SURFACE CELL COLLECTION

Surface cells were collected immediately after coupon collection using a sterile cell lifter (Thermo Fisher Scientific, United States) and by gentle and uniform scratching into 100 mL of a Tris Buffered Saline (TBS) solution (50 mM Tris, 150 mM NaCl, pH 7.6) under sterile conditions ensured by a Bunsen burner, keeping the immediate area sterile. TBS solutions were then stored in ice for transport to the molecular

lab. TBS solutions were filtered through 0.22 µm GTTP polycarbonate membranes (Merck Millipore, United States) which were then transferred to PowerBiofilm® Bead Tubes from the PowerBiofilm DNA extraction kit (MoBio, United States). Control samples were collected under identical conditions and visualized by scanning electron microscopy to ensure removal of the cells attached to the surface.

4.6. DNA EXTRACTION AND SEQUENCING

The DNA extraction was performed according to the manufacturer's instructions of the PowerBiofilm DNA extraction kit (MoBio, United States). The V4-V5 region of the 16S rRNA gene was amplified with the 518F and 926R primers fused with Illumina adapters and sample-specific sets of barcodes and indexes (Nelson et al., 2014). PCR products were visualized on agarose gels and purified with AMPure XP (Agencourt, United States) reagent. DNA concentration was assessed with Quant-iT™ PicoGreen® dsDNA (Invitrogen, United States) prior to pooling the PCR products at equimolar concentration. Sequencing using Illumina MiSeq platform was performed at the Josephine Bay Paul Center (Woods Hole, United States). Sequences were deposited to the European Nucleotide Archive under the accession number PRJEB27599 (http://www.ebi.ac.uk/ena/data/view/PRJEB27599).

4.7. BIOINFORMATICS ANALYSIS

The quality filtering was done following Minoche et al., 2011, recommendations, before merging of paired-end reads with Illumina-Utils python scripts on demultiplexed raw reads (Eren et al., 2013b). OTU delineation was performed with the Swarm algorithm using the default local linking threshold d = 1 (Mahé et al., 2015). The chimera detection and removal were carried out with VSEARCH (Rognes et al., 2016). The Silva NR 132 database (Quast et al., 2013) was used for taxonomic assignment of Swarm representative sequences with Mothur (Schloss et al., 2009). We used the Phyloseq R package to calculate alpha diversity indices and the vegan package to compute beta diversity (with Bray-Curtis indices) and non-metric multidimensional scaling (NMDS) ordination. Stacked bar plots were produced with ggplot2 (Wickham, 2009). We used the two conditions "with ennoblement" (30°C to 38°C) and "without ennoblement" (40°C) to perform biomarker detection with LEfSe (Segata et al., 2011).
A fully reproducible workflow is available at https://loimai.github.io/ennoblement_16S/.

5. RESULTS

5.1. POTENTIAL ENNOBLEMENT ON STAINLESS STEEL AND CATHODIC POLARIZATION CURVES

At the beginning of the incubations, coupons were at - 272 mV (± 6 mV) vs. saturated calomel electrode and after 3 to 5 days of incubation, the OCP increased in all coupons except those incubated at 40°C (Figure 13). The potential ennoblement was highly reproducible among replicates at temperatures from 30°C to 38°C with a mean increase of the electrochemical potential of 470 mV (± 12 mV) for all samples within 4 days of exposure. In contrast, the open-circuit potential for samples immersed at 40°C changed very little over time (+54 mV, ± 8 mV). In our setup, there was thus a critical temperature between 38°C and 40°C under which ennoblement was observed for all samples with similar maximum electrochemical potential values after day 4 (or day 5 at 38°C), whereas ennoblement did not occur above that temperature.

In a subsequent incubation under similar conditions, we carried out a cathodic polarization curve on samples exposed at 36°C and 40°C and we observed a shift of the polarization curve after two weeks of exposure at 36°C that was not observed at 40°C (Figure 14). We estimated the passivation current to be around 0.01 $\mu A/cm^2$ for all conditions, based on these polarization curves.

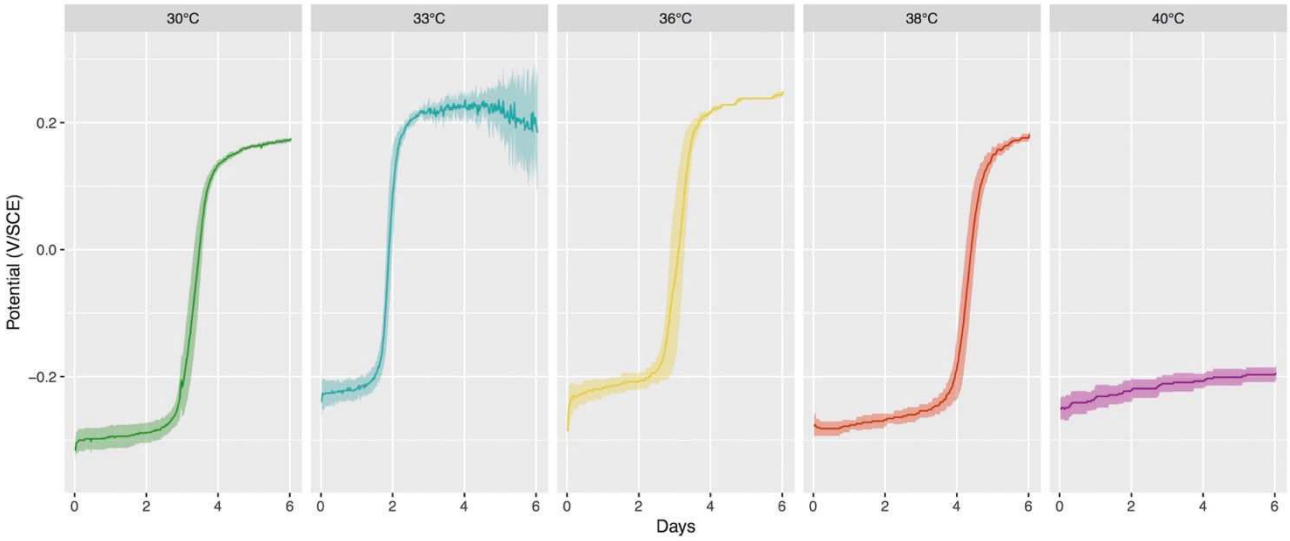

Figure 13. Open-Circuit Potential (OCP) versus time for stainless steel coupons exposed to different temperature of seawater. Mean value and 95% confidence interval for 5 replicates per conditions, or 10 replicates for 30°C as the two sequential series were pooled together. SCE: saturated calomel electrode.

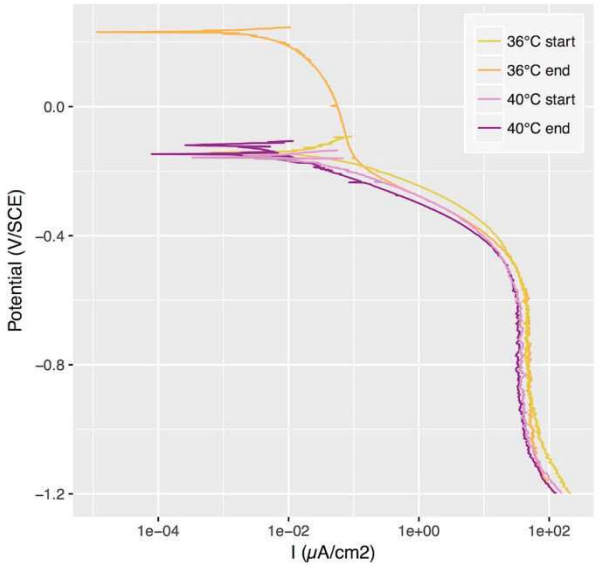

Figure 14. Cathodic polarization curves applied to samples with ennoblement at 36°C and without at 40°C, at the beginning of the exposure and after 2 weeks. SCE: saturated calomel electrode.

5.2. SEM OBSERVATIONS

We used a similar incubation setup at 36°C and 40°C, allowing biomass colonization on immersed stainless steel coupons for observation of surface communities with SEM. We found an average cell density of 11,661 cells/mm^2 (± 773 cells/mm^2) at 36°C, and a lower density of 7,219 cells/mm^2 (± 442 cells/mm^2) at 40°C. Under both conditions, we observed bacilli and coccobacilli, as well as some very long filamentous bacteria, but only at 36°C (see Figure 15).

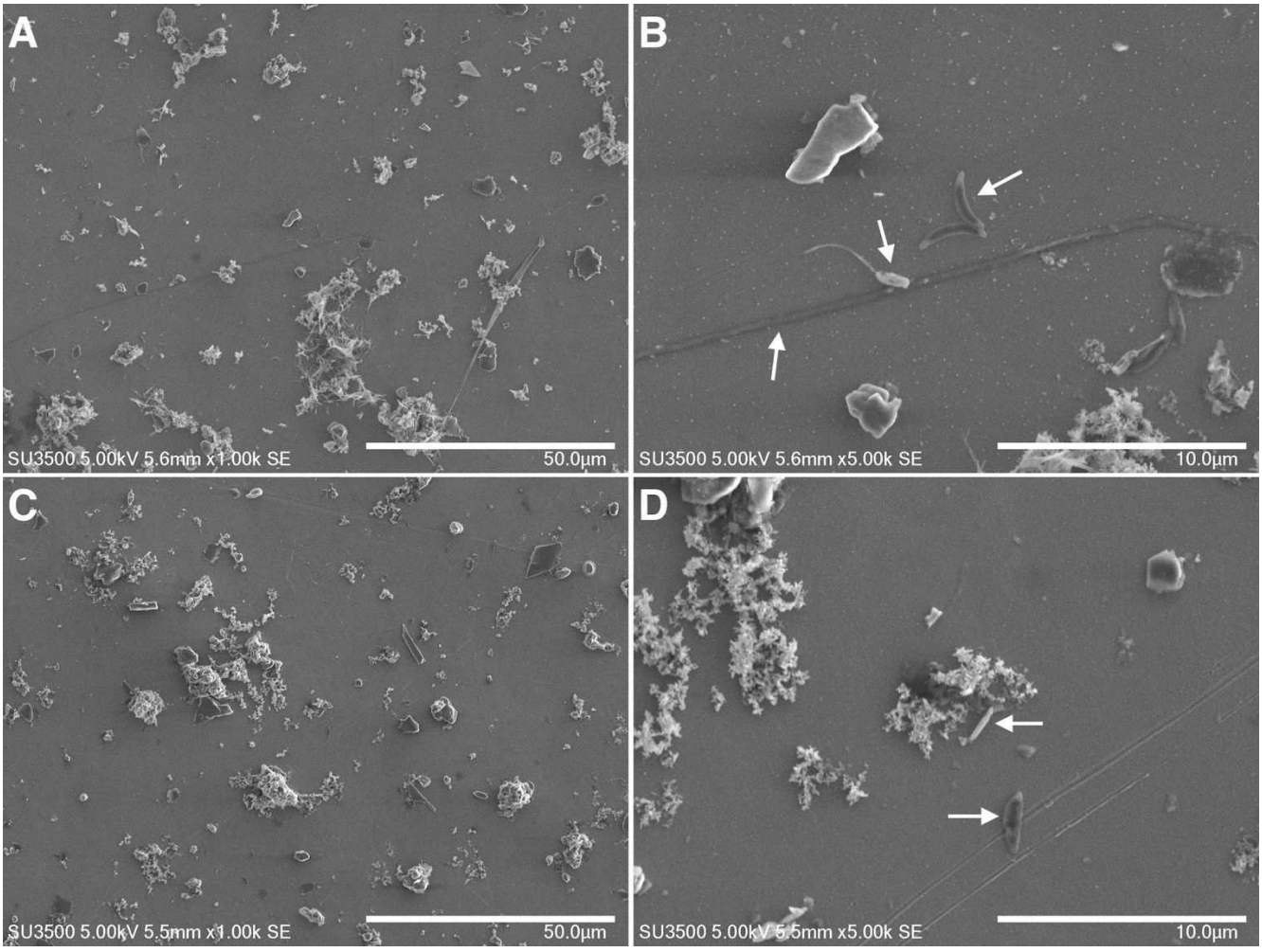

Figure 15. SEM images at 1k (A, C) and 5k (B, D) magnification of stainless steel exposed for one week to seawater and heated to 36°C (A, B) and 40°C (C, D). Some cells are pointed by arrows.

5.3. STAINLESS STEEL BACTERIAL COMMUNITY

We characterized surface bacterial communities for each condition using 16S rRNA amplicon sequencing. We sequenced 36 libraries and obtained 5,597,422 raw sequences of the bacterial 16S rRNA gene V4-V5 region. After quality filtering and paired end read merging, 3,653,018 sequences were retained and clustered into 166,164 operational taxonomic units (OTUs) using the Swarm algorithm with the default local linking threshold d = 1 (Mahé et al., 2015). Putative chimeras where removed with the VSEARCH software (Rognes et al., 2016), leading to a high-quality dataset of 66,892 chimera-free OTUs representing 3,240,780 sequences.

Bacterial 16S rRNA diversity in each sample was compared with a weighted dissimilarity index (Bray-Curtis) in an ordination analysis (Figure 16). Replicate samples collected at the same temperature were clustered and differed significantly from one temperature to another. Therefore, the different bacterial communities that develop at temperatures from 30°C up to 38°C appear able to increase the OCP. In addition, the two coupon series incubated at 30°C during a two weeks interval exhibited high community similarity, showing that surface community assembly was highly reproducible.

We identified microorganisms that were distinctive of the “ennoblement” condition using a biomarker detection analysis with the LEfSe software (Segata et al., 2011). We chose to define the condition with the ennoblement (30°C, 33°C, 36°C, 38°C) as opposed to a lack of OCP change (40°C). We conserved biomarkers with a minimum linear discriminant analysis (LDA) score of 3 resulting in 47 OTUs that were differentially represented during ennoblement. Among these we found mainly *Proteobacteria* including members of the *Oceanospirillales*, *Rhodobacterales*, and Alteromonadales (Figure 17). An OTU affiliated to the genus Oleiphilus genus was remarkably found exclusively in exposures setups between 30°C and 38°C with respective mean relative abundance of 18.41%, 2.67%, 6.76%, 12.40%, and 0.03% at 40°C (Figure 17). However, other Oleiphilus OTUs were also present at 40°C. More strikingly, we also detected the presence of a recently described Proteobacteria ‘*Candidatus* Tenderia electrophaga’ as a very strong biomarker (Figure 17). Members of this candidate genus were found in high relative abundance from 30°C to 38°C with mean relative abundance of 1.54%, 1.58%, 6.64%, and 10.05% with a peak abundance

of 18.4% in a sample replicate at 38°C (Figure 17). In addition, this bacterium was found exclusively in ennoblement conditions, as no other '*Candidatus* Tenderia electrophaga' OTUs were detected at 40°C.

Finally, we examined the abundance of these biomarker bacteria in the pool of colonizing bacteria from natural seawater collected in the vicinity of our set up pump intake, before and after incubation periods. The bacterial composition in seawater was strikingly different from that of the steel surfaces (Figure 16). No sequences of the best ten biomarker OTUs were recovered from seawater, except for two affiliated to an Oleiphilus OTU.

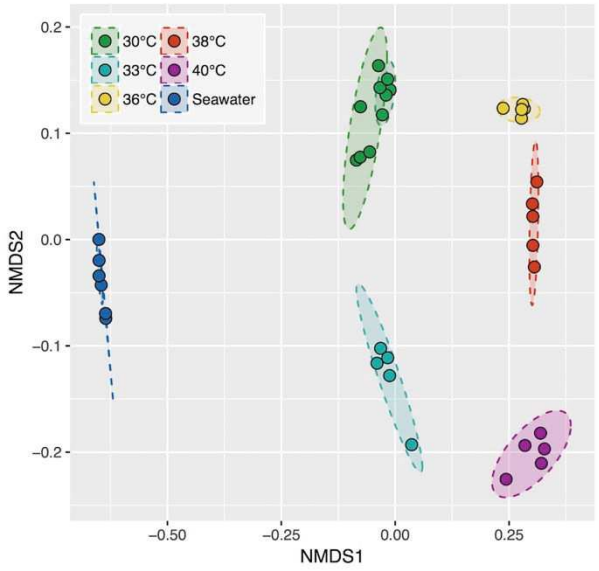

Figure 16. Non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis dissimilarity index. 95% confidence area per condition. Stress: 0.086.

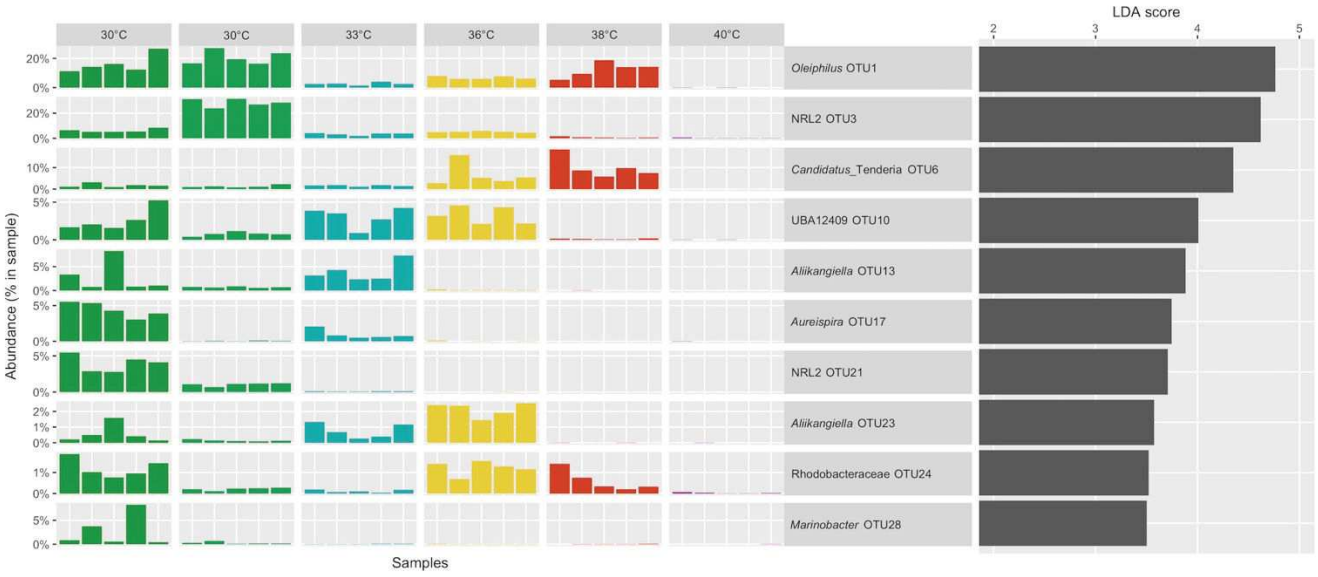

Figure 17. Relative abundance distribution of the ennoblement biomarker OTUs with the 10 best LDA scores. Taxonomic affiliations originate from the Silva 132 database release. Biomarkers were not detected in seawater samples, except for two sequences affiliated to *Oleiphilus* (OTU1).

