Role of mixed species microbial community biofilms in microbially influenced corrosion

Prasanna Jogdeo

2018

Prasanna Jogdeo. (2018). Role of mixed species microbial community biofilms in microbially influenced corrosion. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73530>

<https://doi.org/10.32657/10356/73530>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**ROLE OF MIXED SPECIES MICROBIAL
COMMUNITY BIOFILMS IN MICROBially
INFLUENCED CORROSION**

PRASANNA JOGDEO

Interdisciplinary Graduate School

**Singapore Centre for Environmental Life Sciences Engineering
(SCELSE)**

2018

PRASANNA JOGDEO

**ROLE OF MIXED SPECIES MICROBIAL
COMMUNITY BIOFILMS IN MICROBially
INFLUENCED CORROSION**

PRASANNA JOGDEO

Interdisciplinary Graduate School

**Singapore Centre for Environmental Life Sciences Engineering
(SCELSE)**

A thesis submitted to the Nanyang Technological University in partial
fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Acknowledgements

This four-year journey would not have been possible without the help and support of so many people, to whom I would like to convey my gratitude. Firstly, I would like to express sincere thanks to my supervisor Assoc. Prof. Scott Rice for his guidance, all the fruitful discussions and believing in me to work on this project.

I would also like to thank my co-supervisor, Dr. Enrico Marsili for introducing me to the world of electrochemistry. His encouragement always kept me going. I am obliged to the insightful comments and suggestions from Assoc. Prof. Federico Lauro and Asst. Prof. Diane McDougald, which motivated me to widen my research from various perspectives. I feel very lucky to get such a cool thesis advisory committee. Special thanks to Dr. Sun Shuyang, my Post Doc for his guidance in the initial days of my Ph.D. Cheers to Dr. Florentin and Dr. Gurjeet for their much-needed help with the bioinformatics analysis.

I feel fortunate to have worked in a research-induced environment like Singapore Centre for Environment Life Sciences Engineering, with exposure to a variety of useful resources and an opportunity to interact with experts from interdisciplinary fields. I am very grateful to the St. John's Island National Marine Laboratory, for letting me conduct my experiments at their facility. I also owe one to my interns Martin Saballus, Rosalie Chai, Jessica Less for their help with the experiments.

A big shout-out to my friends and colleagues at SCSELSE and NTU; Anant, Dan, Sakcham, Hari and Sandra for making these four years a wonderful experience. I would also like to thank Siddharth for his help with proof reading and Leena for her support.

A special mention should go to my family. My father Dr. Pramod Jogdeo, who has always been a source of inspiration for me. My mother, Supriya and sister, Amruta for their love and care.

And last but not the least, thanks to my better half Tanvi, for being with me throughout the Ph.D. journey.

Table of contents

Table of contents.....	ii
Abstract.....	vii
List of figures.....	ix
List of tables.....	xiv
Author publications.....	xv
Meetings attended.....	xv
List of abbreviations.....	xvi
Chapter One: Literature Review.....	1
1.1 Fundamentals of corrosion.....	1
1.1.1 Passive films.....	2
1.1.2 Types of corrosion.....	3
1.2 Microbially influenced corrosion - Old problem, no solution.....	5
1.3 Metal surface.....	10
1.3.1 Elemental composition.....	10
1.3.2 The effect of surface and geometry.....	10
1.3.3 Grain boundaries and high-energy areas.....	11
1.4 Biofilms: unexplored dynamics in the MIC communities.....	11
1.4.1 The formation and role of biofilms in MIC.....	12
1.4.2 Corrosion inhibition by biofilms.....	13
1.4.3 The role of extracellular polymeric substances (EPS).....	14
1.5 Interface processes – The interaction between metal, microbes and environment.....	15
1.6 Axenic vs. mixed species biofilms: Increasing complexity.....	16

1.6.1	Axenic culture studies.....	16
1.6.2	Dual species, co-cultures and mixed species communities.....	18
1.7	MIC characterization.....	19
1.7.1	Surface Characterization.....	20
1.7.2	Electrochemical characterization.....	21
1.7.3	Microbial characterization.....	23
1.8	Designing a relevant study system.....	25
Chapter Two: Materials and Methods.....		27
2.1	Introduction.....	27
2.2	Stainless Steel.....	28
2.3	Sample preparation.....	30
2.4	Media.....	31
2.5	Electrochemical techniques.....	34
2.5.1	Open Circuit Potential (OCP).....	34
2.5.2	Polarization techniques.....	35
2.5.3	Linear Polarization Resistance (LPR).....	35
2.5.4	Linear Sweep Voltammetry (LSV).....	36
2.5.5	Electrochemical Impedance spectroscopy (EIS).....	37
2.6	Preliminary environmental experiment.....	39
2.6.1	Sample preparation.....	39
2.6.2	Environmental parameters.....	40
2.6.3	Electrochemical monitoring.....	40
2.6.4	Biofilm visualization.....	40
2.6.5	DNA extraction and sequencing.....	40
2.7	Environmental experiment 2.....	41
2.7.1	Sample preparation.....	41

2.7.2	Environmental parameters	41
2.7.3	Coupon deployment and time points	42
2.7.4	Coupon collection and analysis	42
2.7.5	Surface analysis	43
2.8	Enrichments.....	43
2.8.1	Preparation	43
2.8.2	RNA extraction	43
2.8.3	Metatranscriptomic data analysis.....	44
2.9	Continuous flow cell system	45
2.10	Electrochemical Flow cell (EFC).....	46
2.10.1	Sample preparation and EFC assembly	47
2.10.2	Electrochemical Analysis.....	48
2.10.3	pH and OD	48
2.10.4	Confocal Laser Scanning Microscopy	49
2.11	Flow cell system for biomass characterization	49
2.11.1	Sample preparation	49
2.12	Electrochemical batch reactor system	49
2.12.1	Enriched mixed marine microbial community	49
2.12.2	Corrosion cells setup.....	50
2.12.3	Biofilm visualization and surface analysis	51
2.12.4	Electrochemical analysis.....	52
Chapter Three: Understanding the onset of microbially influenced corrosion (MIC) in equatorial seawater		53
3.1	Introduction	53
3.1.1	Corrosion in seawater	53
3.1.2	MIC in seawater.....	53

3.1.3	Corrosion studies in the marine environment	55
3.2	Results and Discussion.....	56
	Preliminary experiment (September – October 2013).....	56
3.2.1	Biofilm formation on stainless steel coupons in sea water	56
3.2.2	Comparison of potential and biovolume.....	57
3.2.3	Community analysis.....	59
3.3	Study Two (March – April 2017).....	62
3.3.1	Potential (E_{ocp}) over 30 days.....	62
3.3.2	Biofilm formation by a seawater community	64
3.3.3	Comparison of potential and biovolume	66
3.3.4	Community composition analysis.....	67
3.4	Summary	67
Chapter Four: Developing and validating a continuous flow system for MIC characterization		69
4.1	Introduction	69
4.2	Flow cell design	71
4.3	Enrichment of a mixed microbial marine community from sea water	72
4.4	Results and Discussion.....	72
4.4.1	Omics analysis of the enriched community	72
4.4.2	The effect of glucose as a carbon source on the corrosion of SS304	75
4.4.3	Characterization of biofilm communities formed in the presence and absence of glucose	82
4.5	Summary	96
Chapter Five: Developing and validating a fed batch bio-electrochemical system for MIC characterization.....		97
5.1	Introduction	97

5.2	Designing a modified batch reactor system	99
5.3	Results and discussion.....	100
5.3.1	Long-term experiment	100
5.3.2	Short term Experiments	106
5.3.3	Comparison between LPR and LSV techniques.....	111
5.3.4	Effect of different glucose concentrations	112
5.3.5	Comparison of corrosion for mixed species cultures grown with 0.002% glucose or sterile seawater.....	118
5.3.6	Comparison of VNSSFs with and without glucose.....	119
5.4	Summary	121
6	Chapter Six: Conclusions.....	122
6.1	Community changes from planktonic phase to biofilm on the metal surface	123
6.2	Corrosion behavior during the initial stage of MIC	124
6.3	Parameters affecting corrosion behavior.....	125
6.4	Biofilm structure	127
6.5	Summary	128
6.6	Recommendations for future work.....	128
	References.....	131

Abstract

Microbially influenced corrosion (MIC) is a process where microorganisms are involved in the deterioration of materials. It is commonly associated with species rich microbial communities forming biofilms on surfaces, facilitating habitat remodeling through complex metabolic processes, such as the formation of anaerobic zones that facilitate the activity of key organisms such as sulfate reducers and iron oxidizers. MIC significantly impacts many industries such as oil and gas, transportation and logistics resulting in the annual loss of billions of dollars from structural damage and early replacement of infrastructure. Environmental factors such as nutrients, oxygen concentration, and convective fields affect the community composition and microstructure of biofilms, thus determining the corrosion rate of the material. Despite the general understanding that MIC results from community level metabolic activity, most MIC studies focus on single-species, axenic cultures or limited combinations of species.

The aim of this study was to apply an interdisciplinary approach to assess the onset of MIC. Initial experiments to investigate the effect of a mixed community on MIC of stainless steel (SS) in equatorial seawater, showed a strong selection for biofilm-forming microorganisms from the seawater onto the metal surface. This selection happened rapidly within 1 h and the community did not change significantly over the subsequent 7 d of the experiment. Confocal Laser Scanning Microscopy (CLSM) analysis indicated that attachment and biofilm formation begin within a few hours after inoculation. At the same time, the corrosion potential was observed to increase, indicating that ennoblement of the metal had occurred.

Due to challenges in controlling variability in the natural environment, it was essential to design laboratory systems to mimic environment under controlled conditions. Marine mixed microbial communities were enriched from seawater using different artificial seawater formulations and those communities were used as the inocula for subsequent experiments. Electrochemical analysis of coupons incubated with mixed species communities showed a rapid onset of

MIC for stainless steel coupons that coincided with the initial stages of biofilm formation. In contrast to the results from the environmental study, no ennoblement of the stainless steel was observed in the laboratory experiments. Visualization of the metal surfaces and biofilms by confocal laser scanning microscopy (CLSM) and field emission scanning electron microscopy (FESEM) showed distinct changes in the topography of metal surfaces during the onset of MIC. It was additionally observed that the biofilms appeared to preferentially form at the inter grain boundaries and this may be a driving factor in pit formation. The community composition of the biofilms formed on the metal surface were found to depend on the composition of the type of stainless steel used and were significantly different from the planktonic community. The effect of several parameters (e.g., stirring, nutrient concentration, surface polishing and oxygen concentration) on MIC rate were investigated in a systematic manner. The carbon source determined the composition of the microbial community, thus the corrosion rate. Surface polishing affected the topography of the metal surface, which in turn changed the localization of biofilms on the surface as compared to unpolished coupons. A continuous flow cell system was designed to allow for continuous nutrient replenishment, which should more closely reflect the natural environment. As for the batch experiments, the results suggested that nutrient composition plays an important role in biofilm formation and determines MIC rate. The electrochemical impedance of the biofilm formed on stainless steel was consistent with the rapid MIC onset.

This study has attempted to reproduce the environmental conditions in a reliable laboratory setup, while employing different techniques for the analysis of corrosion behaviour due to biofilms. Overall, the results from this study showed that marine communities rapidly colonize metal surfaces and such biofilms may be formed by organisms that specialize in growth as a biofilm. The results also showed that the early corrosion behaviour is primarily driven by nutrient concentration. However, to better understand the relationship between community members and MIC, further mechanistic studies are needed. Such studies will be possible through the combination of electrochemistry,

image analysis and –omics methods to improve our understanding of the role of biofilms in MIC.

List of figures

Figure 1.1 Simplified reaction steps of electrochemical corrosion.....	2
Figure 1.2. Schematic diagrams of different types of corrosion.....	4
Figure 1.3 Schematic representation of factors involved in MIC.....	6
Figure 1.4 Graphical representation of complex MIC processes.....	9
Figure 1.5 The stages of biofilm formation and selected environmental factors that affect each stage of biofilm formation.	12
Figure 1.6 Schematic of MIC mechanisms in biofilms..	16
Figure 2.1 Comparison between polished and unpolished	31
Figure 2.2 Schematic for the environmental experiment at St. Johns Island National Marine Laboratory (SJINML).....	39
Figure 2.3 Deployment of SD and SS304 coupons in the tank at SJINML facility.	42
Figure 2.4 Schematic of the pipeline used for sequence data analysis.	45
Figure 2.5 Schematic representation of the flow cell setup.	46
Figure 2.6 Schematic diagram of the electrochemical flow cell.....	48
Figure 2.7 Assembly of bio-electrochemical batch reactor system.	51
Figure 3.1 CLSM images showing a) attachment of cells at 1 h; b) clustering of cells after 2 d and c) the formation of a biofilm with microcolonies by 7 d.....	57
Figure 3.2 Comparison of potential (mV) and biovolume ($\mu\text{m}^3/\mu\text{m}^2$) after 1 h, 2 d and 7 d of exposure of UNS32750 stainless steel in natural seawater tanks. .	58
Figure 3.3 Comparison of microbial communities in seawater and in biofilms formed on UNSS32750 coupons.	61
Figure 3.4 The open circuit potential as measured for UNS32750 (SD, red line) and UNSS30400 (SS304, grey line) over 30 d exposure.....	63
Figure 3.5 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images for SS304 and SD coupons, retrieved from seawater at 1 h as well as after 2, 7 and 30 d (400X magnification).64	

Figure 3.6 Biovolume and surface area covered by the biomass after 1 h, 2 d, 7 d and 30 d.....	65
Figure 3.7 Comparison of potential (mV) and biovolume (μm^3) for a) SS304 and b) SD.	67
Figure 4.1 Rarefaction plot of observed OTUs in different enrichments..	73
Figure 4.2 The percentage abundance of the top 20 species present in the enrichments of VNSSFs and VNSSFSG.....	75
Figure 4.3 The effect of carbon source on pH a) and OD600 b) on mixed species biofilm communities grown under flowing conditions.	76
Figure 4.4 a) Current density (i) and b) corrosion potential (E_{corr}) of SS304 exposed to different mixed species biofilms grown in VNSSFs or VNSSFSG..	77
Figure 4.5 The capacitance (F/ farad) of biofilm layer vs. time of SS304 exposed to different mixed species biofilms grown in VNSSFs or VNSSFSG.	78
Figure 4.6 The resistance of biofilms formed on SS304 exposed to different mixed species biofilms grown in VNSSFs or VNSSFSG.....	79
Figure 4.7 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images showing biofilm formation on SS304 steel grown in a) VNSSFs or b) VNSSFSG.....	80
Figure 4.8 a) Bio-volume ($\mu\text{m}^3/\mu\text{m}^2$) and b) surface area covered (μm^2) of biofilms on metal coupons grown in either VNSSFs or VNSSFSG.....	81
Figure 4.9 PCoA plot of the enrichment and the biofilm samples on different coupon types for VNSSFs medium.....	83
Figure 4.10 The abundance of the 20 most abundant species in the biofilm samples for three different types of metal coupons where the biofilms were grown in VNSSFs medium..	84
Figure 4.11 Comparison of top 20 species present in VNSSFs enrichment and biofilm samples.....	85
Figure 4.12 PCoA plot of the enrichment and the biofilm samples on different coupon types for VNSSFSG medium.	86

Figure 4.13 The abundance of the 20 most abundant species in the biofilm samples for three different types of metal coupons where the biofilms were grown in VNSSFSG medium.....	87
Figure 4.14 Comparison of top 20 species present in VNSSFSG enrichment and biofilm samples.	88
Figure 4.15 PCA plot of the biofilm samples grouped in relation to the type of metal surface.	89
Figure 4.16 Heat map of significantly different expression of functional pathways in planktonic and biofilm phase for samples from VNSSFSG for three different coupon types.....	91
Figure 4.17 Heat map of significantly different expression of functional pathways in planktonic and biofilm phase for samples from VNSSFSG for three different coupon types.....	93
Figure 4.18 PCA plot for functional classification of biofilm samples for VNSSFSG and VNSSFSG media.....	95
Figure 5.1 Standard large corrosion cell by Bio-logic.....	98
Figure 5.2 a) Current density (i) and b) corrosion potential (E_{corr}) of SS304 over 35 d for the microbial community grown in 3M.....	101
Figure 5.3 CLSM images of the surface of the SS304 coupon after 35 d.	102
Figure 5.4 Intensity profile for reflection of the coupon and the biofilm stained with live-dead staining.	104
Figure 5.5 FESEM images for unpolished SS304 coupons after a 35 d laboratory experiment a) control coupon on 0 d, b) coupon on 35 d after removing the biofilm and c) control coupon after 35 d.	105
Figure 5.6 EDX analysis of SS304 with biofilm on 35 d.	106
Figure 5.7 The effect of nutrient concentration on a) corrosion current density (i) and b) corrosion potential (E_{corr}) of S304 coupons.....	107
Figure 5.8 AFM micrographs of SS304 coupons with different polishing treatments a) Unpolished coupons, b) p600 grit, c) p1000 grit and d) Ra values for surface roughness.	108

Figure 5.9 Effect of surface polishing on a) current density (i) and b) corrosion potential (E_{corr}), for SS304 coupons (Unpolished, p600, p1000, Pre-soaked for 4 days).....	110
Figure 5.10 Effect of shaking speed on a) current density (i) and b) corrosion potential (E_{corr}), for SS304 coupons. (0, 40, 80 rpm). For every reactor, n = 3.	110
Figure 5.11 Comparison between corrosion behaviour of unpolished SS304 coupons, incubated in 3M media and inoculated with mixed microbial community for LSV and LPR techniques.	112
Figure 5.12 Three reactors after 2 d of growth in the presence of 0.2, 0.02 and 0.002% glucose.	113
Figure 5.13 The a) pH and b) OD_{600} for the corrosion reactors incubated with different concentrations of glucose over 7 d.	114
Figure 5.14 a) Corrosion current density (i) and b) corrosion potential (E_{corr}) measured for mixed species cultures cultured under three different glucose concentrations (0.2, 0.02 and 0.002%).	115
Figure 5.15 Polarisation resistance (R_p) over 7 d, measured for mixed species cultures cultured under three different glucose concentrations (0.2, 0.02 and 0.002%).....	116
Figure 5.16 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images showing biofilm formation on SS304 steel in presence of a) 0.2, b) 0.02 and c) 0.002% glucose reactors..	117
Figure 5.17 a) Surface area covered by the biofilm and b) biovolume for reactors with different glucose concentrations. Each bar represents an average of ≥ 3 images at different locations for the same coupon.	117
Figure 5.18 Comparison of a) corrosion current density (i) and b) corrosion potential (E_{corr}), measured for 3M medium with 0.002% glucose, autoclaved seawater inoculated with the 3M enrichment and a sterile seawater control...	119
Figure 5.19 a) pH and b) OD_{600} measured for 7 d corrosion reactors where a mixed species culture was inoculated into VNSSFs and VNSSFSG reactors..	120

Figure 5.20 a) Corrosion current density (i) and b) corrosion potential (E_{corr}),
observed for VNSSFs and VNSSFSG reactors.....121

List of tables

Table 2.1 Summary of the experimental systems used in this study.	27
Table 2.2 Composition of UNS32750, UNSS30400 and UNSS31600 steel coupons.	29
Table 2.3 Recipe for 0.5 X NSS.	32
Table 2.4 Recipe for 3M (Marine minimal medium).	33
Table 2.5 Recipe for VNSS medium.	33
Table 4.1 Pathways that were differentially expressed in biofilms vs. planktonic cells grown in VNSSFs.	91
Table 4.2 Pathways significantly expressed when biofilm and planktonic phase are compared for VNSSFSG with corrected p values for each.	94

Author publications

Jogdeo, P., Chai, R., Shuyang, S., Saballus, M., Constancias, F., Wijesinghe, S.L., Thierry, D., Blackwood, D.J., McDougald, D., Rice, S.A. and Marsili, E., 2017. Onset of Microbial Influenced Corrosion (MIC) in Stainless Steel Exposed to Mixed Species Biofilms from Equatorial Seawater. *Journal of The Electrochemical Society*, 164(9), pp.C532-C538

Meetings attended

- I. International Congress on Marine Corrosion and fouling (ICMCF), Singapore, 6th – 10th July, 2014.
- II. Gordon Research Conference (GRC) on Aqueous Corrosion, New London, NH, US, 10th – 15th July, 2016 (Poster Presentation).
- III. 16th International Symposium on Microbial Ecology (ISME) Montreal, Canada, from 21st - 26th August, 2016 (Poster Presentation).

List of abbreviations

MIC	Microbially Influenced Corrosion
EFC	Electrochemical Flow Cell
NSS	Nine Salts Solution
VNSS	Väätänen Nine Salts Solution
VNSSFSG	Väätänen Nine Salts Solution (1X NSS) with Glucose
VNSSFS	Väätänen Nine Salts Solution (1X NSS) without Glucose
3M	Marine Minimal Medium
OCP	Open Circuit Potential
LPR	Linear Polarisation Resistance
LSV	Linear Sweep Voltammetry
EIS	Electrochemical Impedance Spectroscopy
SRB	Sulphate Reducing Bacteria
CLSM	Confocal Laser Scanning Microscopy
FESEM	Field Emission Scanning Electron Microscope
EDX	Energy-dispersive X-ray spectroscopy

Chapter One: Literature Review

1.1 Fundamentals of corrosion

Metals are used extensively in different environments and for numerous applications from large scale infrastructure such as harbors, bridges, buildings to vehicles, ships, storage tanks, electronic equipment, medical devices, jewelry, coins etc. All metals used in these applications tend to deteriorate when they react with the environment. This corrosion process can be defined as chemical or electrochemical reaction between a metal and its environment, which results in deterioration of the metal and its properties (Davis 2000). It is a natural process of metal returning to its low energy form i.e., metal oxides, the original form in which they are found in the environment. It is a simultaneous transfer of mass and charge across metal/solution interface (McCafferty 2010). This electrochemical process is comprised of oxidation (anodic) and reduction (cathodic) reactions. The anodic reaction describes the dissolution of the metal, where the metal atoms donate electrons and become ions, as can be seen in equation (1.1).

Cathodic reactions take place in the surrounding environment. Electron acceptors involved may differ according to aerobic or anaerobic conditions. These reactions are represented in equations (1.2) to (1.5).

Oxic cathodic reduction reactions

Anoxic cathodic reduction reactions

At the metal surface, these occur as coupled reactions, where certain sites act as anodes, passing the electrons into the environment (Figure 1.1). In the absence of an electron acceptor, these electrons drive the potential towards a negative potential, where dissolution of the metal is retarded and dissolved metal ions are deposited on the metal surface. When the rate of dissolution is equal to the rate of deposition, the metal is at a stable potential. However, these electrons are consumed by various electron acceptors, depending on their availability, in the environment. Additionally, for these reactions to occur, a path of contact between anode and cathode as well as presence of an electrolyte is necessary. An electrolyte is a solution, containing dissolved ions, capable of conducting a current.

Figure 1.1 Simplified reaction steps of electrochemical corrosion; the left side of the panel shows reactions in the absence of dissolved oxygen and on the right, in the presence of dissolved oxygen; the metal (M) donates electrons (e^-) and becomes a dissolved and positively charged ion (Mn^+) at the anode. At the cathode, electrons are accepted by the available electron acceptor. Hydroxide ions (OH^-) react with the metal ions (Mn^+) and precipitate as metallic hydroxides ($M(OH)_n$), indicated by the brown oval spots.

1.1.1 Passive films

Passive films or oxide films are considered as the first line of defense of a metal against corrosion. The composition of the passive film depends on the alloy composition and surface preparation (Compere et al. 1997). In oxygen-replete environments, an oxide film is formed on the metal surface, which protects the

metal from aggressive species such as halides. Chloride ions have the ability to permeate passive films resulting in the initiation of electrochemical reactions. These passive films have self-healing properties, i.e., the oxides are reformed in the presence of oxygen (Machuca et al. 2013). The breakdown of passive films can occur at specific sites on a metal surface, such as around irregularities in topography, discontinuities in the metal surface or uneven deposits of biofilms or debris. The breakdown of the passivating film in small areas on the metal surface results in the formation of pits.

1.1.2 Types of corrosion

Corrosion is classified into several types, of which stress corrosion cracking, uniform corrosion, galvanic corrosion, pitting and crevice corrosion are some important corrosion types (Figure 1.2). In the environment, these types usually overlap with each other (Revie et al. 2011).

Uniform corrosion is often visible for less noble metals and mild steel. It occurs when multiple anodic and cathodic areas are formed on the surface, which leads to an overall breakdown of the passivation layer. It is accepted in some cases, for example for railway rails, where the corrosion products slow the overall corrosion process (Figure 1.2a). Galvanic corrosion occurs at the interface of two different metals or alloys. A more noble metal is protected by a less noble metal, which corrodes only locally at the connection area (e.g.- Zn cathodic protection). This process has been harnessed for cathodic protection, where the more easily corroded material is called a sacrificial anode (Figure 1.2b).

Figure 1.2. Schematic diagrams of different types of corrosion (a) a uniform attack caused by many corrosion elements; brown ovals indicate corrosion products. (b) Galvanic corrosion between a noble metal (left) and a less noble metal (right). (c) Pitting corrosion underneath a small damaged spot of the passivation layer; the red arrows indicate the direction of degradation; brown ovals indicate corrosion products. (d) Crevice corrosion with a small anodic spot at the bottom of the crevice, where the oxygen is depleted. The red arrows indicate the direction of degradation; brown ovals indicate corrosion products.

Pitting is a type of localised corrosion confined to a small area on the surface. It can occur as the passive film breaks down in that fixed small area, usually by chloride ions (McCafferty 2010). It can also be caused by impurities in the metal or the presence of scratches on the metal surface. This small area acts as an anode. A large cathodic area in combination with a very small anodic area

leads to a high local current density at the anode, which results in a fast corrosion process deep into the metal (Figure 1.2c). Crevice corrosion is often visible at assembly points, for example under a bolt head that penetrates the metal surface (Figure 1.2d). The formation of a biofilm on the surface may also result in pitting corrosion, crevice corrosion, selective dealloying, under-deposit corrosion etc., due to metabolic activity of microorganisms (Shalaby et al. 1999). In the marine environment, it has been shown that most severe crevice corrosion occurs in absence of oxygen and in presence of biofilms.

1.2 Microbially influenced corrosion - Old problem, no solution

Microbially Influenced Corrosion (MIC) of metals is a process whereby the microorganisms mediate the deterioration of the metals on which they grow (Muyzer et al. 2008). MIC causes significant economic losses in industries such as oil and gas, shipping, chemical processing, waste water treatment and aviation (Kobrin 1993, Little et al. 2007).

In March 2006, a significant oil spill was detected in the western operating area of the giant Prudhoe Bay oil field on Alaska's North Slope. The estimated volume of leaked oil was around 200,000 gallons and the cleanup cost the company (BP) around 8 million dollars. Upon investigation the leak was attributed to internal corrosion in the bottom of the pipeline, which was caused by presence of microorganisms, mainly SRB (Bailey 2006).

Expeditions to study the RMS Titanic and many other ship wrecks show the activity of a mixed bacterial community on their surfaces. "Rusticles" retrieved from the sites were examined microscopically and revealed that these rusticles were complex structures composed of communities of bacteria and fungi (Cullimore et al. 2000).

The formation of biofilms on metals and alloys is also a health hazard for the public. For example, the corrosion of pipelines in a water distribution system not only results in deterioration of the infrastructure but also compromises the

water quality and may pose a potential health threat if pathogens are present in, and released from, the biofilm (Emde et al. 1992). Bacteria of public health concern such as *Legionella* spp., *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been isolated from corrosion tubercles from water distribution systems (Olson et al. 1984).

Although MIC is often underestimated, it has been suggested that approximately 20% of corrosion cost is due to microbial corrosion (Al Abbas et al. 2013). It is a complex process, which involves the material, biofilm and the environment (Figure 1.3).

Figure 1.3 Schematic representation of factors involved in MIC. MIC occurs only in the presence of suitable microorganisms, environmental conditions, and material properties.

The involvement of microorganisms in the corrosion process was first identified more than 100 years ago (Gaines 1910). Gaines analyzed sulfur-rich corrosion products from anaerobically corroded iron constructions and hypothesized about a connection to the bacterial sulfur cycle. Since then, different theories have been put forth to explain this phenomenon. The role of sulphate-reducing bacteria (SRB) in the corrosion process was first demonstrated and the theory of cathodic depolarization was proposed (von Wolzogen Kühr et al. 1934). According to their theory, an iron surface immersed in water becomes

negatively depolarized, forming anodic areas. Under these conditions, the dissociation of water to protons from the cathodic site is slow and hence is the rate limiting step. However, when SRB are present, they can scavenge the hydrogen atoms to reduce sulphate, resulting in a faster release of hydrogen atoms and hence increasing the rate of corrosion.

Reactions involved –

The overall reaction is

As iron sulphide (FeS) encounters bare metal, it acts as a cathode, where the cathodic reaction i.e., evolution of molecular hydrogen or entry of atomic hydrogen into the metal takes place. According to King and Miller, adsorption of atomic hydrogen by FeS corrosion products is the most critical reaction in SRB induced corrosion (King et al. 1971).

