WA School of Mines: Minerals, Energy and Chemical Engineering

Understanding of Microbiologically Influenced Corrosion in Carbon Steel Pipelines: Towards Developing a Methodology to Assess Probability of Failure

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any another person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: _____

Date: 24/07/2020

Dedication

This thesis is dedicated to my beloved parents, Daisy and Orlando, who have always been there for me and also have taught me to work hard for achieving my goals. This work is also dedicated to my wonderful husband, Eder, who has supported and encouraged me during the challenges I faced these years.

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

Marie Curie (1867-1934)

Abstract

Microbiologically influenced corrosion (MIC) refers to the deterioration of metals caused by the presence and activity of microorganisms on their surfaces. This phenomenon is responsible for about 20% to 40% of corrosion problems in pipelines, representing one of the major asset integrity threats to the global hydrocarbon industry. Although MIC has been widely studied in the last decades, and several models for MIC prediction have been proposed, this phenomenon remains as one of the most unpredictable corrosion mechanisms in the industry. The challenge in making an accurate prediction and assessment of the MIC risk lies in the complex biological, physical and chemical factors involved, as well as in the identification of reliable methods for detection and evaluation of microbial activity and its contribution to corrosion.

This dissertation aimed to advance and improve the way MIC is assessed through the development of a new molecular methodological approach for microbiological evaluation of systems, as well as through the generation of knowledge and understanding of the effect of environmental and operational conditions on MIC processes occurring on carbon steel. The investigation involved field studies and laboratory experimentation, designed and developed to achieve the project objectives; the results of these investigations are presented as individual peer-reviewed publications that comprise the four core chapters of this thesis.

First, we studied the microbiological content of industry samples using traditional DNA-based 16S rRNA next-generation sequencing (NGS). Specifically, we focused on assessing the effect of sample preservation and storage conditions on microbial composition. Samples from an oil production facility were collected and preserved under different conditions, simulating common practices by oil field operators. Microbial composition was compared against reference samples that were processed using optimal preservation protocols for molecular assessment. This study provided

a basic understanding of the implications of sample preservation and storage on the assessment of MIC. We demonstrated that microbiological analysis can underestimate or overestimate the risk of MIC if sampling, preservation, and storage are not properly conducted.

Second, the dissertation focused on the development and implementation of a new DNA/RNA-based complementary methodological approach to evaluate microbial communities in oil production systems with corrosion issues. MIC molecular assessment has been primarily centred on DNA-based analyses. However, the persistence of DNA in dormant or inactive microorganisms means that the analysed communities comprise both active and inactive species. Since MIC is mainly driven by active species, the distinction of the active microorganisms from the total microbial community is crucial. Therefore, the identification of active microorganisms was made through the analysis of RNA amplicons. We demonstrated that DNA results alone can underestimate the active species in oil production systems. The study also highlighted the importance of applying bioinformatics tools to process complex molecular data and to make statistical correlations between microbial ecology and environmental conditions.

The next part of our research focused on developing knowledge on the impact that environmental conditions have on microbial community composition, structure and finally, on MIC. The developed DNA/RNA-based complementary methodological approach was applied to these research studies. Specifically, we evaluated the effect of temperature and sulphur compounds on multispecies biofilms and the resulting MIC. Several prediction models include measurements of sulphate concentration as an essential parameter; however, sulphur compounds other than sulphates have not been considered. The first part of this study focused on evaluating the effect of temperature on biofilms on carbon steel. The temperature range studied (40 °C and 60 °C) was selected as it represents common temperatures in oil production systems. The study showed that biofilms formed at 40 °C caused higher corrosion rates compared to biofilms formed at 60 °C. A correlation was found between corrosion and microbial cell numbers and activity in biofilms. The second part of this study focused on investigating the effect of sulphur compounds on the risk of MIC in carbon

steel. The effect of temperature was included in this investigation to verify conclusions from the previous study using a different microbial consortium. This study showed a different trend in the effect of temperature, which was found to be dependent upon the presence or absence of sulphur compounds. Results indicated that either low or high thermal conditions can be equally corrosive, depending on the microbial species present and nutrients available. Concerning sulphur compounds, we found that when biofilms were formed in the presence of sulphur compounds, the MIC rates were higher, with the highest corrosion rates observed in biofilms grown in the presence of thiosulphate. This study highlighted the importance of including nutrient parameters other than sulphate in models for MIC prediction. The work also demonstrated a correlation between biofilm maturation and MIC severity.

In addition to examining the effect of environmental conditions on MIC, we also studied the impact of operational conditions on multispecies biofilms and resulting MIC. Specifically, we studied the effect of nutrient level (representing different flow regime conditions in oil production facilities) on biofilm characteristics and the subsequent impact on microbial corrosion and biocide effectiveness. Our study involved two different microbial consortia from oilfields. We found that biofilms formed under flowing conditions were more active, dense and robust compared to biofilms exposed to stagnant, nutrient-depleted conditions. In agreement with our previous investigations, results from this study showed that the more active biofilms caused higher corrosion rates. The nutrient level also had an impact on biocide effectiveness since a higher number of cells survived the biocide treatment in biofilms formed under a continuous flow of nutrients. RNA-based sequencing revealed that some microorganisms remained active after biocide treatment. Our findings indicated that RNA-based methods are promising tools for monitoring the effectiveness of mitigation treatments, and for early detection of biocide resistance in industrial systems.

The last part of the project focused on investigating microorganisms from corroded equipment from industry facilities with a history of MIC issues. We aimed at characterising and isolating potential corrosive microorganisms that are not commonly associated with MIC and therefore, not included in MIC prediction models.

For this study, samples from seal rings that failed due to corrosion in a floating production storage and offloading (FPSO) facility were collected, and the microbial diversity profiling was determined. We found that the same type of microorganisms, classified as iron oxidising bacteria (IOB), acid producing bacteria (APB), and nitrate reducing bacteria (NRB), were widespread across different pieces of corroded equipment; therefore, they were proposed to be involved in the corrosion failure. Nine bacterial species related to these groups were isolated, and their ability to induce corrosion was studied. The microbial groups studied are generally not included in MIC prediction models available. However, their potential to cause corrosion of carbon steel was demonstrated. Considering that several of the bacteria recovered from failed rings had not been related to MIC before, the genome characterisation of three of them was included as part of this thesis. Genome analysis indicated that these microbes have genes involved in metabolic pathways relevant to corrosion, providing support to the corrosion potential observed in laboratory experiments using the bacterial isolates.

Overall, this dissertation provides valuable information for improving MIC assessment and prediction through a better understanding of critical parameters affecting MIC that should be considered in MIC prediction models. The dissertation also contributes to the field of MIC through the development of a more suitable methodological approach for MIC assessment and monitoring, which is required to improve corrosion management and asset integrity.

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First of all, I would like to express my sincere gratitude to my primary supervisor, Dr. Laura Machuca, for her countless hours of guidance, knowledge, support, and invaluable contribution to this research. I would also like to extend my sincere gratitude to my co-supervisors, Dr. Kateřina Lepková and Dr. Thunyaluk Pojtanabuntoeng, for their guidance, motivation, and feedback throughout the course of this project.

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List of Publications Included in the Thesis

This thesis is assembled by peer-reviewed publications, which form the core chapters. The relationships between each chapter are described in the Introduction Chapter (Chapter 1).

The research papers are listed below:

Chapter 2 IMPROVING MICROBIOLOGICAL ASSESSMENT

S.J. Salgar-Chaparro and L.L. Machuca. Effect of sample storage conditions on the molecular assessment of MIC, in Proceedings of the Corrosion & Prevention Conference, Paper no. 46. The Australasian Corrosion Association Inc. Nov 11-14 2018, Adelaide, Australia.

S.J. Salgar-Chaparro and L.L. Machuca. Complementary DNA/RNA-based profiling: Characterisation of corrosive microbial communities and their functional profiles in an oil production facility. *Frontiers in Microbiology*, 10: 2587 (2019).

Chapter 3 EFFECT OF ENVIRONMENTAL CONDITIONS

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel, in: Corrosion 2019 Conference & Expo, Paper no. 13343. NACE International. Mar 25-28, Nashville, US.

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms. *Corrosion Science*, 169: 108595 (2020).

Chapter 4 EFFECT OF OPERATIONAL CONDITIONS

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Nutrient level determines biofilm characteristics and subsequent impact on microbial corrosion and biocide effectiveness. *Applied and Environmental Microbiology*, 86 (7) e02885-19 (2020).

Chapter 5 MIC CAUSATIVE MICROORGANISMS

S.J. Salgar-Chaparro, A. Darwin, A. Kaksonen, L.L. Machuca. Carbon Steel Corrosion by Bacteria from Failed Seal Rings at an Offshore Facility. *Scientific Reports*, 62292 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Pseudomonas balearica* strain EC28, an iron-oxidising bacterium isolated from corroded steel. *Microbiology Resource Announcements,* 9:e00275-20 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Draft Genome sequence of *Enterobacter roggenkampii* strain OS53, isolated from corroded pipework at an offshore oil production facility. *Microbiology Resource Announcements*, 9:e00583-20 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Shewanella chilikensis* strain DC57, isolated from corroded seal rings at a floating oil production system in Australia. *Microbiology Resource Announcements*, 9:e00584-20 (2020).

Statement of Contribution by Others

I, Silvia Juliana Salgar Chaparro, as the first author of the publications comprising this thesis was primarily involved in planning and conducting the experiments, data analysis, interpretation and manuscripts preparation. Contributions by the co-authors are mentioned below, and the written statements from the co-authors are included in Appendix 7.

Dr Laura. L Machuca-Suarez significantly contributed supervising the progress of the project, design of all experiments, data interpretation, as well as the preparation and critical review of the manuscripts.

Dr Kateřina Lepková greatly helped with the design of the experiments focused on evaluating the effect of operational conditions in the MIC risk, as well as critical revision of the manuscripts.

Dr Thunyaluk Pojtanabuntoeng greatly helped with the design of the experiments focused on evaluating the effect of operational conditions in the MIC risk, as well as critical revision of the manuscripts.

Dr Adam Darwin provided the industry background and contributed significantly to the conception of the study. Dr Adam Darwin also provided a critical revision of the manuscripts.

Prof. Rolf Daniel facilitated and assisted with the 3-month internship and bioinformatics training carried out in the Genomic and Applied Microbiology & Göttingen Genomics Laboratory, Institute of Microbiology and Genetics Georg-August University Göttingen, Göttingen, Germany. Prof. Rolf Daniel also provided the facilities to conduct the genomes sequencing and a critical revision of the genome announcements manuscripts.

Dr Genis Castillo greatly helped with the bioinformatics analysis of the isolates' genomes and provided a critical revision of the genome announcements manuscripts.

Dr Anja Poehlein conducted the sequencing of the genomes and provided a critical revision of the genome announcements manuscripts.

Dr Anna Kaksonen provided the facilities to conduct microbial isolation and technical support for the isolation of anaerobic bacteria. Dr Anna Kaksonen also provided a critical review of the isolates' manuscript presented in Chapter 5.

This research was funded by Woodside Energy Ltd. Curtin University provided financial assistance to attend national and international conferences. Experiments were conducted at the Curtin Corrosion Centre (CCC), Western Australian School of Mines: Minerals, Energy and Chemical Engineering (WASM-MECE) of Curtin University, Australia. Isolation of bacteria described in Chapter 5 was carried out at the CSIRO Land and Water facilities. Genome sequencing of the bacteria described in Chapter 5 was performed at the Genomic and Applied Microbiology & Göttingen Genomics Laboratory, Institute of Microbiology and Genetics Georg-August University Göttingen, Germany.

Conference Presentations

S.J. Salgar-Chaparro. Assessing the risk of microbiologically influenced corrosion and treatment. Corrosion in Non-Conventional Oil & Gas, ACA Technical Event. 23 May 2017, Brisbane, Australia. *(Presenter).*

S.J. Salgar-Chaparro. MIC prediction models. Microbiologically Influenced Corrosion (MIC) and its Inhibition Workshop, Curtin University. 8-9 Aug 2018, Perth, Australia. *(Presenter).*

S.J. Salgar-Chaparro and L.L. Machuca. Effect of sample storage conditions on the molecular assessment of MIC. Corrosion & Prevention Conference, ACA. 11-14 Nov 2018, Adelaide, Australia. *(Presenter).*

S.J. Salgar-Chaparro and L.L. Machuca. Comparison of DNA and RNA-based 16S rRNA diversity profiling of the microbial community recovered from a Western Australian oil production facility. Australian Microbial Ecology Conference. 13-14 Feb 2019, Perth, Australia. *(Poster).*

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel. Corrosion 2019, Conference & Expo, NACE International. 25-28 Mar 2019, Nashville, United States. *(Presenter).*

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Definitions

Abiotic: characterised by the absence of life or living organisms. Chemical or physical rather than biological.

Biofilm: Collective of one or more types of microorganisms that are embedded within a self-produced matrix of extracellular polymeric substances (EPS) adhere to a surface.

Bioinformatics: is the science of collecting and analysing complex biological data through a wide range of computational techniques.

Biotic: characterised by the presence of life or living organisms.

Carbon steel: is an alloy consisting of iron and up to 2.1% of carbon by weight.

Corrosion: is a process through which refined metals return to their natural oxidation state by interaction with the environment.

Corrosion circuit: is the systematisation of piping components that face similar corrosion mechanisms and is likely to fail in the same way.

Deoxyribonucleic acid (DNA): is a nucleic acid that contains the genetic code.

Mesophile: is a microorganism that grows best in moderate temperature, with an optimum growth range from 20 to 45 °C.

Microbiologically influenced corrosion (MIC): is the metal deterioration promoted by the presence and activity of microorganisms.

Microorganism: is a microscopic organism. In this thesis, these include bacteria and archaea.

Molecular microbiological methods: are methods used to characterise microbial communities in different environments. These are sensitive and reliable, not dependent on cultivation. Molecular methods are based on DNA and RNA analysis.

Next-generation sequencing (NGS): is a high-throughput method used to determine the sequence of nucleotides in a section of DNA or RNA.

Ribonucleic acid (RNA): is a nucleic acid that is transcribed from DNA. It plays a crucial role in regulating gene expression.

Planktonic: is a term used for free-living microorganisms that live in an aquatic environment.

Sessile: is a term used for microorganisms that are attached to a surface.

Thermophile: is a microorganism that grows best at higher than normal temperature, with an optimum growth range from 45 to 122 °C.

Threat: A potential cause of failure.

List of Abbreviations

ADP: Adenosine diphosphate

AEC: Adenylate energy charge

AGRF: Australian Genome Research Facility

AMP: Adenosine monophosphate

APB: Acid producing bacteria

ASV: Amplicon sequence variant

ATP: Adenosine triphosphate

CDT: Cathodic depolarization theory

CFU: Colony-forming units

CLSM: Confocal laser scanning microscopy

CMIC: Chemical MIC

CPF: Central processing facility

DNA: Deoxyribonucleic acid

EDS: Energy-dispersive X-ray spectroscopy

EET-MIC: Extracellular electron transfer MIC

EMIC: Electrical MIC

EPS: Extracellular polymeric substance

FESEM: Field emission scanning electron microscopy

FPSO: Floating production storage and offloading facility

GANB: General anaerobic bacteria

IOB: Iron oxidizing bacteria

IRB: Iron reducing bacteria

MET: Methanogenic archaea

MIC: Microbiologically influenced corrosion

MMM: Molecular microbiological methods

MPN: Most probable number

NCBI: National Centre for Biotechnology Information

NGS: Next-generation sequencing

NMDS: Non-metric multidimensional scaling

NRB: Nitrate reducing bacteria

OTU: Operational taxonomic unit

PBS: Phosphate-Buffered Saline

PCR: Polymerase chain reaction

qPCR: Quantitative PCR

RNA: Ribonucleic acid

SEM: Scanning Electron Microscopy

SPP: Sulphide producing prokaryotes

SRA: Sequence Read Archive

SRB: Sulphate reducing bacteria

THPS: Tetrakis (hydroxymethyl) phosphonium chloride

TOC: Total organic carbon

TPH: Total petroleum hydrocarbons

TRB: Thiosulphate reducing bacteria

VFA: Volatile fatty acids

Chapter 1

Introduction

1.1 Literature Review

1.1.1 Microbiologically Influenced Corrosion (MIC)

Microbiologically influenced corrosion (MIC) is defined as the deterioration of metals that results from the presence and activity of microorganisms on their surfaces [1]. This phenomenon was first identified in 1910 [2], but its significance was not commonly recognised until a few decades ago. It is now well known that the participation of microorganisms in the corrosion process can significantly increase corrosion rates, representing a significant threat to the integrity of industrial infrastructure [3, 4]. MIC can reduce the service life of metal structures and make these vulnerable to structural failure [5]. For instance, a flow line of an oil facility in Western Australia that had been designed to last in service more than 20 years showed deterioration by MIC at the third year of being in service [6]. Similarly, there have been several incidents where MIC has been the root cause of failure in piping systems [7-10]. Although MIC is mainly reported in the hydrocarbon industry, it inflicts considerable damage on an extensive network of industrial infrastructure operating in different environments, including buried systems, marine environments, mining plants, aviation fuel systems, water treatment systems, and nuclear facilities [3, 11-15].

Overall, corrosion generates a substantial economic impact on the world's industry sector [16, 17]. The global cost of corrosion for 2013 was estimated by the NACE international impact study to be approximately 3.4% of the gross national product (GNP), i.e., $2.5 trillion dollars, without including safety and environmental consequences [18]. These costs were later extrapolated by Little et al., [19] to be $2.9 trillion dollars for 2018. Quantification of the global costs associated explicitly with MIC has not been published [19]; however, it has been estimated that MIC contributes between 20% and 40% of total corrosion problems in pipelines [20, 21]. Apart from the financial impact of MIC, this phenomenon can also result in environmental damage, health, safety, reputational, and legal issues for the companies. Therefore, companies must implement corrosion management plans to mitigate the risk of MIC.

MIC is not different from other electrochemical corrosion mechanisms in wet environments where electrons flow through the metal from the anodic to the cathodic sites and are then transferred to a suitable electron acceptor [22]. Microorganisms change the electrochemical conditions at the metal-solution interface by the attachment of cells, biofilm formation, and subsequent release of metabolites, which influence the rates of the reactions at the anode or cathode in the corrosion process [23]. Hence, sessile microorganisms (attached to metal surfaces) are primarily related to MIC, whereas, planktonic microorganisms (floating in the bulk fluid) can modify chemical characteristics of the environment, but are not responsible for MIC [24, 25].

Microorganisms in aqueous environments have a natural tendency to attach to solid surfaces forming biofilms [26]. The development of biofilms is facilitated by the microbial production of extracellular polymeric substances (EPS), which are composed of sticky molecules that enhance the attachment process [1, 3]. Biofilm formation is a universal and widespread process present on different surfaces, including a wide range of metals, and occurs in distinct environmental conditions [25, 27, 28]. Biofilms on metallic surfaces are composed of microbial cells packed with EPS, water, nucleic acids, corrosion products, minerals, and organics [3, 29]. This complex conformation provides advantages to the microorganisms, including protection from environmental stressors [30], greater access to nutrients and other resources accumulated on the surface, more opportunities for interspecies interactions, and superior environmental stability [31]. Since biofilms are formed heterogeneously, these can generate anodic and cathodic areas that can stimulate corrosion [32]. Additionally, microorganisms in biofilms can alter the metal-solution interface by changing the composition and concentration of chemical species, oxygen levels, and pH due to their metabolic activities and the affinity of EPS to bind different molecules [1, 33]. Therefore, microorganisms in biofilms can promote electrochemical reactions, otherwise not favoured under abiotic conditions, leading to MIC [1]. Nonetheless, it has to be noted that biofilms do not always accelerate and promote corrosion, it has also been demonstrated that microorganisms can inhibit or protect metals against corrosion, a phenomenon known as microbiologically

influenced corrosion inhibition (MICI) [16]. MICI has only been observed under laboratory conditions, which appears to be the result of experimental conditions rather than a microbial capability. To date, it has not yet been shown that the presence of microbes can prevent corrosion in a pipeline.

1.1.2 MIC Mechanisms and Causative Microorganisms

MIC has been arduously investigated over the past decades, and several mechanisms have been proposed. Despite all research, MIC mechanisms are still debated and not completely understood, basically because MIC is a complex phenomenon that results from the interaction of several electrochemical, physical, environmental, operational and biological factors [30]. The main mechanisms proposed to explain MIC include cathodic depolarisation, concentration cells, production of corrosive metabolites, removal of protective films, and production of unprotective surface layers [3, 29, 34]. Recently, MIC mechanisms have been reclassified into two broad categories, chemical MIC (CMIC), that considers metal deterioration induced by corrosive metabolites, and extracellular electron transfer MIC (EET-MIC) that refers to the damage caused by direct or indirect uptake of electrons from the metal by specialised microbes [35-37].

Cathodic depolarisation theory (CDT) was the first mechanism proposed by von Wolzogen Kühr and van der Vlugt in 1934 to explain corrosion caused by sulphate reducing bacteria (SRB) [38]. Authors attributed MIC to the microbial utilisation of cathodic hydrogen (H^+) as the electron donor for the reduction of sulphate. SRB can catalyse the recombination of adsorbed atomic hydrogen into hydrogen gas (H_2) using hydrogenase enzymes, which causes an increase of the cathodic reaction rate [35]. Hence, corrosion reactions are indirectly accelerated by SRB through depolarisation of the cathode, which is a rate-limiting step in the corrosion process [29]. This theory was initially favoured by many authors [39-41], but later it suffered

much controversy [42-44]. In 1974, Costello [45] demonstrated that hydrogen sulphide from organotrophic sulphate reduction was a cathodic active compound that could explain the acceleration of cathodic reactions in the presence of SRB, which provoked that the cathodic depolarisation theory became disputable [35].

Concentration cells. Microorganisms can establish several types of concentration cells that can promote localised corrosion [46]. For example, in aerobic environments, biofilms can restrict oxygen diffusion to the metal, creating a differential aeration cell [47]. Areas beneath biofilms have a low oxygen concentration and became anodic, while clean areas surrounding biofilms have higher oxygen concentration and act as a cathode [26]. The differential aeration creates a flow of electrons towards the cathode, which results in corrosion [48]. Metal concentration cells can also be created by biofilm structures. EPS binding properties can accumulate metal ions from the solution or the substratum, which can lead to the formation of galvanic cells on the metal surface [3, 26].

Generation of deposits. Microorganisms, like the iron oxidising bacteria (IOB), that use ferrous iron (Fe^{2+}) as the electron donor in aerobic or anaerobic respiration can cause precipitation of ferric iron (Fe^{3+}) on the metal surface [49-51]. This process leads to the formation of anodic sites underneath the discreet deposits, which can result in under deposit corrosion (UDC) [52, 53].

Dissolution of oxides layers. Microorganisms, like the iron reducing bacteria (IRB), that derive energy from organic compounds and utilise insoluble ferric ions (Fe^{3+}) as electron acceptors, can solubilise Fe [49, 54], accelerating corrosion by removing protective corrosion layers and exposing the metal to further attack by corrosive agents in solution [55].

Chemical MIC (CMIC). CMIC is also called metabolite MIC (M-MIC). Microorganisms can promote corrosion reactions via the formation of aggressive corrosive metabolites such as hydrogen sulphide (H_2S), organic acids, ammonia (NH_3), nitrite, phosphine (PH_3), hydrogen peroxide, and enzymes [3]. Microorganisms that produce H_2S or organic acids during their metabolic activities promote corrosion by acid attack. These metabolites reduce the local pH in the biofilm, which directly relates to an increase in metal dissolution [4, 56]. Microorganisms that can trigger CMIC include SRB [49, 57], thiosulphate reducing bacteria (TRB) [58], sulphur reducing bacteria (S^0RB) [59], acid producing bacteria (APB) [49, 60], sulphur oxidising bacteria (SOB) [61], and nitrate reducing bacteria (NRB) [56, 62].

Extracellular electron transfer MIC (EET-MIC). EET-MIC was initially called electrical MIC (EMIC) [63]. This mechanism is caused by microorganisms that can use the metal as the electron donor for their metabolic reactions [64]. The oxidation reactions are carried out extracellularly since the metal matrix is insoluble. The above means that extracellular electrons released from the metal oxidation must be transported across the cell into the cytoplasm, a process known as EET [52]. There are two routes for EET. One is the direct electron transfer (DET) where microorganisms use cell membrane-bound proteins such as c-type cytochromes and conductive nanowires for transferring electrons over micrometre distances. The other is mediated by electron shuttles such as hydrogen and flavins [65, 66]. Within the microorganisms that have been reported to be able to derive energy from metal iron (Fe^0) are the SRB [67, 68], NRB [69], acetogens [70, 71], methanogens [70, 72, 73].

Microorganisms with the metabolic potential to cause corrosion through the aforementioned corrosion mechanisms are part of the normal microbiota of petroleum reservoirs [74, 75]. Nonetheless, the metabolic reactions conducted by microorganisms in field are limited to the availability of nutrients, electron donors and electron acceptors present in the system [49]. Thus, the presence of specific microbes and the resulting MIC not only depends on biological factors but also on

abiotic factors, including chemical environment, nutrient level, temperature, pH, among others [76].

1.1.3 Management of MIC in the Oil and Gas Industry

MIC is one of the several corrosion threats that need to be managed by oil and gas asset owners to maintain the integrity of the infrastructure for the expected service life [77]. Management of corrosion threats is commonly performed by the implementation of a systematic process that comprises three fundamental steps (Figure 1) [78].

Figure 1. Risk Management Lifecycle

Assessment. During the assessment step, the likelihood of MIC is identified and evaluated for each system in a facility. Systems are usually subdivided into segments that have similar operational and environmental conditions commonly recognised as corrosion circuits [79]. Differences in conditions can change the rate and/or the corrosion mechanism between segments; hence, the MIC probability in each corrosion circuit must be evaluated independently.

The MIC assessment step requires a multidisciplinary approach that considers not only microbiological data but also it requires the data integration of microbiological

data with physical and chemical parameters, corrosion data, material selection and metallurgy, design features, operational history, and mitigation strategies, among others [77, 79]. Data integration and analysis can be used to identify corrosion mechanisms in the system, the probability of failure for each corrosion circuit, and the root cause of damages and failures [77]. Since microorganisms can influence corrosion in different ways, MIC assessment must consider all possible factors that can have an effect on microbial life and the resulting MIC reactions [77]. Therefore, assessing the microbiology of the system is considered the critical step in MIC assessment and the primary method to evaluate the risk of MIC in a particular facility. The microbiological methods commonly used to determine the microbial makeup of oil and gas systems are presented in section 1.1.5. The advantages and setbacks of each method are discussed in the section. Typically, MIC is not properly assessed, which historically leads to inadequate mitigation.

Mitigation. This step consists of the selection of prevention and mitigation strategies to reduce the likelihood of MIC. The most common mitigation strategies applied in oil production equipment and pipelines are chemical treatments, which typically include injection of biocides [52], and mechanical cleaning, e.g., pigging [80]. Other alternatives may be employed at the design stage, for example, materials selection, application of coatings for areas with a higher risk of MIC, such as dead legs, and modification of water chemistry, e.g., seawater desulphation to reduce the risk of souring and MIC [79].

Monitoring. Monitoring activities are implemented to evaluate the efficacy of mitigation strategies employed to reduce the risk of MIC [77]. Corrosion coupons are commonly employed on a regular schedule for obtaining information about the rate of metal loss due to corrosion, which helps for early detection of MIC [79]. These coupons are also used to evaluate the efficacy of biocide treatments by counting sessile bacteria attached to them [19]. Electrochemical probes, such as BIoGEORGE [81] and BIOX [82], that measure electrochemical response to biofilm formation, have

also been used in the oil industry to optimise mitigation treatments [19]. Preferably, monitoring must include microbiological and corrosion data, that provide information about microorganisms, their activities, and the mechanisms driving the corrosion damage in the system [77].

1.1.4 MIC Prediction Models

In order to implement a corrosion management program, oil operators typically use prediction models to determine the probability of failure by different corrosion threats, including MIC [79]. This data supports decision making and the design of mitigation plans and control strategies, particularly to develop targeted mitigation plans for infrastructure at high risk of corrosion failure. Prediction models for abiotic corrosion mechanisms such as CO_2 corrosion have been shown to be accurate and beneficial [37]. Conversely, models developed for MIC prediction remain inaccurate, and therefore, MIC remains as the most unpredictable corrosion mechanism [36]. The underlying reason is that MIC is highly complex; it involves microbial life, and it can be potentially influenced by numerous factors, that act individually or in a synergistic way [83-85]. These possible interactions between chemical, physical and biological factors have made MIC prediction an extremely challenging task in the industry [3, 77, 86, 87].

Hitherto, several MIC prediction models have been proposed. They can be classified into different categories including empirical models, mechanistic models, molecular models and risk-based models [20]. Several input parameters have been included in the models which involve biological, physical and chemical factors. A brief summary of some of the existing MIC prediction models is given below.

- Pots et al., (2002) [88] formulated a model based on biochemical and physicochemical factors relevant to SRB growth. The principal disadvantage of this methodology is the lack of biological parameters leading to the assumption of a contaminated system [89]. This model has served as a starting point for the development of other prediction tools [85, 89, 90].

- Maxwell and Campbell (2006) [89] improved the model developed by Pots et al., [88] by including biological factors such as the number of SRB and the presence of sulphide. The main weakness of this model is that it only considers SRB as the MIC causative microbial group.

- Sooknah et al., (2007) [84] proposed a model to predict the susceptibility of MIC due to the probability of biofilm development. Similar to the model developed by Pots et al., [88], the authors only included chemical and physical parameters leaving out the biological data.

- Allison et al., (2008) [91] created a model based on biological and chemical parameters. The authors include the presence of SRB and heterotrophic bacteria in the system. One of the biggest limitations of this tool is the focus on the planktonic community, which has been demonstrated to have a limited relationship with the biofilm (sessile) community [92-94].

- Sorensen et al., (2012) [93] developed a model founded on the use of molecular microbiological methods to determine the number of cells present in the system. The authors included the presence and activity of sulphate-reducing prokaryotes and methanogenic microorganisms. The key disadvantage of this model is the lack of chemical and physical factors which are critical in determining the rate of corrosion due to their influence on microbial activity. MIC assessment must always consider abiotic factors and the possibility that several corrosion mechanisms can occur simultaneously [77]. Scientists that have implemented this methodology agree that this aspect of the model should be improved [87, 95].

- Skovhus et al., (2016) [87] generated a methodology for evaluating the probability of failure by MIC using the risk-based inspection analysis. This tool includes physical, biological and chemical factors. However, its weakness is the omission of bacterial activity (it only considers bacterial presence). In this way, the detection of corrosive microorganisms in the system alone is not proof of their involvement in corrosion.

- Wang and Jain (2016) [85] also improved the model developed by Pots et al., [88] including biological factors such as the number of cells and the presence of

sulphide. This methodology considers the number of sessile cells to categorise the MIC risk. However, a direct correlation between the number of cells and corrosion rate has not been established; therefore, this practice appears to be inaccurate [77]. Moreover, similar to the model by Skovhus et al. [87], the authors do not consider the activity of the microorganisms identified, i.e., whether the microbial cells identified are active or not. For instance, Al-Shamari et al., [96] demonstrated that the number of microorganisms of three microbial groups commonly associated with MIC did not have a direct correlation with pitting or general corrosion rates.

- Taleb-Berrouane et al., (2018) [97] proposed a more robust model compared to previously proposed models. This model is built upon 60 factors grouped in 20 screening parameters, including design, operating history, microbiological activity, fluid chemistry, among others. Although authors have considered additional parameters not included in previous models, the biological evidence only includes the concentration of SRB and APB.

- Kannan et al., (2020) [98] developed a Bayesian network model, which includes 60 parent nodes. The nodes include tools used to evaluate MIC as well as physical and chemical factors. This model also involves an estimation of the confidence of the result, which provides important information to corrosion engineers unfamiliarised with potential variables that can comprise the model. Similar to other MIC prediction models, this model only evaluates the potential impact of SRB and APB.

The majority of the MIC prediction tools developed until now are centred in the corrosive potential of SRB [20, 87, 99, 100], which is evaluated based on the number of cells and assuming the same bacterial kinetics for the sulphide production. In this sense, bacterial physiological status, i.e., activity, has not been considered [101]. Furthermore, the importance of microbial groups different from SRB in the corrosion process has been ignored in MIC prediction models. Microorganisms belonging to TRB, IOB, IRB, APB, and NRB groups have widely been associated with corrosion [1, 85, 102]. Moreover, the MIC prediction tools available have not considered the cooperative interactions across microorganisms in biofilms and/or their response to

environmental conditions found in oil and gas systems and how this behaviour potentially influence corrosion rates. Therefore, there is ample room for the improvement of MIC assessment and prediction.

1.1.5 Methods for Microbiological Assessment in the Oil and Gas Industry

a. Culture-based methods

Culture-based techniques have been the methods frequently used for assessing and monitoring the presence of microbial groups associated with MIC in the oil and gas industry [103, 104]. Since the National Association of Corrosion Engineers (NACE) published a standard for monitoring the bacterial growth in oilfield Systems [105], the most probable number (MPN) method has been the gold standard method for determining microbial populations in industrial systems [103, 106]. MPN method is based on the determination of the presence or absence of microbial growth in consecutive dilutions of the sample in standard culture media. Currently, the limitations of this method are widely known, and for this reason, oil and gas operators have looked for alternatives. Limitations of culture-based methods include the inability to recover all microorganisms in the community, which leads to the underestimation of the MIC risk [106]. It has been reported that more than 99% of the microorganisms on Earth have not yet been possible to cultivate in the laboratory, and therefore, it is expected that a large portion of the microorganisms in industrial systems will not be recovered using the standard culture media used in the MPN technique [103, 107, 108]. Another limitation of culture-based methods is their inability to provide information about the specific activities and interactions of microorganisms in situ [103]. This data is important to make an association between corrosion damage and the presence of microorganisms, and to differentiate MIC from abiotic mechanisms [77].

b. Culture-independent methods.

Biochemical methods. Biochemical analyses include the detection of biological substances produced by microorganisms such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). These molecules are considered to be suitable for the measurement of viability due to their linear relationship with the cell numbers, therefore have been used to estimate microbial activity and the number of viable microorganisms in the environment and industrial systems [109]. The main setback of this method is that it provides information about the average concentration of these molecules in the community, but cannot differentiate which microbial species are producing them. Moreover, it has been demonstrated that ATP content varies between different species and their growth rates, therefore it has to be considered as a qualitative indicator that reflects total microbial activity in the system.

Microscopy methods. Fluorescent dies that bind to components of the microbial cells have been used to visualise the distribution of microorganisms in biofilms formed over corrosion coupons. The observation of stained samples can be carried out in confocal laser scanning microscopes (CLSM) and fluorescent microscopes [109]. The sample preparation for this analysis is simple; however, it can also have some challenges related to the autofluorescence of other biofilm components that makes it difficult to identify microbial cells [3]. More specialised analyses, such as fluorescence in situ hybridisation (FISH) have been used to overcome these issues, since it uses fluorescent probes with excitation-emission spectra outside of the autofluorescence range. Scanning electron microscopy (SEM) has also been used to observe the distribution of microorganisms and their relationship with corrosion products. This technique provides high-resolution images of biofilms. SEM can be complemented with energy dispersive x-ray spectroscopy (EDS) to identify the elemental composition of corrosion products [109]. An important limitation of this analysis is that the sensitivity is limited to 0.1%, and some of the elements of interest may be below this detection limit.

Molecular methods. In the last decades, molecular microbiological methods have been developed and widely applied to improve the understanding of microbial ecology [108]. These methods have been implemented by the oil and gas industry for the monitoring and management of MIC [102, 110, 111]. Molecular methods are based on the recovery of macromolecules (nucleic acids and proteins) present in each cell without a requirement for laboratory growth of microorganisms, allowing them to overcome the limitation of the culture-based methods [107]. Within these methods, the quantitative polymerase chain reaction (qPCR) has been used to determine the number of microorganisms by amplifying taxonomical and functional gene markers [108]. DNA amplification is detected in real time by measuring fluorescent signal at the end of each PCR cycle (denaturation, annealing, elongation). qPCR reaction mix contains fluorescent dyes or fluorescent probes that bind to dsDNA and make possible to detect the amount of replicated DNA while the reaction occurs. This method has several advantages, including accuracy, sensitivity, and speed, but it also has disadvantages like the inability to distinguish between live and dead cells [112], and the predominantly use of multicopy genes, such as the 16S rRNA [113]. Setbacks of the qPCR can lead to errors in the enumeration of the microorganisms in the system, resulting in an overestimation of the microbial community.

Another molecular method that is state of the art for investigating microbial communities associated with MIC, is the 16S rRNA gene amplicon sequencing [112, 114-117]. 16S rRNA gene is considered the gold standard marker in phylogenetic studies, and has been widely used for microbial community profiling due to the presence of evolutionarily conserved as well as highly diverse regions [108]. The use of advance techniques such as next-generation sequencing (NGS) or high-through sequencing has enabled to obtain results in less time than the traditional Sanger sequencing [25]. NGS utilises parallel sequencing of millions of small fragments of DNA, which generates thousands of megabases of sequence information that must be analysed with computational tools [30]. The use of NGS in MIC studies has led to an increase in the number of microbial species identified as potentially being able to cause or accelerate corrosion [116, 118, 119]. Usually, the microbial characterisation

of oilfield samples has focused on the 16S rRNA gene sequencing, i.e., from the DNA molecule. Although DNA diversity profiling analysis has yielded unprecedented insights into the microbial ecology of corrosive environments, the persistence of DNA in dormant or inactive microorganisms leads to results including both active and inactive species. Presence of inactive and dead cells is more likely expected in industrial systems under mitigation treatments, such as oilfields, which represents a limitation for the application of DNA-based methods. Since corrosion processes are most likely influenced by active species, identification of active microorganisms from the total community would offer a better assessment of the risk of MIC and potentially of resilient microbes developing tolerance to biocide treatments.

The 16S rRNA sequencing, from the RNA molecule, is an alternative method that provides more accurate information of the active members in the community. This analysis consists of the extraction of the total RNA followed by synthesis of cDNA via reverse transcription (RT) PCR and subsequent NGS of the 16S rRNA [120]. RNA-based analysis provides more accurate information of the active members in the community since RNA deteriorates rapidly after cell death [121]. Furthermore, the cellular rRNA concentration usually is well correlated with growth rate and activity of the microorganisms [122]. RNA-based profiling has rarely ever been applied in MIC studies [123, 124], probably because of the challenges involved in this analysis [125]. Unlike DNA, RNA degrades rapidly, mainly because of the ubiquitous presence of RNases that can break down the RNA molecules. Hence, RNA-based analyses require the implementation of preservation protocols to protect field samples, as well as, skilled professionals who can isolate RNA with the purity and quality required for the sequencing process. In spite of the challenges involved in conducting RNA work, the usefulness of the information obtained by this approach and the importance of the accuracy of the MIC assessment to prevent corrosion failures makes it important to further study the suitability of its implementation and application to oilfield and corrosion samples.

The advances on molecular methods based on sequencing have allowed a significant breakthrough in microbial ecology studies, without the need for culturing [126]. Initially, these technologies were expensive to apply; however, in the last decades,

the costs of these have been dramatically reduced, and their use has become popular. Nowadays, the challenge in the application of NGS methods lies on getting relevant meaning out of the obtained data [127]. The high-throughput nature of NGS results on a huge amount of data (hundreds of thousands or even millions of short-read sequences) that needs to be processed using intensive computational power and bioinformatics tools [128, 129]. There are several bioinformatics software that can be used for having a better understanding of the relationship between microorganisms and the environment; though, there are few academic publications in which these have been implemented for the study of MIC. This is because the application of NGS methods in the understanding of MIC requires interdisciplinary integration between biological, computational, metallurgical, and corrosion science.

The most recent advanced molecular methods are the omics-based analyses, which include tools such as metagenomics, metatranscriptomics, metaproteomics and metabolomics [104]. Through the use of these tools, biofilm communities can be studied at both their compositional and functional level [78, 104, 130]. For instance, metagenomics determines and characterises the entire microbial population present in the biofilm, i.e., what type of microorganisms are present, whereas metatranscriptomics, metaproteomics, and metabolomics provide information about the activities being carried out by the microbial community in situ, i.e., gene expression and microbial activity [104, 108]. The application of these methods allows extracting information about biofilm composition and the microbial interactions that could be involved in MIC processes [78, 104, 130]; however, a multi-omics approach will be challenging to implement for regular assessment or monitoring activities involved in MIC management as these technologies are still expensive and require extensive background knowledge for correct interpretation.

1.2 Statement of the Problem

MIC is one of the major asset integrity threats in oil and gas production, transportation, and refining facilities. Despite large research efforts in the field, a full

understanding of this phenomenon has not been achieved. MIC knowledge gaps result in ineffective and inefficient MIC management strategies that lead to early deterioration of assets and reduction of their service life. In order to assess the probability of failure and determine the risk of MIC, several prediction models have been developed to evaluate the susceptibility of the system to this corrosion threat. However, despite the existence of these tools, MIC remains as one of the most unpredictable corrosion mechanisms. The inaccuracy of existing MIC predictive models reflects the need to develop more research focused on filling knowledge gaps that can help improve MIC prediction and management.

Although several attempts have been made to describe MIC related infrastructural failures, the relationship and complex interactions between microbial activities, corrosion, and the physicochemical conditions in the environment are not fully understood. This is mainly because each of these factors has been studied independently, and the interactions among them, as well as their influence on MIC, have not been determined. The dynamic process in oil production systems provides a changing environment to the microbial communities with regards to pH, temperature, pressure, water chemistry and flow velocity, that can result in variations of the microbial makeup and activity throughout facilities, making MIC assessment a difficult task. Understanding the impact that environmental and operational conditions have on the microbial communities, and the resulting extent of corrosion, would allow the identification of critical parameters to be considered in MIC prediction models and the development of improved MIC management strategies.

A critical analysis of the literature on MIC indicated that the inaccuracy of its prediction can also be related to the main focus given to SRB populations. None of the MIC prediction models available considers all the microbiological groups whose corrosive capabilities have been demonstrated. As models have mainly focused on SRB, their focus is also limited to sulphate and do not consider other nutrients that benefit other microbial groups. For instance, low sulphate produced water and desulphated injection water, used in recent facilities to prevent souring and MIC, is known to be limited in SRB; however, other microbial populations can thrive and

potentially cause corrosion [115, 131]. A better understanding of the causative microorganisms, their metabolic activities associated with corrosion and their interactions with other species are required to propose a new prediction model that considers the potential contribution of several microbial groups previously involved in MIC.

MIC research has been usually carried out using single-species biofilms under simplified laboratory conditions. Nonetheless, natural biofilms are multispecies communities that affect electrochemical processes in ways that monocultures cannot simulate. It is known that multispecies communities develop synergistic and competing metabolisms that can promote higher corrosion rates compared to single-species communities. Hence, MIC understanding requires more research with multispecies biofilms, ideally recovered from industrial facilities, which will allow studying interactions among potential corrosive species and the complex metabolic processes that can lead to MIC.

Finally, the literature review also highlights that the common methods used in MIC assessment evaluate the presence and concentration of microorganisms in the system, but they do not evaluate their metabolic state. Since microorganisms can be in a dormant state, it is important to identify the active microbes in the system and the metabolic activities they are carrying out in situ. Therefore, microbiological assessment in oil and gas production systems should include more analyses that reveal the metabolic status of the microbial populations. Likewise, there is a need to implement data mining tools that facilitate data integration and interpretation for an accurate assessment of the MIC risk.

1.3 Aim and Objectives

This investigation aims to improve our understanding of MIC on carbon steel by implementing fundamental research to study the effect of environmental and operational conditions on multispecies biofilms and the resulting risk of MIC.

Likewise, the investigation intends to contribute to the field through the identification of critical aspects, novel MIC causative microorganisms, and more suitable molecular methods that can improve MIC assessment and prediction. On this basis, the specific objectives of this study are as follows:

- Develop a more robust methodological approach that can improve the assessment of microbial communities and can contribute to more effective MIC management in industrial facilities.
- Investigate the effect that environmental conditions, such as temperature and availability of sulphur compounds, have on the composition and structure of microbial communities and the resulting impact on MIC of carbon steel.
- Evaluate the impact that common operating conditions such as flow regime can have on biofilm characteristics and the subsequent effect on MIC and biocide effectiveness.
- Characterise microbial communities in corroded industry equipment and study novel, potential causative microorganisms not considered in existing MIC prediction models.
- Identify critical aspects that influence the risk of MIC in industrial facilities and may improve the accuracy of prediction models if they are considered.

1.4 Significance of the Research and Contribution

Annually, MIC generates millions of dollar losses to the global industry [16, 132]. Costs are related to the repair of corroded infrastructure, and the implementation of MIC management plans to reduce the risk of MIC. This economic impact, together with safety and environmental consequences derived from the loss of assets integrity is a current concern of oil and gas operators. Even though MIC has been studied for several decades [37], fundamental knowledge gaps persist in the understanding of corrosion mechanisms, implicated microorganisms and their metabolic pathways,

and the environmental factors that can lead to MIC of steel. Today, MIC remains highly unpredictable even though several models for its prediction have been proposed.

MIC management includes opportune detection of the microorganisms living in the system. Hence, part of the success of the management process depends on the accuracy of methods used for microbiological assessment and their appropriate execution. In the last decades, methods based on the analysis of the DNA molecule have become popular for the microbiological characterisation of oilfield samples [102, 110, 133]. However, different to the traditional culture-dependent MPN, which is supported by standards that include sampling and preservation protocols [105], at present, there are not standardised protocols for preservation, storage, and processing of samples for molecular assessment. Adequate preservation and storage of samples are essential in maintaining microbial composition and structure of communities as they are in the system. Results from this research provide a base understanding of the implications of this crucial procedure in the MIC assessment.

Microbiological assessment can also be improved if, besides the identification of total microorganisms in the system, information related to their activity can be obtained. It is known that DNA-based analysis cannot discriminate between active and inactive species, and considering that active microorganisms are the ones expected to accelerate corrosion, the need for methods that allow identification of the active members of the microbial community is apparent.

This dissertation proposes a new methodology, including RNA-based sequencing, for identification of active microbial species as part of the MIC assessment, which was successfully applied in this investigation. The approach was demonstrated as an important tool to better characterise the microbial communities in oil production facilities. Likewise, our research described relevant bioinformatics tools that can be implemented in further investigations for improving the understanding of the microbial ecology and the metabolic capabilities of communities in systems experiencing corrosion. Information obtained from this research will assist oil producers in the development and implementation of improved MIC management strategies.

Advancing the understanding of MIC requires further laboratory studies that simulate different field conditions to assess their implications on the risk of MIC. Today, most of the laboratory research on MIC has used monospecific cultures of reference strains, and only a few corrosion studies have been conducted using multispecies microbial consortia recovered from industry assets and corroded structures. Microbial recovery from corroded assets is not always possible due to the difficulty of collecting samples from internal surfaces. However, this research included the recovery of pure microorganisms and multispecies consortia from oil production facilities experiencing corrosion, which were used for laboratory experiments and remain available as a microbial bank at Curtin University for further MIC studies.

Results from this research fill knowledge gaps on the effect that environmental conditions such as temperature, water chemistry and flow regime have on the composition and microbial structure of multispecies biofilms formed on carbon steel, and the subsequent impact on MIC rates. Hence, this research provides valuable information for improving MIC assessment and for refining existing MIC prediction models.

1.5 Overview of the Research Design

This research used several methods to assess MIC in field and laboratory studies. Overall, culture-dependent and culture-independent methods were applied to characterise, enumerate, identify and recover microbial communities from industry assets experiencing corrosion. Microorganisms recovered from oil facilities were exposed to carbon steel samples to form single or multispecies biofilm communities, and their ability to cause corrosion of carbon steel was evaluated. Microbial growth and biofilm formation were conducted in anaerobic reactors sparged with a sterile gas mixture of 80% N_2: 20% CO_2 at a flow rate of 20 ml min^{-1}. Reactors were operated under batch, semi-batch, and continuous flow of nutrients depending on the objective of each experiment. Composition of the test solution, flow of nutrients, temperature conditions, and experimental exposure times varied across the different

laboratory studies depending on the research question (details are given in each Chapter). All conditions were simulated in both biotic reactors (in the presence of microorganisms) and abiotic reactors (without microorganisms) to discriminate baseline abiotic corrosion rates.

1.5.1 Microorganisms and Test Methods Used in This Research

- ***Microbial recovery***

Microorganisms inhabiting different oil production facilities with a history of MIC were recovered and used for the laboratory studies in this research. Enrichment and isolation of single species and multispecies consortia from onshore and offshore oil facilities were performed using traditional microbiological methods (details of the microbial recovery are presented in Appendix 1).

- ***Microbiological methods***

Microbial enumeration: enumeration of microorganisms was conducted through culture-dependent and culture-independent methods. Detection and quantification of microorganisms previously associated with MIC were carried out using the serial dilution method described in the NACE standard TM0194 [105], and the MPN 3-tube method described elsewhere [134]. Enumeration of total bacteria in biofilms was performed using the quantitative polymerase chain reaction (qPCR) method for the detection of the *rpoB* (single copy gen) instead of the commonly used 16S rRNA gene, which is known to have multiple copies.

Microbial activity: microbial activity and adenylate energy charge of planktonic and biofilm communities were estimated through the quantification of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). Concentrations of ATP, ADP, and AMP were determined by luminescence after reaction with luciferin-luciferase using commercial kits (Luminultra Technologies Ltd.).

Microbial community composition: 16S rRNA next-generation sequencing (NGS) from the DNA and RNA molecule was applied to identify the composition of total and active communities in field samples and biofilms grown in laboratory conditions.

- ***Bioinformatics tools***

Several bioinformatics tools were used for the NGS data analysis. The Quantitative Insights Into Microbial Ecology Software - Qiime (v1.9.1 and v2019.4) [135] was the primary tool used for the analysis of the 16S rRNA gene sequences generated with the Illumina MiSeq. Qiime2 plugins, q2-dada2 [136], q2-feature-table, q2-feature-classifier [137], q2-classify-consensus-blast [138], q2-core-metrics-phylogenetic and q2-alpha-diversity were used for raw data processing. Other software applied in the bioinformatics pipeline were: PEAR (v0.9.10 - 64 bit) [139], Cutadapt (v1.10) [140], USEARCH (v10.2) [141], UCHIME [142], and VSEARCH (v1.1.3) [143]. SILVA database (SILVA v132) [144] was used for taxonomic classification. Statistical analyses and graphs were conducted employing R (v3.4.3) [145], and PAST (v3) [146] software. The R packages Venndiagram [147], ggplot2 [148], vegan [149], and indicSpecies [150] were used for microbial ecology analysis. The Tax4Fun [151] and Tax4Fun2 [152] R packages were used to predict the functional profile of microbial communities.

- ***Microscopic analysis of biofilms and corrosion products***

The distribution of live and dead cells within biofilms was determined using the confocal laser scanning microscopy (CLSM). Biofilms were also examined and visualised with field-emission scanning electron microscope (FESEM). The elemental composition of corrosion products was determined with FESEM, coupled with X-ray-spectroscopy (EDS).

- ***Material and metal surface preparation***

Carbon steel coupons with an elemental composition of (weight %): C (0.43 - 0.5), Mn (0.6 - 0.9), Si (0.15 - 0.35), S (0.01 - 0.35), P (0 - 0.035), Cr (0 - 0.40), and Fe (balance) were used in all corrosion studies. Metal surfaces were prepared through wet grinding-polishing, using silicon carbide papers of 80, 120, 320, and 600 grit consecutively (details of the selection of the metal surface preparation are presented in Appendix 2).

- ***Corrosion rates evaluation***

General corrosion rates were measured with the weight loss method described in the ASTM G1 standard [153]. Localised corrosion was assessed using light optical microscopy (LOM) and pitting rates estimated based on the deepest pits found in each sample, as indicated in the NACE standard SP0775 [154]. Pits were measured with a 3D optical profilometer (Alicona InfiniteFocus G4) using the instrument's software (IFM version 3.5).

1.6 Thesis Outline

This thesis is assembled as a hybrid of seven published peer-reviewed journal articles, and two peer-reviewed conference articles. The objectives of this dissertation were addressed through Chapters 2 to 5 as follows:

Chapter 2 is comprised of two (2) studies centred in improving the microbiological assessment of industrial facilities. The first study illustrates the variation of the molecular assessment of microbial communities in oilfield samples as a result of sample storage and preservation conditions. The data generated in this work provided evidence of the importance of sample handling to prevent changes in microbial populations and subsequent accurate assessment of MIC risk.

The second study proposes a new complementary methodological approach coupled with extensive bioinformatics analysis to assess microbial populations potentially involved in MIC of industry assets. The complementary approach consisted of the combined sequencing of the 16S rRNA gene (DNA) and transcripts (RNA) that allowed the identification of not only total but also active species in the system. The suitability of RNA-based sequencing analysis for improving the understanding of MIC was demonstrated in the following chapters.

Chapter 3 is comprised of two (2) studies that focused on evaluating the impact that common environmental conditions have on biofilm communities formed on carbon steel and on the resulting MIC rates. The first study presents the effect of temperature on multispecies biofilms formed on carbon steel and how changes on the active microbial populations can alter the risk of MIC. Our findings showed that active microbial populations varied as a function of the temperature, which resulted in differences in cell numbers, microbial activity, and corrosion rates caused by biofilms formed at different temperatures.

The second study evaluates the impact that sulphur compounds, named sulphate and thiosulphate, have on the risk of MIC in carbon steel. For this research, a different oilfield microbial consortium was used. The effect of temperature was included in this study to verify if a different trend can be observed with a different microbial community. Results from this research showed that active microorganisms varied among different conditions confirming that changes in the environment have an impact on microbial communities and in the subsequent MIC risk.

Chapter 4 presents a comprehensive study on how the nutrient level determines biofilm characteristics and its subsequent impact on microbial corrosion. In this research, two microbial consortia were exposed to a continuous flow of nutrients as well as to stagnant conditions, which represented typical flow regime conditions found in oil production facilities. Biofilms formed under flowing conditions were more active, thicker and robust than biofilms exposed to stagnant, nutrient-depleted

conditions. Biofilms with higher ATP concentration resulted in higher corrosion rates, a pattern that was equally observed in the studies presented in Chapters 3, and suggested that activity level measured as the concentration of ATP is an important parameter to include in the MIC assessment. This work also indicated that nutrient level affects biocide effectiveness since a higher number of biofilm cells survived to treatment when growth was developed under a continuous flow of nutrients.

Chapter 5 is comprised of four (4) studies focused on the identification and characterisation of potential MIC causative microorganisms. The first study presents the microbial composition of biofilm communities in corroded assets suspected of MIC. The study revealed that predominant microorganisms in corroded equipment are associated with microbial groups different from the ones commonly included in MIC prediction models (SRB or methanogens). Nine bacterial species were isolated from corroded seal rings and their potential to cause corrosion studied under laboratory-controlled conditions. This study demonstrated that all isolated microorganisms, which belonged to the IOB, APB, TRB and NRB microbial groups, have abilities to cause corrosion of carbon steel through different mechanisms.

The other three studies are related to the full genome sequence of three bacteria isolates from the aforementioned corroded seal rings. These isolates were selected for further studies and full genome sequence based on the fact that they had not been related to MIC before. Analysis of the genomes allowed to evidence the presence of genes involved in metabolic pathways previously associated with MIC and provided information on the potential MIC mechanisms that these microbes can promote.

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Chapter 2

Improving Microbiological Assessment

This chapter is comprised of two (2) published papers.

S.J. Salgar-Chaparro and L.L. Machuca. Effect of sample storage conditions on the molecular assessment of MIC, in Proceedings of the Corrosion & Prevention Conference, Paper no. 46. The Australasian Corrosion Association Inc. Nov 11-14 2018, Adelaide, Australia.

S.J. Salgar-Chaparro and L.L. Machuca. Complementary DNA/RNA-based profiling: Characterisation of corrosive microbial communities and their functional profiles in an oil production facility. Frontiers in Microbiology, 10: 2587 (2019).

2.1 Study 1

Effect of sample storage conditions on the molecular assessment of MIC

S.J. Salgar-Chaparro and L.L. Machuca. Effect of sample storage conditions on the molecular assessment of MIC, in Proceedings of the Corrosion & Prevention Conference, Paper no. 46. The Australasian Corrosion Association Inc. Nov 11-14 2018, Adelaide, Australia.

An original reprint of the publications is included in Appendix 3

2.2 Study 2

Complementary DNA/RNA-based profiling: Characterization of corrosive microbial communities and their functional profiles in an oil production facility

S.J. Salgar-Chaparro and L.L. Machuca. Complementary DNA/RNA-based profiling: Characterisation of corrosive microbial communities and their functional profiles in an oil production facility. Frontiers in Microbiology, 10: 2587 (2019). https://doi.org/10.3389/fmicb.2019.02587

An original reprint of the publications is included in Appendix 3

Chapter 3

Effect of Environmental Conditions

This chapter is comprised of two (2) published papers.

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel, in: Corrosion 2019 Conference & Expo, Paper no. 13343. NACE International. Mar 25-28, Nashville, US.

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms. Corrosion Science, 169: 108595 (2020).

3.1 Study 3

Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel, in: Corrosion 2019 Conference & Expo, Paper no. 13343. NACE International. Mar 25-28, Nashville, US.

An original reprint of the publications is included in Appendix 4

3.2 Study 4

Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms. Corrosion Science, 169: 108595 (2020). https://doi.org/10.1016/j.corsci.2020.108595

An original reprint of the publications is included in Appendix 4

Chapter 4

Effect of Operational Conditions

This chapter is comprised of one (1) published paper.