6. DISCUSSION

The open-circuit potential is defined by the concentration of the reactants, formal half-cell potential (E°') and the kinetic parameters associated with each half-cell reaction. A change in the cathodic reaction has often been invoked as the only half-cell reaction changed by the presence of microorganisms on the surface of the stainless steel. Indeed, the bacterial community is known to increase cathodic reduction efficiency (Audouard et al., 1994; Holthe R. et al., 1989; Johnsen and Bardal, 1985; Larché et al., 2011; Le Bozec et al., 2001; Mollica and Scotto, 1996; Rogne and Steinsmo, 1996; Zhang and Dexter, 1995). In this study, we were interested in gaining further insight into the bacterial community of the stainless-steel surface immersed in seawater and its electrochemical activity in relation to the potential ennoblement. Previous studies have shown an inhibition of the ennoblement activity above a critical temperature (Dupont et al., 1997; Gümpel et al., 2006; Martin et al., 2003; Scotto et al., 1986; Thierry et al., 2015). In our setting, this critical temperature was between 38°C and 40°C, above which the ennoblement was inhibited despite the continuing presence of bacteria. We used that information to investigate the community composition between ennoblement at lower temperature vs. no ennoblement at higher temperature. A central result of this work is the identification of OTUs affiliated to '*Candidatus* Tenderia electrophaga' that were exclusively present under conditions leading to potential ennoblement, *i.e.* under 40°C and considerably enriched compared to natural seawater.

Other electroactive bacteria have been shown to be able to change the potential of electrode under anaerobic condition (Mehanna et al., 2009b), and a microbial community that was able to do the same in aerobic conditions was described as an electroactive biofilm community (Rimboud et al., 2017). This study is correlative and cannot formally establish a mechanistic link between the detected biomarker and the ennoblement, but the distinctive presence of an electrotroph bacteria in aerobic condition is, to our knowledge, a novel observation and suggest a possible metabolism for potential ennoblement. '*Candidatus* Tenderia electrophaga' can indeed accept electrons from a conductive surface while using oxygen as a terminal electron acceptor (Eddie et al., 2016). This activity is based on its extracellular electron transport system composed of cytochrome c oxidase complexes coupled with the reduction of oxygen and the fixation of carbon dioxide using the Calvin-Benson-Bassham cycle, making it a chemo-

electro-autotroph (Eddie et al., 2017). In that study, type IV pili genes were also proposed to play a role in the extracellular electron transport.

In the original study set up that led to the description '*Candidatus* Tenderia electrophaga' on a biocathode, the biofilm development developed a current density between 0.92 µA/cm^2 and 4.28 µA/cm^2 at a fixed potential of + 66 mV vs SCE (Malanoski et al., 2018). Our experimental set up does not include biocathodes but rather open-circuit conditions, meaning that no current was provided nor drawn to the surface microbial communities. However, a possible source of electrons could be the passivation current produced by the stainless steel. This current is due to the slow oxidation of iron and chromium atoms in the passive layer of the stainless steel, forming a thin film containing iron and chromium hydroxides (Marcus, 2011). Given the polarization curves obtained at 36°C and 40°C we showed that our coupons' passivation current is on the order of magnitude of 0.01 µA/cm^2. These values are of two orders of magnitude lower than those observed at microbial fuel cell biocathodes, but could potentially sustain the growth of electroactive bacteria under open-circuit conditions.

Overall, our results suggest that ennoblement could be explained by the following mechanism: the stainless steel would act as an electron source for electrotrophic bacteria via its passivation current, using extracellular electron transport mechanism coupled to oxygen reduction. This hypothesis is based on results of a study using a metabarcoding approach that comes with its own limitations as the sequenced DNA represent a fragment of the 16S rRNA gene and not the complete genome. Also, the biomarker approach does not consider bacteria that could be present at all temperatures but with a different activity at 40°C that would result in the absence of the potential ennoblement. These limitations would be overcome with the use of metagenomics, to assess the genetic potential of the bacterial communities, and metatranscriptomics to confirm if the actual genes expressed would support our hypothesis.

Alternatively, a model for ennoblement based on local pH change at stainless steel surface has been proposed (Dexter and Chandrasekaran, 2000) and requires the formation of a thick biofilm acting as a strong diffusion barrier. Our SEM observations of bacterial colonization after ennoblement do not

support this hypothesis, as after one week of exposure, the development of attached bacteria was at a very early stage and could be defined as sparse bacterial colonization rather than an actual biofilm. We did not observe a uniform three-dimensional structure of extracellular polymeric substance with embedded bacteria covering the whole surface of the stainless steel.

Another hypothesis invoked the contribution of hydrogen peroxide as a central electron acceptor during ennoblement (Landoulsi et al., 2008c; Scotto and Lai, 1998). H_2O_2 release by heterotrophic bacteria as a byproduct of oxygen respiration however requires a high concentration of electron donor (20 mM of D-Glucose in (Landoulsi et al., 2008b). This does not correspond to our environmental conditions and is thus unlikely a central explanation for ennoblement in natural seawater environments.

Besides '*Candidatus* Tenderia electrophaga', other OTUs were identified as biomarker, especially some bacteria able to use aliphatic hydrocarbons as energy and carbon source like *Oleiphilus* (Yakimov and Golyshin, 2014). These bacteria could originate from the seawater pipes that might be contaminated with a small amount of oil-derived components. This would favor hydrocarbon metabolism and therefore the development of these bacteria. We identified the genus *Marinobacter*, which includes oil degrading species, and which is also found in the cathodic enriched community where '*Candidatus* Tenderia electrophaga' was described by Eddie et al., 2017, and (Wang et al., 2015b). The presence of oil degrading bacteria in potential ennoblement conditions is intriguing, but their role has yet to be defined. Gammaproteobacteria are often reported in oxygen reducing biocathode communities (Milner et al., 2016; Rothballer et al., 2015; Strycharz-Glaven et al., 2013). They were also found to be dominant in this study (SI Figure 1 and 2), and four of the top ten biomarkers are also affiliated to this Gammaproteobacteria (*Oleiphilus*, *Candidatus* Tenderia electrophaga, *Aliikangiella*, *Marinobacter*). We found other biomarkers with poor taxonomical assignment, only to the order level, *e.g.* NRL2 (Alphaproteobacteria). Therefore, no hypotheses can be generated from the presence of these biomarkers.

The risk associated with ennoblement is pitting corrosion as the potential increase reach values close to pitting potential. The use of high grade stainless steel and short exposure time limited the risk of pitting

corrosion in this study. But future work could involve lower grade stainless steel to associate the bacterial communities to pitting corrosion

7. CONCLUSIONS

The rational for this study was the observation of a sharp temperature inhibition around 40°C of stainless steel ennoblement in the temperate seawater of the bay of Brest. We used this property to identify bacteria potentially involved in ennoblement. The detection as a biomarker for ennoblement of '*Candidatus* Tenderia electrophaga', a known electrotroph was a remarkable result. Based on recent literature on '*Candidatus* Tenderia electrophaga' activity, we proposed a new mechanism for ennoblement based on extracellular electron transfer with oxygen as a terminal electron acceptor. The electron donor for this reaction could be the passivation current resulting from slow surface stainless steel oxidation at the passivation layer interface.

CHAPTER II:
INFLUENCE OF DISSOLVED OXYGEN CONTENT

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

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1. RÉSUMÉ

L'anoblissement des aciers inoxydables (*i.e.* l'augmentation du potentiel libre) est associé avec la colonisation bactérienne. Un potentiel libre élevé augmente le risque de corrosion localisée car le potentiel critique de corrosion par piqure/crevasse peut être dépassé, surtout pour des aciers inoxydables de plus faible qualité. Dans cette étude, nous avons étudié l'influence de la teneur en oxygène dissous sur la corrosion caverneuse des aciers inoxydables duplex et super-duplex. Nous avons aussi utilisé le séquençage d'amplicons d'ADN pour identifier les bactéries associées à l'anoblissement. Au-dessus de 100 ppb d'oxygène dissous, on observe l'anoblissement du potentiel libre ce qui induit un risque de corrosion localisée. En-dessous de 100 ppb d'oxygène dissous, on n'observe plus l'anoblissement du potentiel libre et le risque de corrosion localisée est réduit. Nous avons identifié des bactéries associées à l'anoblissement des aciers inoxydables et affiliées à des bactéries décrites comme capable de dégrader des hydrocarbures. Le rôle de ces bactéries n'est pas clair pour l'instant, mais elles pourraient être associées à l'anoblissement des aciers inoxydables en eau de mer.

2. ABSTRACT

The ennoblement of stainless steel (e.g. the increase of open circuit potential) is associated with bacterial colonization. This increases the risk of localized corrosion as the critical pitting/crevice potential can be overcome, especially for lower grades 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 approx. 100 ppb of dissolved oxygen, the ennoblement of open circuit potential was observed leading to an increase risk of localized corrosion. Below approx. 100 ppb of dissolved oxygen, no ennoblement occurred and the risk of localized corrosion was reduced. We identified hydrocarbon degrading bacteria in correlation with the ennoblement of super-duplex stainless steel at saturated DOC. The role of these bacteria is not clear yet but their distribution indicate a possible involvement in stainless steel ennoblement in seawater.

3. 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 the biofilm-induced ennoblement (Audouard et al., 1994; Féron, 2005; Havn T., 1995; Holthe R. et al., 1989; Johnsen R., 1996; Johnsen R. and Bardal E., 1986; Larché et al., 2016; Larché N. et al., 2010; Le Bozec et al., 2001; Rogne T. and Steinsmo U., 1996; Scotto V. et al., 1996; Strandmyr and Hagerup, 1998; Zhang H. J. and Dexter S. C., 1995). The so-called ennoblement is a shift of the open-circuit potential (OCP) of stainless-steel to the noble direction, from below -100 mV/SCE to about +300/+350 mV/SCE. This has been attributed to the development of electroactive bacteria on the passive oxide layer (Audouard et al., 1994; Féron, 2005; Holthe R. et al., 1989; Johnsen R. and Bardal E., 1986; Le Bozec et al., 2001; Rogne T. and Steinsmo U., 1996; Scotto V. et al., 1996; Zhang H. J. and Dexter S. C., 1995). The other significant effect of the electroactive bacteria on passive materials is an increase of the cathodic reduction efficiency (*e.g.* cathodic reduction of dissolved oxygen) (Bergel et al., 2005; Le Bozec et al., 2001). 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 electroactive bacteria that are responsible of the ennoblement and the conditions for which they are promoted. This is particularly important to develop antibacterial strategies in for instance closed or semi-open cooling water circuits (Larché et al., 2013).

The role of temperature on the ennoblement have been investigated, showing that the incubation time for biofilm ennoblement is temperature dependent and can vary from one to two days at tropical temperatures (*i.e.* around 30°C) to about a month at 5°C (Larché et al., 2013). Also, the effect of electroactive bacteria vanishes at higher temperature and no ennoblement has been reported in the North Sea at temperature above 32°C, whereas a temperature limit of about 37°C has been reported in

the bay of Brest (Atlantic Ocean) (Holthe R., 1998; Johnsen R., 1996; Larché et al., 2015; Leballeur et al., 2015). The critical temperature for bacteria-induced ennoblement was above 40°C in the tropical sea of Singapore (Larché et al., 2015). These results suggest that the electroactive bacteria 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 have been investigated in other study and suggests the involvement of electrotrophic bacteria, which are able to catalyze the oxygen reduction with current provided by the cathode (Trigodet et al., 2019).

The effect of dissolved oxygen content (DOC) in natural seawater was shown to have significant influence on the ennoblement and consequently on the localized corrosion risks (Nice et al., 2017). Stainless steels with 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, in case if DOC concentrations are controlled (*e.g.* below 20 ppb) alternatives to highly alloyed grades might then be considered to reduce costs (*e.g*. PREN < 40). This can be the case for treated seawater injection metallic wells where oxygen scavengers are generally used. In this respect, more cost-efficient duplex stainless steels might be considered as alternative to super-duplex stainless steels.

However, at the microbiological scale (*i.e.* bacterial analysis) no data can be found in the literature on the influence of oxygen on bacteria associated with stainless steel. The identification and understanding of the bacteria that are present below and above the critical DOC for ennoblement could help at controlling the effective localized corrosion risk. The objective of the present study was to further investigate the critical dissolved oxygen content promoting bacterial-induced ennoblement and to perform microbiological analysis at low and saturated DOC. The conditions below and above the critical DOC for ennoblement were investigated and compared, both in terms of localized corrosion and on the nature of the bacteria.

4. EXPERIMENTAL PROCEDURE

4.1. MATERIALS

The materials used in this study were duplex stainless steel UNS S31803, and super-duplex stainless steel UNS SS32750. Samples were tested as plates of 100 mm x 50 mm x 10 mm. The nominal composition of the stainless steel is given in Table 2. Prior to exposure, the plates were washed during 20 min in 20% nitric acid and sterilized by autoclaving for 20 min at 121°C (dry cycle). Metallographic investigations were performed on the 2 tested alloys. Results are given in Figure 18. The austenite/ferrite spatial distribution (50/50 with approx. 10 µm austenite spacing) and the microstructure of the inspected alloys showed no abnormal deviations from expected microstructure features (Charles, 1991).

Table 2. Chemical composition of tested passive alloys (%wt – balanced Fe) and PREN.

Elements	C	Mn	S	Ni	Cr	Mo	N	Cu	PREN*
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

** PREN = %Cr + 3,3(%Mo + 0.5%W) + 16%N*

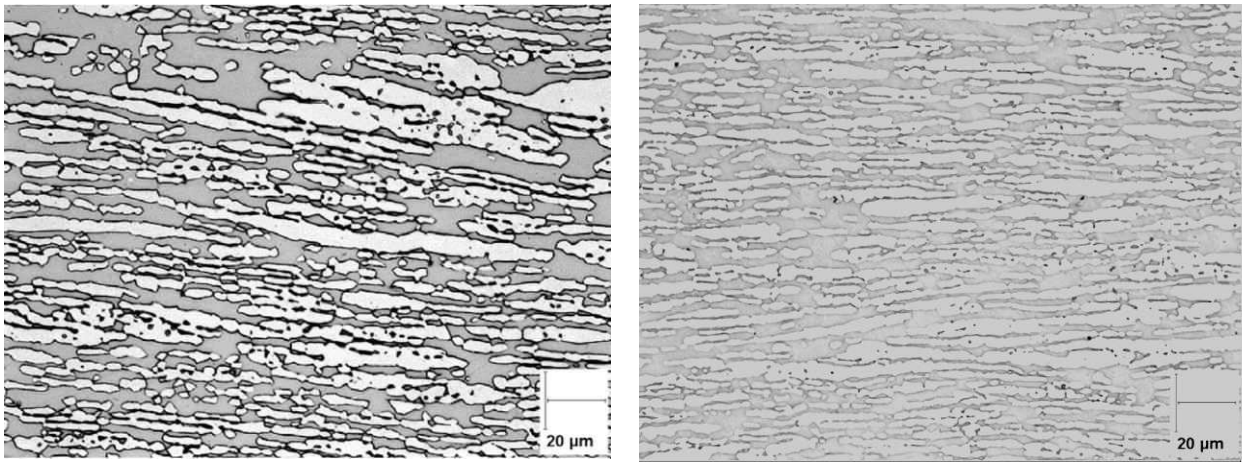

Figure 18. Metallographic cross sections of (left) S31803, (right) S32750.

4.2. OPEN-CIRCUIT POTENTIAL AND CREVICE CORROSION TESTING

The stabilized open-circuit potential (OCP) of the tested alloys have been measured as a function of DOC, in natural seawater. Titanium (grade 2) wires were used for electrical contact allowing OCP measurements. The OCP were measured with high impedance (>$10^{11}\Omega$) data loggers connected to a gel reference electrode Ag/AgCl/KCl. The electrodes were weekly calibrated with a certified saturated calomel electrode (SCE). The criterion for stabilized potential was arbitrarily fixed to no potential evolution of more than +/- 5 mV over 48h (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°C ±0.5°C (regulated by heating bands) and the renewal rate was about one complete renewal per day of the seawater in the cells (300L, *i.e.* about 12 L/h). The used natural seawater showed the standard characteristics of the Atlantic Ocean, with a salinity of 34°/$_{oo}$ ± 1 and pH = 8.1 ± 0.1. Several dissolved oxygen levels were selected from <10 ppb to saturation (6 ppm) in order to draw the OCP *versus* DOC curves and to define the critical DOC for bacterial-induced ennoblement. The DOC was controlled with the use of controlled mass gas flow regulators connected to pure nitrogen and oxygen. The DOC was measured with Light-Dissolved Oxygen (LDO) probes allowing measurement of oxygen trace (< 5 ppb). The principle of the set-up is given in Figure 19.

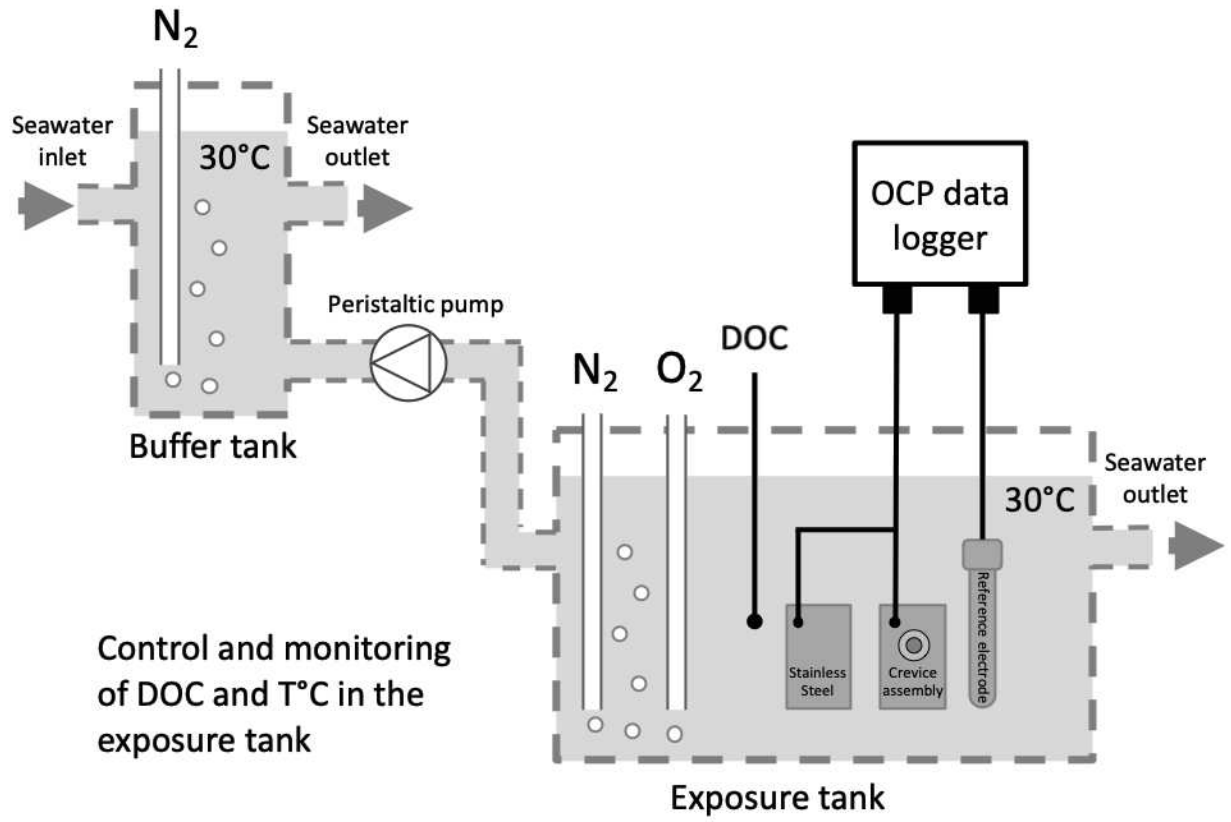

Figure 19. Principle of the set-up for dissolved oxygen control in electrochemical cells.