While studying role of microorganisms in MIC, Johnston and Gaylarde suggested that direct contact of bacteria with the metal surface is required for weight loss to occur (Gaylarde et al. 1980). However, Iverson proposed an alternative corrosive metabolite hypothesis, e.g. phosphorous metabolites produced by bacteria are volatile and do not need a direct contact of bacteria with the metal surface (Iverson 1987). Hamilton (1985) proposed that breaching

of the oxide / passive layer, can lead to MIC. Other MIC mechanisms suggested so far include the effects of differential concentrations of oxygen and nutrients; generation of corrosive metabolites or by-products; alteration of anion ratios and inactivation of corrosion inhibitors (Zuo 2007). Recently, a mechanism, in which corrosive SRB remove electrons directly from the iron surface, has been proposed (Dinh et al. 2004, Enning et al. 2012). Indeed, Most of the MIC mechanisms proposed in literature involve the action of SRB.

In nearly 100 years, since the role of microorganisms in the corrosion process was first shown, there have been many studies of MIC, although these have primarily focused on axenic cultures. While such studies are helpful in understanding individual mechanisms mediated by these bacteria, this approach does not account for the intra- and interspecific complexity in mixed consortia found in the environmental biofilms. As a consequence, the mechanisms responsible for MIC remain unclear, since MIC cannot be linked to a single set of biochemical reactions or a single species (Kip et al. 2015). More likely, the combined microbial metabolic activity of the community, which is intimately linked to the biofilm microstructure heterogeneity and local chemistry, plays a strong role in the MIC process. Recent NACE standards (NACE Std. TM0212-2012) for assessing MIC recognized the shortcomings of investigating the process with specific type of microorganisms and thus included culture independent identification techniques. Recent technological advances in microbial ecology and interface characterization (discussed below) has expanded the tools available for studies of complex communities and their interactions and such tools are now being applied to MIC studies. Figure 1.4 summarizes the complexity of MIC in terms of parameters involved and techniques used in environmental as well as laboratory conditions.

Figure 1.4 Graphical representation of complex MIC processes. Three main factors, the environment, surface properties and microorganisms as well as some of the parameters affecting MIC. Environmental parameters affecting the MIC process are given in blue circles. Green circles summarize different aspects related to microorganisms and green boxes indicate the techniques incorporated for the studies. Similarly, brown circles show the parameters crucial in surface analysis with the techniques used to quantify those in brown boxes. Widely used electrochemical techniques are indicated in purple boxes. The factors that are considered to be important under laboratory conditions are indicated in orange.

1.3 Metal surface

1.3.1 *Elemental composition*

In addition to surface roughness and surface energy, differences between the grain boundary and the grains, bacterial attachment is also affected by the elemental composition. Duplex steels attain their favorable mechanical strength due to the presence of a ferrite (α) phase and high corrosion resistance from an austenite (γ) phase. The presence of chromium, nickel and molybdenum result in the formation of passivation films that protect the metal from aggressive ions like chloride (Xu et al. 2017). Austenitic stainless steels are resistant to corrosion due to the presence of a thin, compact chromium enriched oxide film on the surface (Borenstein 1994). Due to their known physicochemical (Melchers et al. 2004) and microbiological resistance (Fang et al. 2002) to metal deterioration and MIC, stainless steels (SS) are used in many marine components. Molybdenum, when added to the alloy increases the corrosion resistance, as it reduces active sites on the surface by formation of molybdenum oxyhydride or molybdate, which enhances the protective properties of the passive film (Hashimoto et al. 1979). Scott et al. (1991) ranked alloys for their susceptibility to MIC (Scott et al. 1991), which states that alloys with 6 to 9% Mo were prone to MIC and the correlation between presence of Mo and corrosion resistance of the alloy remains unclear.

1.3.2 *The effect of surface and geometry*

In MIC, attachment and colonization of bacteria on the surface is influenced by the substratum as well as the specific mechanisms used by bacteria to perceive and stick to those surfaces (Cook et al. 1988). Initial adhesion of microorganisms starts at a location on the surface where bacteria are sheltered against the shear forces (Gharechahi et al. 2012). For example, defects in the metal surface such as kink sites, edges or screw threads, can change the flow of liquid around the metal surface and facilitate attachment. Furthermore, the metal surface is highly heterogenous due to the presence of surface contaminants associated with impurities. This heterogeneity is also inherent in

the metal, where metal crystals form, often described as grains that are unevenly distributed in the final metal product. These defects change the surface energy of metal atoms (McCafferty 2010). Interestingly, it has been shown that surfaces with lower surface energy are more susceptible to bacterial adhesion, highlighting that the grain distribution can play an important role in the MIC process (Bakterij 2014).

1.3.3 Grain boundaries and high-energy areas

It has been shown that in flowing water systems, bacteria preferentially settle in the depressions of oxide film grain boundaries of SS316 (Geesey et al. 1996). In a similar study, Sreekumari et al. (2001) showed an inverse relationship between the grain size and the area available for bacterial attachment (Sreekumari et al. 2001). Thus, the smaller the grain size, the longer the grain boundary per unit area, which results in more bacterial attachment. A higher percentage of carbon due to carbide precipitation and a reduced percentage of chromium at the grain boundaries (up to 40%) than the adjacent grains (Bruemmer 1989), results in lower corrosion resistance. This elemental segregation at grain boundaries could allow for enhanced attachment of bacteria (Hughes et al. 1989, Sreekumari et al. 2000). As the surface roughness increases, the colonization rate also increases (Baker 1984). For example, Little et al. (1988) observed that porous weld surface shows more attachment than the smooth pipe surface (Little et al. 1988). Furthermore, the number of metastable pits was less on a smooth surface than on a rough surface (Hong et al. 1997). These results suggest that bacterial attachment and biofilm formation is a key step in the MIC process.

1.4 Biofilms: unexplored dynamics in the MIC communities.

Biofilms can be defined as a bacterial population or community encased in extracellular polymeric substances (EPS) adherent to each other and/or to surfaces or interfaces (Costerton et al. 1995). In nature, most of the bacteria are

thought to form biofilms (Stoodley et al. 2002). Several steps are involved in biofilm formation, starting with surface attachment, where bacteria encounter the surface and attach. This is followed by the maturation stage, in which a structured architecture is formed. After maturation, the cell leaves the biofilm in the dispersal stage. The formation and dispersal of a biofilm is highly dynamic and is regulated in response to a range of environmental factors (Toyofuku et al. 2016) (Figure 1.5).

Figure 1.5 The stages of biofilm formation and selected environmental factors that affect each stage of biofilm formation. The environmental cues control the intracellular concentration of second messengers such as cAMP and c-di-GMP. These second messengers control biofilm-related factors such as cell appendages, surface proteins, EPS, and cell motility. QS is also involved in shaping the biofilm by controlling the above biofilm-related factors (Toyofuku et al. 2016).

1.4.1 The formation and role of biofilms in MIC

Biofilm formation associated with MIC can similarly be divided in different stages. The process starts with the adsorption of organic and inorganic macromolecules such as exopolymers, glycoproteins etc. to the surface to form a conditioning film. This conditioning film induces modifications in surface properties such as surface energy, surface tension etc., which enables subsequent bacterial adhesion. Transport of the bacteria to the surface due to fluid dynamics or Brownian motion is followed by reversible and irreversible

attachment of microorganisms (Ford et al. 1990, Compere et al. 1997). Transport of microorganisms to the surface is influenced by pH, temperature, surface energy of the surface, surface hydrophobicity and bacterial growth stage (Gharechahi et al. 2012). Stagnant and low flow conditions can also promote the attachment of bacteria to the surface (Kobrin 1993). The biofilm structure supports a strong attachment of the cells to the surface, enables nutrient diffusion and intercellular communications. It also provides protection against environmental stress, antibiotics and disinfectants and enables intercellular communications (Bakterij 2014). The composition and activity of the microbial communities that form a corrosive biofilm also depends on factors such as temperature (Duncan et al. 2009), availability of carbon substrate (Videla et al. 2009) and electron acceptors.

Biofilms have the capacity to alter the local oxygen concentration (Little et al. 1991), pH levels (Daniels 1980) and ion concentration (Corpe 1970) at the biofilm-metal interface as a consequence of metabolic processes. These changes modify the local environment and directly alter the local corrosion rate (Alasvand Zarasvand et al. 2014). For example, a heterogenous aerobic biofilm on the surface can result in depletion of oxygen under thick films relative to thinner biofilms, and the area with depleted oxygen becomes anodic to more oxygenated area (Ford et al. 1990). No correlation has been found in the number or types of bacteria involved, the type of material affected or the corrosion mechanism involved in the process (Little et al. 2014).

1.4.2 Corrosion inhibition by biofilms

In recent years, several studies have indicated that, while biofilm formation is associated with enhanced corrosion, some biofilms actually protect the metal against corrosion (Jayaraman et al. 1997, Potekhina et al. 1999, Zuo 2007, Videla et al. 2009, Dobretsov et al. 2013, Alasvand Zarasvand et al. 2014). Protection can occur if the biofilm covers the whole surface, which may reduce the cathodic reaction rate (Videla et al. 2009). Typically, such protective biofilms function by inhibiting the activity of, or biofilm formation by, corrosive organisms (Zuo 2007). Some proposed mechanisms include: i)

removal of corrosive substances through microbiological activity, ii) corrosion inhibitors secreted by biofilms, such as poly-aspartate or poly-glutamate containing carboxyl groups with affinity to bind with positive metal ions forming metal-peptide complexes (Örnek et al. 2002), iii) formation of a protective layer by the overproduction of EPS by non-corroding microorganisms, iv) growth of a biofilm on the metal surface, that produces antibiotics, inhibits the biofilm formation by corrosive organisms and v) biofilm secreted surfactants that adsorb on the metal surface, forming a barrier that delays the corrosion process (Videla et al. 2005, Zuo 2007, Alasvand Zarasvand et al. 2014). These studies can have possible applications to explore the use of biofilms to protect the metal surface. As mentioned above, the role of EPS in corrosion inhibition, can be further explored for use as anticorrosion coatings. However, most studies of corrosion inhibition by biofilms have been performed with pure cultures and the roles of these bacteria in a consortium remains poorly understood, which limits their development as MIC control strategies.

1.4.3 The role of extracellular polymeric substances (EPS)

A biofilm is a complex structure that consists of an array of microcolonies encased in EPS excreted by microorganisms. There are often channels between the microcolonies and it is thought that these channels facilitate mass transport of nutrients and waste products into and out of the biofilm (Keevil 2004). EPS affects the metal biofilm interface in three ways: i) it immobilizes water at the interface, ii) it traps metal species (e.g. iron ions) and corrosion products in the biofilm forming organometallic complex (Fe-EPS) (Breur et al. 2002) and iii) it decreases the diffusion of nutrients and soluble corrosion products (Characklis 1981). Adsorbed macromolecules or EPS can alter properties of the surface by modifying the surface charge, wettability and surface energy, thus enhancing or inhibiting MIC (Fletcher et al. 1982, Chongdar et al. 2005). Corrosion is enhanced if the EPS contains acidic groups, which lower the interfacial pH (Ford et al. 1990). A study performed with only EPS showed that a Fe-EPS complex layer was formed and this layer blocked active sites available for corrosion. EIS studies revealed an increase in the corrosion resistance. (Nan et

al. 2015). Enzymes trapped in EPS could also be responsible for the increased corrosion rate (Scotto et al. 1998). Since EPS plays an important role in the corrosion mechanism, it is important to know the chemical composition and its role in corrosion to better understand the role of biofilms in enhancing or reducing corrosion rates.

1.5 Interface processes – The interaction between metal, microbes and environment

Microorganisms affect physicochemical reactions at the metal/liquid interface, either slowing down or accelerating abiotic corrosion processes (Figure 1.6) (Little et al. 2007, Kip et al. 2015). Electrochemical reactions occur at the interface where the metal surface acts as anode donating electrons and the surrounding environment (biofilm and solution) functions as a cathode (Beech et al. 2004). Microorganisms alter the structure of inorganic passive layers and increase metal dissolution (Yuan et al. 2008) with several changes at the interface, such as i) localized high concentration of electrolyte, ii) formation of pH gradients due to metabolic activity of microorganisms, iii) depletion of oxygen at the interphase due to microbial respiration and iv) Selective dissolution of alloying elements (Mansour et al. 2016).

As the macro and microorganisms are adsorbed on the surface, they reproduce, forming colonies to form local cathodes and anodes creating differential aeration cells. These aeration cells can be generated by any factor causing high oxygen concentration in one area and lower in the other. In aerobic conditions, areas under respiring colonies become anodes and the surrounding uncolonized areas become cathodes. The presence of a mature biofilm impedes diffusion of oxygen to cathodic sites, diffusion of aggressive anions to anodic site and chemical metabolites or corrosion products in and out of the biofilm (Little et al. 1991). An increase biofilm biomass is normally associated with a concomitant increase in the number of bacteria within the biofilm, which results in an overall increased metabolic activity and hence, corrosion (Bishop et al. 1999).

Figure 1.6 Schematic of MIC mechanisms in biofilms. A) creation of anaerobic zones, B) concentration of corrosive chemicals, C) concentration of ferrous ions, D) electron conduction away from surface, E) creation of differential aeration zones, F) binding of corrosion promoters and G) disruption of passivating biofilm. The figure is reproduced from (Li et al. 2013).

1.6 Axenic vs. mixed species biofilms: Increasing complexity

1.6.1 Axenic culture studies

MIC studies have primarily used axenic cultures to characterize the corrosion behavior and propose corrosion mechanisms. These studies have focused on bacteria and archaea such as acid producing bacteria (Bogan et al. 2004, Keasler et al. 2010), sulphate-reducing bacteria (SRB) (Dolla et al. 2006, Lambert et al. 2006, Cetin et al. 2009, Enning et al. 2014), sulphur-oxidizing bacteria (SOB) (Tang et al. 2009) and iron precipitating bacteria (Starosvetsky et al. 2001) along with hydrocarbon degrading microorganisms, nitrate-reducing (NRB) methanogens and acetogenic bacteria (Little et al. 2014).

Although SRB are considered to be the organisms that have the greatest role in MIC, many aerobic microorganisms have also been studied for their effect on corrosion behavior on different types of stainless steel. Moradi et al. (2014) observed a negative shift in the corrosion potential and increase in the current

density of 2205 duplex stainless steel in the presence of the marine aerobic bacterium *Pseudalteromonas sp.* (Moradi et al. 2014). In another study, when 304SS was exposed to an aerobic marine *Pseudomonas sp.*, the corrosion rate increased and the resistance of the passive film decreased. Their FESEM images showed extensive micro-pitting corrosion underneath the biofilms. In the absence of bacteria, the passive film was relatively stable and the anodic reaction was under diffusion control (Yuan et al. 2008). Similarly, the facultative anaerobe *Vibrio natriegens*, ubiquitous in the marine environment, showed a decreased potential for the metal surface on which it grew in first 48 h, that was suggested to be due to the establishment of a biofilm, metabolic processes of the microorganism or the establishment of a cathodic reaction (Cheng et al. 2009, Yin et al. 2009).

In contrast, biofilm formation has also been shown to inhibit corrosion (Jayaraman et al. 1997, Zuo 2007, Dobretsov et al. 2013, Alasvand Zarasvand et al. 2014, Kip et al. 2015). Pedersen et al. first observed the inhibitory effect of *Pseudomonas sp.* s9 biofilms. Biofilms of *P. mendocina* (Jayaraman et al. 1997) as well as *V. neocaledonicus* (Moradi et al. 2015) were observed to reduce corrosion rates 10 – 60 fold. The reduction in corrosion rate was associated with the adsorption of bacteria on to the surface that retarded the anodic process by depleting oxygen in the environment through bacterial respiration (Moradi et al. 2015).

These studies highlight that specific bacteria may contribute different MIC mechanisms and in some cases, bacteria may in fact retard corrosion. While much has been learned about the individual mechanisms of corrosion using axenic species, the results from such studies are generally not predictive of corrosion in the natural environment. In natural and engineered systems, microorganisms are typically present as a complex community of different species. These species interact during biofilm formation in many ways, from facilitating attachment of other organisms to cooperative metabolism and thus, it is perhaps not surprising that axenic cultures are not predictive of corrosion mediated by complex communities (Stoodley et al. 2002).

1.6.2 Dual species, co-cultures and mixed species communities

Given that MIC is affected by the aggregate activity of a microbial community, there is a need for corrosion studies to account for this diversity. While most studies have focused on single species cultures, some have used dual species cultures to understand how microbial interactions may alter the corrosion process. For example, a coculture of *V. natriegens* and the SRB *Desulphovibrio vulgaris* has been shown to enhance the corrosion rate of the metal surface under aerobic (Dowling et al. 1988) as well as anaerobic conditions (Gaylarde et al. 1987) as compared to their respective monocultures under static conditions (Gaylarde 1992). However, in a laboratory flow through system, the dual species community did not show a significantly increased corrosion rate, suggesting that a continuous flow system was unsuitable for this co-culture in spite of microbial attachment and multiplication on the surface (Benbouzid R et al. 1991). Jack et al. in 1980 used a consortium of an obligate aerobe (*Bacillus* sp.), a fermentative facultative anaerobe (*Hafnia alvei*) and SRB (*Desulfovibrio gigas*) to monitor the corrosion rate of carbon steel. The monocultures showed an increased corrosion rate initially but then decreased to match to that of the sterile control. A significantly higher corrosion rate was measured for the dual species culture of *D. gigas* and *H. alvei* than the triculture or the other dual species cultures (Jack 1990, Jack et al. 1992). A consortium of the SRB *D. vulgaris* Hildenborough and an acetate producing fermentative anaerobe *Acetobacterium woodii* was grown in a defined medium containing hydrogen, carbon dioxide, and sulfate. *A. woodii* produces acetate, which is further used by *D. vulgaris*. General corrosion rates of metal coupons incubated with *D. vulgaris* in the presence of acetate or of *A. woodii* were higher than in the absence of acetate or *A. woodii*, respectively (Mand et al. 2014). In contrast, *Bacillus* strain B21 was shown to inhibit the growth of the SRB during coculture, which decreased overall sulphide production and hence reduced corrosion (Gana et al. 2011). A mixture of metal oxidizing bacteria (iron and manganese oxidizing bacteria) isolated from the marine environment was used

to study the de-alloying process in which bacteria increased the corrosion rate of SS316, caused segregation of Si elements and depleted Cr and Fe contents in the corrosion products, suggesting that in the presence of these bacteria de-alloying of stainless steel takes place (Moradi et al. 2011).

In addition to reconstituting communities from pure cultures, as described above, some have used undefined cultures as the inoculum for corrosion studies (Scotto et al. 1985, Videla et al. 1992). However, the results obtained were mostly qualitative and were not reproducible, making it difficult to connect the corrosion behavior observed to the microorganisms present. The diversity and metabolic activity of multi-species biofilm exacerbate corrosion through the cascade of biochemical reactions in oxic and anoxic region of biofilms (Zuo 2007). This is further complicated by the recent observations that microbes can either promote or inhibit MIC upon formation of biofilm by one of the three approaches: i) removal of oxygen by microbial aerobic respiration, which results in a differential aeration cell; ii) growth promotion or inhibition of corrosion-causing bacteria, which affects the overall MIC rate and iii) the generation of a protective, poorly conductive layer on the metal surface, which alters the diffusion dynamics of corrosion products (Mansour et al. 2016). While complicated to study, it is clear that experimental systems that enable the use of complex microbial consortia are likely to yield a more holistic understanding of the corrosion process as it occurs in natural and engineered systems.

1.7 MIC characterization

The preliminary method of corrosion investigation is “visual inspection” which is only a guide to determine the most suited measurements. A widely-used approach is monitoring the weight loss for metal which is suitable for corrosion monitoring in long term experiments. Weight is measured for the sample before and after immersion and result is expressed as difference per unit time (Eq. 1),

given as;

$$v = \frac{1}{\rho \Delta t} \frac{\Delta m}{s} \quad \text{Eq. 1}$$

where,

v = corrosion rate cm/s

ρ = material density g/cm³

Δt = duration of the experiment

Δm = mass lost in grams

S = surface area cm²

Although useful for long term corrosion measurements, this method is not suitable for short term experiments or to study the initiation of corrosion. A quartz crystal microbalance can be used to detect minute changes of mass variation from measurement of frequency variation of a quartz resonator (Rahmouni et al. 2005). Such weight loss measurements are suitable for monitoring abiotic corrosion. However, given the complexity of the microbial community and the various potential mechanisms involved in MIC, studies of complex microbial consortia related to this problem will also require an array of techniques to quantify and characterize the corrosion process (Dall'Agnol et al. 2014).

1.7.1 Surface Characterization

As discussed above, the surface characteristics of a metal affects MIC. Characterization of a material surface is very important to understand, relate and interpret the observed corrosion behavior. Techniques such as confocal laser scanning microscopy (CLSM) (Videla et al. 2005, Enning et al. 2012), scanning electron microscopy – energy dispersive x-ray analysis (SEM-EDX) (Enning et al. 2012), atomic force microscopy (AFM), time of flight secondary

ion mass spectrometry (ToF-SIMS) (Beech et al. 1999), x-ray photoelectron spectroscopy (XPS), x-ray diffraction (XRD) (Beech et al. 2005) have been used for surface visualization in corrosion studies (Surman et al. 1996). With CLSM, it is possible to image live cells, which means that the same samples can be imaged multiple times, enabling time course studies of the same samples. SEM-EDX allows a semi-quantitative surface chemistry analysis and a good resolution image can be obtained. An advantage of CLSM over SEM is that the former does not require much sample preparation and in situ analysis can be performed. AFM can also be used in situ, however, this requires contact with samples which might disturb the surface or the biofilm formed and would be considered as a destructive sampling technique (Ilevbare et al. 2004). ToFSIMS is a highly accurate and sensitive technique, which can link the chemical and visual analysis, but since the imaging needs vacuum it is not suitable for in situ analysis or time series studies since it is destructive (Seyeux et al. 2015). Although XPS is a time consuming method, it allows the analysis of surface chemistry quantitatively (Eashwar et al. 2015). XRD is useful in studying corrosion products as it can provide information on phase composition at the surface, but interpretation of biological samples is very difficult. The characterization of a material surface is a complex process. No one parameter can quantify all the characteristics. It includes i) amplitude parameters to investigate peak / valley heights of surface irregularities, ii) spacing parameters to check spacing of irregularities and iii) hybrid parameters which are a combination of both (Kandeil et al. 1989). Metal surface characterization essentially gives information about cause and effect of MIC process. To monitor reactions of interest taking place on the surface, electrochemical characterization is helpful.

1.7.2 Electrochemical characterization

MIC is basically an electrochemical process and hence, changes in the metal surface have been studied using a range of electrochemical techniques, including polarization techniques (Xu et al. 2007), open circuit potential (OCP)

(Faimali et al. 2010), electrochemical impedance spectroscopy (EIS) (Mansfeld et al. 1994), electrochemical noise technique, scanning vibrating electrode technique (SVET) (Little et al. 2007) etc. Potentiostatic methods are advantageous because they can follow the behavior of metal during formation and breakdown of passivating films. The behavior of the metal depends on the potential changes. Most of these methods are implemented by controlling the applied current or potential where the ratio of applied potential and current is called polarization resistance (R_p). Potentiodynamic sweep techniques are simple, with a good correlation with biofilms, but they can alter the microenvironment at the interface, which might result into enhanced corrosion.

EIS is a more accurate method that provides an explanation for the corrosion mechanisms under study. It is used to interpret kinetics and real time interactions between the electrode, biofilm and solution interphases (Al Abbas et al. 2013), however, the system used should be stable and the data obtained is difficult to interpret. A type of EIS, staircase potentiometric electrochemical impedance spectroscopy (SPEIS), where successive impedance measurements are taken in a frequency range during a potential sweep can be used to study the pitting process (Jia et al. 2011).

Electrochemical noise technique, a nondestructive technique (Roberge 1993), records the stochastic fluctuation of current and potential of passing through an interface under potentiostatic or galvanostatic control, typically at low frequency (Chandrasatheesh et al. 2014). It can help to identify the type of corrosion; however, it is very difficult to interpret the data. SVET, when used gives qualitative as well as quantitative data, but it is found to be beneficial in early phase of MIC only. Due to the evolving structure of biofilms, localized measurements are very difficult. Techniques used in this study such as Electrochemical Impedance Spectroscopy, Linear Polarization Resistance and Linear Sweep Voltammetry, are discussed in detail in Chapter 2.

1.7.3 Microbial characterization

1.7.3.1 Community profiling techniques

For the detection, identification and quantification of microorganisms related to corrosion, culture independent techniques are required since culture methods can only explore up to 1-10% of the original population (Beech et al. 2014). Recently, 16S rDNA based methods have been applied to understand microbial diversity of corroded metal surface in natural environments as well as water distribution systems, oil field and gas pipelines (Duncan et al. 2009, Dang et al. 2011, Lenhart et al. 2014). A characterization of microbial community in gas industry pipelines included DGGE and random cloning of 16S rDNA gene sequences amplified for community DNA, which indicated that the 106 bacterial 16S rDNA sequences derived from DGGE bands formed three major clusters: *beta* and *gamma proteobacteria* and *firmicutes* (Zhu et al. 2003). These techniques have also been used to monitor variations in the microbial community in various cultures after different non-toxic corrosion control treatments (magnesium peroxide (MgO_2), hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaOCl)) and different incubation periods. Results showed that the addition of MgO_2 was the most efficient method for corrosion inhibition (almost 70%), which restricts the growth of SRB due to slow release of oxygen from MgO_2 (Chang et al. 2015). While DNA based analyses can reveal the species present, which can be correlated with the corrosion process, DNA based analysis has some limitations. As it detects active and dormant cells equally, it does not reflect the metabolically active microorganisms. This problem is exacerbated by the persistence of extracellular DNA (eDNA) in the biofilm, which can be detected and hence may confound identification of the key corrosion causing organisms (Marty et al. 2012).

1.7.3.2 Meta - omics based techniques

Insight into bacterial communities in terms of diversification and their metabolism is necessary since corrosion is influenced by complex processes of

different microorganisms performing different electrochemical reactions. Interactions with the metal surface on which they attach is complex and varies between species. Metagenomic and metatranscriptomic analyses can be used to characterize the taxonomic composition, metabolic gene content and gene expression profiles of the MIC community (Beech I. B. 2014). In this way, it may be possible to identify the metabolic pathways that are expressed during MIC. The genes and pathways involved in the corrosion process can then potentially be targeted for control strategies. Two platforms are used for metatranscriptomic studies, microarrays and total RNA sequencing. RNA sequencing is advantageous over microarrays since it does not require prior knowledge of genomic sequences necessary for hybridation.

Using a metatranscriptomic approach, the effect of copper (Cu) has been studied and it was observed that the metabolic activity varied according the ability of bacteria to metabolize Cu (Beale et al. 2014). Bacterial metabolic pathways were influenced by presence of metal or as a result of material degradation (Tremaroli et al. 2009). For example, the metabolic profile of bacteria in a biofilm consortium was different from mono-species isolates from the same, which can be because of composition of metal surface, presence other species in the biofilms (intra and interspecies interaction) and varying level of stress due to the environment.

Different analytical platforms such as LC (Liquid Chromatography), LC ToFMS (Time of flight mass spectroscopy), LC-HRT (high resolution ToFMS) etc. would allow more complete metabolome of the bacterial consortia (Beale et al. 2014). There are two major limitations of metatranscriptomics studies, i) total extracted RNA can be up to 99% rRNA and hence can significantly reduce the number of mRNA associated sequencing reads and ii) the lack of reference genomes for the community members often means that many of the mRNA sequences cannot be assigned to an organism or in some cases, even associated with a function (Pascault et al. 2015).

Monitoring MIC at the interface, requires local methods, since the biofilm microstructure and metabolism change across only a few μm . The use of

microelectrodes is an effective way of measuring microenvironments in the biofilm. Oxygen micro-electrodes have been reported to study biofilms, in 10 μm steps using a micromanipulator (Hibiya et al. 2004). Microelectrodes are also available for measuring sulphide, pH, ammonium and redox potential.

1.8 Designing a relevant study system

The study of MIC requires a complex mixture of engineering, material science, chemistry and microbiology expertise. However, to simplify and understand the process, a single aspect of MIC is often studied, e.g. the microbial community, the electrochemical reactions or changes in surface of interest. As a consequence, focused studies often use corrosion systems that are tailored for the types of analysis to be performed, resulting in the use of a wide range of test conditions and experimental systems, which makes it difficult to compare between studies (Wade et al. 2017). The experimental system depends on the scope of the experiments, i.e., the aspect of MIC under investigation. For example, coupons immersed in freshwater or seawater are the optimal system to determine the long-term effects of MIC in those environments as they account for all the environmental variables involved in those habitats (e.g. seafloor). However, such studies typically require longer durations which makes them difficult or impractical, are complex since it is not possible to control all variables and it is usually not possible to manipulate the environment to understand the corrosion process difficult to do long term in situ measurements etc. In addition to these variables, interpretation of the results is often highly complex and it becomes difficult to identify key factors involved in the corrosion process. Variable environmental conditions reduce the reproducibility of the results, which directs researchers towards controlled laboratory systems.