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Nutrient level determines biofilm characteristics and subsequent impact on microbial corrosion and biocide effectiveness. Applied and Environmental Microbiology, 86 (7) e02885-19 (2020).

4.1 Study 5

Nutrient level determines biofilm characteristics and subsequent impact on microbial corrosion and biocide effectiveness

S.J. Salgar-Chaparro, K. Lepkova, T. Pojtanabuntoeng, A. Darwin, L.L. Machuca. Nutrient level determines biofilm characteristics and subsequent impact on microbial corrosion and biocide effectiveness. Applied and Environmental Microbiology, 86 (7) e02885-19 (2020). https://doi.org/10.1128/AEM.02885-19

An original reprint of the publications is included in Appendix 5

Chapter 5

MIC Causative Microorganisms

This chapter is comprised of four (4) published papers.

S.J. Salgar-Chaparro, A. Darwin, A. Kaksonen, L.L. Machuca. Carbon Steel Corrosion by Bacteria from Failed Seal Rings at an Offshore Facility. Scientific Reports, 62292 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Pseudomonas balearica* strain EC28, an iron-oxidising bacterium isolated from corroded steel. Microbiology Resource Announcements, 9:e00275-20 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Draft Genome sequence of *Enterobacter roggenkampii* strain OS53, isolated from corroded pipework at an offshore oil production facility. Microbiology Resource Announcements, 9:e00583-20 (2020).

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Shewanella chilikensis* strain DC57, isolated from corroded seal rings at a floating oil production system in Australia. Microbiology Resource Announcements, 9:e00584-20 (2020).

5.1 Study 6

Carbon Steel Corrosion by Bacteria from Failed Seal Rings at an Offshore Facility

S.J. Salgar-Chaparro, A. Darwin, A. Kaksonen, L.L. Machuca. Carbon Steel Corrosion by Bacteria from Failed Seal Rings at an Offshore Facility. Scientific Reports, 62292 (2020). https://doi.org/10.1038/s41598-020-69292-5

An original reprint of the publications is included in Appendix 6

5.2 Study 7

Complete genome sequence of Pseudomonas balearica strain EC28, an iron-oxidizing bacterium isolated from corroded steel

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Pseudomonas balearica* strain EC28, an iron-oxidising bacterium isolated from corroded steel. Microbiology Resource Announcements, 9:e00275-20 (2020). https://doi.org/10.1128/MRA.00275-20

An original reprint of the publications is included in Appendix 6

5.3 Study 8

Draft genome sequence of Enterobacter roggenkampii Strain OS53, isolated from corroded pipework at an offshore oil production facility

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Draft Genome sequence of *Enterobacter roggenkampii* strain OS53, isolated from corroded pipework at an offshore oil production facility. Microbiology Resource Announcements, 9:e00583-20 (2020). https://doi.org/10.1128/MRA.00583-20

An original reprint of the publications is included in Appendix 6

5.4 Study 9

Complete genome sequence of Shewanella chilikensis Strain DC57, isolated from corroded seal rings at a floating oil production system in Australia

S.J. Salgar-Chaparro, G. Castillo-Villamizar, A. Poehlein, R. Daniel, L.L. Machuca. Complete genome sequence of *Shewanella chilikensis* strain DC57, isolated from corroded seal rings at a floating oil production system in Australia. Microbiology Resource Announcements, 9:e00584-20 (2020). https://doi.org/10.1128/MRA.00584-20

An original reprint of the publications is included in Appendix 6

Chapter 6

Summary, Conclusions and Future work

6.1 Summary and Conclusions

To effectively manage the risk of corrosion in any system, it is essential to clearly understand the unique conditions that may promote it in different assets. Since MIC is one of the corrosion threats most challenging to predict and control in oil and gas production systems, the need to continue advancing its understanding is well known in both academia and industry. At present, several knowledge gaps persist in fundamental aspects of MIC, including all potential causative microorganisms and the mechanisms by which they may trigger corrosion. Furthermore, there is a lack of understanding of how environmental parameters impact the behaviour of biofilm communities and the kinetics of the MIC reactions on carbon steel. This limited knowledge has resulted in inaccurate protocols and practices for the assessment and prediction of MIC in industry.

This investigation aimed to advance and improve the way MIC is assessed. Hence, the first objective of the research was to develop a new methodological approach to improve the assessment of microbial communities in systems with corrosion issues. It is nowadays accepted that molecular microbiological methods are the most suitable methods for microbial characterisation. However, the lack of standards that describe the appropriate process for collection, preservation, storage, and processing of samples for molecular analysis has resulted in oil operators often not aware of the importance of these crucial steps in the microbiological assessment. Biases introduced by sample handling prior to molecular analysis can lead to unreliable data and an inaccurate risk assessment. Therefore, our first study evaluated the effect of different sample storage and preservation conditions on microbial composition by 16S rRNA sequencing. For this, water and sludge samples from an oil facility were collected and stored at room temperature and under refrigeration, simulating common procedures of oil operators. Microbial composition was compared with reference samples that were processed on-site and preserved immediately after collection. Results showed that the relative abundance of the microbial populations, the structure, and functional capability of the community changed as a result of storage conditions. This work demonstrated that microbiological analysis can

underestimate or overestimate the risk of MIC if sampling, preservation, and storage are not executed correctly.

The next step of the research focused on identifying a suitable method to improve the microbiological assessment in corroded systems. MIC molecular assessment has been primarily centred on DNA-based analyses. Although the study of DNA has yielded unprecedented insights into the microbial ecology of corrosive environments, the persistence of DNA in dormant or inactive microorganisms means that the analysed communities comprise both active and inactive species. Since aggressive MIC is likely to be mainly caused by active species, the distinction of the active members in the community should be critical in MIC assessment. We proposed a new complementary approach combining both 16S DNA-based sequencing and 16S RNA-based sequencing. RNA-based analyses provided a better representation of active species since the cellular rRNA concentration is well correlated with growth rate and activity of the microorganisms, and also because RNA deteriorates rapidly after cell death. RNA-based sequencing has become popular in microbial ecology investigations as an approach to generate information of active members in the communities inhabiting different environments. However, the suitability of 16S RNA-based sequencing for identification of active microbial populations involved in corrosion had not been examined before. Our results demonstrated that DNA results alone could lead to underestimation of active species in oil production systems. The benefits of using bioinformatics tools for processing complex molecular data sets were also highlighted in our research. Bioinformatics analyses, such as correlation-based network analysis, functional predictions from 16S rRNA data, and ordination analysis with environmental fitting allowed for the visualisation of differences in microbial communities across an oil facility, as a result of biocide treatment and environmental conditions. Bioinformatics tools applied through our research were essential for understanding the microbial ecology and metabolic capabilities of communities inhabiting complex environments in oil production systems.

The next stage of the investigation focused on addressing knowledge gaps on the impact that environmental and operational conditions found in oil fields have on the behaviour of biofilms and the resulting MIC. To achieve this, we considered it critical

to use microbial consortia from oil fields. Microorganisms were harvested from produced water from three oil production facilities comprising different water chemistries. Microbial consortia were then used to grow multispecies biofilms on carbon steel under different exposure conditions. The first laboratory experiments evaluated the effect of temperature on microbial communities, and the resulting impact on MIC rates. In order to simulate the temperature ranges typically found in oil production facilities, a microbial consortium was exposed to two different temperatures (40 °C and 60 °C) and allowed to form biofilms on carbon steel. Results showed that the active microbial populations in biofilms, as well as corrosion rates and mechanisms, changed in response to temperature. Biofilms developed at mesophilic conditions (40 °C) caused higher corrosion rates compared to biofilms grown at thermophilic conditions (60 °C). Greater corrosion was correlated with higher microbial activity and cell numbers.

The second set of experiments focused on investigating the impact that sulphur compounds, namely sulphate and thiosulphate, have on microbial communities and MIC. Several prediction models include sulphate concentration as an essential input parameter; however, other sulphur compounds such as thiosulphate are generally not considered. Due to the widespread presence of thiosulphate reducing bacteria in oil fields, we considered essential to investigate the role of thiosulphate in the activity of biofilms and the severity of MIC. The effect of temperature on MIC was also evaluated in this study to verify previous findings, this time using a different microbial consortium that exhibited higher diversity of thermophilic microorganisms and methanogens. The microbial consortium used in this study was recovered from a sulphate depleted produced water, which made it more suitable for studying the effect of sulphur compounds. Results showed a different corrosion trend in response to temperature and the nutrients available. In the presence of sulphur compounds, the trend was similar to our previous study were biofilms formed at 40 °C were found to generate higher corrosion rates compared to biofilms developed at 60 °C. Conversely, in the absence of sulphur compounds, biofilms grown at 60 °C caused higher corrosion rates than biofilms formed at 40 °C. Hence, our research indicated that both low or high thermal conditions may be expected to be equally corrosive;

the severity of the process depends on the microbial species present and the nutrients available. With regards to sulphur compounds, our research showed that biofilms caused higher MIC rates when sulphur compounds were present compared to sulphur-absent conditions. The highest corrosion rate was observed when biofilms developed in the presence of thiosulphate alone, which highlights the importance of including other nutrients besides sulphates as an input parameter in MIC prediction models. A correlation between biofilm maturity and the corrosion rate was evidenced in this investigation. Older, mature biofilms caused higher corrosion rates than younger biofilms, suggesting that mitigation treatments should target the prevention of biofilm maturation in field. In this study, the active members of the biofilm community varied among the different experimental conditions, which confirms that changes in the environment have an impact on microbial communities and in the subsequent MIC risk.

The third laboratory study examined the effect that nutrient level has on biofilm characteristics and the subsequent impact on microbial corrosion and biocide effectiveness. For this work, oilfield microorganisms were exposed to either a continuous flow of nutrients or stagnant conditions, which represent typical flow regime conditions found in oil production facilities. Experiments were conducted using two different oilfield microbial consortia to study whether different microbial communities would respond differently to the flow regime. Results showed that biofilms formed under flowing conditions were more active, thicker and robust than biofilms exposed to stagnant, nutrient-depleted conditions. As observed in our previous experimental studies, more active biofilms caused higher corrosion rates. An effect of nutrient level on the effectiveness of biocide was also observed. A higher number of biofilm cells survived the biocide treatment when biofilms were formed under a continuous flow of nutrients. Results of the RNA-based sequencing revealed that some microorganisms related to the *Petrotoga*, *Methanobacterium*, *Methanoculleus*, *Pseudomonas* and *Bacillus* genera remained active after biocide treatment. The identification of active microorganisms after chemical treatment highlights the importance of including RNA-based methods for monitoring the

effectiveness of mitigation plans implemented in industrial systems, and potentially provide the opportunity to detect biocide resistance in a timely manner.

Finally, the last stage of the research focused on the study of microorganisms recovered from equipment that suffered corrosion failure in an oil production facility. One of the common weaknesses found in MIC prediction models is their main focus on SRB. Therefore, this study aimed to gather evidence of unusual MIC causative microorganisms, unlikely to be considered in existing MIC prediction models. For this, corrosion products from seal rings that failed due to corrosion in a floating production storage and offloading (FPSO) facility were collected for microbiological characterisation and bacteria isolation. Biofilm communities on the corroded seal rings were dominated by microorganisms from microbial groups different to SRB such as IOB, APB, and NRB, which were believed to be one of the key contributing factors to the corrosion failure. Nine bacterial species were cultivated and isolated from corrosion products, and their ability to cause corrosion of carbon steel was demonstrated. Since most of the isolated bacteria had not been related to MIC before, the full genome characterisation of three isolates, named *Pseudomonas balearica*, *Shewanella chilikensis*, and *Enterobacter roggenkampii*, was performed to have a better understanding of their metabolic capabilities. Analysis of the genomics data revealed that the three isolates have genes involved in metabolic pathways that have been previously associated with MIC, such as iron utilisation, sulphide production, and nitrate reduction, which provided support to the corrosive potential of these isolates as observed in laboratory experiments.

Overall, this dissertation evaluated critical aspects of MIC and generated valuable information for the understanding and management of this corrosion threat. Results of this research allowed to postulate the following factors as essential aspects for improving MIC prediction and management in oilfields. First, microbiological data must be reliable and meaningful. Second, nutrients required for metabolic activities are critical in determining corrosion rates and should be carefully considered in MIC prediction. Third, physical and chemical factors potentially affecting microbial communities and their activity need to be considered for identifying corrosion circuits in industrial facilities. Fourth, microbial activity, in this study measured as ATP

concentration, should be monitored and included in MIC prediction models. Fifth, mitigation treatments should focus on preventing biofilm maturation. Sixth, the participation of all microbial groups associated with MIC must be considered for the MIC prediction. Seventh, the evaluation of the effectiveness of mitigation strategies to control the risk of MIC should always be included in monitoring plans. Hence, the outcomes of this research contribute to better address the steps of MIC management in the industry.

6.2 Study Limitations

As stated before, MIC is a complex phenomenon where several factors interact at the same time and influence corrosion reactions. To have a better understanding of the conditions that can enhance microbial corrosivity, it is important to study biofilm communities as they form in industrial systems. Although this research used multispecies consortia recovered from oilfields to overcome the limitations of single species biofilms, it has to be acknowledged that the investigations of this research are still laboratory studies that cannot completely simulate field conditions. For example, the diversity and structure of the microbial communities used in the laboratory studies varied compared to the in situ microbial communities from the industrial systems these were recovered from. The variation observed between the microbial species in situ and the ones cultivated in laboratory leaves a gap on the possible behaviour and interaction of non-cultured microorganisms with metal surfaces. Hence, MIC studies developed in field, for instance using biofilm monitoring facilities located on site, would add value to laboratory-based MIC experimentation.

Even though the use of field multispecies microbial communities for MIC studies remains the preferred choice to advance the knowledge of MIC, this approach can also bring some limitations. For example, it remains challenging to obtain the exact same microbial consortium in two different studies. Although the same species can be present in the inoculum, the microbial community structure, i.e., the relative abundances of each species in the community, can vary as a result of variables

associated with culturing, which makes it very difficult to replicate the same conditions multiple times.

Another limitation of this research is the difficulty in establishing the corrosion mechanisms observed in the laboratory studies. Even though likely MIC mechanisms were inferred, it was not possible to identify the root cause of carbon steel deterioration. This limitation was also related to the use of multispecies biofilms, which is known to create a complex environment where the contribution of individual species to the overall corrosion process is impossible to identify. Additional analysis such as metatrascriptomics studies could help address this limitation by revealing microbial pathways in the community and thus, be able to establish associations between microbial activities and accelerated corrosion.

6.3 Future Work

The results of this dissertation can form the foundations for the development of a more accurate methodology to predict MIC risk and to measure the probability of failure due to MIC. This investigation, while filling knowledge gaps about the MIC phenomenon, also raises questions about other potential physical or chemical variables found in industry settings systems that can influence MIC. Further studies to investigate how communities respond to different operational or environmental changes such as oxygen ingress, flow velocity, pressure, and presence of deposits, would complement the information generated in this research and consolidate more robust MIC management systems.

The advantages of implementing RNA-based molecular microbiological methods were widely discussed and demonstrated in this research. This complementary methodological approach could be implemented as a routine practice in industrial facilities for improving microbiological assessment. However, obtaining RNA of high quality can be challenging, and therefore, further work is required for the optimisation of RNA extraction from complex samples such as corrosion products,

oily water, deposits, pig debris and complex biofilms. Once high-quality RNA is obtained, more specialised techniques such as transcriptomics and metatranscriptomics can be applied to laboratory experiments and field samples. These techniques will provide not only information about metabolically active species present on corroded steel but also an unprecedented information about in situ metabolic pathways and gene expression of microorganisms, which could be used to associate up-regulated genes with corrosion rates and mechanisms. The identification of genes directly associated with MIC opens the possibility of establishing bioindicators for detecting corrosion based on gene expression.

Bacterial isolates and multispecies consortia recovered from corroded structures and oilfields that experienced MIC remain available in a microbial bank at Curtin University. These microorganisms can be further investigated in laboratory experiments to understand some of the MIC mechanisms that are yet to be fully understood. Some of the microorganisms were recovered from industrial systems exposed to biocide treatment which indicates that these microbes can potentially display greater tolerance or resistance to antimicrobial substances than other microorganisms. Therefore, these microorganisms can be applied in inhibition studies such as evaluation of biocide efficiency and microbial resistance.

Genome sequences of microbial species recovered from corroded seal rings were released in public databases. Comparative genome studies of species isolated from corroded environments could be conducted in the future to identify genomic differences potentially related to corrosion. Genome sequences can also be used to develop specific probes (primers) to detect the presence of genes associated with metabolic pathways related to increased MIC rates. These sequences can also serve as reference genomes for the data analysis of gene expression studies (transcriptomics or metatranscriptomics) involving these corrosive microorganisms.

Appendices

Appendix 1

Microbial Recovery from Field Samples

Recovery of Microorganisms from Oil Production Systems

1 Introduction

This dissertation comprised the characterisation of microbial communities present in the produced water of three (3) offshore and one (1) onshore oil production facilities experiencing MIC in Western Australia. Microbial consortia from these facilities were recovered in the laboratory for the execution of the studies carried out in this research (Chapters 2 to 4). The chemistry of the produced water in the sampled oil production facilities has some differences, which favoured the recovery of microbial consortia with different species composition and community structure.

2 Methods

2.1 Recovery of microorganisms

Produced water from the oil production facilities was collected in sterile containers and transported to the laboratory at 4°C for the microbial recovery. Samples were inoculated in several culture media to maximise the recovery of the cultivable microbial species. Culture media targeted the growth of sulphate-reducing bacteria (SRB), thiosulphate-reducing bacteria (TRB), methanogenic archaea (MET), acid-producing bacteria (APB), iron-reducing bacteria (IRB), iron-oxidising bacteria (IOB), and general heterotrophic bacteria (GHB). Culture media were prepared following the guidelines and composition described in the NACE standard TM0194 [1] for the MET (methanogenic acid), APB (dextrose phenol red broth), IRB (iron reducing bacteria), IOB (iron oxidizing bacteria), GHB (general aerobic heterotrophic broth); the composition of the media used for the recovery of SRB and TRB was described by Suarez et al., [2]. Salinity of the media was adjusted to 2% with sodium chloride to mimic the conditions microorganisms have in the field. Culture media were incubated

at 40 °C and 60 °C to recover mesophilic and thermophilic microorganisms present in the oil production facilities. For the establishment of one microbial consortium per facility, the culture media that exhibited positive growth were mixed and used as inoculum for the MIC studies.

2.2 16S rRNA diversity profiling

Microorganisms present in the produced water were harvested by filtration through sterile 0.2 µm pore size polyethersulfone membranes. DNA was extracted from filters using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions. DNA concentration was quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies). The hypervariable region V3–V4 of the 16S rRNA gene was amplified using the universal primers 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3'), and sequencing was conducted in a MiSeq® System - Illumina by the Australian Genome Research Facility (AGRF). Raw sequences analysis was performed as described in Chapter 2 [3].

2.3 Chemical characterisation

Chemical analysis of produced water was carried out following US EPA, APHA (American Public Health, 2005), and in-house test methods as described in Chapter 2 [3]. Table 1 describe the methods used for each chemical analysis.

Table 1. Methods used for the chemical analysis of the produced water

pH	pH meter
Conductivity	Conductivity meter
Total petroleum hydrocarbons	Gas chromatography-flame ionization detector
Volatile fatty acids	High performance liquid chromatography
Metals (Fe, Mg, Na, K, Ca)	Atomic absorption spectroscopy
Metals (S, Cr, Cu, Mn, Ni, Zn)	Inductively coupled plasma optical emission spectrometry
Nitrogen, nitrate, nitrite	Colorimetric/Turbidimetric Aquakem System
Sulphate, phosphorous, chloride	Colorimetric/Turbidimetric Aquakem System
Thiosulphate	Iodate/iodide titration
Total organic carbon	High temperature combustion
Alkalinity	Titration

3 Results

Characteristics of the sampled oil production facilities and the microorganisms recovered from each system are described in the next sections.

3.1 Characteristics of the onshore facility

Oil Production facility: Onshore oilfield

Sample: Produced water, injection water, sludge

Average operating temperature: 45 °C

Water chemistry: Low sulphate field – Description in Table 2

Microbial community composition: Description in Table 3

Table 2. Water chemistry of produced water from onshore oilfield

pH	7.02
Conductivity (mS/cm)	59.2
Calcium (mg/L)	490
Iron (mg/L)	4.9
Potassium (mg/L)	170
Magnesium (mg/L)	240
Manganese (mg/L)	0.07
Sodium (mg/L)	14,000
Sulphur (mg/L)	<0.05
Zinc (mg/L)	<0.01
Ammonia-N (mg/L)	43
Chloride (mg/L)	25,000
Sulphate (mg/L)	<1
Thiosulphate (mg/L)	<2
Alkalinity (mg CaCO3/L)	540
Salinity (mg/L)	38,000
Acetic Acid (mg/L)	33
Propionic Acid (mg/L)	28
Total Organic Carbon (mg/L)	56
Total Nitrogen (mg/L)	43
Total Phosphorus (mg/L)	0.15
NOx-N	<0.01
Nitrate-N	<0.01
Nitrite-N	<0.01

Table 3. Microbial community composition of produced water in the onshore oilfield

Caminicella (Clostridiales)	22.5
Thermovirga (Synergistales)	14.6
Methanothermococcus (Methanococcales)	5.5
Uncultured bacterium (Firmicutes)	5.1
Desulfothermus (Desulfovibrionales)	4.9
Pelobacter (Desulfuromonadales)	4.7
Roseospira (Rhodospirillales)	4.4
Thermosipho (Thermotogales)	4.2
Thermoanaerobacter (Thermoanaerobacterales)	3.4
Syntrophomonas (Clostridiales)	3.0
Methanoculleus (Methanomicrobiales)	3.0
Thermococcus (Thermococcales)	3.0
Kosmotoga (Kosmotogales)	2.5
Methanosaeta (Methanosarcinales)	2.4
Acetomicrobium (Synergistales)	2.2
Methylobacterium (Rhizobiales)	1.7
Thermacetogenium (Thermoanaerobacterales)	1.6
Desulfomicrobium (Desulfovibrionales)	1.4

Values correspond to the relative abundance (%) of the genera in the sample. Only dominant microorganisms with relative abundance ≥ 1% are listed in the table.

Samples recovered from this field were used in the studies 1 "*Effect of sample storage conditions on the molecular assessment of MIC*" and 2 "*Complementary DNA/RNA-based profiling: Characterisation of corrosive microbial communities and their functional profiles in an oil production facility".*

3.2 Characteristics of the FPSO facility No. 1

Oil Production facility: FPSO 1

Sample: Produced water from a low-pressure separator

Average operating temperature: 57 °C

Water chemistry: High sulphate field – Description in Table 4

Microbial community composition: Description in Table 5

Table 4: Water chemistry of produced water from FPSO 1

pH	7.82
Conductivity (mS/cm)	41.7
Calcium (mg/L)	400
Iron (total) (mg/L)	2.8
Potassium (mg/L)	319.5
Magnesium (mg/L)	160
Manganese (mg/L)	0.1
Sodium (mg/L)	8750
Zinc (mg/L)	<0.01
Barium (mg/L)	1
Strontium (mg/L)	14
Chloride (mg/L)	17217
Sulphate (mg/L)	924
Thiosulphate (mg/L)	<2
Alkalinity (mg CaCO3/L)	490
Acetic Acid (mg/L)	270
Propionic Acid (mg/L)	33
Total nitrogen (mg/L)	7.3
Total phosphorus (mg/L)	<0.5
NOx-N	<0.01
Nitrate-N	<0.01
Nitrite-N	<0.01

Table 5: Microbial community composition of produced water in FPSO1

Arcobacter (Campylobacterales)	54.9
Desulfotomaculum (Clostridiales)	18.4
Pelobacter (Desulfuromonadales)	8.9
Desulfovibrio (Desulfovibrionales)	4.6
Acetobacterium (Clostridiales)	2.1
Desulfacinum (Syntrophobacterales)	2.1
Desulfocurvus (Desulfovibrionales)	1.9
Desulfobacter (Desulfobacterales)	1.4

Values correspond to the relative abundance (%) of the genera in the sample. Only dominant microorganisms with relative abundance ≥ 1% are listed in the table.

Microorganisms recovered from this field were used in the Study 3 "*Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel*"

3.3 Characteristics of the FPSO facility No. 2

Oil Production facility: FPSO 2

Sample: Produced water from risers

Average operating temperature: 55 °C

Water chemistry: Low sulphate field – Description in Table 6

Microbial community composition: Description in Table 7

Table 6: Water chemistry of produced water from FPSO 2

pH	7.55
Conductivity (mS/cm)	55.5
Calcium (mg/L)	835
Iron (mg/L)	2.35
Potassium (mg/L)	340
Magnesium (mg/L)	400
Manganese (mg/L)	0.43
Sodium (mg/L)	11500
Nickel (mg/L)	0.02
Sulphur (mg/L)	1
Zinc (mg/L)	<0.01
Arsenic	0.005
Ammonia-N (mg/L)	25.5
Chloride (mg/L)	19500
Sulphate (mg/L)	<1
Thiosulphate (mg/L)	6.8
Alkalinity (mg CaCO3/L)	300
Salinity (mg/L)	36000
Total Organic Carbon (mg/L)	2.5
Total Nitrogen (mg/L)	31.5
Total Phosphorus (mg/L)	0.16
NOx-N	<0.01
Nitrate-N	<0.01
Nitrite-N	<0.01

Table 7: Microbial community composition of produced water in FPSO2

Thermovirga (Synergistales)	23.1
Methanobacterium (Methanobacteriales)	23.0
Methanothermobacter (Methanobacteriales)	14.3
Methanothermococcus (Methanococcales)	9.0
Methanoculleus (Methanomicrobiales)	2.5
Thermosipho (Thermotogales)	1.9
Thermoanaerobacter (Thermoanaerobacterales)	1.5
Flexistipes (Deferribacterales)	1.4
Methanocalculus (Methanomicrobiales)	1.2
Methanococcus (Methanococcales)	1.0

Values correspond to the relative abundance (%) of the genera in the sample. Only dominant microorganisms with relative abundance ≥ 1% are listed in the table. Microorganisms recovered from this field were used in the Study 4 "*Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms*

3.4 Characteristics of the FPSO facility No. 3

Oil Production facility: FPSO 3

Sample: Produced water from low-pressure separator

Average operating temperature: 52 °C

Water chemistry: High sulphate field – Description in Table 8

Microbial community composition: Description in Table 9

Table 8: Water chemistry of produced water from FPSO 3

pH	7.01
Conductivity (mS/cm)	44.2
Calcium (mg/L)	330
Iron (mg/L)	<0.01
Potassium (mg/L)	15
Magnesium (mg/L)	210
Manganese (mg/L)	0.08
Sodium (mg/L)	8100
Nickel (mg/L)	<0.01
Sulphur (mg/L)	530
Zinc (mg/L)	0.02
Arsenic	0.003
Ammonia-N (mg/L)	34
Chloride (mg/L)	18000
Sulphate (mg/L)	1800
Thiosulphate (mg/L)	6.9
Alkalinity (mg CaCO3/L)	770
Salinity (mg/L)	28000
Total Organic Carbon (mg/L)	66
Total Nitrogen (mg/L)	96
Total Phosphorus (mg/L)	7.3
NOx-N	<0.01
Nitrate-N	<0.01
Nitrite-N	<0.01

Table 9: Microbial community composition of produced water in FPSO3

Thermovirga (Synergistales)	13.9
Pelobacter (Desulfuromonadales)	12.1
Desulfacinum (Syntrophobacterales)	12.0
Sulfurospirillum (Campylobacterales)	11.3
Methanohalophilus (Methanosarcinales)	6.4
Thermosipho (Thermotogales)	5.3
Thermoanaerobacter (Thermoanaerobacterales)	4.8
Thalassospira (Rhodospirillales)	3.1
Desulfovibrio (Desulfovibrionales)	3.0
Marinobacterium (Oceanospirillales)	2.6
Marispirillum (Rhodospirillales)	2.5
Methylophaga (Nitrosococcales)	2.1
Marinobacter (Alteromonadales)	1.5
Shewanella (Alteromonadales)	1.2
Methermicoccus (Methanosarcinales)	1.2
Desulfoplanes (Desulfovibrionales)	1.2
Arcobacter (Campylobacterales)	1.0
Methanosaeta (Methanosarcinales)	1.0

Values correspond to the relative abundance (%) of genera in the sample. Only dominant microorganisms with relative abundance ≥ 1% are listed in the table.

Microorganisms recovered from this field were used in the Study 5 "*Nutrient Level Determines Biofilm Characteristics and Subsequent Impact on Microbial Corrosion and Biocide Effectiveness"*

3.5 Microbial composition of the oilfield consortia

Table 10: Microbial community composition of oilfield consortia recovered in the laboratory

	Onshore	FPSO1	FPSO2	FPSO3
Acetomicrobium	45.1			1.1
Anaerobaculum			33.2	
Anaerophaga		51.6		4.8
Anaerosalibacter	28.4			
Bacillus				18.7
Desulfonauticus				
Desulfovibrio				66.9
Dethiosulfatibacter		1.1		
Methanoculleus	9.3			
Methanothermobacter			22.0	
Micrococcus	16.2			
Petrotoga		25.2	10.1	3.6
Pseudomonas		13.0		
Pseudothermotoga		4.7		
Thermoanaerobacter			23.9	
Unclassified *Thermoanaerobacterales*			5.0	
Unclassified *Thermotogaceae*			2.8	
Thermovirga		2.4	2.6	4.1

Values correspond to the relative abundance (%) of the genera in the sample. Only dominant microorganisms with relative abundance ≥ 1% are listed in the table.

4 References

[1] TM0194, NACE Standard, field monitoring of bacterial growth in oil and gas systems, Houston, TX, 2014.

[2] E.M. Suarez, K. Lepkova, B. Kinsella, L.L. Machuca, Aggressive corrosion of steel by a thermophilic microbial consortium in the presence and absence of sand, Int. Biodeterior. Biodegrad. 137 (2019) 137-146.

[3] S.J. Salgar-Chaparro, L.L. Machuca, Complementary DNA/RNA-based profiling: characterisation of corrosive microbial communities and their functional profiles in an oil production facility, Front. Microbiol. 10:2587 (2019).

Appendix 2

Metal Surface Preparation Test

Metal Surface Preparation Test

1. Introduction

Surface roughness is an important factor that can affect microbial attachment to metal structures. In this experiment, two (2) methods, sandblasting and grinding-polishing, for the metal surface preparation of carbon steel samples were evaluated in order to select the most suitable method for the preparation of coupons used in the corrosion experiments executed in this research.

2. Objective

Evaluate the use of sandblasting and grinding-polishing for the metal surface preparation of carbon steel coupons used for biofilm growth and MIC studies.

3. Materials and Methods

3.1 Sample preparation

Carbon steel metal samples with an elemental composition of (weight %): C (0.43 - 0.5), Mn (0.6 - 0.9), Si (0.15 - 0.35), S (0.01 - 0.35), P (0 - 0.035), Cr (0 - 0.40), and Fe (balance) were used in the study. A total of 30 samples were cut with dimensions of 1 x 1 x 0.3 cm. Fifteen (15) samples were prepared by sandblasting, and the other fifteen (15) were prepared by grinding-polishing using silicon carbide papers of 80, 120, 320, and 600 grit, consecutively. All coupons were weighted and sterilised by UV radiation for 15 min each side before the experiment set up.

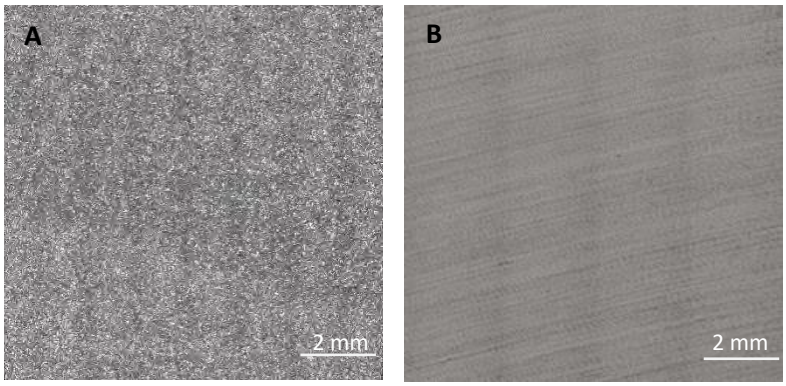

Figure 1. Microscopic image of carbon steel coupons after surface preparation (a) sandblasting; (b) grinding-polishing.

3.2 Test Solution preparation

Supplemented artificial seawater was used as a test solution for the experiment. The composition was as follows: 40 g/L of sea salts (Sigma), 20 mM of Na-lactate, 20 mM Na-acetate, 20 mM Na-formate, 20 mM Na-citrate, 10 mM glucose, 5 g/L bacto casamino acids (BD), 20 mM NH4NO3, 10 mM Na-thiosulphate, 1 mM iron (II) sulphate heptahydrate, and 10 mL/L of Vitamins solution (according to DSMZ medium 141). The pH of the test solution was adjusted to 7.3 with sodium bicarbonate.

3.3 Experiment set up

Sterile serum bottles with a capacity of 100 mL were used for biofilm growth over the metal samples. A volume of 90 mL of the test solution (previously sterilised in the autoclave) was added to the serum bottles, and bottles were sparged with a filter-sterilised gas mixture of N_2-CO_2 (80:20) at a flow rate of 20 mL/min for 40 min. One coupon (previously sterilised) was immersed in each bottle and sparged with the gas mixture for another 10 min. Six bottles (3 with sandblasted coupons and 3 with polished coupons) were inoculated with a thermophilic microbial consortium at a concentration of 1 x 10^6 cells/mL and incubated at 60°C and 100 rpm per 10 days. Another six bottles (3 with sandblasted coupons and 3 with polished coupons) were inoculated with mesophilic microbial consortium at a concentration of 1 x 10^6

cells/mL and incubated at 40°C and 100 rpm per 10 days. Triplicates of abiotic controls were included for each temperature and each surface preparation method.

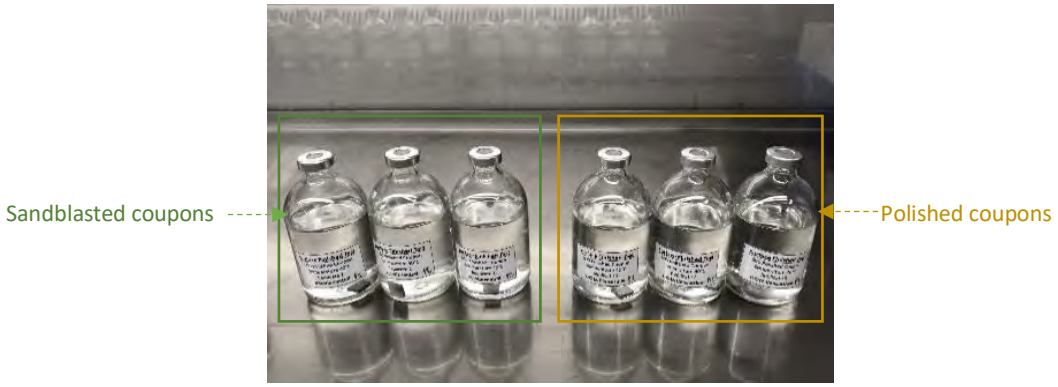

Figure 2. Serum bottles with carbon steel coupons used for the metal surface preparation test.

3.4 Microbial enumeration

After ten (10) days of incubation, biotic bottles evidenced changes in the colour and turbidity of the test solution, which indicated microbial growth. In contrast, abiotic bottles remained clear and colourless (**Figure 3**). One bottle of each condition was selected for direct counting of microorganisms. A total of 10 µL of the solution were taken for counting planktonic cells using a Neubauer counting chamber and a phase-contrast microscope (Nikon Eclipse Ci-L). Enumeration of sessile cells was conducted by immersing the metal sample in 10 mL of PBS buffer. Bacteria were detached from the coupons by vortexing at full speed for 10 s together with sonication for 2 min. After the detachment of cells, 10 µL of PBS buffer were used for direct counting.

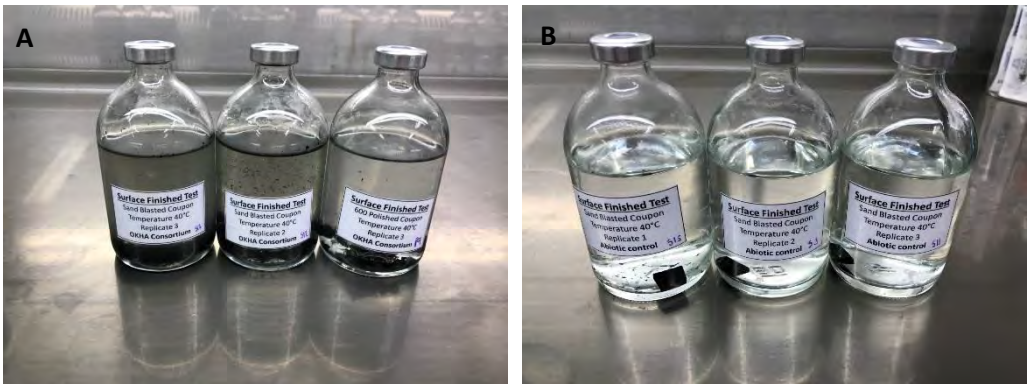

Figure 3. Serum bottles exposed to (a) biotic and (b) abiotic conditions.

3.5 Corrosion measurements

Corrosion rates were estimated from weight loss, as described in the ASTM G1 standard [1]. Coupons were cleaned by immersion in Clarke's solution prepared according to ASTM G1 standard [1]. Coupons were exposed to Clarke's solution in intervals of 30 seconds until a constant weight (within ± 0.2 mg) was obtained for several measurements.

3.6 Surface analysis

Pitting and roughness analyses were conducted with a 3D optical profilometer (Alicona imaging infinite focus microscope IFM G4 3.5). For this, three (3) coupons which surface had been prepared with each method (sandblasting and grinding-polishing) were analysed to determine roughness before the exposure to abiotic and biotic conditions. After the corrosion test, the maximum pit depth of metal surfaces exposed to different conditions was determined.

4. Results

4.1 Microbial enumeration

Direct counting of microorganisms evidenced that the number of planktonic and sessile cells in all conditions was similar (**Table 1**). Results showed that metal surface preparation through sandblasting or grinding polishing did not have an impact on the number of cells that could attach to the metal.

Table 1. Number of planktonic and sessile cells in each condition

Method*	**SB**	**GP**	**SB**	**GP**
Temperature	**40°C**		**60°C**	
Planktonic (cell/mL)	(5.6 ± 0.2) x 10^7	(1.1 ± 0.1) x 10^7	(2.4± 0.2) x 10^7	(2.6 ± 0.3) x 10^7
Sessile (cell/cm^2)	(2.4 ± 0.5) x 10^7	(3.0 ± 0.1) x 10^7	(4.2 ± 0.1) x 10^7	(4.0 ± 0.1) x 10^7

* SB: Sandblasting; GP: grinding-polishing

4.2 Corrosion measurements

Average corrosion rates calculated from the weight loss of the metal samples are presented in **Figure 4**. Corrosion rates of three (3) replicates processed for each condition are shown in **Table 2**.

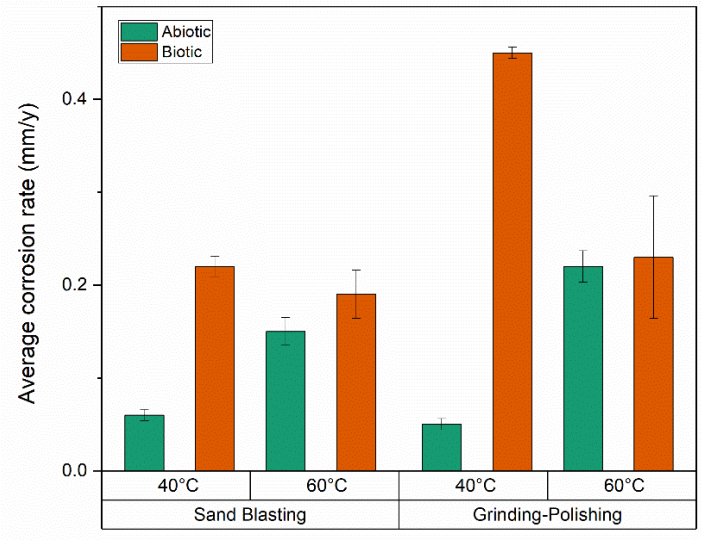

Figure 4. Average corrosion rate calculated from weight loss of metal samples

Results indicated that average biotic corrosion rates were higher than abiotic corrosion in all conditions. It was evidenced that corrosion rates were higher in metal samples prepared with the grinding-polishing method than with the sandblasting method. Greater differences were seen in coupons exposed to the mesophilic microbial consortium (incubated at 40 ^{o}C).

Table 2. Abiotic and biotic corrosion rates calculated from weight loss

Method	Condition	Replicate	Corrosion rate (mm/y)	Average CR (mm/y)
Sandblasting	Abiotic 40°C	1	0.06	0.06
		2	0.06	
		3	0.05	
	Biotic 40°C	1	0.23	0.22
		2	0.23	
		3	0.21	
Grinding-polishing	Abiotic 40°C	1	0.06	0.05
		2	0.05	
		3	0.05	
	Biotic 40°C	1	0.45	0.45
		2	0.44	
		3	0.45	
Sandblasting	Abiotic 60°C	1	0.17	0.15
		2	0.15	
		3	0.14	
	Biotic 60°C	1	0.22	0.19
		2	0.17	
		3	0.18	
Grinding-polishing	Abiotic 60°C	1	0.23	0.22
		2	0.23	
		3	0.20	
	Biotic 60°C	1	0.17	0.23
		2	0.21	
		3	0.30	

4.3 Surface analysis

Roughness profile of coupons prepared by sandblasting and grinding-polishing are shown in **Figure 5**. Average roughness (Ra), RMS roughness (Rq), and the average maximum height of the profile (Rz) measurements were collected for each coupon in three (3) different locations (**Table 3**). Results of roughness analysis indicated that the metal surface prepared by the sandblasting method is more irregular than the metal surface prepared by the grinding-polishing method.

Table 3. Roughness measurements of carbon steel coupons after metal surface preparation

Sandblasting method									
	Coupon 1			**Coupon 2**			**Coupon 3**		
Ra	3.08	2.70	2.78	3.04	2.72	2.41	3.06	2.89	3.22
Rq	3.97	3.52	2.48	3.90	3.40	3.15	3.96	3.69	4.16
Rz	19.53	18.45	17.59	20.09	16.35	17.90	21.26	18.23	20.44
Polishing method									
	Coupon 1			**Coupon 2**			**Coupon 3**		
Ra	0.89	0.86	0.93	1.27	1.24	1.20	1.26	1.19	1.18
Rq	1.10	1.08	1.20	1.58	1.49	1.47	1.58	1.50	1.48
Rz	5.56	5.69	6.16	8.54	8.33	8.10	8.22	8.36	8.50

Ra: roughness average is the arithmetic average of the absolute values of the profile heights over the evaluation length.
Rq: RMS roughness is the root mean square average of the profile heights over the evaluation length
Rz: average maximum height of the profile is the average peak to valley height of the roughness profile.

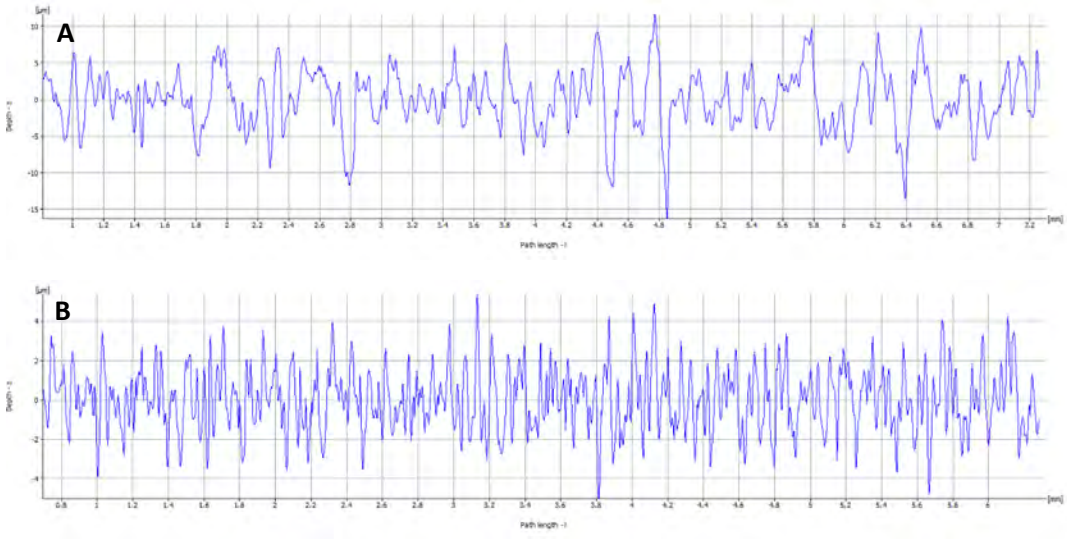

Figure 5. Roughness profile of carbon steel coupons prepared by (a) sandblasting and (b) grinding-polishing.

4.4 Localised corrosion

Average pit depth calculated from the deepest pits found in the three metal samples exposed to each condition is presented in **Figure 6**. Deeper pits were found in metals exposed to biotic conditions than the metals exposed to the abiotic conditions. 3D

optical microscope images of coupons exposed to biotic and abiotic conditions are shown in **Figure 7** to **Figure 10**.

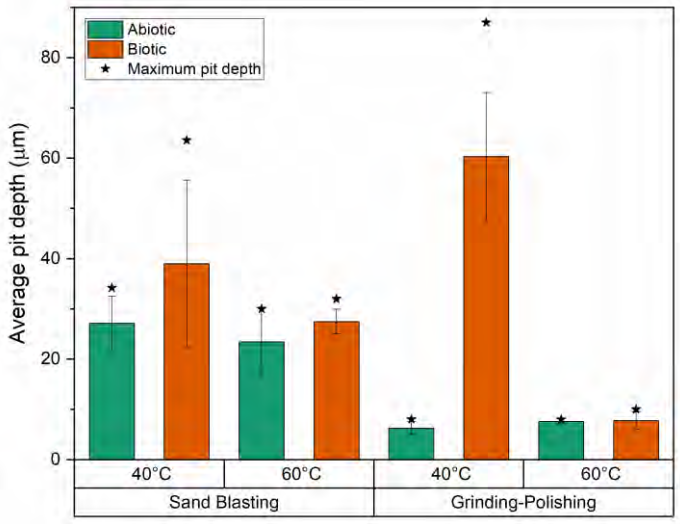

Figure 6. Average and maximum pit depth of carbon steel samples exposed to different conditions

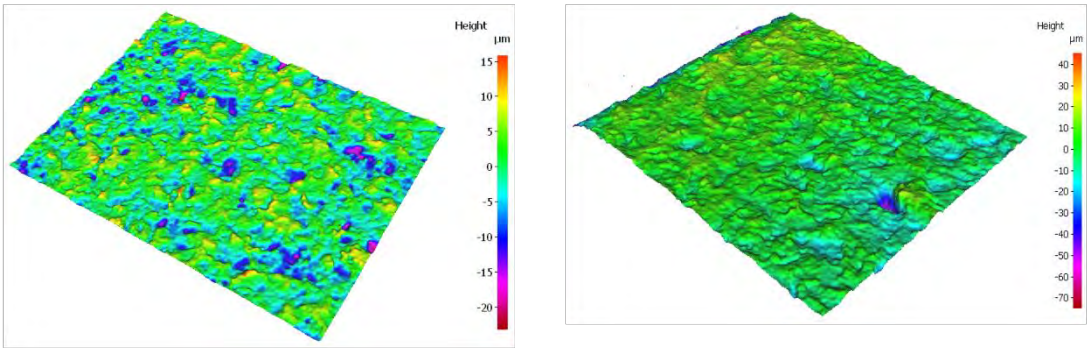

Figure 7. 3D optical microscope image of carbon steel coupons prepared by sandblasting and exposed to (a) abiotic and (b) biotic conditions at 40 ^{o}C.

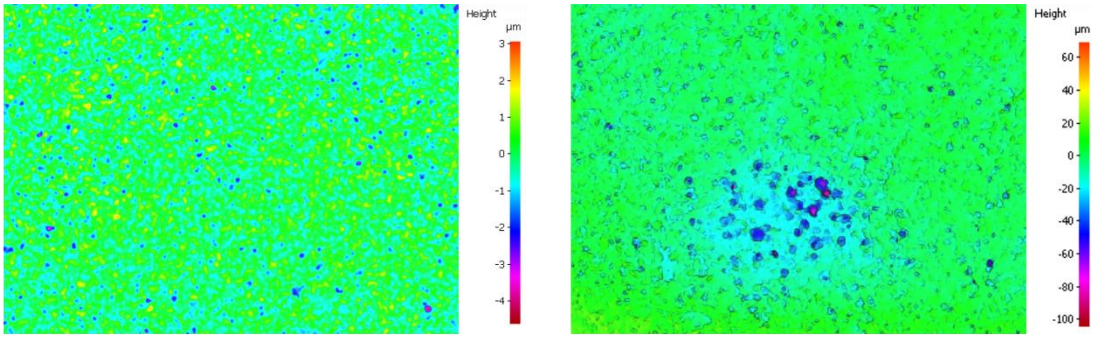

Figure 8. 3D optical microscope image of carbon steel coupons prepared by grinding-polishing and exposed to (a) abiotic and (b) biotic conditions at 40 ^{o}C.

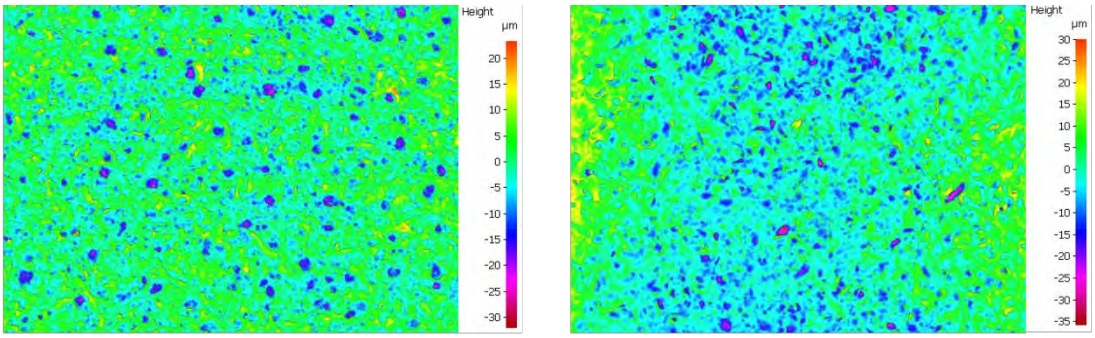

Figure 9. 3D optical microscope image of carbon steel coupons prepared by sandblasting and exposed to (a) abiotic and (b) biotic conditions at 60 ^{o}C.

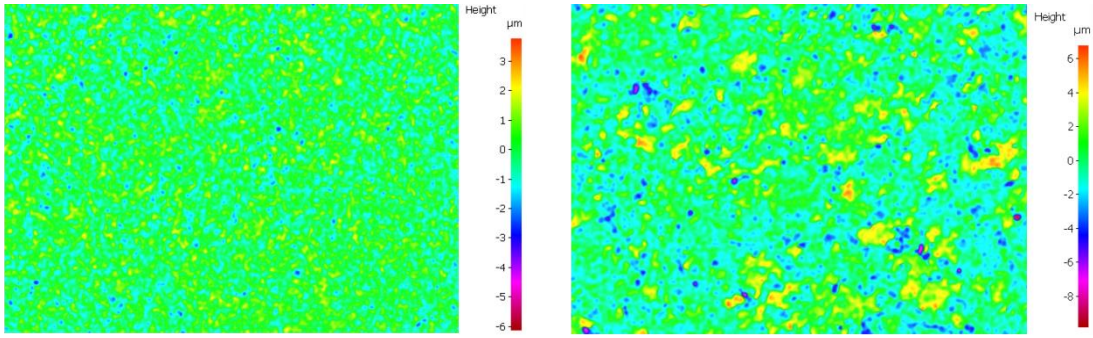

Figure 10. 3D optical microscope image of carbon steel coupons prepared by grinding-polishing and exposed to (a) abiotic and (b) biotic conditions at 60 ^{o}C.

Pitting rates calculated with the maximum pit depth found in metals exposed to microbial consortia are presented in **Figure 11**. To discriminate the effect of the microbial activity from abiotic corrosion, pitting rates obtained in the abiotic conditions were subtracted from the biotic measurements. Hence, marked differences in the metal deterioration of coupons prepared with the different methods were evidenced only with the mesophilic consortium. Higher pitting rates were observed in samples prepared by grinding-polishing.

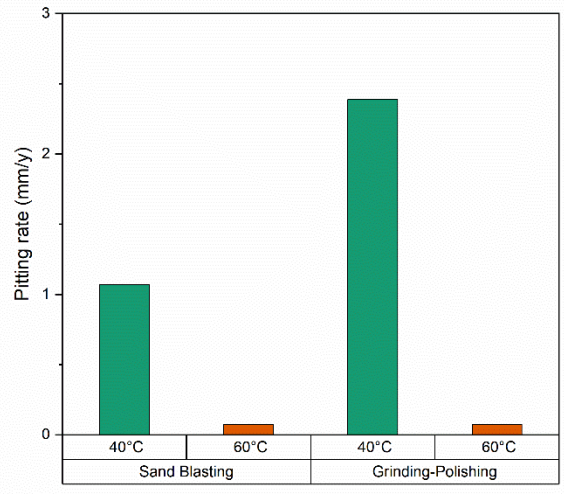

Figure 11. Pitting rate of carbon steel coupons exposed to biotic conditions.

5. Conclusion

Results of this experiment revealed that the surface finish with the sandblasting method is more irregular than the surfaces prepared by the grinding-polishing method. The Rz values of coupons prepared by sandblasting were between 18 to 21 µm, whereas Rz values for samples prepared with grinding-polishing were between 5 to 8 µm. Since MIC is principally a localised phenomenon, pitting analysis is very important to determine the corrosive potential of microorganisms. Therefore, pits above 10 microns in sandblasted samples before the exposure made the pitting analysis more difficult compared with the grinding polishing samples that produced a more regular surface with deepest pits below 10 microns. Additionally, coupons prepared by grinding-polishing exhibited higher corrosion rates than coupons prepared by sandblasting when exposed to the mesophilic consortium, demonstrating that metal roughness had an impact on the severity of the MIC process.

6. References

[1] ASTM, G., Standard practice for preparing, cleaning, and evaluating corrosion test specimens, West Conshohocken, Pensilvania, 2017.

Appendix 3

Original reprint of the publications included in Chapter 2

EFFECT OF SAMPLE STORAGE CONDITIONS ON THE MOLECULAR ASSESSMENT OF MIC

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Curtin Corrosion Centre

SUMMARY: Microbiological surveys play a fundamental role in diagnosing and monitoring microbiologically influenced corrosion (MIC) in oil and gas production systems. Currently, microbiological characterization is being carried out by the implementation of molecular microbiological methods (MMMs) such as the 16S rRNA gene diversity profiling. Molecular characterization of microorganisms provides information to assess the risk of MIC in the production facilities. Even though MMMs have been included in NACE standards, standardized protocols for collection, storage and preservation of oilfield samples have not been written. In this study, the effect of sample storage conditions on the microbial composition, community structure, alpha diversity and functional capability of oilfield samples was investigated. The effect of storage samples at room temperature or refrigeration on the molecular MIC assessment was statistically evaluated by comparison with samples pre-processed and preserved on-site straight upon collection. Sample storage resulted in changes in the relative abundance of the microbial populations, which had a significant impact on the alpha diversity and structure of the community. Likewise, the functional capability of the microbial community in oilfield samples was affected by storage conditions. Abundances of genes associated with corrosive pathways such as sulphate reduction, iron utilization and methanogenesis decreased under conditions evaluated. Results of this research provide evidence of the importance of sample handling for an accurate microbial characterization and subsequent assessment of the MIC risk in industrial systems. Thereby, on-site pre-processing of the samples and addition of nucleic acids preservation solutions is recommended for an optimal microbiological survey, and in cases where this is not feasible, refrigeration is preferred over room temperature storage conditions.

Keywords: Microbiologically Influenced Corrosion (MIC), Molecular Microbiological Methods (MMM), 16S rRNA Microbial Diversity Profiling, oilfield samples, storage conditions.

1. INTRODUCTION

Microbiologically Influenced Corrosion (MIC) is defined as the deterioration of metals that results from the presence and activity of microorganisms on their surfaces. The participation of microorganisms in the corrosion process can significantly increase the corrosion rates, representing a big concern for the integrity of industrial infrastructure particularly oil and gas production facilities (1, 2). Annually, this phenomenon generates millions of dollar losses to the world's industry (3, 4). The most recent NACE report estimated the global cost of corrosion as US$2.5 trillion, without including safety or environmental consequences (5). In fact, several studies have estimated that MIC contributes between 20% and 50% of total corrosion failures (6-9).

A way to prevent corrosion failures is the opportune detection of the threats. This process is usually acknowledged as corrosion management and has been described by Skovhus *et al.*, (10) as a cycle of three fundamental steps: 1) identify the corrosion threats, 2) Mitigate the threats and, 3) Monitor of mitigation effectiveness. The first step of this cycle is essential for the appropriate corrosion control. For the MIC risk, this step refers to an early detection of the presence of corrosive microbes in the system. Periodic microbiological surveys are executed in the oil and gas systems following guidelines of the NACE standard TM-0194 (11, 12).