In parallel, crevice assemblies were used on the plate 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 plate coupons were made of PVDF (20 mm diameter gaskets), designed according ISO 18070:2015 recommendations (ISO, 2015). The gasket pressures of 20 N/mm² was applied on all systems, which is considered as a very severe crevice geometry for the used assembly (Larché et al., 2016). A schematic description of the crevice former for plate geometry is presented in Figure 20. 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 (ISO, 2015). The open-circuit potential of the crevice samples has been continuously monitored using a similar setup as described above for non-crevice samples. The monitoring of the potential allowed to

detect the eventual initiation of localized corrosion, *i.e.* indicated by large potential drop(s). The exposures were stopped when corrosion was visually detected, with maximum exposure duration of 3 months.

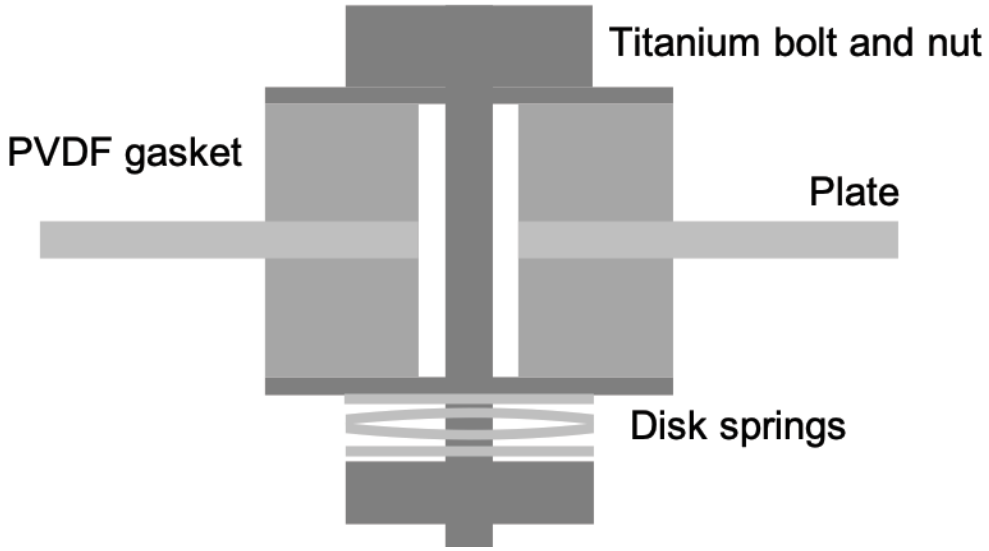

Figure 20. Schematic crevice former assembly based on ISO18070:2015 (ISO, 2015).

For all tested configurations (*i.e.* with and without crevice formers), five replicates have been exposed to allow statistical evaluations. For material affected by corrosion, a pickling step in 20% nitric acid prior to the evaluation was performed.

4.3. BACTERIAL DNA COLLECTION AND SEQUENCING

Many bacteria can settle and form a biofilm on the surface of a stainless steel coupon, but biofilm 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. Glass coupons can be used as non-conductive surface control to be compared with stainless steel bacterial communities.

A similar set up as described in Figure 19 was used to expose super-duplex stainless steel and to collect the bacteria at the surface of the material after two weeks of exposure. The two conditions used were fully aerated around 6 ppm and deaerated water under 10 ppb. Additional 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, United States) by gentle and uniform scratching into 100 mL of a Tris Buffered Saline (TBS) solution (50 mM Tris, 150 mM NaCl, pH 7.6) under sterile conditions ensured by a Bunsen burner that 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, United States) which were then transferred to PowerBiofilm® Bead Tubes from the PowerBiofilm DNA extraction kit (MoBio, United States).

The DNA extraction was performed according to the manufacturer's instructions of the PowerBiofilm DNA extraction kit (MoBio, United States). The V4-V5 region of the 16S 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, United States) reagent. DNA concentration was assessed with Quant-iT™ PicoGreen® dsDNA (Invitrogen, United States) prior to pooling the PCR products at equimolar concentration. Sequencing using Illumina MiSeq platform was performed at the Josephine Bay Paul Center (Woods Hole, United States). Sequences were deposited to the European Nucleotide Archive under the accession number PRJEB30977 (http://www.ebi.ac.uk/ena/data/view/PRJEB30977).

4.4. BIOINFORMATIC ANALYSIS OF THE DNA

The quality filtering of raw sequencing data was carried following recommendations in Minoche et al., 2011 while merging of paired-end reads with Illumina-Utils python scripts on demultiplexed raw reads (Eren et al., 2013b). We then performed OTU delineation with the Swarm algorithm using the default local linking threshold d = 1 (Mahé et al., 2015). We used VSEARCH (Rognes et al., 2016) to detect and

remove chimeras, and the Silva NR 128 database (Quast et al., 2013) for taxonomic assignment of Swarm representative sequences with Mothur (Schloss et al., 2009).

Stacked bar plots were produced with ggplot2 (Wickham, 2009). We used the two conditions “stainless steel with oxygen” and “glass with oxygen” to perform biomarker detection with LEfSe (Segata et al., 2011).

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

4.5. SEM IMAGING

Some coupons were fixed for scanning electron microscopy with 2.5% glutaraldehyde seawater for one hour, 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). The observations were made with a Hitachi SU3500 microscope (Hitachi High-Technologies, Germany).

5. RESULTS

5.1. OPEN-CIRCUIT POTENTIAL 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 21). Similar potentials have been measured for both alloys without pitting corrosion. The OCP decreased from about -100 mV/SCE to -450 mV/SCE in deaerated water when DOC decreased from 100 ppb to <5 ppb. Under the tested conditions, the bacteria-induced ennoblement was observed in the range of 70 ppb to 100 ppb, with an increase in OCP to about +200 mV/SCE. At higher DOC, the stabilized OCP after ennoblement is ranging 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 3. The super-duplex UNS S32750 only showed crevice corrosion at DOC of 600 ppb. At this level, the OCP range of un-creviced 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. The typical aspect of a creviced and not creviced coupon after exposure is given in Figure 22.

Table 3. 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 ISO18070:2015 with gasket pressure of 20 N/mm²

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

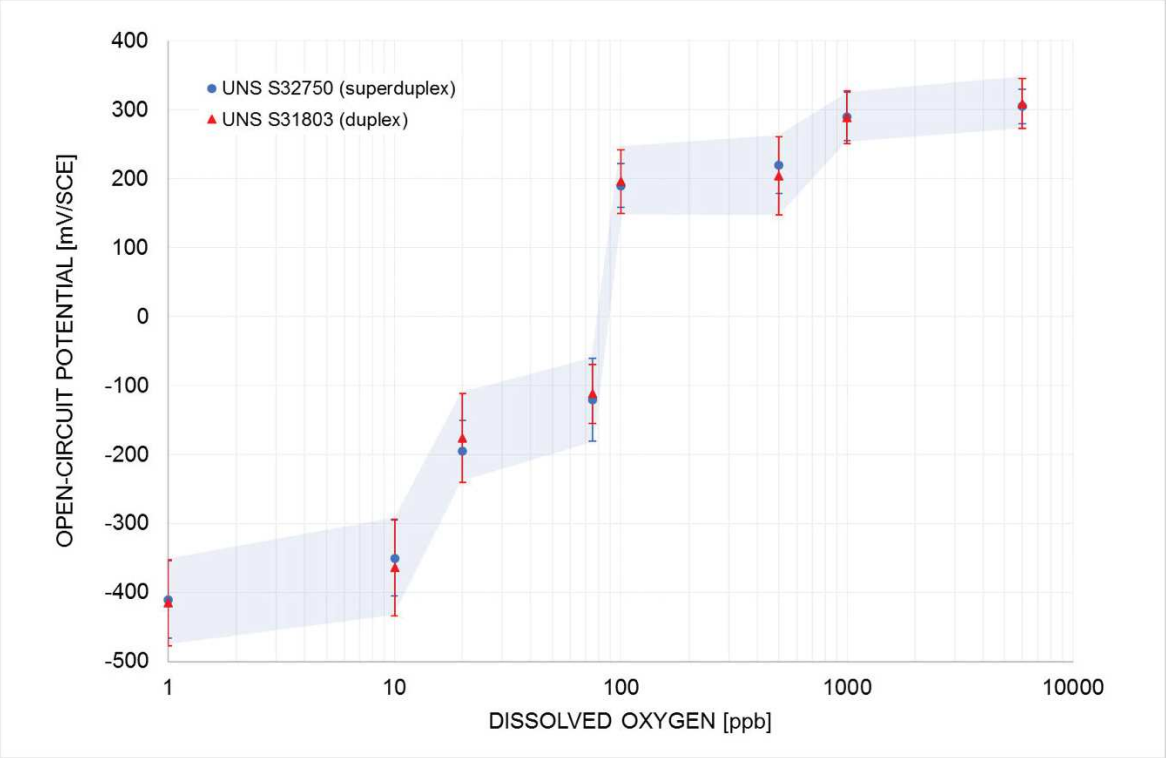

Figure 21. Stabilized OCP *versus* DOC curves in continuously renewed natural seawater at 30°C for stainless steels UNS S31803 and S32570.

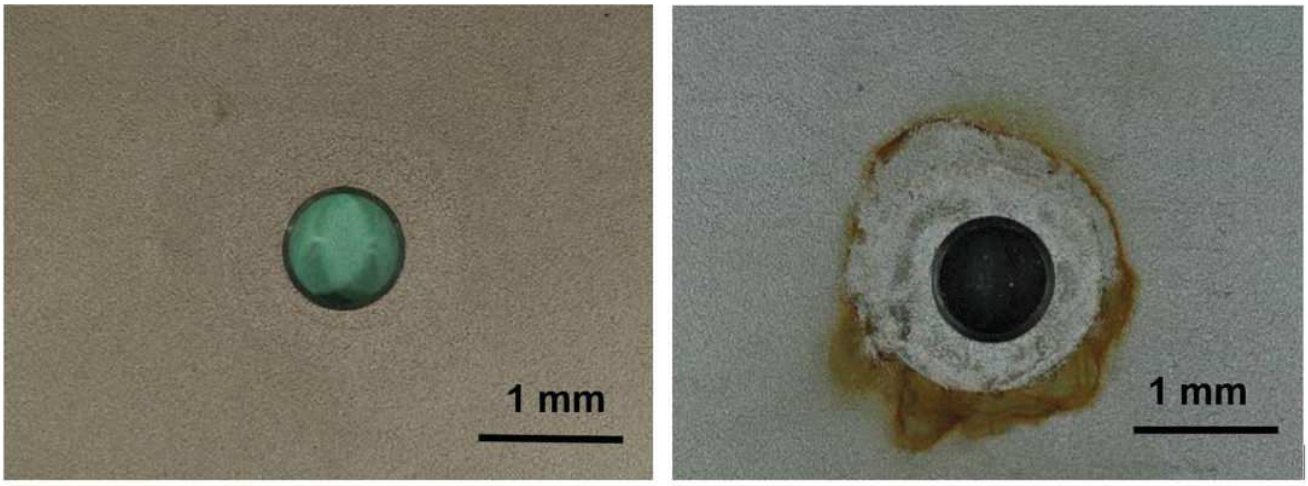

Figure 22. Typical aspect of crevice test specimen after exposure. (Left) without corrosion (S31803 at 100 ppb) — (Right) showing crevice (S31803 at 60 ppb).

5.2. MICROBIOLOGICAL ANALYSES

In parallel, we recorded the potential of stainless steel coupons used for the microbiological analysis during the two weeks of exposure (Figure 23). A typical ennoblement potential up to +300 mV/SCE mediated by bacteria was measured after three days in aerated conditions. In contrast, the potential decreased below -400 mV/SCE at the end of the exposure under 10 ppb of DOC.

In order to characterized 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 operational taxonomic units (OTUs), and 24,695 chimera-free OTUs finally representing 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 to *Muricauda*, Figure 24). Under anaerobic

conditions, the bacterial communities were similar between stainless steel and glass coupons, with only one OTU (affiliated to *Rhodothermaceae*, Figure 24) 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 stainless steel coupons affiliated to the family *Flavobacteriaceae* and the genus *Polycyclovorans*.

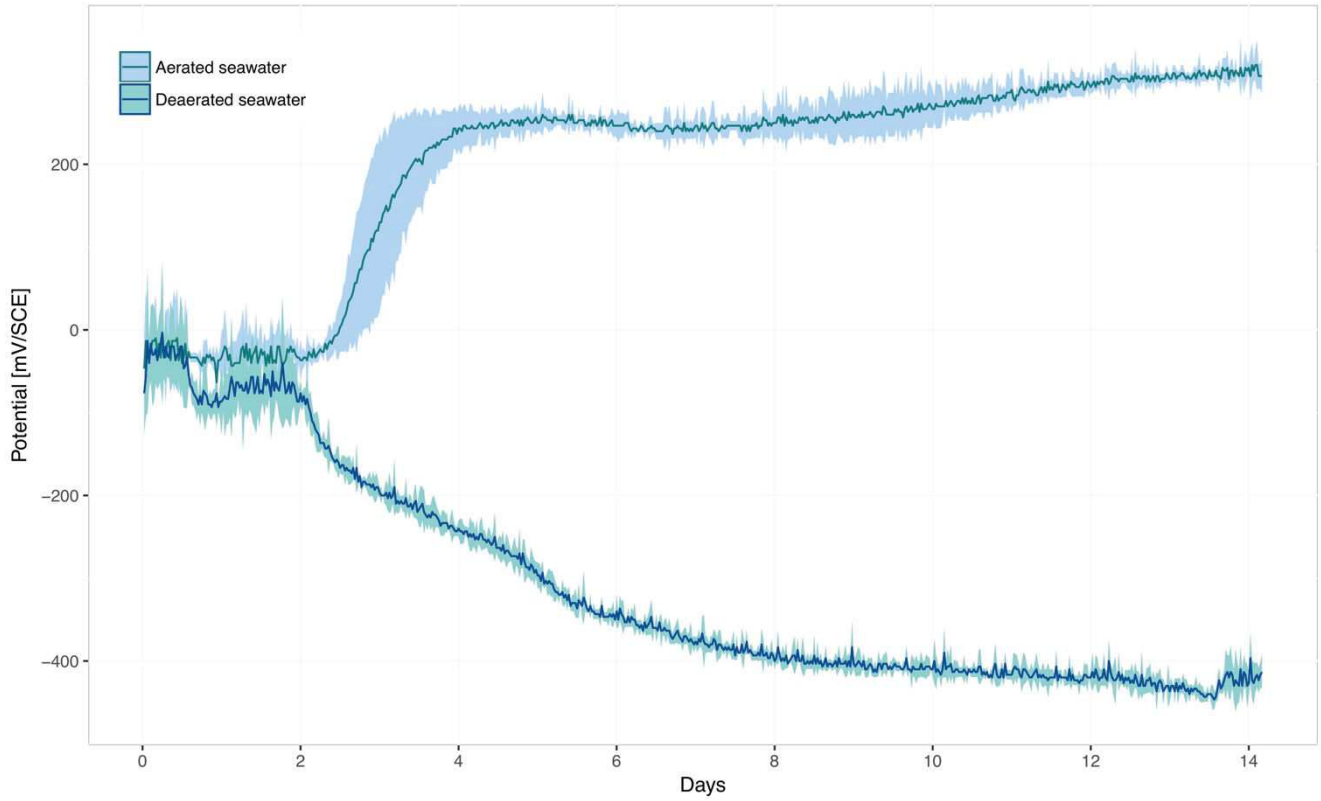

Figure 23. Open circuit potential of UNS S32570 in aerated (approx. 6 ppm) and deaerated (<10ppb) seawater.

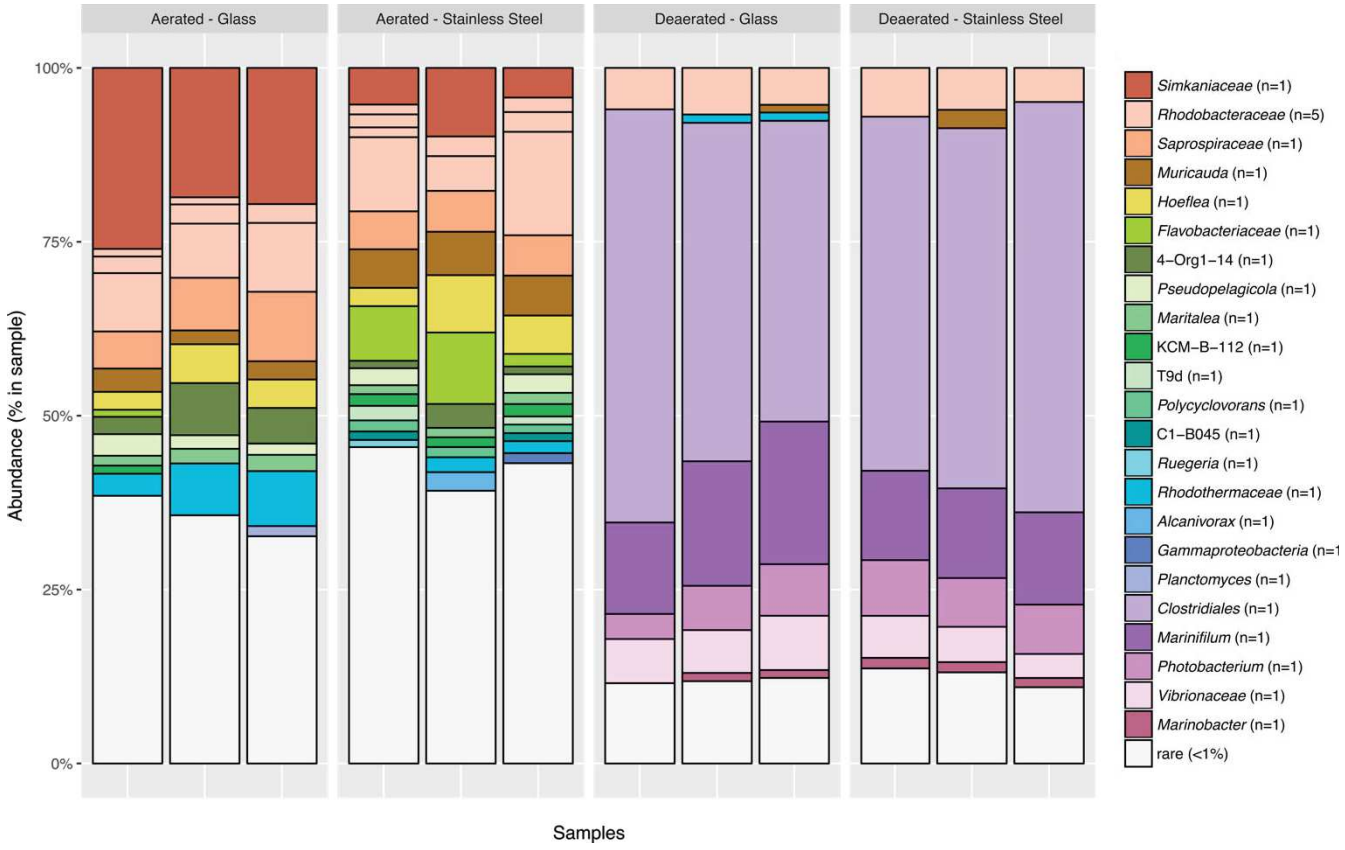

Figure 24. Relative abundance distribution of OTUs above 1% on stainless steel or glass coupons, aerated (approx. 6 ppm) and deaerated (<10ppb) seawater. The taxonomic assignment is represented by the different colors and the number of OTUs with a similar affiliation are shown as (n = x). SS means stainless steel in this figure.

To identify bacteria that are differentially represented on stainless steel and not on the glass coupons, we performed a biomarker detection and retained the ten OTUs with the highest LDA score from LEfSe analysis (Figure 25). These bacteria were all affiliated with microorganisms described as able to degrade hydrocarbon compounds.