Under laboratory conditions, there are several parameters that affect MIC, such as different nutrients or test media, fed batch vs. continuous feed systems, volume of the medium, presence or absence of oxygen etc. Laboratory experiments allow for considerable control over the environmental parameters

and enable continuous monitoring of the electrochemistry and microbiology of the process. It is clear from the literature that MIC in natural and engineered environments is mediated by a consortium of species, growing together in a biofilm encased in a self-produced extra cellular matrix. To better understand the MIC process, it is necessary to focus on mixed species systems. Further, these studies will require a combination of techniques to characterize not only the microbial community function and interaction in this process, but to simultaneously interrogate the changes at the metal surface. Therefore, the aim of this thesis was to investigate microbial communities in relation to MIC. This will be accomplished through the following objectives:

1. To understand and define early stages of MIC in natural marine environment.
2. To enrich a defined marine microbial community from seawater using artificial seawater media and to monitor their MIC behavior in laboratory based system.
3. To Investigate the effect of physical (surface polishing, shaking speed) and chemical parameters (nutrient concentration) on the MIC behavior of a defined marine microbial community.

Chapter Two: Materials and Methods

2.1 Introduction

This study was focused on the early onset of MIC of stainless steel and changes in the microbial community as they form biofilms on the metal surfaces. The effect of different physico-chemical parameters, e.g. medium composition, surface roughness, shaking speed was characterized for the early stage of MIC. The techniques and systems used were selected based on the research goals and hence, different experimental systems (summarized in Table 2.1) were used to investigate the effect of various MIC parameters. A fed batch system and a continuous flow system were designed for laboratory experiments. These were compared with the results from environmental experiments that were performed in a continuous system where seawater was pumped and circulated through 3000 L tanks located at St. Johns Island National Marine Laboratory.

Table 2.1 Summary of the experimental systems used in this study.

Research question	Fed-batch / Continuous flow system	Long-term / Short- term	2/3 electrode system	Small (1 X 1 cm) / Large (3.5 X 4.5 cm) samples
Onset of MIC in the environment	Continuous flow	Both	2	Large
Effect of nutrients	Both	Short-term	3	Small
Effect of sample preparation and shaking	Fed-batch	Short-term	3	Small
Effect of microbial community	Continuous flow	Short-term	3	Large

In this chapter, the materials and methods used for the different experimental systems, along with the specific electrochemical and microbiological techniques used are described in detail.

2.2 Stainless Steel

Stainless steel samples, UNS32750 (SD/Super duplex), UNSS30400 (SS304) and UNSS31600 (SS316) types, were purchased from A plus engineering, Singapore. Elemental composition of these materials is given in Table 2.2. Pitting resistance equivalent number (PREN) gives relative resistance of stainless steel in chloride containing environment. The higher the PREN value, the more resistant is the steel. It is determined using a formula;

$$\text{PREN} = \text{Cr} + 3.3 \text{ Mo} + 16\text{N} \quad (\text{Eq. 2.1})$$

The PREN values for the stainless steels used in this study were UNSS30400 (19.25), UNS32750 (37.7 – 47.6) and UNSS31600 (25.69). PREN values are generally used as an indication of resistance to pitting in seawater. Steels with PREN-values above 32 are considered seawater (corrosion) resistant.

UNSS32750, a duplex stainless steel, was used for experiments in natural seawater under flowing conditions. Duplex steels show favorable mechanical strength due to the ferrite phase and high corrosion resistance due to the austenite phase (Shifler et al. 2005, Ha et al. 2014). For laboratory experiments, the less corrosion resistant SS304 steel was used, to characterize the onset of corrosion behavior, which would be difficult with SD coupons.

Table 2.2 Composition of UNS32750, UNSS30400 and UNSS31600 steel coupons.

Sample	Chemical Composition, % w/w										
	Cr	Ni	Mo	C	N	Mn	Si	Cu	P	S	Fe
UNS S32750	24.0-26.0	6.0-8.0	3.0-5.0	0.03	0.24-0.32	1.2	0.8	0.5	0.035	0.02	Balance
UNSS30400	18.13	8.02	nil	0.02	0.077	1.35	0.35	nil	0.029	0.005	Balance
UNSS31600	16.78	10.21	2.12	0.021	0.12	1	0.51	nil	0.038	0.003	Balance

2.3 Sample preparation

While there are hundreds of sample preparation procedures reported in corrosion studies, the MIC literature often lacks precise information about the sample preparation protocol. Therefore, in this work, we tested several sample preparation procedures, with the goal of increasing the reproducibility of the results.

In experiments described in Chapter 5 (sections 5.3.2.1, 5.3.2.2 and 5.3.2.3), all six surfaces of the 10 X 10 X 2 mm samples were manually polished with abrasion paper of decreasing grit size, p180, p360 and p600. Only for experiments described in section 5.3.2.2, p1000 grit sandpaper was used in addition to p180, p360 and p600. It was observed that the quality of manual polishing was inconsistent and depended upon individual operators.

Therefore, for experiments reported in Chapter 4 (sections 4.4.2, 4.4.3), a mechanical polishing of the samples was preferred, using a pneumatic random orbital sander (Chicago Pneumatic CP7200S). Coupons were polished using p600 grit silicon carbide sandpaper, which has an average particle diameter of $\sim 25\ \mu\text{m}$. This process was faster and more uniform than manual polishing.

An initial microscopic investigation of the coupons showed their surfaces before the experiments were started (Figure 2.1). Non-uniform grain structure and grain boundaries were clearly visible on the unpolished metal surface. Polished coupons showed higher uniformity throughout the samples.

Figure 2.1 Comparison between polished and unpolished SS304 surface. Unpolished coupon surface (left) and surface polished mechanically using the orbital sander fitted with p600 grit sandpaper (right).

For experiments with the fed-batch reactor system reported in Chapter 5 (section 5.3.4), SS304 coupons were polished with p600 grit sandpaper using a Rotoforce-4 grinder (Struers) at the Materials Science facility in NTU. Unsurprisingly, the different sample preparation procedures resulted in different degrees of surface smoothness and therefore only samples that were prepared using the same polishing procedure were compared with each other.

2.4 Media

Throughout the experiments two different media i.e., Marine minimal medium (Table 2.3) and VNSS medium (Table 2.4) were used. The base of both recipes is a nine salt solution (NSS) (Table 2.2) (Neidhardt et al. 1974). Both artificial seawater formulations have been used in studies related to microbially influenced corrosion (Jayaraman et al. 1997, Örnek et al. 2001), although there are variations of the specific media compositions (Rao et al. 2005, Martín-Rodríguez et al. 2014). In preliminary experiments performed in this study, the pH was observed to rapidly decrease and therefore, MOPS buffer (0.04 M) was added to maintain the pH at 8.2.

The carbon source was the main difference for these media, where 3M contained 2 g/L glucose (Table 2.3), which favors fast-growing heterotrophic bacteria. In contrast, VNSS contained 1 g/L peptone and 0.5 g/L yeast extract in addition to 0.5 g/L glucose (Table 2.4). For the VNSS medium, two formulations were used, VNSSFs (without glucose) and VNSSFSG (with glucose). These two media and their respective enrichments were used in Chapter 4 and Chapter 5 (section 5.3.6). For the rest of the experiments in Chapter 5, the enrichment culture was grown in 3M medium.

Table 2.3 Recipe for 0.5 X NSS.

0.5 X NSS (Adjust the pH to 6.5 prior to autoclaving so the pH of sterile medium is 7.5)	
<i>Component</i>	
NaCl	8.8 g
Na ₂ SO ₄	0.735 g
NaHCO ₃	0.04 g
KCl	0.125 g
KBr	0.02 g
MgCl ₂ x 6 H ₂ O	0.935 g
CaCl ₂ x 2 H ₂ O	0.205 g
SrCl ₂ x 6 H ₂ O	0.004 g
H ₃ BO ₃	0.004 g
dH ₂ O	920 mL

Table 2.4 Recipe for 3M (Marine minimal medium).

3M (Marine Minimal Medium)		
920 mL	0.5 X NSS	
40 mL	1 M MOPS (3-morpholinopropane sulfonic acid)	104.65 g / 500 mL Adjust pH to 8.2 and sterile filter
10 mL	0.4 M tricine + 1 mM FeSO ₄ x7H ₂ O	17.93 g tricine / 250 mL 0.07 g FeSO ₄ / 250 mL Sterile filter and adjust pH to 8.2
10 mL	132 mM K ₂ HPO ₄	5.75 g / 250 mL
10 mL	952 mM NH ₄ Cl	12.73 g / 250 mL Adjust pH to 7.8
10 mL	20% glucose w/w	

Table 2.5 Recipe for VNSS medium.

VNSSFS	
To 1 X NSS (per litre) add the following	
Peptone	1.0 g
Yeast extract	0.5 g
Glucose	0.5 g
FeSO ₄ x 7 H ₂ O	0.01 g
Na ₂ HPO ₄	0.01 g

2.5 Electrochemical techniques

The three electrode-setup was used in laboratory experiments throughout the study. This setup gives best results when investigating MIC in aqueous systems. The working electrode was the metal coupon, counter electrode was titanium wire, which does not corrode under the conditions used. Ag/AgCl (sat. KCl) and silver wire electrodeposited with silver chloride layer were used as reference and pseudo-reference electrodes, respectively. The electrodes were assembled in several corrosion cells: i) 120 mL vial for fed-batch experiments; ii) Continuous flow 300 L tank for experiments in natural seawater and iii) 10 mL miniature flow cells, for MIC experiments in biofilms.

Common electrochemical methods used to monitor MIC such as Open Circuit Potential (OCP), Linear Polarisation Resistance (LPR), Linear Sweep Voltammetry (LSV) and Electrochemical Impedance Spectroscopy (EIS) were employed in this study. Where possible, automated sequences were set-up with the multichannel potentiostat to minimize the operator intervention and increase repeatability.

2.5.1 *Open Circuit Potential (OCP)*

Open circuit potential (OCP), also called resting potential, corrosion potential, or equilibrium potential, is the potential at which there is no current flow in the system when no potential is imposed on the system. In the OCP technique, the cell is disconnected from the power amplifier and potential is monitored over time. After sufficient time (e.g., 20-30 min), the potential stabilizes at a value that depends on the environmental conditions (e.g., oxygen concentration, bacterial concentration, etc.) and on the metal coupon surface. This potential was then used as starting point for LSV and LPR analysis. During a MIC process, the OCP will go through various phases, depending on environmental conditions and interface processes. An increase in the OCP indicates passivation of the surface, e.g., formation of an oxidation layer that slow down metal degradation. On the other hand, oxygen scavenging by bacteria in the

planktonic and biofilm phase will decrease OCP, indicating favourable conditions for anaerobic corrosion. Overall, the OCP is not a complete descriptor of the MIC process but is rapidly measured in high-throughput setups.

2.5.2 Polarization techniques

When a material is exposed to a corrosive environment, oxidation and reduction reactions start. E_{corr} is the potential at which these two reactions are at equilibrium. For practical purposes, E_{corr} is very similar to the OCP, assuming the resting time is sufficiently long to allow equilibrium conditions to establish. The system is polarized at a potential lower than OCP/ E_{corr} , and the imposed potential is slowly increased. This potential scan allows the separation of cathodic from anodic reactions and to determine the corrosion current through application of the Tafel equation. The current response is plotted as a function of potential. Since the current can vary by several orders of magnitude, a logarithmic function of current is usually plotted (Stern et al. 1957). When the potential is much lower or much higher than the E_{corr} , the sample corrodes and high corrosion current is observed. Under these conditions, the sample cannot be reused. In all experiments, both anodic and cathodic maximum current were limited to prevent extensive sample damage.

2.5.3 Linear Polarization Resistance (LPR)

The polarisation resistance technique is very rapid, unlike traditionally used weight loss techniques used to measure absolute corrosion rates (McCafferty 2010). While applying this technique, the electrode is scanned through a narrow potential range (25-50 mV) around the E_{corr} and the resulting current is plotted against potential. The corrosion current, I_{corr} , ($\mu\text{A}/\text{cm}^2$), can be calculated using the following equation:

$$I_{\text{corr}} = \frac{\beta_A \beta_C}{2.3 (\beta_A + \beta_C)} \frac{\Delta i}{\Delta E} \quad (\text{Eq. 2.2})$$

Where β_A and β_C are the anodic and cathodic Tafel constants, respectively, and Δi and ΔE are the changes in potential and current, respectively. β_A and β_C are usually assumed equal to 120 mV. Although this is often a first approximation of the actual values, it is realistic at potential values very close to E_{corr} . The polarisation resistance was explained by Stern and Geary for two coexisting electrochemical reactions (Stern et al. 1957) (anodic and cathodic) and can be given as:

$$\frac{\Delta E}{\Delta i} = \frac{2.3 (\beta_A + \beta_C)}{\beta_A \beta_C (I_{corr})} \quad (\text{Eq. 2.3})$$

A high polarisation resistance correspond to a low corrosion current and indicates that the material is resistant to corrosion. During MIC process, LPR decreases as the metal degrades. LPR provides a simple measure of the MIC process at the interface but additional measures are needed to determine the MIC mechanism and details.

2.5.4 Linear Sweep Voltammetry (LSV)

To perform LSV, the electrode is exposed to a broad range of potentials around the corrosion potential E_{corr} . The resulting current is plotted on a logarithmic scale against the potential and this is the Tafel plot. The corrosion current, I_{corr} , is obtained by extrapolating the linear portions of the anodic and cathodic curves obtained. Anodic and cathodic Tafel plots can be described by the equation:

$$\eta = \beta \log \frac{i}{i_{corr}} \quad (\text{Eq. 2.4})$$

Where, η = overpotential or the difference between the potential of the electrode and E_{corr} .

β = Tafel constant and i_{corr} = current at overpotential η .

Rearranging the equation gives:

$$\eta = \beta (\log I - \log i_{\text{corr}}) \quad (\text{Eq. 2.5})$$

This equation suggests that the plot of η Vs $\log i$, should give a straight line with slope β . Tafel constants for each cathodic β_C and anodic reactions β_A are calculated using data analysis software (e.g. EC lab). Due to the broad potential range used, LSV provides additional information such as the trans-passivation potential, the pitting potential, etc. However, LSV is generally considered a destructive technique.

2.5.5 *Electrochemical Impedance spectroscopy (EIS)*

Electrochemical impedance spectroscopy is a non-destructive technique used for a wide range of electrochemical applications. When applied in corrosion related systems, it can provide valuable information such as corrosion rate, electrochemical mechanisms, reaction kinetics and can also detect localised corrosion (Hamdy et al. 2006). Impedance is a complex number with a modulus and a phase shift between sinusoidal applied potential and sinusoidal response current. Impedance is a generalized form of the resistance R , which describes electrical circuit where alternating current flows. The EIS results can be interpreted in terms of equivalent circuits. The complex interfaces of the biofilm, water and metal is described as a series or parallel combination of idealized circuit elements, which include capacitors, resistance and inductances. While the correspondence between a EIS feature and the equivalent circuit element must be supported by independent measures (e.g., microscopy), numerous studies have shown that the appearance and growth of biofilms in MIC and the formation of passivation layer/biofilm interface can be monitored through EIS. In this work, EIS was always performed under resting conditions at E_{corr} . This rather conservative choice ensured that EIS remained a non-destructive technique. During EIS measurements, a sinusoidal voltage signal with a small amplitude was applied and the current signal amplitude and phase shift were recorded (Wang et al. 2006). Common equivalent circuit were used for the interpretation of EIS data.

The real (Z_{Re}) and imaginary (Z_{Im}) components of impedance represent resistive and capacitive components, respectively. Resistance measures the ability of a material to reduce conduction of electrical currents. A higher resistance implies the higher frequency of electrons colliding into the lattice of the material, lowering conductivity of the material. The electrical charge storage ability of a material relative to its size is measured as capacitance. A higher capacitance implies a higher capacity storage of electrical charge of a material. In terms of corrosion processes, high impedance modulus indicates resistance to corrosion. Once MIC onsets, the evolution of its capacitance component can be used to describe biomass accumulation at the interface.

EIS data can be expressed in two different ways. First, the real part of the impedance $Re(Z)$ can be plotted against the imaginary part of impedance $Im(Z)$ in a Nyquist plot. The disadvantage of this plot is that the frequencies are not accessible. Therefore, another representation, the Bode plot, is used in which the modulus of impedance and phase shift are plotted as a function of frequency. If the circuit only consists of a resistor, the phase shift is zero, but if the capacitor and inductor are present, the phase shifts by -90° and 90° respectively.

EIS can be very informative as it describes the relaxation behaviour of the electrochemical system under quasi steady state conditions. In MIC, several electrical, ionic and biological reactions happen at the interphase and every reaction has its own relaxation time. Using EIS, resistance of the electrolyte is detected at higher frequencies and the dissolution phenomenon is detected at lower frequencies. A major challenge faced while using EIS for MIC characterisation is the interpretation of the data. Choosing the right equivalent circuit model becomes difficult due to presence of a biofilm. EIS should be measured in absence of faradaic current, i.e., after sufficiently long rest period at open circuit potential. This improves repeatability, especially for biological systems (Dominguez and Pant, 2012).

2.6 Preliminary environmental experiment

Preliminary environmental experiments were performed at St. Johns Island National Marine Laboratory (SJINML). Metal coupons were immersed in tanks located at SJINML, in which sea water is continuously pumped from depth of 10 meters along the coast. The potential data was generated in collaboration with Prof. Dominique Thierry (personal communication). Coupons retrieved at set time points, were characterised microscopically and biomass obtained was used for community characterisation. Figure 2.2 shows the overall schematic of the experiment performed in natural seawater.

Figure 2.2 Schematic for the environmental experiment at St. Johns Island National Marine Laboratory (SJINML).

2.6.1 Sample preparation

UNSS32750 (SD) coupons were cleaned using 70% ethanol, and subsequently immersed into sand filtered running seawater (Figure 2.3) in aquarium B at the flow rate of 300 L/day at St. John's Island National Marine Laboratory

(SJINML), Singapore. The coupon samples were collected after 1 h, 2 d and 7 d of incubation.

2.6.2 Environmental parameters

The water temperature (26-29°C) and pH (7.8 – 8.2) were monitored throughout the experiment. The evaporation residues (3.7% by weight) were in accordance with average values of seawater composition in the literature (Sverdrup et al. 1942).

2.6.3 Electrochemical monitoring

To measure the open circuit potential (OCP) of the immersed coupons, a multimeter was used. Standard calomel electrode was used as a reference electrode. This two-electrode system differs from the three-electrode setup used in laboratory experiments. However, it was adequate as we measured only the long-term evolution of corrosion potential through OCP.

2.6.4 Biofilm visualization

The coupons were retrieved, rinsed with 0.5X NSS and brought back to SCELSE in petri plates at 4°C. To quantify biofilm formation, coupons were stained with the LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Thermo Fischer Scientific), and imaged using a confocal laser scanning microscope at 400 × magnification (LSM780, Zeiss). Images were collected using two different channels, 488 nm for Syto 9 (live) and 560 nm for propidium iodide (dead). Live cells were visualised as green and dead cells were red. Images were analysed using the image analysis software Imaris version 8.4.

2.6.5 DNA extraction and sequencing

To determine the composition of the biofilm community formed on coupon surfaces, the biofilm biomass was mechanically removed by a sterile spatula, re-suspended in artificial seawater and kept at 4°C for transportation to the

laboratory at NTU. Samples were centrifuged (10000 g for 5 min) and pellets were used for DNA extraction. The genomic DNA of the biofilm community was extracted using the FastDNA[®] SPIN Kit for Soil, PCR was performed using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT) and the purified PCR product was sent to Mr DNA laboratory (<http://www.mrdnalab.com>) for 16S rDNA gene based amplicon sequencing. The genomic DNA of the microorganisms in seawater was also collected and sequenced for comparison. Sequencing results were analysed by QIIME using the standard de novo operational taxonomic unit (OTU) picking approach.

2.7 Environmental experiment 2

2.7.1 Sample preparation

Two different types of steel coupons were used for this experiment: UNSS32750 (SD) and UNSS30400 (SS304). All the coupons used in these experiments were polished mechanically using a Rotoforce-4 grinding machine (Struers) with p600 grit sandpaper. After polishing coupons, the wax was removed using acetone and sonicated for 5 min to remove remaining particulates attached on the surface. Optical microscope was used to visualize the surface of the coupons before deploying them in the sea water.

2.7.2 Environmental parameters

The water temperature (26-29°C) and pH (7.8 – 8.2) were monitored throughout the experiment. The evaporation residues (3.7% by weight) were in accordance with average values of seawater composition in the literature (Sverdrup et al. 1942).

2.7.3 Coupon deployment and time points

The experiment was performed over 30 d. The connection of the copper wire with the metal coupons was sealed using silicone sealant and coupons were left to dry for 90 min. They were then deployed in the tanks at the SJINML facility. The flow rate of the sand filtered seawater was 3000 L/d. A data Acquisition Switch Unit (34972A LXI, Keysight technologies) was used to log the potential data continuously over the duration of the experiment. A two-electrode setup was used as above, with a calomel electrode, as a reference. Coupons deployed in the tanks were placed in such a way that alternate rods had SS304 and SD coupons, as well as on each rod, 4 coupons were placed equidistantly (Figure 2.3)

Figure 2.3 Deployment of SD and SS304 coupons in the tank at SJINML facility.

2.7.4 Coupon collection and analysis

Four coupons for each time point and type of metal were retrieved after 2 h, 2 d, 7 d and 30 d of exposure. Coupons were brought back to SCELSE for further analysis. One coupon on each day was used for biofilm visualization using

confocal microscopy and rest were used for the collection of biomass. For biomass collection, coupons were placed in a beaker with 2 ml 0.5X NSS, sonicated for 5 min, collected in 2 mL microfuge tubes and centrifuged.

2.7.5 Surface analysis

Confocal laser scanning microscopy was used for visualisation of biofilms as mentioned in section 3.3. A reflection technique, with excitation wavelength of 514 nm, which was different than wavelengths used for live (Syto 9 / 488 nm) and dead (propidium iodide / 560 nm) staining, was used to image the topography of the metal surface (Neu et al. 1997).

2.8 Enrichments

2.8.1 Preparation

Two different recipes were adapted to the experimental needs, for marine minimal medium (3M) and vaatanen nine salt solution (VNSS). A seawater sample was collected at SJINML, Singapore. A sterile 5 L bucket was filled with seawater at a depth of approximately half a meter at the shoreline. The sample was immediately transferred to SCELSE and 10 mL of the seawater was inoculated into 20 mL of the different seawater media in a 50 mL tube. The tubes were placed on an orbital shaker at room temperature of approximately 25°C and a rotation speed of 200 rpm. The cultures were split daily and the volume ratio between transferred culture and fresh medium was decreased stepwise. A sample of 2 mL per preparatory culture was centrifuged and the pellet was re-suspended with 1 mL corresponding medium with 25% glycerol. The stock cultures were divided into three tubes and store at -80°C.

2.8.2 RNA extraction

RNeasy mini kit from Qiagen was used to extract RNA from the biomass collected followed by DNase treatment using DNA-free™ Kit from Ambion (Life Technologies). Extracted RNA was purified using the RNA clean and

concentrator kit (Zymo). To assess the quality of RNA, samples were measured using Nanodrop, Qubit and Tape station. After the quality check, samples were sent to the SCELSE sequencing facility for library preparation and RNA sequencing.

2.8.3 Metatranscriptomic data analysis

2.8.3.1 Quality trimming and mRNA/rRNA depletion to use rRNA for ribotagger and mRNA for diamond:

Illumina adaptors, shorter reads (-m 50) and low quality reads (-q 20) were removed using *cutadapt* (version 1.11) (Chen et al. 2014). High quality reads were subjected to *sortmeRNA* analysis (version 2.0) (Kopylova et al. 2015) to extract both rRNA and mRNA.

2.8.3.2 RiboTagger for taxonomic characterization based on rRNA

In order to characterize the taxonomic composition of the microbial communities, rRNA reads were analyzed using a fast tag-based approach, the RiboTagger (Xie et al. 2016). The RiboTagger scripts used in this study can be found at the following link: <http://chaos.bic.nus.edu.sg/ribotagger/manual.html>). Briefly, a universal primer for each hypervariable region, i.e., V4, V5, V6 and V7 of the 16S rRNA was used to scan each sequencing read to obtain 33 nt downstream of the primer, and the 33 nt sequences were defined as the tag of the 16S rRNA gene. Those universal primer sequences are conserved among *bacteria*, *Achaea* and *eukaryotes* (Xie et al. 2016). Short reads that did not cover the full 33 nt regions were discarded. Each different tag (TAG) was used as a signature sequence to represent one operational taxonomic unit (OTU). TAGs that only occurred once (i.e., singletons) were removed from the TAG table prior to random subsampling for normalization.

2.8.3.3 Functional analysis based on RNA seq data

For functional analysis, mRNA sequences were aligned against the NCBI non-redundant (NR) protein database (<ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz>, March 2016) using DIAMOND (version 0.7.10.59, <http://ab.inf.uni-tuebingen.de/software/diamond>) (Buchfink et al. 2015). Aligned reads were assigned to KEGG Ontology (KO) terms using gene id's (GI) to KEGG mapping file implemented in MEGAN (version CE_6_5_5). In order, the lowest common ancestor approach was used to define the taxonomic origin of mRNA reads (LCA, default parameters, MEGAN). Figure 2.4 gives the schematic of the pipeline used for sequence analysis.

Figure 2.4 Schematic of the pipeline used for sequence data analysis.

2.9 Continuous flow cell system

In this study, a novel continuous flow system was developed to investigate the MIC of SS304 steel by a mixed marine microbial community. The flow cell used in the system was designed with help of Mr. Vishal Prajapati and fabricated by Fusion Scientific Glassware, Singapore using autoclavable polycarbonate material. Two designs of the system were used i) the flow cell with a small coupon (10 X 10 X 2 mm) for electrochemical characterization and ii) the flow cell with a larger coupon (35 X 45 X 2 mm) for biomass collection

and community analysis. The same experimental setup was used for both systems (Figure 2.5).

The flow rate was adjusted to approximately 0.48 mL/min. Once assembly of the flow cells was complete, the flow cell was flushed with a sterile medium for 1 h. Then, 1 mL of an inoculum was added and left to incubate for 1 h without flow, before resuming the flow.

Figure 2.5 Schematic representation of the flow cell setup. a) media bottle, b) 0.22 μm air filter, c) tubing, d) peristaltic pump, e) bubble traps, f) flow-cell with a big coupon for biomass collection, g) flow-cell with smaller coupon for electrochemical monitoring, h) VSP potentiostat (Biologic, France) i) computer for data recording and j) tubes running to the waste bottle. Media was pumped at the flow rate of 8 RPM (0.48 mL/min).

2.10 Electrochemical Flow cell (EFC)

For the electrochemical flow-cell (EFC) system, the general setup described above for flow-cell system with a smaller coupon was used (Figure 2.5 g). Detailed assembly of the flow-cell used for electrochemical characterization is

given in Figure 2.6. EFC experiments were carried out in duplicate. Three sets of experiments were carried out for VNSS with glucose (VNSSFSG) and VNSS without glucose (VNSSFs).

2.10.1 Sample preparation and EFC assembly

UNSS30400 (SS304) coupons (10 mm × 10 mm × 2 mm), polished for 30 s with p600 grit paper using an orbital sanding kit (Chicago Pneumatic) were used as the working electrode (WE). The coupons were then attached with titanium wires (0.25 mm diameter, 99.7% trace metal basis, Sigma-Aldrich) and soaked in acetone for 10 min at room temperature. The coupons and attached wires, covered with PVC wire insulator, were then immersed in 96% pure ethanol (molecular grade) and sonicated for 7 min at 37 Hz, 100% power at room temperature. The coupons were air-dried and assembled into the flow cell. To function as a counter electrode (CE), Titanium wire was coiled and connected to the potentiostat. A quasi reference electrode (QRE) was prepared using a silver wire (0.25 mm diameter, annealed, 99.9% metals basis, 0.51 g/m, temper: soft, Alfa Aesar) that was lightly sanded with 600c grit paper, immersed in 6% bleach for 40 min, rinsed with miliQ water and stored in 1 M KCl until used. The potential of the QRE was measured before and after EFC experiments with respect to commercial Ag/AgCl reference electrodes.

The prepared metal coupon and counter electrode were then inserted into the flow cell (Figure 2.6) and secured in place with silicon sealant and two-component epoxy resin. All materials were sterilized by autoclaving at 121°C for 15 min. including media, flow cell assembly and components of the setup. The final assembly was carried out in a sterile environment in a laminar air flow hood and shifted to the bench for connections with potentiostat as well as clamping of tubing to the pump for experiments.

Figure 2.6 Schematic diagram of the electrochemical flow cell. Q.R.E represents the quasi-reference electrode. W.E represents the working electrode, whereby a SS304 metal coupon was used for electrochemical characterization of MIC. C.E represents the counter electrode.

2.10.2 Electrochemical Analysis

Electrochemical measurements were taken before inoculation (BI), 1 h after inoculation (AI), and at 24, 48, 72, 96 and 120 h. Electrochemical Impedance Spectroscopy (EIS), Linear Polarization (LP) and Open Circuit Potential (E_{OCP}) were applied. EIS was measured at E_{OCP} with 10 mV perturbation from 300 kHz to 50 mHz. LP curves were measured from -50 mV to 50 mV vs. E_{OCP} with a scan rate of 0.100 mV/s. Electrochemical data was analyzed using EC lab software version 11.02 (Bio-logic, France)

2.10.3 pH and OD

pH and OD readings were taken every 24 h. pH was measured with Sartorius benchtop pH meter while OD was measured at 600 nm with a Shimadzu spectrophotometer (UVmini-1240).