In the last decades, microbiological characterization of oilfield samples has been mostly carried out by the implementation of traditional culture-based techniques (13-16). However, knowing the limitations of these methods such as the inability to recover all the microorganisms present (17, 18), molecular microbiological methods (MMMs) have been developed and implemented for studying, monitoring and management of MIC (19-25). Microbial diversity profile analysis based in next-generation sequencing (NGS) of the 16S rRNA gene is one of the methodologies being used by the industry to help identify and characterize the total microbial community living in oil and gas production systems (26-30). Apart from the taxonomic classification, 16S sequencing data can be used for predicting the functional capabilities of the community as a proxy to more complex metagenomic analysis (31). Identifying the metabolic potential and the dominant genes in the microbial population living in oilfields could suggest what the microorganisms are doing in the system (32). Nevertheless, it is important to have in mind that analysis based on nucleic acids (DNA or RNA) are susceptible to many biases in the complete procedure of data acquisition. De la Cuesta-Zuluaga & Escobar (33) presented the most common challenges in the 16S rRNA workflow which include steps from sampling to the operational taxonomic unit (OTU) table generation and emphasized on the need of standardized protocols to reduce variations. Although the latest updates of the NACE standards (TM0106, TM0194 and TM0212-2018) have included the MMMs as complementary tests for the monitoring and diagnosing of MIC (34), standardized protocols for collection, storage, preservation and processing of oilfield samples have not been written.

Proper preservation and storage of samples is essential in maintaining the microbial composition and structure of field samples and in the accurate assessment of MIC risk. Transportation of samples from the production facilities can take days, or even weeks, to arrive at specialized laboratories for molecular analysis, time period where the DNA in the samples can change if it is not well preserved (20, 35). Several investigations have focused on the effect of storage conditions on the microbiome analysis of environmental and human samples (36-38). However, there is limited research on the influence of those conditions in the assessment of complex microbial communities in the oilfield systems. A previous study based on culture-dependent methods showed that storage conditions affect significantly the concentration of corrosive microbial groups in the samples (39), nonetheless, the effect on culture-independent methods was not evaluated. To address these knowledge gaps, we assessed the effect of sample storage conditions on the molecular characterization of the microbial composition and structure of water and sludge samples collected from an oilfield facility in Australia. This is the first study that used bioinformatics of the 16S rRNA sequencing data to predict the functional capability of an oilfield microbial community.

2. EXPERIMENTAL DETAILS

2.1 Sample Collection

One sludge sample and six produced oil-water samples were collected from an Australian oil production facility. Sludge sample was collected in a sterile 50 mL Falcon tube. Oil-water samples were decanted in sterile separatory funnels for oil phase removal. The water phase was then divided into two equal volumes. One volume was used for immediate filtration and used as a reference sample. Reference samples were immersed in RNAprotect® Bacteria Reagent, which prevents nucleic acids degradation and gene induction. The remaining volume of three of the water samples was stored under refrigerated conditions (4°C) for one week, whereas the remaining volume of the other three water samples was stored at room temperature for 24 hours (transport period) before refrigeration and processing. Samples were treated as follow.

Room temperature

Water samples: 500 mL of samples PW-1, PW-2 and PW-3 were stored at room temperature ≅ 25°C for 24 h and then stored at 4°C for one week. After refrigeration period, sample was filtered through sterile 0.2 µm pore size membranes and membranes filters were stored at -20°C.

Sludge sample: 5 g of sludge were stored at room temperature ≅ 25°C for 24 h and then stored at 4°C for one week.

Refrigeration

Water samples: 500 mL of samples PW-4, PW-5 and PW-6 were stored at 4°C in sterile glass bottles for one week. After the refrigeration period, samples were filtered through sterile 0.2 µm pore size membranes and membranes filters were stored at -20°C.

Sludge sample: 5 g of sludge were placed in a Falcon tube and stored at 4°C for one week.

Immediate pre-processing (reference samples)

Water samples: 500 mL of samples PW-1, PW-2, PW-3, PW-4, PW-5 and PW-6 were filtered through sterile 0.2 µm pore size membranes, then, membrane filters were immersed in RNAprotect® Bacteria Reagent (QIAGEN) and stored at 4°C for 2 days until arrival to the laboratory. In the laboratory, RNAprotect was washed from the samples and membrane filters were stored at -20°C.

Sludge sample: 5 g of sludge were placed in a Falcon tube with 10 mL of RNAprotect® Bacteria Reagent and stored at 4°C. Sample was processed twice, DNA was extracted after 2 days and after one week of sample storage to determine the effect of time of processing in samples preserved in RNAprotect® solution.

2.2 DNA extraction and quantification.

Water samples

DNA was isolated using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions with the following modifications: filters were placed into the PowerWater DNA Bead Tube containing solution PW1 and heated at 65°C for 10 min before the Vortex step. Total DNA was eluted in 100 µL of free DNA water. DNA concentration was quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies).

Sludge samples

DNA was isolated using the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions with the following modifications: 0.25 g of sludge were placed into the PowerBead Tube containing solution C1 and heated at 65°C for 10 min before the Vortex step. Total DNA was eluted in 100 µL of free DNA water. DNA concentration was quantified as mentioned for water samples.

2.3 16S rRNA gene sequencing

The hypervariable region V3–V4 of the 16S rRNA gene was amplified using the universal primers 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3') (40), and adapters for Illumina MiSeq sequencing were included. Amplicons were pooled in equimolar concentrations and quantified with Quant-iT dsDNA HS assay kit (Life Technologies). PCR products indexing was performed with Nextera XT Index kit (Illumina), and sequencing was completed using the dual index paired-end approach. Both, polymerase chain reaction and sequencing were performed by the Australian Genome Research Facility (AGRF).

2.4 Bioinformatics

The Quantitative Insights Into Microbial Ecology Software (QIIME, v1.9.1) (41) was used for the analyses of the 16S rRNA gene sequences generated with the Illumina MiSeq. Paired-ends reads were merged using PEAR (v0.9.10 - 64 bit) (42) with default parameters. Then, sequences with an average quality score lower than 20 or containing unresolved nucleotides were removed from the dataset. Unclipped primer sequences were also removed by employing Cutadapt (v1.10) (43) using default settings. Afterwards, USEARCH (v10.2) (44) was used for OTU clustering with UNOISE algorithm to dereplicate, remove singletons, and sort all quality-filtered sequences by length (400 bp). Subsequently, OTUs were clustered at 100% sequence identity. Chimeric sequences were removed using UCHIME (45) with SILVA as reference database (SILVA v132) (46). Quality-filtered sequences were mapped to chimera-free OTUs and an OTU table was created using VSEARCH (v1.1.3) (47). Taxonomic classification of the reference sequences (zOTUs) was performed by similarity searches using BLAST against the same database. Sample comparisons were performed at the same surveying effort, utilizing 22,000 by random selection. Species richness, alpha and beta diversity estimates, and rarefaction curves were determined using the QIIME algorithms.

2.5 Statistical analyses

Statistical analyses and graphs were conducted employing R (v3.4.3) (48), and PAST (v3) (49) software. Results of statistical tests were considered significant with $p \leq 0.05$. A t-test was used for comparison of alpha diversity between storage conditions. To visualize the multivariate dispersion of the community composition a non-metric multidimensional scaling (NMDS) was performed based in the Weighted UniFrac distance (50), lines for joining samples collected in the same sampling point were projected onto the ordination, utilizing the function ordiellipse in the Vegan package. Permutational analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were used to test for significant differences in beta diversity, Bray-Curtis distance (51) was used in these tests.

2.6 Prediction of bacterial functional profiles.

The functional profile was predicted by using the R package Tax4Fun (31). Tax4Fun is a software that links the 16S rRNA gene sequences with the functional annotation of sequenced prokaryotic genomes by the identification of the nearest neighbour. Results of Tax4Fun predictions are highly correlated with the functional profiles obtained from whole metagenome sequencing (31).

For this analysis, the genes encoding key enzymes involved in metabolic pathways associated with corrosion were identified in the resulting profiles using their KEGG orthologs. The sum of the abundances of genes selected for each metabolism was used for comparison among storage condition.

3. RESULTS

3.1 Sample codification and storage conditions

Six water samples were collected from distinct locations of an oil production facility. Produced water samples PW-1, PW-2 and PW-3 were stored at room temperature (RT) for 24 hours, whereas PW-4, PW-5 and PW-6 were stored refrigerated (4°C) to determine the impact of the sample storage conditions in the diversity profile of oilfields microbial communities. One portion of sample from each location was processed on-site, immediately after collection, and was preserved in RNAprotect® Bacteria Reagent and used as reference (Ref) in the analysis.

To determine if storage conditions have the same impact on water and solids samples, one sludge sample was collected from a corroded pipe. Similarly to the water samples, a portion of the sample was stored at RT for 24 hours, another portion stored at 4°C for one week, and another placed in RNAprotect solution. Sample in RNAprotect solution was processed twice, after 2 days (Ref) and after one week of being stored at 4°C. This analysis was made to determine if there is an impact of the time of processing in samples preserved in RNAprotect® solution.

3.2 Effect of sample storage on the diversity profile of the microbial community

Water and sludge samples were processed separately. DNA was extracted and V3-V4 region of the 16S rRNA gene was sequenced for determining the microbial composition of the oilfield community. A total of 983,520 (water) and 449,548 (sludge) high-quality sequences were obtained after removal of singletons, chimeras and low-quality sequences. The number of sequences per sample fluctuated from 39,301 to 180,907. Data normalization was executed by subsampling at 22,000 sequences per sample to correct the unequal sequencing depth. After normalizing the number of OTUs ranged from 56 ± 9 to 258 ± 20.

The relative abundance of microbial populations at domain level (Bacteria; Archaea) as a function of storage conditions is shown in Figure 1 for water samples and in Figure 2 for sludge sample. The analysis of the microbial structure at domain level revealed that the relative abundance of the archaea community diminished after storage conditions (both temperatures) with respect to the reference samples (processed on-site). This pattern was seen in all sampling points with higher reduction observed in the water samples stored at room temperature for 24 hours (Figure 1). Sludge sample showed a similar decrease in the archaea community under RT and 4°C storage conditions (Figure 2). The sample preserved for a longer time (1 week) in RNAprotect® Bacteria Reagent did not exhibit significant differences in the community compared to samples preserved in the same solution for shorter time (2 days). This indicates that there is not an impact of storage time on the abundances of bacteria and archaea species in samples preserved in RNAprotect®.

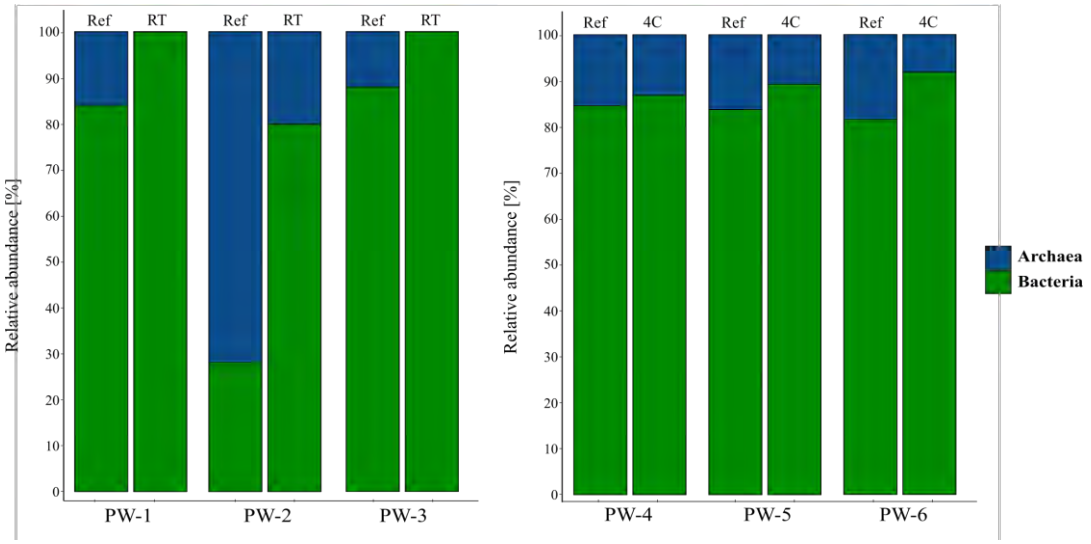

Figure 1 Relative abundance of Bacteria and Archaea in water samples (PW-1 to PW-6) assessed by 16S rRNA sequencing. Sample processed on-site (Ref), sample stored at room temperature 24 hours (RT), sample stored under refrigeration (4C).

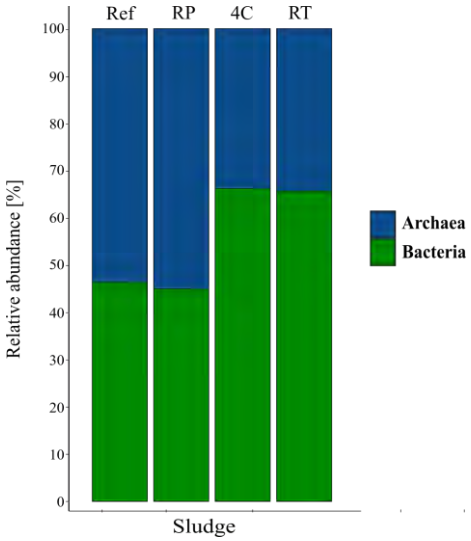

Figure 2 Relative abundance of Bacteria and Archaea in sludge sample assessed by 16S rRNA sequencing. Sample stored in RNAprotect® solution and processed after 2 days (Ref), sample stored in RNAprotect® solution and processed after one week (RP), sample stored sample stored under refrigeration (4C) and sample stored at room temperature 24 hours (RT).

Taxonomic classification of the OTUs was assigned to genus level, species representing <1% of the population were grouped as rare taxa. Bar charts of the diversity profile are presented in Figure 3 for water sample and in Figure 4 for sludge sample. A total of 84% of the species in water samples and 58% of species in sludge sample were classified to the genus level, the remaining 16% and 42%, respectively, belonged to uncultured bacteria living in the oilfield.

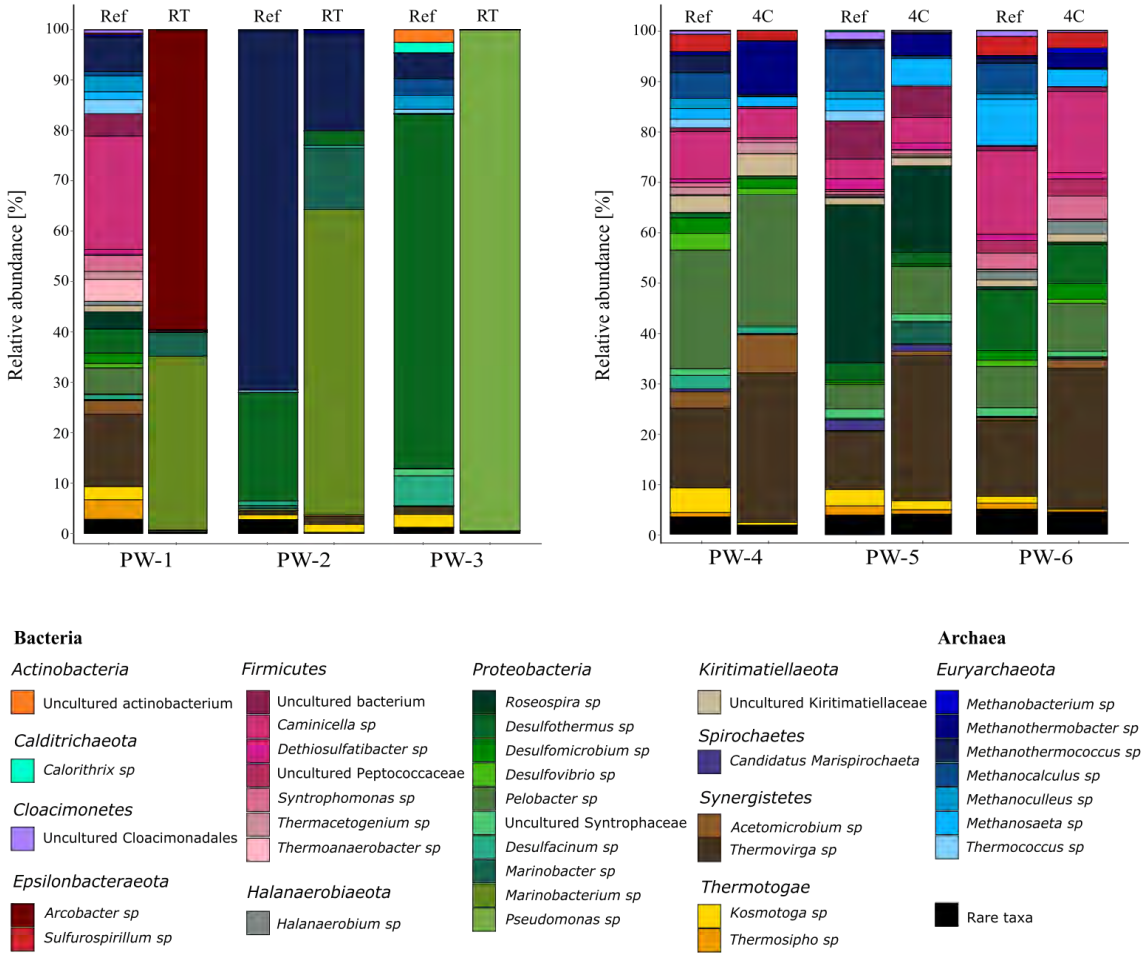

Figure 3 Microbial community composition of water samples at genus level. Sample processed on-site (Ref), sample stored at room temperature 24 hours (RT), sample stored under refrigeration (4C). Bacterial and archaeal genera with relative abundances > 1% are visualized; genus contributing ≤ 1% were summarized as rare taxa.

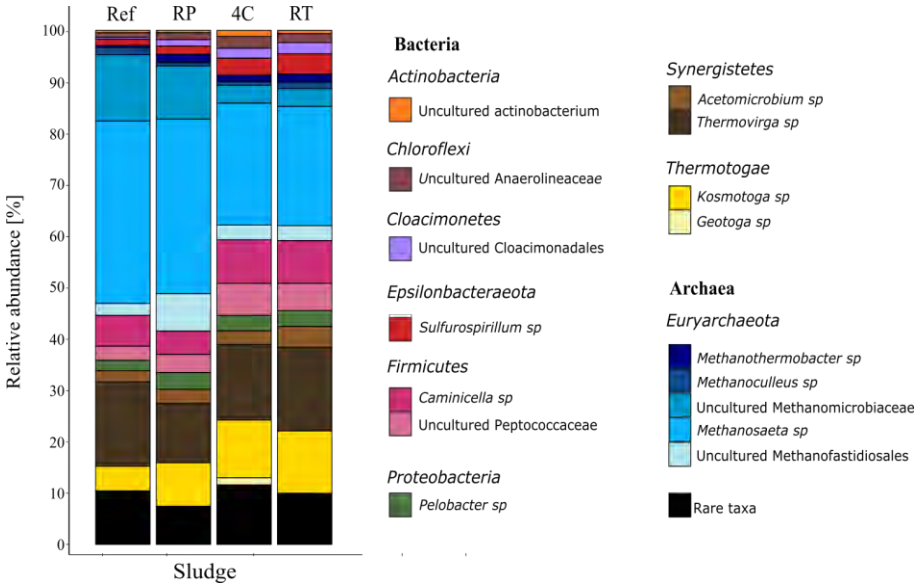

Figure 4 Microbial community composition of sludge sample at genus level. Sample stored in RNAprotect® Bacteria Reagent and processed after 2 days (Ref), sample stored in RNAprotect® Bacteria Reagent and processed after one week (RP), sample stored sample stored under refrigeration (4C) and sample stored at room temperature 24 hours (RT),. Bacterial and archaeal genera with relative abundances > 1% are visualized; genus contributing ≤ 1% were summarized as rare taxa.

Microbial composition analysis in water samples demonstrated marked differences between the samples processed immediately and the samples stored at room temperature for 24 hours. Under this storage condition, the percentage of abundance of the genera *Marinobacter*, *Marinobacterium*, *Arcobacter*, and *Pseudomonas* increased significantly and became the dominant populations in the microbial community. On the other hand, microbial communities of samples stored at 4°C presented variations in the abundances with respect to the microbial composition of references samples, however, those deviations were less significant compared to samples stored at RT. Storage at cooling conditions had a positive effect in genera such as *Methanothermobacter, Pelobacter, Acetomicrobium* and *Thermovirga* which showed an increase in their percentage of abundance in samples but had a negative effect on the genera *Sulfurospirillum, Kosmotoga, Thermosipho, Thermococcus, Methanocalculus, Methanothermococcus* and *Methanoculleus* which exhibited lower abundances compared to the reference sample.

On the other hand, microbial composition analysis in sludge samples showed a different pattern. Both, room temperature and refrigeration conditions had a very similar microbial composition among them but different respect to the reference sample (Figure 4). The main impact was seen in the reduction of relative abundances of methanogenic species such as *Methanosaeta* and the uncultured *Methanomicrobiaceae*. Other genera like *Methanothermobacter, Sulfurospirillum, Kosmotoga* and *Caminicella* showed an increase in their percentage of abundance under storage conditions. Contrary to what was seen in the community structure at domain level, the analysis at genus level revealed that time of sample processing has a slight impact on the microbial composition of samples stored in RNAprotect® Bacteria Reagent. However, variation in storage time of samples stored in RNAprotect resulted in less significant changes compared to storage conditions without preservation solution.

3.3 Effect of sample storage on microbial diversity and richness

The effect of storage conditions on the alpha diversity of oilfield microbial communities was evaluated based on richness (Chao1) and diversity (Shannon) indexes. Richness measures the number of species found in a community whereas diversity combines richness and evenness into a single indicator. This means diversity measures the probability that two randomly selected microorganisms in a community belong to different species.

Analysis of alpha diversity indexes differed significantly between storage conditions in most of the cases ($p \leq 0.05$, T-test) (Table 1). Water samples stored at room temperature presented lower richness values while refrigerated samples had higher richness values compared to the reference samples. In the case of sludge sample, all storage conditions showed higher richness than the reference sample. In terms of Shannon diversity index, the differences between storage conditions and pre-processed samples did not follow a unique pattern of increase or decrease according to the storage conditions. Some stored samples presented higher diversity values than reference samples and some others lower diversity values.

Table 1. Alpha diversity analysis of oilfield samples

Sample	Treatment	Number of sequences	Number of OTUs	Chao1	Shannon
PW-1	Ref	86050	113	105 ± 6	4.6 ± 0.012
PW-1	RT	39301	65	73 ± 7*	1.5 ± 0.005*
PW-2	Ref	55034	45	56 ± 9	1.4 ± 0.137
PW-2	RT	61788	54	57 ± 11	2.0 ± 0.005*
PW-3	Ref	124841	62	57 ± 17	2.0 ± 0.015
PW-3	RT	53015	21	26 ± 12*	0.5 ± 0.003*
PW-4	Ref	141813	142	133 ± 18	4.8 ± 0.010
PW-4	4C	60548	152	153 ± 10*	4.3 ± 0.012*
PW-5	Ref	88721	157	149 ± 12	4.3 ± 0.020
PW-5	4C	65896	214	213 ± 16*	4.6 ± 0.013*
PW-6	Ref	87220	275	258 ± 20	4.9 ± 0.013
PW-6	4C	119293	278	253 ± 19	4.9 ± 0.011
Sludge	Ref	49119	137	146 ± 14	3.0 ± 0.013
Sludge	RP	118463	148	154 ± 8	2.9 ± 0.017
Sludge	4C	180907	152	170 ± 18*	3.4 ± 0.008*
Sludge	RT	101062	148	154 ± 10	3.3 ± 0.009*

(*p<0.05 compared to reference sample).

3.4 Effect of sample storage on microbial structure

The effect of sample storage on the microbial structure was only assessed for water samples due to the number of sludge samples collected not being enough for statistical analysis. NMDS was the method selected for the ordination of samples based on the Weighted Unifrac distance matrix. According to this analysis, the community structure of samples that are ordinated closer to each other are likely to be more similar than those that are ordinated apart.

NMDS of water samples displayed a clear separation of the structure community at room temperature and the reference samples (Figure 5). Consistent with these findings, the PERMANOVA and ANOSIM tests on the Bray-Curtis dissimilarity indicated that there is a significant difference ($p = 0.03$ and $p = 0.01$ respectively) between the beta diversity of room temperature samples and their references. On the contrary, no significant difference was detected in the microbial community structure of refrigerated samples with respect to their references ($_{PERMANOVA}$ $p = 0.30$, $_{ANOSIM}$ $p=0.37$). NMDS displayed a close ordination of the refrigerated and reference samples (Figure 3).

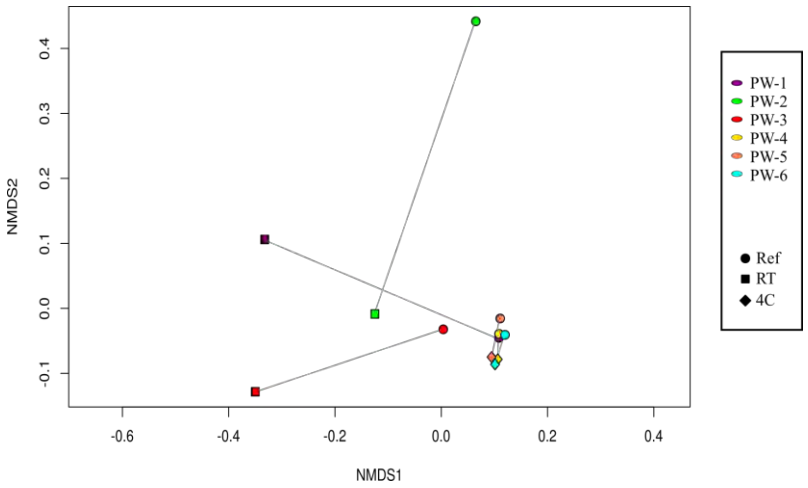

Figure 5 Non-metric multidimensional scaling (NMDS) of microbial community composition in water samples based on weighted Unifrac distance matrix. Storage conditions are represented by shape and sampling points by colours.

3.5 Effect of sample preservation on the functional capability

The functional profile analysis was focused on the microbial metabolic pathways that have been associated with the initiation or acceleration of corrosion processes. Relative abundances of key enzymes encoding genes participating in sulphate reduction, thiosulphate reduction, methanogenesis, acid production, iron oxidation, iron reduction and nitrate reduction were calculated with Tax4Fun R package. The impact of the sample storage on the functional profile of the community followed the same pattern in water (Figure 6) and sludge (Figure 7) samples. Sulphate reduction, methanogenesis and iron oxidation/reduction capabilities were shown to be reduced under both storage conditions compared to reference samples. On the other hand, fermenting and thiosulfate reducing capabilities increased after storage while nitrate reducing capabilities remained the same after storage compared to reference samples. Sludge sample preserved in RNAprotect® solution for a longer time (RP) did not show significant differences in the functional capabilities of the community with respect to the reference.

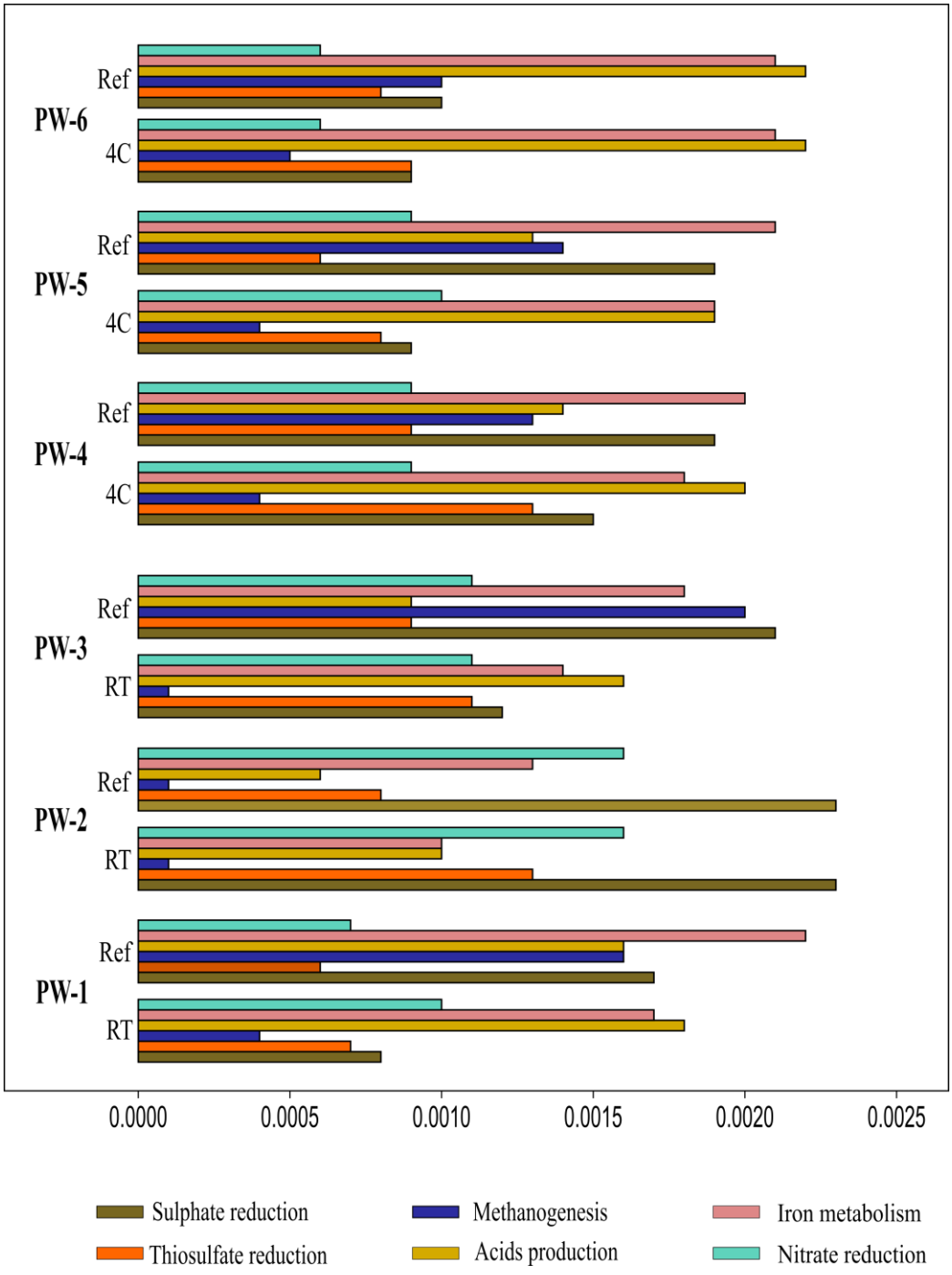

Figure 6 Functional profile of water samples. Relative abundances of key enzymes involved in microbiologically influenced corrosion processes.

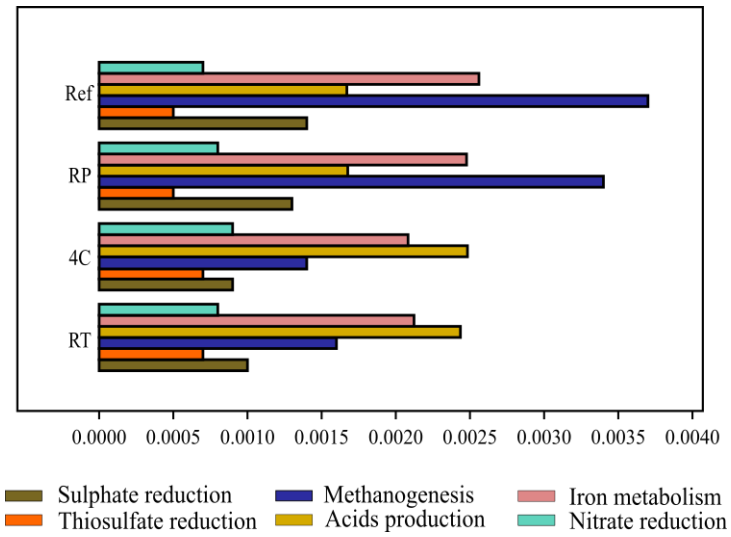

Figure 7 Functional profile of sludge sample. Relative abundances of key enzymes involved in microbiologically influenced corrosion processes.

4. DISCUSSION

Microbiological characterization of samples from remote oil production facilities is a challenging task since samples must be transported long distances after collection and before arriving at the laboratory for analysis. In particular, this is the case when microbiological assessment is done using molecular microbiological methods where sample processing requires more delicate protocols and trained analysists. During transportation, samples are rarely delivered under refrigeration and in most instances, samples are delivered at room temperature. By assessing the impact of storage temperature on the assessment of the microbial community in oilfield samples we provide a base understanding of the implications of this crucial procedure in the MIC assessment.

In this study, the microbial structure of the community was significantly influenced by storage conditions, particularly by room temperature conditions. Predominant populations in the water samples PW-1, PW-2 and PW-3 were replaced by aerobic or aerotolerant mesophilic microorganisms. This can be explained by the fact that during sample collection, samples can be exposed to air developing more favourable conditions for these microorganisms to thrive compared to the oxygen-free environment of the oilfield facilities. At room temperature conditions, microorganisms such as *Marinobacter, Marinobacterium, Arcobacter*, and *Pseudomonas* thrived in the samples and became the major genera in the community. Distinct to the room temperature, refrigeration did not affect considerably the community structure of the water samples. Other genera not related to aerobic respiration, e.g., *Methanothermobacter, Acetomicrobium* and *Thermovirga* exhibited higher relative abundances after storage, however, it is uncertain if this increase was related to the growth of microorganisms or to a higher resistance to the cooling conditions compared to other thermophile species that may be more sensitive to cold temperature and did not survive. According to Canganella & Wiegel, (52) thermophilic anaerobes can survive at mesobiotic and even psychrobiotic environments but cells at these conditions are unlikely to be able to multiply. A previous study developed by Kilbane (39), who investigated the effect of storage conditions on the recovery of microbial groups by serial dilution method reported relatively similar results, showing that higher microbial concentrations were detected on samples stored at room temperature and lower microbial concentrations on samples stored at 4°C. The author attributed the decrease of microorganisms to temperature or metabolic shock and to the depletion of energy reserves during storage.

In terms of alpha diversity and richness, both storage conditions significantly affected the OTUs recovered in the analysis of water and sludge samples. Shannon index (diversity) did not follow a unique pattern of increase or decrease according to the storage condition in both water and sludge samples, the reason for this is unclear but it is likely related to particular characteristics of each sampling point. Richness measured in sludge samples increased under both storage conditions while the pattern was different in water samples. The number of species decreased under room temperature and increased during refrigeration conditions. As mentioned before, room temperature favoured the growth of aerotolerant species generating an uneven distribution of species in the community. On the other hand, cooling conditions resulted in a reduced abundance of mesophilic and thermophilic taxa, all of them classified as strict anaerobes. This phenomenon could be related to temperature sensitivity of species or to the fact that oxygen solubility increases with the decrease of temperature thus harming strict anaerobes by exposing cells to toxic levels of oxygen (53). Lee *et al.,* (20) suggested that increases of DNA concentration during storage conditions could be the result of growing microbes while decreases can be the consequence of the presence of nucleases that degrade the DNA molecules. To avoid the activity of these enzymes and

preserve the DNA and RNA integrity in the samples, immediate freezing of samples in liquid nitrogen or dry ice is the most suitable method. However, when sampling is carried out in remote areas, stable freezing of samples in liquid nitrogen tank or dry ice during transportation is rather impractical and in most cases logistically impossible (54). An alternative to the freezing protocol is the use of nucleic acids preservation solutions such as RNAprotect® Bacteria Reagent that protects the sample from enzymatic degradation and stops bacterial activity.

The functional capability of the community was assessed using Tax4Fun, a novel bioinformatics software that provides data on the potential metabolic capabilities of the microbial community in a sample. Results showed that the functional capability was also affected by storage conditions which is associated with the variations in the relative abundances of microbial populations. Determining the presence and abundance of corrosive microorganisms is the key step in microbiological surveys in terms of MIC prediction. Defining if a microorganism can cause corrosion it is closely related to their metabolic function within a community. Generally, MIC prediction tools are centred in the corrosive activity of sulphate reducing bacteria (55-57) and some have included the effect of methanogenic species (58). Implementing these methodologies for the MIC assessment in samples stored at room temperature or cooling conditions will underestimate or overestimate the risk of MIC because of the effect of these storage conditions have on the abundance of corrosive microorganisms. According to the functional profile analysis realized in this study, sulphate reduction and methanogenesis pathways reduced their abundances under both storage conditions. Other metabolic pathways such as acid production and thiosulphate reduction presented a different pattern, suggesting that these populations have the ability to tolerate aerobic environments. Considering the results of this research and those obtained by Kilbane (39), sample storage conditions have an effect on microbiological surveys executed using both culture-independent and culture-dependent methods.

Overall, results of this research were in agreement with previous studies. For instance, Choo et al., (59) studied the microbiome in faecal samples. The authors detected significant changes in the community structure of samples stored at room temperature and not significant changes in samples stored at 4°C. Similar to our findings, aerotolerant species increased abundances under room temperature conditions, which was also correlated with the exposure of their faecal samples to aerobic conditions. Conversely, some studies have shown no significant effect of storage conditions on the microbial composition (37, 38, 60, 61). However, the difference between this investigation and other published work is the predominance of thermophile microbes in the samples evaluated here. Oilfield microbial communities are mainly constituted by anaerobic thermophile microorganisms whereas most of the studies developed in this area relate to the microbiome of mesophilic communities in soil and human body environments. Considering that temperature is one of the key factor in the microbial growth, the preservation of samples at a different temperature of the sampling point, with exception of freezing, can lead to a complete shift in the microbial community recovered during 16S rRNA gene sequencing. For this reason, results from this work demonstrate that on-site pre-processing along with addition of DNA/RNA preservation solutions is the preferred approach for the molecular assessment of microbial communities in oilfield samples where the field conditions make unlikely the immediate freezing of samples. This research did not evaluate the effect of different preservation solutions on the recovery of the oilfield microbial community and further analysis is required for a complete sampling protocol optimization.

5. CONCLUSIONS

We assessed the effect of sample storage on the assessment of oilfield microbial communities by 16S rRNA sequencing. Results demonstrated that diversity profiling, microbial structure and functional capability of oilfield microbiomes are affected by sample storage conditions, named room and cold temperature conditions, as compared to reference samples pre-processed and preserved immediately on-site. Alpha diversity measured by richness and diversity indexes was significantly affected in most of the samples under both conditions whereas microbial structure was only significantly affected by storage at room temperature. The functional capability of the community was also affected by both storage conditions evaluated. Abundances of genes associated with sulphate reduction, iron utilization and methanogenesis pathways decreased whereas genes related to acid production and thiosulphate reduction increased. Therefore, changes in the microbial composition of oilfield samples due to inadequate sample storage can lead to inaccurate MIC prediction due to the facility and quick response of the microorganisms to environmental changes such as those experienced during sample storage. Due to the above, if on-site freezing of samples is not feasible, on-site pre-processing is the preferred choice for an accurate assessment of the microbiological content in the samples. The on-site pre-processing refers to the sample immersion on a nucleic acids preservation solution, in the case of water samples a previous filtration through membrane filters of 0,2 um pore size is required. When on-site processing is not feasible or practical, samples should be at least stored at 4°C to prevent the growth of mesophilic populations.

6. ACKNOWLEDGMENTS

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Complementary DNA/RNA-Based Profiling: Characterization of Corrosive Microbial Communities and Their Functional Profiles in an Oil Production Facility

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DNA and RNA-based sequencing of the 16S rRNA gene and transcripts were used to assess the phylogenetic diversity of microbial communities at assets experiencing corrosion in an oil production facility. The complementary methodological approach, coupled with extensive bioinformatics analysis, allowed to visualize differences between the total and potentially active communities present in several locations of the production facility. According to the results, taxa indicative for thermophiles and oil-degrading microorganisms decreased their relative abundances in the active communities, whereas sulfate reducing bacteria and methanogens had the opposite pattern. The differences in the diversity profile between total and active communities had an effect on the microbial functional capability predicted from the 16S rRNA sequences. Primarily, genes involved in methane metabolism were enriched in the RNA-based sequencing approach. Comparative analysis of microbial communities in the produced water, injection water and deposits in the pipelines showed that deposits host more individual species than other sample sources in the facility. Similarities in the number of cells and microbial profiles of active communities in biocide treated and untreated sampling locations suggested that the treatment was ineffective at controlling the growth of microbial populations with a known corrosive metabolism. Differences in the results between DNA and RNA-based profiling demonstrated that DNA results alone can lead to the underestimation of active members in the community, highlighting the importance of using a complementary approach to obtain a broad general overview not only of total and active members but also in the predicted functionality.

Keywords: DNA, RNA, 16S rRNA gene, oil production, produced water, injection water, microbiologically influenced corrosion (MIC)

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INTRODUCTION

Corrosion refers to the deterioration of metals that results from its interaction with the environment. It is a natural process that affects several sectors such as production, transportation and refining of hydrocarbons (Hansson, 2011). This phenomenon generates millions of dollar losses to the world's industry every year (Franklin and White, 1991; Kip and Van Veen, 2015). In fact,

the latest estimation of the global corrosion costs quantified as US$2.5 trillion, without including safety or environmental consequences (Koch et al., 2016). From this total cost, microbiologically influenced corrosion (MIC) accounts for almost 20% of external, and 40% of internal corrosion problems in pipelines (Wolodko et al., 2018). MIC is known as the deterioration of metals that results from the presence and activity of microorganisms on their surfaces (Beech and Sunner, 2004). It was first identified in 1963 (Booth and Mercer, 1963), but its significance was not commonly recognized in the same decade (Usher et al., 2014). Nowadays, it is well known that the participation of microorganisms in the corrosion process can significantly increase the corrosion rates, representing a big concern to the integrity of industrial infrastructure, particularly oil and gas facilities (Usher et al., 2014; Xu et al., 2016).

Microorganisms change the electrochemical conditions at the metal/solution interface by the attachment of cells, biofilm formation, and subsequent release of metabolites, which induces or accelerates the corrosion process (Moura et al., 2013). MIC is characterized by a particular morphology of damage – localized pitting (Little and Lee, 2007), with corrosion rates reported up to 10 millimeters per year (Machuca and Polomka, 2018). Remarkably, MIC is not constrained to a unique corrosion mechanism (Lewandowski and Beyenal, 2009; Kakooei et al., 2012; Urquidi-Macdonald and Macdonald, 2014; Li et al., 2018). The main mechanisms described for MIC include the formation of concentration cells, the production of corrosive metabolites, the removal of protective films, and the production of unprotective surface layers (Skovhus et al., 2017). Lately, MIC has been reclassified into two different mechanisms, chemical MIC (CMIC) that considers metal deterioration induced by corrosive chemical species produced via microbial metabolic activity (indirect corrosion), and electrical MIC (EMIC) that refers to the damage caused by direct microbial uptake of electrons from the steel (direct corrosion) (Enning and Garrelfs, 2014). Causative microorganisms have been classified in microbial groups according to their metabolic activities, such as sulfide producing prokaryotes that include sulfate and thiosulphate reducers (Machuca et al., 2017; Machuca and Polomka, 2018), acid-producing (Gu and Galicia, 2012; Gu, 2014), methanogens (Uchiyama et al., 2010), iron-oxidizing (Ashassi-Sorkhabi et al., 2012; Liu et al., 2014), and iron-reducing bacteria (Herrera and Videla, 2009). Microorganisms with these metabolic capabilities are part of the normal microbiota of petroleum reservoirs (Magot et al., 2000; Ollivier and Magot, 2005). Microbial populations in oil reservoirs can reach the surface and colonize the metal infrastructure of the production facilities during the oil and gas extraction process.

Monitoring microbial activity in production facilities is part of the corrosion management of oil and gas industry assets. Microbiological assessment is routinely performed to detect the presence of MIC causative microorganisms and to evaluate the effectiveness of biocide treatments used to mitigate against MIC. Traditionally, culture-based techniques have been used to identify the presence of known corrosive microbial groups in industrial facilities (Muyzer and Marty, 2014; Beale et al., 2016; Machuca and Polomka, 2018). Since culture media cannot recover all microorganisms present in the environment (Keasler et al., 2012; Chakraborty et al., 2014), culture-independent techniques are used to complement cultivation based analyses. In the last decades, molecular microbiological methods have been implemented to improve the understanding of the microbial ecology of a system (Chakraborty et al., 2014). Within molecular methods, 16S rRNA gene amplicon sequencing is the most implemented method to study the biodiversity of oilfield environments (Lin et al., 2014; Wang et al., 2014; Li et al., 2017a; Okoro and Amund, 2018). Despite the disadvantage in the data interpretation due to the variation of 16S gene copy number among species (Crosby and Criddle, 2003; Acinas et al., 2004), the use of this sequencing approach on the biofilm communities recovered from corroded metals has allowed for taxonomic identification of microorganisms likely to be associated with corrosion failures (Vigneron et al., 2016, 2018; Li et al., 2017a). Considering that DNA-based analysis cannot discriminate between active and inactive species, RNA-based analyses have become popular in microbial ecology investigations as an alternative methodological approach to generate information of active members in the communities of different environments (Moeseneder et al., 2005; Bastias et al., 2007; Lillis et al., 2009; Kim et al., 2013). Nevertheless, the suitability of amplicon sequencing of 16S rRNA transcripts for identifying the active microbial populations that may be involved in corrosion of oil production systems has rarely, if ever, been explicitly addressed.

This work aimed to determine whether the application of complementary analysis using amplicon sequencing of the 16S rRNA gene and transcripts would provide relevant information on the microbial communities recovered from an oil production facility with corrosion issues. The production facility chosen for this investigation has exhibited several incidents of pinhole leaks or rapid reduction in wall thickness in pipes and vessels in the last decade. To the present, the causes of the increase in the corrosion rates of assets at the facility are uncertain. Corrosion processes were previously attributed to the presence of deposits called "schmoo," which are a combination of oil, corrosion inhibitor, produced fines and scales (O'Reilly et al., 2016). The formation of this material in the pipe walls reduces the effectiveness of corrosion inhibitors, increasing the risk of corrosion failures. However, the localized corrosion attack evidenced in the facility and the detection of microorganisms previously associated with MIC has raised the concern that microbial activity might also play a part in these corrosion failures. Including the RNA-based sequencing approach in the microbiological assessment of the oil facility helped identify active members in the community. In addition, the comparison of both methodologies through several bioinformatics tools allowed to visualize differences in the profiles between total and active communities, as well as the effect of environmental parameters (local operational conditions) and biocide treatment on their composition. Functional profiles from 16S rRNA data were predicted as a complementary and cost-effective metagenomic pre-study for identifying the metabolic capabilities of the oilfield

communities, and to relate potential differences in the DNA and RNA-based results with predicted functionality.

MATERIALS AND METHODS

Site Description and Sampling

The oil production facility from which samples were taken is located on the north-west coast of Western Australia. The oilfield has been operating for many years and uses water flooding to increase reservoir pressure and thereby stimulate production. Approximately 80,000 barrels of water per day (BWPD) are injected into the reservoir from 268 injector wells. Water used for this practice consists of a mixture of source water extracted from source water wells, and recycled produced water, also known as produced water re-injection (PWRI). Produced fluids extracted by the oil producing wells are transported to satellite stations distributed in the facility, where a biphasic separation is carried out by degasser vessels. Then, the water-oil mixture is transported to the central processing facility (CPF) for further separation of oil and water in low-pressure separator units. After separation, oil is shipped out of the facility and produced water is reinjected into the reservoir. A schematic diagram of the production facility is presented in **Figure 1**.

The assessment of the microbial composition of total and potentially active communities in the facility was carried out by collecting six (6) produced water samples, four (4) deposits samples (schmoo), and one (1) injection water sample. Produced water samples were collected from five (5) satellite stations downstream of the degasser facility and one (1) after the oil-water separator at the CPF. Each satellite station recovers fluid from different oil production wells, while the CPF water sample comingles fluids recovered from the satellite stations in the oilfield. Deposits samples accumulated at 6 o'clock position were collected from four different locations of the pipeline in the produced water recovery system. Deposits were sampled during replacement activities of corroded pipeline downstream the low-pressure separators. All replaced pipeline was covered by approximately 3 cm of schmoo material; only one of the samples was collected in an exact indication of wall thickness loss. The injection water sample was collected from the storage tank, downstream the source water wells. Samples were coded according to the source (produced water = PW, deposits = DP, injection water = IW). Sampling locations are indicated in **Figure 1**. Samples PW1 and PW2 belong to stations with biocide treatment tetrakis (hydroxymethyl) phosphonium chloride (THPS) whereas the other stations were not under chemical treatment. Produced water is also treated with Acrolein (ion dissolver and biocide) in the CPF separators

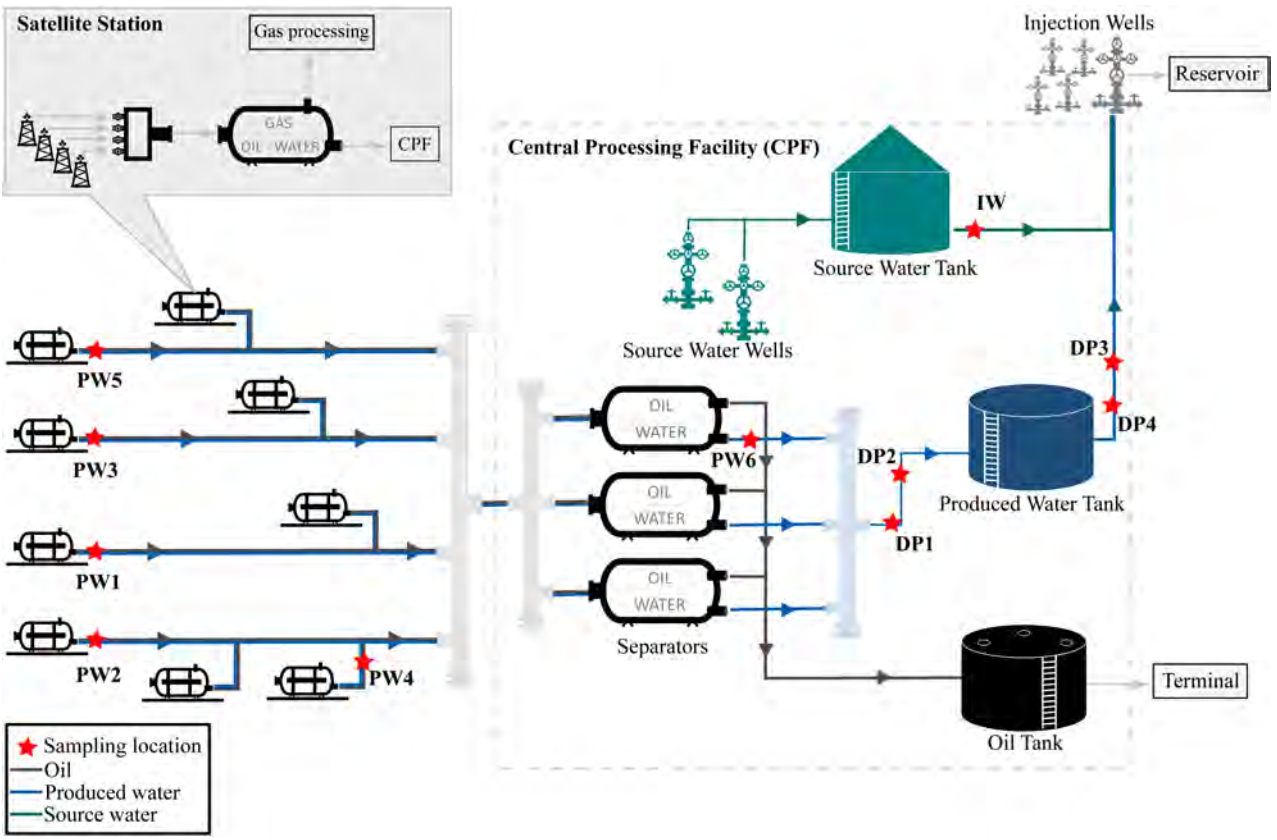

FIGURE 1 | Process flow diagram of the oil production facility.

before entering the produced water recovery system. In this way, PW6 and all deposits are biocide treated samples. Sample DP3 was the only sample collected in an exact indication of wall thickness loss.

Water samples were collected in baked and autoclaved glass containers, after 1 min of line flushing, whereas deposits samples were collected using sterile spatulas and placed in DNase/RNase free sterile plastic containers. Six samples were collected from each sampling location and processed separately to obtain triplicates for the DNA and RNA analysis, respectively. All containers were tightly sealed to avoid oxygen intrusion and immediately transported to the oilfield production laboratory for processing and preservation within maximum 30 min after collection. Oil-water samples were decanted in sterile separatory funnels for oil phase removal, and 500 mL of water were filtered through sterile 0.2 μm pore size membranes to harvest microbial cells. Deposits and filter membranes were immersed in RNAprotect® Bacteria Reagent (QIAGEN) and transported at 4°C to a research facility (2 days after collection) for further processing. Upon arrival, RNAprotect was washed from the samples with diethyl pyrocarbonate (DEPC) treated water, and samples were stored at −80°C until DNA/RNA extractions were conducted (maximum 1 week upon collection).

Chemical Characterization

Chemical analysis of produced water and deposits (performed by a certified commercial laboratory) were carried out following US EPA, APHA (American Public Health Association, 2005), and in-house test methods. Analyses included: (a) pH (Thermo Scientific, Orion Star A329 pH probe and meter); (b) conductivity (Thermo Scientific Orion 5-Star Conductivity meter); (c) total petroleum hydrocarbons (TPH) by Gas Chromatography-Flame Ionization Detector (GC-FID) (US EPA 3510C); (d) volatile fatty acids (VFA) by High Performance Liquid Chromatography (HPLC) (in-house method); (e) metals Fe, Mg, Na, K, Ca by atomic absorption spectroscopy (AAS) (APHA 3030 and 3110) and S, Cr, Cu, Mn, Ni, Zn by inductively coupled plasma optical emission spectrometry (ICP- OES) (APHA 3030 and 3120); (f) total nitrogen, total phosphorus, nitrate-N, chloride and sulfate measured using an automated Colorimetric/Turbidimetric Aquakem System (APHA 4500); (g) thiosulphate measured using an in-house method involving standardized iodate/iodide titration following by formaldehyde pre-treatment; (h) total organic carbon (TOC) by the high-temperature combustion method (APHA 5310B), and (i) alkalinity by titration (APHA 2320).

Microbial Enumeration

The number of cultivable sulfide-producing prokaryotes (SPP), acid-producing bacteria (APB), iron-reducing bacteria (IRB), and iron-oxidizing bacteria (IOB) were determined by the serial dilution method described in the standard test method NACE TM0194 (NACE International, 2014), which is the most widely used technique in the industry for monitoring oil field microbes. For counting SPP microorganisms, a culture media described elsewhere was used (Suarez et al., 2019a). Other microbial populations were evaluated using culture media suggested in the standard mentioned above. The serial dilution method consists of preparing 10-fold dilutions of the sample into liquid media. For this, 1 mL of water or 1 g of deposits was inoculated in a glass vial with 9 mL of culture medium and diluted six times (10^6). Each serial dilution was conducted in duplicate. Culture vials were incubated at the temperature found *in situ* during sample collection (40°C), for a total of 28 days. Positive growth was determined by changes in the culture media as per NACE TM0194 guidelines.

Nucleic Acids Extraction

To assess the potential involvement of the microbial communities in the corrosion failures experienced in the oil production facility, DNA and RNA-based profiling were used for the molecular characterization of the total and potentially active microorganisms, respectively. DNA was extracted from water samples using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions with the following modification: filters were placed into the PowerWater DNA Bead Tube containing solution PW1 and heated at 65°C for 10 min before the Vortex step. RNA was extracted using the RNeasy PowerWater Kit (QIAGEN) following the manufacturer's instructions. DNA and RNA concentrations were quantified fluorometrically with the Qubit dsDNA and RNA HS Assay kits (Life Technologies). Afterward, total RNA was treated with DNase I (Thermo Scientific) to remove remaining DNA. To verify the complete removal of DNA, a PCR reaction targeting the 16S rRNA gene was performed. Subsequently, RNA was purified and concentrated by using the RNeasy MinElute Cleanup kit (QIAGEN). Purified RNA was converted to cDNA by using the QuantiTect Reverse Transcription kit (QIAGEN). DNA and RNA from deposits were extracted as mentioned for water samples but employing the DNeasy PowerSoil and RNeasy PowerSoil Kits (QIAGEN), respectively. Despite several attempts with modified conditions, the extraction of high-quality RNA from the IW sample or cDNA synthesis from DP1 – DP4 samples failed.

Library Preparation and Sequencing

Polymerase chain reaction (PCR) and sequencing were performed by the Australian Genome Research Facility. PCR amplicons were generated using the primers 341F (5′ CCTAYGGGRBGCASCAG 3′) and 806R (5′ GGACTACNNGGGTATCTAAT 3′) (Yu et al., 2005). Thermocycling was completed with an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 master mix (Life Technologies, Australia) for the primary PCR. The first stage PCR was cleaned using magnetic beads, and samples were visualized on 2% Sybr Egel (Thermo-Fisher). A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were cleaned again using magnetic beads, quantified by fluorometry (Promega Quantifluor) and normalized. The equimolar pool was cleaned a final time using magnetic beads to concentrate the pool and then measured using a High-Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pool was diluted to 5 nM and molarity was confirmed again

using a High-Sensitivity D1000 Tape. This was followed by sequencing on an Illumina MiSeq instrument with a V3 (600 cycles) kit (Illumina).