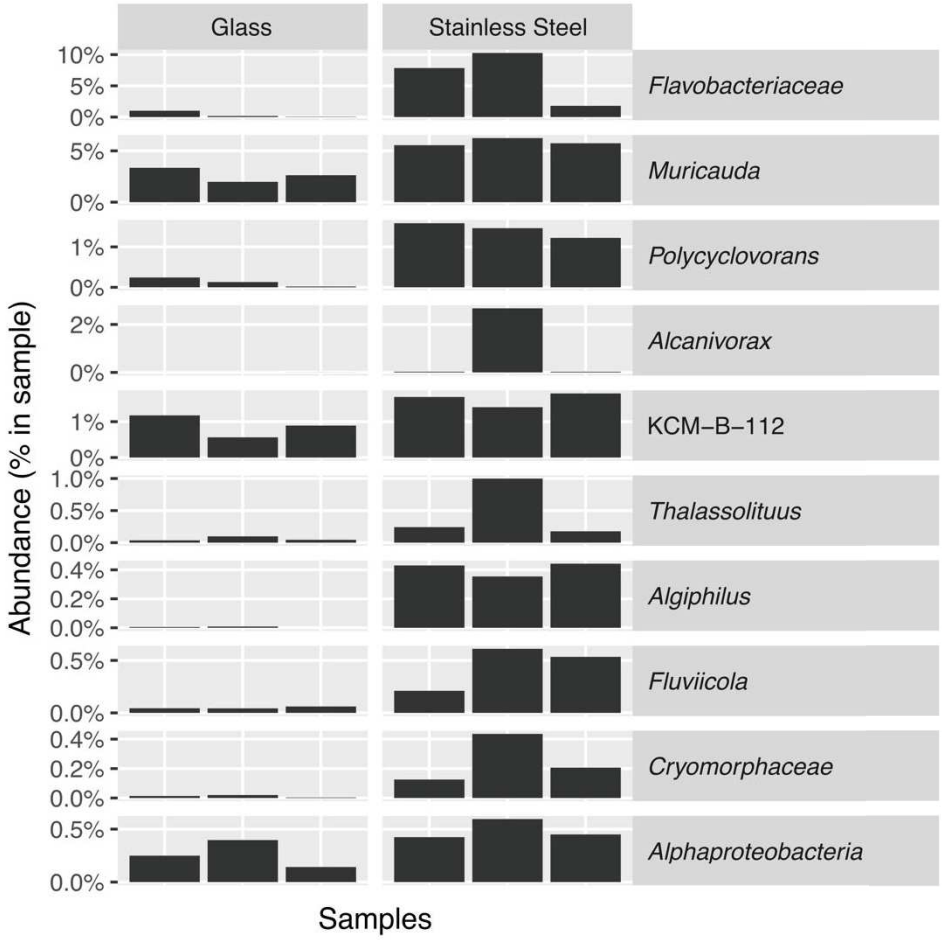

Figure 25. Relative abundance distribution of the ten best biomarker OTUs of the stainless steel under aerobic conditions. SS means stainless steel in this figure.

The bacterial colonization of the UNS S32750 under both conditions are shown in Figure 26. Various morphology of bacteria can be observed under the two distinct oxygen content conditions: bacillus, coccus, segmented and non-segmented filamentous bacteria. The bacterial morphology and coverage of the surface is relatively similar between the two conditions.

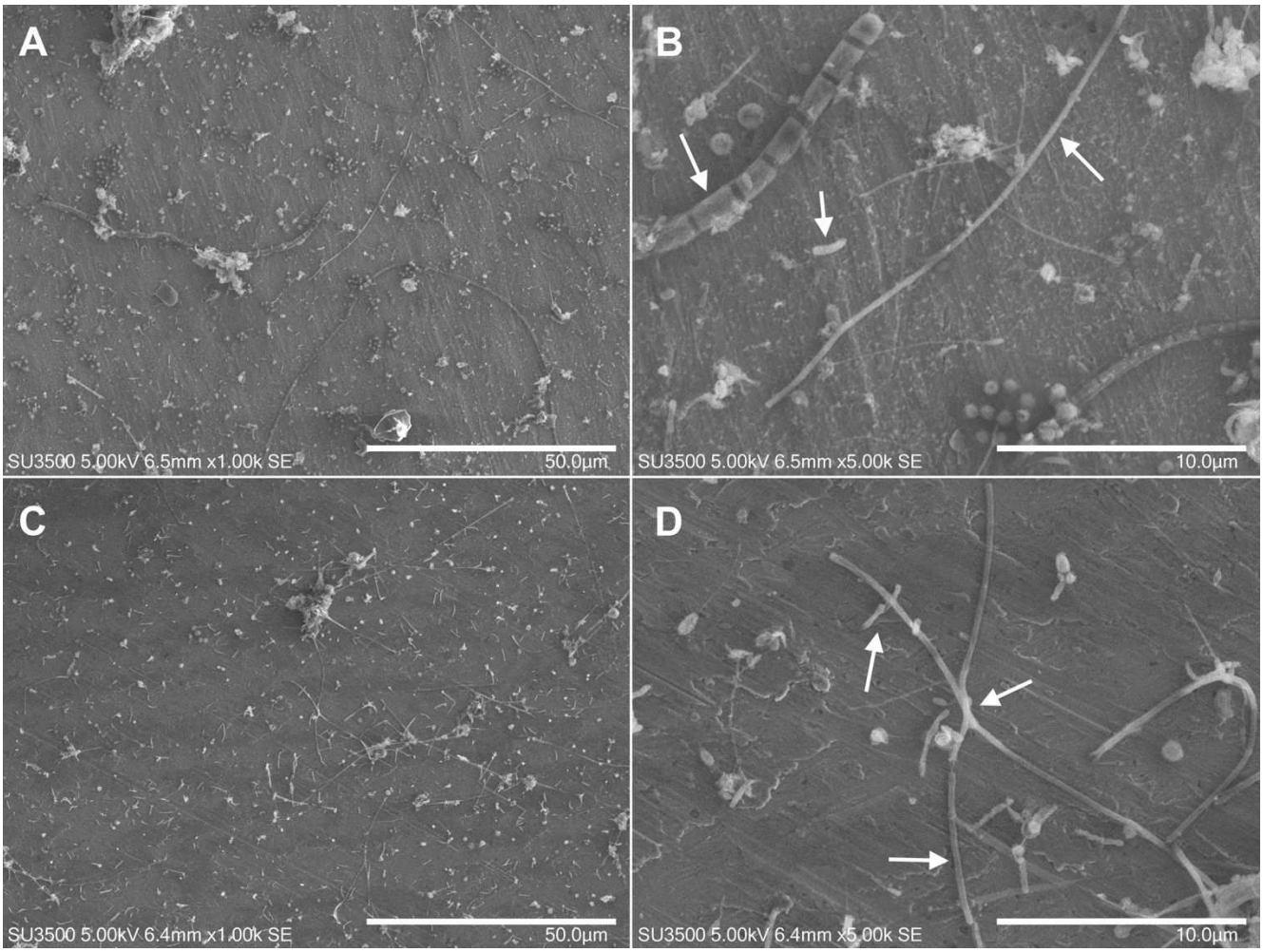

Figure 26. Scanning electron microscopy micrographs at 1K (A, C) and 5K (B, D) magnification. UNS532750 after two weeks of exposure, in aerobic (A, B) and anaerobic (C, D) conditions.

6. DISCUSSION

Low DOC below 70-100 ppb prevent the bacteria-induced ennoblement of stainless steel, limiting the risk 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 to 6 ppm), the ennoblement of OCP increased the risk of crevice corrosion and higher grade of stainless steel should be considered.

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 others 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 coherent 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 (Trigodet et al., 2019). A member of the *Alcanivoraceae* family was also found in a biocathode experiment where bacteria are fed by a cathode as sole electron donor source (Wang et al., 2015b). 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. Their recurrence in our studies and their presence in another study which involves electroactive bacteria indicate that they might have a role in the ennoblement.

Another biomarker of our study, *Muricauda,* was also identified by (Wang et al., 2015b), in a biocathode biofilm.

In this study we propose a list of bacteria associated with ennoblement (Figure 25), 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 is 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.

7. CONCLUSIONS

The dissolved oxygen content impact the open circuit potential and the bacterial communities. It was found that it exists a critical DOC between 70 and 100 ppb below which no bacteria-induced ennoblement is measured. 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, the biofilm-induced ennoblement is measured, increasing the risk of localized corrosion, and therefore requesting the use of more resistant alloys. The bacteria identified 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 (Trigodet et al., 2019), and associated with electroactive bacteria by other authors (Wang et al., 2015b). Future studies should consider and further investigate the role of hydrocarbon degrading bacteria to understand their relation with the ennoblement of stainless steel.

CHAPTER III: METAGENOMIC EVIDENCE OF ELECTROTROPHIC BACTERIA

Metagenomic evidence of an electroactive bacteria associated with stainless steel ennoblement

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Draft paper, additional results required before submission to publication.

1. RÉSUMÉ

Les microorganismes peuvent augmenter le potentiel libre des aciers inoxydables immergés en eau de mer par un phénomène que l'on appelle anoblissement. Le changement de potentiel, de l'ordre de plusieurs centaines de millivolts, augmente les risques de corrosion localisée. Malgré l'importance de ce phénomène, on ne connait que peu de choses concernant les mécanismes microbiens associés. Nous avons étudié le rôle des bactéries électrotrophes par métagénomique et avons comparé des conditions de potentiels libre et contrôlés. Nous avons reconstitué un génome, issu de l'assemblage métagénomique, proche du génome de '*Candidatus* Tenderia electrophaga' (ANI: 97.1%) et nous avons identifié les mêmes systèmes présumés de transport d'électron extracellulaire. Cette bactérie a été enrichie sur une biocathode lors d'une autre étude, mais nous l'avons identifiée sur des aciers inoxydables en condition de potentiel libre. Ces résultats supportent un nouveau modèle pour expliquer l'anoblissement dans lequel les bactéries électrotrophes seraient capables d'influencer le potentiel libre.

2. ABSTRACT

Microorganisms increase the open-circuit potential of stainless steel immersed in seawater in a phenomenon called ennoblement. This change of potential of several hundreds of millivolts raises the chance of localized corrosion. Despite the importance and impact of ennoblement, little is known about the actual microbial mechanisms involved. We investigated the role of electroactive bacteria with metagenomics and by comparing open circuit with controlled potential conditions. We recovered a metagenome-assembled genome close to the genome of '*Candidatus* Tenderia electrophaga' (ANI: 97.1%) and we identified the same putative extracellular electron transport system. This bacterium was previously enriched in a biocathode system, but we found it in open circuit condition on stainless steel. The results support a model in which electroactive bacteria would be able to modulate the open circuit potential, and consequently mediate the ennoblement.

3. INTRODUCTION

Metals and alloys immersed in oxic seawater can form an electrochemical cells in which the oxidation of the metal is the anodic reaction, and the reduction of oxygen is the cathodic reaction. In the case of stainless steel, various oxides (Fe, Cr, Mo) will form a passive layer that considerably reduce the electron flow between the anode and cathode, and provide corrosion resistance. Stainless steel exhibit a measurable electrochemical potential between these two half-cells and is called open circuit potential (OCP) as no external current are provided. The stainless steel ennoblement is a well-known phenomenon which consists in the increase of the OCP in time (Mollica and Trevis, 1976). This increase of OCP is usually in the range of + 400-500 mV. The central problem with ennoblement is the enhance probability of pitting and crevice corrosion as the OCP gets closer to the pitting corrosion potential (Mollica, 1992; Zhang and Dexter, 1995). The ennoblement is associated with the colonization and development of microorganisms on the stainless steel surface (Gümpel et al., 2006; Le Bozec et al., 2001; Motoda et al., 1990; Scotto and Lai, 1998; Wei et al., 2005).

Stainless steel are of critical importance in marine constructions and despite the importance of ennoblement, little is known about the microbial mechanisms involved. An increased oxygen reduction efficiency, also referred as a cathodic depolarization, have been often measured (Audouard et al., 1994; Holthe et al., 1989; Johnsen and Bardal, 1985; Larché et al., 2011; Le Bozec et al., 2001; Mollica and Scotto, 1996; Rogne and Steinsmo, 1996; Thierry et al., 2015; Zhang and Dexter, 1995), but there is not yet a unifying mechanism that could explain the ennoblement for all reported conditions.

In a previous study using 16S rRNA marker genes and a temperature gradient experiment, we identified '*Ca.* Tenderia electrophaga' as a biomarker of ennoblement (Trigodet et al., 2019). '*Ca.* Tenderia electrophaga' is an electrotrophic bacteria enriched in a biocathode system where it uses the electrode as electron donor to reduce oxygen in the medium (Eddie et al., 2016). It is also an autotrophic bacterium, able to fix carbon dioxide. The role of electroactive bacteria in ennoblement has not been fully addressed yet, but they have been shown to be able to change OCP in other condition than seawater (Mehanna et al., 2009a; Rimboud et al., 2017). Electroactive bacteria are able to use extracellular electron acceptor (electrogenic) or donor (electrotrophic) with the mean of dedicated extracellular

electron transport (EET) systems, *e.g.* outer membrane cytochromes or electron transport shuttles (flavins, phenazines) (Philips et al., 2015).

In this study, we aim at identifying electrotroph bacteria that can grow on stainless steel surfaces during ennoblement. Our working hypothesis is that among all bacteria developing at this interface, only the electroactive ones will respond to controlled potential modulation. We thus exposed stainless steel coupons under both OCP and potentiostatic conditions that inhibited electrotrophic bacteria. Using genome resolved metagenomic approaches, we could identify population genome differentially responding to potential modulation and inspect their potential metabolism.

4. MATERIAL AND METHODS

4.1. MATERIAL

We used super-duplex stainless steels (S32750) coupons as described in Trigodet et al., 2019. Plates of 100 mm x 50 mm x 10 mm (French Corrosion Institute, France) with a nominal composition of 25.1% Cr, 7.0% Ni, 3.8% Mo, 0.13% Cu, 0.29% N, completed with Fe. The pitting resistance equivalent numbers is 42.3 (%Cr + 3,3(%Mo + 0.5%W) + 16%N). Prior to the experiment, the coupons were washed for 20 min in 20 % nitric acid and sterilized by autoclaving for 20 min at 121°C (dry cycle). Additional glass coupons of the same size were used as non-conductive surface control to discriminate between microorganisms with a general surface-attached lifestyle as compared to the more specifically stainless steel bound bacteria.

4.2. EXPERIMENTAL SET-UP

Our experimental design included 5 sets of samples composed of coupons in open-circuit or controlled potentials, and glass plates. The potential conditions and the rationale for each condition are summarized in Table 4: (1) open circuit potential for 13 days; (2) open circuit potential for 12 days, then the potential was decreased by 100 mV under the last OCP value; (3) same as for (2) but the potential was raised by 50 mV above the last OCP value; (4) the potential was imposed to +300 mV vs. SCE for the 13 days, (5) glass coupons.

We exposed all coupons simultaneously (2017-12-07) in a 300L seawater tank fed with renewed fresh seawater at an approximated 12L/h rate. Stainless steel samples were held by a titanium wire to measure the open-circuit potential with an Ag/AgCl reference electrode. The electrodes were calibrated with saturated calomel electrode (SCE) REF421 (Radiometer, France). The use of titanium wires has been documented in previous works and inhibits galvanic corrosion at the point of contact with stainless steel (Espelid, 2003). We used potentiostat (Microstat from Sycopel) to impose specific potentials in a three electrodes system with a stainless steel as the working electrode, titanium wire as counter electrode, and Ag/AgCl as a reference electrode. Five replicates were used for all conditions and exposed for 13

days at 36°C. Measurements of potential were recorded every 30 min for three of five replicates due to the number of channels available. The rest of the replicates were manually checked regularly during the experiment and did not differ from the recorded samples.

Table 4. Conditions summary and rationale. OCP for Open Circuit Potential

Condition	Material	Potential	Rationale
1	Stainless steel	OCP	Standard ennoblement microbial communities
2	Stainless steel	OCP and cathodic depolarization -100 mV vs OCP for the last 24h.	Enhanced electrotrophic activity during the last 24h
3	Stainless steel	OCP and anodic depolarization +50 mV vs OCP for the last 24h	Reduced electrotrophic activity during the last 24h
4	Stainless steel	Potential fixed at +300 mV vs Ag/AgCl	Prevent electrotrophic bacteria colonization
5	Glass	-	Non-conductive control surface

4.3. CELL COLLECTION

Surface cells were collected after 13 days for immersion using a sterile cell lifter (Thermo Fisher Scientific, United States) and by gentle and uniform scratching into 100 mL of a Tris Buffered Saline (TBS) solution (50 mM Tris, 150 mM NaCl, pH 7.6) under sterile conditions ensured by a Bunsen burner, keeping the immediate surrounding area sterile. TBS solutions were then stored in ice for transport to the molecular lab. TBS solutions were filtered through 0.22 µm GTTP polycarbonate membranes (Merck Millipore, United States) which were then transferred to PowerBiofilm® Bead Tubes from the DNeasy PowerBiofilm extraction kit (Qiagen, Germany). We previously collected control samples that were visualized by scanning electron microscopy to ensure removal of the cells attached to the surface (results not shown).

4.4. DNA EXTRACTION, METAGENOMIC LIBRARY PREPARATION AND SEQUENCING

We used the DNeasy PowerBiofilm kit for DNA extraction following the manufacturer's instructions. We used the TruSeq Nano DNA LT Library Preparation Kit to prepare libraries with 100 ng of DNA input for metagenomics sequencing on an Illumina NextSeq performed at the Josephine Bay Paul Center (Woods Hole, United States).

4.5. ASSEMBLY, BINNING AND FUNCTIONAL ANNOTATION

We used the snakemake (Köster and Rahmann, 2012) metagenomic workflow proposed in the Anvi'o software v5.3 (Eren et al., 2015).

The quality filtration of the demultiplexed raw reads was carried out with Illumina-Utils python scripts (Eren et al., 2013b) following recommendations in Minoche et al., 2011. A co-assembly of all samples was performed with MEGAHIT v1.1.1 (Li et al., 2016) with the option --meta-sensitive. We used Anvi'o to profile the contigs with Prodigal v2.6.3 (Hyatt et al., 2010) for genes identification, and HMMER3 v3.1b2 (Mistry et al., 2013) to identify the single-copy core genes collections for bacteria (Campbell et al., 2013), archaea (Rinke et al., 2013) and eukaryotes (Simão et al., 2015). We used Centrifuge v1.0.1 (Kim et al., 2016) and the NCBI's NT database for gene-level taxonomic annotation.

Short reads were mapped back to the contigs with Bowtie2 v2.3.1 (Langmead and Salzberg, 2012) and the recruited reads were sorted into BAM files with samtools v1.3.1 (Li et al., 2009). Anvi'o was used to estimate the coverage and detection of each contigs based on the BAM files, and combine the mapping profiles into a merged profile.

Contigs were automatically binned with CONCOCT (Alneberg et al., 2014) into a constrained number of bins (n = 80) to minimize the fragmentation error before manual refinement of each bins with the anvi-refine-bin command. We identified bins as either high quality metagenome-assembled genomes (MAGs) (> 90 % completion, < 5 % redundancy) or medium quality MAGs (> 50 % completion, < 10% redundancy) (Bowers et al., 2017).

We assigned functions to the genes identified by Prodigal with the NCBI COG (Galperin et al., 2014) and Pfam v32.0 (El-Gebali et al., 2019) using anvi-run-ncbi-cog and anvi-run-pfams from Anvi'o. We also used the KEGG databases with GhostKOALA (Kanehisa et al., 2016).