2.10.4 Confocal Laser Scanning Microscopy

Coupons from the EFC were retrieved, washed with NSS and imaged using confocal laser scanning microscopy as mentioned in section 4.4. Quantitative image analysis was performed using IMARIS version 8.4 (Bitplane) to quantify biovolume and surface coverage.

2.11 Flow cell system for biomass characterization

2.11.1 Sample preparation

UNSS30400 (SS304), UNSS31600 (SS316) and UNS32750 (SD) coupons (3.5 X 4.5 X 0.2 cm) were polished for 30 s with p600 grit paper using an orbital sanding kit (Chicago Pneumatic). After polishing, coupons were first soaked in acetone for 10 min at room temperature then immersed in 96% pure ethanol (molecular grade) and sonicated for 7 min at 37 Hz, 100% power, and room temperature. The coupons were air-dried and assembled into the flow cell. The flow cell was connected with silicon tubing and autoclaved at 121°C for 15 mins along with other components of the set up. The final assembly was carried out in a sterile environment under a laminar air flow hood and shifted to the bench for connections with potentiostat as well as clamping of tubing to the pump for the experimental run.

2.12 Electrochemical batch reactor system

2.12.1 Enriched mixed marine microbial community

The enriched microbial community for laboratory experiments was obtained by inoculating 10 mL of coastal seawater into minimal marine medium (3M). After

one week of sub-culturing, frozen stock of the mixed microbial community was prepared and used for laboratory experiments.

2.12.2 Corrosion cells setup

A 150 mL glass vial with a septum cap was used as a reactor. A 1 mm hole was drilled in the middle of the septum cap and a glass tube with 2 mm diameter was inserted through the hole as a bridge for the reference electrode. The end of the tube inside the reactor was closed by a glass diaphragm which was fixed by a shrink hose. At the other side of the glass tube, filled with 1 M KCl, a 3 mL syringe body was fixed in place by a silicone tube. The corrosion cells were fitted with three SS coupons (1 cm X 1 cm X 0.2 cm) as working electrodes, a Ti coil was used as a counter electrode and an Ag/AgCl (saturated KCl) as a reference electrode. Titanium was used as a conductor due to its strong self-passivating property. The thick passivation layer gives the wires an inert surface and suppresses a galvanic reaction with the sample during the whole experiment.

The part of the wires inside the reactor, approximately 5 cm, were protected from contact with one another by rubber isolation. Four wires were threaded through the septum. The assembly was autoclaved and inserted in the 150 mL vial. The media volume used for the experiments was 120 mL. In the following experiments, all electrochemical potentials were reported with respect to Ag/AgCl (saturated KCl). 1 mL of the enriched mixed microbial community was inoculated into the 120 mL corrosion cells. The corrosion cells were incubated on a rotary shaker at 0 to 80 RPM, at room temperature (~22°C) for 7 or 35 d. Every 2nd and 4th day for the 7 d experiments and every 5 d for the 35 d experiments, 50% of the spent growth medium was replaced with fresh growth medium to reduce the concentration of suspended cells, provide fresh nutrients and maintain circum-neutral pH.

Figure 2.7 Assembly of bio-electrochemical batch reactor system. a) Inlet line with filter, b) potentiostat connections, c) PVC wire insulator covering, d) coiled titanium wire (CE), e) 150 mL glass vial, f) Ag/AgCl electrode (Reference), g) syringe, h) exhaust line with filter i) septum, j) glass bridge, k) 3x (10m X 10 X 2 mm) sample coupons [WE] and l) diaphragm.

2.12.3 Biofilm visualization and surface analysis

The biofilms on the coupon surfaces were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fischer Scientific) and imaged by CLSM at 400× magnification (LSM780, Zeiss). The reflection technique was used here to visualize the metal surface (Neu et al. 1997). The images obtained were analyzed using Imaris 8.4 (Bitplane), to obtain data for the surface area covered and biovolume on the metal surface. Three dimensional reconstructions of the biofilms were used for the intensity profile analysis for different Z-slices.

Field Emission Scanning Electron Microscopy (JEOL-7600F CEE, NTU) was performed on the metal coupons at the end of the experiment, after 35 d. Samples were prepared by lyophilisation. Samples were subsequently platinum sputter coated and FESEM images were collected (JEOL 7600F). Energy-dispersive X-ray spectroscopy was also performed on the same samples for elemental analysis of the surface of the coupon using EDX facility (Oxford X-max 80 mm²) at CEE, NTU. Atomic force microscopy (AFM) was performed to assess surface roughness of polished coupons using Bioscope Catalyst AFM (Bruker), in tapping mode.

2.12.4 Electrochemical analysis

In the following experiments, a few electrochemical techniques were performed using multichannel potentiostat (VSP biologic, France). LSV was performed at a scan rate of 0.166 mV/s from -800 to -100 mV and corrosion current density (I_{corr}) and corrosion potential (E_{corr}) were calculated using Tafel equation in EC lab software. LPR was performed at the scan rate of 0.100 mV/s for a range of ± 50 mV around E_{corr} . R_p fit analysis was used in EC-lab software to obtain corrosion potential, corrosion current density and polarisation resistance.

Chapter Three: Understanding the onset of microbially influenced corrosion (MIC) in equatorial seawater

3.1 Introduction

3.1.1 Corrosion in seawater

Seawater is often considered to be one of the most harsh environments for materials, especially metals. Localized corrosion, such as pitting and crevice corrosion is a concern for metallic materials immersed in natural seawater. Within a few hours of a metal surface being exposed to seawater, the formation of very small pits begins (Melchers 2016). These pits grow deeper for a few weeks and then start increasing in diameter, resulting in mixture of larger and smaller pits and unaffected areas on the surface that act as a cathodic nodes (Melchers 2016). Seawater is highly corrosive because of the high chloride content that makes it an efficient electrolyte for the electrochemical corrosion reactions (Guedes Soares et al. 2011). In seawater, the high concentration of Cl^- ions shifts the critical potential towards the negative direction (Little 2003). The critical potential is the potential below which localized corrosion reaction is not stable (Revie et al. 2011). The hydroxide (OH^-) ions are attracted to the anodic metal surface and lead to the formation of an aggressive acid chloride solution. The passive film loses its stability and anodic dissolution increases. Due to its hygroscopic nature, the presence of salts on the surface continuously attracts moisture, although the metal is not immersed, thus increasing the corrosion rate.

3.1.2 MIC in seawater

A key factor from natural seawater, relevant to corrosion, is the presence of microorganisms, as they grow on the metal surface, forming non-uniform biofilms and hence affecting electrochemical reactions. In the marine environment, the deterioration of materials due to MIC affects industries such as oil pipelines, shipping etc. and incurs significant costs associated with the

repair and replacement of infrastructure. Marine biofouling can be divided into micro and macrofouling. Macrofouling is caused by organisms such as barnacles or oysters and microfouling is due to microorganisms including bacteria, fungi and microalgae (Little et al. 2013). Any material exposed to seawater is colonized by micro and macroorganisms rapidly (Wahl 1997). Usually, bacteria are the first colonizers of the immersed surface (Scotto et al. 1998). The formation of a biofilm is mainly governed by the nature of the surface as well as nutrient availability (Ford et al. 1990). Elevated nutrient levels in areas such as harbours, estuaries and areas with discharges from industrial and agricultural wastes affect microbial growth, which results in increased fouling (Melchers 2016). In aerobic marine habitats, biofilm formation starts with an aerobic phase, in which aerobic microorganisms grow on the surface, depleting the local amount of dissolved oxygen. This results in the formation of anoxic conditions in the inner parts of the biofilm, where bacteria such as SRB can grow (Melchers et al. 2005). The formation and maturation of the biofilm affects the electrochemical state of the metal (Dhar et al. 1982, Wang et al. 2004).

The presence of a biofilm causes a positive shift in the equilibrium potential of the exposed metal (Olesen et al. 2000) and while this phenomenon (ennoblement) has been extensively studied, no unifying explanation for this process has been described (Little et al. 2013). Mollica et al. (1984) suggested that when stainless steel is exposed to natural sea water, biofilm formation facilitates ennoblement and enhances the oxygen reduction reaction (Mollica et al. 1984, Boillot et al. 2014). In natural seawater, the E_{corr} for high Mo steel, shifted by 150 mV in the noble direction for one day and showed very little increase in next 3 d. However, in sterile seawater no changes were seen for the potential. It was suggested that carbohydrates and proteins in the biofilm play important role in ennoblement (Wang et al. 2004). To explain the role of biofilms in the ennoblement process, different mechanisms have been suggested, including i) microbial colonisation ii) production of extracellular substances (Mollica et al. 1976), iii) formation of the organometallic complexes, iv) production of specific enzymes by the microorganisms (Scotto

et al. 1985, Scotto et al. 1998) and v) production of inhibitors by bacteria retained in the biofilm matrix.

3.1.3 Corrosion studies in the marine environment

There have been many studies of MIC in marine environments. Scotto et al. (1985) immersed stainless steel in natural seawater and confirmed that the biofilms forming on the surface in natural sea water alter the oxygen reduction kinetics, possibly due to enzymatic catalysis (Scotto et al. 1985). Carbon steel (type 25) was immersed in natural and sterile tropical seawater conditions to monitor the corrosion behaviour and the mechanical properties of steel. It was observed that the presence of a biofilm accelerated the corrosion and after immersion for a year, average corrosion depth in natural seawater was 2.6 times that of sterile seawater (Xiao et al. 2010).

Most studies have focused on long term experiments in order to study effect of environmental parameters such as temperature, nutrient levels and the presence of microorganisms and to predict the material durability (Melchers 2016). Based on such long-term studies, the corrosion behaviour is often assumed to be linear with time. However, some studies have shown that the behaviour is in fact highly non-linear in the first few days (~5-7) of exposure and becomes linear after the formation of a stable corrosion product layer (Melchers et al. 2004). For example, Terry and Edyvean (1986) suggested that early stages of corrosion are dominated by the formation of biofilms by macro/microorganisms and formation anodic and cathodic areas (Terry et al. 1986). However, there remains a lack of data correlating role of microorganisms with respect to the early corrosion behaviour. To understand the initiation of biofilm formation in the marine environment and their correlation with electrochemical behaviour of the metal surface, two different hypotheses were proposed:

1. Specific planktonic community from seawater initiate biofilm formation on the metal surface.
2. The attachment of bacteria to the metal surface and formation of biofilm over time correlates with changes in the corrosion potential.

To address these hypotheses, experiments were planned to identify the microbial community that colonizes metal surfaces, selection of the biofilm community from seawater and to determine if there is a correlation to the change in potential.

3.2 Results and Discussion

Preliminary experiment (September – October 2013)

3.2.1 Biofilm formation on stainless steel coupons in sea water

Coupons were imaged 1 h after immersion in the seawater tank and it was observed that individual bacterial cells were already attached to the coupon surface (Figure 3.1 a) and covered $16.3 \pm 4.4\%$ of the surface. On day 2, the biofilm had grown to multiple layers of bacterial cells (Figure 3.1 b). After 7 d incubation, the steel coupons were covered ($25.8 \pm 13.4\%$) with a biofilm that included bacterial cells clustered in microcolonies (Figure 3.1 c). All the biofilms consisted primarily of live cells and there were relatively few dead cells observed. The average thickness increased from $0.4 \mu\text{m}$ to $7 \mu\text{m}$ over 7 d.

Figure 3.1 CLSM images showing a) attachment of cells at 1 h; b) clustering of cells after 2 d and c) the formation of a biofilm with microcolonies by 7 d. Images show 3D projections of the area images (212 X 212 μm). Magnification 400X.

3.2.2 *Comparison of potential and biovolume*

The E_{corr} of unpolished UNS32750 coupons immersed in seawater increased and then stabilized after 2 d at 268 ± 8 mV (Figure 3.2), indicating that ennoblement of the metal surface had occurred. The increase in the potential was consistent with previous experiments by Motoda et al. (1990), in which 18 different types of stainless steels were immersed in coastal seawater, a sharp increase in potential to ~ 400 mV was observed, in the first 10 d (Motoda et al. 1990). Xu et al. (2015) also observed ennoblement (up to 300 mV) for SS316L in 5 d of immersion in marine environment (Xu et al. 2015). Similar ennoblement was also observed by Eashwar et al. (2015) in flowing seawater under equatorial conditions, where the E_{corr} increased to 350 mV vs. Ag/AgCl (Eashwar et al. 2015). The potential was compared with the biovolume

measured from CLSM images. The potential increased in 2 d and remained stable until 7 d. However, the biomass increased by 3.5 fold from 1 h to 2 d and further increased 2.5 fold until 7 d (Figure 3.2). The results suggest that, as the biofilm formed, the potential of the coupons increased. This is consistent with results reported by Scott et al. (1985), where SS coupons were immersed in seawater and when the microorganisms were killed using sodium azide, the potential decreased to what was observed for coupons immersed in sterile seawater (Scotto et al. 1985). The role of biofilms in establishing ennoblement has also been reported by several other studies (Mollica et al. 1984, Wang et al. 2004). However, further work would be required to understand relationship between microbial attachment and the potential increase in first two days of exposure.

Figure 3.2 Comparison of potential (mV) and biovolume ($\mu\text{m}^3/\mu\text{m}^2$) after 1 h, 2 d and 7 d of exposure of UNS32750 stainless steel in natural seawater tanks. The potential at each time point is the average of ≥ 3 replicate coupons. Biovolumes were calculated as the average of 3 images per coupon.

3.2.3 Community analysis

The DNA sequences were clustered at > 97% similarity. Approximately 600 different OTUs were observed in the sequencing datasets, although most were present at very low abundances (<0.01%). The data indicated a selection of specific organisms from the seawater that became enriched on the coupon surface. The microbial community composition of the biofilm formed on the metal surface was compared with the seawater in the immersion tank and the two communities were observed to be significantly different after 1 h of exposure (Figure 3.3). The biofilm community composition remained relatively consistent thereafter, with no significant change between 1 h and 7 d of incubation. The biofilms contained 64% less of an operational taxonomic unit (OTU) that was identified as an unknown *Proteobacteria*, which was abundant in seawater. The dominance of proteobacteria in coastal seawater has been reported previously. For example, Lee et al. (2008) investigated changes in bacterial community on steel surface for 36 hours of immersion in Sacheon harbour, Korea. They observed that *Gammaproteobacteria* dominated the samples for first 9 h, whereas by the end of 36 hours, *Alphaproteobacteria* became dominant (Lee et al. 2008). Similarly, Cyanobacteria showed a 90% reduction in abundance in the biofilm community after 1 h, relative to their abundance in seawater. Indeed, Cyanobacteria represented less than 0.02% of the biofilm community on the SD coupons.

Gammaproteobacteria such as *Aestuariibacter* and *Alteromonas* were abundant in sea water community and decreased by 95% in abundance in the biofilm formed on 1 h exposure. In contrast, *Gammaproteobacteria* from the orders *Pseudomonadales* and *Xanthomonadales*, were enriched as the biofilm formed. The abundance of *gammaproteobacteria* has been reported before for marine biofilms formed on carbon steel (Bermont-Bouis et al. 2007). McBeth et al. (2011) also reported the presence of *Beta* and *Gammaproteobacteria* in biofilms formed on mild steel immersed in marine environments along with *Epsilonproteobacteria*, which were not observed in this study (McBeth et al. 2011). Positive selection was also seen from sea water into the 1 d biofilm for

members of the genus *Varivorax* (Boone et al. 2005). On day 7, around 60% of the biofilm was populated by *Gammaproteobacteria*. *Rugeria*, a gram negative *Alphaproteobacteria*, was less abundant in biofilm community until 2 d after which, its abundance increased almost 50 fold for the remaining 5 d.

It should be noted that, although some members of class *Deltaproteobacteria* such as *Myxococcales* and *Bdellovibrionales* were present in the biofilms at very low abundance, no SRB related organisms were observed in the sequence data. A study performed by Lanneluc et al. (2015) used carbon steel immersed in marine environment for 1-8 weeks and observed that SRB were detected only after 1 month of immersion (Lanneluc et al. 2015). In a different study, Bermont-Bouis et al. (2007) reported for carbon steel immersed in seawater for 1-8 months, that although *Desulfovibrio* appeared in the biofilm after one month, they dominated the biofilm only after 8 months of exposure (Bermont-Bouis et al. 2007). Thus, the absence of SRB here is not surprising.

Overall, these results suggest that colonisation of a surface by a mixed seawater community is driven by a subset of Proteobacteria found in the seawater community. These early colonisers may then allow for other bacteria, such as the SRB to colonise later, once they modify the local niche, e.g. reduced oxygen concentrations.

Figure 3.3 Comparison of microbial communities in seawater and in biofilms formed on UNSS32750 coupons. a. The most abundant OTU's (>10%) in the samples are shown to give a general overview of the differences between the communities. b. OTUs that could not be assigned to taxa have been removed for presentation and hence, the total percentage does not reach 100%. Each OTU, defined at the 97% identity threshold, is presented as different colors and their percentage contribution to the communities is indicated on the y-axis.

3.3 Study Two (March – April 2017)

Based on the preliminary studies, an additional corrosion experiment was designed to replicate the experiment described above. Further, this study was modified to include an additional metal surface and extended the experimental time to 30 d. The metals selected were UNS30400 (SS304) and UNS32750 (SD) since SS304 is less corrosion resistant (Hashimoto et al. 1979). All the coupons used in these experiments were polished mechanically using a Rotoforce-4 (Struers) with p600 grit sandpaper. Moreover, in the preliminary study, the potential was measured only at discrete timepoints, 1 h, 2 d and 7 d, where the maximum increase was observed in first 2 d, after which time the potential remained stable. This suggested that ennoblement occurred very early in the experiment. Therefore, this study was conducted using continuous potential monitoring to better define the ennoblement behaviour and to correlate that with biofilm development.

3.3.1 *Potential (E_{ocp}) over 30 days*

The potential for both types of steel, SS304 and SD, was between -100 to -150 mV at the beginning of the experiment. A significant difference was seen in the E_{ocp} behaviour measured for SS304 and SD over 30 d. For SS304, the potential increased to ~ 100 mV over the first 4 d and remained stable until day 7. After 7 d, there was a sudden increase to approximately 150 mV and then remained constant for the remainder of the experiment (30 d). In contrast, the potential for SD coupons reached 200 mV by 7 d, and then decreased to, and remained at, 50 mV by the end of 30 d (Figure 3.4 a). The shift of the potential towards a positive value suggests that these materials were undergoing ennoblement (Motoda et al. 1990, Ito et al. 2002). In 1998, Neville and Hodgkiess, characterised the corrosion behaviour of different types of steels and alloys immersed in natural seawater off the west coast of Scotland, at a temperature range of 6 - 11°C. They observed that for all the samples, there was a positive shift in potential (~50 mV) in initial 12 d. The reduction in potential after that

was more for super-duplex steel as compared to austenitic steels (Neville et al. 1998).

The OCP measurements had large standard deviations (Figure 3.4a) and this could be attributed to several factors. One potential source of interference with the measurements could be the formation of bubbles in the holding tank due to the high flow rate. The presence of bubbles can interrupt the electrical continuity of seawater, thus causing an unstable potential reading. Additionally, for two of the SS304 coupons, it was noted that the silicon sealant at the connection between the Cu wire and SS coupon was damaged, exposing the bimetallic junction to seawater. It has been shown that the exposure of the connection to seawater can induce galvanic corrosion between Cu wires and stainless steel decreasing the potential of SS304 (Mak 2002). Therefore, the samples where the connection were damaged were removed from the analysis, and the subsequent data analysis shows that both remaining SS304 coupons stabilize at ~ 300 mV. A clear trend with time was seen, that was different for SS304 and SD (Figure 3.4 b).

Figure 3.4 The open circuit potential as measured for UNS32750 (SD, red line) and UNSS30400 (SS304, grey line) over 30 d exposure. OCP was measured continuously against a commercial Ag/AgCl reference electrode. Every point represents the average of ≥ 4 replicates. Pink and grey shaded areas around the lines depict the standard deviation observed for $n \geq 4$ samples. a) without removal of faulty SS304 samples and b) after removal of faulty SS304 samples.

3.3.2 Biofilm formation by a seawater community

To better understand the relationship between the development of a biofilm on the metal surface and the change in potential, confocal imaging was performed at various time points (2 h as well as 2, 7 and 30 d) (Figure 3.5). After 1 h of exposure, only a few cells were observed attached to the surface for both SS304 and SD. On day 2, the number of cells attached to the surface increased for both metals. Images collected on day 7 show the formation of microcolonies on the surface. However, by 30 d, the biomass appeared to have decreased.

Figure 3.5 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images for SS304 and SD coupons, retrieved from seawater at 1 h as well as after 2, 7 and 30 d (400X magnification). Live cells stained with syto 9 appear green and dead cells stained with propidium iodide appear red. Small panels on top and on right of every image show x/z and y/z planes respectively, which shows the thickness of the biofilm.

The image data was quantified (Figure 3.6), which showed that the biofilm biomass for SS304 increased by around 400% from 1 h to 7 d but decreased by 60% from 7 d to 30 d. On day 7, the amount of biomass present on the SS304 surface was significantly higher (~ 65%) than on SD. The amount of dead

biomass was very low compared to the live biomass and consistent throughout the experiment (Figure 3.6 a). The surface area covered by the biofilm showed a similar trend as observed for the biovolume. For SS304 coupons, the surface area covered almost doubled from 1 h to 2 d and further increased 3 fold from 2 d to 7 d and decreased by 30% by the end of the experiment. Unlike SS304, a gradual increase was seen for SD over the course of the experiment (Figure 3.6 b).

Figure 3.6 Biovolume and surface area covered by the biomass after 1 h, 2 d, 7 d and 30 d. a) Comparison of biovolume ($\mu\text{m}^3/\mu\text{m}^2$) between SS304 and SD coupons. b) Comparison of surface area coverage (μm^2). Light green and light red bars indicate live and dead cells for SS304, respectively. Dark green and dark red represent live and dead cells for SD coupon. Each measurement is the average of ≥ 3 images per coupon.

The increase in the biovolume for first 7 d for SD in this experiment was compared with the biovolume data from preliminary experiment. For the preliminary experiment (Section 2.1), the number of live cells increased 3.5 fold from 1 h to 2 d and further increased by 2.5 fold until 7 d. Whereas in this study, the live biomass increased by 50% from 1 h to 2 d and further increased by 1.5 fold from 2 d until 7 d. The percentage of dead biomass on 7 d was

around 12% of the total biomass, whereas for the preliminary study the dead cells represented ~ 1% of the total biomass. The higher biovolume on 7 d for the preliminary study can be due to the lower flow rate of seawater through the tanks. For example, it has been previously shown that the flow of the medium affects the biofilm composition and microstructure in the environment (Dobretsov et al. 2013)

3.3.3 Comparison of potential and biovolume

For both types of steel coupons (SS304 and SD), the relationship of the potential measured over 30 d (2 h and 2, 7 and 30 d) to the biovolume measured from the CLSM images was compared (Figure 3.7). Although not significant, SS304 showed more ennoblement as compared to SD over 30 d. For SS304, potential increased from -120 mV to -50 mV in the first 2 d and this was associated with a 7-fold increase in biovolume of the biofilm. The potential subsequently increased to 140 mV by the end of the experiment (30 d) at which time, the biovolume had decreased by approximately 60% (Figure 3.7 a). For SD coupons, the potential increased from -150 mV to 10 mV in the first 7 d, when the increase in the biovolume was around 2.5 fold. Unlike SS304, the potential increased to ~60 mV by the end of the experiment and biovolume decreased by only 30% (Figure 3.7 b). Although it is clear that ennoblement was observed as a result of biofilm attachment, the relationship between the potential increase and biovolume observed remains unclear.

Figure 3.7 Comparison of potential (mV) and biovolume (μm^3) for a) SS304 and b) SD.

3.3.4 Community composition analysis

Due to time limitations, the community analysis could not be performed, but samples from seawater and community formed on the coupons after 2 h, day 2, day 7 and day 30 have been collected and stored at -80°C for future analysis. It will be interesting to see the effect of coupon composition on the community, as seen in the laboratory experiments.

3.4 Summary

Community analysis from preliminary study showed a significant selection for the community from natural seawater to the biofilm formed on the metal surface. Time course data for the biofilms suggest that the community formed after 7 d of development largely resembled the community that initially colonized the surface. Visualization of the biofilms formed on SD coupons over a period of 7 days in environmental conditions by CLSM (Figure 3.2) matches with the classical biofilm model (Stoodley et al. 2002).

Ennoblement was observed to occur primarily in the first 48 h as the community initiated biofilm formation. Although not statistically significant based on the results presented here, a general trend indicated that SS304 showed more ennoblement than SD in a 30 d experiment. Flow rate also affected the ennoblement as observed from differences between the two experiments. The initial steep increase in ennoblement was observed to occur in the first 48 h, however, the relationship between the initial biofilm formation and ennoblement remains unclear. In part, this relationship could not be better resolved due to the limited biofilm analysis. Thus, incorporation of more time points between 1 h and 2 d, will be beneficial to further study this phenomenon. Future work would therefore focus on these early time points and indeed, as a result of these observations, subsequent laboratory studies (Chapters 4 and 5) have focused on the initial stages of corrosion to better define these relationships.

In the marine environment, parameters such as temperature, salinity, flow rate, microbial community affects the corrosion behaviour. Variations in these parameters per season, on and offshore industrial activities and geographical area makes the reproducibility in these experiments questionable. Moreover, the application of characterisation techniques is cumbersome in environmental setup. Therefore, to understand the mechanisms involved in MIC, experiments should be performed with fixed parameters such as surface treatments, pH, temperature, microbial community etc. It can be achieved in laboratory with a suitable study system. The next two chapters present two such systems that were adapted from traditional systems used, to achieve more reproducibility, a higher throughput and easy application of several interdisciplinary techniques to study MIC.

Chapter Four: Developing and validating a continuous flow system for MIC characterization

4.1 Introduction

Batch MIC experiments allow for rapid characterization and initial screening of the process parameters, including coupon polishing, nutrient profile, etc. However, the biofilm structure is dependent on fluid-dynamic conditions. For example, shear stress and flow conditions have been shown to influence biofilm thickness and microstructure affecting diffusion vs. reaction processes in viable biofilms (Heitz et al. 1996, Ismail et al. 2002) .

In the environment, several parameters affect MIC, including water currents, nutrient gradients, pH, temperature, and microbial community inducing variability over time in corrosion measurements. The precise control of these parameters is not feasible in natural environments. Furthermore, in natural settings, MIC is a slow process and thus requires longer duration experiments. Therefore, MIC is often studied in laboratory systems that mimic the environment, where the parameters affecting the corrosion can be controlled and monitored. In addition to the increased control over various parameters, laboratory experiments can be completed in shorter time frames, as accelerated microbial growth can be induced by increasing the nutrient concentration. For example, in marine habitats, comparatively low biomass is present and the amount of organic nutrients is very low, but in a laboratory system, this can be overcome using artificial seawater media. The choice of using natural seawater (Little et al. 2014), artificial seawater (Venzlaff et al. 2013), augmented seawater (Dinh et al. 2004, Cheng et al. 2009) or synthetic media has been widely debated in the research community (Little et al. 2014). While artificial seawater does not mimic the complexity of natural seawater and hence, always has some limitations, its composition is highly reproducible and can be easily amended for specific experimental purposes.

For laboratory systems, it is also easier to implement the techniques required for characterization of the MIC process. Electrochemical, imaging and meta-omic techniques can be easily adopted in the laboratory conditions. An additional advantage of laboratory based systems is that the ability to control parameters can increase the reproducibility of experiments.

Most of the studies related to MIC are performed in a batch or a fed-batch system. However, such systems inevitably have limitations associated with the depletion of oxygen and nutrients in the bulk liquid as well as the accumulation of waste metabolites. Flow-through systems avoid some of these limitations by the continuous supply of nutrients and allow for the removal of potentially inhibitory waste products, which would mimic the natural environment where oceans would enable almost infinite dilution. The formation of a biofilm on a surface in such flow-through system allows for the establishment of local nutrients and oxygen gradients that would enable the formation of complex communities, with aerobic and anaerobic metabolism in different parts of the biofilm. Different designs have been used as per the requirements of the experiments. A flow-cell system was proposed to study biofilm formation in continuous flow system (Pedersen 1982), which was later modified to incorporate metal coupons and investigate the biofilm formation on metal surfaces (Kielemoes et al. 2000). However, the system was complicated and with a high volume to surface ratio for investigation of role of biofilms in microbial corrosion.

Similarly, Ismail et al. (2002) investigated how the aerobic strain *Pseudomonas fragi* protect mild steel from corrosion in continuous flow conditions. Biofilms of this organism inhibited corrosion almost tenfold. At low flow rates, both planktonic and biofilm microorganisms contributed to MIC. At higher flow rates, only the bacteria from the biofilm were associated with the MIC inhibition (Ismail et al. 2002). This is related to previous research, where it was shown that high flow velocities, and thus stronger shear forces, did not prevent biofilm formation, but rather resulted in thinner and more robust biofilms (Heitz et al. 1996) as well reduced density of planktonic bacteria.

Recently, the effect of marine biofilms on 316 L type stainless steel in natural and sterile seawater was studied by Xu et al. (2015). They deployed the coupons in a 10 L system fed with natural coastal waters. OCP results showed that natural seawater caused higher potential ennoblement as compared to sterile seawater. EIS performed for coupons in aerated and deaerated conditions revealed that corrosion inhibition by aerobic biofilms was enhanced in presence of oxygen (Xu et al. 2015). Duncan et al. (2014) constructed a continuous flow system to investigate the effect of SRB on pitting corrosion. The system was built using PVC pipes to mimic pipeline system and coupons were placed at different points in the system. The media was circulated at controlled flow rate through the pipes. An enriched hydrogen sulphide (H₂S)-producing bacterial community dominated by SRB was used as an inoculum (Duncan et al. 2014). Although this setup mimics the actual conditions in oil pipelines, it does not allow precise control of flow conditions and shear forces.