Bioinformatics and Statistical Analysis

The Quantitative Insights Into Microbial Ecology Software (QIIME, v1.9.1) (Caporaso et al., 2010) was used for the analyses of the 16S rRNA gene sequences generated with the Illumina MiSeq. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (v0.9.10 - 64 bit) (Zhang et al., 2014) with default parameters. Then, Primers were identified and trimmed with Cutadapt (v1.10) (Martin, 2011) using default settings. Afterward, USEARCH (v10.2) (Edgar, 2010) was used for quality filtering, dereplication, denoising, and clustering into zero-radius operational taxonomic units (zOTUs) with the UNOISE3 algorithm. Chimeric sequences were removed using UCHIME (Edgar et al., 2011) with SILVA as reference database (SILVA v132) (Yilmaz et al., 2014). Filtered sequences were mapped to chimera-free OTUs, and the zOTU table was created using VSEARCH (v1.1.3) (Rognes et al., 2016). Taxonomic classification of the reference sequences (zOTUs) was performed by similarity searches using BLAST against the same SILVA database. Species richness, alpha and beta diversity estimates were determined using the QIIME algorithms. Sample comparisons were done at the same surveying effort, utilizing 34,532 by random selection.

Statistical analyses and graphs were conducted employing R (v3.4.3) (R Core Team, 2014), and PAST (v3) (Hammer et al., 2001) software. Results of statistical tests were considered significant with $p \leq 0.05$. The statistical analyses implemented depended on the normality of the data in each variable. Shapiro–Wilk test (Shapiro and Wilk, 1965) was used to determine data distribution and homogeneity of variance. To test differences in variables with normal distribution we used analysis of variance (ANOVA) followed by Tukey's multiple comparisons (Tukey, 1949). For those variables with a non-normal distribution, we used the Kruskal–Wallis test followed by Dunn's multiple comparisons. The shared microbial zOTUs among communities in the produced water, injection water and deposits were investigated using "VennDiagram" R package (Boutros Paul and Chen, 2011). Relative abundances of specific microbial groups in the total and active communities were studied at phylum and order level, whereas differences in the microbial composition of all sample sources was investigated at genus level. Bart charts of the microbial communities with phylogenetic groups with relative abundances equal or greater to 1% in at least one sample were created using the "ggplot2" R package (Wickham, 2016).

To visualize the multivariate dispersion of the community composition a non-metric multidimensional scaling (NMDS) analysis was performed based on the Weighted UniFrac distance (Lozupone and Knight, 2005), lines for joining samples collected in the same sample source were projected onto the ordination, utilizing the function *ordiellipse*. The effect of environmental parameters on the microbial community was analyzed using the *envfit* function and projected into the ordination with arrows. *Ordiellipse* and *envfit* functions are contained in the "vegan" R package (Oksanen et al., 2015). Permutational analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were used to test for significant differences in beta diversity, Bray–Curtis distance (Bray and Curtis, 1957) was used in these tests. To identify the microbial orders associated with the produced water at the different stations or within each sample source, an analysis based on the point biserial correlation coeÿcient was performed using *multipatt* in the "indicSpecies" R package (De Caceres and Legendre, 2009). For visualization, a network was generated using stations or sample source as source nodes, and the bacterial orders as target nodes. All taxa with significant associations were visualized in the networks. The network was performed using the edge-weighted spring embedded layout algorithm in Cytoscape (v3.5) (Shannon et al., 2003), with the edge weight corresponding to the association strength of each order with each sampling location.

The functional profile of the microbial communities was predicted using the "Tax4Fun" R package (Aßhauer et al., 2015). FTU (fraction of taxonomic units unexplained) values of the prediction were relatively low in most of the samples (FTU x̄ 26) which indicated that the majority of the zOTUs were included in the functional prediction. Comparison of the functional profiles predicted from the DNA and RNA-based sequencing was performed using the average of the relative abundance predicted per pathway in all samples. Linear discriminant analysis (LDA) effect size was employed through LEfSe v1.0 (Segata et al., 2011) to identify KEGG pathways as significant biomarkers of the microbial communities and sample sources. For this analysis, the alpha parameter significance threshold for the Krushkal–Wallis (KW) test implemented among classes in LEfSe was set to 0.05 and the logarithmic LDA score cut-off was set to 2.0. All analyses were performed through the Galaxy server (Goecks et al., 2010).

RESULTS

Chemical Characterization

The chemical composition of the water and deposit samples is shown in **Tables 1**, **2**, respectively. A clear difference in the chemistry of produced water and injection water was evidenced. Injection water contained lower levels of petroleum hydrocarbons (TPH), organic carbon (TOC) and volatile fatty acids (VFAs), as well as less total dissolved solids as indicated by the conductivity. Produced water samples exhibited similar characteristics in the different sampling locations, pH close to neutrality, high salinity, and similar content of organic compounds. The main difference in the chemical composition of produced water samples was the concentration of metals. PW1, PW2, and PW5 reported higher levels of iron and manganese. Additionally, PW2 displayed higher levels of zinc, as well as the presence of sulfate. Chromium, copper, nickel, sulfur, and thiosulphate were not detected in any of the water samples. On the other hand, deposits samples exhibited more variation in chemical composition among them. Different to

TABLE 1 | Chemical composition of produced water and injection water samples.

Compound/element[a]	LOD[b]	Produced water						Injection water
		Stations					CPF	Tank
		PW1	PW2	PW3	PW4	PW5	PW6	IW
pH	–	6.75	6.95	7.34	7.18	7.03	7.02	6.92
Conductivity (mS/cm)	–	60.1	60.3	68.3	65.7	65.2	59.2	48.0
TPH[c] C6-9 (mg/L)	0.02	2.2	0.64	1.1	2.3	44	4.4	<0.02
TPH C10-14 (mg/L)	0.02	27	8.7	10	25	210	43	0.74
TPH C15-28 (mg/L)	0.04	31	9.4	9.5	31	200	44	0.17
TPH C29-36 (mg/L)	0.04	1.7	0.28	0.19	3.0	20	2.6	<0.04
TPH C > 36 (mg/L)	0.04	0.29	0.08	<0.04	0.30	5.4	0.34	<0.04
Calcium (mg/L)	0.1	390	530	570	610	750	490	500
Iron (mg/L)	0.01	10	11	4.0	3.7	12	4.9	0.55
Potassium (mg/L)	0.1	190	370	170	160	170	170	310
Magnesium (mg/L)	0.1	190	270	370	310	370	240	140
Manganese (mg/L)	0.01	0.16	0.15	0.06	0.08	0.15	0.07	0.07
Sodium (mg/L)	0.1	13,000	13,000	15,000	14,000	14,000	14,000	9,800
Zinc (mg/L)	0.01	0.16	1.7	<0.01	0.30	0.04	<0.01	0.07
Ammonia-*N* (mg/L)	0.02	45	42	52	47	47	43	41
Chloride (mg/L)	5	22,000	23,000	28,000	27,000	26,000	25,000	20,000
Sulfate (mg/L)	1	<1	2	<1	<1	<1	<1	<1
Alkalinity (mg CaCO3/L)	5	720	520	610	410	450	540	580
Salinity (mg/L)	10	37,000	39,000	44,000	42,000	42,000	38,000	31,000
Acetic Acid (mg/L)	1	66	39	69	29	67	33	<1
Propionic Acid (mg/L)	2	29	24	60	53	36	28	<2
TOC[d] (mg/L)	1	96	64	100	73	97	56	< 1
Total Nitrogen (mg/L)	0.2	45	42	52	47	47	43	41
Total Phosphorus (mg/L)	0.01	0.11	0.38	0.12	0.25	0.06	0.15	0.10

[a]Chromium, Copper, Nickel, Sulfur, NOx-N, Nitrate-N, Nitrite-N, and thiosulphate were not detected in any of the samples. [b]LOR, Limit of Detection. [c]TPH, Total Petroleum Hydrocarbons. [d]TOC, Total Organic Carbon.

produced water, deposits exhibited high levels of sulfur, sulfate and thiosulphate. Samples also contained high levels of petroleum hydrocarbons and metals such as iron and manganese, the last two probably associated with under-deposit corrosion (UDC). Nitrogen oxides, nitrate, and nitrite were not detected in any of the samples.

Microbial Enumeration

Serial dilution analysis indicated that microbial populations typically monitored by the oil and gas industry and previously associated with corrosion are present in all the sources evaluated in concentrations of 10–10^6 Bact/mL-g. Sulfide producing microbes and APB were widespread across the oil production facility whereas iron utilizing microorganisms were present only in a few locations, as shown in **Table 3**. Stations treated with biocide (PW1 and PW2) reported similar levels of microorganisms than other stations without biocide treatment (PW3, PW4, and PW5). Microorganisms were also detected in the deposits samples despite the chemical treatment applied to the system. In fact, sample DP4 exhibited the highest concentration of sulfide producing and acid producing microorganisms of all samples collected.

Microbial Molecular Characterization

Characteristics of the 16S rRNA Datasets

A total of 3,991,895 (DNA-based) and 2,926,183 (RNA-based) sequence reads were obtained from the MiSeq sequencing. After removal of low-quality sequences, chimeras and singletons 3,536,010 (DNA-based) and 2,010,504 (RNA-based) high-quality sequences were used for the diversity profiling analysis. The number of sequences in each sampling location ranged from 34,532 to 203,508 DNA-based, and from 73,419 to 168,184 RNA-based. After rarefaction analysis with normalized sequences per sample (34,532), we obtained 493 zOTUs from DNA-based (ranged from 54 ± 2 to 285 ± 4 per sample) and 287 from RNA-based (ranged from 116 ± 5 to 198 ± 8 per sample). Reads and zOTU counts for the individual sample replicates are summarized in **Supplementary Table S1**. The Good's coverage index of 0.99 (±0.005) for both sequencing approaches indicated that the datasets enclose all major microbial groups inhabiting the oil production facility (**Supplementary Figure S1**).

The shared microbial zOTUs analysis indicated that there are unique and shared zOTUs in each sample source (**Supplementary Figure S2**). Shared zOTUs between the three sources was 65, accounting for 78% of the total community

TABLE 2 | Chemical composition of deposits samples collected in the produced water system.

Compound/element[a]	LOD[b]	DP1	DP2	DP3
TPH[c] C6–9 (mg/Kg)	0.2	5,900	22000	26000
TPH C10–14 (mg/Kg)	0.2	22,000	74000	87000
TPH C15–28 (mg/Kg)	0.4	1,400	83000	100000
TPH C29–36 (mg/Kg)	0.4	<0.4	8100	12000
TPH C > 36 (mg/Kg)	0.4	<0.4	3200	5300
Calcium (mg/Kg)	10	160	7100	1500
Chromium (mg/Kg)	1	11	120	77
Copper (mg/Kg)	1	7	1300	36
Iron (mg/Kg)	1	330,000	66000	58000
Potassium (mg/Kg)	10	<10	820	790
Magnesium (mg/Kg)	10	30	1100	970
Manganese (mg/Kg)	1	2,900	470	260
Sodium (mg/Kg)	10	<10	18000	36000
Nickel (mg/Kg)	1	8	260	170
Sulfur (mg/Kg)	10	77,000	41000	31000
Zinc (mg/Kg)	1	<1	680	1900
Total Nitrogen (mg/Kg)	10	430	13000	28000
Total Phosphorus (mg/Kg)	1	3	11000	11000
Ammonia-N (mg/Kg)	10	30	60	70
Chloride (mg/Kg)	10	1,700	6400	8000
Sulfate (mg/Kg)	10	40,000	10	10
Thiosulphate (mg/Kg)	2	600	82	140
Propionic Acid (mg/Kg)	2	120	<2	<2
Formic Acid (mg/Kg)	2	<2	30	70
TOC[d] (mg/Kg)	0.1	10	14	19

[a]*NOx-N, Nitrate-N and Nitrite-N were not detected in any of the samples.* [b]*LOR, Limit of Detection.* [c]*TPH, Total Petroleum Hydrocarbons.* [d]*TOC, Total Organic Carbon.*

TABLE 3 | Enumeration of microorganisms associated to MIC corrosion.

Sample	SPP[a]	APB[b]	IRB[c]	IOB[d]
PW1	10^3	10^2	<10	<10
PW2	10^2	10^2	10^1	10^3
PW3	10^3	10^2	<10	<10
PW4	10^3	10^2	<10	<10
PW5	10^2	10^1	<10	<10
PW6	10^2	10^2	<10	<10
DP3	10^3	10^1	<10	<10
DP4	10^6	10^3	<10	<10
IW	10^2	<10	<10	10^1

[a]*SPP, Sulfide-producing prokaryotes;* [b]*APB, Fermenting acid-producing bacteria;* [c]*IRB, Iron reducing bacteria;* [d]*IOB, Iron oxidizing bacteria.*

in the injection water, 15% of deposits, and 17% of produced water. Most of the zOTUs were simultaneously detected in the produced water and deposits (263 zOTUs accounting for 53% of the total zOTUs detected). However, deposits hosted more individual species than other sample sources (105 zOTUs). Based on the Venn diagrams, unique zOTUs were also detected in the satellite stations and deposits when the same sample source was compared. Only 22% (82 zOTUs) of the total zOTUs detected in produced water were shared in all stations. Stations with biocide treatment (PW1 and PW2) exhibited more individual species than stations without biocide treatment. Similar to produced water, only 29% (125 zOTUs) of the total zOTUs detected in deposits were common in all samples. Shared zOTUs analysis between DNA and RNA-based profiling showed that most of the microorganisms present in produced water were detected with both sequencing approaches (325 zOTUs accounting for 82% of the total zOTUs detected in produced water). The detection of unique zOTUs with the DNA-based approach indicated that not all microorganisms in the system are active. Likewise, the detection of unique zOTUs with the RNA-based approach indicated that the system host rare taxa that are highly active.

Comparison of DNA and rRNA Amplicon Libraries

Total and potentially active microbial communities in the produced water were dominated by Bacteria. Relative abundances of Archaea were higher in the RNA-based profiling than in the DNA-based profiling. Same dominant phyla were detected with both methodological approaches. However, the relative abundances of the phyla detected in each sampling location varied between DNA and RNA-based profiling (**Figure 2A** and **Supplementary Table S2**). The trend of variation in the abundance of each phylum was similar in all samples. Dominant phyla were *Proteobacteria* (28% DNA, 35% RNA), *Firmicutes* (24% DNA, 18% RNA), *Euryarchaeota* (19% DNA, 30% RNA), *Synergistetes* (16% DNA, 8% RNA), *Thermotogae* (7% DNA, 1% RNA), *Kiritimatiellaeota* (2% DNA, 4% RNA), and *Epsilonbacteraeota* (1% DNA, 2% RNA). At the order level, *Methanococcales, Methanosarcinales,* and *Desulfovibrionales* were more abundant in the RNA-based than in the DNA-based profiling (**Figure 2B** and **Supplementary Table S3**). *Clostridiales, Synergistales, Kosmotogales*, and *Thermotogales* orders showed the opposite trend. Dominant orders such as *Desulfuromonadales, Rhodospirillales,* and *Methanomicrobiales* presented similar abundances in the total and potentially active communities.

Microbial Community Composition of the Three Sample Sources at the Genus Level

DNA-based profiling of the abundant genera found in the different sampling locations of the oil production facility is shown in **Figure 2C** (complete list of genera is available in the **Supplementary Table S4**). Results indicated that produced water and deposits samples contained similar populations with differences in their abundance among samples. Results also evidenced that the microbial community existing in the injection water was markedly different from the other sample sources. An overview of the main microbial genera found in the production samples (water and deposits) allowed to differentiate two communities. The first type of community was evidenced in deposits samples DP2 and DP3. The community was principally composed by *Methanosaeta* (x̄ 35%), *Thermovirga* (x̄ 20%), *Methanoculleus* (x̄ 8%), and *Caminicella* (x̄ 6%) genera. The second type of community was found in all other samples, main species were *Caminicella* (x̄ 16%), *Thermovirga* (x̄ 14%), *Pelobacter* (x̄ 8%), *Roseospira* (x̄ 7%), *Methanothermococcus*

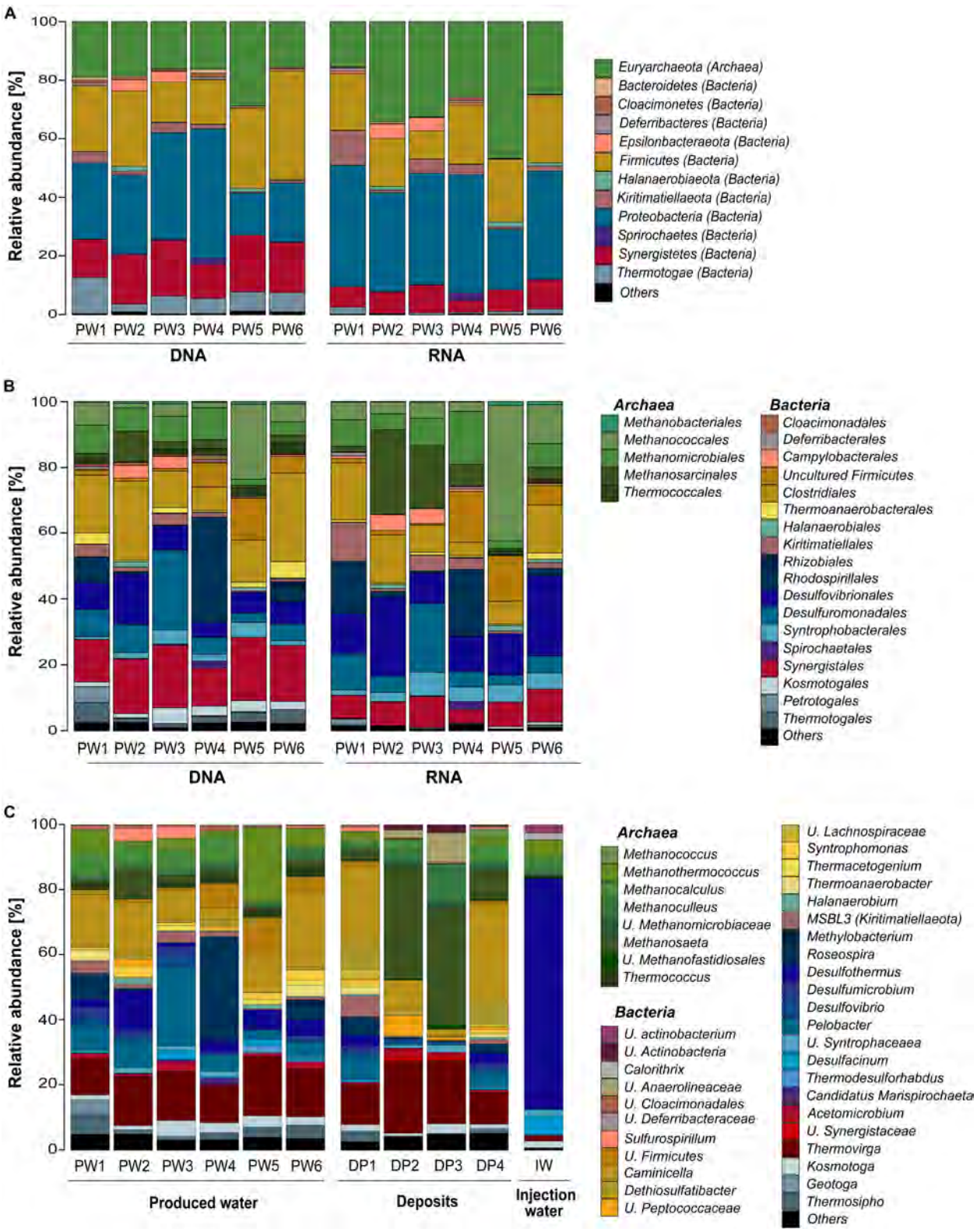

FIGURE 2 | Microbial community composition in the oilfield retrieved from 16S rRNA gene and transcripts sequencing. **(A)** Total and active microbial communities in produced water samples at phylum level. **(B)** Total and active microbial communities in produced water samples at order level. **(C)** Total microbial community in produced water, deposits and injection water samples at genus level. U., unclassified. Phylogenetic groups accounting for <1% of all classified sequences were summarized in the artificial group "Others." Results from the three replicates collected in each sampling location were pooled together.

(x̄ 6%), *Desulfothermus* (x̄ 5%), and *Methanocalculus* (x̄ 5%). Differently, the microbial community in injection water was dominated *Desulfothermus* (x̄ 71%), *Desulfacinum* (x̄ 6%), *Methanothermococcus* (x̄ 5%), *Methanocalculus* (x̄ 3%), and *Methanoculleus* (x̄ 3%).

Alpha Diversity Analysis of the Microbial Community

Comparison of the biodiversity between total and potentially active communities in produced water samples showed significant ($p \leq 0.05$, *t*-test) differences in the alpha diversity measurements. The richness index (Chao1) of the microbial community was highest for the RNA-based profiling and lowest for the DNA-based profiling (**Figures 3A,B**). Conversely, the diversity index (Simpson) showed the opposite pattern (**Figures 3D,E**). Statistical analysis of the alpha diversity also revealed significant differences in the biodiversity among sampling locations ($p \leq 0.05$, ANOVA). Unexpectedly, stations with biocide treatment (PW1 and PW2) displayed higher richness than stations without biocide treatment under both sequencing approaches. A similar pattern was evidenced in diversity indices with the DNA-based analysis but slightly different from the RNA-based. The diversity of the potentially active community in station PW1 was the highest, whereas diversity in PW2 and PW5 were the lowest.

Alpha diversity metrics calculated from the DNA-based sequencing were significantly different ($p \leq 0.05$, ANOVA) among sample sources and sampling locations (**Figures 3C,F**). In the comparison of sample sources, deposits presented the highest Chao1 richness, followed by produced water, and injection water. Simpson diversity measurements were similar between deposits and produced water, and lower in the injection water. Looking at the biodiversity among samples from the same source, both the samples from produced water and samples from deposits presented significant variations in the alpha diversity indices calculated.

Environmental Factors Affecting the Microbial Community Structure

Non-metric multidimensional scaling ordination analysis showed differences in the microbial community structure of the produced water samples (**Figures 4A,B**). Greater differences were observed with the RNA-based sequencing approach, which showed a clear separation of the sampling points. The two-way PERMANOVA and two-way ANOSIM tests confirmed that the community structure between sequencing approaches, as well as among sampling locations had significant differences ($p = 0.0001$). We tested the correlation of physicochemical characteristics and fitted them onto the ordination to determine what properties were correlated to the total and active community composition. The structure of the total community (DNA based) was strongly influenced by concentrations of TPH ($p = 0.001$), iron

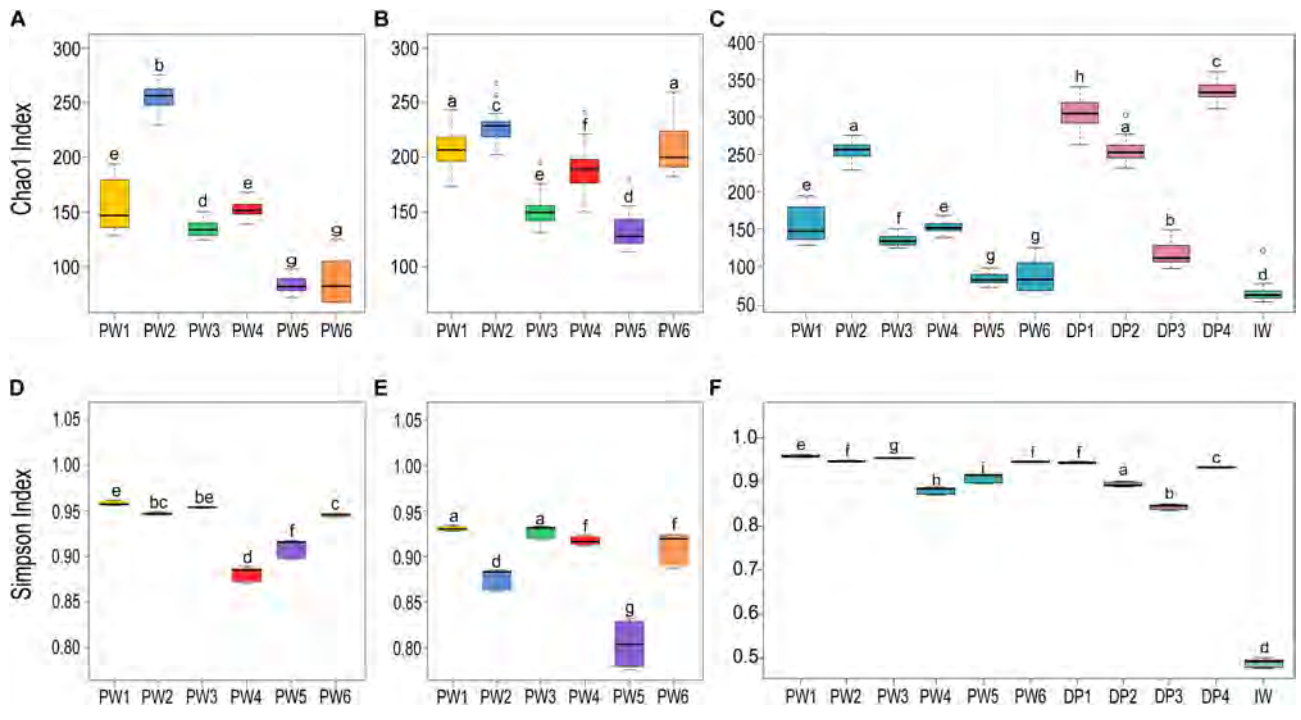

FIGURE 3 | Alpha diversity indices of microbial communities in the different sampling locations. **(A,C,D,F)** Richness (Chao1) and diversity (Simpson) indices of the total communities. **(B,E)** Richness and diversity indices of the active communities. Boxes are extended from the 25–75th percentiles, the line in the box is plotted at the median. Whiskers represent the smallest and the largest value. ANOVA followed by Tukey's multiple comparison tests was used to determine differences among stations. Samples with the same letter indicate that the diversity indices were not significantly different ($p > 0.05$).

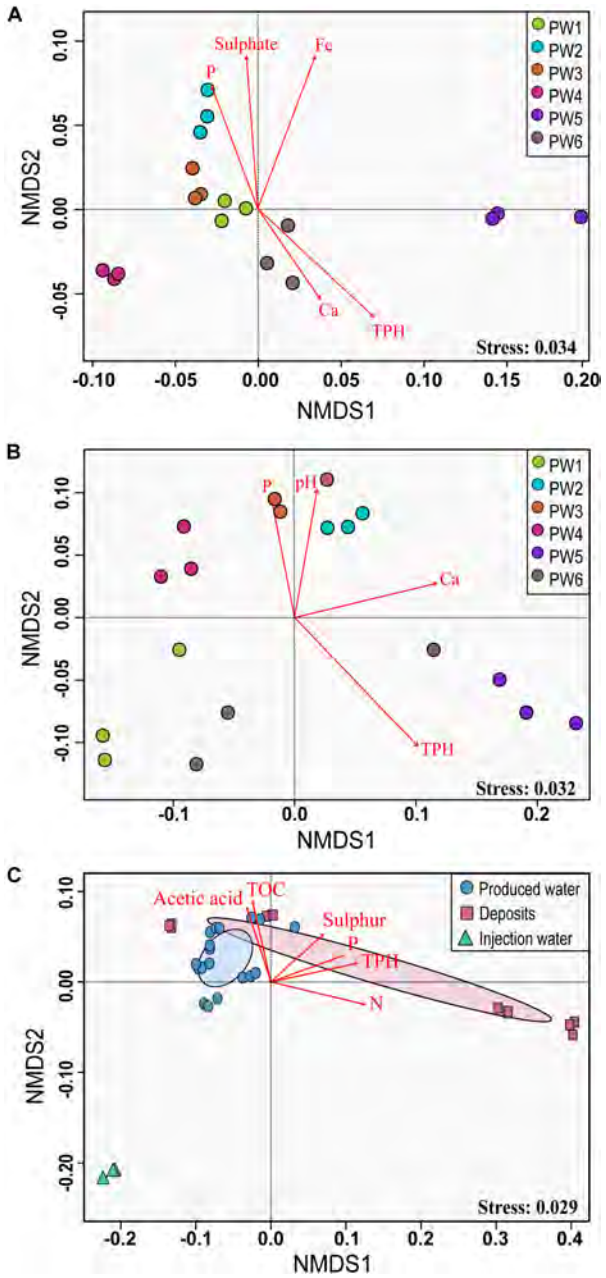

FIGURE 4 | Non-metric multidimensional scaling (NMDS) analysis of the microbial communities. **(A)** NMDS for total microbial communities in produced water samples. **(B)** NMDS for active microbial communities in produced water samples. **(C)** NMDS for total microbial communities in all sampling locations. The analysis was based on weighted UniFrac distance matrices. Environmental parameters that were significantly correlated ($p \leq 0.05$) to microbial community structure are indicated by arrows.

(p = 0.001), sulfate (p = 0.006), and phosphorous (p = 0.008). On the other hand, the structure of the potentially active community (RNA based) was greatly influenced by the concentration of TPH and calcium (p = 0.002 and 0.007, respectively), followed by pH and phosphorous (p = 0.022 and 0.033, respectively).

Non-metric multidimensional scaling ordination with all sampling locations confirmed differences in the microbial community structure according to the sample source (**Figure 4C**). Injection water samples created a separated cluster away from deposits and produced water samples. According to the PERMANOVA and ANOSIM tests, differences were significative (p = 0.004 and p = 0.006, respectively). Pairwise comparison evidenced that the significant differences in the microbial structure were only related to the injection water samples. The comparison of the microbial structure in produced water and deposits samples showed no significant differences (p = 0.07). Correlation between environmental variables with the total microbial composition of all sampling points showed that concentration of TPH, nitrogen, phosphorous, TOC, sulfur and acetic acid impacted considerably the structure of the total community (p = 0.001, 0.001, 0.004, 0.015, 0.025, 0.031, respectively).

Association Networks of Specific Taxa With Sampling Location

Association networks indicated that 85% of the total orders detected in the active community had significant biserial correlation coeÿcients with the sampling location ($p \leq 0.05$). Most of the orders were associated with stations with biocide treatment PW1 and PW2 indicating that the environment at these locations favors the activity of several microorganisms (**Figure 5A**). The majority of the orders significantly associated with each station belong to the same microbial groups (sulfate reducing bacteria, fermenting bacteria, and methanogens).

The correlation-based association analysis of the orders significantly associated with the sample source (**Figure 5B**) was consistent with the multivariate analysis (**Figure 4C**). None of the orders that were significantly associated with the injection water was significantly associated with another sample source. The majority of the orders were significantly associated with only one source suggesting that the microbial community structure in the oilfield is driven by the specific conditions along the facility. Only 8% of the orders were significantly associated with both production sample sources.

Functional Profile Prediction

Tax4Fun analysis applied to infer the metagenomic content of the total and active communities in produced water predicted the presence of 6422 KEGG Orthologs (KO) across all samples (**Supplementary Table S5**). LEfSe analysis indicated that 43 from the 280 pathways found, were significantly different between communities recovered with both sequencing approaches (**Supplementary Table S6**). Level 2 KO predicted from the DNA and RNA-based profiling are presented in **Figure 6**. Overall, the functional structure of the communities was dominated by metabolism-related KEGG pathways, especially that of carbohydrates, amino

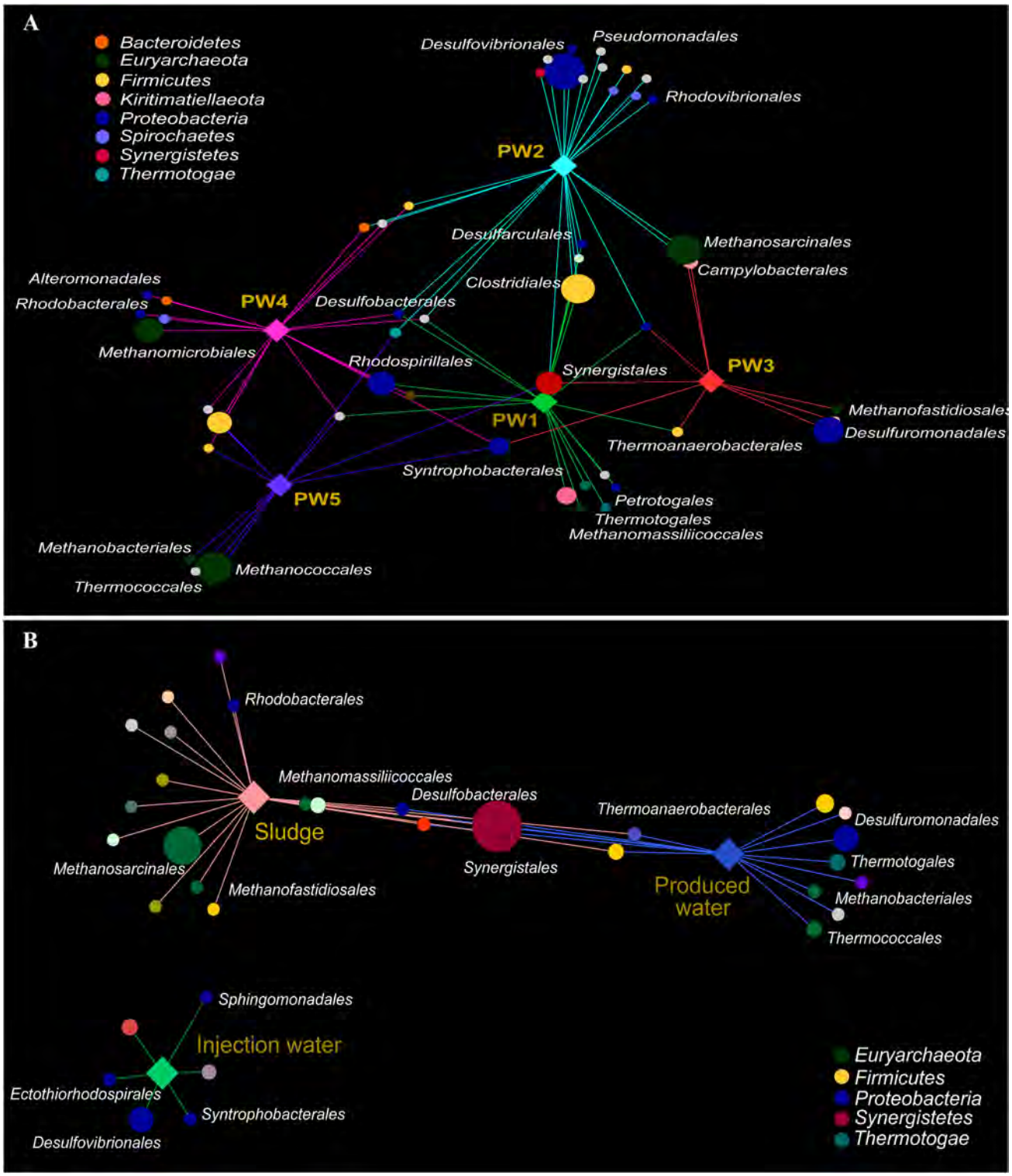

FIGURE 5 | Correlation-based association network between microbial community (order level) and sampling location. **(A)** Association network of active community with produced water stations. **(B)** Association network of total community with sample sources. Only statistical significant microbial orders are visualized ($p \leq 0.05$). The size of each node is proportional to the taxon relative abundance and the edge width corresponds to the association strength of each taxon with the sampling location. Color of nodes contributes to prominent microbial phyla. Hub nodes and edges are colored according to sampling location.

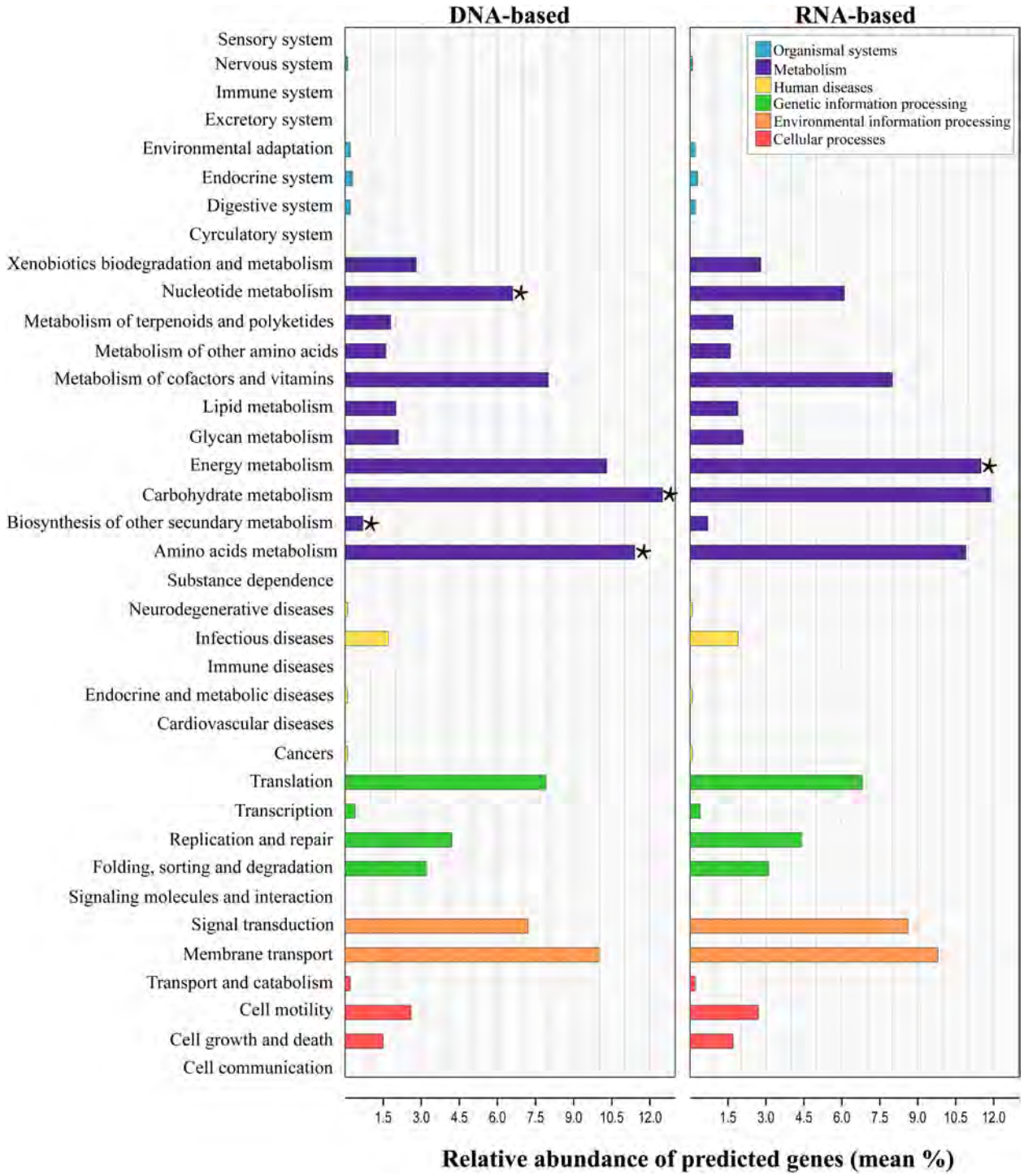

FIGURE 6 | Prediction-based abundance of genes involved in KEGG level 2 categories in total and active communities in the produced water samples. Significant ($p \leq 0.05$) biomarkers identified with the linear discriminant analysis (LDA) effect size are indicated by a star (⋆).

acids, nucleotide, energy, cofactors and vitamins. Other dominant KEGG categories predicted were environmental and genetic information and processing, principally in pathways related to signal transduction, membrane transport, and translation. Genes related to cellular processes, human diseases and organismal systems were predicted with lower abundances. LEfSe analysis at this level showed that metabolic pathways related to carbohydrate, amino acids, nucleotide and

biosynthesis of other secondary metabolites were biomarkers of the DNA-based analysis, whereas energy metabolism was a biomarker of the RNA-based analysis. Within the energy metabolism, the methane metabolism pathway was the most abundant.

Linear discriminant analysis test applied for the identification of the metabolic KEGG pathways associated with the sample source showed that 46 of the 139 pathways predicted were significantly differentially abundant among sources (**Supplementary Table S7**). According to the results, pathways related to the metabolism of amino acids and xenobiotics were significantly enriched in the deposits and production water samples, whereas pathways related to the energy and carbohydrate metabolism were enriched in injection water samples. It has to be noted, that the results from predicted functional profile based on 16S rRNA data can deviate from metagenomics profiling since taxonomic identification does not necessarily relates to the presence of functional genes. Additionally, zOTUs derived from unknown taxa limit this prediction. In this sense, the predicted metabolic pathways identified here remain to be validated by metagenomics studies in future.

DISCUSSION

DNA Versus RNA-Based Diversity Profiling

The comparison between the DNA and RNA-based diversity profiles indicated that not all the microbial community members were active and that not all the active members were detected with the DNA approach. Predominant orders in the community were recovered with both methodologies, but significant differences in their relative abundance were evidenced. One of the main differences detected was the reduction in the abundances of all thermophilic orders (*Thermotogales, Thermococcales, Thermoanaerobacterales, Synergistal*es, *Petrotogales,* and *Kosmotogales*) in the RNA approach. The temperature at the time of sampling in all analyzed locations was approximately 40°C, which could explain the lower abundances of thermophiles in the active communities. Thermophilic microorganisms are commonly associated with native populations in oil reservoirs (Ollivier and Magot, 2005; Song et al., 2017), however, temperature gradients generated during the production process favor the growth of mesophilic microorganisms at lower temperatures (Li et al., 2017b). The effect of the temperature variations on the community structure of active populations has been previously evaluated (Salgar-Chaparro et al., 2019). The authors pointed out that temperature has a significant impact in shaping the microbial composition of oilfield systems which, in turns, affects its corrosivity. Other microbial orders that exhibited a decrease in the relative abundances with the RNA-based approach were *Clostridiales, Rhodospirillales,* and *Synergistal*es. Several species that belong to these taxonomic groups have been associated with oil degradation, which is one of the principal metabolisms in oil reservoirs along with fermentation, methanogenesis and sulfate reduction. It has been reported that oil-degrading microorganisms need to be in contact with the petroleum hydrocarbons to be able to use them as electron donors, while also being in contact with the water phase for reaching electron acceptors such as sulfate, nitrate, or ferric iron (Pannekens et al., 2019). Therefore, considering that the water cut in all sampling locations was close to 95%, it is inferred that the oil fraction in the fluid could have been a limiting factor for the microbial growth of these populations, which might provide an explanation to their lower abundances in the RNA-based approach compared to the DNA-based approach. Conversely, sulfate reducers like *Desulfuromonadales, Desulfovibrionales,* and *Syntrophobacterales,* as well as, methanogens such as *Methanobacteriales, Methanococcales, Methanomicrobiales*, and *Methanosarcinales* showed an increase in their abundance in the active communities. Higher abundance of these populations in the RNA-based analysis suggests that the oil production facility provides suitable conditions for the metabolic activities of these particular groups.

Alpha and beta diversity analysis of the total and active populations confirmed that differences in communities recovered with both sequencing approaches were significant. Higher diversity values obtained with the DNA-based analysis are the result of recovering active, dormant and dead cells when studying the DNA molecule. Conversely, RNA-based analysis only retrieves information about the active cells, thereby lower values of biodiversity are often obtained. Similar results have been described in other studies where a comparison of the DNA and RNA sequencing profile was carried out (Angel et al., 2013; Kim et al., 2013; De Vrieze et al., 2016; Inkinen et al., 2016; Li R. et al., 2017). Dissimilarities in the diversity profile of total and active communities were also reflected in the predicted functional capability. Fundamentally, genes involved in energy metabolism, principally the genes related to methane metabolism were significantly enriched in the RNA-based sequencing approach. Higher capability for using methane pathways resulted from the increase in the relative abundance of methanogens in the active communities.

Differences between the DNA and RNA-based results highlighted the importance of using a complementary methodological approach for studying microbial populations. It has to be noted that RNA-based methods have disadvantages linked to more laborious extraction procedures, the susceptibility of RNA to degradation, presence of multiple copies of ribosomes per cell, and the existence of rRNA reserves in dormant cells (Angel et al., 2013). Even considering these drawbacks, RNA-based methods can better reflect the active members of oilfield communities compared to DNA surveys, which is essential for assessing the potential involvement of microorganisms in corrosion since only metabolically active microorganisms can cause MIC. Identification of active microorganisms in oilfield systems also provides relevant information with regards to the eÿc acy of biocide treatments. The detection of active microorganisms after exposure to biocide treatments indicates ineÿciency of treatments or

inadequate treatment dosages, which can lead to the emergence of resistant communities. Therefore, early detection of microbial activity in industrial facilities would help optimize mitigation strategies to control MIC.

Microbiological Assessment in the Oil Production Facility

The microbial community recovered from the water and deposits samples provided a general representation of the planktonic and sessile populations inhabiting the production system. Microbiological characterization using the complementary approach indicated that total and active microbial communities in the oilfield were dominated by bacteria in a proportion that ranged from 50 to 80%. Taxonomic identification of the sequencing reads revealed that several of the microorganisms present in the oil facility have been previously reported in other oil production facilities and corrosive environments.

Main corrosion damages in the oil facility have been reported downstream satellite stations and water-oil separation. As mentioned before, systems with major corrosion problems are under chemical treatment with biocides (THPS or Acrolein) to reduce the risk of MIC. Nonetheless, produced water samples with THPS treatment showed similar microbial composition to the samples without biocide treatment. Likewise, microbial enumeration showed a similar concentration of microorganisms in treated and non-treated stations. In the same way, deposits samples that are treated with Acrolein reported the same or greater microbial concentrations than produced water. All these findings suggest that the biocide treatments, which are intended to inactivate all microorganisms present, are not effective against microorganisms in the facility. THPS biocide is widely used in the industry due to its adequate characteristics such as low toxicity, broad-spectrum activity and ability to dissolve ferrous sulfide deposits (Conlette, 2014). However, it is known that the persistent use of the same biocide chemical will lead to the selection of resistant microorganisms over time (Li et al., 2016). Moreover, biocide chemicals can adsorb onto deposits, which will result in underdosing of chemical treatments, therefore reducing the biocide residual concentration required to maintain microbiological control. Likewise, there are microorganisms that can degrade THPS at sub-lethal concentrations, using it as a nutrient for growth (Vorholt et al., 2000). THPS degradation produces formaldehyde under aerobic conditions and methanol under anaerobic conditions (Sharma et al., 2018). Since oil production systems are anaerobic environments, methanol is the most probable byproduct in THPS degradation. This molecule can be used as a carbon source by several of the methanogens detected in the oilfield. Additionally, THPS dissociation releases sulfate and phosphorus to the environment, both also used by many microorganisms in their metabolic functions (Sharma et al., 2018). In fact, station PW2 that undergoes biocide treatment had a higher phosphate concentration compared to other stations and was the sole station where sulfate was detected. It can be speculated that the presence of these additional components in the produced water treated with THPS is associated with the higher richness values measured compared to stations without chemical treatment.

Microbiological analysis of the deposits samples recovered from different locations in the produced water pipework showed significant variations in the community structure among samples. Differences were also detected in the chemical composition of the deposits, particularly relating TPH, iron, sulfur, and sulfate concentrations. It is known that a non-homogeneous environment generates microniches with dominant populations adapted to the local conditions (Korona, 1996), which could explain the variability in the microbial communities recovered from the same system. Compared to produced water samples, deposits samples presented a higher number of zOTUs and richness values. More zOTUs in the deposits may be related to species accumulation over time in the biofilm communities living in the deposits, whereas water samples only reflect the community in the fluid at the time of sampling. In addition, other studies have shown that extracellular DNA (eDNA) can potentially be adsorbed in deposits or surfaces over time. eDNA can remain in the environment as part of sediment particles that can preserve it from degradation (Dell'anno and Corinaldesi, 2004; Torti et al., 2015; Corinaldesi et al., 2018). Indeed, corrosion products have been nominated as a repository of eDNA in the Makama et al. (2018) investigation, who found eDNA in biofilm-free corroded surfaces. Due to the diÿculty of extracting good quality RNA from the deposits samples, it was not possible to determine if the higher richness detected in these samples was a consequence of recovering eDNA preserved in the schmoo. According to the chemical composition, deposits had high levels of metals which correlates with active corrosion evidenced in the produced water recovery system. Likewise, high levels of total sulfur and sulfur compounds including sulfate, thiosulphate were detected in deposits. The source of these sulfur compounds remain unclear since both produced and injection waters did not have considerable concentrations of such compounds (<10 ppm of sulfate/thiosulphate). It is plausible to expect that small amounts of those compounds can also be adsorbed and accumulate in deposits over time (Suarez et al., 2019b). It has been documented that high doses of THPS can lead to the precipitation of the sulfate introduced by the THPS, which cause scale formation downhole (Li et al., 2016). The presence of these additional nutrients in the deposits is expected to attract more species and result in higher richness, as evidenced in this study.

As mentioned before, the production facility has been flooded for over 30 years to stimulate the reservoir and increase oil recovery. Long-term water injection can modify the indigenous microbial community structure in oil systems (Zhang et al., 2012; Lenchi et al., 2013; Gao P. et al., 2015). Apart from the microbiological contamination, water injection is problematic to oil reservoirs by providing nutrients and electron acceptors to the resident microorganisms (Magot et al., 2000; Varjani and Gnansounou, 2017). For evaluating the impact of this practice in the indigenous oilfield microbial community, and determining if the populations detected were the result of microbial contamination during water injection, the source water used

in the injection system was also characterized. Microbiological analysis showed a clear difference in the microbial structure between production water and injection water which suggests that the secondary recovery practices have not had a significant impact on the community widespread in the facility. Moreover, species association analysis confirmed that none of the orders found in the injection water sample was significantly associated with the produced water or the deposits samples. Similarly, chemical analysis of the samples showed that source water contains lower levels of ions, metals and organics compared to produced water; therefore, it is not providing additional nutrients to the community in the reservoir. These results are in disagreement with similar investigations in other oilfields, where significant changes in the microbial structure of the oil reservoir were evidenced after the water injection (Gao P. et al., 2015; Gao P. K. et al., 2015). The likely explanation of this phenomenon is that the ratio of an external source for water injection is only 5:95 source water:produced water. The above indicates that potentially corrosive microbial populations found in the oil production facility are likely coming directly from the reservoir.

Dominant microorganisms found active in the facility belong to three microbial groups, sulfate reducers, fermenters, and methanogens; all of these previously associated with MIC processes (Machuca et al., 2017; Machuca and Polomka, 2018; Vigneron et al., 2018; Suarez et al., 2019a). Microbial interactions among these populations have been previously studied (Raskin et al., 1996; Morris et al., 2013; Ozuolmez et al., 2015; Sela-Adler et al., 2017). Syntrophic interactions between fermenters (H_2 producing microbes) and methanogens (H_2 consuming microbes) are widely known. Due to the physiological versatility of several sulfate reducers, similar interactions can occur between fermenters and sulfate reducers, which can use the H_2 produced by fermenters as electron donor in respiration, or methanogens and sulfate reducers, which can also have the capability to obtain energy from fermentation providing nutrients such as H_2 and acetate to methanogenic species (Morris et al., 2013). Co-existence of methanogens and sulfate reducers is usually reported in oil production facilities (Lenchi et al., 2013; Lin et al., 2014; Varjani and Gnansounou, 2017), however, microbial interactions appear to be affected by the presence of sulfate (Oremland and Polcin, 1982; Okoro and Amund, 2018). In the presence of high sulfate concentrations, sulfate reducers compete with methanogens for the availability of nutrients. Conversely, low sulfate content environments, such as the produced water studied here, favor syntrophic interactions (Paulo et al., 2015). Thus, the increase in the relative abundances of methanogens and sulfate reducers in the active communities might be related to a symbiotic relationship.

Multispecies biofilms formed by same microbial groups found in this investigation have been reported to accelerate UDC of steel (Larsen and Hilbert, 2014; Machuca et al., 2017; Shukla and Naraian, 2017; Suarez et al., 2019a). It is known that different interactions between microorganisms might induce a cascade of biochemical reactions that cause more severe corrosion than single-species biofilms (Kip and Van Veen, 2015). In the presence of deposits, microbial cells are attracted to these particles as they provide nutrients, a suitable environment for the synergistic interactions of sessile communities, and also protection from shear forces and biocides (Kagarise et al., 2017). Microbial communities can interact with the deposits changing their properties, such as making them more electroactive or precipitating new corrosive species in the metal surface, which favors the formation of microenvironments and differential cells that can cause localized corrosion (Machuca et al., 2011). The existence of a schmoo layer previously suggested that corrosion is taking place in the oilfield assets by UDC mechanisms. Nonetheless, the detection of active microbial populations with reported corrosive metabolisms in this investigation provided more evidence to the hypothesis that the corrosion processes in the facility may be the result of UDC enhanced by MIC mechanisms. Further laboratory investigations simulating the oilfield conditions are required to study the possible MIC-UDC mechanisms involved.

CONCLUSION

Complementary analysis of DNA and RNA-based amplicon sequencing allowed to assess differences in the microbial composition of total and active communities in the oil facility. It was demonstrated that DNA results alone could lead to underestimation of active members in the community. By implementing the RNA-based sequencing, it was found that not all microorganisms in the communities were active whereas other community members showed an increase in their relative abundances, which is proposed to be related to higher activity. A better characterization of active microorganisms can improve the understanding, mitigation and prediction of MIC processes. Moreover, this methodological approach can be used to evaluate the impact that operational conditions like temperature and water chemistry have on microbial activity and community structure. The reduction on the relative abundances of thermophilic species in the active community seen in this study was likely to be related to the decrease in temperature from the reservoir to the oil production facility. In addition, this study showed the detection of active microorganisms at biocide treated locations, which added to the identification of similar microbial composition and cells concentration in locations without biocide treatment suggesting poor eÿc acy of the mitigation treatments. Bias in the DNA-based analysis resulted in an underestimation of the predicted capability of the community for using methane pathway in the energy metabolism, which was correlated with the lower abundance of the methanogenic microorganisms in the total community. The detection of active microorganisms with reported corrosive metabolisms provided more evidence that microorganisms might have been involved in the localized corrosion detected in oil production assets.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the National Centre for Biotechnology Information (NCBI), under the accession number PRJNA525758 (http://www.ncbi.nlm.nih.gov/bioproject/525758).

AUTHOR CONTRIBUTIONS

SS-C and LM designed the experiments. SS-C conducted the sampling, executed the experiments, carried out the microbial analysis, and prepared the manuscript with the contribution of LM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02587/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix 4

Original reprint of the publications included in Chapter 3

Paper No.
13343

NACE INTERNATIONAL CORROSION Conference & Expo 2019

Investigating the Effect of Temperature in the Community Structure of an Oilfield Microbial Consortium, and its Impact on Corrosion of Carbon Steel

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ABSTRACT

Crude oil and formation water in oil reservoirs host a variety of microorganisms. The community structure of these microbial populations depends on the environmental conditions. Petroleum reservoirs are generally characterized by high temperatures, favouring the activity of thermophilic microorganisms. Nonetheless, temperature decreases after the oil-water extraction process and along the oil production facilities. The effect of this temperature fluctuation from thermophilic conditions to mesophilic conditions on the microbial composition has been investigated using a microbial consortium recovered from a Western Australian oilfield. Next-Generation Sequencing (NGS) of 16S rRNA gene was employed to study the changes in the microbial profile of the total and active community cultured at 40 °C and 60 °C. Additionally, carbon steel coupons were exposed to the oilfield microbial consortium for determining the impact of the temperature on biofilm structure and corrosivity. Results showed noticeable differences in the relative abundance of the species and their corrosive behaviour at the two temperatures. The mesophilic community was predominantly composed by *Proteobacteria, Firmicutes, Synergistetes,* and *Bacteroidetes* phyla whereas the dominant phyla in the thermophilic community were *Thermotogae* and *Synergistetes.* In terms of corrosion, mesophiles increased three (3) times the corrosion rate with respect to the abiotic control while thermophiles reduced it two (2) times. This analysis suggests that microbiologically influenced corrosion rates and mechanisms may vary at different processing locations in oil facilities due to temperature-driven variation of abundant species.

Key words: Microbiologically Influenced Corrosion (MIC); Mesophiles, Thermophile, 16S rRNA profiling, DNA, RNA.

INTRODUCTION

Petroleum reservoirs harbour an abundant diversity of microorganisms. These environments are characterized for presenting extreme conditions for life such as high pressure, salinity and temperature.[1] Despite the hostile conditions, several microbial species linked to different metabolic groups have been detected inhabiting oil formations.[2] It has been reported that the microbial community structure in reservoirs is affected by a variety of factors such as organics, electron donors and acceptors available, salinity, and temperature.[3-5] Potential electron donors and carbon sources in reservoirs are hydrogen, volatile fatty acids, petroleum hydrocarbons or inorganic electron donors.[6] These molecules are natural constituents of these environments and are normally present in an abundant concentration which facilitates the sustenance of life.[7]

Microorganisms in the oil reservoirs are extracted from the earth together with the oil-water produced fluids. In this way, microorganisms colonise the oil production facilities generating biofilm structures that can induce microbiologically influenced corrosion (MIC) processes. Microorganisms in oilfield production facilities can also result from contamination by drilling operations, use of seawater during subsea and topsides flushing operations and seawater injection into the oil reservoir for pressure support.[8] Microorganisms cause deterioration of metals by their interaction with the environment altering the electrochemical conditions in the metal-solution interface.[9] Some metabolic activities that have been associated with an increase in the corrosion rate are sulphate reduction, thiosulphate reduction, iron reduction, iron oxidation and acid production.[10] Contamination of the production facility with microorganisms is practically inevitable. For this reason, oilfield operators have to establish corrosion management strategies to control the risk of MIC.