The Anvi'o pangenomic workflow was used twice: a first time to compare MAG_79_1 with '*Ca.* Tenderia electrophaga', and a second time to include six other known electroactive bacteria: *Acidithiobacillus ferrooxidans* ATCC 23270, *Geobacter sulfurreducens* PCA, *Mariprofundus ferrooxydans* PV-1, *Rhodopseudomonas palustris* CGA009, *Shewanella oneidensis* MR-1, *Sideroxydans lithotrophicus* ES-1. DIAMOND (Buchfink et al., 2015) was used to compare similarity between amino acid sequences and the MCL algorithm (Van Dongen and Abreu-Goodger, 2012) identified the gene clusters with the default parameters. ANI scores were generated with the anvi-compute-ani command in Anvi'o.

5. RESULTS

5.1. OCP AND POTENTIAL

Coupons potentials were highly reproducible among the replicates and conditions (Figure 27). At the beginning of the exposure, the mean OCP for the condition (1)(2)(3) was -110 mV (± 9 mV) vs. SCE and increased after 3-4 days to reach + 212 mV (± 3 mV) vs. SCE at 21 days. The sample were collected simultaneously after 13 days.

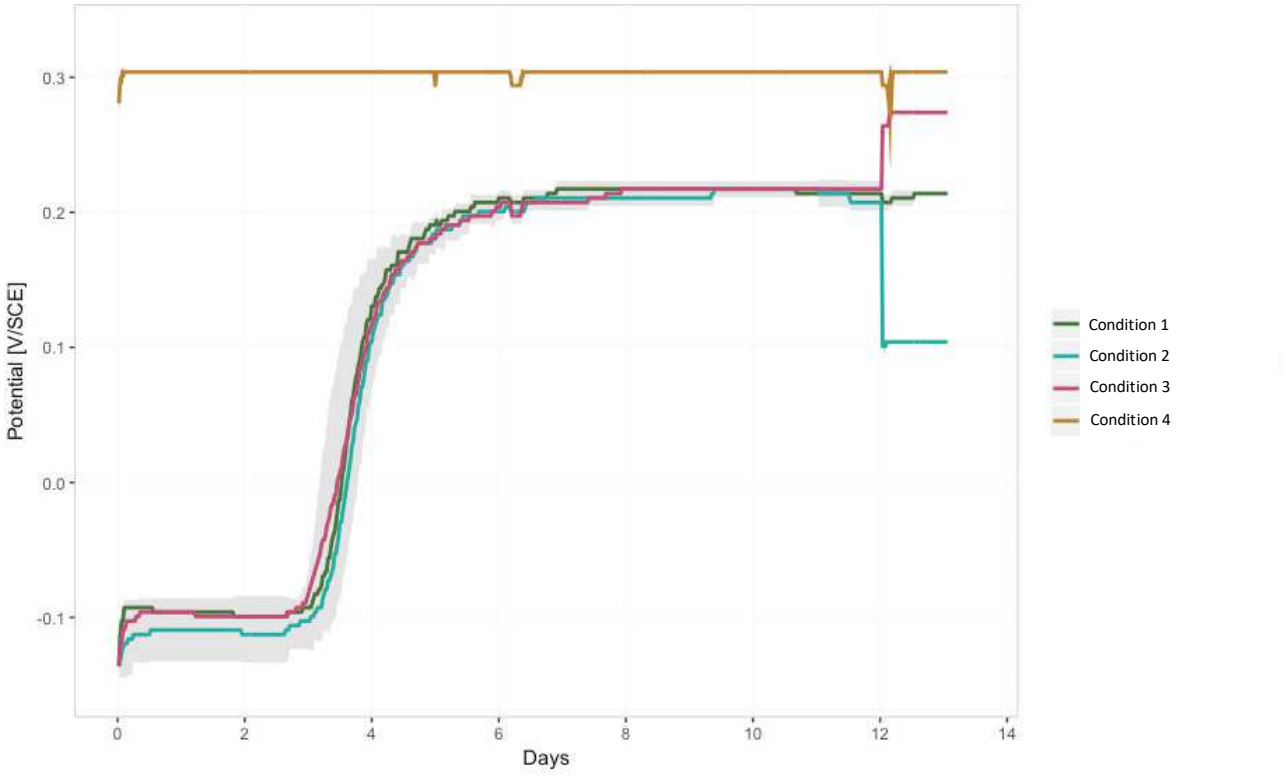


Figure 27. Open-Circuit Potential (OCP) versus time. Condition (1) in green, (2) in blue, (3) in red, (4) in brown. 95% confidence area is shown in grey

5.2. METAGENOME AND MAG_79_1

We sequenced 25 metagenomic libraries for a total of 938 734 769 raw reads. After the quality filtering, 893 678 555 sequences were retained for the assembly with MEGAHIT resulting in 310 167 contigs (N50: 28 970 bp). The 80 bins automatically made with CONCOCT were refined into 397 bins of which 230 were qualified as medium quality MAGs (> 50% completion, < 10% redundancy) and 82 high quality MAGs (> 90% completion, < 5% redundancy)(Table 5).

We represented the 82 high quality metagenome-assembled genomes (MAGs) recovered from the metagenomic analysis, and their distributions in different samples and conditions (Figure 28.A). Most MAGs were present in all conditions, with a high reproducibility among replicates. We found only one MAG with a systematic differential average abundance according to the conditions of collection, named MAG_79_1 hereafter. Its distributions was limited to the condition 1-2-3, with no or few sequences from condition 4, and 5 mapping to this MAG. We observed a weak coverage abundance in the replicate 5 of condition 5, which can be explained by the very high sequencing depth of this particular sample (190 102 712 reads for that sample, as compared to a median number of reads of 66 230 940). This could had led to some sparse read mapping to MAG_79_1. We further inspected MAG_79_1 contigs distribution across replicates and conditions and found that first, the split coverage was homogenous across the entire MAG, and secondly, that the differential relative abundance of each contig in the MAG was consistent with the pattern observed between conditions at the MAG level (Figure 28.B).

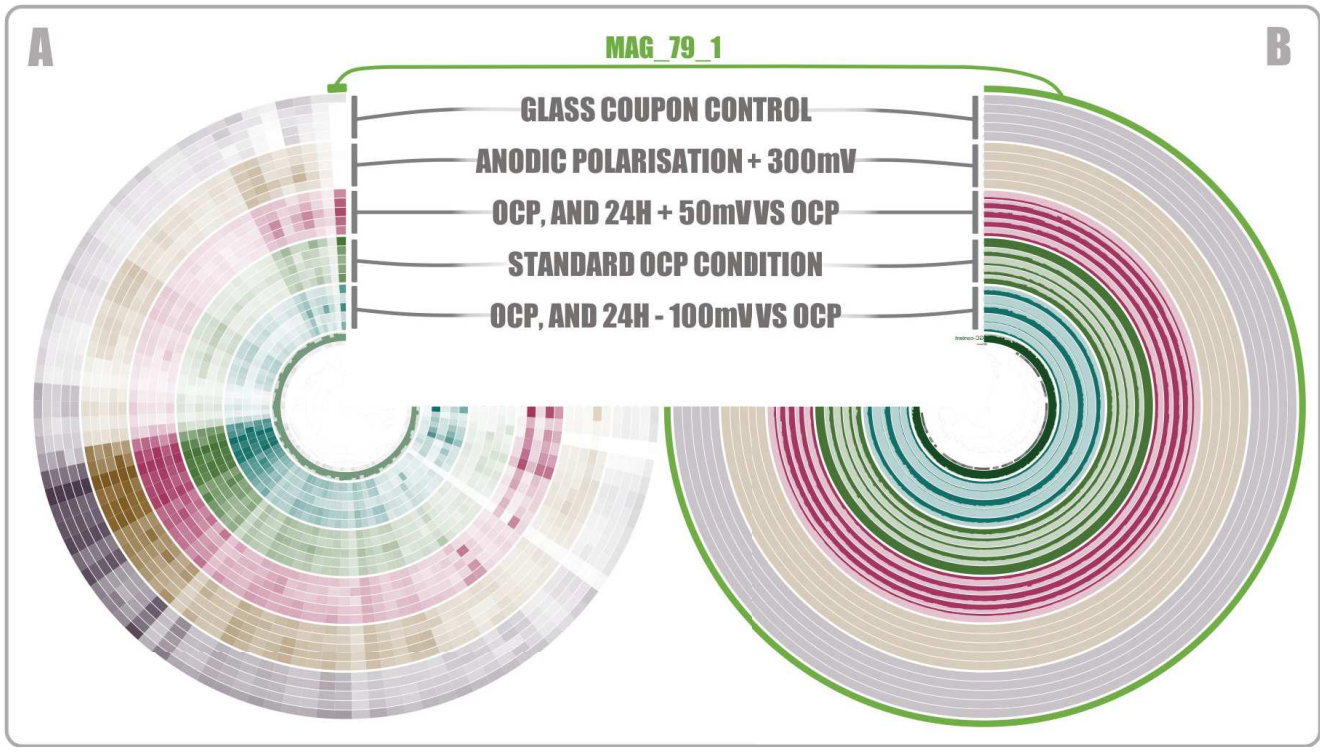


Figure 28. Overview of the metagenomic high quality MAGs with a close up view of MAG_79_1. (A) Representation of the 82 high quality MAGs obtained by co-assembly. The central cladogram orders the MAGs according to their distribution across samples, and layers represent MAG coverage in each sample. Replicate samples are grouped in successive layers and colored per condition. The color intensity represent the mean coverage of the metagenomic dataset. (B) Leaves of the central cladogram represent MAG_79_1 splits—*i.e.* contigs split to a maximum size around 20kb—and each layer represent mapping results from each sample on these splits. The colored bars represent the max normalized ratio, which means that—for each split—the bar will be full for the sample with the maximum coverage value and the rest will be scaled to this value.

Table 5. MAGs estimates. Includes percentage abundance and redundancy based on single occurrence of SCGs.

MAG	Percent completion	Percent redundancy	Length (total nucleotide)	Number of contigs	GC content (%)
MAG_08_5	99,28	0,72	4 374 965	16	38,18
MAG_08_6	100,00	0,00	2 456 650	17	34,32
MAG_12_2	94,24	2,16	8 128 786	141	72,34
MAG_12_9	96,40	2,16	3 693 717	42	70,04
MAG_15_11	96,40	0,00	3 182 842	20	37,35
MAG_15_12	94,24	0,72	4 661 729	110	41,92
MAG_15_13	97,12	1,44	2 517 375	49	40,41
MAG_15_6	94,96	0,00	3 215 308	17	41,17
MAG_15_8	99,28	0,72	2 389 712	36	41,13
MAG_15_9	95,68	0,00	3 664 209	82	40,68
MAG_16_1	99,28	0,72	6 687 775	40	67,65
MAG_16_5	97,84	0,72	7 637 324	66	68,81
MAG_18_1	92,09	3,60	3 605 072	503	61,53
MAG_18_2	94,96	4,32	7 968 514	207	59,36
MAG_21_1	97,84	3,60	6 047 675	42	74,06
MAG_21_6	97,84	2,88	8 397 558	82	73,33
MAG_22_1	95,68	2,16	6 224 484	116	50,85
MAG_23_1	90,65	1,44	5 111 139	207	66,29
MAG_24_1	96,40	4,32	10 236 754	219	58,61
MAG_24_2	96,40	2,16	7 620 534	33	61,03
MAG_24_3	93,53	2,16	11 216 601	781	57,46
MAG_25_1	91,37	1,44	4 697 012	90	64,95
MAG_26_1	92,81	0,72	3 432 703	18	58,75
MAG_26_2	98,56	1,44	3 517 871	28	52,24
MAG_26_3	94,96	4,32	9 208 356	119	55,52
MAG_26_4	94,96	2,88	8 732 674	77	53,05
MAG_27_12	97,12	0,72	5 178 160	17	47,77
MAG_27_3	97,12	1,44	3 667 014	18	50,39
MAG_27_5	100,00	0,72	2 173 130	4	48,04
MAG_27_7	97,84	3,60	8 429 996	74	46,43
MAG_27_9	96,40	3,60	8 606 967	136	47,20
MAG_30_3	98,56	0,00	2 668 939	30	66,18
MAG_35_1	94,24	1,44	3 158 629	21	56,17
MAG_35_3	98,56	0,72	4 484 090	15	53,16
MAG_35_6	100,00	1,44	3 018 169	19	58,27
MAG_35_7	100,00	2,16	3 217 303	37	56,67
MAG_36_2	92,81	1,44	4 480 751	286	59,31
MAG_37_1	97,84	2,88	3 934 433	33	62,20
MAG_37_3	96,40	2,88	3 604 092	35	61,71
MAG_37_4	90,65	0,00	4 379 334	50	62,63
MAG_38_4	96,40	1,44	4 298 806	40	69,26
MAG_38_5	93,53	0,72	3 471 607	71	67,40

MAG_39_3	96,40	4,32	8 679 367	187	56,91
MAG_40_1	96,40	0,72	4 647 562	29	63,00
MAG_40_2	97,84	0,72	4 587 803	25	59,91
MAG_40_3	97,84	2,88	3 741 146	31	66,60
MAG_40_4	93,53	1,44	3 729 922	140	61,25
MAG_41_1	97,84	0,00	5 026 076	113	51,77
MAG_41_4	96,40	4,32	5 131 634	122	52,84
MAG_42_1	94,96	0,72	4 838 003	68	63,18
MAG_42_4	100,00	0,00	4 969 077	36	62,96
MAG_43_1	97,84	0,72	5 653 894	157	63,07
MAG_44_3	96,40	0,72	2 299 892	122	70,03
MAG_44_8	90,65	1,44	6 090 985	45	72,31
MAG_47_1	96,40	4,32	11 239 207	231	63,03
MAG_49_2	91,37	2,16	6 518 943	552	62,93
MAG_49_5	92,81	0,00	2 815 088	20	57,13
MAG_50_1	97,12	0,72	4 521 388	444	69,15
MAG_50_2	93,53	0,72	5 471 393	67	69,53
MAG_51_1	91,37	2,88	4 669 985	110	61,95
MAG_52_2	100,00	1,44	3 592 927	46	61,05
MAG_52_3	92,81	3,60	3 312 015	247	61,92
MAG_53_1	99,28	0,00	3 664 050	18	41,51
MAG_53_8	99,28	0,72	2 652 143	57	41,12
MAG_54_1	97,84	1,44	4 785 343	296	54,81
MAG_56_2	97,84	4,32	3 488 162	170	59,10
MAG_58_1	97,84	4,32	7 485 708	66	66,76
MAG_59_1	99,28	1,44	5 529 655	211	50,07
MAG_60_1	90,65	0,72	2 170 795	235	55,29
MAG_62_2	99,28	2,16	3 992 441	14	57,76
MAG_62_3	92,09	0,00	3 598 107	35	52,17
MAG_63_1	99,28	2,16	2 056 898	56	41,93
MAG_63_4	100,00	0,72	4 929 574	76	45,38
MAG_63_5	98,56	1,44	3 540 408	136	45,52
MAG_65_1	98,56	0,72	3 157 094	56	46,16
MAG_65_2	92,09	1,44	8 647 825	61	46,33
MAG_66_2	91,37	0,00	2 722 436	16	50,01
MAG_71_1	100,00	0,72	2 485 250	8	44,37
MAG_71_2	100,00	0,00	3 313 306	26	44,69
MAG_71_3	97,84	0,00	2 006 140	131	40,26
MAG_74	95,68	1,44	6 663 405	99	54,09
MAG_79_1	100,00	0,72	3 768 194	81	58,36

5.3. COMPARATIVE GENOMIC

A partial 23S rRNA gene was recovered from the MAG_79_1 and the best match corresponded to '*Ca.* Tenderia electrophaga' (96% similarity). To investigate the relation between MAG_79_1 and '*Ca.* Tenderia electrophaga', we used the average nucleotide identity (ANI). This ANI score was 97.1% identity for a coverage of 82.8%. Using the pangenomic workflow of Anvi'o, using the total 7 055 genes calls, we defined 3 762 gene cluster, with 2 909 shared between the two, 444 unique for MAG_79_1 and 409 unique for 'Ca. Tenderia electrophaga', including both genome and plasmid (Figure 29).

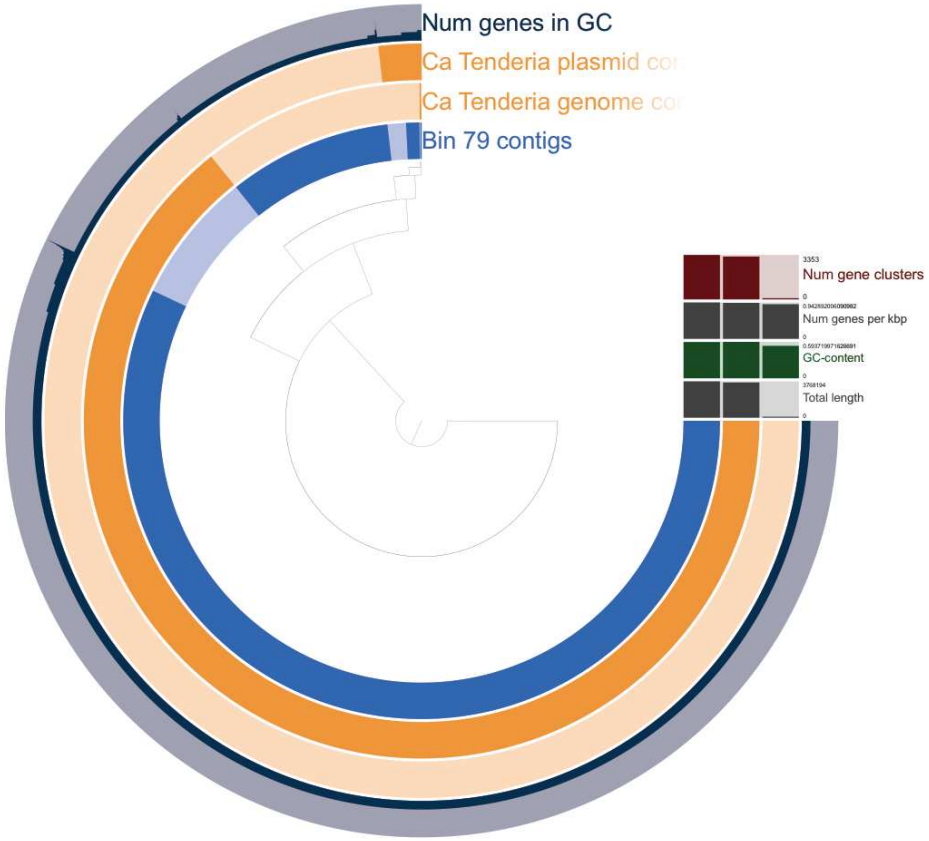


Figure 29. Pangenome analysis of MAG_79_1 and '*Ca.* Tenderia electrophaga'. The genome and plasmid of '*Ca.* Tenderia electrophaga' are in orange and MAG_79_1 in blue. 2 909 gene clusters are shared (blue and orange overlapping), 444 unique for MAG_79_1 (only blue) and 409 unique for '*Ca.* Tenderia electrophaga' (only orange).

5.4. FUNCTIONAL ANNOTATION

5.4.1. ELECTRON TRANSFER SYSTEMS

With three annotation databases (KEGG, COG, Pfam), we identified 68 cytochromes in MAG_79_1, of which 65 were shared with the genomes of '*Ca.* Tenderia electrophaga'. The cytochrome genes not shared between the genome and MAG_79_1 were functionally redundant with the shared cytochromes. We selected genes related to electron transfer system, and carbon fixation, shared by both MAG_79_1 and '*Ca.* Tenderia electrophaga' in Table 6.