4.2 Flow cell design

Recent studies have shown that controlled growth of the biofilm formed on the metal surface under continuous flow conditions and repeatable morphology and composition of the biofilm are very important to characterize MIC. Such conditions are achieved in commercially available polycarbonate flow cells, developed by DTU systems biology, Denmark (Crusz et al. 2012). DTU flow cells allow rapid growth of biofilms and easy microscopic characterization. Miniature flow cells are also useful in electrochemical MIC characterization, as the electrode potential is approximately constant on each point of the metal coupon, thus giving noise-free results. Based on this, a novel electrochemical flow cell was designed for MIC experiments. The flow cell design is described in detail in Chapter 2 section 9 (Figure 2.5). This flow cell facilitated biofilm growth on the metal surface under controlled conditions such as flow rate, composition of medium, inoculum, etc. The flow cell can also be used for

electrochemical characterization when the inserted coupon is attached to a potentiostat via Titanium (Ti) wires.

4.3 Enrichment of a mixed microbial marine community from sea water

In environmental studies, it is difficult to achieve reproducibility, due to variability in the community that arise because of differences in temperature and pH as well as seasonal variations such as storms or heavy rainfall, industrial or domestic discharge in coastal areas, etc. A reproducible starting community, for experiments to characterize MIC will help increase the repeatability of the experiments. To achieve a reproducible, defined starting community, seawater was inoculated into two different artificial seawater media with 5 different variations (3MFS, 3MHS, VNSSF, VNSSFSG, VNSSHSG) to create enrichments to be used as inocula for laboratory experiments. (Chapter 2 Section 2.8).

The media variations tested here primarily differed in the carbon source or carbon source concentration. For example, 3M medium contained 2 g/L glucose, which favors fast growing heterotrophic bacteria. In contrast, VNSS contained 1 g/L peptone and 0.5 g/L yeast extract in addition to 0.5 g/L glucose. The degradation of complex carbon sources usually favors slow-growing bacteria and resulted in several by-products.

4.4 Results and Discussion

4.4.1 Omics analysis of the enriched community

After the enrichments were obtained, RNA was extracted and sequenced as described in Chapter 2, section 8. A rarefaction plot describing the observed OTUs in all the enrichments was constructed that indicated the sequencing

depth was near saturation (Figure 4.1). Cultures grown in VNSS medium without glucose (VNSSFSG) showed the highest number of species, followed by 3M and VNSS medium with glucose (VNSSFSG). The least diverse community was VNSSFSG, possibly due to inhibition by the high salt content in the media and the presence of glucose, which might favour growth of heterotrophic bacteria that outcompete slower growing organisms. PCoA plot generated by calculating the community distance using Bray-Curtis dissimilarity index showed that the enriched communities were significantly different from each other (data not shown).

Figure 4.1 Rarefaction plot of observed OTUs in different enrichments. The media formulations used were, 3MFS – 3M with 1X NSS, 3MHS – 3M with 0.5X NSS, VNSSFSG – VNSS with 1X NSS without glucose, VNSSHSG – VNSS with 0.5X NSS with glucose and VNSSFSG – VNSS with 1X NSS with glucose.

Enrichments for VNSSFSG (highest diversity) and VNSSFSG (lowest diversity) are compared below, as they were also used in the following experiments to see the effect of glucose in the media on biofilm formation and corrosion behavior of SS304 coupons. Taxonomic evaluation showed that, the enrichments were dominated by *Vibrio spp.* It has been shown that *Vibrio spp.* are some of the most abundant, culturable bacteria from the marine environment (Heidelberg et al. 2002). For VNSS in presence of glucose, it was observed that *Vibrio fortis*, a

Gram-negative *Gammaproteobacteria* ubiquitous in marine environment, was the most abundant and formed around 34% of the community (Figure 4.2). However, in absence of glucose (VNSSFs) it formed only 9% of the entire community. *Vibrio parahaemolyticus* was most abundant in VNSSFs, and represented almost 33% of the community, whereas formed only 1% of the community in presence of glucose. *V. parahaemolyticus* is a Gram-negative, halophilic facultative aerobe found naturally in marine and brackish saltwater, known to cause gastroenteritis (Daniels et al. 2000). Molitoris et al. (1985) had reported abundance of this species from tropical seawater from coasts of Jakarta, Indonesia (Molitoris et al. 1985). VNSSFSG showed 2 and 3 fold increases in the abundance of *V. diabolicus* and *V. vulnificus*, respectively, as compared to VNSSFs. *V. diabolicus* is a facultative anaerobic, heterotrophic *Gammaproteobacteria*, isolated from deep-sea vents. It has been shown to produce exopolysaccharides containing uronic acids and hexosamines in presence of glucose in batch cultures (Raguns et al. 1997). *V. vulnificus*, a *Gammaproteobacteria* which produces a capsule of polysaccharides for its protection, is a lactose fermenting human pathogen found in marine environment (Oliver et al. 1983). *Zunongwangia profunda*, a Gram-negative, strict aerobe, originally isolated from deep-sea sediment known to secrete large amounts of polysaccharides (Liu et al. 2011), was present only in VNSSFs enrichment. *Vibrio cambellii*, another marine pathogen was present in both enrichments forming around 7 – 9% of the community *Pseudoalteromonas lipolytica*, a marine *Gammaproteobacteria* was found only in VNSSFSG. This strain, able to hydrolyse lipids, has been reported previously in estuarine environments (Xu et al. 2010). Along with these prominent species pathogens like *Salmonella enterica*, *Typhlorella asinigenitalis*, *Vibrio cholerae* were also present in both enrichments in low abundances. VNSSFs and VNSSFSG enrichments were used as an inoculum in this chapter and Chapter 5 (section 5.3.6), primarily to determine the effect of glucose on MIC and because they represented the greatest difference in community diversity between the various enrichments.

Figure 4.2 The percentage abundance of the top 20 species present in the enrichments of VNSSF and VNSSFSG.

4.4.2 *The effect of glucose as a carbon source on the corrosion of SS304*

4.4.2.1 *Growth of mixed species biofilms using media with and without glucose as a carbon source*

The sequencing data indicated that VNSSF supported the most diverse community, which was significantly different from VNSSFSG. The presence of glucose might favour the growth of heterotrophic bacteria that outcompete

organisms that otherwise might be able to grow when only peptone and yeast extract were provided. To assess the effect of this diversity on the corrosion of SS304, these communities were inoculated into the electrochemical flow cell system and corrosion was monitored as the biofilms were grown with VNSSFSG or VNSSFS for 5 d. When grown the presence of glucose (VNSSFSG), the pH decreased from 8.2 to 7.5 in the first 24 h of incubation, possibly due to the growth of heterotrophs converting glucose into organic acids (Figure 4.3 a). The decrease in pH was consistent with pH drop observed by Javed et al. (2014) for medium with glucose, when *E. coli* were grown in presence and absence of glucose on carbon steel (Javed et al. 2014).

When VNSSFS was used as a substrate, the pH decreased only slightly, from 8.2 to 7.9 within the first 24 h. For both conditions, the pH did not change further after the first 24 h. The optical density of the effluent from the biofilm was used as a proxy for the growth of the biofilm and for both media. OD for both media increased in 1 d after inoculation and remained constant over 5 d of the experiment (Figure 4.3 b).

Figure 4.3 The effect of carbon source on pH a) and OD600 b) on mixed species biofilm communities grown under flowing conditions. Biofilms were grown full salts marine medium with either glucose (VNSSFSG) or without glucose (VNSSFS), where the carbon sources are peptone and yeast extract. Values are the mean of ≥ 3 replicate experiments.

To characterise the change in corrosion behaviour over time, the open circuit potential E_{OCP} was measured, and linear polarization resistance was measured from -50 mV to 50 mV vs. E_{OCP} with a scan rate of 0.100 mV/s. Linear polarisation resistance (LPR) data showed that the corrosion current density (i) increased rapidly in the first 24 h for both media, with and without glucose (Figure 4.4 a). Although not significant, the corrosion current was higher in VNSSFSG compared to VNSSFs after the first 24 h of incubation. For VNSSFSG, the corrosion current reached a maximum of 25 ± 11 nA on day 4 and was unchanged at the end of the experiment on day 5. In contrast, the corrosion current for biofilms grown without glucose in the medium, showed initial current density increase in the first 24 h, achieved a maximum of 12 nA after 72 h and this did not change over the course of the rest of the experiment. The corrosion potential, E_{corr} decreased 2 h after inoculation for both media, with and without glucose. Subsequently, E_{corr} decreased gradually only in the glucose-containing medium, while it increased in the absence of glucose after 72 h (Figure 4.4 b). An increase in E_{corr} , from 72 h to 96 h corresponded with a reduction of current density, suggesting that the biofilm may be protective.

Figure 4.4 a) Current density (i) and b) corrosion potential (E_{corr}) of SS304 exposed to different mixed species biofilms grown in VNSSFs or VNSSFSG. E_{corr} was measured against Ag/ AgCl quasi reference electrode. Each point shows the average of ≥ 3 biological replicates.

Electrochemical impedance spectroscopy was used to determine the impedance of the biofilm. The data was analyzed with two time constants in series, to fit the data to monitor resistance and capacitance of the biofilm. Capacitance Q3 increased with time in both media. After 96 h, however, Q3 of the biofilm in the VNSSFSG was slightly higher than Q3 for VNSSFS. It is likely that Q3 stabilized after 96 h for both media, as the biofilm thickness reached the maximum value allowed by the shear stress in the EFC (Figure 4.5).

Figure 4.5 The capacitance (F/ farad) of biofilm layer vs. time of SS304 exposed to different mixed species biofilms grown in VNSSFS or VNSSFSG. Each point shows the average of ≥ 3 biological replicates.

The resistance (R3) was not significantly different between VNSSFSG and VNSSFS after 96 h (Figure 4.6). A slight increase was observed in both media 2 h after inoculation, which can be the result of attachment of bacteria on the metal surface. Despite a uniform biofilm formed on the surface as observed on day 5 (Figure 4.7), a significant fluctuation in resistance was observed for VNSSFS. The increase in the resistance after 72 h corresponded to the decrease in current density (Figure 4.4 a). To explain these fluctuations, more time points for biofilm visualisation would be required to understand correlation between structure of the biofilm and resistance.

Figure 4.6 The resistance of biofilms formed on SS304 exposed to different mixed species biofilms grown in VNSSFs or VNSSFSG. Each point shows the average of ≥ 3 biological replicates.

A two-interface electrical circuit model, in series and parallel, was used to fit the EIS data. These two models imply two possible scenarios, where for a circuit in series, the biofilm forms over an undamaged passivation layer and for a parallel model, the biofilm is in contact with the passivation layer as well as the metal surface. Since the actual system cannot be represented entirely by any of the above systems, the best fit was chosen. A bode plot, in which modulus of impedance and phase shift are plotted as a function of frequency, was used to determine the best fit for the data to highlight both high and low frequency charge transfer processes. The fit was particularly ensured at low frequencies, limited to 20-50 MHz where biofilm-related corrosion reactions are predominant. The high frequency points were excluded by the simulation if $Z < 0$, depending on the inductance of the Ti coil used for coupon connections.

4.4.2.2 *Biofilm visualisation*

To observe the structure and localisation of biofilms, coupons were retrieved from the flowcells after 5 d, stained with the live dead reagents and observed by confocal laser scanning microscopy (Figure 4.7). Biofilms in VNSSFs were more uniform and evenly distributed over the coupon surface. In contrast,

biofilms formed in the presence of glucose were more clustered and thicker than the biofilms grown in VNSSFSG. While the biofilms formed in the presence of glucose were more heterogeneous in terms of surface coverage, they formed dense microcolonies that were thicker than the biofilms formed when glucose was omitted from the medium. The surface coverage of biofilms grown in VNSSFSG were 70% higher than for VNSSFSG (Figure 4.8 b). Heterogeneity of biofilm has been reported to cause loss of passivity of the surface oxide film, facilitating diffusion of active and product ions (Beech et al. 2000, Videla 2001). Yuan et al. (2007) reported presence of heterogeneous biofilms formed by marine, aerobic *Pseudomonas* on 70/30 Cu-Ni alloy and their SEM images revealed the occurrence of micropitting underneath bacterial clusters (Yuan et al. 2007).

Figure 4.7 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images showing biofilm formation on SS304 steel grown in a) VNSSFSG or b) VNSSFSG. Biofilms were stained with the BacLight live-dead reagents, where live cells appear green and dead cells appear as red. Separate wavelength (514 nm) than that used for syto9 (live) (488 nm) and propidium iodide (dead) (561 nm) was used for reflection of the metal surface. The panels on the top and right edges of every image show the x/z and y/z planes respectively, which shows the thickness of the biofilm.

Biofilms grown in the absence of glucose showed significantly higher biomass (~180%) than in the presence of glucose (Figure 4.8 a). Javed et al. (2014) reported a higher corrosion rate of carbon steel in presence of *E. coli*, when immersed in medium containing glucose as compared to medium without glucose, which could be due to production of acidic by products by the bacterial cells, in presence of glucose (Javed et al. 2014). The formation of acid metabolites can rupture the passive films exposing the metal surface to the environment and also increase the supply of oxidising agents (protons) in the environment. (Edyvean et al. 1991). The fraction of dead cells was considerably lower (5%) in the absence than in the presence of glucose (20% dead cells). Due to high variability, an unpaired t test with Welch's correction was performed to ascertain the significance, which showed that the biovolume and surface area were both significantly higher for VNSSFs than VNSSFSG ($p < 0.0001$).

Figure 4.8 a) Bio-volume ($\mu\text{m}^3/\mu\text{m}^2$) and b) surface area covered (μm^2) of biofilms on metal coupons grown in either VNSSFs or VNSSFSG. Green columns indicate the live biomass and red indicates dead cells. The figure shows the combined analysis of images from two experiments for each media ($n \geq 8$).

4.4.3 Characterization of biofilm communities formed in the presence and absence of glucose

Based on the results above, different microstructures of colonies formed due to different medium compositions was correlated with different corrosion behaviours. Therefore, to determine if the communities present in the two media differ and to correlate those differences in community composition, total RNA was extracted from the biofilms for community analysis. It has been observed that in the marine environment, the composition of the metal substratum influences the formation rate and cell distribution of biofilms during first few hours of incubation (Marszalek et al. 1979). For example, Kielemoes et al. (2000) showed that when SS304 and SS316 stainless steels were compared for surface colonisation, a higher percentage of the surface of SS304 was colonized, possibly due to the difference in the chemical composition, since the presence of molybdenum in SS316 increases the corrosion resistance (Kielemoes et al. 2000). In addition, preliminary experiments performed here (results not shown) to determine the effect of metal composition on the selection of biofilm community members showed that the biofilm community on SD coupons was significantly different from that of SS304 and SS316. In contrast, the communities established on SS304 and SS316 were very similar. The reason for that could be difference of microstructure and composition between austenitic and duplex steels. Those results suggested that the metal type may select for different community members. Therefore, experiments were also designed to compare the impact of carbon source (VNSSFs and VNSSFSG) and metal type (SS304, SS316 and SD), on the community composition when inoculated with an enrichment of marine microorganisms.

4.4.3.1 Taxonomic classification for VNSSFs

Enrichment and biofilm samples were compared to observe the selection of community from planktonic culture to biofilm and the effect of different metal types (SS304, SS316 and SD). PCoA plot constructed using Bray-Curtis dissimilarity index suggested that biofilm samples were significantly different from the enrichment (Figure 4.9).

Figure 4.9 PCoA plot of the enrichment and the biofilm samples on different coupon types for VNSSF medium.

When the percentage abundance of read counts for 20 most abundant species from the enrichment and biofilm samples was compared (Figure 4.10), it was observed that the inoculum and the biofilm community was largely dominated by *Vibrio* spp. *V. parahaemolyticus* was the most abundant in the enrichment. Its abundance remained constant in biofilm samples related to SD coupons and increased a by approximately 25 and 50% for SS304 and SS316 samples respectively. *V. parahaemolyticus* is a Gram-negative, halophilic facultative aerobe found naturally in marine and brackish saltwater. It is known to cause gastroenteritis (Daniels et al. 2000) . *Vibrio hepatarius* was enriched from the inoculum in the biofilm. The abundance was highest for SD samples and decreased by around 30% in the other two. In contrast, the abundance of *V. diabolicus*, *V. fortis* and *Vibrio campbellii* decreased by approximately 50, 25 and 98%, respectively, as the biofilm formed. *V. cholerae* was equally abundant in the enrichment as well as in the biofilm samples. *V. vulnificus* formed around

4% of the enrichment, but its abundance decreased by 80, 85 and 90% for SS304, SD and SS316 respectively as the biofilms were formed.

Figure 4.10 The abundance of the 20 most abundant species in the biofilm samples for three different types of metal coupons where the biofilms were grown in VNSSF medium. The bar on the left is the community diversity of the inoculum.

It was observed that seven species were common in all the samples suggesting they were selected either positively or negatively from the enrichment to form a biofilm (Figure 4.11). Eleven species were found only in the enrichment. Six

species were common to all the biofilm samples but they were not abundant in the enrichment, suggesting that although these species are not abundant in the planktonic phase, they are positively selected when the biofilm is formed. Four species were selected only on SS304 coupons followed by two species each for SS316 and SD, which were not abundant in the enrichment. These results suggest that composition of the substrate also plays an important role in selecting the community which forms a biofilm.

Figure 4.11 Comparison of top 20 species present in VNSSFS enrichment and biofilm samples; a) Venn diagram with 20 most abundant species in enrichment as well as biofilm samples for three coupon types for VNSSFS (green – enrichment, yellow – SS304 coupon biofilm, blue – SS316 coupon biofilm samples, red – SD coupon biofilm samples, b) eleven species present in VNSSFS enrichment only, c) seven species present in all the samples and d) species present only in biofilm samples.

4.4.3.2 Taxonomic classification for VNSSFSG

Enrichment and biofilm samples were compared to observe the selection of community from planktonic culture to biofilm and the effect of different metal types (SS304, SS316 and SD). PCoA plot constructed using Bray-Curtis dissimilarity index suggested that biofilm samples were significantly different from the enrichment (Figure 4.12).

Figure 4.12 PCoA plot of the enrichment and the biofilm samples on different coupon types for VNSSFSG medium.

When the VNSSFSG enrichment was used to form biofilms on the metal surfaces, it was observed that *V. parahaemolyticus* showed a strong, positive selection from the enrichment during biofilm formation. In enrichment, it formed only 1% of the community, which increased significantly in biofilm samples to 25, 19 and 14% for SS316, SS304 and SD coupons respectively. *Photobacterium gaetbulicola* also showed a similar trend as it formed 4.5, 5 and 7% of the biofilm communities for SD, SS316 and SS304, in spite of being very

low abundant in the enrichment. *P. gaetbulicola* is a Gram-negative, motile, lipolytic bacterial strain isolated from west coast of Korea. The abundance of *V. fortis* and *V. diabolicus* remained relatively constant in the enrichment and biofilm samples. *V. vulnificus* decreased in abundance in the biofilms compared to the enrichment.

Figure 4.13 The abundance of the 20 most abundant species in the biofilm samples for three different types of metal coupons where the biofilms were grown in VNSSFSG medium. The bar on the left is the community diversity of the inoculum.

A total of eleven species were exclusive to the enrichment and were not observed in the biofilm (Figure 4.14). Four species were common to all the biofilm samples, irrespective of the metal composition. Some species, which were not abundant in the enrichment samples, were found to be highly abundant in biofilms, suggesting these species may be specialists in biofilm formation or may have an affinity for growth on the metals. Of these organisms, 7 were only enriched in the presence of SS304, four were specific for SS316 and 4 were specific for SD coupons.

Figure 4.14 Comparison of top 20 species present in VNSSFSG enrichment and biofilm samples; a) Venn diagram with 20 most abundant species in enrichment as well as biofilm samples for three coupon types for VNSSFSG (green – enrichment, yellow – SS304 coupon biofilm, blue – SS316 coupon biofilm samples, red – SD coupon biofilm samples), b) eleven species present in VNSSFSG enrichment only, c) seven species present in all the samples and d) species present only in biofilm samples.

PCA plots based on the taxonomic classification of community present in the biofilm samples for both VNSSF and VNSSFSG, depict overall community differences among samples (Figure 4.15). Samples cluster significantly according to the media used, indicating that presence or absence of glucose in the media had a significant effect on community of the biofilm. For VNSSFSG, SD samples cluster separately from the other two steel types. However, effect of metal composition is not clear and requires further investigation.

Figure 4.15 PCA plot of the biofilm samples grouped in relation to the type of metal surface. Green oval indicates samples for VNSSF and the red oval indicates samples in VNSSFSG. The ovals here are for convenience of grouping samples and do not represent any statistical significance

A preliminary analysis of functional classification of the samples according to KEGG (Kyoto encyclopedia of genes and genomes) annotations was performed. The aligned reads were assigned to KEGG ontology terms (KO) using gene id's (GI) to KEGG mapping file implemented in MEGAN. Samples

were compared according to phase (biofilm and planktonic) for both media separately, to determine the differences in functional pathways expressed between the two phases.

4.4.3.3 Functional classification based on mRNA for the enrichment cultured in VNSSFs

The comparison of planktonic and biofilm phases in VNSSFs indicated that the ABC transporter pathway and arginine and proline metabolic pathways were expressed significantly higher in the biofilm samples as compared to planktonic samples (Table 4.1). ABC transporter pathways are essential for primary and secondary membrane transport, i.e. supply of external nutrients and removal of unwanted compounds by bacteria (Wilkins 2015). Arginine and proline metabolism is a key pathway responsible for arginine and proline biosynthesis from glutamate and has been shown to be upregulated as the biofilm is formed (Yadav et al. 2012). Pathways related to DNA replication and beta alanine metabolism were also more highly expressed in the biofilm phase. The implication of this is unclear as it has been shown in the literature that DNA replication pathways are usually suppressed when a biofilm is formed since the bacteria are dividing less than under planktonic growth conditions (Svensäter et al. 2001). The role of these pathways in corrosion mechanism is unknown. Gomez-alvarez et al. (2012), performed metagenomic analysis on biofilm formed on corroded concrete waste water pipe biofilm and reported that, proteins involved in sulfur and nitrogen metabolism showed the most significant differences between biofilms (Gomez-Alvarez et al. 2012).

Table 4.1 Pathways that were differentially expressed in biofilms vs. planktonic cells grown in VNSSFs.

Pathway		p-values (corrected)
Metabolism	Porphyrin and chlorophyll metabolism	0.011152442
Environmental Information Processing	ABC transporters	0.012390654
Metabolism	Arginine and proline metabolism	0.021097833
Genetic Information Processing	DNA replication	0.038328268
Metabolism	beta-Alanine metabolism	0.042131493

Figure 4.16 Heat map of significantly different expression of functional pathways in planktonic and biofilm phase for samples from VNSSFs for three different coupon types. Samples coloured green in the dendrogram represent planktonic phase and in light blue represents biofilm phase.

4.4.3.4 *Functional classification based on mRNA for the enrichment cultured in VNSSFSG*

A similar analysis of VNSSFSG samples was performed (Figure 4.17). Results showed that the glycolysis/ gluconeogenesis pathway was more highly expressed in the planktonic phase, which was surprising as several studies in the literature suggest that this pathway tends to be more highly expressed during biofilm formation (Li et al. 2015). A similar trend was also seen for citrate cycle pathway, which was also more highly expressed in the planktonic phase compared to the biofilm. RNA degradation pathway was more highly expressed in the biofilm phase. Lipopolysaccharide synthesis (LPS) pathway was more highly expressed in the biofilm phase as well. It has been shown before that this pathway plays an important role in biofilm formation (Beloin et al. 2008). Expression of this pathway could be linked to the positive selection of *V. diabolicus*, a polysaccharide secreting bacteria (Raguénès et al. 1997).

The arginine and proline metabolism pathway was more highly expressed in the biofilm phase irrespective of the medium used. Lopez et al. (2015), while studying the mechanisms of biofilm formation by *P. aeruginosa*, reported that inhibition of reactions related to arginine and proline metabolism pathway decreased biofilm formation, suggesting their importance in biofilm formation (Lopez et al. 2015).

Genes associated with homologous recombination and DNA repair were more highly expressed in biofilm phase. This pathway has been shown to be related with DNA repair processes in bacteria. It is possible that this is an indication that these biofilms are experiencing some DNA damaging stress, such as reactive oxidative or nitrogen stress. For example, it has been shown that mature biofilms of *P. aeruginosa* undergo cell death as a result of increased activity of the denitrification pathway and the production of oxidizing compounds such as peroxyxynitrite (Barraud et al. 2006).

Figure 4.17 Heat map of significantly different expression of functional pathways in planktonic and biofilm phase for samples from VNSSFSG for three different coupon types. Samples coloured green in the dendrogram represent planktonic phase and in light blue represents the biofilm phase.

Table 4.2 Pathways significantly expressed when biofilm and planktonic phase are compared for VNSSFSG with corrected p values for each.

Pathway		p-values (corrected)
Metabolism	Arginine and proline metabolism	0.000985
Metabolism	Lipopolysaccharide biosynthesis	0.002854
Genetic Information Processing	Homologous recombination	0.006131
Metabolism	Glycolysis / Gluconeogenesis	0.009749
Metabolism	Propanoate metabolism	0.012294
Metabolism	Biotin metabolism	0.020909
Genetic Information Processing	Ribosome biogenesis in eukaryotes	0.027798
Genetic Information Processing	RNA degradation	0.040715
Metabolism	Geraniol degradation	0.042612
Metabolism	Arachidonic acid metabolism	0.046393
Metabolism	Fructose and mannose metabolism	0.04643
Metabolism	Citrate cycle (TCA cycle)	0.04983

PCA for functional classification of biofilm samples, as the media were compared against each other, showed that samples cluster according to the two media (Figure 4.18). Interestingly, it can also be seen that biofilm samples from SS304 in both media are clustered close to each other as compared to biofilm samples from SS316 and SD coupons. Functional pathways expressed in biofilm samples of SS316 and SD in VNSSFSG are significantly different from that of VNSSFS.

Figure 4.18 PCA plot for functional classification of biofilm samples for VNSSFS and VNSSFSG media. Red oval represents group of samples corresponding to VNSSFSG. Green oval represents VNSSFS samples. Three different colours of the samples for each media represent samples obtained from three different coupon types. The ovals here are for convenience of grouping samples and do not represent any statistical significance.

4.5 Summary

The effect of glucose concentration on the corrosion behavior of SS304 was observed in a novel continuous flow system. The system allowed for the continuous quantification of the corrosion behavior as the biofilms formed on the metal surface. CLSM images suggested that the inclusion of glucose as a carbon affected the structure and species composition of the biofilm. VNSSFSG grown biofilms formed more uniform biofilms on the metal surface whereas, VNSSFSG forms patchy and thicker biofilm, which can induce formation of nutrient and oxygen concentration cells within the biofilm. Taxonomic analysis performed using RNA sequencing for planktonic phase in the flowcell and biofilms scraped from coupons surface suggest significant changes in the community, which is influenced by presence or absence of glucose. The metal composition also plays an important role in selection of the community. Preliminary functional analysis did not show significant expression of corrosion related pathways. Most of the pathways expressed were related to carbohydrate and lipid metabolism, suggesting that the biofilm community was reorganizing metabolism, most likely to optimize growth as the cell numbers increase and the primary carbon source becomes limiting. Further work will need to focus on determining which organisms within the biofilm are differentially expressing these genes to better understand community interactions and energy flow within the community.

Chapter Five: Developing and validating a fed batch bio-electrochemical system for MIC characterization

5.1 Introduction

In the previous chapter, a continuous flow cell system was designed and used to characterise the corrosion behaviour of stainless steel materials in the presence of mixed species consortia. This system was shown to be suitable for the study of the MIC behaviour under continuous flow of media, which is more relevant to the natural environment and facilitated biomass collection for community characterisation. However, this flow through system was time consuming to set up and difficult to maintain over long experimental time frames. Further, one flow cell could accommodate only one metal coupon due to the complexity of the electrochemical assembly. As a consequence of some of these limitations, the flow through system was not well suited to test a wide range of different parameters to determine how they influence MIC. Therefore, a simpler system would be advantageous to study a broader range of conditions more rapidly. Thus, a fed batch system was also developed in parallel for monitoring the corrosion of stainless steel in the presence of a mixed microbial marine community.

A fed batch system is commercially available (Figure 5.1), that is relatively easy to set-up and that allows for control of the environmental conditions (e.g., temperature and headspace composition). However, it is limited in that each corrosion cell holds only a single metal coupon, thus decreasing the throughput and also the volume of the planktonic biomass largely exceeds the concentration of biofilm; thus, the MIC process at the metal surface is confounded by the high number of planktonic cells present relative to the biofilm.