Apart from the microbial evidence, several physicochemical factors have to be considered when assessing the risk of MIC. One of the major factors that can control the growth and activity of microorganisms is the temperature.[3] Within oil production systems, it is common to have a gradient of temperature from thermophilic conditions to mesophilic conditions.[11] Most reservoirs are high-temperature environments that favour the growth of thermophilic microbes.[12] However, during the different stages in the oil production and processing, the temperature of the fluid decreases thus reaching mesophilic conditions that not only reduce the activity of the thermophilic microbes coming from the reservoir but also, they can favour the proliferation of a mesophilic microbial consortium. The effect of operational changes such as temperature variations on the active microbial community is not completely understood. A first approach for evaluating the response of the microbial community to the temperature gradient in an oil production system was made by Li et al.[13] Authors found differences in the corrosion rates and microbial composition of produced water incubated for long periods at different temperatures (37 ºC, 55 ºC, and 65 ºC). Additional testing for determining the impact of operating temperature changes on the microbial structure is relevant to understand the risk of MIC in oil production facilities. This study was conducted to evaluate the effect of temperature on microbial community structure and activity at conditions simulating oil production facilities. Corrosion analysis was included to evaluate how the changes in the microbial community influence the corrosion processes at carbon steel.

EXPERIMENTAL PROCEDURE

Sampling and microbiological characterization

Oilfield microbial consortium recovery

Produced water was collected from a low pressure (LP) separator of a Western Australian oilfield production facility during shutdown activities. The average operating temperature of the fluid in the outlet of this facility is 57 ºC, with a minimum and maximum reported of 40 ºC and 65 ºC respectively. During downtime periods temperature decreases to ambient conditions, and the temperature of the

produced water at the time of sampling was 33 ºC. Collected water was inoculated in several culture media to maximise the recovery of the cultivable microbial species. Culture media used targeted the growth of sulphide producing prokaryotes (SPP), iron-oxidising bacteria (IOB), iron reducing bacteria (IRB), acid producing bacteria (APB), and general heterotrophic anaerobic bacteria (GANB). All culture media were incubated at two different temperatures 40 °C and 60 °C. This was done to recover mesophilic and thermophilic microorganisms present in the oil production facility. Culture media that exhibited positive growth were used for the establishment of the oilfield microbial consortium which was composed by the mixture of mesophilic and thermophilic species.

16S rRNA profiling of produced water from LP separator

Microbial cells present in the produced water from LP separator were harvested by filtration of 200 mL of water through sterile 0.2 µm pore size polyethersulfone membranes. Microorganisms grown in the culture media were harvested by centrifugation of 10 mL of solution at 3,260 x *g* for 30 min. DNA was extracted from filters and pellets using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions. DNA concentration was quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies). The hypervariable region V3–V4 of the 16S rRNA gene was amplified using the universal primers 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3'), and sequencing was conducted in a MiSeq® System - Illumina by the Australian Genome Research Facility (AGRF).

Evaluation of the effect of temperature changes in the community structure and its impact on corrosion of carbon steel

Sample preparation

Carbon steel (1030 grade) round coupons with an exposed area of 3.14 cm^2 (diameter 2 cm) were used for evaluating the effect of temperature in the microbial composition and its corrosivity. To have a constant exposed surface area, only the face up side of the coupons was in contact with the test solution, the other sides of the coupons were electro-coated with a protective epoxy (Powercron 6000CX, PPG Industrial coatings). Coupons for electrochemical measurements were connected with electrical leads. Working surface of each coupon was wet ground using silicon carbide papers of 80, 120, 320, and 600 grit, consecutively. The polished specimens were washed with Milli-Q water, degreased with acetone, washed with ethanol, and dried with nitrogen gas. Prior to corrosion testing, coupons were weighed and sterilised by 15 min of ultraviolet (UV) radiation at both sides.

Test conditions

Corrosion testing was conducted by immersion of carbon steel coupons in fed-batch reactors (reactors with replenishment of nutrients). Four (4) reactors were set up in this study, two biotic (in the presence of the oilfield consortium), and two abiotic (in the absence of the oilfield consortium). Experiments were conducted in 2 L glass cells, under anaerobic conditions by continuous sparging of a gas mixture of 20% CO_2: 80% N_2. Sterilised synthetic produced water supplemented with nutrients suitable for growth of the consortia was used as a test solution. The composition of the test solution was as follows: 10 mM $CaCl_2.2H_2O$, 7 mM $MgCl_2.6H_2O$, 7 mM KCl, 410 mM NaCl, 8 mM Na_2SO_4, 4 mM $Na_2S_2O_3.5H_2O$, 0.5 mM K_2HPO_4, 5 mM NH_4Cl, 0.2 mM $SrCl_2.6H_2O$, 0.01 mM $BaCl_2.2H_2O$, 0.06 mM $FeCl_2.4H_2O$, 29 mM $NaHCO_3$, 2.5 mM D-glucose, 15 mM Na-formate, 6 mM Na-lactate, 18 mM Na-acetate, and 10 mL/L of vitamins solution (German Type Culture Collection, DSMZ, media 141). The water chemistry of the produced water in LP separator is presented in Table 1. Abiotic and biotic reactors were heated to two different temperatures, 40 °C for mesophilic conditions and 60 °C for thermophilic conditions. Temperature and agitation (250 rpm) were sustained throughout the test. Biotic reactors were inoculated with the microbial consortium recovered from the oilfield at the two different temperatures in a concentration of 3.2 x 10^5 cells/mL. To maintain the microbial activity of biotic reactors, the test

solution was replenished (50% of total volume) every five days with fresh test solution and active microbial cells of the oilfield consortium in a concentration of 1 x 10^5. The test duration was 15 days.

Test monitoring

During the test, adenosine triphosphate (ATP) measurements using the Quench-Gone Organic Modified (QGO–M) test kit (Luminultra Technologies Ltd.) were conducted periodically to monitor the activity of planktonic cells. Open circuit potential (OCP) was measured with the Reference 600+ potentiostat (GAMRY Instruments). A double junction silver/silver chloride (Ag/AgCl) electrode was used as the reference electrode with KCl ≈3 M as reference electrolyte. Two working electrodes were included in each reactor for electrochemical replicates.

Planktonic and sessile cells were enumerated by the extinction dilution method according to the instructions in the NACE standard TM0194-2014.[14] Two culture media were used for the quantification of SPP and APB present in the biotic test reactors. On the last day of the test, 1 mL of the test solution from each biotic reactor was inoculated in each dilution series for planktonic estimation. At the same time, coupons were suspended in sterile phosphate buffer solution (PBS), and sessile microorganisms were detached by vortexing at full speed for 10 sec together with sonication during 2 minutes in cycles of 15 seconds on and 10 seconds off. 1 mL of PBS was used as inoculum of each dilution series for sessile estimation. Serial dilutions were performed in duplicates with a final dilution of 1 x 10^{10}. Culture media was incubated at the temperature of the reactor (40 or 60 °C) for 28 days. SPP and APB growth was visually evaluated by changes in the colour of the medium. Positive growth was reported when the SPP medium turned black due to iron sulphide (FeS) precipitation resulting from the bacterial reduction of sulphate or thiosulphate to sulphide, and APB medium turned yellow due to the decrease in the pH by the acids produced as a result of microbial activity.

Planktonic and sessile DNA/RNA diversity profiling

Once the corrosion tests were finished, planktonic and sessile cells were recovered from the biotic reactors in triplicates. Planktonic cells were harvested by centrifugation of 50 mL of test solution at 3,260 x *g* for 30 min. Sessile cells were detached from the metal surface by immersion in sterile 2:1 RNAprotect® Bacteria Reagent: PBS-Tween 20 (0.1% w/v), and sonication during 2 minutes in cycles of 15 seconds on and 10 seconds off. After biofilm detachment, cells were pelleted by centrifugation at 3260 x g for 30 min, and preserved at -80 ^{0}C. DNA and RNA were extracted using the DNeasy PowerBiofilm and RNeasy PowerBiofilm Kits (QIAGEN). DNA extracted was used as a template for DNA amplicon sequencing, and RNA extracted was used as a template for cDNA synthesis and subsequent sequencing of the 16S rRNA gene. Amplification was conducted with the same primers mentioned above.

The Quantitative Insights Into Microbial Ecology Software (QIIME, v1.9.1)[15] was used for the analyses of the 16S rRNA sequences. Other bioinformatics software such as PEAR (v0.9.10 - 64 bit)[16], Cutadapt (v1.10)[17], and USEARCH (v10.2)[18] were run for sequencing data for quality filtering and operational taxonomic unit (OTU) assignment of each sequence. After the OTU table generation, VSEARCH (v1.1.3)[19] software was used to determine the taxonomy association of the OTUs by comparison with SILVA 132[20] database. Taxonomy table was subsequently visualised with R-studio.[21]

Corrosion measurements and surface analysis

After biofilm detachment, coupons were cleaned by immersion in Clarke's solution in intervals of 30 sec to constant weight. An unexposed coupon was also cleaned in Clarke's solution equal number of times to measure the metal removed by the cleaning process. The corrosion rates were determined from the metal weight loss method according to the formula in the standard ASTM G1.[22] Coupons surfaces were also analysed using a 3D optical profilometer (Alicona imaging infinite focus microscope IFM G4 3.5).

Biofilm imaging

Biofilms formed over the metal surface of biotic reactors were examined by field emission scanning electron microscope (FESEM) coupled with an energy-dispersive X-ray detector for EDS mapping. After 15 days of exposure to corrosive conditions, coupons were removed from the reactors and fixed for 22 h in a phosphate buffered solution containing 2.5% glutaraldehyde and 0.15% alcian blue. Later, coupons were washed with PBS and dehydrated with an ethanol series (30%, 50%,70 %, 80%, 90%, 95%, and 100%). Dehydrated coupons were dried under nitrogen flow and kept in a desiccator until analysis.

Table 1
Water chemistry of produced water from LP separator

pH	7.8
Conductivity (mS/cm)	41.7
Chloride (mg/L)	17217
Sodium (mg/L)	8750
Potassium (mg/L)	319.5
Calcium (mg/L)	400
Magnesium (mg/L)	160
Barium (mg/L)	1
Strontium (mg/L)	14
Iron (total) (mg/L)	2.8
Sulphate (mg/L)	924
Bicarbonate alkalinity (mg/L)	490
Carbonate alkalinity (mg/L)	<5
Total alkalinity (as CaCO3) (mg/L)	490
Total phosphorus (mg/L)	<0.5
Phosphate (mg/L)	<1
Total nitrogen (mg/L)	7.3
Nitrate (mg/L)	<1
Nitrite (mg/L)	<1

RESULTS AND DISCUSSION

Microbial community composition in produced water from LP separator

Microorganisms were detected in the produced water of LP separator. Positive growth was evidenced in several culture media at both temperatures of incubation, however, more microbial groups were detected at 40 °C than at 60 °C as presented in Table 2.

Table 2
Microbial groups detected in produced water from LP separator at the two different temperatures

Microbial group	Growth at 40 °C	Growth at 60 °C
Sulphide producing prokaryotes	Positive	Positive
Iron-oxidising bacteria	Positive	Positive
Iron reducing bacteria	Positive	Positive
Acid producing bacteria	Positive	Negative
General heterotrophic anaerobic bacteria	Positive	Negative

DNA was extracted from the produced water sample as well as from culture media that exhibited positive growth, and microbial composition was determined by 16S rRNA gene sequencing. A total of 1,368,861 high-quality sequences were obtained after removal of singletons, chimeras and low-quality

sequences. These sequences were clustered into 147 OTUs, most of them associated with Bacterial taxa. LP separator water was dominated by three bacterial phyla, *Epsilonbacteraeota* (48%), *Proteobacteria* (25%), and *Firmicutes* (22%). Other eight (8) bacterial phyla *(Thermotogae Synergistetes, Bacteroidetes, Acetothermia, Atribacteria, Cloacimonetes, Marinimicrobia, Actinobacteria)* and one (1) archaeal phylum *(Euryarchaeota)* were also detected in the produced water albeit at low abundance. Although culture media could recover most of the microorganisms present in the oil system, the structure of the community in each medium had significant differences with respect to the produced water sample. These differences are associated with the specific nutrients provided in each solution that favour the growth of particular microbial groups, and the limitation in mimicking the exact conditions that microorganisms have in the field.[23] The dominant genera identified in the LP separator water and cultures are presented in Figure 1 and Table 3. Only genera with relative abundances equal or greater to 1% are shown. Genus contributing less than 1% were summarised as rare taxa and are not described in this work.

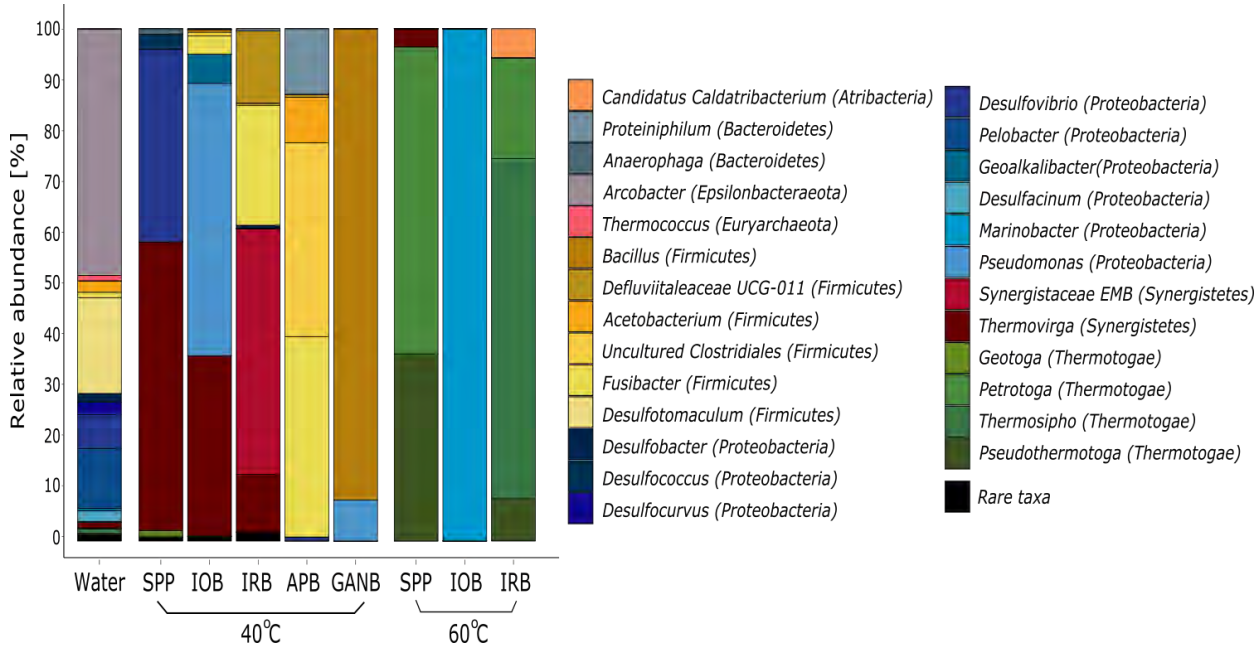

Figure 1: Microbial community composition at genus level in produced water sample and cultures from the same sample

Results from molecular microbiology analysis and cultivation at the two test temperatures indicate that the produced water contains both mesophilic and thermophilic microorganisms. At the time of sampling, the microbial community in the produced water was dominated by mesophilic species, which is likely to be the result of the production shutdown activities that lead to lower temperatures (33 ºC) in the system compared to those typically found during production (57 ºC). The presence of microorganisms with optimal growth temperatures different to those found in oil reservoirs and production facilities has been previously reported.[24] For instance, Junzhang et al.[25] found a large number of mesophilic microorganism in high-temperature reservoirs. Authors pointed out that this is a common consequence of long term water injection into the reservoir for pressure support. Injected water decreases the temperature of the reservoir at the injection well locations resulting in a greater geothermal temperature gradient between the perimeter of water injection wells and deep oil reservoirs. Besides the temperature decrease, flood water also introduces nutrients, oxygen and foreign microbes into the reservoir. Microorganisms injected are normally mesophiles that find appropriate conditions for proliferation in the temperature gradient generated increasing the microbial diversity in the reservoir. The presence of mesophilic and thermophilic microorganisms in produced water suggests that the changing temperatures observed through the oil production system and during operations upsets can play a key role in the development of MIC processes in the facility.

Table 3
Relative abundance of dominant genera detected in produced water sample and cultures from the same sample

Phylum	Genus	Relative Abundance (%)								
		Water	SPP 40ºC	IOB 40ºC	IRB 40ºC	APB 40ºC	GANB 40ºC	SPP 60ºC	IOB 60ºC	IRB 60ºC
Atribacteria	*Candidatus Caldatribacterium*	0.1								5.7
Bacteroidetes	*Proteiniphilum*			0.2	0.4	**12.8**				
Bacteroidetes	*Anaerophaga*		1							
Epsilonbacteraeota	*Arcobacter*	**48.1**								
Euryarchaeota	*Thermococcus*	1								
Firmicutes	*Bacillus*						92			
Firmicutes	Unclassified *(Defluviitaleaceae)*				**14.1**	0.6				
Firmicutes	*Acetobacterium*	2.2		0.5	0.1	8.9				
Firmicutes	Uncultured *(Clostridiales)*			0.7	0.3	37.8				
Firmicutes	*Fusibacter*	1		3.5	**23.4**	39.1				
Firmicutes	*Desulfotomaculum*	18.7								
Proteobacteria	*Desulfobacter*	1.7			0.6					
Proteobacteria	*Desulfococcus*		2.9							
Proteobacteria	*Desulfocurvus*	2.4								
Proteobacteria	*Desulfovibrio*	6.6	37.6			0.7				
Proteobacteria	*Pelobacter*	11.7								
Proteobacteria	*Geoalkalibacter*	0.5		5.7						
Proteobacteria	*Desulfacinum*	2.1								
Proteobacteria	*Marinobacter*	0.1							99.9	
Proteobacteria	*Pseudomonas*			**53.2**			8			
Synergistetes	Unclassified *(Synergistaceae)*				**47.9**					
Synergistetes	*Thermovirga*	1.1	**56.3**	**35.1**	**11.3**			3.6		
Thermotogae	*Geotoga*	0.1	1.1	0.2						
Thermotogae	*Petrotoga*		0.3					**59.9**		**19.7**
Thermotogae	*Thermosipho*	0.9								**66.4**
Thermotogae	*Pseudothermotoga*	0.3						**36.5**		8.2
Rare Taxa		1.4	0.8	1	1.9	0.1			0.1	0.1

Most of the dominant genera identified in the produced water belong to the sulphide producing prokaryotes group which can use sulphate, thiosulphate or elemental sulphur as an electron acceptor during anaerobic respiration. This finding is correlated with the presence of high concentration of sulphate (≈9 mM) in the produced water (Table 1). In addition to the reduction of sulphur compounds, several of the microorganisms identified have alternative metabolisms such as iron reduction, nitrate reduction or acid production. Microorganisms with such metabolic capabilities were recovered in culture media and used to generate the oilfield microbial consortium for evaluating the effect of temperature on the microbial community structure and its impact on MIC.

Effect of the temperature in the corrosive behaviour of the oilfield consortium

The average corrosion rates calculated from weight loss of the carbon steel coupons exposed to biotic and abiotic conditions at different temperatures are shown in Figure 2. The results showed that temperature has a significant impact on the biotic and abiotic corrosion processes. Under the conditions studied, microbial corrosion was higher at 40 C compared to 60 C whereas abiotic corrosion was much higher at the 60 C temperature. Differences in the corrosion rates of carbon steel exposed to 40 ^{0}C and 60 ^{0}C were statistically significant among biotic and abiotic conditions ($p = 0.008$ and $p = 0.02$, respectively). In this regard, at mesophilic conditions, a three-fold increase in the abiotic corrosion rate

was observed in the presence of microorganisms. In contrast, at thermophilic conditions the presence of microorganisms halved the comparable abiotic corrosion rate. The reduction of the corrosion rates by microbial activity is associated with MIC inhibition (MICI). MICI has been demonstrated in several laboratory studies in the past two decades.[26-29] Similar to MIC, the mechanisms of MICI are not fully understood. Some of the mechanisms described at present are a) formation of protective layers that hinder the corrosion reactions, b) removal of corrosion agents, e.g. oxygen depletion by microbial respiration, and c) production of corrosion inhibiting substances. Despite this phenomenon being often observed in the laboratory, it is difficult to infer that same processes happen under field conditions in proportions that can make a real impact on the corrosion inhibition of carbon steel facilities.

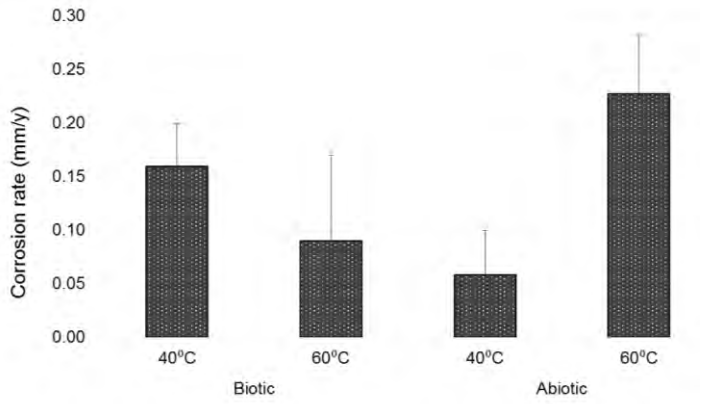

Figure 2: Average corrosion rates by weight loss of carbon steel in synthetic produced water at 40 °C and 60 °C under biotic and abiotic conditions.

Macroscopic analysis by visual inspection revealed that all coupons exposed to the synthetic produced water presented a deposits layer (Figure 3a and 3b). Test solution in biotic reactors became black (Figure 3c) which is linked to the sulphide production by the sulphate, thiosulphate or sulphur reducing bacteria in the consortium. Hydrogen sulphide reacts with the dissolved iron producing iron sulphide (FeS) precipitates that have a characteristic black colour.[30] EDS analysis confirmed the presence of iron and sulphur elements which were massively distributed over the surface. In contrast, the test solution in abiotic reactors did not change colour (Figure 3d) and the deposits observed were scale deposits, principally calcite (determined by XRD analysis-data not shown). Precipitation of scales rises with an increase in the temperature due to an increase in precipitation kinetics. In this way, coupons exposed to 60 ^{0}C showed a thicker layer of deposits (Figure 3b). The thick and heterogeneous scales layer formed in the coupons exposed to a higher temperature can favour under deposit corrosion at the edges of the deposited and non-deposited surface. According to Huang et al.[31] deposits facilitate crevice formation and pitting by providing a local environment that is chemically and physically different from the areas not covered with the precipitated material. Similarly, Pandarinathan et al.[32] argued that deposits can create a diffusion barrier for the transport of ions which results in the creation of micro-environment that led the material more susceptible to localised corrosion.

3D optical surface imaging and pit profile measurements were conducted to identify damaged zones and localised corrosion. Images from the entire surface and localised attack of carbon steel coupons under biotic and abiotic conditions are shown in Figure 4. The coupons surfaces evidenced deterioration after the exposure time. The pattern of the damage was dissimilar between biotic and abiotic reactors. The deepest pits (60 µm) were detected in the abiotic reactor under thermophilic conditions. In the absence of bacteria at 60 °C, pits were displayed at the edges between the deposited and non-deposited surface which suggests an under deposit corrosion mechanism. However, in the presence of bacteria, the damage was irregularly distributed over the metal surface. The above suggests that microorganisms changed either the deposition pattern or the characteristics of the

deposits thus shifting the corrosion mechanism. The surface of the steel exposed to biotic conditions was completely covered by corrosion products and scales (Figure 3) which prevented the formation of the interface area evidenced in the coupons of the abiotic reactor. In contrast, carbon steel exposed to mesophilic conditions did not show a significant difference in the localised corrosion and pit depth between abiotic and biotic reactors. Reactors at 40 °C exhibited only shallow pits of maximum 14 µm depth.

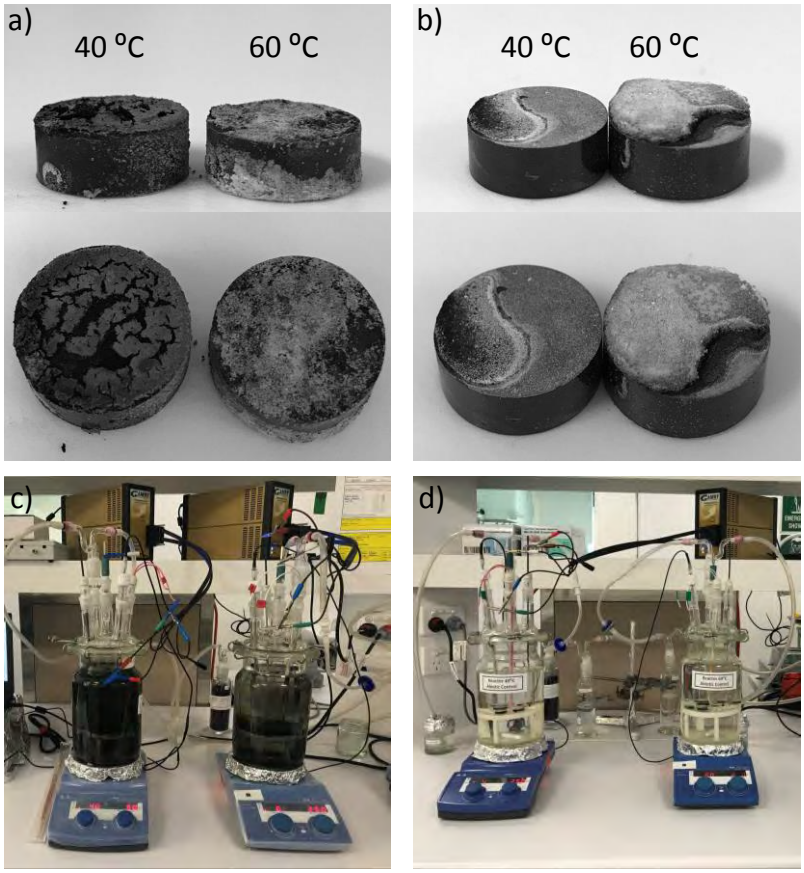

Figure 3: Corrosion testing of carbon steel in synthetic produced water at 40 °C and 60 °C. a) Coupons appearance after 15 days of exposure to biotic conditions. b) Coupons appearance after 15 days of exposure to abiotic conditions. c) Blackening of test solution by hydrogen sulphide production in biotic reactors. d) Colourless test solution in abiotic reactors.

The open circuit potential variations for biotic and abiotic systems are shown in Figure 5a. The E_{ocp} as a function of time data revealed that the microbial growth substantially shifted the E_{ocp} towards noble values in the reactor at mesophilic conditions. The shift to positive potential correlates with the ATP concentration measured over time (Figure 5b). Replenishment of nutrients in the test solution clearly showed a significant impact on the mesophilic microbial activity which also influenced the OCP. This shift in the E_{ocp} has been previously observed in carbon steel and has been associated with microbial activity.[33] Conversely, the E_{ocp} of the abiotic reactor exposed to 40 ^{0}C showed a slight steady shift towards negative direction which confirms that the microbial activity and the corrosion potential had a correlation in the test. At 60 ^{0}C there was no significant microbial activity, and the E_{ocp} of the abiotic and biotic reactors showed a similar pattern. The E_{ocp} remained more or less steady during the first days of the test and then presented a shift to a positive direction. The shift to positive values presented in the reactors at thermophilic conditions may have been an effect of the scales deposition over time which is also associated with electrochemical changes over the metal surface.

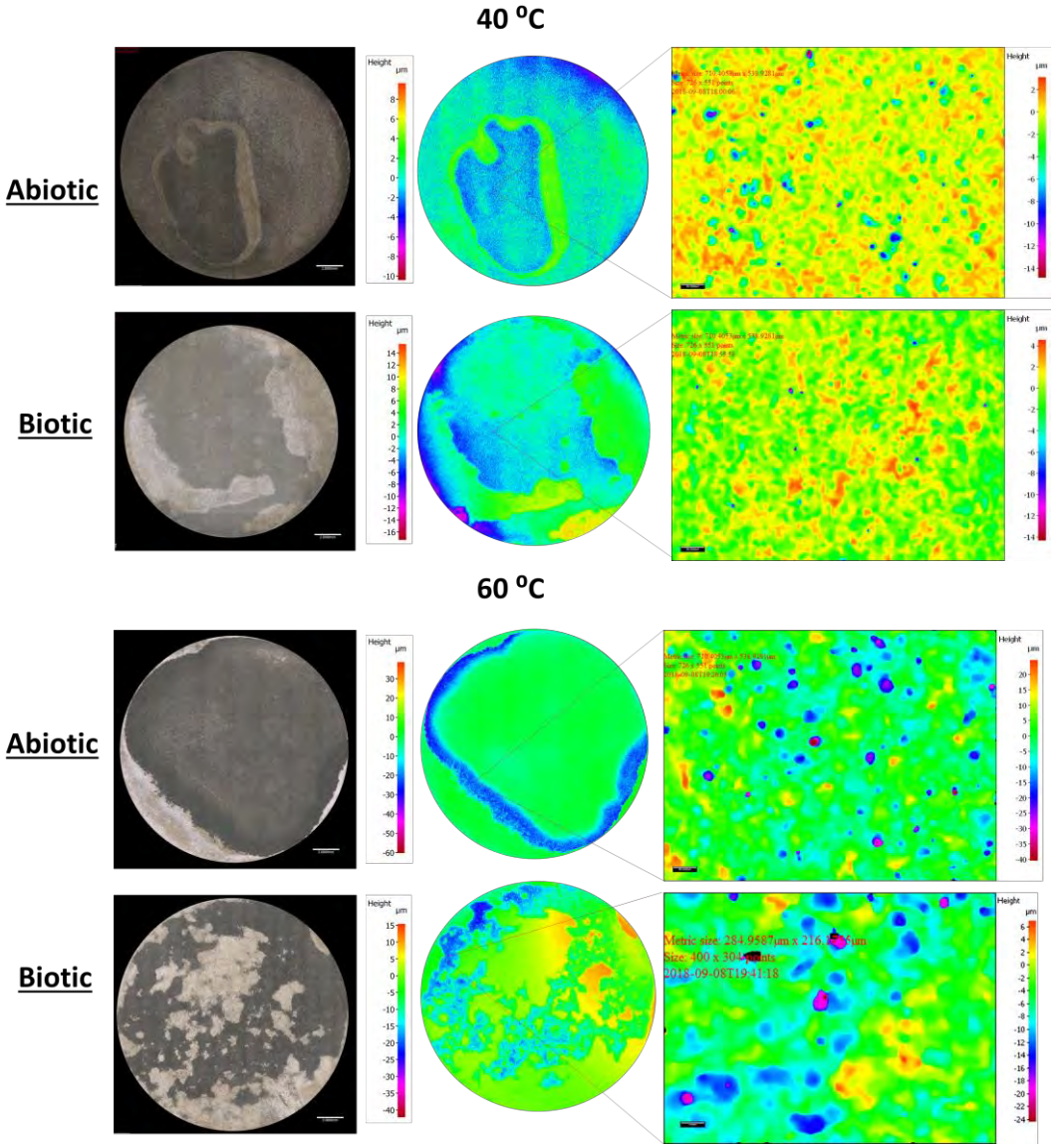

Figure 4: Visible-light microscopy images showing surface profiles of carbon steel coupons

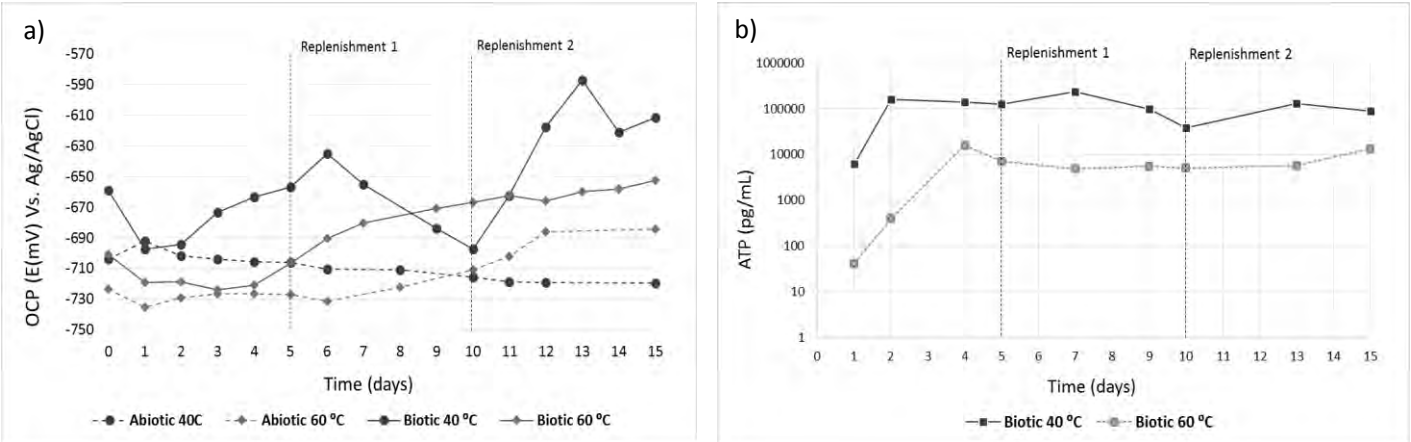

Figure 5: Corrosion potential and microbial activity over time. a) Average OCP values of carbon steel under biotic and abiotic conditions. b) ATP fluctuation in biotic reactors.

The dissimilarities between the corrosion rates and metal damage observed in biotic reactors might be associated with the differences in the metabolic activity of the microorganisms. The microbial community in the reactor at 40 ^{0}C produced considerably more ATP than the microbial community present in the reactor at 60 ^{0}C. Lower ATP values found in the thermophilic reactor might be the result of microbial growth inhibition, lower ATP yield in the metabolic reactions, or higher maintenance requirements. It is known that thermophiles have low growth rates, low growth yields, and are also susceptible to growth inhibition by product or substrate at low concentrations.[34] Additionally, thermophiles have lower efficiency in energy conversion compared with mesophiles due to an increased proton permeability of the cell membrane that makes microorganisms to invest more energy in maintenance.[35] Less energy expenditure in maintenance activities allows mesophiles to have a higher growth rate and more energy available to carry out other activities such as biomass generation. The cells enumeration analysis executed after 15 days of testing evidenced that the mesophilic reactor had higher levels of SPP and APB planktonic cells than the thermophilic reactor suggesting that the low levels of ATP measured at higher temperature were likely a consequence of a lower growth rate or growth inhibition (Figure 6). With the aim of confirming a possible growth inhibition as consequence of the lack of some of the most important nutrients due to the precipitation phenomenon presented at 60 ^{0}C, the concentration of dissolved carbon (C), nitrogen (N), and phosphorous (P) was measured in the solution of abiotic reactors after the 15 days of the testing. Results of chemical analysis presented similar values for thermophilic and mesophilic conditions (C = 1400 mg/L and 1500 mg/L, N = 130 mg/L and 140 mg/L, P = 6.3 mg/L and 7.4 mg/L) which discards that the lower growth rate evidenced in thermophilic conditions was related to an inhibition by a limited concentration of these nutrients. Higher metabolic rates of the mesophilic community lead to greater production of metabolites such as hydrogen sulphide or organic acids which in turn could be the cause of the higher corrosion rates seen in this study at 40 ^{0}C.

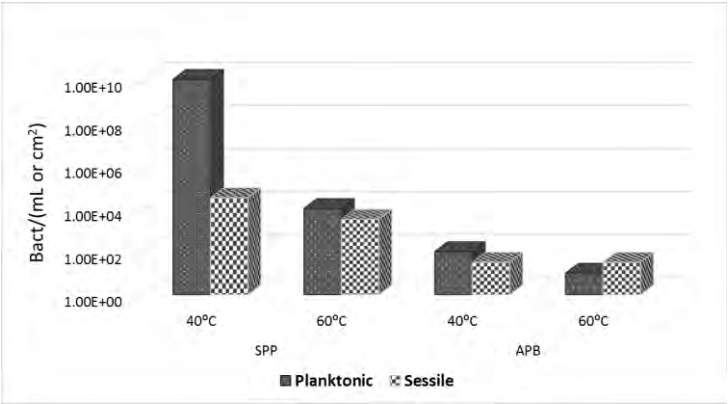

Figure 6: SPP and APB concentration of planktonic and sessile cell in biotic reactors at the completion of the exposure.

In contrast to our results, Li et al.[13] found that thermophilic conditions were more aggressive to the carbon steel coupons than mesophilic conditions. The differences in the results of both studies are associated with the differences in the microbial consortia used. Li et al. evaluated a community with a high abundance of archaea species, which are predominantly thermophiles, whereas the community evaluated in this study was mainly composed of sulphide producing prokaryotes, most of them reported as mesophilic species in the literature. In this sense, the results obtained in this study cannot be generalised to all oilfield systems since different produced waters have different types and abundance of microorganisms. It has been reported that in environments with a low concentration of sulphate, fermentation and methanogenesis become the dominant microbial metabolism.[36, 37] There are numerous investigations that have shown evidence of direct or indirect participation of methanogens in corrosion.[38-40] In this sense, corrosion reactions of systems with low sulphate concentration could be mainly mediated by methanogens and due to the majority of methanogenic microorganisms found in oil reservoirs are thermophilic species, higher corrosion rates might be expected at higher temperatures.

Effect of the temperature on the community structure of an oilfield consortium

Total and active microorganisms present in the biotic reactors were identified through DNA and RNA profiling, respectively. Overall 668,165 high-quality sequences were obtained after quality filtering and bioinformatics processing of the raw reads. These sequences were taxonomically classified into 27 bacterial genera. Only the genera with relative abundances equal or greater to 1% are presented in Figure 7. On average, 21 genera were detected in the DNA analysis of the planktonic and sessile populations of the biotic reactors. In comparison, RNA analysis showed a marked reduction of the genera detected in both planktonic and sessile population of the reactor exposed to 60 °C (only 11 genera were detected active). Lower richness in the RNA analysis compared with DNA analysis is in agreement with other studies that have implemented a DNA/RNA based approach.[41-43] 16S gene sequencing of the DNA molecule provides information of the whole community including dormant, spores and dead cells whereas sequencing of the RNA molecules delivers information only related to active cells.[42]

DNA and RNA based profiling revealed noticeable differences in the microbial composition of the community established at each temperature. The reactor incubated at 40 °C primarily contained genus related to *Proteobacteria, Firmicutes, Synergistetes,* and *Bacteroidetes* phyla whereas the dominant phyla in the reactor incubated at 60 °C were *Thermotogae* and *Synergistetes*. The sole genus that was detected to be significantly active at both temperatures was *Thermovirga* sp. This microorganism has previously been found to be involved in MIC processes of oil and gas production facilities.[44-46] *Thermovirga* is a moderately thermophilic bacterium with an optimal growth temperature of 58 °C.[47] Nevertheless, it has been detected in mesophilic environments living in syntrophy with mesophilic H_2 scavenging partners.[7] *Thermovirga* is classified as a fermenting bacteria, but it can also reduce elemental sulphur to hydrogen sulphide. In the 40 °C reactor, *Thermovirga* was accompanied by the sulphate reducing bacteria *Desulfovibrio*, the thiosulphate reducing bacteria *Dethiosulfatibacter* and other fermenting bacteria (*Anaerophaga* and uncultured *Firmicutes).* On the other hand, all the microorganisms detected at 60 °C had a fermentative metabolism with the ability to reduce thiosulphate or elemental sulphur (*Petrotoga, Thermosipho,* and *Pseudothermotoga*).

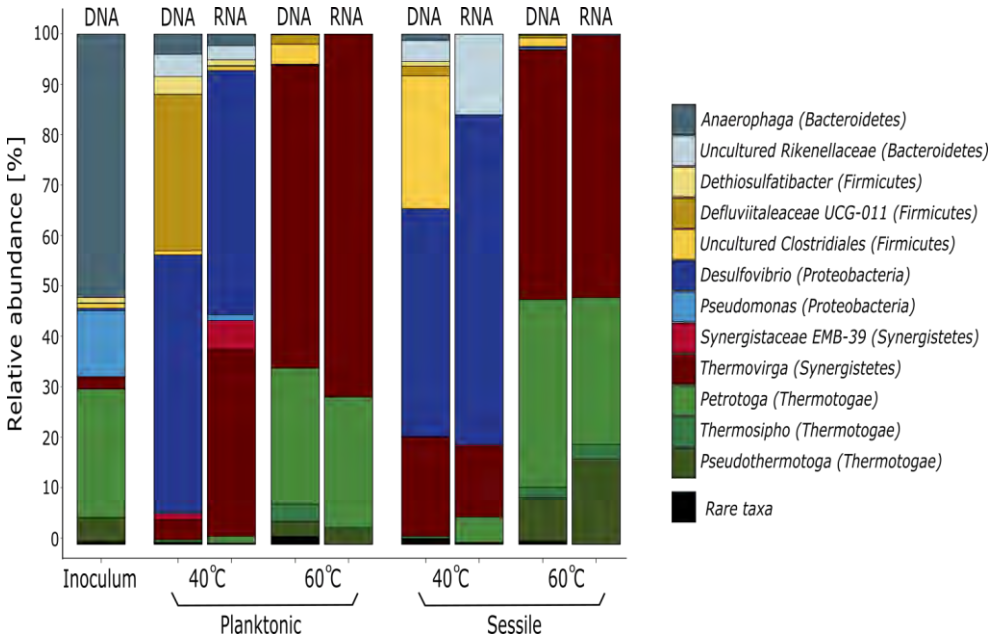

Figure 7: Microbial community composition at genus level for planktonic (bulk fluid) and sessile (biofilm on carbon steel) communities assessed at the completion of the exposure. *DNA:* DNA-based microbial profile, *RNA*: RNA-based microbial profile. *40 °C* and *60* °C: Test temperature.

The microbial structure of total and active communities was similar in the reactor exposed to thermophilic conditions and varied more in the reactor incubated at mesophilic conditions. Differences found at mesophilic conditions reflect the dynamic interaction among the species of a community and the quick response of microorganisms to the changing environment. As mentioned before, microorganisms in the reactor at 40 ºC showed a positive response to the nutrients replenishment by increasing the energy production (Figure 5b). After three days of nutrients addition, ATP concentration decreased until the next replenishment of the test solution. This fluctuation on the ATP production is directly related to the nutrients availability which has an impact on the community structure. Changes in the environment therefore produce favourable conditions for some species in the community and unfavourable conditions for others generating an effect on the abundance of the species. For example, at the time of sampling, microorganisms related to the *Firmicutes* phylum were present in the mesophilic community in considerable concentration (DNA relative abundance of planktonic 35% and sessile 29%) but these were not active (RNA relative abundance of planktonic 2% and sessile 0%) (Figure 7 – yellow bars). This fact is most likely the result of nutrients limitation in the test solution. Cells for DNA and RNA analysis were harvested after five days of nutrients replenishment, and according to the ATP measurements, activity in the reactor at 40 ^{0}C had started to decrease at that point. Conversely, activity in the reactor at 60 ^{0}C continued to increase at the time of sampling, and this is reflected by a similar structure in the total and active community. These results show the benefits of including RNA analysis for the correct estimation of the microbial species that could be facilitating the MIC processes in the system as a result of their activity and microbial metabolism.

FESEM images and EDS mapping of the metal surface of biotic reactors are shown in Figure 8. Micrographs also revealed differences in the microbial community composition. Different bacteria morphology was observed in the biofilms. Mesophilic microorganisms generated a dense biofilm with overlapping material and microbial cells. Cells had a rod shape consistent with the most abundant microorganism detected (*Desulfovibrio*). Round cells with the appearance of spores embedded in the biofilm were also seen, however, further analysis for characterising these structures was not executed. Conversely, the thermophilic microorganisms created a biofilm mat colonised by microbial cells grouped in chains. It is known that *Petrotoga* can create chains up to 30 mm and *Thermovirga* chains of two to five cells. EDS maps are provided for each biofilm, major elements detected were Fe, S, Ca, and P.

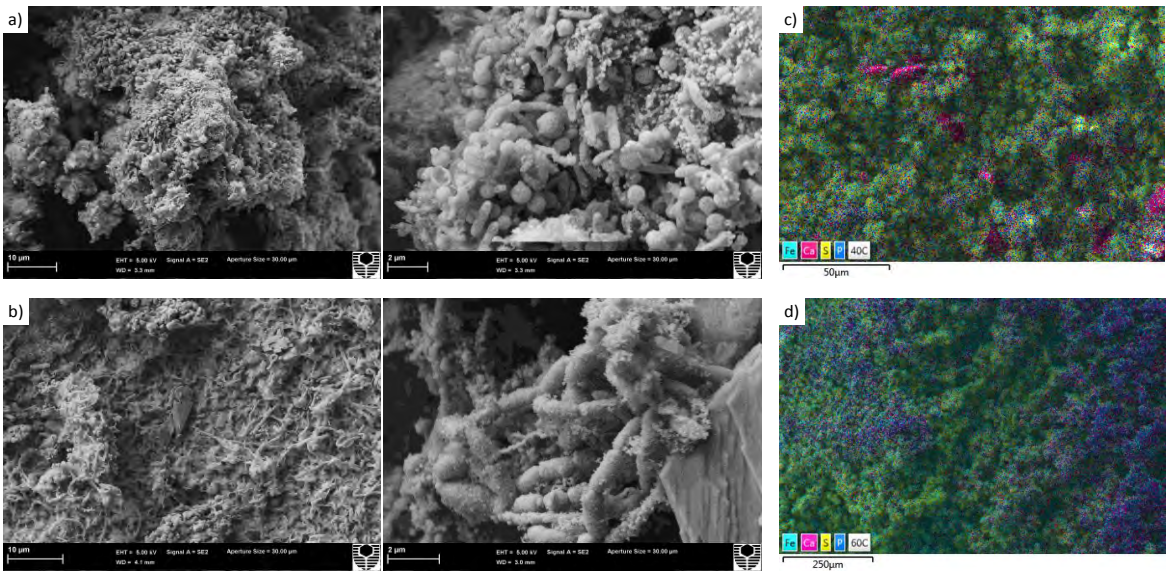

Figure 8: FESEM micrographs and EDS maps of biofilms formed over carbon steel coupons at two different temperatures. a) Biofilm formed at 40 ^{0}C. b) Biofilm formed at 60 ^{0}C. c) EDS map of coupon exposed to 40 ^{0}C. d) EDS map of coupon exposed to 60 ^{0}C.

CONCLUSIONS

The effect of temperature in the community structure of an oilfield microbial consortium and its impact on corrosion of carbon steel was investigated. The main findings of the study are as follows:

1. Microbial analysis revealed that produced water recovered from the LP separator in an oil production facility hosts mesophilic and thermophilic microorganisms. Moreover, this facility contains microorganisms that can be active at different temperatures, such as the one identified in this study *Thermovirga sp*. which was significantly active at 40 ^{0}C and 60 ^{0}C.
2. Corrosion testing demonstrated that temperature has a significant impact in shaping the microbial structure, which also has an effect on the corrosion of carbon steel. Under the conditions evaluated a decrease in fluid temperature increased the risk of MIC in the system by favouring the activity of *Desulfivibrio sp*. a sulphate reducing microorganism widely reported to cause corrosion. It is important to emphasise that different communities to the ones studied here can produce different results.
3. Temperature affected both the abiotic and biotic corrosion processes of carbon steel. In abiotic conditions higher temperature favoured under deposit corrosion by the precipitation of scales that created a heterogeneous layer over the metal surface. In biotic conditions, microorganisms increased three (3) times the corrosion rate under mesophilic conditions but reduced it two (2) times under thermophilic conditions. Reduction of corrosion rate in thermophilic conditions was related to MIC inhibition possibly associated with changes in the distribution or characteristics of corrosion products and scales precipitated at a higher temperature.
4. Microbial activity measured by the ATP production was significantly different between the mesophilic and thermophilic consortium. Considerably higher levels of ATP were detected in the mesophilic reactors compared to thermophilic reactors. The lower energy conversion efficiency of thermophilic organisms was considered a contributing factor.
5. DNA/RNA based profiles pointed out differences in the microbial structure of the planktonic and sessile community of the oilfield consortium in response to temperature. Dominant phyla detected at 40 °C were different from the dominant phyla at 60 °C. Additionally, RNA analysis revealed significant variations between the total and active microorganisms in the mesophilic conditions. Principal differences were evidenced in the microorganisms belonging to the *Firmicutes* phylum which were detected abundantly in the DNA analysis but were not active in the reactor. These dissimilarities highlight the importance of including the analysis of the active microbes for the correct understanding of the microbial interactions with the environment.

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Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms

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ABSTRACT

The effect of sulphur compounds and temperature on the microbiologically influenced corrosion of carbon steel has been assessed using multispecies biofilms. Biofilm composition, community structure and activity level shifted in response to environmental conditions, which resulted in substantial differences in pitting susceptibility of carbon steel. Microorganisms enhanced pitting when sulphur compounds were available, but there was not a distinctive pattern on the effect of temperature. In average, old biofilms caused greater pitting than young biofilms. Functional profiles predicted from 16S rRNA data revealed that biofilms had similar capabilities regardless of environmental conditions and the extent of corrosion associated with them.

1. Introduction

Microbiologically Influenced Corrosion (MIC) refers to the degradation of metals that results from the activity of the microorganisms that are in close contact with the metal surface. Microorganisms attach to the metal surface forming biofilm structures that modify the electrochemical conditions at the metal/solution interface, changing the kinetics of the corrosion process [1]. This corrosion phenomenon can significantly compromise the integrity of many industry assets including those of oil and gas production, transportation and refining facilities, marine engineering and shipping, aviation, recirculated cooling systems, and water treatment facilities [2,3]. It has been estimated that MIC contributes to almost 40 % of internal corrosion and 20–30 % of external corrosion problems in pipelines [4,5]. Most MIC is induced by a localised attack where deep penetration into the base metal is observed [2]. Complete wall penetration of a pipe leads to several consequences that include environmental damage, health and safety (fire, exposure to toxic fluids), financial (loss of production), reputational and legal issues for the companies. Considering the vast negative impacts caused by corrosion, risk assessment and management plans are typically implemented by the industry for preservation of equipment and facilities; nevertheless, the complex and unpredictable nature of MIC makes this phenomenon more challenging to control [6].

Several mechanisms have been proposed since the first time microorganisms were associated with corrosion [7–10]. MIC mechanisms have been classified into two broad categories, extracellular electron transfer MIC (EET-MIC) that is instigated by direct or indirect microbial uptake of electrons from the metal, and chemical MIC (CMIC) that is caused by corrosive metabolites produced by the microorganisms during their metabolic activities [11,12]. Additionally, microorganisms can also accelerate corrosion initiated by other mechanisms or damage the protective mineral films formed over the metal surface [13]. Several groups of microorganisms have been implicated in corrosion including sulphate-reducing bacteria [14,15], sulphate-reducing archaea [16], thiosulphate-reducing bacteria [17], acid-producing bacteria [18], methanogenic archaea [19,20], iron-oxidising bacteria [21,22], iron-reducing bacteria [23], and nitrate-reducing bacteria [24,25]. Considering that more than one microbial group is generally present in a natural biofilm, more than one MIC mechanism can potentially take place at the same time, making this phenomenon difficult to understand and predict. In fact, the most aggressive forms of MIC have been reported in the presence of multispecies biofilms [26,27]. This is because microorganisms are known to establish synergistic interactions that involve communication with other microbes within biofilms, which facilitate their growth and proliferation. These interspecies interactions can result in substantial corrosion rates of carbon steel as reported by Lee et al. [28] in their laboratory study when a mixed culture of iron-oxidizing and iron-reducing bacteria caused more corrosion than single species cultures. Similarly, Ke et al. [29] found that a mixed culture of *Vibrio* sp. and iron-oxidising bacteria promoted greater corrosion of

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carbon steel compared to single species. The authors concluded that *Vibrio* sp. caused corrosion by decreasing the pH locally in the biofilm due to organic acid production. Subsequently, the iron bacteria isolate oxidised the dissolved ferrous ion to ferric ion, promoting the anodic reaction.

Although several microbial groups can influence corrosion, the sulphate reducing bacteria (SRB) has been the predominant group studied and considered in risk prediction models [30]. Likewise, MIC assessment and monitoring have primarily relied on the detection and enumeration of SRB [31–33] and the concentration of sulphate in produced water [3,34]. However, MIC prediction based solely on the activity of this sulphidogenic group seriously underestimates the risk of MIC. In particular, this approach is not suitable for low-sulphate environments where microbial communities are dominated by methanogens, oil-degrading, fermenting, and nitrate-reducing microorganisms [35–37]. Some of these microorganisms can produce sulphide by the reduction of sulphur compounds other than sulphates, such as thiosulphate and elemental sulphur, causing similar corrosion reactions than the ones described for SRB. Additionally, other MIC mechanisms not related to sulphide generation can take place in these low-sulphate environments. For example, methanogenic microorganisms can couple electro-methanogenesis with iron oxidation causing EET-MIC [19,38–40] Likewise, nitrate-reducing microorganisms can cause EET-MIC by taking electrons from the metal as it has been described for *Pseudomonas aeruginosa* and other nitrate-reducing species [24,41,42]. Thus, the probability of corrosion by these microbial mechanisms has been largely ignored [43] even though there is existing evidence of their association with corrosion [17,27,44].

In oil production facilities, microbial activity is a dynamic process that can be affected by several variables, including operating temperatures, water chemistry and flow velocity, among others [45–47]. Operational changes in a production facility can induce a shift in the structure and function of microbial communities in response to the changing environment [36]. It is a common practice in these facilities to flood the reservoir with water for increasing crude oil recovery [44]. This practice can alter the reservoir environment by adding chemical species such as sulphates and organic matter that could be used by the indigenous microbial community. Particularly, low-sulphate reservoirs are susceptible to microbial community changes as a result of this practice. A previous investigation on the microbiome of a low-sulphate production facility showed that microbial community composition varied across wells with formation waters considered pristine as compared to seawater flooded wells [36]. An increase in the sulphate concentration of the seawater flooded wells was reported by the authors; however, the resulting impact of fluid chemistry changes on the risk of MIC was not studied.

Understanding the risk of MIC in low-sulphate environments and the impact that changes in the water chemistry have on the activity of existing microbial communities is essential for improving current MIC prediction models, and to account for environments where SRB are not the main risk. Therefore, this study was conducted to evaluate the risk of MIC in carbon steel as a function of sulphur compounds, named sulphate and thiosulphate, in the environment. For this purpose, multispecies biofilms were formed on the steel surface using a microbial consortium from field. This consortium was recovered from an oil production facility in Western Australia that experienced corrosion failures attributed to MIC despite undetectable sulphate levels measured in its produced water. As there is a gradient of temperature, from 60 ^{0}C to 40 ^{0}C across the production facility, which is expected to have an effect on microbial community composition and activity [48], the effect of the temperature on MIC was also assessed in this investigation. Adenosine triphosphate (ATP) levels and 16S rRNA sequencing targeting metabolically active populations (RNA-based) were used to evaluate the impact of environmental conditions on biofilm activity and community structure on carbon steel, respectively. Amplicon sequencing from the RNA molecule provides more accurate information of the active members in the community than the commonly used DNA-based sequencing analysis [49]. RNA analysis allows discrimination of the active members in the community since RNA deteriorates rapidly after cell death and its abundance is well correlated with the microbial growth rate [50]. This methodological approach has recently been applied in MIC investigations [49,51] and, in agreement with the present study, it has proven very useful for the analysis of microbial communities in complex MIC processes. Additionally, this study evaluated the effect of biofilm maturation on the MIC process by determining corrosion rates and biofilm community composition at two different exposure times. Sequencing data of the active populations was processed using bioinformatics tools to predict the functional capability of the biofilms by profiling the relative abundance of genes within the microbial community. This data was then used to correlate microbial metabolic activity with pitting corrosion at the different exposure conditions.

2. Materials and methods

2.1. Oilfield microbial consortium

Produced water samples were collected in sterile containers from the risers of a floating production storage and offloading (FPSO) facility located on the Australian North West Shelf. Samples were inoculated in several culture media to maximise the recovery of the cultivable microbial species present in the facility. Culture media targeted the growth of sulphate-reducing bacteria (SRB), thiosulphate-reducing bacteria (TRB), methanogenic archaea (MET), acid-producing bacteria (APB), iron-reducing bacteria (IRB), iron-oxidising bacteria (IOB), and general anaerobic bacteria (GANB). Culture media was prepared according to the guidelines of the standard test method NACE TM0194 [52]. The temperature of the fluid was 55 °C at the time of sampling; however, all culture media were incubated at two different temperatures 40 °C and 60 °C, to recover mesophilic and thermophilic microorganisms present in the oil production facility. Culture media that exhibited positive growth were used for the establishment of the oilfield microbial consortium which was composed by the mixture of the cultivable mesophilic and thermophilic species recovered in the laboratory.

2.2. Sample preparation and corrosion evaluation

Carbon steel round coupons with an exposed area of 1.27 cm^2 were used for the corrosion evaluation. Elemental composition (weight %) of the metal samples was: C (0.43 - 0.5), Mn (0.6 - 0.9), Si (0.15 - 0.35), S (0.01 - 0.35), P (0 - 0.035), Cr (0 - 0.40), and Fe (balance). Only one side of the coupons was used as a working surface for the corrosion evaluation; the other sides were electro-coated with a protective epoxy (Powercron 6000CX, PPG Industrial coatings). The exposed surface of each coupon was wet ground using silicon carbide papers of 80, 120, 320, and 600 grit, consecutively. The polished specimens were washed with Milli-Q water, degreased with acetone, washed with ethanol, and dried with nitrogen gas. Before immersion, the coupons were weighed and sterilised by 15 min of ultraviolet (UV) radiation.