Wang et al., 2015b, identified an undecaheme c-type cytochrome in '*Ca.* Tenderia electrophaga' with the Pfam motif PF09699 and two genes coding for this undecaheme cytochrome were found in MAG_79_1. The Cyc2-like cytochrome identified in '*Ca.* Tenderia electrophaga' was not annotated as a cytochrome, but was found using blast and was identical in both genomes, but only 26.09% identity and a cover of 46% against the Cyc2 protein from *Acidithiobacillus ferrooxidans*. Four cytochromes peroxidase catalyzing a two electron reduction of hydrogen peroxide to water, were shared between the two genomes.

Five genes coding for molybdopterin oxidoreductases were identified and are thought to be related to the alternative complex III, a putative electron transfer system in *Mariprofundus ferrooxydans* (Singer et al., 2013).

Four electron transfer complexes were complete: the cbb3 cytochrome with the ccoNOP genes, the cytochrome bc1 with the PetABC genes, the NADH-quinone oxidoreductase with 14 Nuo genes, and the succinate dehydrogenase with the SdhCDAB genes. The two genes CydA and CydB for a cytochrome bd ubiquinol oxidase were present, but the gene CydX was missing.

In addition to the electron transfer systems, all the genes for the F-type ATPase for ATP generation using the proton-motive force were also found.

5.4.2. CARBON FIXATION

Five genes encoding for the large (RbcL) and small (RbcS) subunit of the RuBisCO enzyme were found, a key component of the Calvin-Benson-Bassham (CBB) cycle for CO2 fixation. Using the KEGG pathway reconstruction tool, only the fructose-1,6-bisphosphatase was missing from the CBB cycle. But the enzyme was found with the COG annotation making the cycle complete.

5.4.3. OTHER

The full phosphate transport system (pstSCAB, phoU) identified in *M. ferrooxidans* (Barco et al., 2015) was found in both genomes. Other transport system includes molybdate, nitrate-nitrite, iron, zinc, cobalt, nickel, heme, phospholipid, lipopolysaccharide, lipo-oligosaccharide.

Some genes related to the nitrogen cycles were identified, like the genes NirBD associated with dissimilatory nitrite reduction to ammonia and the genes NasAB for the assimilatory nitrate reduction. Regarding the sulfur cycle, the genes Sat and CysCHJI were associated with assimilatory sulfate reduction.

Chemotaxis and flagella assembly genes were detected as well as surface and cell contact genes which allow the production of EPS and confirm the biofilm lifestyle of the bacterium.

Genes that were only found in '*Ca.* Tenderia electrophaga' genome could have been missed during the assembly of MAG_79_1 contigs, but genes specific to MAG_79_1 were interesting as they included the full urease operon with seven Ure genes, an ABC transporter for urea, and a transporter of phosphonate. The other genes that were exclusive to either MAG_79_1 or '*Ca.* Tenderia electrophaga' were not critical for most metabolic pathways identified in both genomes.

Table 6. A selection of genes shared between MAG_79_1 and '*Ca.* Tenderia electrophaga' and compared to other electroactive bacteria. The putative electron transfer system genes and carbon fixation genes are represented, with the number of copies and KEGG accessions. Dark green boxes are genes that belong to the same gene cluster, light green are similar gene annotation.

Functions	Number of genes	Kegg Accession	*M. ferrooxidans*	*S. lithotrophicus*	*A. ferrooxidans*	*R. palustris*	*S. oneidensis*	*G. sulfurreducens*
Cytochrome c oxidase cbb3-type								
ccoN (subunit I)	4	K00404	✓	✓		✓	✓	
ccoNO (subunit I/II)	1	K15862						
ccoO (subunit II)	2	K00405	✓	✓		✓	✓	
ccoP (subunit III)	1	K00406	✓	✓		✓	✓	✓
Molybdopterin oxidoreductase	5	K00184	✓	✓				
Cytochrome bc1								
petA	1	K00411	✓	✓	✓	✓	✓	
petB	1	K00412	✓	✓	✓		✓	✓
petC	1	K00413	✓	✓	✓		✓	
NADH-quinone oxidoreductase								
nuoA	1	K00330	✓	✓	✓	✓	✓	✓
nuoB	1	K00331	✓	✓	✓	✓	✓	✓
nuoC	1	K00332	✓	✓	✓	✓		✓
nuoD	1	K00333	✓	✓	✓	✓		✓
nuoE	1	K00334	✓	✓	✓	✓	✓	✓
nuoF	1	K00335	✓	✓	✓	✓	✓	✓
nuoG	1	K00336	✓	✓	✓	✓	✓	✓
nuoH	1	K00337	✓	✓	✓	✓	✓	✓
nuoI	1	K00338	✓	✓	✓	✓	✓	✓
nuoJ	1	K00339	✓	✓	✓	✓	✓	✓
nuoK	1	K00340	✓	✓	✓	✓	✓	✓
nuoL	2	K00341	✓	✓	✓	✓	✓	✓
nuoM	1	K00342	✓	✓	✓	✓		✓
nuoN	1	K00343	✓	✓	✓	✓	✓	✓
Succinate dehydrogenase								
SdhA	1	K00239	✓	✓		✓	✓	✓
SdhB	1	K00240	✓	✓		✓	✓	✓
SdhC	1	K00241	✓	✓		✓	✓	✓
SdhD	1	K00242		✓		✓	✓	
Cytochrome bd ubiquinol oxidase								
CydA	1	K00425	✓	✓	✓	✓	✓	✓
CydB	1	K00426	✓	✓	✓	✓	✓	✓
Cytochrome peroxidase	4	K00428	✓				✓	✓
RuBisCO								
rbcL (large chain)	2	K01601	✓	✓	✓	✓		
rbcS (small chain)	3	K01602	✓	✓	✓	✓		

5.5. COMPARISON WITH OTHER ELECTROACTIVE BACTERIA

We did some additional comparative genomics with the genomes of other described electroactive bacteria: *Acidithiobacillus ferrooxidans* ATCC 23270, *Geobacter sulfurreducens* PCA, *Mariprofundus ferrooxydans* PV-1, *Rhodopseudomonas palustris* CGA009, *Shewanella oneidensis* MR-1, *Sideroxydans lithotrophicus* ES-1. Some of the genes involved in electron transfer and carbon fixation were compared. Table 6 summaries the similarity with the MAG_79_1 and '*Ca.* Tenderia electrophaga'.

Both *M. ferrooxidans* and *S. lithotrophicus* shared molybdopterin oxidoreductases. The former shared the cbb3 cytochrome complex, and the later had a quite similar cytochrome bc1 complex and NADH-quinone oxidoreductase. *A. ferrooxidans* also had a resembling NADH-quinone oxidoreductase, but lacks cbb3 cytochrome complex and succinate dehydrogenase. These three bacteria all shared the same RuBisCO genes for carbon dioxide fixation. *R. palustris* had a similar cytochrome bd ubiquinol oxidase and the two model electroactive bacteria *S. oneidensis* and *G. sulfurreducens* had less in common with MAG_79_1 and '*Ca.* Tenderia electrophaga' than the other genomes compared here.

6. DISCUSSION

6.1. ELECTROACTIVE BACTERIA AND ENNOBLEMENT

With the presence of MAG_79_1 and its putative metabolism inferred from '*Ca.* Tenderia electrophaga', we add new evidence to support a model we proposed to explain the ennoblement of stainless steel by bacteria. In this model, electrotrophic bacteria can reduce oxygen with the electron drawn from the passive film of the stainless steel, and consequently increase the OCP. This hypothesis is coherent with the cathodic depolarization observed in other studies where the bacteria ability to reduce oxygen is proposed as the reason for the ennoblement (Audouard et al., 1994; Holthe et al., 1989; Johnsen and Bardal, 1985; Larché et al., 2011; Le Bozec et al., 2001; Mollica and Scotto, 1996; Rogne and Steinsmo, 1996; Thierry et al., 2015; Zhang and Dexter, 1995). The ennoblement occurs in open circuit condition, *i.e.* without any external current provided, but the source of electrons used in the oxidation reaction could be found in the very slow renewal of the passive film (hydr)oxides (iron, chromium) that provides the corrosion resistance to the stainless steel (Marcus, 2011). We had shown that the passive current was two orders of magnitudes lower than in biocathode systems used for the description of '*Ca.* Tenderia electrophaga' (Malanoski et al., 2018; Trigodet et al., 2019), which makes the presence of a putative electrotrophic bacteria in open circuit condition a remarkable result.

With this hypothesis, the growth of electrotrophic bacteria rely on the stainless steel passive current. The current provided in anodic polarization (condition 4) was in the reverse direction of the passive current, and glass coupons are a non-conductive material. MAG_79_1 was not found in these two conditions, which support the idea that they use the passive current in OPC conditions, and therefore support the model described above to explain ennoblement.

This experiment does not prove that MAG_79_1 was responsible for the ennoblement, but that it reacted to the OCP/+300 mV conditions. To prove the role of bacteria like MAG_79_1 or '*Ca.* Tenderia electrophaga' in the ennoblement, we would need to use pure culture, or enrichment of these bacteria instead of the natural seawater community as the inoculum for stainless steel colonization.

6.2. COMPARISON OF MAG_79_1 WITH '*CA.* TENDERIA ELECTROPHAGA'

Classical taxonomic assignation often rely on the comparison of 16s rRNA marker gene, but the assembly of metagenomics sequences often fail in very conserved region of the genomes such as ribosomal RNA. Here, we had a non-complete 23S rRNA which matched to '*Ca.* Tenderia electrophaga' and the average nucleotide identity (ANI) between the two genomes was 97.1% for an alignment coverage 82.8%. This score was quite high and within the range of the same species, with and ANI over 96% (Kim et al., 2014). MAG_79_1 was composed of 81 contigs, therefore some parts of the genome were missing and would have contributed to the ANI score.

With the very high proportion of shared genes cluster, we can assume that MAG_79_1 has potentially the same metabolism which is confirmed by the presence of the same electron transfer genes for both genome (Figure 30). Likewise, other electroactive bacteria had some similarity with MAG_79_1.

Inner membrane possible electron transfer systems

The aerobic iron oxidizing bacteria *M. ferrooxidans* and *S. lithotrophicus* shared a similar cbb3 cytochrome complex to ultimately reduce oxygen and create a proton-motive force for ATP generation (Barco et al., 2015; Emerson et al., 2013). They also share cytochrome bc1 and a possible 'uphill' electron transport system to ultimately regenerate NADH (Bird et al., 2011), used for carbon dioxide fixation, with a NADH-oxidoreductase complex. Some molybdopterin oxidoreductases were shared with *M. ferrooxidans* and are thought to be involved in the Alternative Complex III, another element of the electron transfer system (Singer et al., 2011, 2013). The cytochrome bd, and the succinate dehydrogenase are also part of the electron transfer chain of *S. lithotrophicus* (Emerson et al., 2013) and found in the genome of MAG_79_1 and '*Ca.* Tenderia electrophaga'.

Outer membrane possible electron transfer systems

The outer membrane constitute the electron entry point and several proteins could be involved. The Cyc2-like protein identified in MAG_79_1 and '*Ca.* Tenderia electrophaga' had low similarity with the Cyc2 of *A. ferrooxidans* but metatranscriptomics experiment on '*Ca.* Tenderia electrophaga' shown the relative importance of that protein with extracellular electron transfer (Eddie et al., 2017). This

cytochrome is the key component of *A. ferrooxidans* extracellular electron transfer (Valdés et al., 2008). The presence of an undecaheme cytochrome could also be an evidence for a 'cytochrome porin' model (Richardson et al., 2012) already observed in other electroactive bacteria such as *G. sulfurreducens* OmcB/OmcC (Liu et al., 2014), *S. oneidensis* MtrA/MtrB (Coursolle and Gralnick, 2012), *R. palustris* PioA/PioB (Bose et al., 2014; Jiao and Newman, 2007) and *S. lithotrophicus* MtoA/MtoB (Liu et al., 2012).

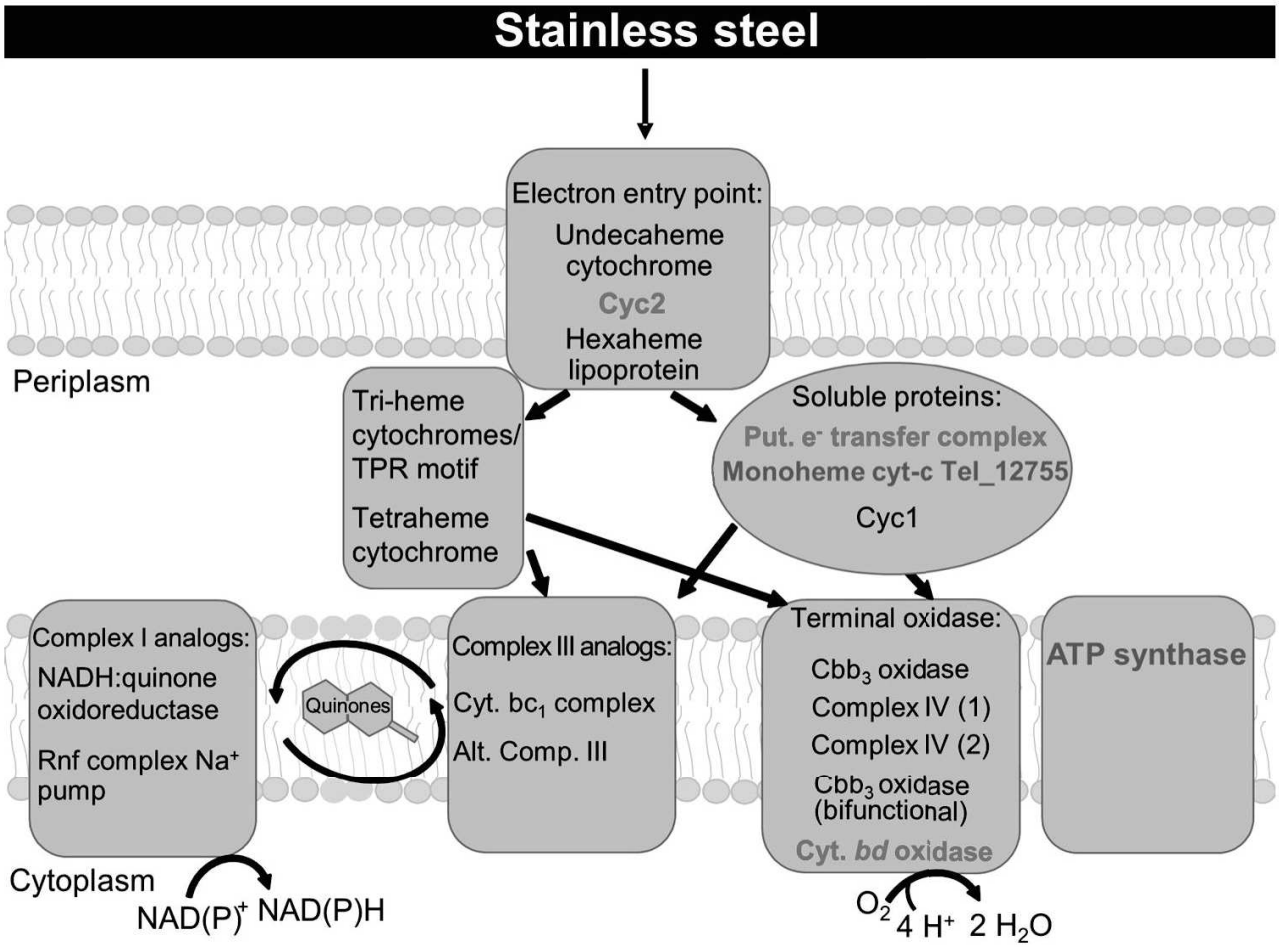


Figure 30. Schematic potential route for extracellular electron transfer in MAG_79_1. All proteins in this figure are both found in MAG_79_1 and '*Ca.* Tenderia electrophaga'. Adapted from (Eddie et al., 2017).

6.3. IRON OXIDIZING BACTERIA AND ENNOBLEMENT

The estimation of the free Gibbs energy for the reduction of oxygen (+ 820 mV vs. SHE, + 576 mV vs. SCE) with an electrode at the potential of + 212 mV vs. SCE (-nFΔE°, F = 96 485 J) is - 35.12 kJ/mol e^-, which is in the range of the iron oxidizing bacteria (Bird et al., 2011; Emerson et al., 2010; Ferguson and Ingledew, 2008). The relative similarity with known iron oxidizing bacteria like *M. ferrooxydans* and *S. lithotrophicus* indicates that MAG_79_1 could likely oxidize Fe(II) to Fe(III). Therefore, in the passive film composition, Fe(II)/Fe(III) ratio is more likely to be influenced than other elements.

7. CONCLUSIONS

Overall, this experimental design was successfully used to recover and identify electroactive bacteria, but a strong part of this study rely on metatranscriptomic data to assess the expressed genes under the different conditions, especially for 2-3 for which a current was use to either increase or decrease the potential 24 hours before sampling. These results are being processed and could provide key answers to the role of the putative electron transfer genes identified in MAG_79_1 and '*Ca.* Tenderia electrophaga'.

We did a co-assembly of all sample to increase the chance to reconstruct population genomes, we could also have used reference based assembly which uses reference genomes to better assemble similar genome expected from a metagenomic dataset.

We have selected the condition selective for '*Ca.* Tenderia electrophaga' after Trigodet et al., 2019 (seawater heated to 36˚C). However, we observed the ennoblement in other conditions, which suggest the presence of alternative, yet unknown, electrotrophic bacteria. The role of electrotroph in the environment remains poorly explored and we provide an efficient method to detect them. With this, identifying and comparing genomes of electrotrophs and the diversity of electron transport pathways constitute an accessible task and a new challenge for environmental microbiology.

DISCUSSION AND PERSPECTIVES

We used amplicon sequencing to describe the bacterial communities collected on stainless steel exposed under different conditions (temperature, oxygen content), and biomarkers detection to identify the bacteria most likely associated with ennoblement. There were two major types of bacteria associated with ennoblement: electrotrophic and hydrocarbon degrading bacteria. The presence of the former indicated a new bacterial mechanism for ennoblement, driven by oxygen reduction with electron drawn from the stainless steel passive film. This explanation fit with the enhanced oxygen reduction due to bacteria on the surface of stainless steel that has been observed by many authors (Audouard et al., 1994; Holthe et al., 1989; Johnsen and Bardal, 1985; Larché et al., 2011; Le Bozec et al., 2001; Mollica and Scotto, 1996; Rogne and Steinsmo, 1996; Thierry et al., 2015; Zhang and Dexter, 1995).

We confirmed the presence of the electrotrophic bacteria identified in amplicon sequencing with the use of metagenomics in an experiment with fixed vs. open circuit potential conditions. We recovered a metagenome-assembled genome closely related to the electrotrophic bacteria '*Ca.* Tenderia electrophaga', previously enriched on a biocathode. These results strongly indicates that electrotrophic bacteria could be responsible for the ennoblement.

Future studies on the ennoblement of stainless steel should consider these results, especially the role of electrotrophic bacteria. For that reason, the following chapter discuss the results generated during the thesis and the general perspectives that ensue.

1. EXPLORE NEW ENVIRONMENTS

1.1. FOR THE ENNOBLEMENT

Despite the advent of molecular methods for the description of microbial diversity in the environment, the lack of information on the functional role of most of the discovered taxa described by metabarcoding remains a major obstacle to the understanding of their role and activity. We were thus fortunate to identify '*Ca.* Tenderia electrophaga' in the temperature gradient experiment because it had been

described as a putative electrotroph (Eddie et al., 2016) and we were therefore able to propose a new mechanism for the ennoblement. According to this hypothesis, electrotrophic bacteria should be associated with ennoblement all the time, not only in the heated seawater condition of our experiments. The ennoblement is a phenomenon observed world-wide (Scotto and Lai, 1998), in marine and freshwater systems (Gümpel et al., 2006; Thierry et al., 2015), cold or tropical regions (Bardal et al., 1993; Martin et al., 2003; Mattila et al., 2000), and even deep sea. Hence, we should also investigate ambient temperature seawater to better understand which bacteria are naturally responsible for the ennoblement in the Bay of Brest.