This commercial system is comprised of a three-electrode system, in which the desired metal acts as a working electrode (WE), Ag/AgCl or the calomel

electrode is a common reference electrode (RE) and the system includes a counter electrode (CE) (Moradi et al. 2014, Moradi et al. 2015, Batmanghelich et al. 2017). Similar systems have been used to monitor the electrochemical behaviour of surfaces and the effect of biofilms by electrochemical impedance spectroscopy (Dowling et al. 1988, Jia et al. 2011), the electrochemical noise technique (Zaveri et al. 2007, Chandrasatheesh et al. 2014, Moradi et al. 2014, Moradi et al. 2015), as well as several polarisation techniques.

Figure 5.1 Standard large corrosion cell by Bio-logic. This cell is commonly used for accurate characterization of corrosion in large samples. Image reproduced from (<http://www.bio-logic.net/en/accessories/corrosion-cell-kit/standard-corrosion-cells>).

The system has also been modified in a number of ways to suit the needs of different researchers, including changing the surface to volume ratio, headspace, geometry of the working electrodes, electrical connections etc. For example, Venzlaff et al. (2013) used a 250 mL duran bottle as reactor with a large (2 X 4 cm) electrode of pure iron as a WE, a similar electrode as a CE and a standard Ag/AgCl RE, with a surface to volume ratio of 5 mL/cm², colonised by sulphate reducing bacteria to compare the biological catalysis of SRB and abiotic effect of FeS on the corrosion behaviour. They observed that the

corrosion current was reduced upon inactivation of bacteria, supporting the role of biocatalysis in SRB induced corrosion (Venzlaff et al. 2013).

Yuan et al. (2007) used a semi continuous system to study the effect of an aerobic marine *Pseudomonas* strain on coupons of a 70/30 Cu-Ni alloy. They used a conical flask to grow bacterial cultures in which the alloy samples were exposed to the bacteria. After the prescribed exposure time, samples were transferred to a three-electrode system by mounting them on polyvinylidene fluoride (PVDF) holders in a volume of 500 mL for electrochemical characterisation, concluding that the corrosion rate was higher in presence of bacteria. Xu et al. (2015) immersed SS316L coupons in natural and artificial sea water in laboratory conditions. For electrochemical measurements, coupons were transferred from sea water tank to a three-electrode system with 316L SS (WE), a 2.0 cm $\times$ 2.0 cm platinum foil (CE) and an Ag/AgCl electrode (RE). Their results indicated that corrosion was inhibited by the biofilms formed by aerobic marine bacteria.

5.2 Designing a modified batch reactor system

There are no set guidelines in the literature about the construction of the three electrode system used for corrosion monitoring. The design of such systems largely depend on aerobic/anaerobic conditions required, the material used and the experimental conditions prescribed. One of the key features of an experimental system is the ability to include multiple replicates to address the issue of reproducibility. Therefore, in this study a fed batch reactor system was developed with three-working electrodes in the same environment. Square metal coupons were used to facilitate electrode pre-treatment and imaging at the end of the experiment. The volume/surface ratio was fixed at 14, thus increasing the contribution of the biofilm to the overall MIC process. A detailed description of the system is given in Chapter 2 (section 2.12). The choice of the optimal experimental time, depends on the objectives of the experiment. Previous studies have shown that anaerobic microorganisms begin to become

active only after 1 month exposure of metal in coastal seawater (Lanneluc et al. 2015). Up to that point, the corrosion behaviour is mainly governed by aerobic microorganisms, and their role in reduction of dissolved oxygen. Since the mixed enrichments cultured in the laboratory mainly consisted aerobic microorganisms, the duration of the initial laboratory study was set at 35 d. Results for this long-term experiment indicated that a drastic decrease in the potential occurred upon inoculation and that the current density increased in first few days. Based on these observations, subsequent experiments focused on shorter experimental time frames. The short-term experiments focused on assessing the effect of parameters such as nutrient concentrations, polishing pre-treatments and shaking speed of the reactor.

5.3 Results and discussion

5.3.1 Long-term experiment

5.3.1.1 Electrochemical characterization

The corrosion behaviour was monitored for three unpolished UNS30400 (SS304) coupons (10 X 10 X 2 mm) as working electrodes in 3M medium with 0.2% glucose as the carbon source inoculated with an enrichment culture grown in 3M. After inoculation, the E_{corr} decreased almost instantly to -650 mV, decreased further to -700 ± 10 mV by 10 d and then remained stable over 35 d. In the abiotic controls, E_{corr} decreased gradually to -500 ± 3 mV after 25 d and then remained constant (Figure 5.2 b). It is interesting to note that the potential trend observed in this experiment was similar to that recorded in anaerobic corrosion tests involving SRB (Sheng et al. 2007, Yuan et al. 2013). However, the likelihood of corrosion for SS304 cannot be predicted only by the decrease in corrosion potential (E_{corr}) (Little et al. 2008). The current density (i), increased to $6 \mu\text{A}/\text{cm}^2$ in 10 d and then remained constant over 35 d ($n=3$). In contrast, the current density of the sterile controls remained very low ($\sim 0.2 \mu\text{A}/\text{cm}^2$) throughout the experiment (Figure 5.2 a). The low corrosion current observed for the samples exposed to the mixed species community was

comparable with previous studies on aerobic corrosion and was consistent with the lack of anaerobic microorganisms, particularly SRB in the starter community (Lanneluc et al. 2015). The large variation across technical replicates could be attributed to the microstructural variability of the metal surface which affects localisation of bacteria affecting corrosion behaviour. It has been previously shown that a variable substratum structure due to a lack of uniform pre-treatment can affect amount of localised bacteria on the surface (Geesey et al. 1996, Sreekumari et al. 2001).

Figure 5.2 a) Current density (i) and b) corrosion potential (E_{corr}) of SS304 over 35 d for the microbial community grown in 3M. SS304 coupons were used as working electrodes. For each reactor $n=3$.

5.3.1.2 Surface visualization

After 35 d, the coupons were retrieved from the reactors and CLSM and SEM were used to visualise the biofilm and coupon surface. An advantage of using CLSM is that a 3D image of hydrated biofilm can be obtained, which gives insight into structure and localisation of the biofilm. Since it does not require sample pre-treatment, unlike SEM, the living biofilm can be observed, without altering its structure (Beech et al. 1999). Using this approach, an uneven grain structure was observed on the material surface (Figure 5.3 b). Biofilms were approximately 15 μm thick and were localised in the grain boundaries (Figure

5.3 a). Very few dead cells were observed in the biofilm, possibly due to the biofilms being thinner and as a consequence of the replenishment of media every 5 d throughout the experiment. The presence of biofilms in the grain boundaries was further analysed using intensity profile analysis (see below) to determine if the biofilms formed preferentially around the grain boundaries. Similar localisation of bacteria in grain boundaries was observed by Sreekumari et al. (2001) while investigating biofilm formation of *P. aeruginosa* on SS304 L coupons. They observed increased attachment at the grain boundaries rather than on the grains themselves during the initial phases of colonisation (Sreekumari et al. 2001).

Figure 5.3 CLSM images of the surface of the SS304 coupon after 35 d. Images a) and b) represent the same area on the coupon surface. a) The metal surface with a biofilm (seen in green) and b) only the reflection of the surface ($400\times$ magnification).

5.3.1.3 Intensity profile analysis

To investigate the localisation of biofilm on the metal surface, intensity profile analysis was performed. Intensity profiles of biomass (green) and coupon surface (red) were plotted. For convenience and to serve as a reference point, the left edge of the image was set as the 0 point and a scale of 35 μm , as

indicated by the red arrow drawn on the CLSM, was included for 5 slices of the representative image (Figure 5.4). Using this technique, when the surface is 'in focus' or is present in the confocal plane imaged, the red fluorescence intensity will be higher. If in that position, the metal surface is below the plane of illumination, there will be less fluorescence signal. It was observed that the biofilm was localised to the inter grain boundaries. For example, for slice 3 of the CLSM image, moving along the red arrow from left to right, it was observed that at the position between 10 μm to 25 μm showed a higher green intensity (cells) than the red (surface reflectance). In contrast, from 25 μm to the far right edge of the image at 35 μm along the horizontal axis, a higher red intensity was observed, suggesting the presence of a grain structure rather than the inter grain boundary. In the same position, there was less green fluorescence, indicating that there was less biomass present. Thus, where there is a high biomass signal, green, the red fluorescence tended to be low (grain boundary) and when the red fluorescence was high, there tended to be less biomass (grain surface). Recently, Li et al. (2016) used CLSM to visualise live and dead bacterial cells as well as to measure the depth of pits on HDSS2707 samples and observed that in the presence of an aerobic marine strain of *P. aeruginosa*, very small pits were formed after 7 and 14 d (Li et al. 2016). It has previously been observed that microorganisms adhere preferentially to pre-existing pits or to locations that show some degree corrosion or damage (Little et al. 1999, Li et al. 2013). It has also been shown that bacteria preferentially colonized the grain boundaries on stainless steel (Sreekumari et al. 2001), indicating that intergranular MIC might contribute to the overall corrosion. However, intergranular corrosion (IGC) can also have chemical causes, hence further investigation is required to distinguish between pitting as a consequence of MIC relative to abiotic processes (Chung et al. 1999).

Figure 5.4 Intensity profile for reflection of the coupon and the biofilm stained with live-dead staining. Images 1-5 on the left, show consecutive slices in the z-stack. The red arrow indicates the position on the coupon that was imaged for the cell intensity and the reflectance from the coupon surface, along the width of the coupon, 35 μm . The intensity profile shown in the graphs on the right is across the red arrow drawn across the image. Ch1-T1 (green) shows the intensity profile for Syto-9 stained biofilms and Ch1-T3 (red) shows the profile for the reflection of the metal surface.

FESEM images of the coupons on 0 d, revealed a relatively small grain structure with shallow boundaries as compared to coupons imaged on 35 d after the biofilm was removed (Figure 5.5 a, b). A widening of the grain boundaries was not observed for coupons exposed to sterile medium for 35 d (Figure 5.5 c), suggesting that microbial activity was responsible for the increased pit size or depth. Xu et al. (2017) used CLSM 3D images to measure pit dimensions on the 2205 DSS surfaces in presence and absence of *P. aeruginosa* biofilms. They observed an increase in depth and surface diameter of pits in the presence of biofilms after 14 d (Xu et al. 2017). Geesey et al. (1996) performed AFM analysis on SS316L exposed to bacteria and showed that the grain boundaries harbored bacteria. They also observed the depletion of chromium (Cr) and iron (Fe) in the boundaries, due to bacterial colonization and they hypothesised that the bacteria promoted the localized attack on the alloy (Geesey et al. 1996).

Figure 5.5 FESEM images for unpolished SS304 coupons after a 35 d laboratory experiment a) control coupon on 0 d, b) coupon on 35 d after removing the biofilm and c) control coupon after 35 d.

Energy dispersive x-ray spectroscopy (EDX) analysis was used to determine the elemental composition at the surface (Figure 5.6). An abundance of carbon was observed at the grain boundaries as determined by the EDX analysis (Figure 5.6 b). Such localised carbon was not observed in the sterile control and this suggested that the carbon detected was associated with the biofilm that can be visualized at the grain boundaries (Figure 5.4). When the weight percentage of different elements on the surface was compared over time compared to the

sterile controls, it was observed that abiotic control coupons showed a lower carbon content and higher Fe and Cr compared to those with biofilms. The higher percentage of Na and Cl on 35 d control coupons could be due to the deposition of salts on the surface.

Figure 5.6 EDX analysis of SS304 with biofilm on 35 d. The red images represent the elemental carbon present on the surface. Blue shows the presence of iron on the surface.

5.3.2 Short term Experiments

In the 35 d experiments, it was observed that both the current (i) and E_{corr} stabilized within the first 10 d (Figure 5.2 a) and therefore, further experiments were performed over 7 d to focus on the onset of corrosion. By limiting the experimental time frame to 7 d, it also becomes possible to test a range of different parameters such as nutrient composition, shaking and different polishing treatments.

5.3.2.1 Effect of nutrient composition on initial corrosion

To determine the effect of nutrient concentration on the initial corrosion behaviour, the corrosion of coupons exposed to a mixed species consortia inoculated into sterile filtered seawater (~0.002% glucose (Lyman et al. 1940)) or 3M medium (0.2% glucose) was compared. The I_{corr} and E_{corr} calculated using Tafel plots suggest there was a higher corrosion behaviour when the communities were grown in 3M medium than in seawater. In 3M medium, the current density reached $10.2 \pm 2 \mu\text{A}/\text{cm}^2$ on 7 d, whereas it was $< 0.1 \mu\text{A}/\text{cm}^2$

for the seawater (Figure 5.7 a). The enhanced corrosion under high nutrient concentration can be caused by the lack of a protective biofilm on the metal surface, or by the low redox potential in the bulk phase, or a combination of the above. The effect of organic and inorganic media was investigated by Rajasekar and Ting (2011) on SS304 in the presence of two aerobic bacteria *B. megaterium* and *Pseudomonas sp.* They found that growth in an inorganic medium with phosphate salts inhibited corrosion by forming an inhibiting layer on the metal surface, whereas, an organic medium promoted bacterial growth and accelerated corrosion (Rajasekar et al. 2011). The higher corrosion activity for the 3M grown communities could be due to increased microbial growth (planktonic as well as biofilm) (Figure 5.7).

Figure 5.7 The effect of nutrient concentration on a) corrosion current density (i) and b) corrosion potential (E_{corr}) of S304 coupons. Coupons were incubated in either 3M medium (0.2% glucose) or sterile natural seawater and were either inoculated with a mixed species consortium or were not inoculated (sterile controls). All coupons were polished with p600 grit sandpaper and the shaking speed was 40 rpm. For all reactors, $n=3$.

5.3.2.2 The effect of surface treatment (polishing) on initial corrosion

To assess the effect of surface treatment, coupons were manually polished with p600 grit and p1000 grit sandpaper. Atomic force microscopy analysis showed that the surface roughness (R_a) of unpolished, p600 polished and p1000

polished SS304 coupons were of 185 ± 20 , 173 ± 50 and 93.2 ± 5 nm, respectively (Figure 5.8). From the Ra values, it can be seen that although Ra values for unpolished and p600 polished coupons were similar, visually, the p600 polished coupons had a more uniform surface. Surface roughness has been studied before for its effect on bacterial attachment and a minimum Ra value (the arithmetical mean value of the deviation of the profile) of $0.8 \mu\text{m}$ or less was recommended in food industries to reduce bacterial attachment (Hilbert et al. 2003).

Figure 5.8 AFM micrographs of SS304 coupons with different polishing treatments a) Unpolished coupons, b) p600 grit, c) p1000 grit and d) Ra values for surface roughness. Ra values represent the average of 4 replicates.

Following polishing, the coupons were soaked in sterile 3M for 4 d to obtain a stable passivation layer and then inoculated with the mixed species marine community. The surface preparation neither affected the initial corrosion potential nor the corrosion density (Figure 5.9). Corrosion potentials for unpolished, p600 and p1000 polished coupons decreased rapidly after inoculation to around -450 mV and then decreased gradually to approximately -600 mV until 7 d. In contrast, for the unpolished control E_{corr} decreased gradually and reached -340 mV by 7 d (Figure 5.9 b). The corrosion current density for all of the inoculated samples increased initially for 2 d. For unpolished coupons, the current density increased to $5 \pm 0.6 \mu\text{A}/\text{cm}^2$ in first 2 d and remained almost stable until 7 d. For p600 and p1000 polished coupons, the current density increased to approximately $8 \mu\text{A}/\text{cm}^2$ after 2 d, which then showed a slight increase for p1000 and a slight decrease for p600 by the end of the experiment (Figure 5.9 a). The current density for p600 was not significantly different from the current density observed in the previous experiment (Figure 5.7 a), which suggested that pre-soaking did not affect the corrosion behaviour significantly for 7 d. Hilbert et al. (2003) investigated the effect of different surface polishing methods resulting in Ra values in the range of 0.0 to 0.001 μm , on microbial attachment and corrosion resistance of steel. They found that, between those values, the surface finish did not affect the bacterial attachment. However, it was observed that as the Ra value decreased, the resistance to pitting corrosion increased (Hilbert et al. 2003).

Figure 5.9 Effect of surface polishing on a) current density (i) and b) corrosion potential (E_{corr}), for SS304 coupons (Unpolished, p600, p1000, Pre-soaked for 4 days). For all reactors, $n=3$.

5.3.2.3 Effect of shaking speed on initial corrosion

Mass transport limitations affect biofilm development, the biofilm life cycle (Kraigsley et al. 2008) and community composition (Lawrence et al. 2004). As oxygen is rapidly depleted in both biofilm and planktonic phases due to bacterial respiration, it is likely that the passive film on the stainless steel weakens, thus making the surface more vulnerable to corrosion (Chen et al. 2016). Shaking increases aeration and nutrient delivery to the biofilm (McDaniel et al. 1969) and thus facilitates the removal of the reaction products from the metal surface, enhancing the corrosion current. Without shaking, the current density was 47% and 52% lower than with 40 rpm and 80 rpm shaking, respectively, after 7 d (Figure 5.10 a). Similarly, E_{corr} without shaking was higher by 200 mV than with shaking, indicating that diffusional limitations determine the onset of MIC (Figure 5.10 b). However, E_{corr} and I_{corr} did not change when the shaking speed was increased from 40 to 80 rpm. Thus, while shaking was important in modulating the E_{corr} and I_{corr} , the specific shaking speed was not important under the conditions used here.

Figure 5.10 Effect of shaking speed on a) current density (i) and b) corrosion potential (E_{corr}), for SS304 coupons. (0, 40, 80 rpm). For every reactor, $n = 3$.

It has been shown that uniform biofilms obstruct oxygen diffusion and inhibit corrosion (Little et al. 2002), whereas, patchy or non-uniform biofilms can facilitate diffusional gradients within the biofilm increasing transport of oxygen to the metal surface increasing corrosion (Videla 2001). Therefore, in order to better understand the link between shaking speed and the local oxygen concentration at the biofilm-substratum interface, oxygen micro sensors could be used to understand the effect of diffusional limitations from biofilm structure in enhancing or reducing the corrosion current.

5.3.3 *Comparison between LPR and LSV techniques*

Linear Sweep Voltammetry (LSV) was used to obtain the current density and corrosion potential using the Tafel plot. However, it was realised that the potential range applied in the LSV analysis can damage the sample and enhance the corrosion process. During LSV, the coupons were exposed to the potential range of 300 mV to -600 mV for analysis before inoculation and a range of 100 mV to -900 mV after inoculation as well as on days 2, 4 and 7 at a scan rate of 0.166 mV/s. For LPR analysis, the potential range was set at ± 50 mV around the E_{ocp} at a scan rate of 0.100 mV/s. The results clearly indicate that LSV perturbed the working electrodes, resulting in a higher corrosion current and a slightly lower corrosion potential. The current density for LPR analysis reached up to $0.3 \mu\text{A}/\text{cm}^2$, whereas for LSV based analysis it reached $8 \mu\text{A}/\text{cm}^2$ by 7 d. The corrosion potentials measured during LSV were slightly lower than LPR at all time points (Figure 5.11). Therefore, LPR was used for further experiments instead of using LSV.

Figure 5.11 Comparison between corrosion behaviour of unpolished SS304 coupons, incubated in 3M media and inoculated with mixed microbial community for LSV and LPR techniques. For each technique two reactors each were used (TR1 and TR2) (n=3 for each reactor).

5.3.4 Effect of different glucose concentrations

As shown above, it was concluded that the glucose concentration of 3M determines the growth of the biofilm and its structure. Thus, MIC is influenced by the carbon source (Section 2.2.1). Following the initial comparison with the seawater, three glucose concentrations, 0.2%, 0.02% and 0.002%, were tested to determine the impact of carbon source concentration, and hence biomass accumulation, in short-term experiments. It has been shown that for *E. coli* biofilms on steel surface, as the concentration of glucose increased from 0 to 0.4 g/L, corrosion of the steel increased. However, the corrosion rate slowed down when the glucose concentration was further increased to 4 g/L, suggesting a saturation limit for further increase in corrosion behaviour (Javed et al. 2014).

To monitor bacterial growth in the reactors, OD_{600} (to determine relative cell densities) and pH were measured daily and it was observed that the OD_{600} was directly correlated with glucose concentration (Figure 5.12). For example, the reactors with 0.2% glucose showed the highest OD_{600} in the range of 0.4 to 0.6 on 2, 4, and 7 d (Figure 5.13 b). The pH decrease for the 0.2% samples can be correlated with the higher OD_{600} . This was likely to be due to the growth of heterotrophs converting glucose into organic acids as the oxygen concentration

decreased and this would result in a reduction in pH (Figure 5.13 a). In contrast, the OD_{600} remained very low, at around 0.1 and 0.04 for 0.02% and 0.002% glucose, respectively. As expected, the pH values for 0.02% and 0.002% glucose did not show a significant decrease. For the 0.02% glucose reactor, the pH decreased from 8.2 to 7.8 after inoculation and remained constant and for 0.002% glucose, the pH remained constant at 8.2 throughout the experiment. The lack of significant pH change for the two reactors with the lower glucose concentrations was most probably due to the low amount of biomass present. The low biomass would be less likely to deplete oxygen in the medium and hence repress fermentative metabolism. If the bacteria did initiate fermentation due to oxygen depletion, it is possible that the amounts of organic acids produced were not sufficient to influence the pH.

Figure 5.12 Three reactors after 2 d of growth in the presence of 0.2, 0.02 and 0.002% glucose.

Figure 5.13 The a) pH and b) OD₆₀₀ for the corrosion reactors incubated with different concentrations of glucose over 7 d.

The corrosion potential (E_{corr}) for all of the reactors decreased within the first 48 h. The E_{corr} for 0.2% glucose grown cultures further decreased to approximately -400 mV after 7 d, which was the lowest for the three glucose concentrations. Increase in the potential was observed in the presence of 0.002% glucose from 4 d to 7 d, from ~ -200 mV to ~ -50 mV (Figure 5.14 b). The corrosion current density for the 0.2% glucose cultures increased to $0.12 \mu\text{A}/\text{cm}^2$ on day 4 and then decreased by almost 50% by day 7. The current density for the 0.002% glucose cultures was the lowest on day 7 at $0.01 \mu\text{A}/\text{cm}^2$ (Figure 5.14 a).

Figure 5.14 a) Corrosion current density (i) and b) corrosion potential (E_{corr}) measured for mixed species cultures cultured under three different glucose concentrations (0.2, 0.02 and 0.002%). For each reactor, $n=3$.

The polarisation resistance (R_p) for all three reactors decreased rapidly upon inoculation within the first 2 h and remained stable for 4 d. R_p increased almost 6-fold for the 0.002% glucose reactor from 4 to 7 d (Figure 5.15). The ratio of the applied potential and current response is given as polarization resistance (Stern et al. 1957). This "resistance" has an inverse relationship with the corrosion rate. A higher polarization resistance indicates a lower corrosion rate. A decrease in corrosion current was observed for the 0.002% glucose cultures from 4 to 7 d (Figure 5.14 a), which corresponded with the increasing polarisation resistance.

Figure 5.15 Polarization resistance (R_p) over 7 d, measured for mixed species cultures cultured under three different glucose concentrations (0.2, 0.02 and 0.002%). For each reactor, $n=3$.

5.3.4.1 Biofilm and surface visualisation

On day 7, one coupon from each reactor was used to visualise the biofilm. In the presence of 0.002% glucose (Figure 5.16 c), individual cells were observed to be attached to the metal surface. For the 0.02% culture, clusters of microorganisms were visible on the surface (Figure 5.16 b). Biofilms formed in the presence of 0.002% glucose covered a greater percentage of the coupon surface compared to biofilms formed with 0.02 and 0.2% glucose (Figure 5.17 a). However, the total biovolume on 7 d for the 0.002% glucose cultures was 25% and 30% less than for coupons from the reactors grown with 0.2 and 0.02% glucose, respectively. The number of dead cells was the lowest at 17% of the total biovolume for the 0.002% glucose containing reactor, whereas it was around 30% for the other two glucose concentrations (Figure 5.17 b).

Figure 5.16 Orthogonal view from different planes (x/y, x/z or y/z) of CLSM images showing biofilm formation on SS304 steel in presence of a) 0.2, b) 0.02 and c) 0.002% glucose reactors. Biofilms were stained with the BacLight live-dead reagents, where live cells appear green and dead cells appear as red. Separate wavelength (514 nm) than that used for syto9 (live) (488 nm) and propidium iodide (dead) (561 nm), was used for reflection of the metal surface. The small panels on top and on right of every image show the x/z and y/z planes, respectively, which indicate the thickness of the biofilm.

Figure 5.17 a) Surface area covered by the biofilm and b) biovolume for reactors with different glucose concentrations. Each bar represents an average of ≥ 3 images at different locations for the same coupon.

5.3.5 *Comparison of corrosion for mixed species cultures grown with 0.002% glucose or sterile seawater*

The results for the mixed species cultures grown with 0.002% glucose were compared with seawater inoculated with the 3M enrichment and a control were compared for corrosion behavior. LPR was used for electrochemical analysis before inoculation, after inoculation, day 2, 4 and 7. The corrosion potential for the inoculated seawater showed a slight decrease after inoculation but then showed ennoblement from -194 ± 40 mV to 83 ± 25 mV. For the reactor with 0.002% glucose, E_{corr} decreased to -236 ± 8 mV until 4 d, but then increased to -50 ± 3 mV by 7 d. The sterile seawater control showed ennoblement throughout the experiment (Figure 5.18 b).

The corrosion current density for the inoculated seawater showed an initial increase in the first 2 d and then decreased to $0.002 \mu\text{A}/\text{cm}^2$ by 7 d, matching the current density for the sterile control reactor, which remained constant throughout the experiment. Similarly, for the 0.002% glucose reactor, the current density increased rapidly from 0.002 to $0.03 \mu\text{A}/\text{cm}^2$ after inoculation, remained stable until day 4 and decreased to $0.0016 \mu\text{A}/\text{cm}^2$ on day 7, which was similar to the inoculated seawater. The initial decrease in polarization resistance (data not shown) was consistent with the increased current density and since the polarization resistance increased from 2 to 7 d, the decrease in the current density could be observed (Figure 5.18 a).

Figure 5.18 Comparison of a) corrosion current density (i) and b) corrosion potential (E_{corr}), measured for 3M medium with 0.002% glucose, autoclaved seawater inoculated with the 3M enrichment and a sterile seawater control. Each point represents $n = 3$.

5.3.6 Comparison of VNSSFs with and without glucose

To further study the effect of glucose in the electrochemical batch reactor, VNSSFs medium with and without glucose (VNSSFSG) was used. LPR and EIS were used for electrochemical analysis before and after inoculation on days 2, 4 and 7. The VNSSFSG reactor showed the most growth. A decrease in OD_{600} in the VNSSFs reactor after days 2 and 4 and in the VNSSFs reactor after day 2 was consistent with the 50% media change on those days. The pH decreased for both VNSSFs reactors with (8.2 to 7.5) and without glucose (8.2 to 7.8) and this correlated with the increase in OD_{600} . The pH for the VNSSFSG culture, decreased to 7.7 after inoculation and remained lower than for the VNSSFs culture, and this was most likely due to the higher growth of heterotrophs converting glucose into organic acids (Figure 5.19). While it is likely that the pH alone did not contribute significantly to metal degradation and corrosion, the lower pH can be correlated with lower oxygen concentration, as well as with depletion of the passivation layer on the metal coupon.

Figure 5.19 a) pH and b) OD₆₀₀ measured for 7 d corrosion reactors where a mixed species culture was inoculated into VNSSFS and VNSSFSG reactors. On days 2 and 4, the OD and pH measurements were taken before the 50% media change.

The corrosion potential of the VNSSFS reactor decreased to ~ -50 mV upon inoculation, whereas for VNSSFSG it remained higher at ~ 150 mV. For both reactors, the E_{corr} decreased rapidly over the course of the experiment and reached around -400 mV after 7 d (Figure 5.20 b). The corrosion current density (i) for VNSSFSG reached a maximum of $0.14 \pm 0.3 \mu\text{A}/\text{cm}^2$ after 4 d and then decreased by $0.2 \mu\text{A}/\text{cm}^2$ over next 3 d (Figure 5.20 a). The higher current density in VNSSFSG could be due to uneven biofilm formation on the coupon surface (data not shown). Patchy or non-uniform biofilms that are undergoing aerobic respiration induce differential aeration cell formation, where anodic areas are formed below thicker colonies due to higher respiration activity and low oxygen concentration and cathodic areas are formed below thinner colonies, thus promoting corrosion (Zuo 2007). In contrast, for the VNSSFS reactor, which had uniform and thinner biofilms, the current density increased for the first 2 d, but then decreased for the remainder of the experiment.

Figure 5.20 a) Corrosion current density (i) and b) corrosion potential (E_{corr}), observed for VNSSFs and VNSSFSG reactors. Each point represents average of $n = 3$.

5.4 Summary

The onset of MIC for stainless steel coupons exposed to mixed microbial biofilms enriched from seawater was characterised to determine the relative impacts of surface polishing, shaking speed of the reactor and nutrient composition, using the fed batch bioelectrochemical reactor. As compared to environmental conditions, laboratory conditions can provide more reproducibility. The corrosion current density increased in glucose rich 3M as compared to sterile seawater. Indeed, the corrosion potential was directly correlated with the nutrient concentration. This is possibly due to oxygen consumption by the rapidly growing community and this may weaken the oxide layer on the stainless steel surface. In contrast to changing the nutrient concentration, increasing the shaking speed from 40 RPM to 80 RPM did not significantly change the corrosion rate. Similarly, there was no measurable impact of polishing on the corrosion behaviour under the conditions tested here. CLSM, SEM imaging and EDX analysis showed the accumulation of a biofilm at the grain boundaries. The pit dimensions were observed to have increased after 35 d in the presence of a biofilm, suggesting that the biofilm plays a role in the initiation of localised corrosion.