Corrosion experiments were conducted in Anaerobic CDC Biofilm Reactors (Biosurface Technologies Corporation). Five different conditions, described in Table 1, were simulated in the reactors to evaluate the effect of nutrients and temperature on the microbial composition and corrosive behaviour of oilfield microorganisms. The exact conditions were evaluated under the same sterile setups, without microorganisms, to discriminate the abiotic corrosion reactions. Sterilised synthetic produced water supplemented with nutrients suitable for the growth of the consortium was used as a test solution. The composition of the synthetic produced water was as follows: 1.4 mM $CaCl_2x2H_2O$, 1.5 mM $MgCl_2x6H_2O$, 2 mM K_2HPO_4, 1.7 mM KH_2PO_4, 410 mM NaCl, 5 mM NH_4Cl, 59 mM $NaHCO_3$, 2.5 mM D-glucose, 30 mM Na-formate,

Table 1
Experimental conditions evaluated in the corrosion experiments.

Condition	Experiment code[a]	Microbial Consortium	Temperature	Sulphur compounds
1	B-40C-NS	Present	40 °C	NIL
	A-40C-NS	Absent		
2	B-40C-S	Present	40 °C	$S_2O_3^{2-}$ and
	A-40C-S	Absent		SO_4^{2-}
3	B-60C-NS	Present	60 °C	NIL
	A-60C-NS	Absent		
4	B-60C-S	Present	60 °C	$S_2O_3^{2-}$ and
	A-60C-S	Absent		SO_4^{2-}
5	B-60C-S2	Present	60 °C	$S_2O_3^{2-}$
	A-60C-S2	Absent		

[a] B – biotic, A – abiotic, 40C – 40 °C, 60C – 60 °C, NS – without sulphur compounds, S – with sulphate and thiosulphate, S2 – with thiosulphate.

6 mM Na-lactate, 18 mM Na-acetate, and 10 mL/L of vitamins solution and trace elements solution (German Type Culture Collection, DSMZ, media 141). Sulphur compounds, 8 mM Na_2SO_4 and 4 mM $Na_2S_2O_3x_5H_2O$ were added when the effect of these compounds was evaluated. The initial pH of the test solution was 7.3, which increased after microbial growth up to 7.56 (Fig. S1). Anaerobic conditions were maintained by continuous sparging of a gas mixture of 20 % CO_2: 80 % N_2 (20 mL min^{-1}). The baffle within the reactor was set to 50 rpm to maintain a homogeneous solution throughout the test. Biotic reactors were inoculated with the microbial consortium recovered from the oilfield in a concentration of 1.9×10^7 cells/mL. After an initial 48 h of operation under batch condition, in which no additional nutrients were added to the reactors, continuous flow of fresh test solution was initiated at a rate of 0.27 mL min^{-1} which replaced 110 % of the synthetic produced water daily.

2.3. Analytical methods

2.3.1. Corrosion measurements and surface analysis

Corrosion measurements were conducted after 21 days of metal exposure to different conditions. Corrosion rates in biotic reactors were also determined after 12 days of metal exposure, to evaluate MIC at two different stages of biofilm maturation. For the corrosion evaluation, three metal samples from each reactor were removed and cleaned using Clarke's solution, as described in the ASTM G1 standard [53]. The corrosion rates were estimated from the metal mass loss according to the same standard. Coupons surfaces of the three replicates were analysed using a 3D optical profilometer (Alicona imaging infinite focus microscope IFM G4 3.5). The average pit depth was obtained from 10 deepest pits measured on each sample. Pitting rate was calculated using the maximum pit depth found in each condition, as described in the NACE SP-0775 standard practice [54].

2.3.2. Microbial activity

ATP was determined by luminescence after reaction with luciferin-luciferase using the Quench-Gone Organic Modified (QGO–M™) test kit (Luminultra Technologies Ltd.) in planktonic cells and the Deposit & Surface Analysis (DSA™) test kit (Luminultra Technologies Ltd.) in sessile (attached to steel) cells. ATP measurements were conducted periodically to monitor the activity of planktonic cells during the testing period. ATP was extracted from 10 mL of the bulk test solution from each reactor following the kit's manufacturer instructions. ATP from sessile cells on the metal samples after 21 days of exposure was determined on one coupon from each condition. The coupons were gently washed with sterile phosphate-buffered solution (PBS) to remove unattached cells and then immersed in the lysis solution of the kit. Coupons were vortexed at full speed for 10 s and sonicated during 2 min to help detach cells while in contact with the lysis solution. ATP measurements were collected using the PhotonMaster™ Luminometer (Luminultra Technologies Ltd.), and ATP content was calculated from the measured luminescence by comparing it with a standard. Microbial activity of planktonic cells is expressed as cATP representing only the intracellular ATP of living cells. On the other hand, microbial activity of biofilms is expressed as tATP, which denotes total ATP in the biofilms, including ATP from living cells in addition to the potential presence of ATP released from dead cells.

2.3.3. Microbial quantification

Direct cell counts of planktonic (bulk solution) microorganisms were performed periodically using Neubauer counting chamber and a phase-contrast microscope (Nikon Eclipse Ci-L) at 100X magnification. Microorganisms observed in the central square of the chamber were tallied and multiplied by the appropriate dilution factor to estimate cells per mL.

Quantitative polymerase chain reaction (qPCR) method was used to determine the abundance of bacteria in biofilms. Cells were recovered from the metal surface by coupon immersion in 3 mL of sterile PBS containing 0.1 % w/v of Tween 20. Microorganisms were detached by vortexing at full speed for 10 s together with sonication during 2 min in cycles of 15 s on and 10 s off. After biofilm detachment, cells were pelleted by centrifugation at 15,000 x *g* for 5 min. DNA was extracted using the DNeasy PowerBiofilm Kit (QIAGEN) according to the manufacturer's instructions. Extracted DNA was resuspended in 100 µL sterile nuclease-free water and quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies). Total Bacteria were estimated quantifying the number of copies of the *rpoB* gene using the primers rpoB-f-4/rpoBr-2 described elsewhere [55]. qPCR reactions were performed using the QuantiFast SYBR Green PCR Kit (QIAGEN). Each PCR reaction was performed in a total volume of 25 µL following the manufacturer's instructions. qPCR reactions were carried out in a CFX96 Real-Time PCR Detection System (BioRad) with the following cycling parameters, an initial denaturing step at 95 °C for 5 min followed by 40 cycles consisting of 10 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and 30 s of extension at 72 °C. Fluorescent data collection and data analysis were carried out using the BioRad CFX Manager 3.1 (BioRad). Standard curve was created by plotting logarithmic values of the ten-fold serial dilution (10^9 to 10^1 copies per microliter) of the standard versus the threshold cycle (Ct) values generated from the qPCR analysis. Genomic DNA from *Shewanella oneidensis* was used as a standard template for bacterial quantification. All samples, including standards and negative controls, were performed in triplicate reactions.

2.3.4. Biofilm imaging

The metal surface of coupons exposed to biotic reactors was examined by field emission scanning electron microscope (FESEM) coupled with energy-dispersive X-ray spectroscopy (EDS) to confirm biofilm formation and to generate the element composition mapping. The analysis was performed using a Zeiss NEON high-resolution scanning electron microscope. Before the analysis, biofilms were fixed for 22 h in PBS containing 2.5 % glutaraldehyde and 0.15 % alcian blue. Then, coupons were washed with PBS and dehydrated through ethanol series (30, 50, 70, 80, 90, 95, and 100 % v/v ethanol-water) for 10 min interval. Dehydrated coupons were dried under nitrogen flow, coated with a platinum layer (5 nm thick), and stored in a vacuum desiccator until analysis. Aztec® 3.0 software (Oxford Instruments NanoAnalysis) was used for FESEM/EDS data analysis.

2.3.5. Biofilm microbial composition

After exposure of the metal samples to the test solution for a period of 12 and 21 days, sessile cells were detached from the metal surface by immersion in sterile 2:1 RNAprotect® Bacteria Reagent: PBS-Tween 20 (0.1 % w/v), and sonication during 2 min in cycles of 15 s on and 10 s off. After biofilm detachment, cells were pelleted by centrifugation at 15,000 x *g* for 5 min and preserved at -80 °C. RNA was extracted using the RNeasy PowerBiofilm Kit (QIAGEN) following the manufacturer's

instructions. RNA concentrations were quantified fluorometrically with the Qubit RNA HS Assay kit (Life Technologies). Afterwards, total RNA was treated with Turbo DNA-free kit (Invitrogen) to remove remaining DNA. Verification of the complete removal of DNA was performed by a PCR reaction targeting the 16S rRNA gene. Subsequently, RNA was purified by using the RNeasy MinElute Cleanup kit (QIAGEN). Purified RNA was converted to cDNA by using the SuperScript™ IV First-Strand Synthesis System (Invitrogen).

Polymerase chain reaction (PCR) and sequencing were performed by the Australian Genome Research Facility (AGRF). PCR amplicons were generated using the primers 341 F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3') [56]. Thermocycling was completed with an Applied Biosystem 384 Veriti and using AmpliTaq Gold 360 master mix (Life Technologies, Australia) for the primary PCR. The first stage PCR was cleaned using magnetic beads, and samples were visualised on 2% Sybr Egel (Thermo-Fisher). A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were cleaned again using magnetic beads, quantified by fluorometry (Promega Quantifluor) and normalised. The equimolar pool was cleaned a final time using magnetic beads to concentrate the pool and then measured using a High-Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pool was diluted to 5 nM and molarity was confirmed again using a High-Sensitivity D1000 Tape. This was followed by sequencing on an Illumina MiSeq instrument with a V3 (600 cycles) kit (Illumina).

The raw reads were demultiplexed and assigned to respective samples according to their barcodes by AGRF. The resulting fastq.gz files were imported into QIIME2 (2019.4) [57] to carry out all downstream analysis. Bioinformatics analysis was performed as described elsewhere [51].

2.3.6. Functional profile

The functional profile of the active microbial community was predicted using the R package Tax4Fun2 [58]. The analysis was focused on comparing the relative abundance of predicted genes encoding key enzymes involved in the metabolism of the predominant microbial groups detected as active in the biofilm communities. It has to be noted that the predicted functional profile only refers to the metabolic potential of the community, but it does not indicate which of the identified pathways are being used by the community.

2.3.7. Statistical analysis

Corrosion rates and measurements were performed in triplicate, and results are presented as the mean ± standard deviation. Kruskal-Wallis analysis was implemented to test if there were statistically significant differences in the corrosion rates and microbial diversity among conditions. PAST (v3) [59] software was used for the statistical analysis and results were considered significant with p-value ≤ 0.05. The microbial composition of active species at each sampling period and each condition was performed in duplicate. The beta diversity group significance test was done using PERMANOVA and pairwise tests in QIIME2 (2019.4). A non-metric multidimensional scaling (NMDS) analysis to visualise the multivariate dispersion of the community composition was conducted employing R (v3.4.3) [60]. NMDS was performed based on the Weighted UniFrac distance [61], lines for joining samples collected in the same reactor were projected onto the ordination, utilising the function ordiellipse.

2.4. Sequence data deposition

The 16S rRNA sequences were deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under bioproject number PRJNA56420.

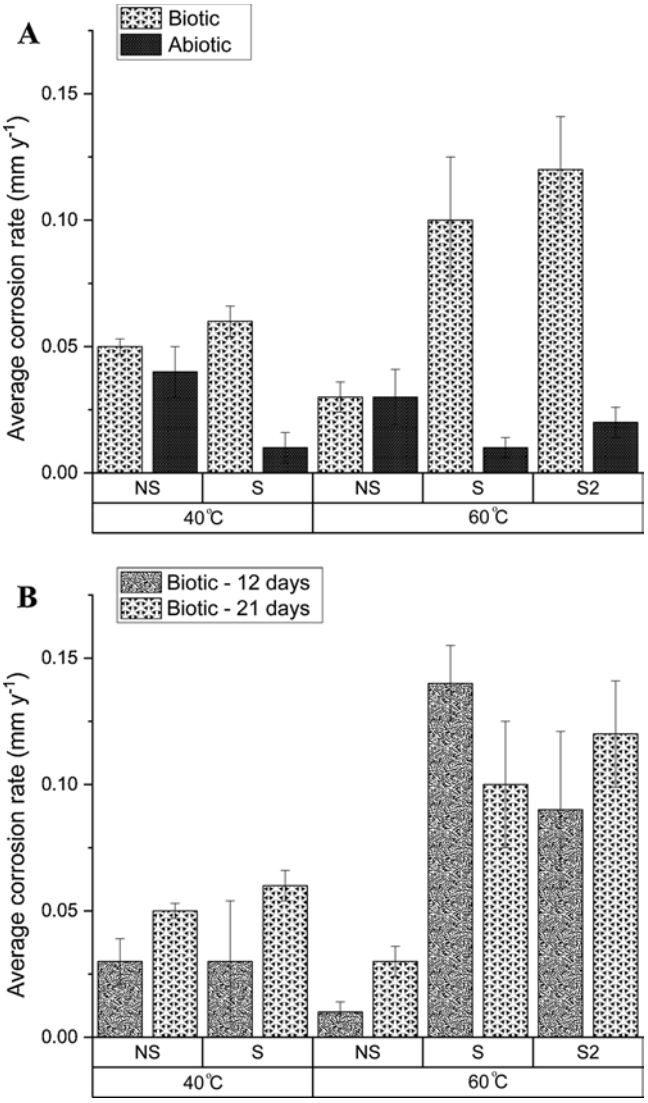

Fig. 1. Average corrosion rates calculated from weight loss of the metal samples. (A) Corrosion rates of coupons exposed for 21 days to biotic and abiotic conditions. (B) Corrosion rates of coupons exposed for 12 days and 21 days to biotic conditions. NS: Sulphur compounds absent, S: sulphate and thiosulphate present, S2: thiosulphate present.

3. Results

3.1. MIC of carbon steel as a function of temperature and presence of sulphur compounds

3.1.1. General corrosion rates

Average corrosion rates calculated from the coupons mass loss after the exposure period to the different conditions are presented in Fig. 1. In order to determine the impact of the microorganisms in the corrosion phenomenon, corrosion rates obtained in sterile controls were subtracted from the biotic corrosion rates. Results evidenced that microorganisms increased corrosion rates of carbon steel in all the conditions evaluated; however, differences with the controls were only statistically significant in the metals exposed to sulphur compounds in the test solution ($p \leq 0.05$, Table S1). The presence of sulphur compounds resulted in higher mass losses triggered by the microbial activity. Variation of temperature conditions also resulted in changes in the corrosivity of the microbial consortium. At 60 ^{0}C, biofilms resulted in higher corrosion rates in the presence of sulphur compounds, whereas in their absence biofilms at 40 ^{0}C caused higher corrosion rates.

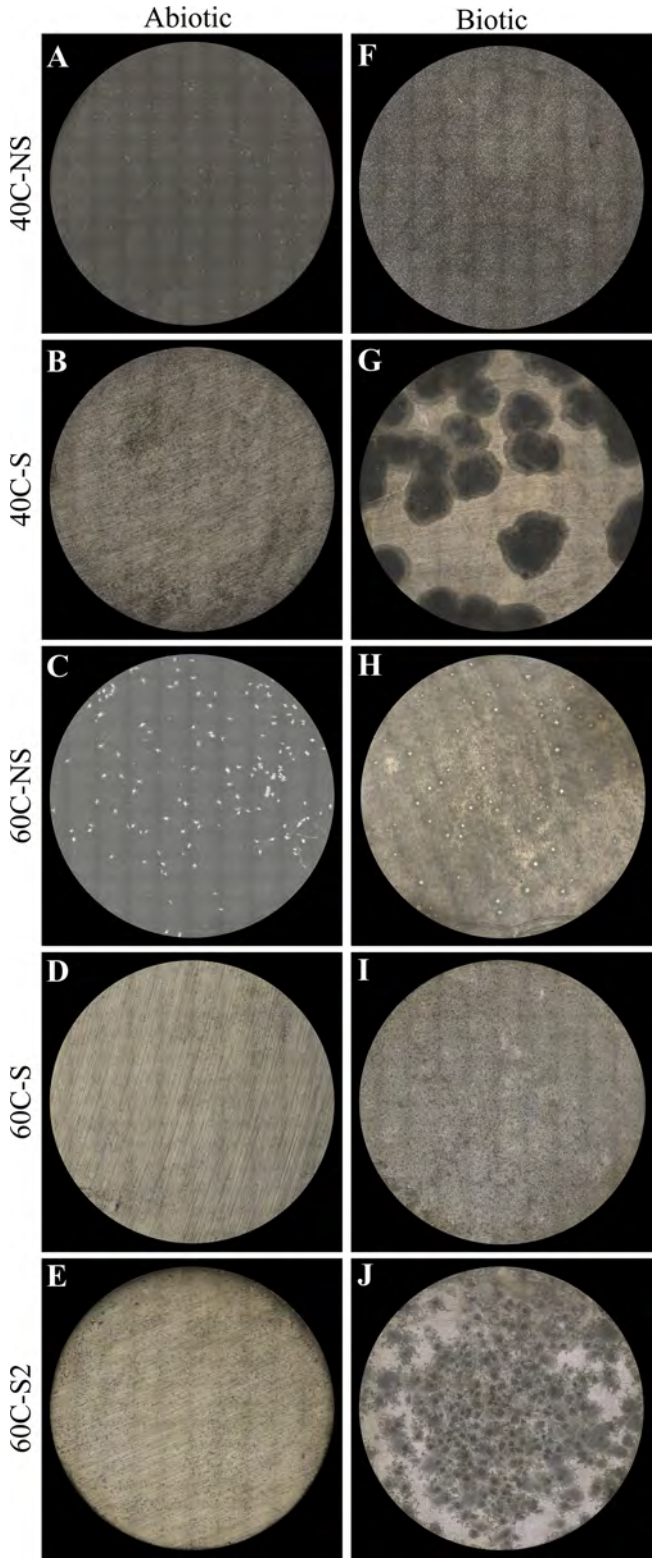

Fig. 2. Images of coupons surfaces after 21 days of corrosion testing. (A, F) coupons exposed to 40 ^{0}C without sulphur compounds, (B, G) coupons exposed to 40 ^{0}C with sulphate and thiosulphate, (C, H) coupons exposed to 60 ^{0}C without sulphur compounds, (D, I) coupons exposed to 60 ^{0}C with sulphate and thiosulphate, (E, J) coupons exposed to 60 ^{0}C with thiosulphate.

Corrosion rates measured at different exposure times showed that in most cases, older biofilms caused higher corrosion rates than younger biofilms (Fig. 1b).

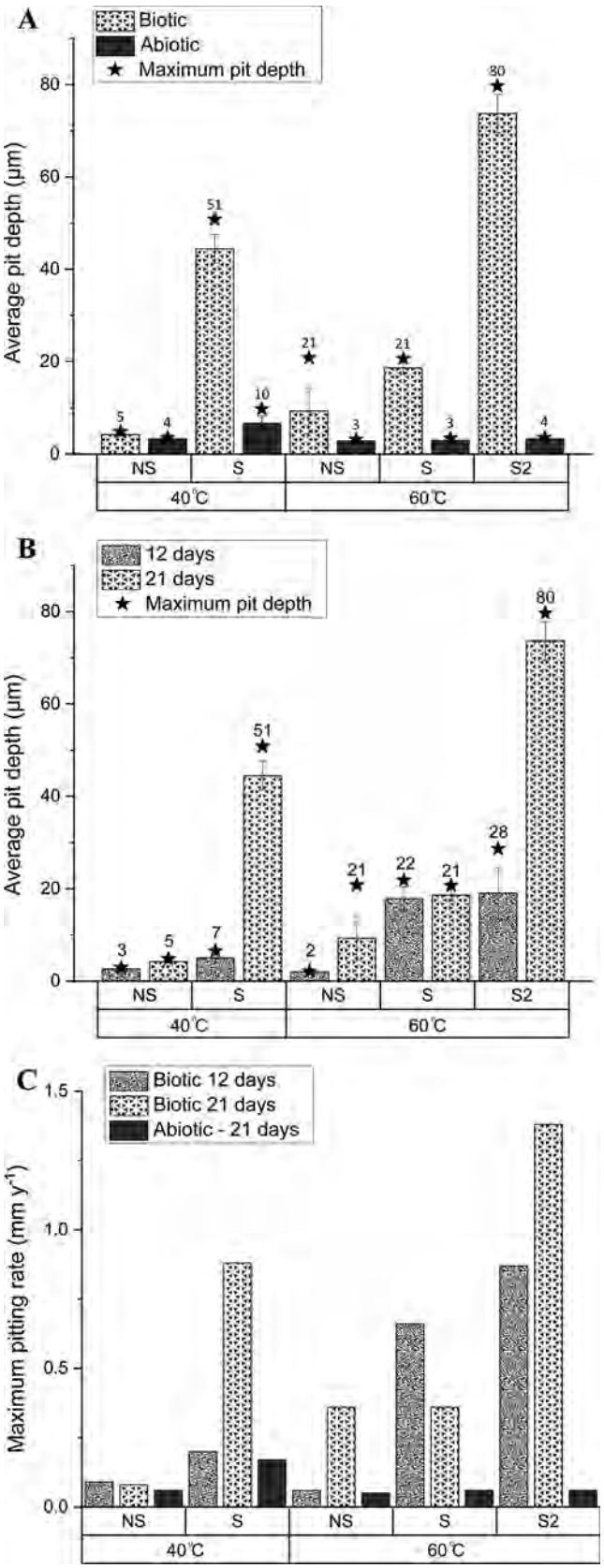

Fig. 3. Average pit depths and pitting rate calculated from the maximum pit depth. (A) Average pit depth of coupons exposed for 21 days to biotic and abiotic conditions. (B) Average pit depth of coupons exposed for 12 days and 21 days to biotic conditions. (C) Pitting rate of coupons exposed to biotic conditions for 12 and 21 days, and abiotic conditions for 21 days. NS: Sulphur compounds absent, S: sulphate and thiosulphate present, S2: thiosulphate present.

3.1.2. Pitting corrosion

Surface profilometry analysis revealed differences in the localised corrosion damage among the different experimental conditions evaluated (Fig. 2). Average pit depth, calculated from the ten (10) deepest pits found in three coupons of each reactor, maximum pit depths, and pitting rates are presented in Fig. 3. Deeper pits were visualised in the presence of microorganisms in all conditions. Differences in the depth of the pits versus the controls were significant in most of the cases except in the metals exposed to 40 ^{0}C without sulphur compounds ($p \leq 0.05$, Table S1). Biofilms generated deeper pits in the presence of sulphate and/or thiosulphate as compared to conditions lacking sulphur compounds.

The effect of temperature varied as a function of sulphur content in the solution. Marked deterioration of the metal surface was seen in the samples exposed to the 40 ^{0}C reactor only when sulphate and thiosulphate were present (B-40C-S, Fig. 2g) and, in the 60 ^{0}C reactor with only thiosulphate (B-60C-S2, Fig. 2j). Nonetheless, the morphology of the corroded areas was different between these conditions. Larger areas (area: $\bar{X}$ 400 μm) of localised corrosion were visualised at the lower temperature, compared to the numerous pits of smaller radius (area: $\bar{X}$ 11 μm) and deeper cavities observed at the higher temperature. Differences in the surface deterioration between samples exposed to 60 ^{0}C in the presence of sulphur compounds (B-60C-S and B-60C-S2) were also evidenced. Deeper pits were measured in the presence of thiosulphate only, whereas, in the presence of sulphate and thiosulphate, pits were less deep, but a greater deterioration of the surface was observed. In the absence of sulphur compounds, only a few pits (pit frequency: $\bar{X}$ 2 / cm^{2}) were detected at 60 ^{0}C, and no pits were seen at 40 ^{0}C. When comparing corrosion rates as a function of biofilm age for the most corrosive conditions found (B-40C-S and B-60C-S2), it was seen that pit growth increased significantly in the last days of exposure, from day 12 to day 21 (Fig. 3b), indicating that the more mature biofilms induced the greater pitting corrosion. Only the B-60C-S condition showed pit passivation in older biofilms.

Pitting rates calculated from the maximum pit depth found in each condition are shown in Fig. 3c. The highest pitting rate was measured in the metals exposed to higher temperature with thiosulphate as the only sulphur compound present in solution. According to the NACE standard practice SP0775 [54], the pitting rates caused by the microbial consortium were classified from low to severe corrosion depending on the evaluated conditions (Table 2). The availability of sulphur compounds in the environment resulted in high (B-60C-S = 0.31 mm y^{-1}) and severe (B-40C-S = 0.71 mm y^{-1}, B-60C-S2 = 1.32 mm y^{-1}) pitting rates due to microbial activity. High pitting rates were also evidenced in the absence of sulphur compounds only when the consortium was grown at a higher temperature (B-60C-NS = 0.31 mm y^{-1}). Typical 3D-images of the pitting corrosion evidenced in the metal samples exposed to biotic conditions are presented in Fig. 4.

3.2. Biofilm imaging

FESEM micrographs and EDS maps of selected areas of the biofilms are shown in Fig. 5. Micrographs revealed the presence of different cell morphologies within the biofilm communities, including long rod-

Table 2
Pitting rates induced by the biofilm community.

Condition	Pitting rate (mm y^{-1})	Categorisation[a]
B-40C-NS	0.02	Low
B-40C-S	0.71	Severe
B-60C-NS	0.31	High
B-60C-S	0.30	High
B-60C-S2	1.32	Severe

[a] Category according to the NACE standard SP0775 [49].

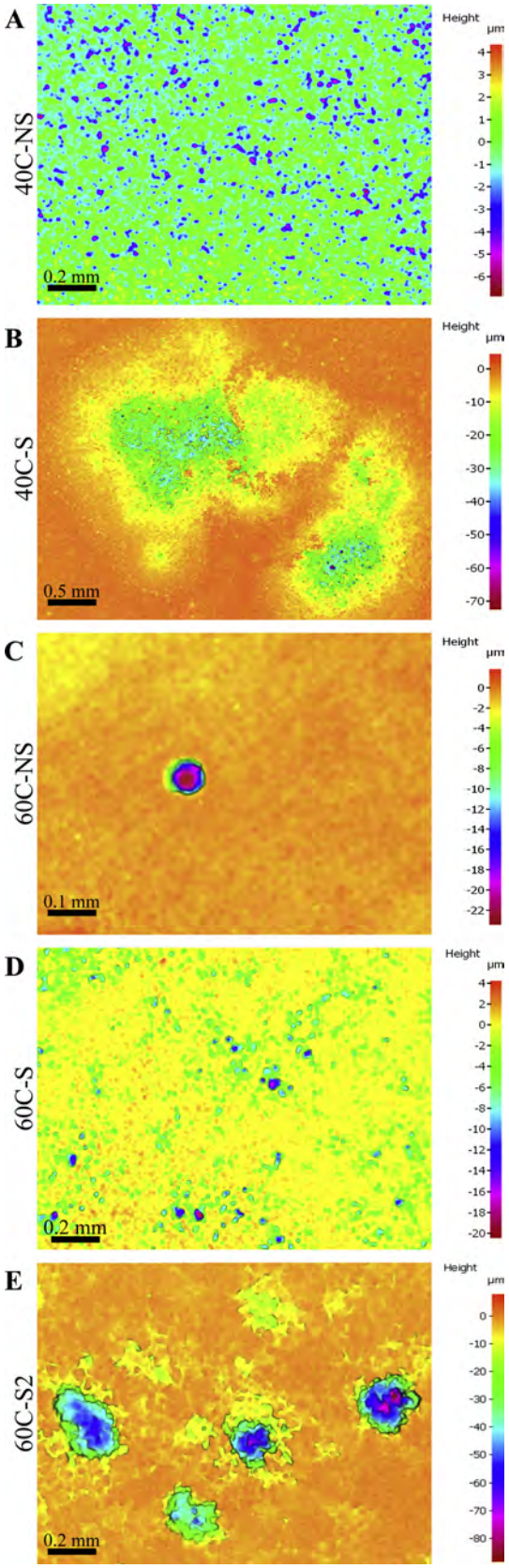

Fig. 4. 3D optical microscope surface images of carbon steel exposed to biotic conditions for 21 days. (A) Biofilm formed at 40 ^{0}C without sulphur compounds. (B) Biofilm formed at 40 ^{0}C with sulphate and thiosulphate. (C) Biofilm formed at 60 ^{0}C without sulphur compounds. (D) Biofilm formed at 60 ^{0}C with sulphate and thiosulphate. (E) Biofilm formed at 60 ^{0}C with thiosulphate.

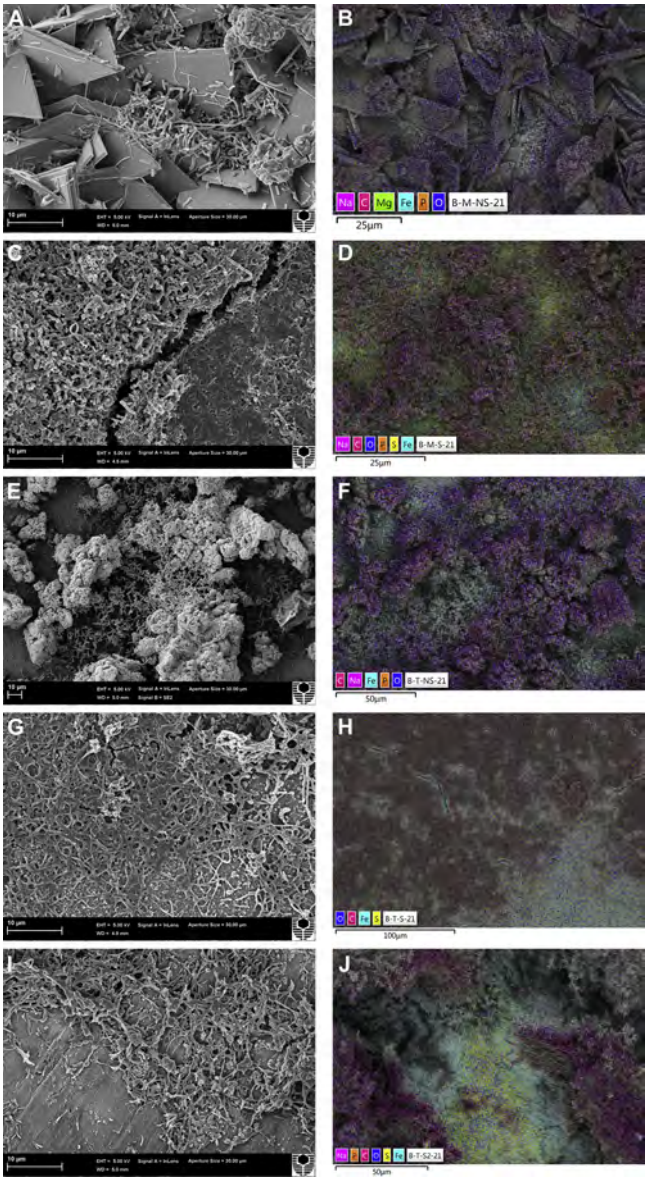

Fig. 5. SEM micrographs and EDS elemental maps of carbon steel coupons exposed to different biotic conditions. (A, B) Biofilm formed at 40 ^{0}C without sulphur compounds. (C, D) Biofilm formed at 40 ^{0}C with sulphate and thiosulphate. (E, F) Biofilm formed at 60 ^{0}C without sulphur compounds. (G, H) Biofilm formed at 60 ^{0}C with sulphate and thiosulphate. (I, J) Biofilm formed at 60 ^{0}C with thiosulphate.

shaped cells (> 10 μm). Biofilm structure and distribution over the metal surface varied among the different conditions. Biofilmed metal samples exposed in the reactors with sulphur compounds in the test solution showed a dense layer of deposits, corrosion products and microbial cells. In contrast, in the reactors without sulphur compounds, the corrosion products layer over the metals was less dense, and more patchy regions of microbial cells and deposits were evident. Elemental composition of biofilms and surrounding areas indicated that the major elements present on the biofilms were iron, phosphorous, carbon, oxygen and sulphur, the last only detected abundantly in samples exposed to reactors containing sulphate and/or thiosulphate. Sulphur was mapped together with iron, suggesting the presence of iron sulphides (FeS) which was correlated with the blackening of the test solution.

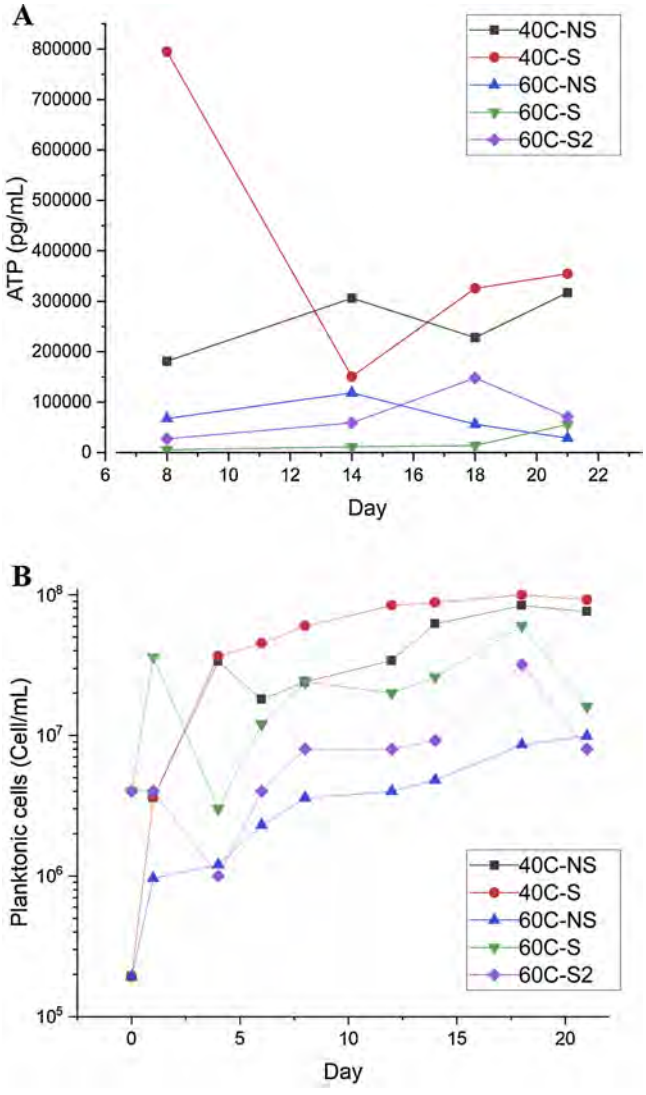

Fig. 6. Microbial activity and cell numbers of planktonic microorganisms during the experimental period. (A) cATP levels in planktonic cells. (B) Planktonic cells numbers in the test solution.

3.3. Microbial activity

ATP levels of planktonic cells are shown in Fig. 6a. The cATP fluctuation revealed that microbial activity of planktonic cells varied during the experimental period. Higher cATP levels were always detected in planktonic cells thriving at 40 ^{0}C compared to cells exposed to 60 ^{0}C. The effect of sulphur compounds on the activity levels of planktonic cells did not follow a distinctive pattern. Analysis of sessile (biofilm) cells indicated that biofilms produced higher tATP levels in the presence of sulphur compounds as compared to conditions lacking sulphur compounds (Table 3). Comparison of the ATP levels produced

Table 3
tATP levels in biofilms after 21 days of testing.

Condition	tATP pg/cm^2
B-40C-NS	149,550
B-40C-S	1′513,760
B-60C-NS	1,960
B-60C-S	24,485
B-60C-S2	222,085

Table 4
Bacterial cell numbers in biofilms after 21 days of testing.

Condition	Bacteria (*rpoB*) cells/cm^2
B-40C-NS	(1.3 ± 0.1) x 10^8
B-40C-S	(9.9 ± 0.1) x 10^8
B-60C-NS	(3.9 ± 0.2) x 10^7
B-60C-S	(1.8 ± 0.6) x 10^6
B-60C-S2	(2.5 ± 1.5) x 10^6

Errors represent standard deviation from 3 replicates.

at 60 ^{0}C in the presence of sulphur compounds indicated that in the presence of sulphate and thiosulphate, planktonic and sessile cells of the microbial consortium were considerably less active than in the only presence of thiosulphate. Microbial activity levels also varied between biofilms grown at different temperatures; higher tATP levels were measured for biofilms grown at 40 ^{0}C compared to biofilms grown at 60 ^{0}C.

3.4. Microbial quantification

Similar to the activity levels, planktonic cell numbers in the test solution fluctuated over the experimental time (Fig. 6b). Higher cell numbers were measured in the lower temperature reactors (up to two orders of magnitude) compared to the reactors at 60 ^{0}C, although cell counts were not directly proportional with cATP levels. A similar pattern was observed in the biofilm communities, higher cells numbers were detected in the reactors at lower temperature, and the tATP levels were not directly proportional to the number of cells. Results of the sessile bacterial quantification are presented in Table 4.

3.5. Microbial composition

Active microbial populations in the biofilms recovered from the carbon steel surfaces were identified through RNA-based sequencing of the 16S rRNA transcripts. A total of 4,728,847 raw-reads were retrieved from the Miseq sequencing. After quality filtering, singletons, and chimera removal through the bioinformatics processing, 1,881,832 sequences were used for the taxonomic classification of cells attached to the metals. High-quality sequences were clustered into 325 ASVs. Subsequently, ASVs were taxonomically classified into 18 classes and 48 genera, 15 of them remaining as unclassified/uncultured microorganisms. Rarefaction analysis was used to assess the microbial diversity in the biofilms. The curves showed differences in the numbers of ASVs among the conditions, indicating that the variation in the environmental conditions caused an impact on the microbial composition of biofilms (Fig. S2). In order to reduce the bias of an unequal number of sequences, normalisation was performed by subsampling method at 39,014 sequences per sample. Kruskal-Wallis test of the diversity indices Chao1 and Shannon confirmed that differences observed in the alpha diversity of the biofilm communities were statistically significant ($p \leq 0.05$). Greater differences were observed between mesophilic (B-40C-NS and B-40C-S) and thermophilic (B-60C-NS and B-60C-S2) communities. The diversity indices values are presented in Table S2.

Diversity profiling analysis was carried out at the genus level. Only the genera with relative abundances equal or greater to 1% in at least one of the samples are presented in Fig. 7. The complete list of the active microorganisms recovered from biofilms attached to the carbon steel coupons is presented in Table S3. Results of the diversity profiling analysis revealed that the microbial community composition in the biofilms varied in response to environmental conditions. At 40 ^{0}C, the active microbial community in the biofilms was comprised of mesophiles and thermophiles. However, at 60 ^{0}C, only thermophiles were detected active in the biofilms. The dominant active genera detected in the reactors at 40 ^{0}C were *Thermovirga, Petrotoga, Methanobacterium, Acetomicrobium, Halocella*, *Pseudomonas, Anaerophaga* and, *Desulfovibrio*. The genera *Pseudomonas* and *Anaerophaga* were only detected abundantly in the reactor without sulphate and thiosulphate in the test solution. *Desulfovibrio* genus was only detected abundantly in the reactor with the presence of sulphate and thiosulphate. Biofilms formed over carbon steel at 60 ^{0}C were dominated by *Thermovirga, Acetomicrobium* and, *Thermoanaerobacter* in reactors without sulphur compounds, and by unclassified Desulfovibrionales, *Petrotoga, Acetomicrobium, Methanothermobacter* and, *Thermoanaerobacter* in reactors with sulphur compounds. Even though most of the dominant microorganisms were detected in the different conditions evaluated, changes in their relative abundances in the biofilm community were visualised (Fig. 7). Variation in the microbial composition of biofilms formed at different periods was also observed with the sequencing analysis. Microorganisms such as *Halocella*, *Pseudomonas*, *Anaerophaga,* and the unclassified Rhizobiales showed a decrease in their abundances during biofilm maturation. In contrast, the genera such as *Methanobacterium*, *Desulfovibrio*, *Thermoanaerobacter,* and the unclassified Desulfovibrionales increased their abundances. Differences in the relative abundances of the microorganisms in the biofilms produced changes in the microbial community structure. NMDS analysis used to visualise the β-diversity among the microbial communities is presented in Fig. 8. Ordination analysis demonstrated differences in the active microbial community structure of biofilms formed at different conditions. Greater differences were observed between biofilms formed at 40 ^{0}C and 60 ^{0}C. A PERMANOVA test indicated that the biofilm community structure in each condition is significantly different from the other conditions evaluated (p-values ≤ 0.05, Table S4). Only the biofilm communities of the two reactors incubated at 60 ^{0}C with sulphur compounds available presented a similar structure (p-value > 0.05).

3.6. Functional profile

The relative abundances of some of the predicted KEGG pathways are shown in Fig. 9. The complete list of the functional capabilities predicted for the biofilm communities is presented in Table S5. The analysis was centred in the abundance of genes involved in carbohydrate metabolism, sulphur metabolism, and methane metabolism. Higher abundance of genes involved in carbohydrate and sulphur metabolisms were predicted in microbial communities grown at 60 ^{0}C (Fig. 9a and b). Contrarily, the relative abundance of genes involved in methane metabolism was predicted in lower abundance compared to the microbial communities grown at 40 ^{0}C (Fig. 9c).

For the carbohydrate metabolism, it was noticed that biofilm communities formed in the presence of sulphur compounds had a lower abundance of genes involved in this metabolic pathway, compared with the communities grown in their absence. The capability of the microbial communities for using sulphur compounds in energy metabolism was present in all conditions. Although sulphur compounds were not present in some reactors, genes related to the sulphur metabolism were predicted in all active microbial communities grown over the carbon steel samples. The relative abundance of predicted genes involved in sulphate and thiosulphate reduction was lower in reactors without sulphur compounds, however, a marked difference was only observed between communities exposed to the higher temperature (Fig. S3). It is important to emphasise that predictive functional profiling using 16S rRNA data can deviate from metagenomics profiling and gene annotation, considering that taxonomic identification does not necessarily reflect the presence of functional genes. Nevertheless, it has been shown that the metabolic potential of communities from different environments studied with this bioinformatics technique is in good agreement with those derived from the metagenomic analysis [58,62].

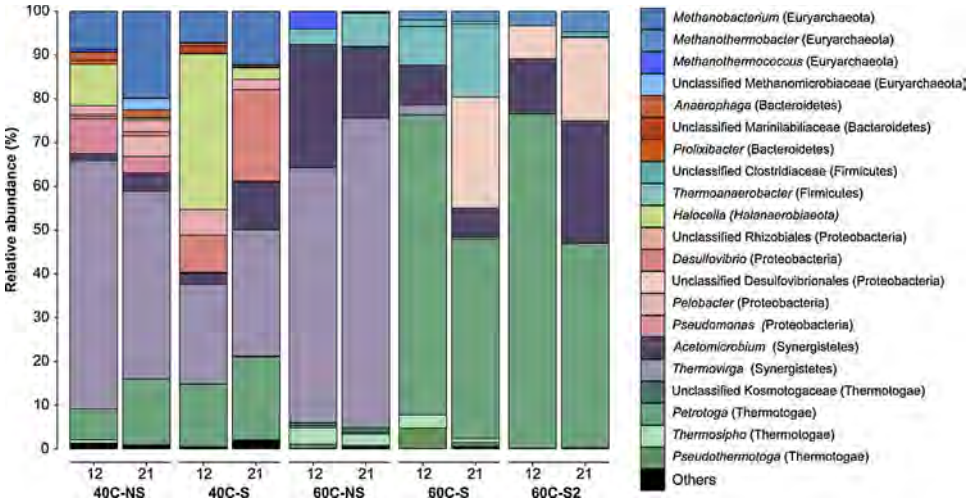

Fig. 7. Relative abundance of active microorganisms in biofilms formed over carbon steel coupons at different conditions. Classification at genus level from 16S rRNA sequencing. Genera accounting for < 1% of all classified sequences were summarised in the artificial group "Others". 12: biofilms recovered after 12 days, 21: biofilms recovered after 21 days, 40C: 40 ^{0}C, 60C: 60 ^{0}C, NS: Sulphur compounds absent, S: sulphate and thiosulphate present, S2: thiosulphate present. Each bar represents the results from the two replicates evaluated in each condition which were pooled together by the sum of reads at the same sequencing effort.

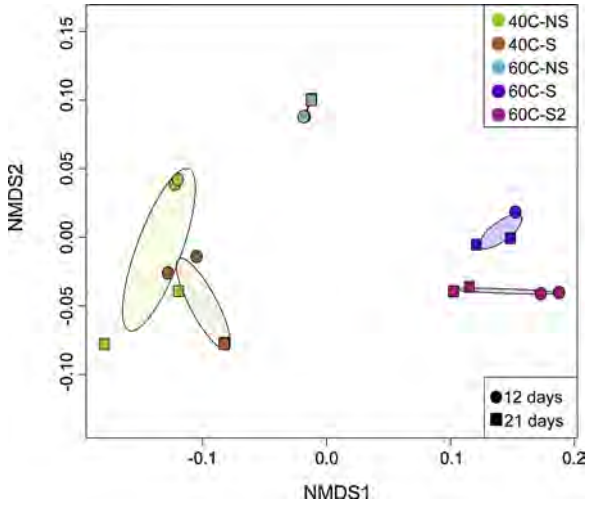

Fig. 8. Non-metric multidimensional scaling (NMDS) of the active microbial community recovered from biofilms formed under different conditions.

4. Discussion

4.1. MIC of carbon steel as a function of temperature and presence of sulphur compounds

This study assessed the effect of sulphur-containing compounds and temperature on MIC by biofilms from an oilfield consortium. Results confirmed that the corrosive behaviour of oilfield microorganisms is significantly affected by environmental conditions. Average corrosion rates and maximum pitting rates varied among the experimental conditions. Changes in the microbial corrosivity under the different conditions were attributed to differences in microbial composition, community structure, and metabolic activities of the biofilms in response to the environmental conditions. Specifically, these biofilm properties changed in response to the addition of electron acceptors to the solution and the temperature conditions. Sulphate and thiosulphate are used by the sulphide producing prokaryotes as the final electron acceptor during anaerobic respiration [37], thiosulphate can also be used as electron sink by some thermophilic fermenting microbes [63]. The presence of these compounds in the test solution stimulated the activity of the bacterial communities in biofilms, which produced higher ATP levels compared to biofilms grown without sulphur compounds in the solution. Likewise, the relative abundance of sulphate reducing bacteria such as *Desulfovibrio* and the unclassified Desulfovibrionales, and of thiosulphate reducing bacteria such as *Acetomicrobium, Thermoanaerobacter,* and *Petrotoga* increased when sulphate and/or thiosulphate were available in the solution. The availability of sulphur compounds and the concomitant presence of sulphidogenic microorganisms in biofilms resulted in sulphide production, evidenced by blackening of test reactors after a few days of exposure. Hydrogen sulphide reacts with the dissolved iron, producing iron sulphide (FeS) precipitates that have a characteristic black colour [17]. The EDS mapping confirmed the presence of sulphur element in the biofilm, which was detected along with iron in the elemental maps. FeS precipitates can be protective to the metal, but they can also induce galvanic corrosion, acting as a cathode to the steel [7,15]. It has also been shown that FeS is a semiconducting material that plays a significant role in interspecies electron transfer, a phenomenon that mediates electron flow through the biofilm and promotes syntrophic interactions across

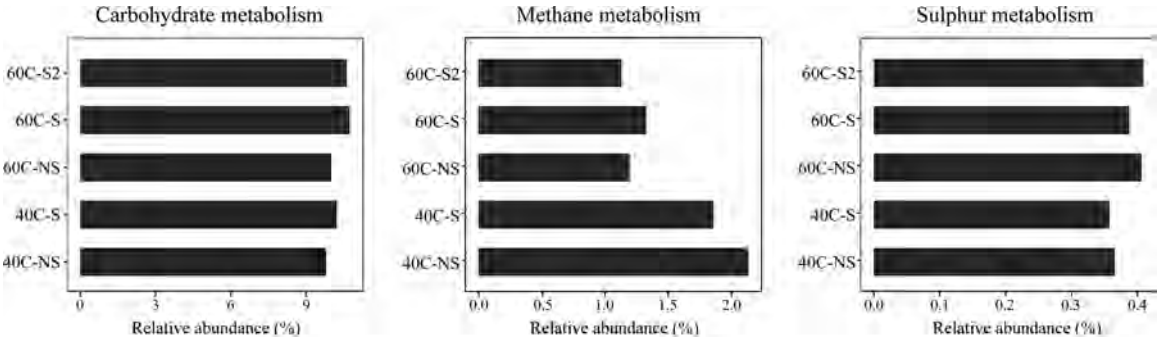

Fig. 9. Relative abundance of predicted genes involved in the carbohydrate metabolism, methane metabolism, and sulphur metabolism. Prediction based on 16S rRNA data from biofilm communities after 21 of corrosion testing.

microorganisms in the consortium. Recently, the effect of sulphate availability on the corrosivity of an anaerobic long-chain paraffin-degrading consortium was studied by Liang et al. [64]. The authors reported similar results to the findings of this investigation, where sulphate addition led to the proliferation of sulphate reducing microbes in the consortium and an increase in the corrosion rates of carbon steel by MIC. Variation of the microbial corrosivity due to modification of the nutrients has been previously reported by Javed et al. [65]. The authors stated that changes in the components of the test solution resulted in acceleration or inhibition of the corrosion processes by a single species biofilm. The authors explained the differences in the corrosive behaviour due to changes in metabolic activity and the corrosivity of metabolic by-products, which could vary from acidic (accelerated corrosion) to alkaline (inhibited corrosion) depending on the components added to the test solution [65]. Overall, the results of this investigation indicates that the addition of sulphur compounds to oilfields with low-sulphate produced water increases the risk of MIC. However, it has to be noted that in the absence of sulphur compounds microorganisms can also generate localised corrosion as it has been demonstrated in this investigation.

The effect of temperature on MIC has been previously investigated. However, previous studies do not show a distinctive trend. Li et al. [66] found that in the presence of bacteria, carbon steel samples exhibited higher corrosion rates at 55 ^{0}C than at 37 ^{0}C. Conversely, Salgar et al. [48] reported that microbial consortium was more aggressive to carbon steel when incubated at 40 ^{0}C than at 60 ^{0}C. The differences in the results of both studies were linked to the differences in the microbial consortia used. Li et al. evaluated a community with a high abundance of archaeal species, which were predominantly thermophiles, whereas Salgar et al. evaluated a community mainly composed of sulphide producing prokaryotes, most of them reported in the literature as mesophilic species. The results of the present study corroborate previous findings; temperature changes in oilfield facilities can lead to changes in the microbial composition and community structure, which can consequently result in different MIC processes. Corrosion rates (general and localised corrosion) in this investigation presented a different trend when evaluating the effect of temperature. While 60 ^{0}C condition induced higher mass losses in the presence of sulphur compounds, 40 ^{0}C condition induced higher pitting rates. Differences in the corrosion rates can be the result of dissimilarities in the corrosion mechanisms. According to the results of this study, temperature gradients in the oil production facilities promote the formation of thermal niches throughout the production process. Specific microbial communities grown at each niche can have different corrosive behaviour depending on the type of microorganisms and the nutrients available in the fluids (water chemistry), which will ultimately dictate the metabolic activities of said microorganisms. In this sense, a decrease in the temperature of the fluid can lead to an increase or decrease in the microbial corrosivity with water chemistry (nutrients) representing the most important factor in predicting the risk of MIC

4.2. Microbial composition and metabolic activity as drivers for MIC

Many of the microorganisms found active in the biofilm communities developed in this study have been previously implicated in MIC [17,26,27,44,67]. Most of the cultivable microorganisms recovered from the produced water belonged to genera with species classified as thermophiles, i.e., microorganisms classified within these genera can tolerate high temperatures, which is consistent with the temperature found in the facility at the sampling location (55 °C). Interestingly, thermophilic bacteria such as *Thermovirga*, *Thermosipho*, *Acetomicrobium*, and *Petrotoga* were also found active when evaluated at 40 °C, showing that some microorganisms can grow in a wide range of temperature. It has to be noted that despite the high temperature of the fluid at the sampling location, mesophilic microorganisms such as *Desulfovibrio*, *Pseudomonas*, and *Methanobacterium*, were still detected and cultivated in the laboratory. Detection of microorganisms with optimal growth temperatures different from those found at the sampling locations in oil reservoirs and production facilities has been previously reported [68]. For example, Junzhang et al. [69] reported a large number of mesophilic species in high-temperature reservoirs. Presence of mesophiles in high-temperature oilfields has been related to water injection into the reservoir for pressure support. Injected water introduces foreign microbes that are mostly mesophiles. Some of these microbes can remain dormant in the production fluids and have the ability to proliferate when appropriate temperature conditions are reached in the oil facilities.

The presence of a single abundant active microorganism to which the corrosivity could be attributed to was not evidenced. Instead, several microorganisms were found equally active (comparable relative proportions) in the biofilm communities that induced high pitting rates, which suggests that MIC was the result of synergistic interactions among different microbial species in the biofilm and not a process dominated by a single type of microorganism. Multispecies biofilms have been reported to lead to higher corrosion rates compared to single species biofilms, which indicates that MIC is the result of multiple processes and interactions between taxonomically and metabolically different microorganisms [70,71]. In this study, the pitting rates induced by biofilms were classified as severe according to the NACE categorisation. The marked increase of the pitting rate by older biofilms revealed that the age of the biofilm plays a significant role in the MIC process. Changes in the microbial composition and community structure of the active species in the biofilm suggested that there was a variation in the synergistic and antagonistic interactions during the maturation time. At 40 ^{0}C, younger biofilms showed higher abundances of genera related to surface colonisation and exopolymeric substances (EPS) production, essential processes in the biofilm development. Likewise, a higher abundance of the *Halocella* genus was also evidenced in young biofilms, which are species known to produce bacteriocins substances that inhibit or kill other microorganisms. Conversely, older biofilms at the same thermal condition showed an increase in the activity of the *Acetomicrobium* and *Desulfovibrio* genera, both reported as corrosive species [17,37,72]. The proliferation of these populations was higher in the presence of sulphur compounds which correlates with the increase in corrosion rates; however, the factors that led to the microbial succession remain unknown. In contrast, at 60 ^{0}C the dominant species remained active in both young and old biofilms; however, a variation in the biofilm community structure was also detected in older biofilms, with an observed increase in the proportion of an unclassified Desulfovibrionales in the biofilms grown with sulphur compounds. Although there is a pattern in the increase of these sulphate-reducing microorganisms and the increase in the corrosion rate, results from this study cannot conclude that these populations were the only biofilm species implicated in MIC since other populations were also active in the biofilms.

Functional prediction of the microbial taxa identified in biofilms shows that the active communities have the potential to carry out a number of different but complementary metabolic processes that could have influenced the corrosion of carbon steel. Dominant microorganisms in the corrosive biofilms were mainly fermenting species, which could have developed synergistic interactions with H_2 consuming microorganisms, such as sulphate reducers, methanogens or acetogens, also detected active in the biofilms. The predicted microbial capability for using sulphur compounds in the energy metabolism was higher in reactors incubated at 60 ^{0}C, however, the abundance of genes was similar in biofilms grown in the presence and absence of these substances. These results suggest that these populations have the ability to adapt to the environment and to switch electron acceptors for their metabolism.

Sulphidogenic microorganisms have been listed as one of the most physiologically versatile microorganisms in petroleum reservoirs, with the capability of oxidizing more than 100 different compounds coupled

with the reduction of several molecules [73]. Considering that no other electron acceptors for the anaerobic respiration were added to the test solution, the metabolic activities in the conditions that lack sulphur compounds were restricted to glucose fermentation and CO_2 reduction (methanogenesis or homoacetogenesis). Additionally, in an active carbon steel corrosion process, microorganisms can use iron oxides generated in this process as electron acceptors for their metabolisms. A limitation in electron acceptors in solution was correlated with lower ATP levels in biofilms. However, despite the low activity levels observed in the biofilms grown in the 60 ^{0}C reactor lacking sulphur compounds, microorganisms still induced high pitting rates. Differences in biofilm activity as measured by ATP content were not only observed as a response to the nutrients conditions but also to temperature conditions. When comparing the microbial activity of populations grown with the same nutrients, lower ATP levels were always measured at 60 ^{0}C as compared to 40 ^{0}C conditions. Dissimilarities in the ATP production between mesophiles and thermophiles were also observed in a previous investigation with a different microbial consortium [48]. Low ATP levels in thermophiles have been attributed to lower growth rates, lower growth yields, and higher microbial inhibition compared to mesophiles [74]. Furthermore, it has been suggested that thermophiles have lower efficiency in energy conversion due to an increased proton permeability of the cell membrane that forces microorganisms to invest more energy in maintenance [75]. The microbial quantification of planktonic and sessile cells confirmed that microorganisms exposed to higher temperature had lower cell numbers compared to microorganisms exposed to a lower temperature which suggests that differences in microbial activity at different thermal conditions were related to growth rate. It was also identified that ATP concentration was not directly proportional to cell numbers. The concentration of ATP per cell can vary between microbial species, within the same species, and in the same cell according to the metabolic state or growth phase [76]. Additionally, ATP yield also depends on the cellular process used for its production. For instance, in the aerobic respiration 32–36 molecules of ATP are derived from glucose oxidation, whereas in the glucose fermentation, only 2–4 molecules are generated [77]. Although ATP levels varied according to temperature, biofilms recovered from the most corroded metals exhibited the highest ATP levels detected in each thermal condition, which indicated a relationship between the microbial activity and microbial corrosivity. This relationship was also evidenced in the reactors incubated at 60 ^{0}C in the presence of sulphur compounds. Microbial activity was higher in the presence of thiosulphate only, which was the condition that displayed the highest pitting rates.