We carried out a bacterial community analysis at ambient seawater temperature (11°C, February) at the very beginning of this project. We exposed and collected 25 samples in 5 seawater tanks to assess the assembly of the bacterial communities. The samples collected were quite similar and the assembly rather deterministic and we identified mainly *Flavobacteriaceae* and one hydrocarbon degrading bacteria: Oleiphilus (Figure 31). The conclusions are similar to the low DOC experiment (Chapter II): we did not identify known electrotrophic bacteria, but we did not prove their absence either. Most of the taxonomic affiliations, from the 16S rRNA, were poorly resolved due to reference databases lacking bacteria from similar environments.

Nevertheless, in the Chapter III, we used controlled potential conditions vs. OCP to identify electrotrophic bacteria from the rest of the surface attached bacterial community. We successfully recovered a MAG, very close to '*Ca.* Tenderia electrophaga', a known electrotroph. This protocol can thus be used to identify other electrotroph bacteria in any condition suitable for the ennoblement. And their systematic presence would considerably support our model to explain the ennoblement. Conversely, we could use this protocol in conditions that inhibits the ennoblement (low DOC, temperature above 40°C) to show the absence of electrotroph bacteria.

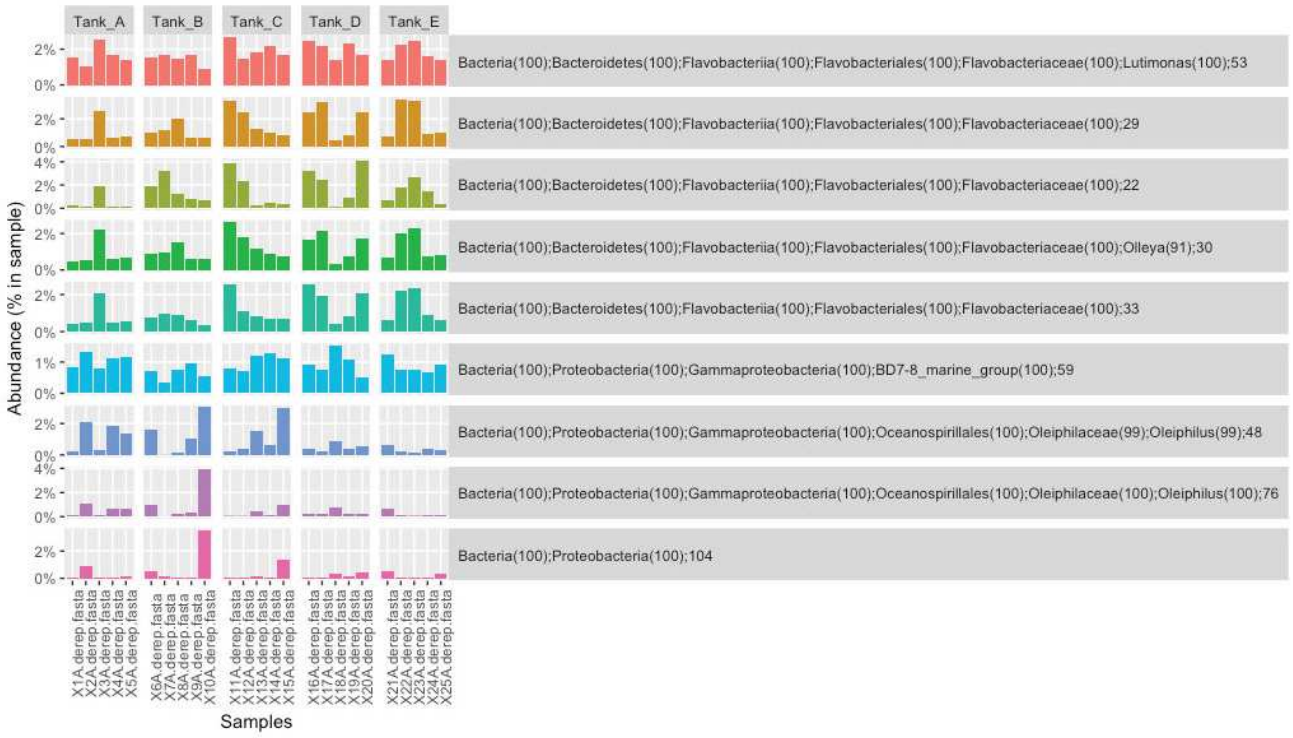


Figure 31. Relative abundance distribution of the most abundant bacteria collected on stainless steel exposed for two weeks in seawater (11°C).

1.2. ENVIRONMENTAL OCCURRENCE OF '*CA.* TENDERIA ELECTROPHAGA'

At the time of writing this thesis, it was the first identification of '*Ca.* Tenderia electrophaga' in another system than the enrichment from which it was originally described. It is interesting to investigate the natural habitat of that bacteria for two reasons: find new electrotrophic bacteria that can be useful for microbial fuel cells studies, and to compare stainless steel bacterial communities to the natural community of '*Ca.* Tenderia electrophaga' and identify microbial partners or factors that relate to the ennoblement.

The only bacterial inoculum used in this thesis was the seawater, therefore a bacterium like '*Ca.* Tenderia electrophaga' should be present in that seawater. A bacterial survey has been carried out by Lemonnier et al. (in prep) spanning 3 years with a bi-monthly sampling in the immediate vicinity of our pumping

system intake. Some OTUs were affiliated to '*Ca.* Tenderia electrophaga' in this time series and their distribution is shown in Figure 32. The abundance was quite low with a maximum of 0.25% relative abundance in June 2017, but a remarkable seasonal pattern can be observed with higher abundance from April to September. The occurrence of these OTUs correlate with higher seawater temperature, but also with the phytoplanktonic blooms that first arise around April—with the high nutrient and light availability—and recur during the summer season.

These OTUs were affiliated to '*Ca.* Tenderia electrophaga' but with a sequence similarity of only 90% for the V4-V5 region of the 16S rRNA—compare to the 100% similarity from the OTU of the temperature experiment (Chapter I). This affiliation was still the best considering the actual databases of 16S rRNA (Silva NR 132) but interpretation of these OTUs was limited to a correlation with the biologically productive period of the year. Also, this database is only about the pelagic (free-living) part of the microbial diversity and not bacteria with a sessile lifestyle. Hence, it is possible that electrotrophs such as' *Ca.* Tenderia electrophaga', due to their attached lifestyle metabolic requirement, are more dominant in the particulate fraction of the marine microbial community. We will soon be able to test this hypothesis with the future release of time series amplicon data for this fraction (Lemonnier et al. in prep). However, we cannot also discard the possibility for '*Ca.* Tenderia electrophaga' to belong to the very rare biosphere of the marine bacteria, with local enrichment in our experimental facility due to repeated stainless steel exposition over the years.

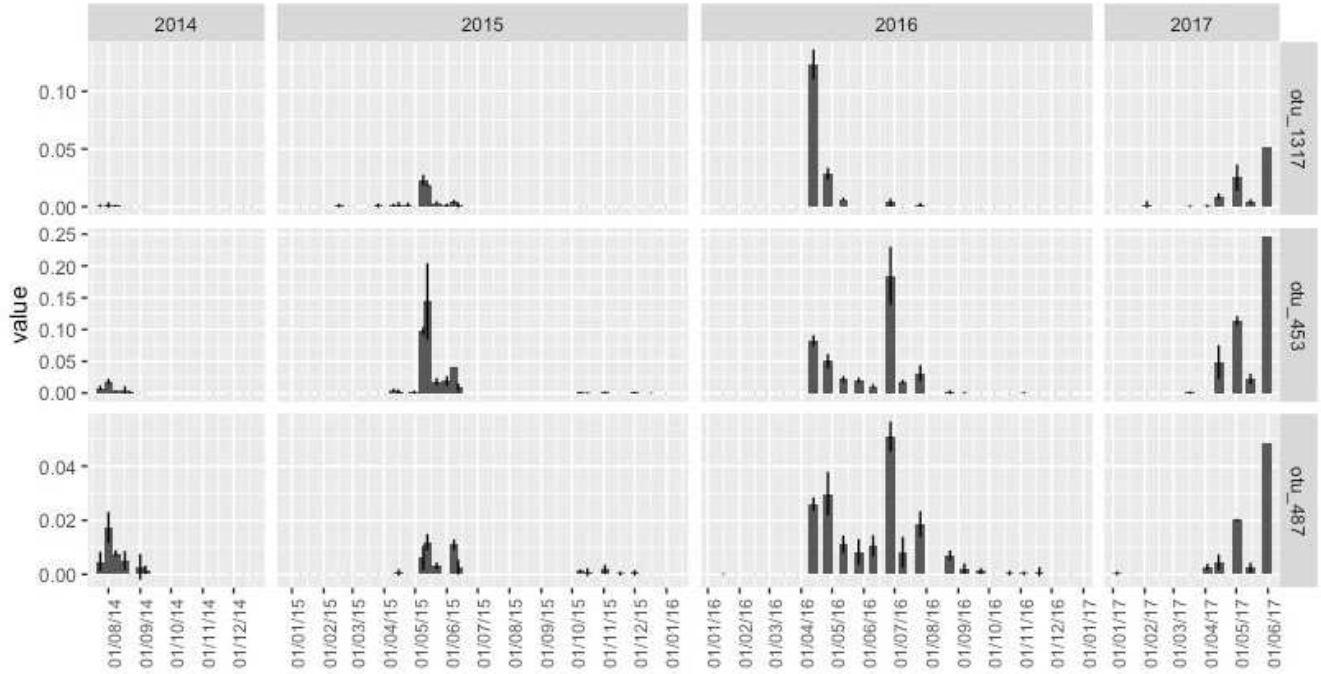
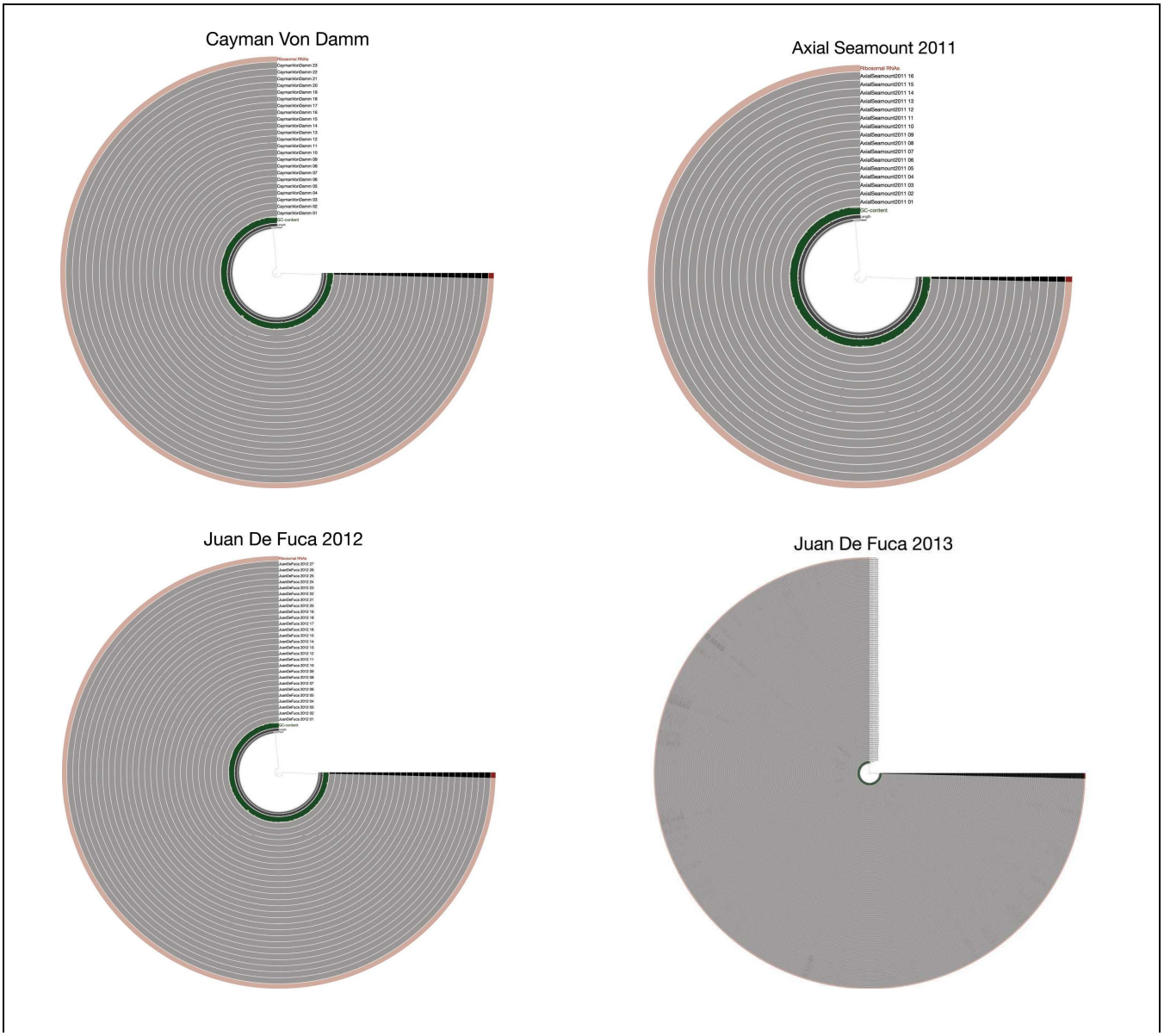


Figure 32. Relative abundance of OTUs affiliated to '*Ca.* Tenderia electrophaga' in a time series of seawater collected at the pump used as intake for our experiments

In addition, the putative extracellular electron transfer system in MAG_79_1 or '*Ca.* Tenderia electrophaga' was quite close to iron oxidizing bacteria. Currently only two neutrophilic aerobic iron oxidizing bacteria have been characterized: *Mariprofundus ferrooxyd*ans in seawater (Singer et al., 2011) and *Sideroxydans lithotrophicus* in fresh water (Emerson et al., 2013). The former has been discovered in the Loihi Seamount, in Fe-rich microbial mats associated with hydrothermal venting (Emerson et al., 2007). We used several publicly available metagenomics data of hydrothermal vents (Courtine, 2017) and mapped these sequences to the genome of '*Ca.* Tenderia electrophaga' (Figure 33) to study its presence in these systems. It did not recruit any metagenomic sequences from hydrothermal vents, which was a surprising result given the amount of iron available in these systems, and the great expectation to find iron oxidizing bacteria (Emerson et al., 2010). These results are in strong contrast with the clear enrichment of '*Ca.* Tenderia electrophaga' on our stainless steel coupons. The natural niche of '*Ca.* Tenderia electrophaga' thus remains elusive. However, the increasing availability of marine

environmental metagenomes may bring new answers on the distribution of '*Ca.* Tenderia electrophaga', as well as extracellular electron transfer pathway carrying microorganisms in a near future.



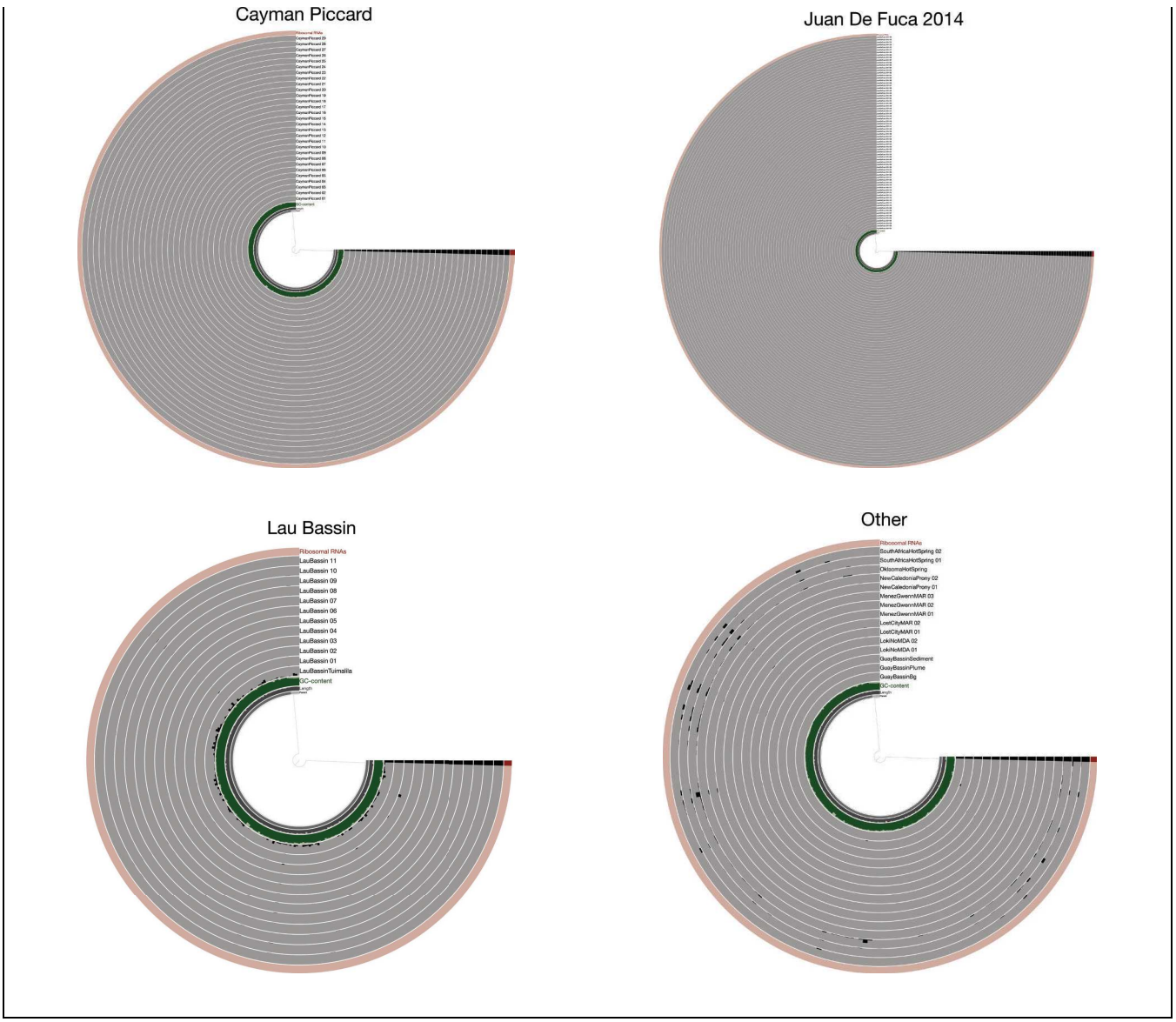


Figure 33. Metagenomes recruitment on '*Ca.* Tenderia electrophaga'. Each layer represent a sample from a metagenomic data set. The gray color means there are no metagenomic sequenced that mapped to '*Ca.* Tenderia electrophaga' genome.

2. CONFIRM THE ROLE OF ELECTROTROPHIC BACTERIA

In the Chapter III, we found that electrotrophic bacteria are associated with stainless steel ennoblement, but did not yet formally prove that they were responsible for that increase of potential. The only way to confirm their role would be to use pure culture of these bacteria in controlled conditions, as opposed to the tanks which only rely on seawater for bacteria input.

Obviously, the first bacteria strain to use would be '*Ca.* Tenderia electrophaga'. It has not yet been isolated (Eddie et al., 2016), but it has been enriched in a community used on biocathode. That community can be used as inoculum for stainless steel coupons. The observation of an OCP increase could further support that electrotrophic bacteria activity mediates the stainless steel ennoblement. Another interesting bacteria would be *Mariprofundus ferrooxydans* as it is a marine iron oxidizing bacteria able to switch to an electrotrophic lifestyle (Summers et al., 2013), and therefore represents a good candidate.