6 Chapter Six: Conclusions

The primary aim of this work was to understand the onset of MIC, in aerobic marine environments. A secondary goal was to develop and validate laboratory systems for multi method monitoring of MIC process. According to Melcher's phenomenological model (Melchers et al. 2005), corrosion in the marine environment starts with the aerobic phase, where aerobic microorganisms colonise the metal surface. In this phase, the corrosion rate is governed by reduction of oxygen in the environment. The formation of thick biofilms and corrosion products begin to appear at the end of this phase, with the gradual establishment of anaerobic conditions. Under these conditions, sulphate reducing bacteria (SRB) begin to grow. It is generally accepted, and our work confirmed this, that the aerobic phase of corrosion is characterised by a low corrosion current, mostly due to bacterial action. However, the role of mixed species biofilm formation under aerobic conditions, in the early phase of the corrosion process remains poorly understood (Melchers 2016).

In Chapter three, biofilm formation, changes in microbial community and the MIC process of stainless steels in the presence of a natural mixed marine community were studied. However, environmental experiments face a lack of reproducibility due to variable environmental parameters such as flow, pH, temperature and temporal variations in the microbial community in the feed water. A fed-batch bioelectrochemical system, as discussed in Chapter five, is easy to assemble and monitor, thus increasing the experimental throughput. In our experiments, we inoculated the same mixed microbial marine community to reduce the variability associated with using natural microbial communities that vary in the starting community. A continuous flow system, described in Chapter four, mimics the environmental conditions, to avoid issues associated with batch and fed-batch systems, such as nutrient and oxygen depletion. This flow through system also facilitates focusing on the role of the biofilm, as the planktonic cells are continually flushed out of the system.

6.1 Community changes from planktonic phase to biofilm on the metal surface

Most of the research related to MIC has focussed on axenic cultures in laboratory systems. However, such studies do not mimic the environmental conditions in which a microbial community facilitates acceleration or inhibition of the corrosion process (Kip et al. 2015). Advances in culture independent and meta-omics techniques have allowed for detailed investigation of the microbial community associated with MIC, allowing for a better understanding of the types and abundance of different organisms in the community, their specific functions and their activity (Beech et al. 2014, Eckert et al. 2016). Molecular microbiological methods have been widely used to understand biofilm communities related to oil field pipelines (Whitby et al. 2011). Culture independent methods were also used to identify and characterize the biofilm communities related to acid mine drainage system in the Richmond Mine in northern California (Baker et al. 2003). Using these approaches, it was possible to not only define the species that comprised the biofilms associated with the acidic waters, but to also assign biochemical functions to those organisms as well as the potential metabolic interactions between community members. This highlights the potential of a full systems biology approach to define an ecological system and its function.

In the environmental system reported here, there was a clear selection for specific community members from the natural seawater to establish the biofilms on the metal surface. This suggests that biofilm formation is driven by organisms that may specialise in the rapid formation of biofilms and that not all organisms in the community are equally capable of biofilm formation. Further, the composition of those biofilms was observed to be relatively stable over the 7 d of the experiment. This process of selection for biofilm-specific organisms was observed in environmental and flow cell experiments that were inoculated with the abovementioned mixed species enrichment. This also highlights that the laboratory bioreactor in some ways replicates the biofilm community succession process observed in the natural seawater experiments. This

observation has also been reported for waste-water communities (Luo et al. 2017). In those biofouling experiments, it was observed that the bacteria that colonise the membrane surface represented only a subset of the sludge community and that some colonising bacteria were rare or at relatively low abundances in the sludge. One might argue that some bacteria show enhanced growth or fitness when provided with a surface for biofilm formation.

6.2 Corrosion behavior during the initial stage of MIC

In the preliminary experiment (Chapter 3 section 3.2.1), following immersion in seawater, the OCP of super duplex stainless steel (SD) increased and reached ~ 270 mV in 2 d and remained stable for 7 d. This was consistent with experiments in tropical environments, where SD initially showed ennoblement at approximately 300 mV in 3-5 d of immersion and remained stable until 50 d, (Thierry et al. 2014). Xu et al. (2015) also observed potential ennoblement for SS316L immersed in natural seawater. In those experiments, the OCP remained constant for the first 2 d in natural and sterile seawater and then increased to 330 mV after 5 d and remained stable for rest of the experiment (15 d) (Xu et al. 2015). In the experiments reported here, it was observed that as the microorganisms begin to attach onto the material surface, potential ennoblement occurred, particularly in the first 48 h. Therefore, incorporating time points between 1 h and 2 d will be beneficial to further study this phenomenon. The correlation between formation of biofilm (and its structure) and potential ennoblement still remains unclear. Short term experiments with continuous electrochemical measurements and additional time points are recommended to understand this phenomenon in detail.

When both SS304 and SD coupons were exposed to natural seawater, SS304 showed higher potential ennoblement than SD coupons in 30 d. Al-Muhanna et al. (2010) observed the effect of temperature variation on corrosion behaviour of four alloys, including SS304, in seawater from gulf of Kuwait. They reported that the potential of SS304 increased after 40 d, possibly due to slow biofilm

growth during winter ($\sim 18^{\circ}\text{C}$) and/or reduced biological activity, and increased to -150 mV in the next 20 d. Then, the potential decreased for 60 d and reached -350 mV , as the temperature of water increased during summer ($\sim 33^{\circ}\text{C}$) (Al-Muhanna et al. 2010). However, such effects of temperature were not seen in our experiments, as the temperature remained constant at $\sim 29^{\circ}\text{C}$ throughout the experiment.

Interestingly, potential ennoblement was only observed for the experiments conducted with natural seawater in the environment (Chapter 3) or when sterile seawater was used in the laboratory batch reactor (Chapter 5). This suggests that the artificial seawater used here, where ennoblement did not occur, lacks some essential component, e.g., specific salts, organic compounds or microorganisms, which contribute to the ennoblement process in natural seawater. These observations are consistent with other laboratory based MIC studies. For example, Moradi et al. (2014) observed a negative shift in the potential of 2205 duplex steel in the presence of a marine *Pseudoalteromonas* sp. in a laboratory batch system (Moradi et al. 2014). When carbon steel was exposed to *Vibrio neocaledonicus*, a less negative shift compared to the control experiment was observed, suggesting inhibition of corrosion process. They concluded that MIC was inhibited as the bacteria slowed the anodic dissolution process through attachment (Moradi et al. 2015). It has also been shown that biofilm formation of a marine facultative anaerobe *Vibrio natriegens* on stainless steel lowered the E_{corr} , which could be due to establishment of biofilm and production of metabolites by bacteria (Cheng et al. 2009).

6.3 Parameters affecting corrosion behavior

The environmental experiments presented in this thesis suggest that the flow rate affected the formation of the biofilms, which in turn affected potential ennoblement. For SD coupons, at a flow rate of 300 L/d, the potential reached 270 mV in 2 d and remained stable until 7 d. In contrast, at a flow rate of 3000 L/d, the potential ennoblement was delayed and eventually reached $\sim 200\text{ mV}$

on 7 d. The amount of biofilm formed was higher for the lower flow rate (300 L/d) over the 7 d of the experiment. These results suggest that the flow rate affects the microbial attachment as well as the corrosion behavior of the immersed metal.

In addition, it was clear that the carbon source concentration also played an important role in mediating MIC and this effect was greater than the effect of polishing or stirring, neither of which had an impact on MIC under the conditions tested here. This suggests therefore that organic carbon is the dominant variable for biofilm growth and MIC. It was shown previously that increasing the nutrient concentration from 0.1 to 0.5 g/L glucose, increased the biofilm formation of *Pseudomonas putida* isolated from a paper machine, however, higher concentration of glucose (1 g/L) reduced the biofilm accumulation rate due to higher detachment (Rochex et al. 2007). The results obtained here from linear polarization (LP), electrochemical impedance spectrometry (EIS) and open circuit voltage (OCV) analysis showed that readily available organic nutrients (glucose) enhance the bacterial growth and thus the MIC rates, likely resulting in pH and oxygen concentration gradients across the biofilm.

Two different polarization techniques were used to monitor corrosion behavior. For LP measurements, the coupon was exposed to a narrow range of potentials across the E_{OCP} (± 50 mV). In contrast, for LSV the metal was exposed to a fixed, but broader, range of potentials. It was observed that the current density measured for LSV was higher compared to LP for experiments performed under the same conditions. These differences may be due to exposure of the metal to high potential that initiate pitting corrosion or low potential where hydrogen is produced. Since LP was performed across a very narrow range of potentials it most likely does not damage the samples, thus returning a more repeatable description of the onset MIC process.

6.4 Biofilm structure

Visualization of the biofilms formed on SD coupons over a period of 7 d in environmental conditions by CLSM (Figure 3.2) and it was observed that few cells were attached on the surface after 1 h, aggregates of bacteria after 2 d and aggregates of bacteria were observed on 7 d. This matches the classical biofilm formation model (Stoodley et al. 2002). In the laboratory system, CLSM images of SS304 coupon on 35 d, from the long-term laboratory experiment, show localization of biofilm in the inter grain boundaries, which was confirmed by intensity profile analysis. EDX analysis also showed higher concentration of carbon in inter grain boundaries, suggesting the presence of biofilms. Widening of inter grain boundaries, as observed from FESEM images on 35 d, suggested that localization of biofilms in these boundaries can lead to formation of pits.

Patchy biofilms were observed for coupons from flow cell system in presence of glucose, for which higher corrosion current was also recorded. It has been shown that, when the surface of the metal is incompletely covered, the biofilm will facilitate oxygen and chemical concentration cells, thus increasing the likelihood of localised corrosion (Dexter 1993). Increased cathodic reduction efficiency, i.e., reduction of dissolved oxygen, promotes corrosion reactions and increases the risk of localized corrosion. Yuan et al. (2008) reported that *Pseudomonas* forms a patchy biofilm embedded in EPS on metal surfaces, which impedes oxygen diffusion and creates O₂ concentration cells. If the biofilm covers the whole surface, it leads to decreased cathodic reaction rates, which in turn inhibits the corrosion rate (Videla et al. 2009). The observed higher corrosion current in flow cells with VNSSFSG may also have been related to the production of acid as a consequence of fermentative metabolism once oxygen became limiting within a thick biofilm. Acidic metabolites can increase cathodic processes, by rupturing passive films and hence increase the supply of protons to the surface (Edyvean et al. 1991). Better monitoring of gradients of pH, nutrients and oxygen within the biofilm using microelectrodes,

will be beneficial to study the effect of microenvironment on the overall corrosion rate (Billings et al. 2015).

6.5 Summary

Microbially influenced corrosion (MIC) is a complex phenomenon and despite years of research, it is not well understood. Axenic culture studies are valuable in that they can provide a detailed understanding of species-specific corrosion mechanisms, but do not account for inter- and intraspecific reactions that increase the complexity of corrosion mechanisms in the environment. However, studies of complex microbial communities are difficult, in part because of issues of reproducibility of the community as well as the possibility of multiple corrosion mechanisms occurring when multiple species are present. Therefore, it is important to develop approaches that enable the use of mixed species communities to address corrosion in a systematic fashion. The work presented here, demonstrates that it is possible to use defined mixed species inocula to study the initial stages of corrosion. The results highlighted that carbon source concentration was a key variable in controlling the corrosion rate and that the community that forms a biofilm on a metal surface is rapidly selected from the planktonic phase and is stable for several weeks. It was also observed that ennoblement is a feature of metals immersed in natural seawater but not in artificial seawater media. The approaches developed here will enable future studies that focus on the factors that lead to ennoblement under natural conditions. Further, these approaches can be used to better define the genetic and metabolic processes that lead to MIC as well as defining how microbial communities interact with metals in seawater conditions.

6.6 Recommendations for future work

The work presented in this thesis has highlighted some methodological problems with MIC characterization, due to the poor repeatability of biofilm growth and low corrosion rates observed on stainless steel. Further investigation of the initial corrosion process is needed in order to determine the

relevance of MIC vs. abiotic corrosion. It is likely that local characterisation methods, such as local EIS, high resolution microscopy and micro sensors experiments will allow for enhanced sensitivity in measuring the initial pitting process as well as how biofilms accumulate on high energy features of the metal surface (e.g., grain boundaries).

In this work, the impact of MIC on stainless steel caused by an aerobic mixed microbial community grown in defined marine media was characterised. The results showed that the glucose concentration was a key factor for MIC under the conditions used here, suggesting a non-specific corrosion mechanism related to local anaerobic conditions in the biofilms. For example, it is likely that as the community consumed oxygen through aerobic respiration of glucose, they community shifted towards fermentative metabolism, which would release organic acids that could attack the metal surface. While this MIC mechanism is relevant to nutrient-rich seawater, other MIC process are likely to contribute to corrosion in reduced nutrient environments (e.g., sediments, deep seawater, oligotrophic marine environments). Therefore, it will be essential to also study MIC under such conditions with the same community to characterise the full potential of different MIC mechanisms. This might lead to a unified MIC theory, assuming that the variety of chemical reactions that relate to MIC can be reconciled under a small set of mechanisms.

MIC processes are radically different in the natural environment, where the high microbial diversity and the micronutrients dissolved in seawater result in faster corrosion rates. Therefore, additional work must be done in reproducible experimental systems, with a large number of samples and extensive microbiological and metallurgic observations.

The laboratory experiments presented in this thesis were performed for seven days to monitor biofilm formation and initial corrosion behaviour. Further experiments should be planned carefully also to monitor long-term behaviour, taking precautions to avoid blockage of the flowcell system, and to include a

sufficient number of replicates to facilitate imaging of coupons and biofilms at regular time intervals.

Finally, there is a lack of studies that focus on the effect of community composition on MIC. Meta-omics and other microbial ecology methods should be implemented to determine the evolution of the community in relation to MIC rates, e.g., determining which species take advantage of MIC to increase their survival and abundance in the mixed community as well as to determine individual functions of community members as they relate to MIC.

References

- Al-Muhanna, K. and K. Habib (2010). "Corrosion behavior of different alloys exposed to continuous flowing seawater by electrochemical impedance spectroscopy (EIS)." Desalination **250**(1): 404-407.
- Al Abbas, F. M., R. Bhola, J. R. Spear, D. L. Olson and B. Mishra (2013). "Electrochemical Characterization of Microbiologically Influenced Corrosion on Linepipe Steel Exposed to Facultative Anaerobic *Desulfovibrio* sp." Int. J. Electrochem. Sci **8**: 859-871.
- Alasvand Zarasvand, K. and V. R. Rai (2014). "Microorganisms: Induction and inhibition of corrosion in metals." International Biodeterioration & Biodegradation **87**: 66-74.
- Bailey, A. (2006). BP: learning from oil spill lessons. Petroleum News. Anchorage, AK. **Vol. II No. 20**.
- Baker, B. J. and J. F. Banfield (2003). "Microbial communities in acid mine drainage." FEMS Microbiology Ecology **44**(2): 139-152.
- Baker, J. (1984). "Factors affecting the bacterial colonization of various surfaces in a river." Canadian journal of microbiology **30**(4): 511-515.
- Bakterij, A. (2014). "An overview of the influence of stainless-steel surface properties on bacterial adhesion." Materiali in tehnologije **48**(5): 609-617.
- Barraud, N., D. J. Hassett, S.-H. Hwang, S. A. Rice, S. Kjelleberg and J. S. Webb (2006). "Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*." Journal of bacteriology **188**(21): 7344-7353.
- Batmanghelich, F., L. Li and Y. Seo (2017). "Influence of multispecies biofilms of *Pseudomonas aeruginosa* and *Desulfovibrio vulgaris* on the corrosion of cast iron." Corrosion Science **121**: 94-104.
- Beale, D., P. Morrison, C. Key and E. Palombo (2014). "Metabolic profiling of biofilm bacteria known to cause microbial influenced corrosion." Water Science and Technology **69**(1): 1-8.
- Beech, I., V. Zinkevich, L. Hanjansit, R. Gubner and R. Avci (2000). "The effect of *Pseudomonas* NCIMB 2021 biofilm on AISI 316 stainless steel." Biofouling **15**(1-3): 3-12.

- Beech I. B. , S. M., Gaylarde C. C. , Smith W. L. , Sunner J. (2014). Biofilms and biocorrosion. Understanding Biocorrosion: Fundamentals and Applications: 33-50.
- Beech, I. B. and C. C. Gaylarde (1999). "Recent advances in the study of biocorrosion: an overview." Revista de microbiologia **30**(3): 117-190.
- Beech, I. B. and J. Sunner (2004). "Biocorrosion: towards understanding interactions between biofilms and metals." Current opinion in Biotechnology **15**(3): 181-186.
- Beech, I. B., J. A. Sunner and K. Hiraoka (2005). "Microbe-surface interactions in biofouling and biocorrosion processes." Int Microbiol **8**(3): 157-168.
- Beech, I. B., M. Szttyler, C. Gaylarde, W. Smith and J. Sunner (2014). "Biofilms and biocorrosion." Understanding Biocorrosion. Oxford, UK: Woodhead Publishing: 33-56.
- Beloin, C., A. Roux and J.-M. Ghigo (2008). "Escherichia coli biofilms." Current Topics in Microbiology and Immunology **322**: 249-289.
- Benbouzid R, N. D, M. Conte, J. Guezennec and D. Prieur (1991). "Monitoring of a Vibrio natriegens and Desulfovibrio vulgaris marine aerobic biofilm on a stainless steel surface in a laboratory tubular flow system." Journal of applied bacteriology **71**(3): 244-251.
- Bermont-Bouis, D., M. Janvier, P. Grimont, I. Dupont and T. Vallaey (2007). "Both sulfate ~~-reducing~~ and Enterobacteriaceae take part in marine biocorrosion of carbon steel." Journal of applied microbiology **102**(1): 161-168.
- Billings, N., A. Birjiniuk, T. S. Samad, P. S. Doyle and K. Ribbeck (2015). "Material properties of biofilms—a review of methods for understanding permeability and mechanics." Reports on Progress in Physics **78**(3): 036601.
- Bishop, P. L. and T. Yu (1999). "A microelectrode study of redox potential change in biofilms." Water science and technology **39**(7): 179-185.
- Bogan, B. W., B. M. Lamb, G. Husmillo, K. Lowe, J. R. Paterek and J. J. Kilbane II (2004). "Development of an environmentally benign microbial inhibitor to control internal pipeline corrosion." Gas Technology Institute, Final Report.

- Boillot, P. and J. Peultier (2014). "Use of Stainless Steels in the Industry: Recent and Future Developments." Procedia Engineering **83**: 309-321.
- Boone, D. R., M. Goodfellow, F. A. Rainey, K.-H. Schleifer and P. Vos (2005). Bergey's Manual® of Systematic Bacteriology: Volume Two The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria, Bergey's Manual Trust.
- Borenstein, S. (1994). Microbiologically influenced corrosion handbook, Elsevier.
- Breur, H., J. de Wit, J. Van Turnhout and G. Ferrari (2002). "Electrochemical impedance study on the formation of biological iron phosphate layers." Electrochimica acta **47**(13): 2289-2295.
- Bruemmer, S. (1989). Grain boundary chemistry and intergranular failure of austenitic stainless steels. Materials Science Forum, Trans Tech Publ.
- Buchfink, B., C. Xie and D. H. Huson (2015). "Fast and sensitive protein alignment using DIAMOND." Nat Methods **12**(1): 59-60.
- Cetin, D. and M. L. Aksu (2009). "Corrosion behavior of low-alloy steel in the presence of *Desulfotomaculum* sp." Corrosion Science **51**(8): 1584-1588.
- Chandrasatheesh, C., J. Jayapriya, R. George and U. K. Mudali (2014). "Detection and analysis of microbiologically influenced corrosion of 316 L stainless steel with electrochemical noise technique." Engineering Failure Analysis **42**: 133-142.
- Chang, Y.-J., C.-H. Hung, J.-W. Lee, Y.-T. Chang, F.-Y. Lin and C.-J. Chuang (2015). "A study of microbial population dynamics associated with corrosion rates influenced by corrosion control materials." International Biodeterioration & Biodegradation **102**: 330-338.
- Characklis, W. (1981). "Bioengineering report: Fouling biofilm development: A process analysis." Biotechnology and Bioengineering **23**(9): 1923-1960.
- Chen, C., S. S. Khaleel, H. Huang and C. H. Wu (2014). "Software for pre-processing Illumina next-generation sequencing short read sequences." Source Code Biol Med **9**: 8.

- Chen, D., E.-H. Han and X. Wu (2016). "Effects of crevice geometry on corrosion behavior of 304 stainless steel during crevice corrosion in high temperature pure water." Corrosion Science **111**: 518-530.
- Cheng, S., J. Tian, S. Chen, Y. Lei, X. Chang, T. Liu and Y. Yin (2009). "Microbially influenced corrosion of stainless steel by marine bacterium *Vibrio natriegens*:(I) Corrosion behavior." Materials Science and Engineering: C **29**(3): 751-755.
- Chongdar, S., G. Gunasekaran and P. Kumar (2005). "Corrosion inhibition of mild steel by aerobic biofilm." Electrochimica Acta **50**(24): 4655-4665.
- Chung, Y. and L. Thomas (1999). Comparison of MCI pit morphology with non-MIC chloride induced pits in types 304/304L/E308 stainless steel base metal welds, NACE International, Houston, TX (United States).
- Compere, C. and N. Le Bozec (1997). Behaviour of stainless steel in natural seawater. The First Stainless Steel Congress in Thailand, Bangkok, Thailand, December 15-17, 1997.
- Cook, P. E. and C. C. Gaylarde (1988). "Biofilm formation in aqueous metal working fluids." International Biodeterioration **24**(4-5): 265-270.
- Corpe, W. A. (1970). "Attachment of marine bacteria to solid surfaces." Adhesion in biological systems: 73-87.
- Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber and H. M. Lappin-Scott (1995). "Microbial biofilms." Annual Reviews in Microbiology **49**(1): 711-745.
- Crusz, S. A., R. Popat, M. T. Rybtke, M. Cámara, M. Givskov, T. Tolker-Nielsen, S. P. Diggle and P. Williams (2012). "Bursting the bubble on bacterial biofilms: a flow cell methodology." Biofouling **28**(8): 835-842.
- Cullimore, D. and L. Johnston (2000). "Science and RMS Titanic: a biological odyssey." Voyage **32**: 172-176.
- Dall'Agnol, L. and J. Moura (2014). "Sulphate-reducing bacteria (SRB) and biocorrosion." Understanding Biocorrosion: Fundamentals and Applications: 77.
- Dang, H., R. Chen, L. Wang, S. Shao, L. Dai, Y. Ye, L. Guo, G. Huang and M. G. Klotz (2011). "Molecular characterization of putative biocorroding

- microbiota with a novel niche detection of Epsilon ~~Zeta~~ proteobacteria in Pacific Ocean coastal seawaters." Environmental microbiology **13**(11): 3059-3074.
- Daniels, N. A., L. MacKinnon, R. Bishop, S. Altekruze, B. Ray, R. M. Hammond, S. Thompson, S. Wilson, N. H. Bean, P. M. Griffin and L. Slutsker (2000). "Vibrio parahaemolyticus Infections in the United States, 1973–1998." The Journal of Infectious Diseases **181**(5): 1661-1666.
- Daniels, S. (1980). "Mechanisms involved in sorption of microorganisms to solid surfaces." Adsorption of microorganisms to surfaces: 7-58.
- Davis, J. R. (2000). Corrosion: Understanding the basics, ASM International.
- Dexter, S. C. (1993). "Role of microfouling organisms in marine corrosion." Biofouling **7**(2): 97-127.
- Dhar, H., D. Howell and J. M. Bockris (1982). "The use of in situ electrochemical reduction of oxygen in the diminution of adsorbed bacteria on metals in seawater." Journal of The Electrochemical Society **129**(10): 2178-2182.
- Dinh, H. T., J. Kuever, M. Mußmann, A. W. Hassel, M. Stratmann and F. Widdel (2004). "Iron corrosion by novel anaerobic microorganisms." Nature **427**(6977): 829-832.
- Dobretsov, S., R. M. Abed and M. Teplitski (2013). "Mini-review: Inhibition of biofouling by marine microorganisms." Biofouling **29**(4): 423-441.
- Dolla, A., M. Fournier and Z. Dermoun (2006). "Oxygen defense in sulfate-reducing bacteria." Journal of biotechnology **126**(1): 87-100.
- Dowling, N., J. Guezennec, M. Lemoine, A. Tunlid and D. White (1988). "Analysis of carbon steels affected by bacteria using electrochemical impedance and direct current techniques." Corrosion **44**(12): 869-874.
- Duncan, K. E., L. M. Gieg, V. A. Parisi, R. S. Tanner, S. G. Tringe, J. Bristow and J. M. Suflita (2009). "Biocorrosive thermophilic microbial communities in Alaskan North Slope oil facilities." Environmental science & technology **43**(20): 7977-7984.
- Duncan, K. E., B. M. Perez-Ibarra, G. Jenneman, J. B. Harris, R. Webb and K. Sublette (2014). "The effect of corrosion inhibitors on microbial communities

associated with corrosion in a model flow cell system." Applied microbiology and biotechnology **98**(2): 907-918.

Eashwar, M., G. Sreedhar, A. Lakshman Kumar, R. Hariharasuthan and J. Kennedy (2015). "The enrichment of surface passive film on stainless steel during biofilm development in coastal seawater." Biofouling **31**(6): 511-525.

Eckert, R. B. and T. L. Skovhus (2016). "Advances in the application of molecular microbiological methods in the oil and gas industry and links to microbiologically influenced corrosion." International Biodeterioration & Biodegradation.

Edyvean, R. and H. Videla (1991). Biofouling and MIC interactions in the marine environment: an overview. Microbial Corrosion. 2 nd EFC Workshop.

Emde, K. M. E., D. W. Smith and R. Facey (1992). "Initial investigation of microbially influenced corrosion (MIC) in a low temperature water distribution system." Water Research **26**(2): 169-175.

Enning, D. and J. Garrelfs (2014). "Corrosion of Iron by Sulfate-Reducing Bacteria: New Views of an Old Problem." Applied and Environmental Microbiology **80**(4): 1226-1236.

Enning, D., H. Venzlaff, J. Garrelfs, H. T. Dinh, V. Meyer, K. Mayrhofer, A. W. Hassel, M. Stratmann and F. Widdel (2012). "Marine sulfate -reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust." Environmental microbiology **14**(7): 1772-1787.

Faimali, M., E. Chelossi, G. Pavanello, A. Benedetti, I. Vandecandelaere, P. De Vos, P. Vandamme and A. Mollica (2010). "Electrochemical activity and bacterial diversity of natural marine biofilm in laboratory closed-systems." Bioelectrochemistry **78**(1): 30-38.

Fang, H. H. P., L.-C. Xu and K.-Y. Chan (2002). "Effects of toxic metals and chemicals on biofilm and biocorrosion." Water Research **36**(19): 4709-4716.

Fletcher, M. and K. Marshall (1982). Are solid surfaces of ecological significance to aquatic bacteria? Advances in microbial ecology, Springer: 199-236.

Ford, T. and R. Mitchell (1990). The ecology of microbial corrosion. Advances in microbial ecology, Springer: 231-262.

- Gaines, R. H. (1910). "Bacterial Activity as a Corrosive Influence in the Soil." Industrial & Engineering Chemistry **2**(4): 128-130.
- Gana, M. L., S. Kebbouche-Gana, A. Touzi, M. A. Zorgani, A. Pauss, H. Lounici and N. Mameri (2011). "Antagonistic activity of Bacillus sp. obtained from an Algerian oilfield and chemical biocide THPS against sulfate-reducing bacteria consortium inducing corrosion in the oil industry." Journal of Industrial Microbiology & Biotechnology **38**(3): 391-404.
- Gaylarde, C. and J. Johnston (1980). "The importance of microbial adhesion in anaerobic metal corrosion." Microbial adhesion to surfaces. Ellis Horwood, Chichester: 511-513.
- Gaylarde, C. C. (1992). "Sulphate-reducing bacteria which do not induce accelerated corrosion." International biodeterioration & biodegradation **30**(4): 331-338.
- Gaylarde, C. C. and H. A. Videla (1987). "Localised corrosion induced by a marine vibrio." International biodeterioration **23**(2): 91-104.
- Geesey, G., R. Gillis, R. Avci, D. Daly, M. Hamilton, P. Shope and G. Harkin (1996). "The influence of surface features on bacterial colonization and subsequent substratum chemical changes of 316L stainless steel." Corrosion Science **38**(1): 73-95.
- Gharechahi, M., H. Moosavi and M. Forghani (2012). "Effect of Surface Roughness and Materials Composition." Journal of Biomaterials and Nanobiotechnology **3**(04): 541.
- Gomez-Alvarez, V., R. P. Revetta and J. W. S. Domingo (2012). "Metagenome analyses of corroded concrete wastewater pipe biofilms reveal a complex microbial system." BMC Microbiology **12**: 122-122.
- Guedes Soares, C., Y. Garbatov and A. Zayed (2011). "Effect of environmental factors on steel plate corrosion under marine immersion conditions." Corrosion Engineering, Science and Technology **46**(4): 524-541.
- Ha, H.-Y., M.-H. Jang, T.-H. Lee and J. Moon (2014). "Interpretation of the relation between ferrite fraction and pitting corrosion resistance of commercial 2205 duplex stainless steel." Corrosion Science **89**: 154-162.