With regards to MIC mechanisms, chemical corrosion reactions implicated in the MIC processes observed in this study can be suggested from the metabolic capabilities of the active microorganisms constituting the biofilms. Such metabolic capabilities include 1) CO_2 and acid production from carbohydrate fermentation, 2) sulphide production from sulphate, thiosulphate or elemental sulphur reduction, 3) reduction of iron oxides formed by corrosive agents such as CO_2, 4) EPS production by the sessile cells during biofilm formation and maturation. The potential corrosion mechanisms, reactions derived from these microbial activities are summarised in Table 5. All capabilities described above can induce CMIC; however, EET-MIC cannot be disregarded considering that species of some of the genera detected in the study can uptake electrons from the metallic surfaces. Although EET-MIC has predominantly been reported when microorganisms are exposed to starvation condition, i.e., without electron donors other than the bare metal [13,24,72,78], EET-MIC can potentially still occur in systems with a continuous flow of nutrients. For example, microorganisms in the bottom of the biofilm might have difficulties in reaching the electron donors in the solution due to the diffusion barrier generated by the corrosion products and biofilm itself, forcing them to use the metal as the electron donor. Since pitting density and pits morphology were different in the most corrosive condition found at each thermal scenario, it is suggested that the corrosion reactions caused by the same consortium varied as a response to changes in the environmental conditions. Further analyses are required to confirm the MIC mechanisms evidenced in each condition, within these, the in situ measurements of the biofilm pH would indicate if acidic microenvironments were created by the metabolic activities of fermenting microbes in the biofilm. Specialised analysis like metatranscriptomics that reveals information about genetic expression could be applied to pinpoint the specific metabolic pathways used by microbial communities in response to environmental changes and corrosive scenarios.

5. Conclusion

Results from this investigation show that changes in the environment can induce changes in the microbial activity, microbial composition and community structure of biofilms and the resulting risk of microbiologically influenced corrosion. The main findings of this investigation are as follows:

1 The 16S rRNA profiling based on the RNA molecule that was used to identify only the active microbial populations in biofilms indicated that microorganisms in the oilfield consortium responded to the environmental conditions.
2 The presence of sulphur compounds (sulphate and thiosulphate) significantly increased the average corrosion rates and pitting rates induced by multispecies biofilms at both thermal conditions. Higher corrosivity was related to the higher microbial activity observed in the presence of these compounds, which resulted in the production of hydrogen sulphide, generated from the sulphate and thiosulphate reducing metabolisms. Fermentative microorganisms, many of them with the capability of producing sulphide from thiosulphate or elemental sulphur reduction, were the predominant active populations in the biofilms in all conditions. The highest pitting rate was obtained in the presence of thiosulphate as the only sulphur compound present in solution. This highlights the importance of evaluating sulphur compounds other than sulphate in production fluids to assess more accurately the risk of MIC.
3 The effect of temperature on the risk of MIC did not follow a distinctive pattern. Depending on the microbial species present in the environment, low or high thermal conditions can be equally corrosive. Based on the above, the inclusion of the temperature parameter in MIC prediction models should also consider both the chemistry (nutrients) and microbiology of the system.
4 Microbial activity measured by ATP levels was affected by the nutrients and temperature conditions; 1) the biofilm community produced higher ATP levels in the presence of sulphur compounds than in their absence as a result of having more electron acceptors in the solution for metabolic activities; 2) the biofilm community produced higher ATP levels at a 40 ^{0}C than at 60 ^{0}C, which was correlated with cell numbers. This result suggests growth rate differences between mesophiles and thermophiles.
5 A correlation was found between biofilm maturity and corrosion rates; older biofilms triggered higher corrosion rates than younger biofilms. Changes in the microbial composition and community structure were evidenced as part of the biofilm maturation. Factors influencing the microbial succession and the associated corrosivity changes require further investigation.
6 Microbial genera detected in an active state during the exposure to different environmental conditions of temperature and nutrients demonstrates the adaptability and versatile physiology of oilfield microorganisms. This adaption and versatility resulted in different mechanisms and rates on corrosion.

Role of the funding source

The authors declare that Woodside Energy Ltd. contributed

Table 5
Potential corrosion mechanisms induced by the metabolic activities of the oilfield microbial consortium.

Corrosion mechanism		Microbial activity involved	Corrosion Reaction	Reference
CMIC	Acid corrosion	Acid production: organic acids released during fermentation.	$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2$	[3,79]
	CO_2 corrosion	CO_2 production: CO_2 released during fermentation.	$Fe^0 + CO_2 + H_2O \rightarrow FeCO_3 + H_2$	[79]
	H_2S corrosion [a]	Sulphide production: HS released during sulphate, thiosulphate or sulphur reduction. $HS^- + H^+ \rightarrow H_2S$	$Fe^0 + H_2S \rightarrow FeS + H_2$	[79]
	Galvanic corrosion Fe^0/FeS_x [a]	Sulphide production - HS released during sulphate, thiosulphate or sulphur reduction. FeS precipitates act as a cathode to the steel. $Fe^{2+} + HS^- \rightarrow FeS + H^+$	$Fe^0 + 2H^+ \xrightarrow{FeS} Fe^{2+} + H_2$	[11,80]
EET-MIC	Extracellular electron transfer	Iron oxidation - Sulphate reduction [a]	$3Fe^0 + SO_4^{2-} + 3HCO_3^- + H_2O \rightarrow FeS + FeCO_3 + 5HO^-$ $4Fe^0 + SO_4^{2-} + 3HCO_3^- + 5H^+ \rightarrow FeS + 3FeCO_3 + 4H_2O$	[81,82]
		Iron oxidation - Methanogenesis	$4Fe^0 + 5HCO_3^- + 5H^+ \rightarrow CH_4 + 4FeCO_3 + 3H_2O$ $4Fe^0 + CO_2 + 4HCO_3^- + 4H^+ \rightarrow CH_4 + 4FeCO_3 + 2H_2O$	[39,81]
		Iron oxidation - Acetogenesis	$4Fe^0 + 6HCO_3^- + 5H^+ \rightarrow CH_3COO^- + 4FeCO_3 + 4H_2O$ $4Fe^0 + 2CO_2 + 4HCO_3^- + 4H^+ \rightarrow CH_3COOH + 4FeCO_3 + 2H_2O$	[39,81]
Remotion of passivation layers		Iron reduction – Fe(III) used as an electron acceptor during anaerobic respiration. $Fe^{3+} + e^- \rightarrow Fe^{2+}$		[83]

[a] Mechanism only possible in conditions with sulphur compounds available.

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CRediT authorship contribution statement

Silvia J. Salgar-Chaparro: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **Katerina Lepkova:** Conceptualization, Supervision, Writing - review & editing. **Thunyaluk Pojtanabuntoeng:** Conceptualization, Supervision, Writing - review & editing. **Adam Darwin:** Conceptualization, Supervision, Resources, Writing - review & editing. **Laura L. Machuca:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.corsci.2020.108595.

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Appendix 5

Original reprint of the publication included in Chapter 4

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

ENVIRONMENTAL MICROBIOLOGY

Nutrient Level Determines Biofilm Characteristics and Subsequent Impact on Microbial Corrosion and Biocide Effectiveness

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ABSTRACT The impact that nutrient level has on biofilm characteristics, biocide effectiveness, and the associated risk of microbiologically influenced corrosion (MIC) was assessed using multispecies biofilms from two different oilfield consortia. A range of microbiological, microscopy, and corrosion methods demonstrated that the continuous flow of nutrients for the microbial growth resulted in higher activity, thickness, and robustness of the biofilms formed on carbon steel, which induced greater localized corrosion compared to biofilms formed under batch, nutrient-depleted conditions. Despite of the differences in biofilm characteristics, biofilms displayed comparable susceptibilities to glutaraldehyde biocide, with similar $\log_{10}$ reductions and percent reductions of microorganisms under both nutrient conditions. Nevertheless, nutrient replenishment impacted the effectiveness of the biocide in controlling microbial populations; a higher concentration of cells survived the biocide treatment in biofilms formed under a continuous flow of nutrients. Complementary DNA-/RNA-based amplicon sequencing and bioinformatics analysis were used to discriminate the active within the total populations in biofilms established at the different nutrient conditions and allowed the identification of the microbial species that remained active despite nutrient depletion and biocide treatment. Detection of persistent active microorganisms after exposure to glutaraldehyde, regardless of biofilm structure, suggested the presence of microorganisms less susceptible to this biocide and highlighted the importance of monitoring active microbial species for the early detection of biocide resistance in oil production facilities.

IMPORTANCE Microbiologically influenced corrosion (MIC) is a complex process that generates economic losses to the industry every year. Corrosion must be managed to prevent a loss of containment of produced fluids to the external environment. MIC management includes the identification of assets with higher MIC risk, which could be influenced by nutrient levels in the system. Assessing biofilms under different nutrient conditions is essential for understanding the impact of flow regime on microbial communities and the subsequent impact on microbial corrosion and on the effectiveness of biocide treatment. This investigation simulates closely oil production systems, which contain piping sections exposed to continuous flow and sections that remain stagnant for long periods. Therefore, the results reported here are useful for MIC management and prevention. Moreover, the complementary methodological approach applied in this investigation highlighted the importance of implementing RNA-based methods for better identification of active microorganisms that survive stress conditions in oil systems.

KEYWORDS carbon steel, microbiologically influenced corrosion, nutrient level, biofilms, microbial activity, biocide, 16S rRNA

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Microorganisms have a natural tendency to attach to wet surfaces producing extracellular polymeric substances (EPS) that initiate or enhance the attachment process. Surface colonization is followed by the formation of biofilm structures that provide several ecological advantages to the microorganisms over planktonic growth (1). Sessile microorganisms have greater access to nutrients and other resources accumulating at surfaces, superior environmental stability, enhanced opportunities for interactions such as horizontal gene transfer and cometabolism, and protection from physical disturbances and antimicrobial compounds (2). Microorganisms can attach to different surfaces, organic and inorganic, and in distinct environmental conditions. Hence, in industrial assets, biofilms have been found on a wide range of metals and alloys, including carbon steel (3), stainless steel (4), aluminum (5), and copper (6), among others. The metabolic activities of the microorganisms attached to the metallic surfaces can alter the physical and chemical properties of these materials, changing their susceptibility to corrosion (7). This process where microorganisms modify the kinetics of corrosion reactions is known as microbiologically influenced corrosion (MIC) (8). Microorganisms can initiate, accelerate, or facilitate corrosion reactions by several mechanisms, including the formation of concentration cells, the production of corrosive metabolites, the dissolution of protective layers, the uptake of electrons directly from the metal, and the production of layers that are not protective (9). Most MIC is localized in nature occurring at the substratum-electrolyte interphase, where deep penetration into the base metal is observed (10). This phenomenon generates economic losses principally to the oil and gas industry, which have been estimated in billions of dollars per year in the United States alone (11). In addition to material deterioration, microorganisms forming biofilms may cause other issues, such as biological fouling, heat transfer loss, and a decrease in product quality in several industries.

Biofilms on metallic surfaces are mainly composed of microbial cells that are packed with EPS, corrosion products, nucleic acids, traces of inorganic minerals, and some organics adsorbed from the bulk fluid. Biofilms are heterogeneous with microcolonies of cells separated by interstitial channels, voids, and pores that allow diffusion and transport of energy sources and waste products (12, 13). This heterogeneity influences biofilm activity by its effect on the rates of nutrient transport and consumption (14). Likewise, the structure and morphology of biofilms can vary with the growth rate of microorganisms and the types and levels of energy sources available (13). The physiology of sessile cells is particularly complex and is markedly different from those growing free in the bulk fluid (planktonic). Moreover, the physiological status of the microorganisms conforming the biofilm is diverse and is determined by the location of the cells within the multiple layers of the biofilm (15). It has been proposed that microorganisms located in the upper regions of the biofilm are more active due to greater accessibility to nutrients and greater ease of discharging of metabolic waste products compared to cells at the bottom of the biofilm (15).

In oil production facilities, microorganisms find sufficient essential nutrients and carbon sources, including petroleum hydrocarbons, volatile fatty acids, and other fermentation products distributed across the production system through produced water (16). Nonetheless, design features and operational conditions in these facilities may create zones with different flow regimes, such as dead legs, that limit the availability of such nutrients (17). Higher flow velocity can provide an increased supply of nutrients for biofilms formed on metal surfaces, but it may also result in biofilm removal (13, 18). In contrast, the sections of equipment and piping that remain stagnant for long periods during operation allow microorganisms to settle but also provide less flow of nutrients. Environments with an abundant supply of nutrients would be expected to favor attachment and biofilm formation; however, previous studies have shown a greater tendency to form biofilms under low-nutrient or starvation conditions (18). Likewise, in cases of electrical MIC, microorganisms have been shown to switch from soluble electron donors to metallic iron as the only energy source to carry out microbial activities under starving conditions (19). This mechanism has been shown to lead to higher corrosion rates in the laboratory (20). However, under

these conditions biofilms are known to operate under stress and are unable to multiply because metal does not provide the carbon that cells need for growth (20). Although under starvation cells can scavenge nutrients from the EPS and dead cells, reductions in cell counts are frequently observed when carbon sources are depleted (19, 21). In the field, MIC has been mainly associated with low velocity or infrequently flooded systems, dead legs and pipelines with solid deposit accumulation that cannot be adequately cleaned. The increased MIC risk in these areas has been related to the more favorable conditions for microbial settlement and biofilm formation (22); nonetheless, the availability of nutrients is expected to play a significant role in biofilm activity and the resulting MIC rates. However, the relationship between nutrient availability and the risk of MIC remains undefined for stagnant areas. Since the majority of MIC found in oilfields is attributed to corrosive metabolites (23), it is implied that the presence of nutrients in the system that allow microorganisms to carry out metabolic activities and proliferate is critical for catalytic MIC to occur.

In addition to the effect of nutrients on MIC, it is also unclear whether the biofilm physiological status plays a role in the effectiveness of biocide treatments used to prevent MIC. It has been reported that the restriction of essential nutrients to microorganisms can markedly affect their susceptibility to antimicrobial substances (24, 25). Several researchers have argued that actively growing cells are more susceptible to antibiotics than those cells that are slowly or not actively growing. The greater antimicrobial resistance in such cells within the biofilm has been attributed to the less-permeable membranes present in slow-growing cells (15). Moreover, biofilm structure can also play an important role in biocide efficacy by affecting the diffusion of antimicrobial compounds through biofilm layers and reducing the concentration of antimicrobial compounds reaching the cells at the bottom of biofilms. Therefore, these cells will be exposed to sublethal concentrations of biocidal compounds and will have sufficient time to switch on the expression of antimicrobial-resistant factors and antimicrobial-degrading enzymes (26). Hence, it is important to identify the environmental stresses that are likely to influence the physiology of the microorganisms in industrial systems to develop better approaches and practices to prevent the pervasive and detrimental corrosion caused by biofilms.

This investigation was conducted to identify changes in biofilms in response to nutrient level and to examine how these changes influence the severity of MIC and the effectiveness of glutaraldehyde, a chemical biocide commonly used to prevent MIC in the industry. Considering that natural and industrial environments are colonized by multispecies consortia and that multispecies biofilms have been poorly studied so far, this study was conducted using multispecies biofilms from microbial consortia recovered from oilfields. Two microbial consortia from different oilfields were exposed to the same conditions separately to determine whether different microbial communities will display similar response to the nutrient regime. The variation in the microbial composition of biofilms on carbon steel was studied by complementary DNA-/RNA-based amplicon sequencing from the 16S rRNA gene and transcripts, as used elsewhere (27). The physiological status of the microbial communities was assessed by the estimation of the adenylate energy charge (AEC), which provided information about the metabolic state of the cells before and after biocide evaluation. Biofilm morphology and thickness were studied via visible-light, confocal, and scanning electron microscopy analyses, and the results were correlated with corrosion rates. Dissimilarities found in the biofilms developed on metals exposed to different nutrient regimes provided valuable information about the variation expected in microbial communities throughout oil production facilities, which should be accounted for when assessing the risk of MIC and for establishing MIC mitigation and monitoring strategies.

RESULTS

Effect of nutrient replenishment on biofilm characteristics. (i) Biofilm microscopy analysis. Field emission scanning electron microscopy (FESEM) morphological views of the biofilms formed by both microbial consortia over carbon steel samples

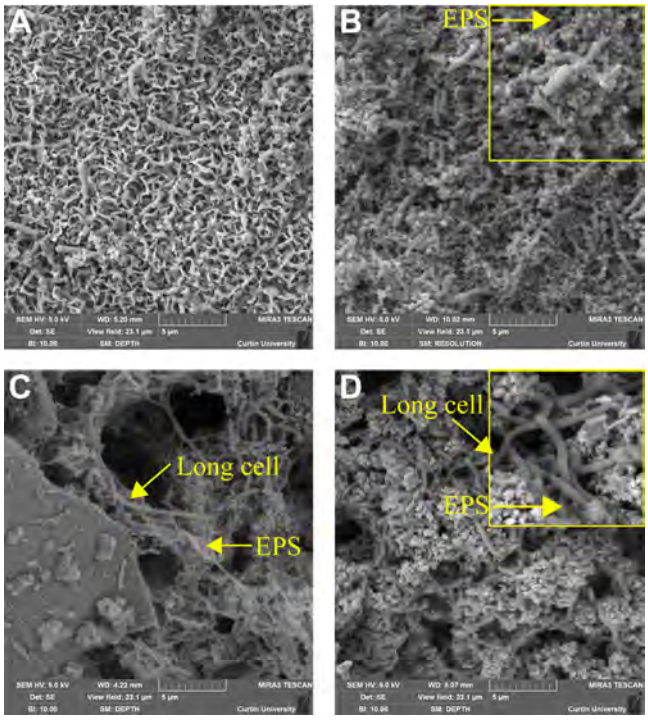

FIG 1 FESEM views of the biofilms/corrosion products formed over carbon steel coupons at different nutrient conditions. (A) Consortium 1 under batch conditions; (B) consortium 1 with continuous replenishment; (C) consortium 2 under batch conditions; (D) consortium 2 with continuous replenishment.

exposed to different nutrient conditions are shown in Fig. 1. Biofilms grown under batch conditions contained fewer cells (Fig. 1A and C) than biofilms grown under a continuous flow of nutrients (Fig. 1B and D). The same result was obtained for the two microbial consortia evaluated. A particular pattern in biofilm distribution was observed in microbial consortium 1. Cells exposed to the batch conditions were embedded in a thin layer of corrosion products; no EPS-like structures were evident on the surface (Fig. 1A). In contrast, biofilms formed by the same consortium under continuous nutrient replenishment exhibited microbial cells interconnected by organic structures compatible with pili filaments or EPS (Fig. 1B). Biofilms formed by microbial consortium 2 were denser in corrosion products and cells than the biofilm of consortium 1 at each nutrient condition. Different from consortium 1, cells in the biofilms formed under batch conditions for consortium 2 were embedded on EPS-like structures (Fig. 1C), which were also evident in the biofilms under continuous replenishment. As expected, dissimilarities in the morphology of microbial cells were also evidenced using FESEM analysis. The presence of long rod cells was detected in microbial consortium 2 (Fig. 1C and D), a morphology that was not visualized in microbial consortium 1, which indicated differences in microbial composition between consortia.

Figure 2 shows the confocal laser scanning microscopy (CLSM) images of the biofilms with differentiation of live and dead cells by the fluorescent dye. This analysis revealed differences in the structure and distribution of live and dead cells in the biofilms as a result of nutrient replenishment. As observed in the FESEM analysis, denser biofilms were observed on steel coupons exposed to a continuous replenishment of nutrients for both consortia (Fig. 2B and D); z-stack images indicated that thicker biofilms were formed under these conditions than under batch conditions (Fig. 2A and C). Likewise, live and dead staining indicated that biofilms grown under batch conditions had a higher density of dead cells, a difference that was more marked in biofilms formed by microbial consortium 1. It was also noticed that, for both consortia, thicker

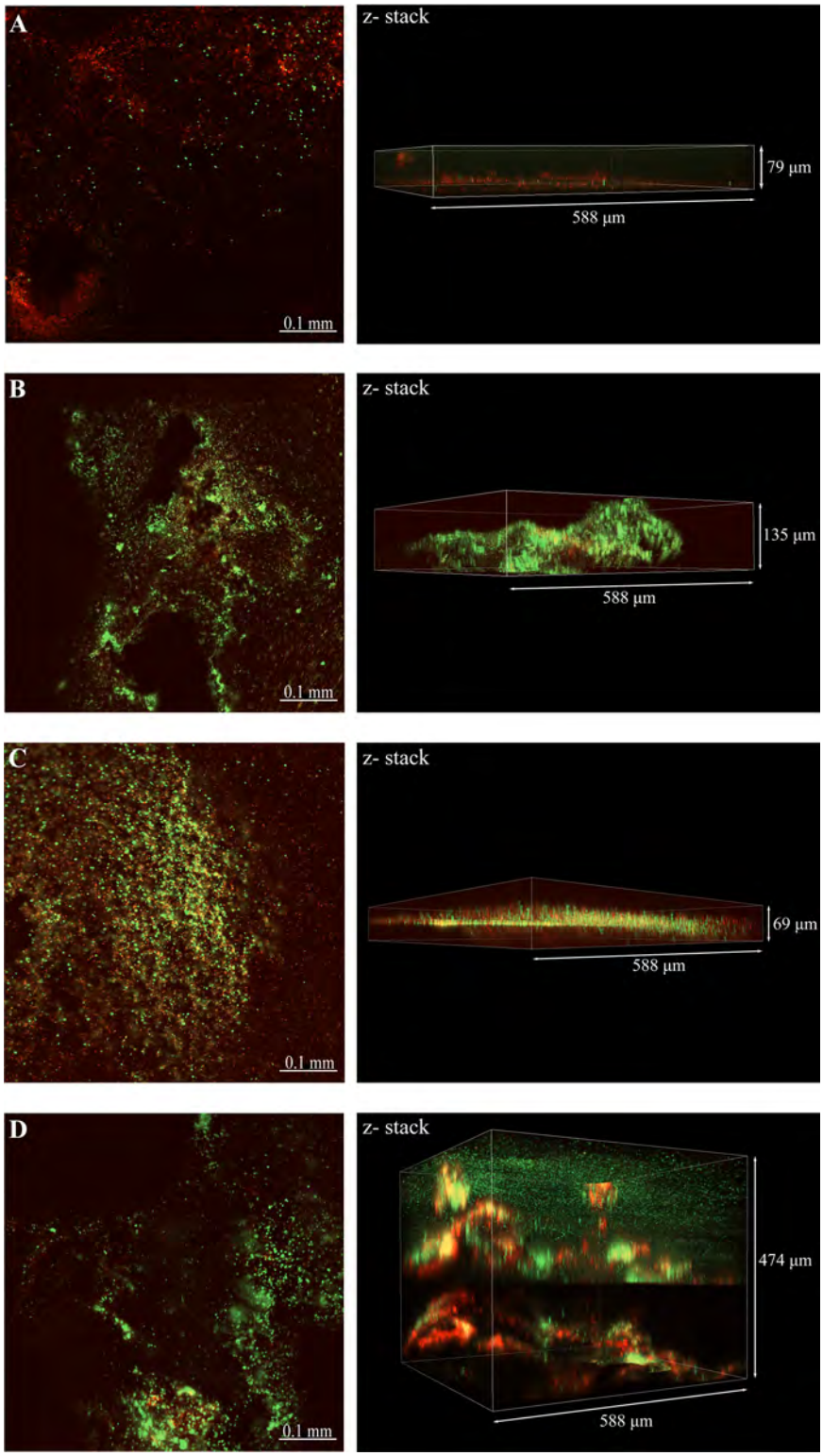

FIG 2 CLSM images of biofilms formed over carbon steel coupons at different nutrient conditions. (A) Consortium 1 under batch conditions; (B) consortium 1 with continuous replenishment; (C) consortium 2 under batch conditions; (D) consortium 2 with continuous replenishment. Live cells, green stain; dead cells, red stain.

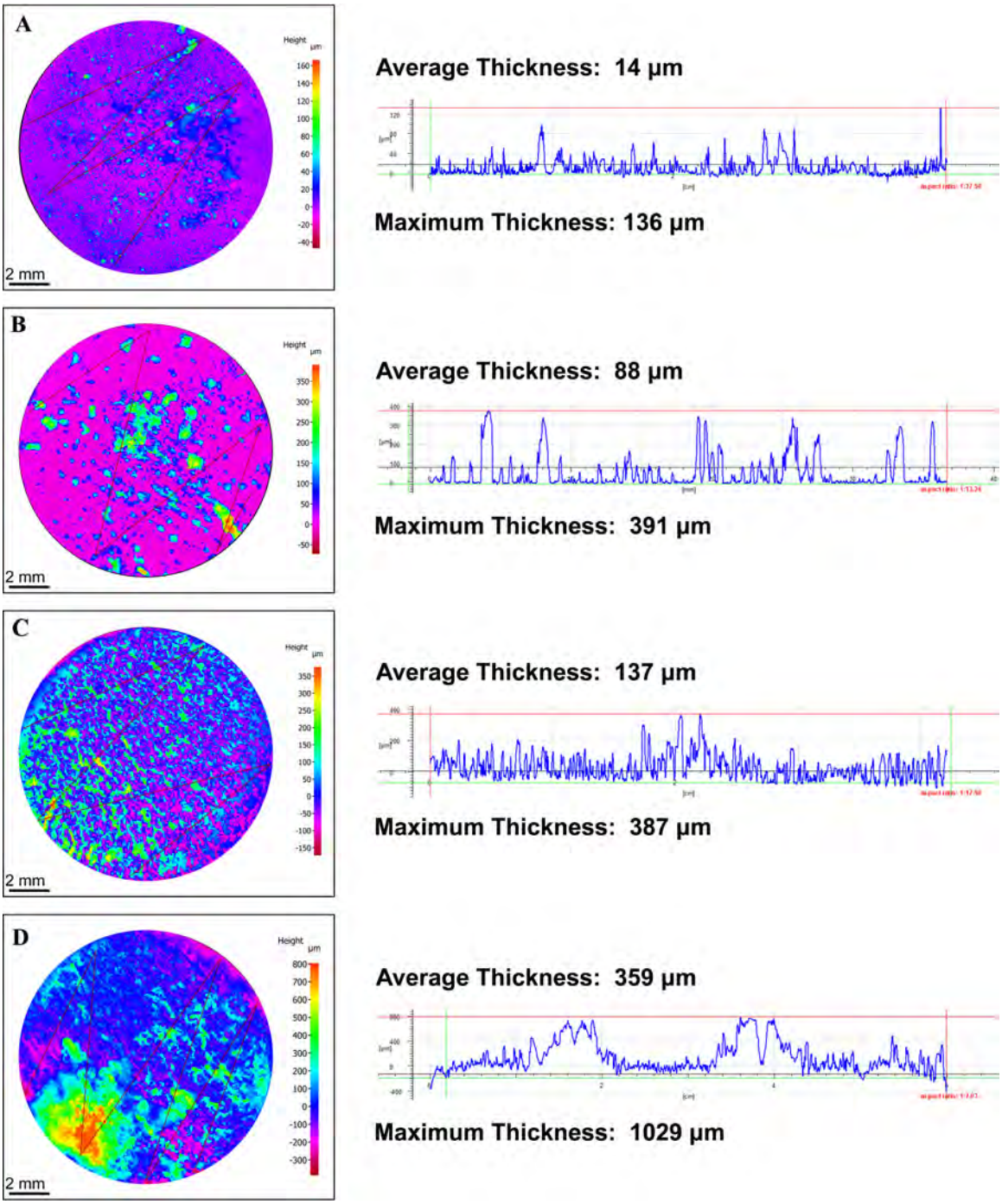

FIG 3 3D reconstruction of the corrosion products and biofilm profiles formed over the surface of carbon steel coupons under different nutrient conditions. (A) Consortium 1 under batch conditions; (B) consortium 1 with continuous replenishment; (C) consortium 2 under batch conditions; (D) consortium 2 with continuous replenishment.

biofilms formed under continuous replenishment of nutrients had a higher concentration of dead cells at the bottom layers of the biofilm than at the top layers.

The heterogeneous distribution of the biofilms over the metal surface did not allow us to conduct measurements of the maximum biofilm thickness using CLSM analysis. Therefore, the thickness of wet biofilms was not determined. Three-dimensional (3D) reconstruction of the corrosion products and biofilm profiles (dried samples) was performed with a 3D profilometer microscope; the images are shown in Fig. 3. This

TABLE 1 Most probable numbers of viable cells in biofilm communities exposed to different nutrient conditions[a]

Consortium	Condition	MPN SPP (cells/cm^2)	95% confidence limit		MPN ANA (cells/cm^2)	95% confidence limit	
			Lower	Higher		Lower	Higher
1	Batch	1.50E+05	3.70E+04	6.30E+07	7.49E+09	1.70E+09	1.50E+12
	Continuous	4.60E+06	9.00E+05	9.20E+09	2.10E+11	4.00E+10	9.03E+13
2	Batch	2.40E+05	4.20E+04	2.40E+08	1.50E+09	3.70E+08	6.30E+11
	Continuous	1.10E+10	1.80E+09	4.51E+13	>1.1E+12	4.20E+11	

[a]Most probable numbers (MPN) were determined from three-tube serial dilutions. SPP, sulfide-producing prokaryotes; ANA, total anaerobic microorganisms.

analysis revealed differences in the distribution of the corrosion products and biofilms formed over the metal surface by each microbial consortium. The results of the 3D analysis confirmed that consortium 2 created thicker biofilms that, together with corrosion products, covered the complete surface of the steel samples (Fig. 3C and D). In contrast, consortium 1 formed thinner biofilms that were irregularly distributed over the metal surface (Fig. 3A and B). Differences in biofilm thickness formed by the same consortia but under different nutrient conditions were also confirmed via 3D profiling; thicker biofilms were seen in reactors with a continuous replenishment of nutrients (Fig. 3B and D). The same pattern was observed for the two microbial consortia evaluated.

(ii) Microbial concentration. Most probable numbers (MPNs) of biofilm microbial communities revealed that the continuous replenishment of nutrients stimulated biomass production and biofilm growth, with 1 to 3 orders of magnitude more cells compared to batch conditions (Table 1). Similar results were obtained for the two microbial consortia evaluated. The concentration of sulfide-producing prokaryotes (SPP) was significantly higher in the biofilms formed by consortium 2 under nutrient replenishment, with 4 orders of magnitude more cells than the biofilm formed by consortium 1 under the same conditions. Microorganisms in consortium 2 were recovered from the floating production storage and offloading (FPSO) with high sulfate concentration in the produced water, which could explain the greater numbers of microorganisms with the capability of using sulfate for energy acquisition. Enumeration of planktonic cells showed that the concentration of SPP in the continuous reactors was lower than in the batch reactors (see Table S4 in the supplemental material), which could be related to the contribution of nutrients to the synergistic or antagonistic interactions among species within biofilms. The accurate concentration of anaerobic cells in planktonic communities could not be determined since the highest serial dilution vials showed positive growth.

(iii) Microbial activity. ATP, ADP, and AMP concentrations and the corresponding energy charge measured in the biofilm communities are presented in Table 2. The results indicate that biofilms grown under batch conditions had lower concentrations of ATP, ADP, and AMP than biofilms exposed to continuous replenishment of nutrients, which was correlated with the lower biomass found under batch conditions. Statistical

TABLE 2 Average adenosine nucleotide concentrations and adenylate energy charges of the biofilm communities grown under different nutrient conditions

Parameter	Avg ± SD[a]			
	Consortium 1		Consortium 2	
	Batch	Continuous	Batch	Continuous
ATP (pg/cm^2)	765 ± 520	21,523 ± 19,098	634 ± 359	81,594 ± 38,026
ADP (pg/cm^2)	896 ± 651	17,362 ± 10,781	157 ± 128	82,181 ± 28,115
AMP (pg/cm^2)	1,269 ± 925	4,057 ± 289	357 ± 175	33,520 ± 3,526
Total adenylates (ng/cm^2)	2.9 ± 2.1	42.9 ± 3.0	1.1 ± 0.6	197.3 ± 69
AEC	0.49 ± 0.13	0.67 ± 0.08	0.61 ± 0.21	0.61 ± 0.05
% ATP	26 ± 11.3	50 ± 9.9	54 ± 27.8	40 ± 5.1
% ADP	31 ± 0.7	40 ± 3.9	15 ± 14.0	42 ± 1.4
% AMP	43 ± 13.5	10 ± 6.0	31 ± 14.9	18 ± 4.2

[a]Errors represent standard deviations from three independent replicates.

analysis revealed that differences in the adenosine nucleotide concentrations between batch and continuous replenishment biofilms were significant ($P \leq 0.05$; see Table S5 in the supplemental material). Similar results were obtained for both microbial consortia evaluated. Analysis of the physiological status of the biofilms indicated that microbial communities were under stress conditions (Table 2); all of the microbial populations had an AEC below 0.75. Greater stress was observed in the biofilm formed under batch conditions by consortium 1 with AEC below 0.5. Differences in the relative proportion of the three nucleotides were observed in the biofilms as a result of nutrient availability. Higher AMP proportions were measured in the biofilms formed by both consortia under batch conditions, suggesting a major proportion of dormant cells under this condition. Differences in the ATP proportion between batch and continuous replenishment were not consistent in both consortia. Consortium 1 showed a lower ATP proportion under batch conditions than under continuous replenishment, whereas consortium 2 showed the opposite pattern. It should be noted that even though consortium 2 exhibited a higher ATP proportion when exposed to batch conditions than when exposed to continuous replenishment conditions, the total proportion of ATP and ADP in the continuous reactor was higher than that in the batch reactor, indicating more stored energy in reactors with replenishment of nutrients, as seen for consortium 1.

The concentration of the adenosine nucleotides in planktonic cells showed a pattern similar to that observed in sessile populations; higher concentrations of ATP, ADP, and AMP were observed in cells exposed to a continuous replenishment of nutrients (see Table S6 in the supplemental material). The AEC values of planktonic cells in batch reactors of both consortia was below 0.5, which indicated that microorganisms were under severe stress. In contrast, planktonic cells in reactors with continuous replenishment had higher AEC; however, as detected in the biofilm communities, AEC values were still below 0.75. It was noticed that planktonic communities had a higher concentration of adenosine nucleotides than biofilm communities in both consortia, indicating higher cell numbers in an active state in the test solution compared to microbial cells attached to the metal surface.

(iv) Biofilm community composition. Total and active microorganisms in biofilms grown under different nutrient conditions were identified through DNA and RNA-based amplicon sequencing. A total of 1,705,081 high-quality sequences were obtained after bioinformatics processing of the raw reads. These sequences were taxonomically classified into 29 microbial genera. Only the genera with relative abundances of $\geq$1% are presented in Fig. 4. The complete list of biofilm microbial composition is presented in Table S7 in the supplemental material. Molecular identification of the microorganisms showed that both biofilms exhibited similar microbial populations, with variations related to the presence of methanogenic species in consortium 1 that were not detected in the consortium 2. Differences in the relative abundances of dominant genera and in the composition of the low-abundance microorganisms were also noted. The results of the alpha diversity analysis indicated that biofilms grown under continuous replenishment of nutrients were more diverse (Table S8), suggesting a more complex community when a higher level of nutrients was available in the reactor.

Comparison of the DNA and RNA profiles showed differences in the relative abundances of the total and active populations in each consortium under the conditions evaluated, which were also reflected in the NMDS ordination analysis (Fig. 5). Total communities in microbial consortium 1 were dominated by *Desulfovibrio* spp. (80 and 63%, batch and continuous mode, respectively), whereas, in the active communities this genus had a lower relative abundance (20 and 37%, batch and continuous mode, respectively). Other genera showed the opposite pattern, i.e., lower abundances in the total community and higher abundances in the active community. Substantial differences in the biofilm microbial composition as a result of nutrients were observed in consortium 1. For this consortium, the genera *Thioalbus*, *Methanobacterium*, and *Methanoculleus* were only detected abundantly in the communities formed under batch

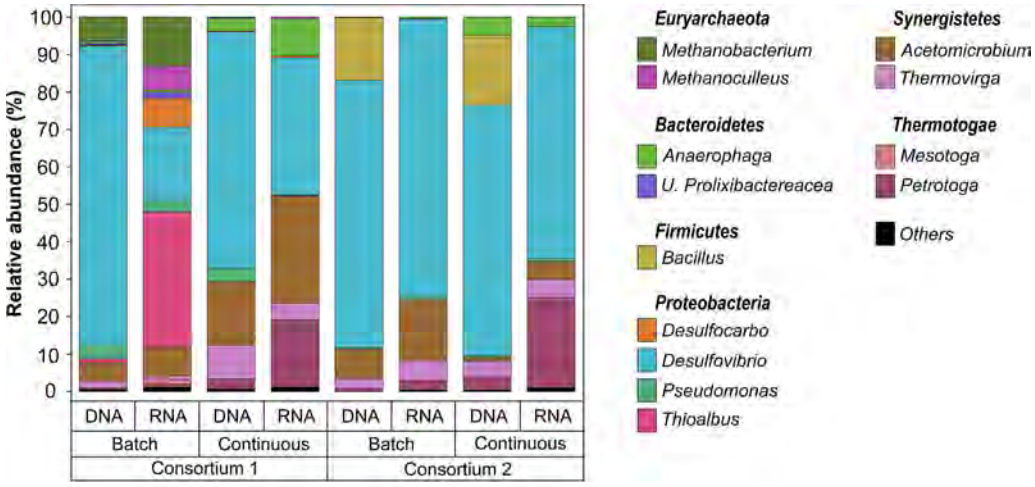

FIG 4 Total (DNA-based) and active (RNA-based) microbial community composition of biofilms exposed to different nutrient conditions. The results show the mean relative abundances of microbial communities classified at the genus level from 16S rRNA sequencing ($n = 2$). Genera with relative abundances lower than 1% in all samples were summarized in the artificial group "Others."

conditions. These microorganisms, together with *Desulfovibrio*, *Acetomicrobium*, and *Desulfocarbo*, were the most dominant genus in the active community. In contrast, the active biofilm community formed under continuous mode was dominated by *Desulfovibrio*, *Acetomicrobium*, *Petrotoga*, and *Anaerophaga*. Total and active biofilm communities in consortium 2 under both conditions were equally dominated by *Desulfovibrio*. The most considerable difference between total and active communities was observed in the relative abundances of the genera *Bacillus*, *Acetomicrobium*, and *Petrotoga*. The last two showed a higher abundance in the active communities, a pattern similar to that observed in microbial consortium 1. In contrast, *Bacillus* that was one of the dominant populations in the total communities of both conditions was not detected abundantly in the active communities. The main difference in the biofilm composition of consortium 2 as a result of the nutrient level was associated with the increase in the relative abundance of the genera *Petrotoga* and *Anaerophaga* in the continuously replenished reactor, a pattern similar to that observed in microbial consortium 1.

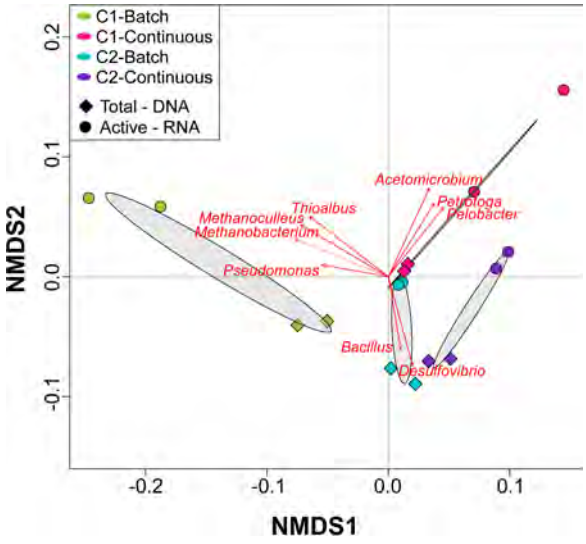

FIG 5 NMDS of total and active microbial communities in biofilms exposed to different nutrient conditions. Dominant microorganisms that were significantly correlated ($P \leq 0.05$) to microbial community structure are indicated by arrows.

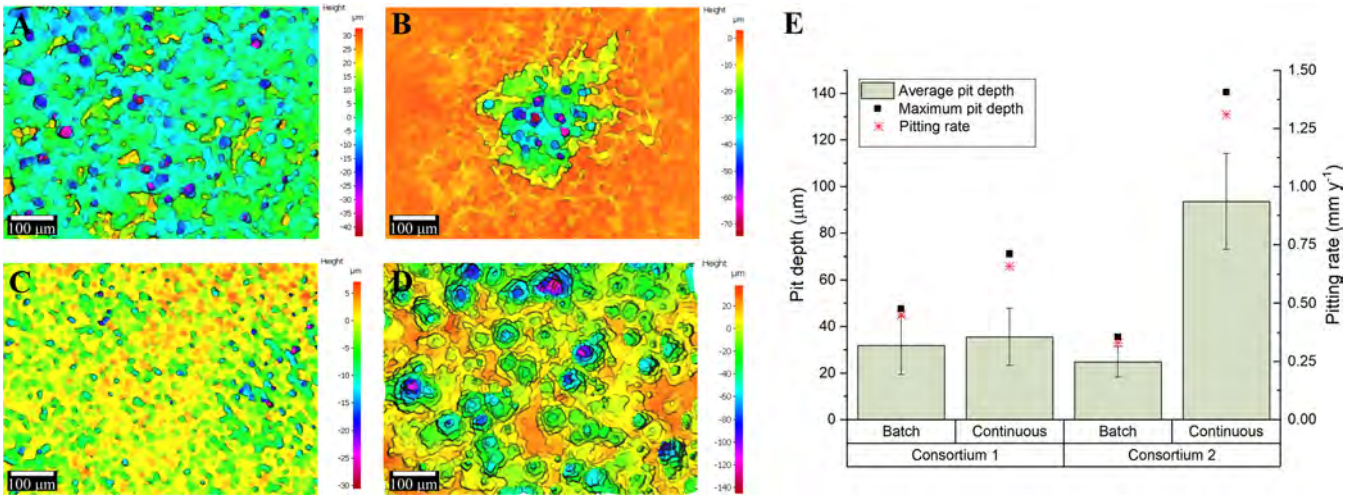

FIG 6 Localized corrosion analysis of carbon steel coupons exposed to different nutrient conditions. (A to D) 3D optical microscope surface images of carbon steel coupons. (A) Consortium 1 under batch conditions; (B) consortium 1 with continuous replenishment; (C) consortium 2 under batch conditions; (D) consortium 2 with continuous replenishment. (E) Average pit depths and pitting rates calculated from the maximum pit depth. Error bars represent standard deviations of the 10 deepest pits found in three independent replicates.

Effect of nutrient replenishment on microbial corrosion. (i) Corrosion measurements. Surface profilometry analysis revealed that biofilms from the two different consortia triggered localized corrosion under both nutrient conditions (Fig. 6). The average pit depth (calculated from the ten deepest pits found in the three coupons analyzed from each reactor), the maximum pit depths, and the pitting rates are shown in Fig. 6E. For the two consortia, deeper pits were found in reactors with a continuous replenishment of nutrients than in reactors under batch conditions; however, the difference in the pit depth according to the nutrient level was only significant for consortium 2 ($P \leq 0.05$; Table S5). The extent of MIC was different for each consortium; under batch conditions consortium 1 caused higher pitting rates than did consortium 2, whereas under a continuous replenishment of nutrients consortium 2 resulted in more localized corrosion compared to consortium 1. The abiotic reactor did not show evidence of localized corrosion damage (pits < 10 μm) (see Fig. S1 in the supplemental material), which indicated that the surface deterioration observed in the metal samples was the result of microbial activity. The average corrosion rates calculated from the coupon's mass loss are presented in Table S9. The results showed a different trend in both consortia. Microorganisms of consortium 2 induced higher general corrosion rates when continuous replenishment of nutrients was established. In contrast, microorganisms of consortium 1 triggered similar general corrosion rates independently of nutrient levels.

(ii) Cross-sectional analysis of corrosion products. Cross-sectional images of corrosion products and biofilms attached to the metal samples also showed differences in distribution and thickness of biofilms in response to nutrient conditions (Fig. S2). Likewise, corroded areas under the biofilms were examined by cross-sectional analysis. It was seen that coupons exposed to the microbial consortium 2 exhibited greater corroded areas than coupons exposed to consortium 1 under both nutrient conditions, which was supported by the higher mass loss evidenced in the coupons exposed to consortium 2 (Table S9). The elemental maps of the cross-sectional surface analysis are shown in Fig. 7. The major elements detected in coupons exposed to all conditions were iron, sulfur, and oxygen. In the coupons exposed to microbial consortium 2, phosphorous and calcium were also detected (Fig. S3). Corroded areas of all coupons were mainly covered by Fe and O, whereas the top layers were mainly composed of Fe and S. Layered images of coupons exposed to microbial consortium 2 showed a higher concentration of phosphorous in the top layers of the coupon exposed to batch conditions compared to coupons exposed to the continuous flow of nutrients; this

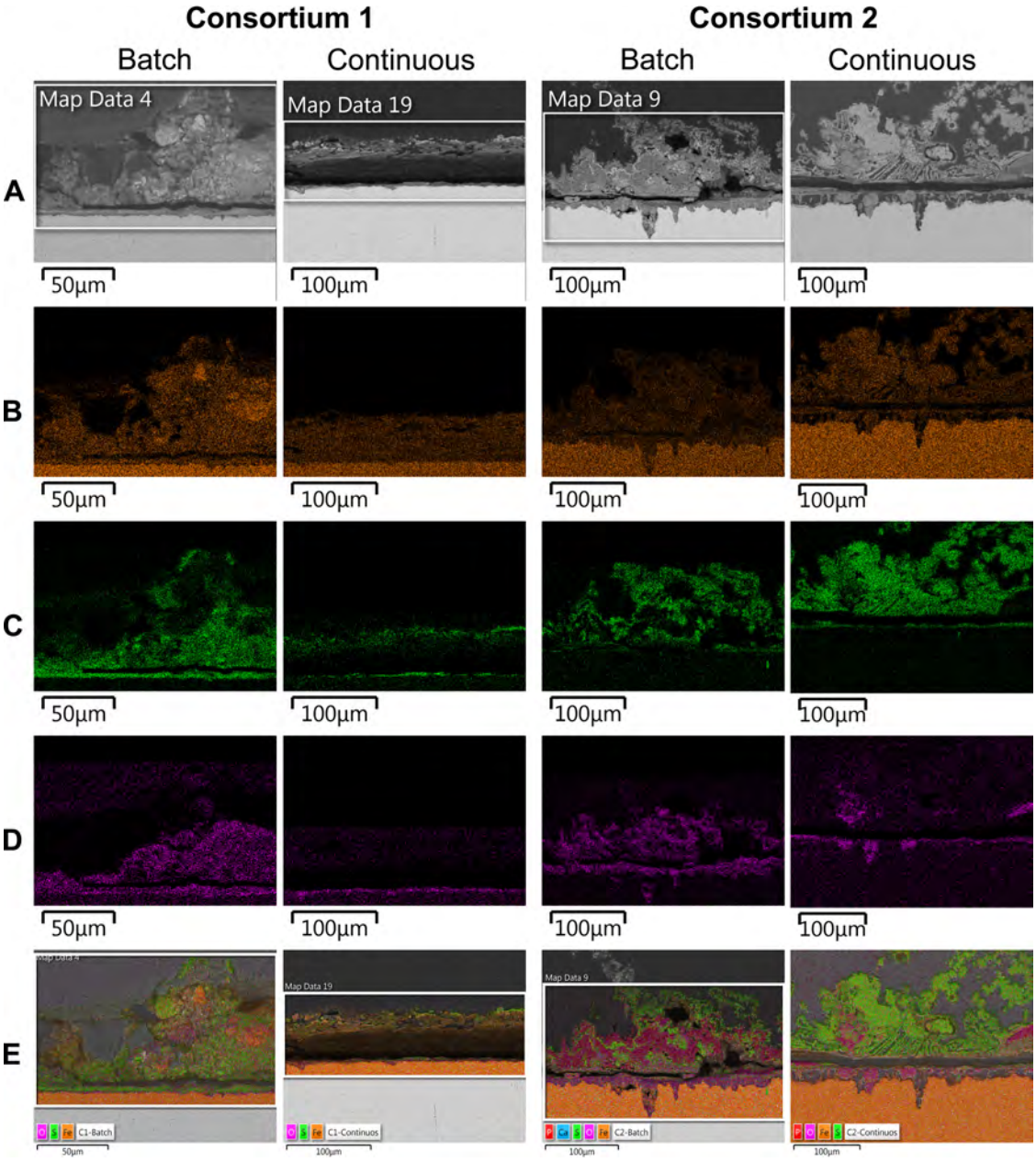

FIG 7 EDS-elemental mapping of cross-sectioned coupons exposed to different nutrient conditions. (A) SEM image; (B) iron map; (C) sulfur map, (D) oxygen map; (E) combined elemental map.

element was mapped in same areas with oxygen. A carbon distribution map could not be obtained due to the carbon-based nature of the epoxy resin used for mounting of the samples.

Effect of nutrient replenishment on biocide effectiveness. (i) Microbial concentration. The microbial concentrations of SPP and other anaerobic microorganisms in the biofilms after the biocide treatment are presented in Table 3. The results show that glutaraldehyde treatment reduced considerably the concentration of viable microorganisms in biofilms before the treatment, killing 99.99% of the initial population with maximum $\log_{10}$ reduction of 8 orders under all conditions (Table 4). Similar results were observed for both consortia and both nutrient conditions. However, differences in the number of viable cells remaining after biocide treatment were observed based on nutrient conditions. Biofilms formed in batch reactors showed a lower number of viable

TABLE 3 Most probable numbers of viable cells for biofilm communities after biocide treatment[a]

Consortium	Condition	MPN SPP (cells/cm^2)	95% confidence limit		MPN ANA (cells/cm^2)	95% confidence limit	
			Lower	Higher		Lower	Higher
1	Batch	3.60E+00	1.70E−01	6.48E+01	3.94E+01	9.00E+00	7.74E+03
	Continuous	2.80E+02	8.70E+01	2.63E+04	1.22E+03	3.70E+02	6.30E+05
2	Batch	< 3		9.50E+00	7.40E+00	1.30E+00	1.48E+02
	Continuous	2.80E+02	8.70E+01	2.63E+04	4.32E+03	9.00E+02	9.20E+06

[a]The MPNs were determined from three-tube serial dilutions. SPP, sulfide-producing prokaryotes; ANA, total anaerobic microorganisms.

residual cells than biofilms formed under continuous nutrient replenishment (Table 3), which indicated that higher biocide effectiveness in controlling the biofilm populations was achieved under nutrient-depleted conditions. The same results were obtained from both microbial consortia.

(ii) Microbial activity. The ATP concentration in all biofilms was reduced after exposure to glutaraldehyde biocide (Table 5). A higher percentage of reduction in ATP levels was observed in biofilms grown under continuous replenishment of nutrients (72 and 84%, consortia 1 and 2, respectively) than in those under batch conditions (27 and 29%, consortia 1 and 2, respectively). As expected, the total concentration of adenosine nucleotides was reduced with the biocide treatment, which was likely related to the release of these molecules during the cell membrane lysis of the microorganisms killed by the treatment. Measurements of the AEC in biofilms after biocide treatment were below 0.5 in all communities, indicating that on average, microorganisms that remained viable in biofilms were senescent or severely stressed by the presence of the biocide. Similar results were observed for both nutrient conditions, although it was noticed that the AEC of the biofilm of consortium 1 formed under batch conditions, which was already under severe stress before the treatment, was not considerably changed by biocide treatment. It was seen that biocide exposure caused an increase in the proportion of AMP in the biofilms formed under nutrient replenishment, whereas this proportion was reduced in the biofilms formed under batch, nutrient-depleted conditions; same results were observed for both consortia. Changes in the AMP proportion are associated with variation in dormancy and/or stress-responsive processes within microbial populations; therefore, the disparity observed in the variation on the proportion of this molecule between nutrient conditions suggested an impact of the nutrient availability in the population affected by the biocide.

(iii) Biofilm community composition. A total of 1,351,660 high-quality sequences were obtained after bioinformatics processing of the raw reads from the DNA and RNA-based amplicon sequencing of biofilms after biocide. These sequences were taxonomically classified into 52 microbial genera. Only the genera with relative abundances of ≥1% are presented in Fig. 8. Molecular identification of the microorganisms showed that biocide shifted the relative abundances of the predominant populations in the biofilm communities, causing changes in the community structure (Fig. 9). More microbial genera were detected in the biofilm communities after biocide treatment, which might be related to the reduction in the cell concentration of dominant species

TABLE 4 Log_{10} reductions and percent reductions for biofilm communities after biocide treatment[a]

Consortium	Condition	Log_{10} reduction		% reduction	
		SPP	ANA	SPP	ANA
1	Batch	4.62	8.24	99.99	99.99
	Continuous	4.22	8.15	99.99	99.99
2	Batch	5.38	8.31	99.99	99.99
	Continuous	7.59	8.48	99.99	99.99

[a]SPP, sulfide-producing prokaryotes; ANA, total anaerobic microorganisms.

TABLE 5 Average adenosine nucleotide concentrations and adenylate energy charges of biofilm communities after biocide treatment

	Avg ± SD[a]			
	Consortium 1		Consortium 2	
Parameter	Batch	Continuous	Batch	Continuous
ATP (pg/cm^2)	560 ± 109	3,425 ± 472	451 ± 54	22,521 ± 2,008
ADP (pg/cm^2)	1,132 ± 513	10,500 ± 1,484	1,285 ± 132	64,410 ± 9,353
AMP (pg/cm^2)	726 ± 457	3,654 ± 714	644 ± 109	48,978 ± 5,221
Total adenylates (ng/cm^2)	2.4 ± 1.0	17.6 ± 2.6	2.4 ± 0.1	135.9 ± 16.5
AEC	0.48 ± 0.05	0.49 ± 0.01	0.46 ± 0.03	0.49 ± 0.003
% ATP	24 ± 1.5	19 ± 0.4	19 ± 1.9	17 ± 0.7
% ADP	47 ± 0.4	60 ± 1.3	54 ± 2.9	47 ± 1.2
% AMP	29 ± 1.1	21 ± 1.1	27 ± 4.7	36 ± 0.6

[a]Errors represent standard deviations from three independent replicates.

susceptible to the treatment, allowing the detection of more species with lower abundance (rare taxa) in the biofilm. The proportion of the genera *Desulfovibrio*, *Acetomicrobium*, *Thioalbus*, and *Thermovirga* in the total and active communities was reduced after glutaraldehyde exposure, indicating susceptibility of these populations to the chemical. An opposite pattern was observed for the genera *Petrotoga*, *Methanobacterium*, *Methanoculleus*, *Pseudomonas*, and *Bacillus*, which showed higher relative abundances, especially in the active communities after biocide treatment. These findings suggested that these populations were less susceptible to biocide treatment. It is important to clarify that a higher relative abundance after chemical treatment does not mean proliferation or growth of these populations during biocide exposure; increases are likely related to changes in the proportions of dominant microorganisms in the remaining viable cells due to the lysis of other dominant species that were more susceptible to the treatment. In addition, microbial composition analysis revealed that the reduction in the relative abundance of the *Desulfovibrio* genus in the active communities was greater in biofilms formed under batch conditions than in biofilms formed in the nutrient replenishment scenario. Likewise, it was noticed that the increase in the relative abundance of *Pseudomonas* and *Petrotoga* in the active community after biocide injection was associated with nutrient conditions. *Pseudomonas* abundance increased more significantly in biofilms formed in batch reactors, whereas *Petrotoga* showed higher abundance in biofilms formed under continuous

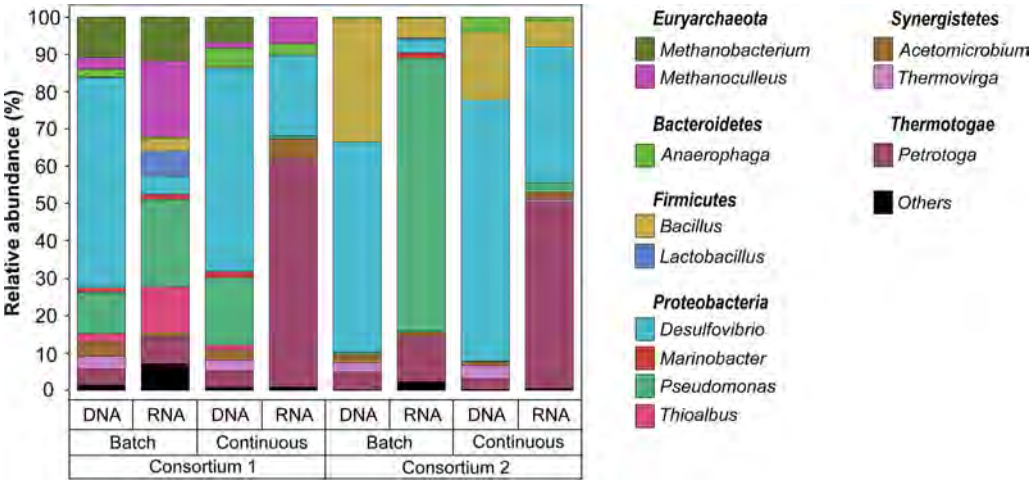

FIG 8 Total (DNA-based) and active (RNA-based) microbial community composition of biofilms after biocide treatment. The results show the mean relative abundances of microbial communities classified at the genus level from 16S rRNA sequencing ($n = 2$). Genera with relative abundances lower than 1% in all samples were summarized in the artificial group "Others."