Another possibility would be to enrich the electrotroph already present on stainless steel during ennoblement. In this case, we would need to create biocathode with cathodically polarized stainless steel to act as an electron donor, at a much higher rate than the passive film current and assess the ability of that enrichment to trigger the ennoblement in a controlled system and in open circuit conditions.

3. OTHER ENVIRONMENTAL FACTORS INFLUENCING THE ENNOBLEMENT

Some conditions inhibit the ennoblement of stainless steel, such as critical seawater temperature (40°C in Brest) (Chapter I), or low dissolved oxygen content (Chapter II). Interestingly, seawater renewal rate was also critical for the apparition of the OCP increase.

In the course of this work, this was observed in a separate experiment in which we wanted to perform sterile incubation of stainless steel coupons to confirm that ennoblement was related to the presence of

bacteria. We used samples immersed in a 20L Nalgene bottle that we could close and sterilize by autoclaving (Figure 34). The seawater in this bottle would not be renewed. Prior to a sterile experiment, we carried out a positive control with stainless steel samples exposed in a 20L seawater bottle and heated at 30°C. Interestingly, the potential never increased despite the use of natural seawater. Of course, that experimental design was not further developed as the potential never changed with natural seawater. As a result, seawater renewal was clearly necessary for electrotrophic activity and we could invoke three reasons for that requirement: (1) a need for rare bacteria from the seawater, (2) a need to renew nutrients, (3) or to eliminate waste products. Later, in another experiment involving 300 L tanks, the seawater renewal had stopped working for few days but the ennoblement was still observed. The ratio between the tanks and the coupon size was completely different and a small pump in the 300 L tanks ensured continuous seawater flow which could be enough to counter the lack of renewal.

Identifying the factors lost by the lack of renewal is essential to design solutions to prevent the development of these bacteria. It was shown that the phosphate transport is really important for *Mariprofundus ferrooxydans* as the phosphate could get chelated by the iron(III) oxides produced by the iron oxidation process and therefore would quickly be depleted (Barco et al., 2015). '*Ca.* Tenderia electrophaga' has the same phosphate transport system (Chapter III). A depletion of phosphate due to the lack of renewal and/or mechanical flow at the stainless steel interface could be a limitation for the growth of these bacteria. To address this question, we could collect bacteria in a time series associated with a decreasing renewal rate correlate their activity (with metatranscriptomics) to identify the factors limiting the ennoblement.

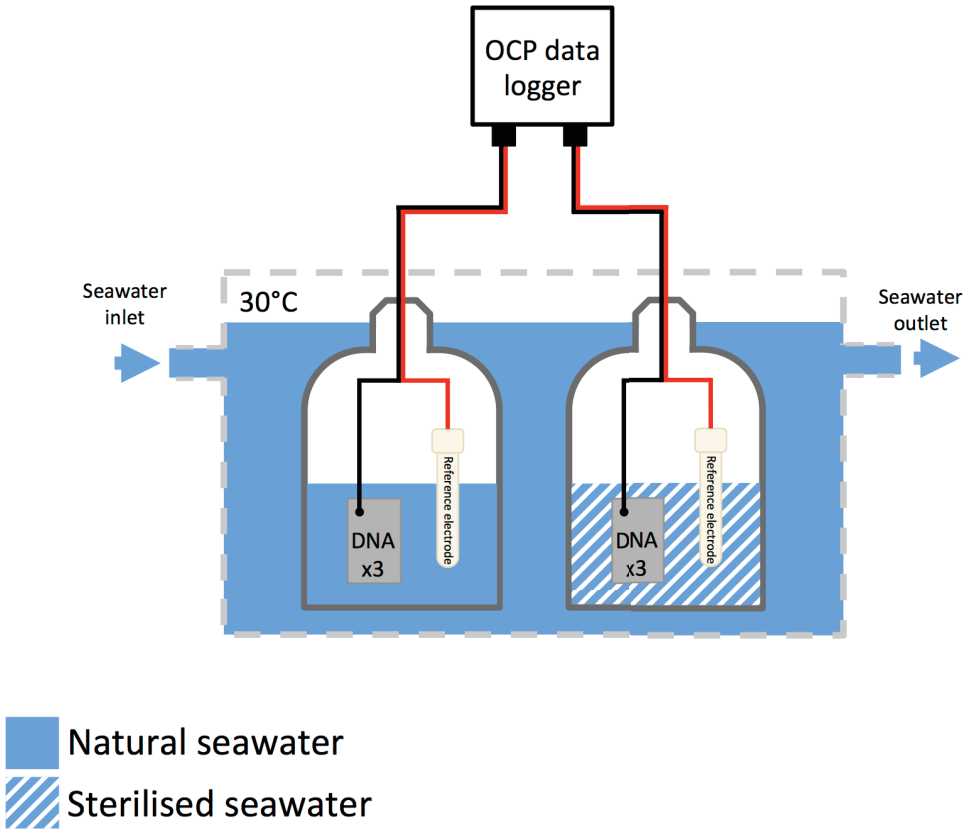


Figure 34. Experiment design for sterile control on ennoblement.

4. HYDROCARBON DEGRADING BACTERIA

Hydrocarbon degrading bacteria are usually found in oil contaminated environments and use either saturated aliphatic or aromatic hydrocarbons as source of carbon or energy (Brzeszcz and Kaszycki, 2018; Das and Chandran, 2011). This type of bacteria was the second most significant bacteria associated with ennoblement in our experiments. They could be related to an oil contamination of the seawater pumping and tubing system, but their recurrence in our dataset remains a remarkable result. The main hydrocarbon degrading bacteria in our datasets included *Oleiphilus*, *Alcanivorax* and *Marinobacter*. They are defined as obligate hydrocarbonoclastic bacteria (OHCB) (Yakimov et al., 2007) which means that

they only rely on hydrocarbon compounds as energy and carbon source and are unable to use monomeric sugars as growth substrates. The genes involved in the n-alkane degradation have been summarized in three categories depending on the length of the alkane (van Beilen and Funhoff, 2007). Short chain (C2-C4) alkane degradation involves soluble or membrane bound methane monooxygenase while medium chain (C5-C17) degradation rely on soluble cytochrome P450 and integral membrane non-heme iron monooxygenases, such as AlkB. The latter requires soluble electron transfer protein named rubredoxin and rubredoxin reductase to transfer electron from NADH to eventually reduce oxygen and hydroxylates the terminal methyl group of the alkane (Rojo, 2009). Similarly, the cytochrome P450 requires a ferredoxin and a ferredoxin reductase to transfer electron from NADH for alkane hydroxylation (van Beilen and Funhoff, 2005). For long chain (>C17), use of dedicated monooxygenase like AlmA from *Acinetobacter*, and LadA from *Geobacillus* (Wang and Shao, 2013). The genome of *Oleiphilus messinensis* ME102 contains the cytochrome P450 and AlkB genes for medium chain alkane degradation (Golyshin et al., 2002; Toshchakov et al., 2017), as well as for *Alcanivorax borkumensis* SK2 (Schneiker et al., 2006; Yakimov et al., 1998) and *Marinobacter hydrocarbonoclasticus* SP17 (Duran, 2010) (Duran, 2010).

The bioremediation of oil contaminated environment can use these bacteria, either by bioaugmentation with the addition of known hydrocarbon degrading bacteria to supplement the existing microbial community, or by biostimulation of the local hydrocarbon degrading bacteria with addition of nutrients and oxygen (Suja et al., 2014, 201). Bioelectrochemical systems are used to increase their natural rate of hydrocarbon degradation in soils with anodes as electron acceptor to replace the oxygen which can be quickly depleted (Morris and Jin, 2007). Bacteria used in these experiments are anaerobic bacteria able to oxidize hydrocarbon compound and transfer electron extracellularly to an electrode (Morris et al., 2009). Some typical electrogenic bacteria like *Geobacter spp*. are able to degrade hydrocarbon compound and transfer electron to an anode (Zhang et al., 2010; Zhou et al., 2016). In these bioremediation conditions, bacteria use an anode as electron acceptor, *i.e.* they are electrogenic and not electrotrophic. Nonetheless, it has been shown that *G. sulfurreducens* and *S. oneidensis* are able to switch from an electrogenic to an electrotrophic metabolism. Hence it is possible that hydrocarbon

degrading bacteria could use anodes to reverse their extracellular electron transfer to draw electron from a cathode.

In the same line of evidence, hydrocarbon degrading bacteria have been associated with biocathode and with '*Ca.* Tenderia electrophaga' (Wang, 2015b). *Marinobacter* (Wang et al., 2015a), *Labrenzia* (Wang et al., 2016), and *Alcanivoraceae* (Wang, 2015b) were all found in the '*Ca.* Tenderia electrophaga' enrichment community. The isolated *Marinobacter* was even able to form a biofilm on cathode and produce a maximum current that was two orders of magnitude lower than the biocathode consortium (Wang et al., 2015b). The role of hydrocarbon degrading bacteria in the context of ennoblement is not clear yet. Additional work is required to either prove or invalidate the role of these bacteria with ennoblement.

CONCLUSIONS

The stainless steel ennoblement has been studied for 40 years, but for the first time we used a molecular microbial ecology approach to better understand the microorganisms involved and their metabolisms associated with this increase of potential which eventually increase the risk of localized corrosion. As a result, we proposed a model that can explain the ennoblement based on electrotrophic bacteria and their ability to reduce oxygen with electrons drawn from the stainless steel passive film. This hypothesis is supported by the cathodic depolarization (enhanced oxygen reduction rate) reported in the literature, and the presence of an OTU and a metagenome-assembled genome affiliated to '*Ca.* Tenderia electrophaga', a known electrotrophic bacteria enriched in a biocathode community. This model must be further tested by finding other electrotroph associated with ennoblement in other conditions/geographical localizations, and with pure culture of electrotrophic bacteria on stainless steel to confirm their role in mediating ennoblement.

A challenge in electromicrobiology is to go beyond the two model bacteria *Geobacter* and *Shewanella* to address the taxonomic and mechanistic diversity of electroactive bacteria, but also elucidate their role in the environment (Nealson and Rowe, 2016). Currently the diversity of electrotrophic bacteria remain poorly explored (Logan et al., 2019) while various environments suitable for their lifestyle have been identified like hydrothermal vents system (Emerson, 2016) or marine sediments (Dale et al., 2015) which have conductive mineral such as iron oxides. Our identification of an electrotrophic bacteria in open circuit condition, as opposed to bioelectrochemical system, offers new perspectives about their ability to uses conductive surface in natural environments and their role in biogeochemical process, especially with an ability to fix carbon dioxide and therefore provide organic matter to sustain heterotrophic bacteria.

We successfully developed a protocol to identify the electrotrophic bacteria present during ennoblement. This represents a new tool to investigate the presence of these bacteria in all conditions the ennoblement can be observed to support and confirm our model, but also to explore the diversity of electrotrophic bacteria and increase our knowledge on these bacteria influencing ennoblement.

This is the first time that the importance of electrotrophic bacteria in ennoblement has been investigated. This works represent the basis for future studies dedicated to the identification of possible solution to avoid the ennoblement. Future works should focus on the electrotrophic bacteria and their metabolism to design solutions and eventually selectively prevent their activity, hence prevent the ennoblement and the inherent risk of localized corrosion.

PERSONAL PERSPECTIVES

An important aspect of this work was the first collaboration between the Laboratory of Microbiology of Extreme Environments and the French Corrosion Institute. My challenge was to associate the knowledges and skills of microbiology and electrochemistry of stainless steel to design meaningful experiments. Nothing had been done between the two institutes before, so we had to create everything, form the microbial collection on stainless steel to the bioinformatic pipeline on our lab server. This collaboration successfully led to the identification of the bacteria associated with ennoblement, and we proposed a new explanation for the ennoblement with electrotrophic bacteria that would reduce oxygen with electron drawn from the stainless steel passive film.

In the end, from microbiology to electrochemistry, everything is about electron transfer.

Cela fait 40 ans que l'anoblissement de l'acier inoxydable est étudié, et pour la première fois nous avons utilisé une approche de biologie moléculaire et d'écologie microbienne pour mieux comprendre quels microorganismes, ainsi que leurs métabolismes, sont impliqués dans l'élévation du potentiel libre qui augmente les risques de corrosion localisé. C'est ainsi que, pour expliquer l'anoblissement, nous avons proposé un modèle basé sur des bactéries électrotrophes et leur capacité à réduire de l'oxygène avec des électrons provenant du film passif de l'acier inoxydable. Cette hypothèse est renforcée par la dépolarisation cathodique (augmentation du taux de réduction de l'oxygène) déjà décrite dans la littérature, ainsi que la présence d'un OTU et d'un génome issu d'assemblage métagénomique affilié à '*Ca.* Tenderia electrophaga', une bactérie électrotrophe enrichie dans une communauté bactérienne sur une biocathode. Ce modèle doit être mis à l'épreuve en recherchant d'autres bactéries électrotrophes associé à l'anoblissement dans d'autres conditions et autres zones géographiques, et par l'utilisation de culture pure de bactéries électrotrophes pour confirmer leur rôle dans l'anoblissement.

Le prochain défi en électro-microbiologie sera de dépasser les deux modèles que sont Geobacter and Shewanella pour s'intéresser à la diversité taxonomique et fonctionnelle des bactérie électroactives, mais aussi explorer leur rôle dans l'environnement (Nealson and Rowe, 2016). Actuellement, la diversité des bactéries électrotrophes est peu connue (Logan et al., 2019) malgré l'identification d'environnements propices à leur métabolisme comme les cheminées hydrothermales (Emerson, 2016) ou les sédiments marins (Dale et al., 2015) où l'on trouve des minéraux conducteurs comme les oxydes de fer. La présence d'une bactérie électrotrophe en condition de potentiel libre, par opposition aux système bio-électrochimiques, offre de nouvelles perspectives à propos de leurs capacités à utiliser des surfaces conductrices en condition naturelle, ainsi que sur leur rôle dans les processus biogéochimiques. Surtout avec une capacité à capter et fixer le dioxyde de carbone, et donc apporter de la matière organique pour les bactéries hétérotrophes.

Nous avons réussi à développer un protocole pour l'identification des bactéries électrotrophes présentes pendant l'anoblissement. Cela représente un nouvel outil pour étudier la présence de ces bactéries dans toutes les conditions compatibles avec l'anoblissement pour soutenir et confirmer notre modèle, mais

aussi explorer la diversité des bactéries électrotrophes et améliorer nos connaissances sur ces bactéries capables d'induire l'anoblissement.

C'est la première fois que l'importance des bactéries électrotrophes est étudié dans le contexte de l'anoblissement. Ces travaux représentent le fondement pour de future études dédiées au développement de solutions contre l'anoblissement. Les prochaines études pourront être dirigées vers les bactéries électrotrophes et leurs métabolismes pour concevoir des solutions pour inhiber leur croissance de manière sélective, et donc inhiber l'anoblissement et le risque de corrosion localisée.

PERSPECTIVES PERSONNELLES

Un aspect important de cette thèse est la collaboration entre le Laboratoire de Microbiologie des Environnements Extrêmes et l'Institut de la Corrosion. Mon défi était d'associer les connaissances et compétence de microbiologie et électrochimie des aciers inoxydables pour proposer des expériences pertinentes. C'est la première fois que les deux instituts ont collaboré, il a donc fallu tout mettre en place : du protocole de collection des bactéries sur les aciers inoxydables, à la maintenance et développement d'un serveur de bioinformatique. Cette collaboration a permis l'identification des bactéries associées à l'anoblissement, et nous avons pu proposer une nouvelle explication au phénomène avec des bactéries électrotrophes qui seraient capables de réduire de l'oxygène avec des électrons provenant du film passif de l'acier inoxydable.

Pour résumer, de la microbiologie à l'électrochimie, tout n'est qu'une affaire de transfert d'électrons.

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UNIVERSITE SCIENCES BRETAGNE DE LA MER LOIRE ET DU LITTORAL

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Titre : Caractérisation électrochimique et moléculaire des biofilms électroactifs sur acier inoxydable en milieu marin

Mots clés : anoblissement, écologie microbienne, bactérie électroactives, métagénomique

Résumé : Les microorganismes sont capables d'augmenter le potentiel libre des aciers inoxydables en eau de mer via un phénomène que l'on appelle anoblissement. Cette élévation de plusieurs centaines de millivolts du potentiel augmente le risque de corrosion localisé. L'anoblissement a été étudié pendant plus de 40 ans, et malgré son importance, les mécanismes microbien responsables du phénomène n'ont pas été identifiés. Nous avons combiné l'écologie microbienne et l'électrochimie pour étudier la diversité des bactéries associées à l'anoblissement des aciers inoxydables. La température de l'eau de mer ainsi que la teneur en oxygène dissous sont des facteurs qui influencent l'anoblissement et nous les avons utilisés pour identifier la fraction bactérienne associée au changement de potentiel. L'anoblissement est inhibé par une température critique de l'eau de mer (au-dessus de 38°C/40°C) et par une teneur basse en oxygène dissous.

A l'aide du séquençage d'amplicons ADN, nous avons identifié des unités taxonomiques opérationnels (OTUs) comme biomarqueurs de l'anoblissement. Certaines étaient affiliées à des bactéries capables de dégrader des hydrocarbures, et une OTU était affiliée à '*Candidatus* Tenderia electrophaga', une bactérie électrotrophe capable de réduire l'oxygène avec des électrons provenant d'une électrode. Nous avons étudié le rôle de ces bactéries avec des conditions a potentiels fixés et libres avec une approche de métagénomique. Nous avons reconstitué un génome issu d'assemblage métagénomique (MAG) très proche de '*Candidatus* Tenderia electrophaga' et associé à l'anoblissement.

Avec ces résultats, nous avons proposé un nouveau mécanisme bactérien pour expliquer l'anoblissement : les bactéries électrotrophes seraient capables de réduire de l'oxygène avec des électrons provenant du film passif de l'acier inoxydable, et ainsi influencer le potentiel libre et donc l'anoblissement.

Title : Electrochemical and molecular characterization of electroactive biofilms on stainless steel in marine environment

Keywords : ennoblement, microbial ecology, electroactive bacteria, metagenomic

Abstract : Microorganisms increase the open-circuit potential of stainless steel immersed in seawater in a phenomenon called ennoblement. This change of potential of several hundreds of millivolts raises the chance of localized corrosion. The ennoblement has been studied for more than 40 years, and despite the importance and impact of ennoblement, little is known about the microbial mechanisms responsible for the phenomenon. We have combined microbial ecology and electrochemistry to investigate the diversity of surface attached bacteria associated with stainless steel ennoblement. Seawater temperature and dissolved oxygen content are factors that influence the ennoblement and we used them to infer the bacterial fraction associated with the phenomenon. The ennoblement is inhibited by a critical seawater temperature (above 38°C/40°C) and low dissolved oxygen content.

With DNA amplicon sequencing, we identified operational taxonomic units (OTUs) that were biomarkers of the ennoblement. There were some OTUs affiliated to hydrocarbon degrading bacteria, and one OTU affiliated to '*Candidatus* Tenderia electrophaga', an electrotrophic bacteria able to reduce oxygen with electrons from an electrode. We investigated the role of electrotrophic bacteria with potentiostatic and open circuit conditions and with metagenomics we recovered a metagenome-assembled genome (MAG) very close to '*Candidatus* Tenderia electrophaga' associated with the ennoblement.

From these results, we proposed a new bacterial mechanism to explain the ennoblement: electrotrophic bacteria would be able to reduce oxygen with electron drawn from the stainless steel passivation film, hence influencing the open circuit potential and therefore the ennoblement.