- Hamdy, A. S., E. El-Shenawy and T. El-Bitar (2006). "Electrochemical impedance spectroscopy study of the corrosion behavior of some niobium bearing stainless steels in 3.5% NaCl." International Journal of Electrochemical Science **1**(4): 171-180.
- Hashimoto, K., K. Asami and K. Teramoto (1979). "An X-ray photo-electron spectroscopic study on the role of molybdenum in increasing the corrosion resistance of ferritic stainless steels in HCl." Corrosion Science **19**(1): 3-14.
- Heidelberg, J., K. Heidelberg and R. Colwell (2002). "Seasonality of Chesapeake Bay bacterioplankton species." Applied and Environmental Microbiology **68**(11): 5488-5497.
- Heitz, E., H.-C. Flemming and W. Sand (1996). Microbially influenced corrosion of materials: scientific and engineering aspects, Springer-Verlag.
- Hibiya, K., J. Nagai, S. Tsuneda and A. Hirata (2004). "Simple prediction of oxygen penetration depth in biofilms for wastewater treatment." Biochemical Engineering Journal **19**(1): 61-68.
- Hilbert, L. R., D. Bagge-Ravn, J. Kold and L. Gram (2003). "Influence of surface roughness of stainless steel on microbial adhesion and corrosion resistance." International Biodeterioration & Biodegradation **52**(3): 175-185.
- Hong, T. and M. Nagumo (1997). "Effect of surface roughness on early stages of pitting corrosion of Type 301 stainless steel." Corrosion Science **39**(9): 1665-1672.
- Hughes, M. N. and R. K. Poole (1989). "Metals and Micro-organisms."
- Ilevbare, G., O. Schneider, R. Kelly and J. Scully (2004). "In situ confocal laser scanning microscopy of AA 2024-T3 corrosion metrology I. Localized corrosion of particles." Journal of The Electrochemical Society **151**(8): B453-B464.
- Ismail, K. M., T. Gehrig, A. Jayaraman, T. Wood, K. Trandem, P. Arps and J. Earthman (2002). "Corrosion control of mild steel by aerobic bacteria under continuous flow conditions." Corrosion **58**(5): 417-423.
- Ito, K., R. Matsushashi, T. Kato, O. Miki, H. Kihira, K. Watanabe and P. Baker (2002). Potential ennoblement of stainless steel by Marine Biofilm and Microbial Consortia Analysis. CORROSION 2002, NACE International.

- Iverson, W. P. (1987). "Microbial corrosion of metals." Advances in applied microbiology **32**: 1-36.
- Jack, R., D. Ringelberg and D. White (1992). "Differential corrosion rates of carbon steel by combinations of *Bacillus* sp., *Hafnia alvei* and *Desulfovibrio gigas* established by phospholipid analysis of electrode biofilm." Corrosion Science **33**(12): 1843-1853.
- Jack, R. F. (1990). The effects of increased bacterial metabolic diversity on the corrosion of carbon steel, University of Tennessee, Knoxville.
- Javed, M. A., P. R. Stoddart, E. A. Palombo, S. L. McArthur and S. A. Wade (2014). "Inhibition or acceleration: Bacterial test media can determine the course of microbiologically influenced corrosion." Corrosion Science **86**: 149-158.
- Jayaraman, A., E. Cheng, J. Earthman and T. Wood (1997). "Axenic aerobic biofilms inhibit corrosion of SAE 1018 steel through oxygen depletion." Applied microbiology and biotechnology **48**(1): 11-17.
- Jia, Z.-j., C.-w. Du, C.-t. Li, Z. Yi and X.-g. Li (2011). "Study on pitting process of 316L stainless steel by means of staircase potential electrochemical impedance spectroscopy." International Journal of Minerals, Metallurgy, and Materials **18**(1): 48-52.
- Kandeil, A. Y. and M. Y. Mourad (1989). "Effect of surface texture on corrosion behaviour of steel." Surface and Coatings Technology **37**(2): 237-250.
- Keasler, V., B. Bennett and H. McGinley (2010). Analysis of bacterial kill versus corrosion from use of common oilfield biocides. 2010 8th International Pipeline Conference, American Society of Mechanical Engineers.
- Kielemoes, J., F. Hammes and W. Verstraete (2000). "Measurement of Microbial Colonisation of Two Types of Stainless Steel." Environmental Technology **21**(7): 831-843.
- King, R. and J. Miller (1971). "Corrosion by the sulphate-reducing bacteria." Nature **233**(5320): 491-492.
- Kip, N. and J. A. van Veen (2015). "The dual role of microbes in corrosion." ISME J **9**(3): 542-551.

- Kobrin, G. (1993). A practical manual on microbiologically influenced corrosion, Nace International.
- Kopylova, E., L. Noe, C. Da Silva, J. F. Berthelot, A. Alberti, J. M. Aury and H. Touzet (2015). "Deciphering metatranscriptomic data." Methods Mol Biol **1269**: 279-291.
- Kraigsley, A., P. Ronney and S. Finkel (2008). "Hydrodynamic effects on biofilm formation." Retrieved May 28.
- Lambert, T. W., V. M. Goodwin, D. Stefani and L. Stroscher (2006). "Hydrogen sulfide (H₂S) and sour gas effects on the eye. A historical perspective." Science of the total environment **367**(1): 1-22.
- Lanneluc, I., M. Langumier, R. Sabot, M. Jeannin, P. Refait and S. Sablé (2015). "On the bacterial communities associated with the corrosion product layer during the early stages of marine corrosion of carbon steel." International Biodeterioration & Biodegradation **99**: 55-65.
- Lawrence, J. R., M. R. Chenier, R. Roy, D. Beaumier, N. Fortin, G. D. W. Swerhone, T. R. Neu and C. W. Greer (2004). "Microscale and Molecular Assessment of Impacts of Nickel, Nutrients, and Oxygen Level on Structure and Function of River Biofilm Communities." Applied and Environmental Microbiology **70**(7): 4326-4339.
- Lee, J.-W., J.-H. Nam, Y.-H. Kim, K.-H. Lee and D.-H. Lee (2008). "Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces." The Journal of Microbiology **46**(2): 174-182.
- Lenhart, T. R., K. E. Duncan, I. B. Beech, J. A. Sunner, W. Smith, V. Bonifay, B. Biri and J. M. Suflita (2014). "Identification and characterization of microbial biofilm communities associated with corroded oil pipeline surfaces." Biofouling **30**(7): 823-835.
- Li, H., E. Zhou, D. Zhang, D. Xu, J. Xia, C. Yang, H. Feng, Z. Jiang, X. Li, T. Gu and K. Yang (2016). "Microbiologically Influenced Corrosion of 2707 Hyper-Duplex Stainless Steel by Marine Pseudomonas aeruginosa Biofilm." **6**: 20190.
- Li, K., M. Whitfield and K. J. Van Vliet (2013). "Beating the bugs: Roles of microbial biofilms in corrosion." Corrosion Reviews **31**(3-6): 73-84.

- Li, Z., Y. Chen, D. Liu, N. Zhao, H. Cheng, H. Ren, T. Guo, H. Niu, W. Zhuang, J. Wu and H. Ying (2015). "Involvement of glycolysis/gluconeogenesis and signaling regulatory pathways in *Saccharomyces cerevisiae* biofilms during fermentation." Frontiers in Microbiology **6**: 139.
- Little, B. (2003). "A perspective on the use of anion ratios to predict corrosion in Yucca Mountain." Corrosion **59**(8): 701-704.
- Little, B., J. Lee and R. Ray (2007). "A review of 'green' strategies to prevent or mitigate microbiologically influenced corrosion." Biofouling **23**(1-2): 87-97.
- Little, B. and R. Ray (2002). "A perspective on corrosion inhibition by biofilms." Corrosion **58**(5): 424-428.
- Little, B., R. Ray, J. Lee, T. Liengen, D. Feron, R. Basseguy and I. Beech (2014). "Understanding marine biocorrosion: experiments with artificial and natural seawater." Understanding Biocorrosion: Fundamentals and Applications, eds.: 329-340.
- Little, B., R. Ray, P. Wagner, J. Jones ~~-M. Lee~~ and F. Mansfeld (1999). "Spatial relationships between marine bacteria and localized corrosion on polymer coated steel." Biofouling **13**(4): 301-321.
- Little, B., P. Wagner and J. Jacobus (1988). The impact of sulfate-reducing bacteria on welded copper-nickel seawater piping systems, DTIC Document.
- Little, B., P. Wagner and F. Mansfeld (1991). "Microbiologically influenced corrosion of metals and alloys." International Materials Reviews **36**(1): 253-272.
- Little, B. J. and J. R. Depalma (2013). "Marine biofouling." Materials for Marine Systems and Structures: Treatise on Materials Science and Technology **28**: 89.
- Little, B. J. and J. S. Lee (2014). "Microbiologically influenced corrosion: an update." International Materials Reviews **59**(7): 384-393.
- Little, B. J., J. S. Lee and R. I. Ray (2008). "The influence of marine biofilms on corrosion: A concise review." Electrochimica Acta **54**(1): 2-7.
- Liu, S.-B., L.-P. Qiao, H.-L. He, Q. Zhang, X.-L. Chen, W.-Z. Zhou, B.-C. Zhou and Y.-Z. Zhang (2011). "Optimization of fermentation conditions and

- rheological properties of exopolysaccharide produced by deep-sea bacterium *Zunongwangia profunda* SM-A87." Plos one **6**(11): e26825.
- Lopez, F. G., J. Reifman and A. Wallqvist (2015). "Biofilm Formation Mechanisms of *Pseudomonas aeruginosa* Predicted via Genome-Scale Kinetic Models of Bacterial Metabolism." PLOS Computational Biology **11**(10): e1004452.
- Luo, J., P. Lv, J. Zhang, A. G. Fane, D. McDougald and S. A. Rice (2017). "Succession of biofilm communities responsible for biofouling of membrane bio-reactors (MBRs)." PLoS One **12**(7): e0179855.
- Lyman, J. and R. H. Fleming (1940). "Composition of sea water." J. mar. Res **3**(2): 134-146.
- Machuca, L. L., S. I. Bailey, R. Gubner, E. L. J. Watkin, M. P. Ginige, A. H. Kaksonen and K. Heidersbach (2013). "Effect of oxygen and biofilms on crevice corrosion of UNS S31803 and UNS N08825 in natural seawater." Corrosion Science **67**: 242-255.
- Mak, A. (2002). "Corrosion of Steel, Aluminum and Copper in Electrical Applications." General Cable.
- Mand, J., H. S. Park, T. R. Jack and G. Voordouw (2014). "The role of acetogens in microbially influenced corrosion of steel." Frontiers in Microbiology **5**(268).
- Mansfeld, F., G. Liu, H. Xiao, C. Tsai and B. Little (1994). "The corrosion behavior of copper alloys, stainless steels and titanium in seawater." Corrosion science **36**(12): 2063-2095.
- Mansour, R. and A. M. Elshafei (2016). "Role of Microorganisms in Corrosion Induction and Prevention."
- Marszalek, D. S., S. M. Gerchakov and L. R. Udey (1979). "Influence of substrate composition on marine microfouling." Applied and environmental microbiology **38**(5): 987-995.
- Martín-Rodríguez, A. J., A. González-Orive, A. Hernández-Creus, A. Morales, R. Dorta-Guerra, M. Norte, V. S. Martín and J. J. Fernández (2014). "On the influence of the culture conditions in bacterial antifouling bioassays and biofilm properties: *Shewanella* algae, a case study." BMC microbiology **14**(1): 102.

- Marty, F., J. F. Ghiglione, S. Paisse, H. Gueune, L. Quillet, M. C. van Loosdrecht and G. Muyzer (2012). "Evaluation and optimization of nucleic acid extraction methods for the molecular analysis of bacterial communities associated with corroded carbon steel." Biofouling **28**(4): 363-380.
- McBeth, J. M., B. J. Little, R. I. Ray, K. M. Farrar and D. Emerson (2011). "Neutrophilic iron-oxidizing "Zetaproteobacteria" and mild steel corrosion in nearshore marine environments." Applied and Environmental Microbiology **77**(4): 1405-1412.
- McCafferty, E. (2010). Introduction to corrosion science, Springer Science & Business Media.
- McDaniel, L. E. and E. G. Bailey (1969). "Effect of Shaking Speed and Type of Closure on Shake Flask Cultures." Applied Microbiology **17**(2): 286-290.
- Melchers, R. and R. Jeffrey (2004). "Influence of water velocity on marine immersion corrosion of mild steel." Corrosion **60**(1): 84-94.
- Melchers, R. E. (2016). Principles of Marine Corrosion. Springer Handbook of Ocean Engineering. M. R. Dhanak and N. I. Xiros. Cham, Springer International Publishing: 111-126.
- Melchers, R. E. and R. Jeffrey (2004). "Influence of Water Velocity on Marine Immersion Corrosion of Mild Steel." Corrosion **60**(1): 84-94.
- Melchers, R. E. and R. Jeffrey (2005). "Early corrosion of mild steel in seawater." Corrosion Science **47**(7): 1678-1693.
- Molitoris, E., S. Joseph, M. Krichevsky, W. Sindhuhardja and R. Colwell (1985). "Characterization and distribution of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated in Indonesia." Applied and environmental microbiology **50**(6): 1388-1394.
- Mollica, A. and A. Trevis (1976). "Correlation between the formation of a primary film and the modification of the cathodic surface of stainless steel in seawater." Proceedings of 4th Int. Cong. Marine Corrosion and Fouling, Antibes, France: 351-355.
- Mollica, A., A. Trevis, E. Traverso, G. Ventura, V. Scotto, G. Alabiso, G. Marcenaro, U. Montini, G. De Carolis and R. Dellepiane (1984). Interaction

- between biofouling and oxygen reduction rate on stainless steel in seawater. 6th International Congress on Marine Corrosion and Fouling, Athens.
- Moradi, M., J. Duan, H. Ashassi-Sorkhabi and X. Luan (2011). "De-alloying of 316 stainless steel in the presence of a mixture of metal-oxidizing bacteria." Corrosion science **53**(12): 4282-4290.
- Moradi, M., Z. Song and X. Tao (2015). "Introducing a novel bacterium, *Vibrio neocaledonicus* sp., with the highest corrosion inhibition efficiency." Electrochemistry Communications **51**: 64-68.
- Moradi, M., Z. Song, L. Yang, J. Jiang and J. He (2014). "Effect of marine *Pseudoalteromonas* sp. on the microstructure and corrosion behaviour of 2205 duplex stainless steel." Corrosion Science **84**: 103-112.
- Moradi, M., T. Xiao and Z. Song (2015). "Investigation of corrosion inhibitory process of marine *Vibrio neocaledonicus* sp. bacterium for carbon steel." Corrosion Science **100**: 186-193.
- Motoda, S., Y. Suzuki, T. Shinohara and S. Tsujikawa (1990). "The effect of marine fouling on the ennoblement of electrode potential for stainless steels." Corrosion science **31**: 515-520.
- Muyzer, G. and A. J. Stams (2008). "The ecology and biotechnology of sulphate-reducing bacteria." Nature Reviews Microbiology **6**(6): 441-454.
- Nan, L., K. Yang and G. Ren (2015). "Anti-biofilm formation of a novel stainless steel against *Staphylococcus aureus*." Materials Science and Engineering: C **51**: 356-361.
- Neidhardt, F. C., P. L. Bloch and D. F. Smith (1974). "Culture medium for enterobacteria." Journal of bacteriology **119**(3): 736-747.
- Neu, T. R. and J. R. Lawrence (1997). "Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy." FEMS Microbiology Ecology **24**(1): 11-25.
- Neville, A. and T. Hodgkiess (1998). "Comparative study of stainless steel and related alloy corrosion in natural sea water." British Corrosion Journal **33**(2): 111-120.

- Olesen, B., R. Avci and Z. Lewandowski (2000). "Manganese dioxide as a potential cathodic reactant in corrosion of stainless steels." Corrosion science **42**(2): 211-227.
- Oliver, J. D., R. A. Warner and D. R. Cleland (1983). "Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment." Applied and Environmental Microbiology **45**(3): 985-998.
- Olson, B. H. and L. A. Nagy (1984). "Microbiology of potable water." Advances in applied microbiology **30**: 73-132.
- Örnek, D., A. Jayaraman, B. Syrett, C.-H. Hsu, F. Mansfeld and T. Wood (2002). "Pitting corrosion inhibition of aluminum 2024 by *Bacillus* biofilms secreting polyaspartate or γ -polyglutamate." Applied microbiology and biotechnology **58**(5): 651-657.
- Örnek, D., A. Jayaraman, T. Wood, Z. Sun, C. Hsu and F. Mansfeld (2001). "Pitting corrosion control using regenerative biofilms on aluminium 2024 in artificial seawater." Corrosion science **43**(11): 2121-2133.
- Pascual, N., V. Loux, S. Derozier, V. Martin, D. Debroas, S. Maloufi, J.-F. Humbert and J. Leloup (2015). "Technical challenges in metatranscriptomic studies applied to the bacterial communities of freshwater ecosystems." Genetica **143**(2): 157-167.
- Pedersen, K. (1982). "Method for studying microbial biofilms in flowing-water systems." Applied and environmental microbiology **43**(1): 6-13.
- Potekhina, J., N. Sherisheva, L. Povetkina, A. Pospelov, T. Rakitina, F. Warnecke and G. Gottschalk (1999). "Role of microorganisms in corrosion inhibition of metals in aquatic habitats." Applied microbiology and biotechnology **52**(5): 639-646.
- Raguénès, G., R. Christen, J. Guezennec, P. Pignet and G. Barbier (1997). "*Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*." International Journal of Systematic and Evolutionary Microbiology **47**(4): 989-995.

- Rahmouni, K., M. Keddad, A. Srhiri and H. Takenouti (2005). "Corrosion of copper in 3% NaCl solution polluted by sulphide ions." Corrosion science **47**(12): 3249-3266.
- Rajasekar, A. and Y.-P. Ting (2011). "Role of inorganic and organic medium in the corrosion behavior of *Bacillus megaterium* and *Pseudomonas* sp. in stainless steel SS 304." Industrial & Engineering Chemistry Research **50**(22): 12534-12541.
- Rao, D., J. S. Webb and S. Kjelleberg (2005). "Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*." Applied and environmental microbiology **71**(4): 1729-1736.
- Revie, R. W. and H. H. Uhlig (2011). Uhlig's corrosion handbook, John Wiley & Sons.
- Roberge, P. (1993). "Analysis of spontaneous electrochemical noise for corrosion studies." Journal of applied electrochemistry **23**(12): 1223-1231.
- Rochex, A. and J. M. Lebeault (2007). "Effects of nutrients on biofilm formation and detachment of a *Pseudomonas putida* strain isolated from a paper machine." Water Research **41**(13): 2885-2892.
- Scott, P., M. Davies and J. Goldie (1991). "Ranking alloys for susceptibility to MIC; A preliminary report on high-Mo alloys." Materials Performance;(United States) **30**(1).
- Scotto, V., R. Di Cintio and G. Marcenaro (1985). "The influence of marine aerobic microbial film on stainless steel corrosion behaviour." Corrosion Science **25**(3): 185-194.
- Scotto, V. and M. Lai (1998). "The ennoblement of stainless steels in seawater: a likely explanation coming from the field." Corrosion science **40**(6): 1007-1018.
- Scotto, V. and M. E. Lai (1998). "The ennoblement of stainless steels in seawater: a likely explanation coming from the field." Corrosion Science **40**(6): 1007-1018.
- Seyoux, A., S. Zanna, A. Allion and P. Marcus (2015). "The fate of the protective oxide film on stainless steel upon early stage growth of a biofilm." Corrosion Science **91**: 352-356.

- Shalaby, H., A. Hasan and F. Al-Sabti (1999). "Effects of inorganic sulphide and ammonia on microbial corrosion behaviour of 70Cu–30Ni alloy in sea water." British Corrosion Journal **34**(4): 292-298.
- Sheng, X., Y.-P. Ting and S. O. Pehkonen (2007). "The influence of sulphate-reducing bacteria biofilm on the corrosion of stainless steel AISI 316." Corrosion Science **49**(5): 2159-2176.
- Shifler, D., S. Fujimoto, H. Kihira, F. Martin and D. Shifler (2005). "Corrosion in Marine and Saltwater Environments." The Electrochemical Society, Inc., Nueva Jersey, Estados Unidos: 345-354.
- Sreekumari, K. R., K. Nandakumar and Y. Kikuchi (2001). "Bacterial attachment to stainless steel welds: significance of substratum microstructure." Biofouling **17**(4): 303-316.
- Sreekumari, K. R., M. Ozawa, K. Tohmoto and Y. Kikuchi (2000). "Adhesion of Bacillus sp. on stainless steel weld surfaces." ISIJ International **40**(Suppl): S54-S58.
- Starosvetsky, D., R. Armon, J. Yahalom and J. Starosvetsky (2001). "Pitting corrosion of carbon steel caused by iron bacteria." International biodeterioration & biodegradation **47**(2): 79-87.
- Stern, M. and A. L. Geary (1957). "Electrochemical polarization I. A theoretical analysis of the shape of polarization curves." Journal of the electrochemical society **104**(1): 56-63.
- Stoodley, P., K. Sauer, D. G. Davies and J. W. Costerton (2002). "Biofilms as complex differentiated communities." Annu Rev Microbiol **56**: 187-209.
- Surman, S. B., J. T. Walker, D. T. Goddard, L. H. G. Morton, C. W. Keevil, W. Weaver, A. Skinner, K. Hanson, D. Caldwell and J. Kurtz (1996). "Comparison of microscope techniques for the examination of biofilms." Journal of Microbiological Methods **25**(1): 57-70.
- Svensäter, G., J. Welin, J. Wilkins, D. Beighton and I. Hamilton (2001). "Protein expression by planktonic and biofilm cells of Streptococcus mutans." FEMS microbiology letters **205**(1): 139-146.
- Sverdrup, H. U., M. W. Johnson and R. H. Fleming (1942). The Oceans: Their physics, chemistry, and general biology, Prentice-Hall New York.

- Tang, K., V. Baskaran and M. Nemati (2009). "Bacteria of the sulphur cycle: an overview of microbiology, biokinetics and their role in petroleum and mining industries." Biochemical Engineering Journal **44**(1): 73-94.
- Terry, L. and R. Edyvean (1986). "Recent investigations into the effects of algae on corrosion." Studies in environmental science **28**: 211-229.
- Thierry, D., N. Larché, C. Leballeur, S. Wijesinghe and T. Zixi (2014). "Corrosion potential and cathodic reduction efficiency of stainless steel in natural seawater." Materials and Corrosion.
- Toyofuku, M., T. Inaba, T. Kiyokawa, N. Obana, Y. Yawata and N. Nomura (2016). "Environmental factors that shape biofilm formation." Bioscience, biotechnology, and biochemistry **80**(1): 7-12.
- Tremaroli, V., M. L. Workentine, A. M. Weljie, H. J. Vogel, H. Ceri, C. Viti, E. Tatti, P. Zhang, A. P. Hynes and R. J. Turner (2009). "Metabolomic investigation of the bacterial response to a metal challenge." Applied and environmental microbiology **75**(3): 719-728.
- Venzlaff, H., D. Enning, J. Srinivasan, K. J. J. Mayrhofer, A. W. Hassel, F. Widdel and M. Stratmann (2013). "Accelerated cathodic reaction in microbial corrosion of iron due to direct electron uptake by sulfate-reducing bacteria." Corrosion Science **66**: 88-96.
- Videla, H. A. (2001). "Microbially induced corrosion: an updated overview." International biodeterioration & biodegradation **48**(1-4): 176-201.
- Videla, H. A. and W. G. Characklis (1992). "Biofouling and microbially influenced corrosion." International Biodeterioration & Biodegradation **29**(3): 195-212.
- Videla, H. A. and L. K. Herrera (2005). "Microbiologically influenced corrosion: looking to the future." International microbiology **8**(3): 169.
- Videla, H. A. and L. K. Herrera (2009). "Understanding microbial inhibition of corrosion. A comprehensive overview." International Biodeterioration & Biodegradation **63**(7): 896-900.
- von Wolzogen Kühr, C. A. H. and L. Van der Vlugt (1934). De grafiteering van gietijzer als electro-biochemisch proces in anaerobe gronden.

- Wade, S., M. Javed, E. Palombo, S. McArthur and P. Stoddart (2017). "On the need for more realistic experimental conditions in laboratory-based microbiologically influenced corrosion testing." International Biodeterioration & Biodegradation **121**: 97-106.
- Wahl, M. (1997). "Living attached: aufwuchs, fouling, epibiosis." Fouling organisms of the Indian Ocean: biology and control technology. AA Balkema, Rotterdam: 31-83.
- Wang, W., X. Li, J. Wang, H. Xu and J. Wu (2004). "Influence of biofilms growth on corrosion potential of metals immersed in seawater." Materials and Corrosion **55**(1): 30-35.
- Wang, W., J. Wang, H. Xu and X. Li (2006). "Electrochemical techniques used in MIC studies." Materials and Corrosion **57**(10): 800-804.
- Whitby, C. and T. L. Skovhus (2011). Applied microbiology and molecular biology in oilfield systems, Springer.
- Wilkins, S. (2015). "Structure and mechanism of ABC transporters." F1000Prime Reports **7**: 14.
- Xiao, W., CHAI Ke, YANG Yuhui and W. Jinyi (2010). "EFFECT OF MICROBE ON THE CORROSION BEHAVIORS AND MECHANICAL PROPERTIES OF 25 CARBON STEEL IN TROPICAL SEAWATER CONDITION." Journal of Chinese Society for Corrosion and protection **30**(5): 359-363.
- Xie, C., C. L. W. Goi, D. H. Huson, P. F. R. Little and R. B. H. Williams (2016). "RiboTagger: fast and unbiased 16S/18S profiling using whole community shotgun metagenomic or metatranscriptome surveys."
- Xu, C., Y. Zhang, G. Cheng and W. Zhu (2007). "Localized corrosion behavior of 316L stainless steel in the presence of sulfate-reducing and iron-oxidizing bacteria." Materials Science and Engineering: A **443**(1): 235-241.
- Xu, D., J. Xia, E. Zhou, D. Zhang, H. Li, C. Yang, Q. Li, H. Lin, X. Li and K. Yang (2017). "Accelerated corrosion of 2205 duplex stainless steel caused by marine aerobic *Pseudomonas aeruginosa* biofilm." Bioelectrochemistry **113**: 1-8.

- Xu, F.-l., J.-z. Duan, C.-g. Lin and B.-r. Hou (2015). "Influence of Marine Aerobic Biofilms on Corrosion of 316L Stainless Steel." Journal of Iron and Steel Research, International **22**(8): 715-720.
- Xu, X.-W., Y.-H. Wu, C.-S. Wang, X.-H. Gao, X.-G. Wang and M. Wu (2010). "Pseudoalteromonas lipolytica sp. nov., isolated from the Yangtze River estuary." International Journal of Systematic and Evolutionary Microbiology **60**(9): 2176-2181.
- Yadav, M. K., S. K. Kwon, C. G. Cho, S.-W. Park, S.-W. Chae and J.-J. Song (2012). "Gene expression profile of early in vitro biofilms of Streptococcus pneumoniae." Microbiology and Immunology **56**(9): 621-629.
- Yin, Y., S. Cheng, S. Chen, J. Tian, T. Liu and X. Chang (2009). "Microbially influenced corrosion of 303 stainless steel by marine bacterium Vibrio natriegens: (II) Corrosion mechanism." Materials Science and Engineering: C **29**(3): 756-760.
- Yuan, S., B. Liang, Y. Zhao and S. Pehkonen (2013). "Surface chemistry and corrosion behaviour of 304 stainless steel in simulated seawater containing inorganic sulphide and sulphate-reducing bacteria." Corrosion Science **74**: 353-366.
- Yuan, S., S. Pehkonen, Y. Ting, E. Kang and K. Neoh (2008). "Corrosion behavior of type 304 stainless steel in a simulated seawater-based medium in the presence and absence of aerobic pseudomonas NCIMB 2021 bacteria." Industrial & Engineering Chemistry Research **47**(9): 3008-3020.
- Yuan, S., F. Xu, S. Pehkonen, Y. Ting, E. Kang and K. Neoh (2008). "Biocorrosion behavior of titanium oxide/butoxide-coated stainless steel." Journal of The Electrochemical Society **155**(5): C196-C210.
- Yuan, S. J., A. M. F. Choong and S. O. Pehkonen (2007). "The influence of the marine aerobic Pseudomonas strain on the corrosion of 70/30 Cu–Ni alloy." Corrosion Science **49**(12): 4352-4385.
- Zaveri, N., R. Sun, N. Zufelt, A. Zhou and Y. Chen (2007). "Evaluation of microbially influenced corrosion with electrochemical noise analysis and signal processing." Electrochimica Acta **52**(19): 5795-5807.

Zhu, X. Y., J. Lubeck and J. J. Kilbane (2003). "Characterization of Microbial Communities in Gas Industry Pipelines." Applied and Environmental Microbiology **69**(9): 5354-5363.

Zuo, R. (2007). "Biofilms: strategies for metal corrosion inhibition employing microorganisms." Appl Microbiol Biotechnol **76**(6): 1245-1253.