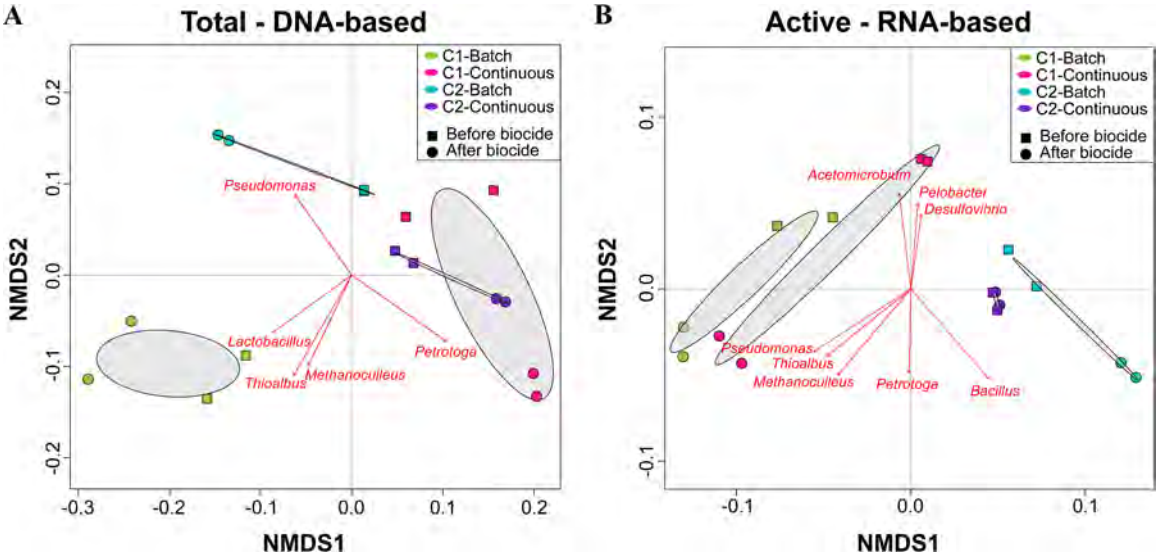

FIG 9 NMDS of total (A) and active (B) microbial communities after biocide treatment. Dominant microorganisms that were significantly correlated ($P \leq 0.05$) to microbial community structure are indicated by arrows.

nutrient replenishment. These findings may indicate that nutrient replenishment could have played a role in the survival of these microorganisms despite biocide treatment.

DISCUSSION

We evaluated here the impact that nutrients have on biofilm characteristics and the subsequent impact on carbon steel corrosion and biocide effectiveness. Biofilms formed by two different microbial consortia from different oilfield production facilities were used to assess whether a similar response to nutrient level can be expected from different microbial communities. Assessing the impact of dynamic flow on biofilm activity and the resulting effectiveness of biocide treatments is important in order to understand the risk of MIC through oil production facilities, which contain piping sections exposed to continuous operational flow (nutrient replenishment) and sections that remain stagnant for long periods of time.

The results of this investigation demonstrated that biofilm morphology, thickness, biomass, activity, and composition are determined by the availability of nutrients in the environment. It was shown that both microbial consortia produced thinner biofilms with lower biomass content under batch conditions compared to biofilms formed under continuous replenishment of nutrients (Fig. 1). Less-dense biofilms observed in batch reactors are the result of exposing the microorganisms to an environment limited in essential nutrients necessary for cell growth. Moreover, a relationship between nutrient level and microbial corrosivity was found; nutrient replenishment resulted in more corrosive conditions and the formation of more corrosion products, which contributed to biofilm thickness.

ATP concentration in biofilms was significantly affected by nutrient availability; replenishment led to an increase in microbial activity and viability. However, AEC measurements carried out to determine the physiological status of biofilms indicated that all biofilm communities, regardless of the nutrient conditions, were under some level of stress. In batch reactors, stress can be directly associated with a lack of nutrients. However, for reactors operating under continuous replenishment, stress was inferred to be associated with limited diffusion of nutrients and waste accumulation due to biofilm thickness. CLSM analysis (Fig. 2) showed that such biofilms had a high concentration of dead cells in areas close to the metal surface, indicating that the cells at the bottom layers of biofilms corresponded to the stressed portion of the biofilms. It has been reported that cells in the deepest areas of the biofilm are exposed to more nutrient-depleted conditions compared to upper layers of the biofilm due to the

diffusion barrier generated by EPS and other biofilm components (15, 28), as well as the consumption of nutrient during the metabolic activities carry out by cells at the periphery of the biofilm (29). EPS-like structures that could have generated this diffusion barrier were observed more abundantly in the metal surfaces of coupons exposed to both consortia under continuous replenishment (Fig. 1). Hence, our results support the view that a significant portion of the biofilms would experience nutrient limitation regardless of the availability of nutrients in the bulk solution.

Analysis of microbial composition and structure of biofilms showed dissimilarities in the total and active microbial communities for each microbial consortium and at different nutrient conditions. RNA-based sequencing showed that despite the limited nutrients available in the batch reactors, both consortia had microbial species that were metabolically active in the biofilms. Of particular interest is the detection of active methanogens such as *Methanobacterium* and *Methanoculleus* only in the batch reactor of consortium 1. Methanogenic species have been shown to display metabolic capabilities, including extracellular electron transfer and interspecies electron transfer under starvation conditions (30, 31). The lower abundance of these methanogenic species in biofilms formed in continuous flow reactors can be related to an inhibition by the sulfate-reducing bacterium *Desulfovibrio*, one of the dominant genera in these biofilms. Methanogens and sulfate reducers are known to compete for energy sources, with sulfate-reducing microbes outcompeting the methanogens in sulfate-rich environments (32). Like methanogens, the genus *Thioalbus* was a dominant microorganism only detected in biofilms formed in batch conditions of consortium 1. *Thioalbus* are chemolithoautotrophic microorganisms that can use inorganic compounds such as sulfur or thiosulphate to obtain energy and bicarbonate as a carbon source (33); these metabolic capabilities could explain their dominance in the batch reactor. Conversely, fermenting microorganisms showed an opposite pattern; the genera *Acetomicrobium*, *Petrotoga*, *Anaerophaga*, and *Thermovirga* increased their abundances in the continuously replenished reactors, which indicated that these populations were favored by the availability of nutrients, most likely organic sources needed to conduct fermentation. Substantial differences observed in the beta diversity of active communities in biofilms (Fig. 5), which were based on nutrient availability pointed out an expected shift in biofilm community composition along oil production facilities in response to flow regime.

Influence of nutrient level on the severity of microbial corrosion. Experimental results demonstrated that MIC was directly correlated with nutrient availability. Biofilms with a permanent flow of nutrients triggered higher pitting rates compared to biofilms with a nutrient-limited environment. Same corrosion behavior was observed for the two microbial consortia, which provided strong support to the findings of this investigation. These results are consistent with previous studies with single-species biofilms of *Desulfovibrio desulfuricans* (34). The authors of that study found that *D. desulfuricans* in nutrient-replenished reactors decreased the polarization resistance and accelerated corrosion rates to a greater extent than did the microorganisms in batch reactors. Similarly, Javed and coworkers (35) evaluated the difference in the corrosivity of *Escherichia coli* between nutrient-replenished and static batch reactors, finding that culture medium replenishment produced an increase in microbial corrosion rates. The authors of both of these studies attributed the differences in the MIC rates to the higher number of bacteria present in the most corrosive scenario. Although the number of microorganisms has been demonstrated to influence the severity of MIC (36), we attribute the differences in the microbial corrosivity to the higher number of cells in an active state, measured as the ATP concentration, under continuous replenishment of nutrients. Biofilms in continuously replenished reactors exhibited 28 times (consortium 1) and 129 times (consortium 2) more ATP than biofilms in batch mode reactors. Moreover, the severity of the corrosion was correlated with the ATP concentration; the highest pitting rate and localized corrosion were observed in the coupons exposed to the most active biofilm community (consortium 2, continuous), and the lowest pitting

rate was observed in the coupons exposed to the less active biofilm community (consortium 2, batch).

The impact of microbial activity on the pitting rates found in this study is supported by the data from the microbiological characterization of biofilms. For example, the microbial compositions of the active populations in consortium 2 biofilms under both nutrient conditions were similar, although at different relative abundances, indicating that differences in the corrosivity were not related to specific microbial species present but to the level of metabolic activity carried out by these active populations. Microbial consortium 2 was constituted by similar relative proportions of sulfate-reducing bacteria (74 and 62%) and sulfidogenic fermenters (24 and 34%) under both nutrient conditions, and yet the corrosion rates were markedly different. Instead, corrosion rates were more likely to be influenced by the increased production of corrosive metabolites when nutrients were more available. Nonetheless, it is important to note that even for biofilms formed under nutrient replenishment, the majority of cells in close contact with the metal surface remained under stressed conditions, which suggests a possible nutrient limitation and accumulation of waste products due to the biofilm thickness. Waste accumulation in a sulfidogenic-fermentative biofilm can lead to the generation of concentration cells of sulfides and acidic species which are corrosive to the metal. In addition, species from the genus *Desulfovibrio*, which was the predominant microorganism in all biofilms regardless of the nutrient conditions, are very versatile and can use several molecules as energy source, including metallic iron under starvation conditions (20, 37). Biofilm communities with higher metabolic states compared to the planktonic communities in a batch reactor exposed to consortium 2 suggests that microorganisms in the biofilms used the metal, corrosion products, or biofilm components for their metabolic activities. When iron oxidation is coupled to the sulfate reduction, sulfate-reducing bacteria can accelerate anodic and cathodic reactions, causing electrical MIC (EMIC) and chemical MIC (CMIC) at the same time (38). Hence, the deterioration evidenced in the carbon steel samples could have been the result of different corrosion mechanisms that took place by the activity of microorganisms attached to the metal surfaces.

Moreover, SEM-EDS analysis demonstrated the presence of at least three different types of corrosion products. Areas with a bigger concentration of Fe and S indicated the presence of iron sulfides, suggesting that MIC was likely to be driven by biogenic H_2S production carried out by sulfate, thiosulphate, and sulfur reducing microorganisms, which were all found active in biofilms. Corroded areas in the coupons contained a greater concentration of Fe and O, indicating the presence of iron oxides and probably iron carbonates, which could be related to the activity of the fermenting microorganisms (*Petrotoga*, *Thermovirga*, and *Acetomicrobium*) (39) that can create microenvironments with acidic conditions due to the release of organic acids and CO_2 during metabolic activities. Apart from these two different areas, coupons exposed to consortium 2 showed areas with Fe, P, and O, suggesting the presence of iron phosphides. One of the mechanisms previously related to the activity of sulfate-reducing bacteria is associated with the production of extremely corrosive phosphorus compounds through phosphate reduction (40). Phosphine is one of the reported phosphorous compounds generated during the biotic reduction of phosphate, which can lead to the production of iron phosphide as a corrosion product. Although this corrosion process is controversial, these elements were identified in the corroded areas of the metals exposed to consortium 2 that had a dominance of the *Desulfovibrio* genus in the biofilms.

The lower pitting rates evidenced in the nutrient-limited environment were attributed to low microbial activity and the lack of electron donors and carbon sources but also to the deficiency of electron acceptors that could have restricted the use of the metal as a source of electrons. Xu and Gu (20) demonstrated that a carbon source reduction of 100% reduced carbon steel corrosion rates caused by *Desulfovibrio*, which was attributed to the formation of a weak biofilm under these conditions. However, other studies have shown that most corrosive scenarios can be achieved when there is a total reduction of carbon sources, which allegedly triggered an electrical MIC mech-

anism (21). Nevertheless, such experiments were carried out for very short periods of exposure, only 7 days, which may not provide enough time to observe a reduction in the metabolic status of the microorganisms and consequent reduction in corrosion rates with time in the nutrient-depleted scenarios. In addition, these studies are typically assessed under a limitation of electron donors but not of electron acceptors, which does not represent any industry setting since equipment exposed to prolonged starvation (or stagnation) is typically depleted of all essential nutrients, including electron acceptors.

The results presented here suggest that facilities experiencing long periods of stagnation will limit metabolic activity in biofilms which will result in lower MIC rates compared to flow conditions experiencing nutrient replenishment. Reactors with a continuous flow of nutrients are the practical equivalent of piping in which produced fluids are normally flowing. The greatest localized corrosion found under these conditions suggests that MIC failures are most likely to occur when a higher level of nutrients is available unless the microbial activity is controlled by routine biocide treatments. Nevertheless, it has to be highlighted that even though batch reactors showed a reduced pitting rate compared to the continuous reactors, the pitting rates evidenced in batch reactors (0.5 and 0.33 mm year^{-1}) were still categorized as severe according to the NACE standard practice SP0775 (41). Batch reactors are the equivalent of piping that is only in occasional service, such as dead legs. Lower pitting rates under these conditions indicate that MIC failures would take longer to occur than under the equivalent flowing conditions provided the flow is below the critical flow for detachment.

Effect of nutrient replenishment on biocide effectiveness against biofilms. Biocide treatment is the most commonly used and effective mitigation strategy to prevent MIC. However, the ability of microorganisms to respond and adapt to harsh environments (42) typically results in the emergence of tolerance and the resistance of species to a variety of antimicrobial substances (43). It has been reported that the metabolic state of microorganisms can determine their response and susceptibility to antimicrobial substances; therefore, understanding how oilfield communities respond to biocide treatment when exposed to different nutrient conditions is essential for the optimization of chemical treatments.

This investigation showed that biofilms exposed to continuous replenishment of nutrients displayed a bigger percentage of reduction in microbial activity when exposed to biocide, measured as the ATP concentration, compared to biofilms formed under batch conditions. However, the microbial log reduction and the percent reduction estimation indicated that glutaraldehyde biocide had similar efficacy in biofilm communities exposed to different nutrient conditions. Hence, a greater reduction in ATP concentration observed in the biofilms of continuous reactors was associated with a greater reduction in the number of viable microorganisms under this condition, since these were present in greater numbers before the treatment. Although differences in the susceptibility of biofilms to biocide treatment based on nutrient conditions were not observed, we show here that biocide effectiveness against biofilms (measured as the number of cells that survived the treatment) was affected by nutrient conditions. According to a common industry practice, a bacterial growth threshold of 1,000 CFU/ml, mainly measured as planktonic cells, is used to classify oil systems under control for MIC (44). Differences in the number of cells between planktonic and sessile communities have been discussed by other researchers (45); hence, microbial control in this investigation was only measured in sessile populations. None of the biofilm communities in the continuously replenished reactors was below this threshold after the mitigation treatment. In contrast, reactors under batch conditions had a cell concentration considered "under control." This suggests that biofilm regrowth after the chemical treatment in systems with continuous replenishment of nutrients will take place more easily due to the higher number of viable cells remaining in biofilms and the availability of essential nutrients for microbial growth.

Detection of viable microorganisms in biofilm communities after treatment with glutaraldehyde at a concentration where planktonic cells showed complete inhibition highlighted the importance of studying biofilms when assessing biocide effectiveness and mitigation treatments. The increased tolerance of biofilm communities to biocide compounds has been associated with a number of properties found in biofilms (46). Among them, the diffusion barrier generated by the EPS layers can prevent biocides from reaching microorganisms at the bottom of biofilms (47). Moreover, the reaction of the antimicrobial molecules with EPS components during biocide penetration decreases the concentration of the chemical that reaches microorganisms within the biofilm, which are then exposed to sublethal concentrations of the biocide (47). Biocide sublethal concentrations can lead to the survival of exposed cells and the development of antimicrobial resistance (43). Hence, we propose that differences in the effectiveness of glutaraldehyde against biofilms formed under a continuous flow of nutrients could have been related to the presence of thicker biofilms that protected a major proportion of biofilm cells, which might not have been exposed to an inhibitory concentration of the chemical.

The effectiveness of an antimicrobial substance is also highly dependent on the type of microorganism, i.e., the genus and species and sometimes even the strain (32). The persistence of microorganisms after their contact with antimicrobial substances has been associated with the presence of resistance mechanisms that can be either intrinsic or acquired (46). Although it is not possible with the results available to confirm the presence of resistance mechanisms in the surviving species, the identification of the same active genera after biocide treatment in all reactors regardless of nutrient conditions suggests that these microorganisms are less susceptible to glutaraldehyde than other genera that showed a marked decrease in relative abundance in biofilms.

Glutaraldehyde is an electrophilic biocide that reacts with thiol and secondary amine groups in proteins present in the outer layers of bacterial cell walls, leading to cell wall damage and cytoplasmic coagulation (48). This biocide has a broad spectrum of activity against bacteria, but to our knowledge, there are no studies of its efficacy in archaea. The differences in the cell wall and cell membrane between archaea and bacteria have been used to explain the ineffectiveness of some antibiotics against archaea (49). This could also be the reason for the detection of active methanogens in the community that survived the treatment. Likewise, it has been reported that Gram-positive bacteria are less susceptible than Gram-negative bacteria to biocides similar to glutaraldehyde (50), which could explain the presence of active *Bacillus* and *Lactobacillus* in biofilms after the treatment. Specific resistance mechanisms for *Petrotoga* have not been described; however, *Petrotoga* has been detected in gas formations treated with glutaraldehyde (51). This bacterium has an outer-sheath-like structure that might provide physical characteristics that confers resistance to biocides (52). The resistance of some *Pseudomonas* species to glutaraldehyde has been related to the presence of genetic mechanisms, specifically, the expression of efflux pumps and the induction of modulators of biofilm formation (53). Efflux pumps can be induced when microorganisms are under stress conditions, which contributes to higher bacterial resistance of stressed populations to antimicrobial substances and could be the reason for the greater increase in the relative abundance of *Pseudomonas* in biofilms formed in batch reactors. Although physical characteristics have been the primary mechanisms described for glutaraldehyde resistance in bacterial biofilms (53), future studies are needed to identify whether the microorganisms that survived the treatment have resistance mechanisms that make them less susceptible to glutaraldehyde or whether a diffusion barrier prevented their contact with lethal doses of the chemical.

Conclusions. This investigation demonstrated that the nutrient level affects biofilm characteristics and the resulting corrosive behavior and biocide effectiveness. Microorganisms grown under nutrient replenishment conditions produced a higher sessile biomass and created thicker biofilms. The constant flow of nutrients allowed biofilms to be more active and cause greater localized corrosion. Higher corrosivity was attributed

to the presence of nutrients that allowed microorganisms to remain metabolically active during the experimental period, potentially constantly producing corrosive metabolites compared to biofilms formed under nutrient-limited environment. The experimental results also demonstrated differences in the biocide effectiveness at controlling microbial populations; more microorganisms survived the glutaraldehyde treatment in the continuously replenished reactors, which was associated with thicker biofilms established under this condition. Biofilm layers can generate a diffusion barrier that prevents that biocide reaches the microorganisms at the bottom of the biofilm. Finally, the complementary DNA-/RNA-based profiling revealed changes in the total and active microbial community composition and structure of biofilms as a result of nutrient level and biocide treatment and also suggested the presence of microbial species that are less susceptible to glutaraldehyde in the oilfield consortia evaluated.

MATERIALS AND METHODS

Oilfield microbial consortia. Microbial consortia used in this study were recovered from two different floating production storage and offloading (FPSO) facilities located on the Australian North West Shelf. The oilfields were selected based on their substantial differences in the produced water chemical composition, aiming to recover microorganisms living under different nutritional conditions. The main difference in the chemistry between the two produced waters was the sulfate and sulfur content, which were absent in produced water from FPSO 1 but present in considerable concentrations in water from FPSO 2. In addition, the produced water from FPSO 2 exhibited a higher concentration of essential nutrients such as phosphorus, nitrogen, and total organic carbon compared to the produced water from FPSO 1. The chemical composition of the produced water from the two FPSOs is presented in the Table S1 in the supplemental material. Cultivable microorganisms recovered from produced water in these facilities were grown in the laboratory at 40°C employing different culture media to maximize the recovery of the diverse microbial populations. The culture media targeted different populations, including sulfate- and thiosulfate-reducing bacteria, methanogenic archaea, acid-producing bacteria, iron-reducing bacteria, and iron-oxidizing bacteria, separately. The composition of the culture medium is presented in Table S2. After 28 days of incubation, culture media that exhibited positive growth were mixed in equal proportions for the establishment of one microbial consortium from each FPSO.

Evaluation of the effect of nutrient replenishment on biofilm characteristics. (i) Sample preparation. Carbon steel round coupons of 1.27-cm^2 exposed surface area were used for biofilm formation. Steel used had the following elemental composition (weight %): C (0.43 to 0.5), Mn (0.6 to 0.9), Si (0.15 to 0.35), S (0.01 to 0.35), P (0 to 0.035), Cr (0 to 0.40), and Fe (balance). Samples were electrocoated with a protective epoxy (Powercron 6000CX; PPG Industrial coatings) to limit the working surface to only one side of the coupons. The working surface in each coupon was wet ground to a 600-grit finish using silicon carbide paper. The samples were then washed with Milli-Q water, degreased with acetone, washed with ethanol, and dried with nitrogen gas. Before immersion, the samples were sterilized by a 15-min exposure to UV radiation.

(ii) Test conditions. To determine the effect of nutrient level on biofilm characteristics, sterile coupons were fixed in reactors and exposed separately to both microbial consortia for 40 days. Four anaerobic CDC biofilm reactors (Biosurface Technologies Corporation) operating in batch or continuous mode were used for the experiments. Anaerobic conditions were maintained throughout the test by continuous sparging of a gas mixture of 20% CO_2 and 80% N_2. Synthetic produced water supplemented with nutrients was used as a test solution for the growth of the consortia. The test solution had the following composition: 1.4 mM $CaCl_2{\cdot}2H_2O$, 1.5 mM $MgCl_2{\cdot}6H_2O$, 2 mM K_2HPO_4, 1.7 mM KH_2PO_4, 410 mM NaCl, 5 mM NH_4Cl, 59 mM $NaHCO_3$, 8 mM Na_2SO_4, 4 mM $Na_2S_2O_3{\cdot}5H_2O$, 10 mM sodium pyruvate, 30 mM sodium formate, 6 mM sodium lactate, 20 mM sodium acetate, and 10 ml/liter of vitamin solution and trace elements solution (German Type Culture Collection [DSMZ], medium 141). The pH of the test solution was adjusted to 7.3 $\pm$ 0.2 with deoxygenated $NaHCO_3$ solution (100 mM). Agitation of the baffle in each reactor was set to 50 rpm to maintain a homogeneous solution throughout the test. The temperature in each reactor was set to 40 $\pm$ 1°C. Reactors were inoculated with the microbial consortia at using concentrations of 10^6 cells/ml. All reactors were operated in a batch mode for the first 72 h to allow settlement and facilitate biofilm formation. After this period, two reactors were switched to continuous flow of fresh test solution at a rate of 0.21 ml min^{-1}, which replaced 50% of the total volume (600 ml) of test solution daily. The other two reactors remained under batch mode, i.e., without nutrient replenishment, for the duration of the test.

(iii) Microscopic examination of biofilms. *(a) Field emission scanning electron microscopy*. Biofilm morphology was examined under a Tescan Mira-3 field emission scanning electron microscope. At the completion of immersion, coupons were gently rinsed with sterile anaerobic phosphate-buffered solution (PBS), and biofilms were fixed for 22 h with a 2.5% glutaraldehyde fixative solution containing 0.15% alcian blue. After fixation, coupons were rinsed again with PBS and dehydrated using a series of ethanol gradient solutions (30, 50, 70, 80, 90, 95, and 100%) for 10 min each. Dehydrated coupons were dried under nitrogen flow for 2 days, coated with a platinum layer (5 nm thick), and stored in a vacuum desiccator until imaging. Biofilms were visualized at an emission voltage of 5 kV.

(b) Confocal laser scanning microscopy. The distribution of live and dead cells within biofilms was studied using CLSM. Coupons were gently rinsed with sterile anaerobic PBS and stained using the

FilmTracer Live/Dead biofilm viability kit (Invitrogen) according to the manufacturer's instructions. Before imaging with a Nikon A1RMP confocal and multiphoton phosphorus, coupons were rinsed with sterile deionized water to remove the excess of dyes. Images were obtained with a 20× water lens objective. The dyes used stained live cells with a green-fluorescent color (SYTO 9) and dead cells with a red color (propidium iodide). The z-stacked images were analyzed using Nikon Elements AR software.

(c) 3D optical profilometer. 3D profiling of the biofilms and corrosion products attached to the carbon steel surfaces was reconstructed with an Alicona imaging infinite focus microscope IFM G4 3.5. The images were used to assess the distribution of the biofilms over the coupons and to estimate biofilm thickness for each condition.

(iv) Microbial viability in biofilms via MPN enumeration. The numbers of sulfide-producing prokaryotes (SPP) and total anaerobic microorganisms (ANA) in biofilms were determined by a culture-dependent MPN method using culture medium SPP described in Table S2. After exposure, biofilmed coupons were gently washed with sterile anaerobic PBS to remove unattached cells and immersed in Falcon tubes containing 10 ml of PBS solution. The cells were detached from the metal by sonication as described elsewhere (54). Portions (1 ml) of PBS sonicate suspension were inoculated into 9 ml of culture media, and then 10-fold serial dilutions up to 10^{12} were performed in triplicate for the MPN estimation. Serial dilution vials were incubated at 40°C for 28 days, and positive growth was determined by visual inspection of changes in the turbidity and the color of the culture media. Positive vials were confirmed by phase-contrast microscopy (Nikon Eclipse Ci-L). The microbial concentration was determined using the three-tube standard table for MPN (55). The number of planktonic cells in the bulk fluid of the reactors was determined by the MPN method described above; in this case, 1 ml of the bulk test solution was used for each serial dilution. The detection limit of the MPN method was 3 MPN cm^{-2} for sessile cells and 0.3 MPN ml^{-1} for planktonic cells.

(v) Adenylate energy charge estimation. ATP, ADP, and AMP play an important role in the energy metabolism of all living cells. These adenosine nucleotides are linked to a chain of three, two, or one phosphate groups, respectively. The hydrolysis of the bonds between phosphate groups in these molecules provides a large amount of free energy to the microorganisms that use it to develop other cellular processes (56). The ATP content of the cell varies depending on its level of activity; actively growing cells have a higher ATP content than stressed cells. Hence, ATP concentration has been used to determine the viable biomass (57); however, the ratio of ATP, ADP, and AMP concentrations is thermodynamically more important than the absolute concentration of ATP for identifying the physiological and nutritional status of microorganisms (58). This ratio was defined as adenylate energy charge (AEC) by Atkinson and Walton (59) and corresponds to the degree to which the adenylate system is charged with donatable phosphate groups (ranging between 0 when it is all AMP and 1 when it is all ATP) (60). It is generally accepted that AEC values of >0.75 correspond to actively growing microorganisms (i.e., no stress involved), values between 0.5 and 0.75 correspond to microorganisms in a stationary growth phase (i.e., microorganisms partially stressed), and values below 0.5 correspond to senescent or dormant microorganisms (i.e., microorganisms severely stressed) (61).

The concentrations of ATP, ADP, and AMP in biofilms were determined by luminescence after reaction with luciferin-luciferase using the AXP assay and the Quench-Gone Organic Modified (QGO–M) test kits (Luminultra Technologies, Ltd.). All assays were performed according to the manufacturer's instructions. Coupons were gently rinsed with PBS and then immersed in a Falcon tube containing 10 ml of PBS solution. Coupons were then vortexed at full speed for 10 s and sonicated for 2 min to help with cell detachment. PBS containing detached cells (sessile cells) was processed with the QGO–M kit. The ATP, ADP+ATP, and AMP+ATP concentrations were determined by measuring luminescence with a PhotonMaster luminometer (Luminultra Technologies, Ltd.) before and after biocide treatment. The AEC in biofilms was calculated according to the following formula: AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP). The AEC of planktonic cells in each reactor was determined following the same method described before; in this case, 10 ml of the bulk test solution was processed with the QGO–M kit.

(vi) Microbial community composition. The microbial composition of the total and active communities in the biofilms was determined by the 16S rRNA gene and transcript sequencing. This complementary methodological approach allows identifying the relative abundances of all microorganism in the biofilm by analysis of the total DNA and the relative abundance of active microorganisms in the biofilm at the time of sampling by analysis of the RNA molecule (27). For this, six coupons from each reactor were collected, rinsed with PBS, and immersed in sterile 2:1 RNAprotect bacteria reagent (Qiagen)–PBS-Tween 20 (0.1% wt/vol). Sessile cells were detached by sonication as described elsewhere (54). Cells were harvested by centrifugation at 15,000 × *g* for 5 min at 4°C and preserved at −80°C until nucleic acid extraction.

(a) Nucleic acid extraction and cDNA synthesis. Extraction of the DNA and RNA molecules was performed with DNeasy PowerBiofilm and RNeasy PowerBiofilm kits (Qiagen), respectively, as recommended by the manufacturer, with minor modifications. Cell lysis was performed in a FastPrep-24 5G instrument at 6.5 m/s for 40 s, and nucleic acids were eluted with 60 μl of nuclease-free water. The DNA and RNA concentrations were quantified fluorometrically with the Qubit dsDNA and RNA HS assay kits (Life Technologies). Subsequently, total RNA was treated with a Turbo DNA-free kit (Invitrogen) to remove the remaining DNA. To verify the complete removal of DNA, a PCR targeting the 16S rRNA gene was performed using 27f and 1492r primers, described elsewhere (62). RNA was purified by using an RNeasy MinElute cleanup kit (Qiagen) and converted to cDNA by using a SuperScript IV first-strand synthesis system (Invitrogen).

(b) Library preparation and sequencing. Library preparation and sequencing were performed by the Australian Genome Research Facility (AGRF). Briefly, DNA and cDNA were used to amplify the V3 and V4 regions of the 16S rRNA gene by PCR. Amplicons were generated by using the primers 341F (5′-CCTA YGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (63). PCR was performed in Applied

Biosystem 384 Veriti system following standard PCR protocol in 50-μl reactions for 29 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s. PCR products were indexed in a second PCR using TaKaRa *Taq* DNA polymerase (Clontech). Reactions were purified and paired end sequenced on an Illumina MiSeq instrument with a V3 (600 cycles) kit (Illumina).

(c) Bioinformatics analysis. The Qiime2 (version 2019.4) (64) software pipeline was used for data analysis. Reads were demultiplexed and assigned to respective samples according to their barcodes by the AGRF. Next, the q2-dada2 (65) plugin was implemented for quality control, trimming, dereplication, and chimera removal. Based on the demux-summary.qzv file, forward reads were truncated at 280 bases and reverse reads were truncated at 220 bases. Afterwards, low-frequency sequences were removed by using the q2-feature-table plugin. Representative sequences were taxonomically classified using the q2-feature-classifier (66) and q2-classify-consensus-blast (67) plugins against the SILVA database version 132 (68). The alpha and beta diversity metrics were calculated using diversity q2-core-metrics-phylogenetic and q2-alpha-diversity plugins. To visualize the multivariate dispersion of the community composition under each condition, a nonmetric multidimensional scaling (NMDS) analysis was conducted employing R (v3.4.3) (69). NMDS was performed based on the weighted UniFrac distance (70); lines for joining samples collected in the same reactor were projected onto the ordination, utilizing the function ordiellipse.

Evaluation of the effect of nutrient replenishment on microbial corrosion. (i) Corrosion measurements and surface analysis. Corrosion analysis was carried out on three coupons from each reactor after 40 days of biofilm growth. Corrosion products and biofilms were removed from the surface using Clarke's solution, as described in the ASTM G1 standard (71). Corrosion rates were determined by the gravimetric technique that considers the weight loss and surface area of the metal samples (71). Surface profilometry analysis, including measurements of pit depth, was conducted using a 3D optical profilometer (Alicona imaging infinite focus microscope IFM G4 3.5). The average pit depth in each condition was obtained from the 10 deepest pits measured in each coupon. The pitting rate was calculated using the maximum pit depth found in each condition, as described in the NACE SP0775 standard practice (41). To discriminate microbial corrosion from abiotic corrosion, an additional reactor was set up and maintained under sterile conditions and surface profilometry analysis performed to obtain baseline corrosion in the absence of microorganisms.

(ii) Cross-sectional analysis of corrosion products. Cross-sectional imaging and elemental analysis of the corrosion products and biofilms were done by scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (SEM-EDS). One coupon was extracted from each reactor on day 40 and immediately placed in a glass cell for drying under nitrogen flow for 5 days. Subsequently, coupons were mounted in Epofix resin and one of the sides was dry polished to reveal the cross-sectional profile. Samples were coated with a platinum layer (5 nm thick), and surface analysis was performed using a Zeiss Neon high-resolution scanning electron microscope. Images were collected using 20 kV and the backscatter detector. Aztec 3.0 software (Oxford Instruments NanoAnalysis) was used for the data analysis.

Evaluation of the effect of nutrient replenishment on biocide effectiveness. (i) MIC. Glutaraldehyde solution grade I (25% in water) was used as the antimicrobial substance for the evaluation of biocide susceptibility in biofilms formed on carbon steel. To select the doses for the chemical treatment, an MIC test in planktonic cells was performed. Glass vials containing 50 ml of synthetic produced water (described above under "Test conditions") were supplemented with different concentrations of glutaraldehyde (0, 50, 100, 200, 400, 1,000, 2,000, and 2,500 ppm) and were inoculated with both microbial consortia at a concentration of 10^6 cells/ml. Glass vials were incubated at 40°C for 28 days, and then microbial cells were enumerated using a Neubauer counting chamber and a phase-contrast microscope (Nikon Eclipse Ci-L) at $\times$100 magnification. Complete microbial inhibition was only observed in the vials containing a concentration of 2,500 ppm of glutaraldehyde (Table S3). All analyses were performed in triplicate.

(ii) Biocide effectiveness against biofilms on carbon steel. Biofilms grown for a period of 40 days under the conditions mentioned previously were exposed to 2,500 ppm of glutaraldehyde biocide. For this, the synthetic produced water used as a test solution for microbial growth was washed out from reactors and replaced with sterile anaerobic PBS to avoid biocide degradation upon contact with planktonic cells and metabolic products in the test solution and also to the prevent growth of biofilm cells during biocide exposure. A concentration of 2,500 ppm of glutaraldehyde in PBS was added to each reactor operating at 40°C for a total of 4 h of contact time. After this period, biocide-treated biofilms were analyzed for viability, metabolic activity, and microbial composition, as detailed above. Cell enumeration was used to evaluate the efficacy of the treatment under each condition. The $\log_{10}$ reduction and the percent reduction of microbial cells were calculated according to the ASTM standard E2315 (72).

Statistical analysis. Statistical differences in the mean concentration of adenosine nucleotides found in biofilms and pit depths found in the coupons exposed to different nutrient conditions were analyzed by using PAST (v3) (73) software. The results of statistical tests were considered significant if the *P* value was $\leq$0.05. The statistical analyses implemented depended on the normality of the data in each variable. The homogeneity of variance was determined by the Shapiro-Wilk test (74). To test differences in variables with normal distribution, analysis of variance, followed by Tukey's multiple comparisons (75), was implemented. For variables with a nonnormal distribution, the Kruskal-Wallis test (76) was applied.

Data availability. The 16S rRNA sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive under BioProject number PRJNA57919.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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Appendix 6

Original reprint of the publications included in Chapter 5

SCIENTIFIC REPORTS
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Carbon steel corrosion by bacteria from failed seal rings at an offshore facility

Silvia J. Salgar-Chaparro[1], Adam Darwin[2], Anna H. Kaksonen[3] & Laura L. Machuca[1]✉

Corrosion of carbon steel by microorganisms recovered from corroded seal rings at an offshore floating production facility was investigated. Microbial diversity profiling revealed that communities in all sampled seal rings were dominated by *Pseudomonas* genus. Nine bacterial species, *Pseudomonas aeruginosa* CCC-IOB1, *Pseudomonas balearica* CCC-IOB3, *Pseudomonas stutzeri* CCC-IOB10, *Citrobacter youngae* CCC-IOB9, *Petrotoga mobilis* CCC-SPP15, *Enterobacter roggenkampii* CCC-SPP14, *Enterobacter cloacae* CCC-APB1, *Cronobacter sakazakii* CCC-APB3, and *Shewanella chilikensis* CCC-APB5 were isolated from corrosion products and identified based on 16S rRNA gene sequence. Corrosion rates induced by the individual isolates were evaluated in artificial seawater using short term immersion experiments at 40 °C under anaerobic conditions. *P. balearica*, *E. roggenkampii*, and *S. chilikensis*, which have not been associated with microbiologically influenced corrosion before, were further investigated at longer exposure times to better understand their effects on corrosion of carbon steel, using a combination of microbiological and surface analysis techniques. The results demonstrated that all bacterial isolates triggered general and localised corrosion of carbon steel. Differences observed in the surface deterioration pattern by the different bacterial isolates indicated variations in the corrosion reactions and mechanisms promoted by each isolate.

Corrosion is a ubiquitous problem that affects almost all industrial sectors including oil and gas production, transportation and refining facilities[1,2], mining[3], marine engineering and shipping[4,5], industrial water systems[6], food processing plants[7], nuclear industries[8], among others. This phenomenon occurs via electrochemical reactions, where electrons are released from the metal at anodic sites and are gained at cathodic sites[9]. Although assessment of the cost of corrosion is difficult, the NACE International IMPACT study estimated the global cost of corrosion as US$2.5 trillion in 2013[10]. Microbiologically influenced corrosion (MIC) has been estimated to contribute at least 20% to 40% of the total corrosion costs[11,12]. The loss of integrity of industrial infrastructure can result in substantial economic, environmental, health, safety and technological consequences[13].

MIC is a type of corrosion in which the deterioration of metals occurs due to the presence and activity of microorganisms[14]. Microorganisms initiate, facilitate or accelerate corrosion reactions by altering the electrochemical conditions in the metal-solution interface[15]. Compared to other forms of corrosion, MIC is highly unpredictable and occurs at rates as high as 10 mm year^{-1}[16]. Early detection of MIC is difficult due to its localised nature and the wide range of environmental conditions and associated microorganisms[17]. MIC has been proposed as the cause of failure in many significant incidents in the hydrocarbon industry such as the propane tank leak and explosion in Umm Said NGL Plant (Qatar)[18], the natural gas leak and explosion in Carlsbad (New Mexico)[19,20], and the oil spill in the Prudhoe Bay oil field (Alaska North Slope)[21].

Microorganisms promote corrosion through their metabolic activities, and several mechanisms have been proposed in the literature to explain MIC. The main mechanisms include the formation of concentration cells, the production of corrosive metabolites, the dissolution of protective films, the creation of unprotective surface layers, and the uptake of electrons directly from the metal[22]. Various microorganisms have been implicated in MIC; these are commonly categorised in groups according to their metabolic capabilities. Main groups related with MIC include sulphate-reducing bacteria and archaea[23,24], thiosulphate-reducing bacteria[25], acid-producing bacteria[26], iron-oxidising bacteria[27], iron-reducing bacteria[28], nitrate-reducing bacteria[29], and methanogenic

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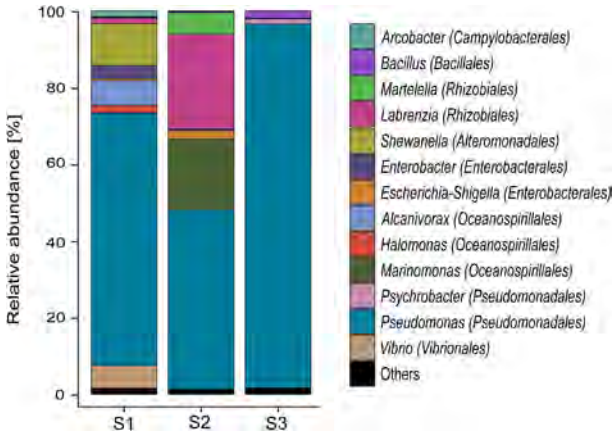

Figure 1. Microbial community composition of failed seal rings at the genus level based on partial 16S rRNA gene sequencing. Phylogenetic order is indicated in parentheses. Genera with relative abundances lower than 1% in all samples were summarised in the artificial group "Others". S1: Sample 1; S2: Sample 2; S3: Sample 3. Figure generated using ggplot2 version 3.1.0 (R package). https://cran.r-project.org/web/packages/ggplot2/.

archaea[30]. Therefore, MIC is a complex phenomenon that can be triggered by several microorganisms with different metabolic capabilities.

Prevention of MIC in the industry requires the control of the growth and activity of microorganisms, which, provided the right environmental conditions exist, can thrive and initiate or accelerate corrosion of equipment and pipelines. Biocide treatment is the traditional method used in the oil and gas industry to mitigate MIC. However, there remain challenges associated with MIC mitigation, mainly due to the formation of recalcitrant biofilms, where antimicrobial compounds are known to be less effective[31]. Lower biocide efficacy has been attributed to the diffusion barrier generated by the biofilm structure, which results in cells close to the metal surface being exposed to sublethal biocide concentrations and the consequent development of biofilm resistance[32]. When biofilms have formed, MIC mitigation becomes a challenge to corrosion engineers; corrosion failures due to MIC can occur despite the application of biocide treatment[33–36].

Although MIC has been studied for many decades[37], fundamental knowledge gaps persist in the understanding of the processes leading to MIC on steel materials. Several laboratory studies have included corrosion experiments with reference strains[38,39], or strains isolated from various environments[25,40]. However, only a few corrosion studies have been conducted using microorganisms recovered from corroded equipment. Since microorganisms are characterised by high genetic diversity and genotypic variation occurs as a result of environmental adaptation[41,42], the isolation and study of microorganisms from corroded equipment provide a unique opportunity to expand our knowledge of MIC through the investigation of novel species implicated in corrosion, their metabolic capabilities and their potential role in corrosion.

In oil and gas pipelines and all piping systems, a pressure test called hydrostatic testing is normally carried out to inspect the system for leaks, to evaluate its integrity, and to validate that the system can operate under desired conditions[2,43]. During hydrostatic testing carried out on a floating production storage and offloading (FPSO) facility located on the Australian North West Shelf, several leaks were detected at piping system seal rings. These rings were exposed to production fluids during regular operation (~ 80 °C) for over five years. However, the system had been depressurised, flushed and filled with seawater (previously treated with oxygen scavenger and biocide) to conduct a hydrostatic test. This treated seawater was left in place for about four (4) months before hydrotesting was performed. The present investigation describes the microbial community identified in the corroded seal rings, as well as the isolation and identification of cultivable species within the community. The ability of the isolates to instigate MIC of carbon steel is examined and discussed.

Results

Microbial community composition in corrosion products from failed seal rings. Microbial community composition in corroded seal rings was investigated with 16S rRNA gene sequencing. Miseq sequencing generated a total of 789,041 raw-reads, however, after the bioinformatics processing (quality filtering, singletons, and chimera removal) only 275,501 high-quality sequences were used for the taxonomic classification of the microorganisms present in the three collected samples. Data analysis indicated that all sequences belonged to the Bacteria domain, most of them affiliated to the *Proteobacteria* phylum (~ 98% relative abundance). Other phyla detected in lower abundance included *Firmicutes*, *Bacteroidetes, Cyanobacteria,* and *Epsilonbacteraeota*. Taxonomic classification with Silva 132 database showed the presence of 37 different genera in the microbial communities. The genera with relative abundances equal or greater to 1% in at least one of the samples are presented in Fig. 1. The complete list of microorganisms identified in the microbial community of corroded seal rings is given in Table S1.

DNA-based diversity profiling revealed differences in the microbial community composition among samples. Higher diversity was observed in sample 1 (Shannon index = 3.7) and lower diversity in sample 3 (Shannon index 2.1). Despite the differences observed in the relative abundances of dominant genera across samples,

Isolate ID	Accession no.	Best match in GenBank database (Accession no.)	Similarity %
CCC-APB1	MT215169	*Enterobacter cloacae* subsp. *cloacae* strain ATCC 1,304 (CP001918.1)	99.84
CCC-APB3	MT215170	*Cronobacter sakazakii* strain ATCC 29,544 (NR_118449.1)	99.7
CCC-APB5	MT215171	*Shewanella chilikensis* strain JC5 (NR_117772.1)	99.78
CCC-SPP14	MT215176	*Enterobacter roggenkampii* strain DSM 16,690 (CP017184.1)	99.93
CCC-SPP15	MT215177	*Petrotoga mobilis* strain SJ95 (CP000879.1)	99.84
CCC-IOB1	MT215172	*Pseudomonas aeruginosa* strain JCM 5,962 (MK796437.1)	100
CCC-IOB3	MT215173	*Pseudomonas balearica* strain SP1402 (NR_025972.1)	99.71
CCC-IOB9	MT215174	*Citrobacter youngae* strain NCTC13709 (LR134485.1)	99.42
CCC-IOB10	MT215175	*Pseudomonas stutzeri* strain ATCC 17,588 = LMG 11,199 (MT027239.1)	100

Table 1. Identification of bacteria isolated from corroded seal rings using 16S rRNA gene sequences.

the microbial communities in all sampled seal rings were dominated by *the Pseudomonas* genus. Although the production fluid in the offshore facility is transported at high temperature (~ 80 °C), none of the genera detected has been classified as thermophilic microorganism. Conversely, all genera identified in the samples have been classified as mesophiles, and most of them have also been associated with marine environments and hydrocarbon degradation (*Martelella, Shewanella, Alcanivorax, Halomonas,* and *Marinomonas)*. Most of the microorganisms detected in the seal rings are classified as aerobic species, although many of them can survive without oxygen (facultative anaerobes).

Isolation, molecular identification and phylogenetic analysis. The three culture media used for the cultivation of sulphide producing prokaryotes (SPP), acid-producing bacteria (APB) and iron-oxidising bacteria (IOB) exhibited positive growth after four weeks of incubation. Nine different colony morphologies were observed on solid media, and all of the strains were rod-shaped Gram-negative bacteria (Table S2). Colonies were transferred at least three times to the same medium for complete purification. Comparison of the nucleotide sequences of the 16S rRNA gene with previously published sequences in the National Center of Biotechnology Information (NCBI) allowed the identification of the isolated bacteria to species level. The similarities and species identified by BLASTn analysis are given in Table 1. Three of the nine isolated microorganisms were classified into the *Pseudomonas* genus, which was found to be the most abundant genus in the microbial communities identified in corroded seal rings (Fig. 1). Other genera found as part of the microbial community and that were cultivated in the laboratory were *Enterobacter* and *Shewanella*. Bacteria belonging to the genera *Cronobacter, Petrotoga,* and *Citrobacter* were isolated from the seal rings even though they were not detected through DNA analysis, possibly because they were present in low abundances.

A phylogenetic tree was constructed to analyse the relationships among the sequences of the isolated species and sequences from related organisms available in the GenBank database. The optimal tree with the sum of branch length = 6.61426233 is shown in Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Microbiologically influenced corrosion. *Corrosion screening test.* Carbon steel was exposed to isolated bacteria for 7 days to characterise their corrosive behaviour under anaerobic conditions. Surface profilometry analysis (Fig. 3) showed that in the presence of bacterial isolates except for CCC-IOB3 (*P. balearica*), the metal surface exhibited greater deterioration compared to the abiotic control. The weight loss in each coupon after exposure to abiotic and biotic conditions is shown in Table S3. The corrosion rate and pitting rate calculated from the weight loss and maximum pitting depth, respectively, measured for each isolate are shown in Fig. 4. Most of the isolates significantly increased the corrosion rates and pitting rates of carbon steel coupons as compared to abiotic control (Table S4). The corrosion rates triggered by the isolated bacteria showed distinct trends among species. Isolates such as CCC-APB3 (*C. sakazakii*), CCC-APB5 (*S. chilikensis*), and CCC-SPP14 (*E. roggenkampii*) induced higher general corrosion rates than pitting rates. In contrast, the isolates CCC-APB1 (*E. cloacae*), CCC-SPP15 (*P. mobilis*), CCC-IOB1 (*P. aeruginosa*), CCC-IOB9 (*C. youngae*), CCC-IOB10 (*P. stutzeri*) induced higher pitting rates than general corrosion rates. Different to all other isolates, carbon steel exposure to CCC-IOB3 (*P. balearica*) resulted in corrosion inhibition. Optical microscopy revealed that after 7 days of carbon steel exposure to *P. balearica*, the metal surface was completely covered by a biofilm layer. Instead, for the other isolates, carbon steel was covered by patchy biofilms (Fig. S1). The corrosion rates influenced by the isolated bacteria were classified from low to severe (Table S5), according to NACE SP0775 standard[44].

MIC studies with selected bacterial isolates. Three of the isolated bacteria were selected for further corrosion investigation. The selection was based on the results of the corrosion screening test and the importance of studying microorganisms associated with different microbial groups. Microorganisms selected were CCC-IOB3 (*P. balearica*), CCC-SPP14 (*E. roggenkampii*), and CCC-APB5 (*S. chilikensis*). The three isolates belong to genera that have been previously associated with MIC; however, none of the specific species identified has been related to MIC before. Corrosion experiments consisted of exposing carbon steel coupons to active cultures of each

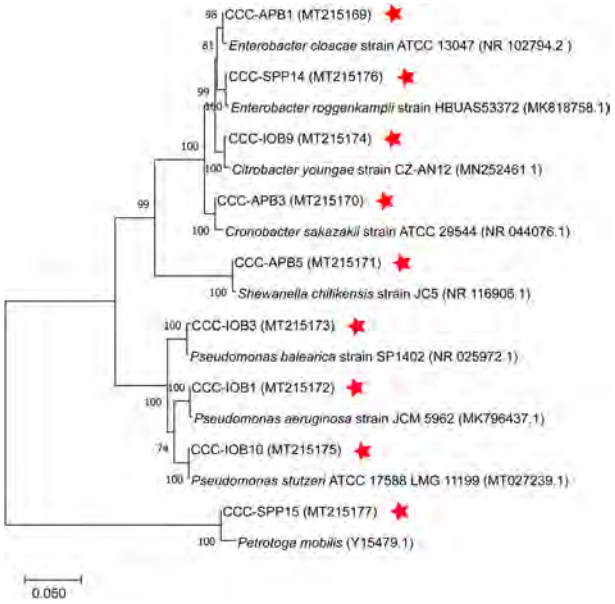

Figure 2. Neighbour-joining tree based on partial 16S rRNA gene sequences, showing phylogenetic relationships between isolates and related species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is displayed next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accession numbers are given in parentheses. Red star indicates bacteria isolated in this study. Figure generated using MEGAX. https://www.megasoftware.net/.

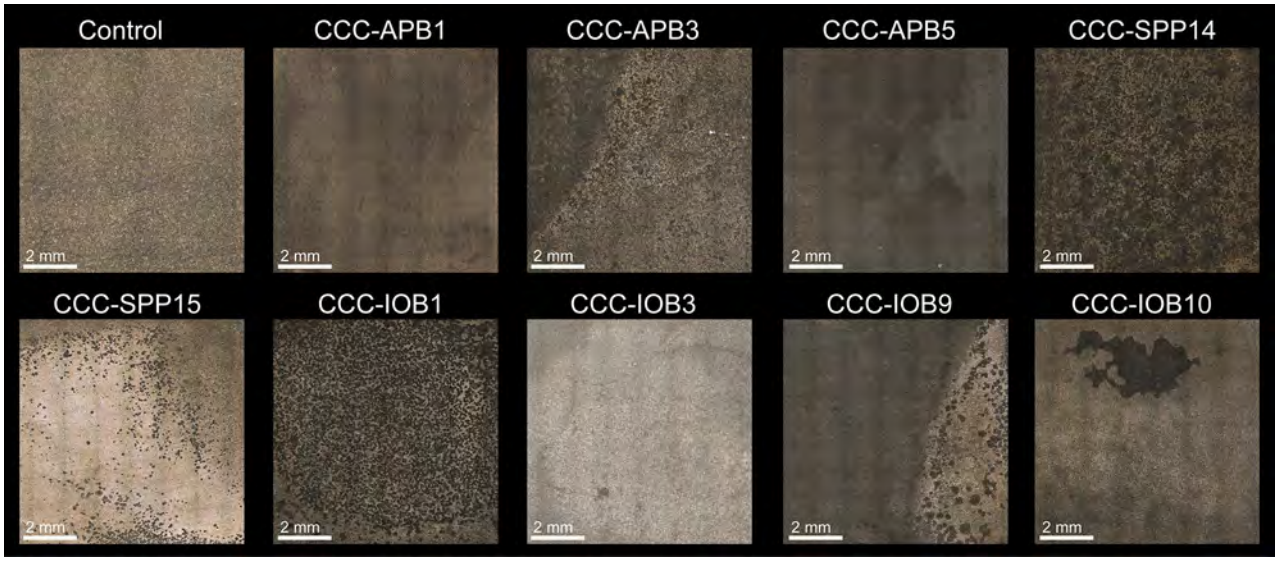

Figure 3. Microscope images of the surfaces of carbon steel coupons exposed for 7 days to bacterial isolates recovered from corroded seal rings. Control: coupon exposed to sterile test solution without bacteria. Images taken with the Alicona InfiniteFocus G4 at a lens magnification of ×5 (IFM version 3.5).

isolate for 21 days. During the immersion period, planktonic cell numbers and solution pH were frequently monitored (Fig. S2). It was evident that the metabolic activities of the isolates altered the pH of the solution. *P. balearica* and *S. chilikensis* increased the pH, whereas *E. roggenkampii* decreased it. Meanwhile, the pH of the control test without bacteria remained stable during the test. The growth pattern of planktonic cells in the reactors as estimated by microscopic counting is shown in Fig. S2. The fluctuation in the number of cells detected throughout exposure was influenced by the solution replenishment that removed part of the population every 3 days. However, at the end of the test, the three isolates reached similar cell numbers in the bulk medium. On the other hand, the numbers of cells estimated to be attached to the coupons (sessile) at the end of the immersion period based on quantitative polymerase chain reaction (qPCR) varied among isolates. The highest cell density was reached by *S. chilikensis* (2.96×10^9 cell cm^{-2}), followed by *E. roggenkampii* (1.73×10^8 cell cm^{-2}) and finally,

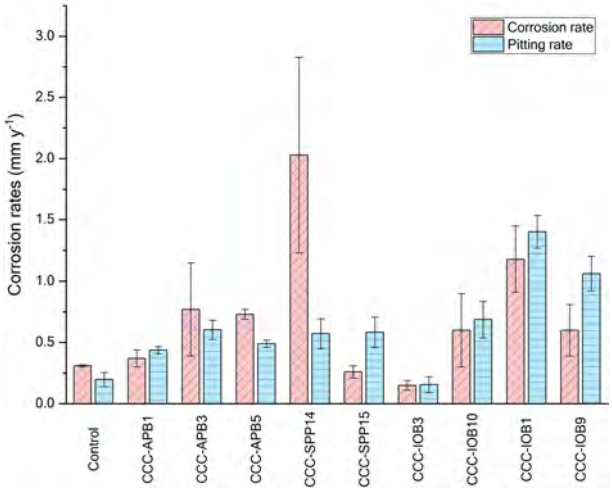

Figure 4. Corrosion rates of carbon steel during exposure to bacteria isolates for 7 days. Corrosion rate was calculated from the average weight loss of three coupons. Pitting rate was calculated from the maximum pit depth found in each replicate. Figure generated using OriginPro version 2020 (OriginLab Corporation). https://www.originlab.com/.

by *P. balearica* (1.45×10^5 cell cm^{-2}). At the end of the test, nitrites were detected in the bulk test solution of the three biotic reactors, which indicated that all isolates reduced nitrate. However, the presence of H_2S was only detected in the reactor with *S. chilikensis*. Detection of sulphide and blackening of the test solution indicated that this isolate also reduced thiosulphate.

After retrieval of coupons from reactors, differences in the metal surface were observed among the tests. Coupons exposed to the isolates were covered by layers of corrosion products and cells that varied in colour and thickness. The surface analysis showed differences in deterioration and morphology of damage for the various isolates (Fig. 5). It was noted that all isolates caused MIC, including the *P. balearica* isolate that had resulted in corrosion inhibition in the short term corrosion test. Results of the corrosion measurements indicated that *P. balearica, E. roggenkampii* and *S. chilikensis* promoted MIC under the studied conditions (Fig. 6). The weight loss in coupons exposed to abiotic and biotic conditions is shown in Table S6. According to the Kruskal–Wallis test, there were significant differences in the corrosion rates and pit depth among coupons exposed to the three isolates and the control ($p < 0.05$). Isolates were shown to increase the corrosion rate and pitting rate of carbon steel (Table S7). Coupons exposed to *E. roggenkampii* exhibited the highest corrosion rate, whereas coupons exposed to *S. chilikensis* exhibited the deepest pits.

Biofilms formed on the carbon steel coupons were observed under the field emission scanning electron microscope (FESEM) (Fig. 7). The micrograph of the coupon exposed to abiotic conditions revealed the presence of a thin layer of corrosion products derived from corrosion reactions promoted by components in the test solution (Fig. 7a). Micrographs of the three bacterial isolates showed their ability to form biofilms over the metal surface (Fig. 7b-d). Apart from bacteria cells, deposits and corrosion products were also observed on the metal surface. Thicker layers of corrosion products and cells were seen in biofilms of *S. chilikensis* and *E. roggenkampii*. The cross-sectional profile also showed differences in the distribution of layers and the elemental maps of deposits and corrosion products over coupons exposed to biotic conditions (Fig. 8). Samples exposed to *S. chilikensis* and *E. roggenkampii* showed thicker layers of corrosion products. The major elements detected in coupons exposed to the three isolates were oxygen and iron. Carbon was also abundant in all samples; however, since the mounting resin was carbon-based, it was not possible to discriminate carbon from biological material. Apart from these three elements, phosphorous was one of the major elements on coupons exposed to *E. roggenkampii*, and sulphur one of the major elements on coupons exposed to *S. chilikensis*.

Discussion

Several seal rings from an in-service offshore oil production facility failed during hydrostatic testing due to corrosion. This testing is a common practice to assess piping systems integrity before service, during service, or for qualification to increase the operating pressure in in-service pipelines[45]. This investigation comprised the characterisation of the microbial community present in corroded seal rings and the recovery of cultivable microorganisms for MIC laboratory investigation. Previous studies have described the risk of MIC associated with poor hydrotesting practices[46–48]. This is because the water used for the test is taken from natural systems (sea, rivers, lakes), which have a high concentration and diversity of microorganisms that can contaminate the facility if the water is not effectively treated[2,47]. The primary issue with hydrotesting is that the water used is typically left stagnant in the system for extended periods. Whilst the test lasts approximately 10 h, the water used may remain in place for months, in some cases years[49,50], promoting the development of biofilms under the stagnant conditions.

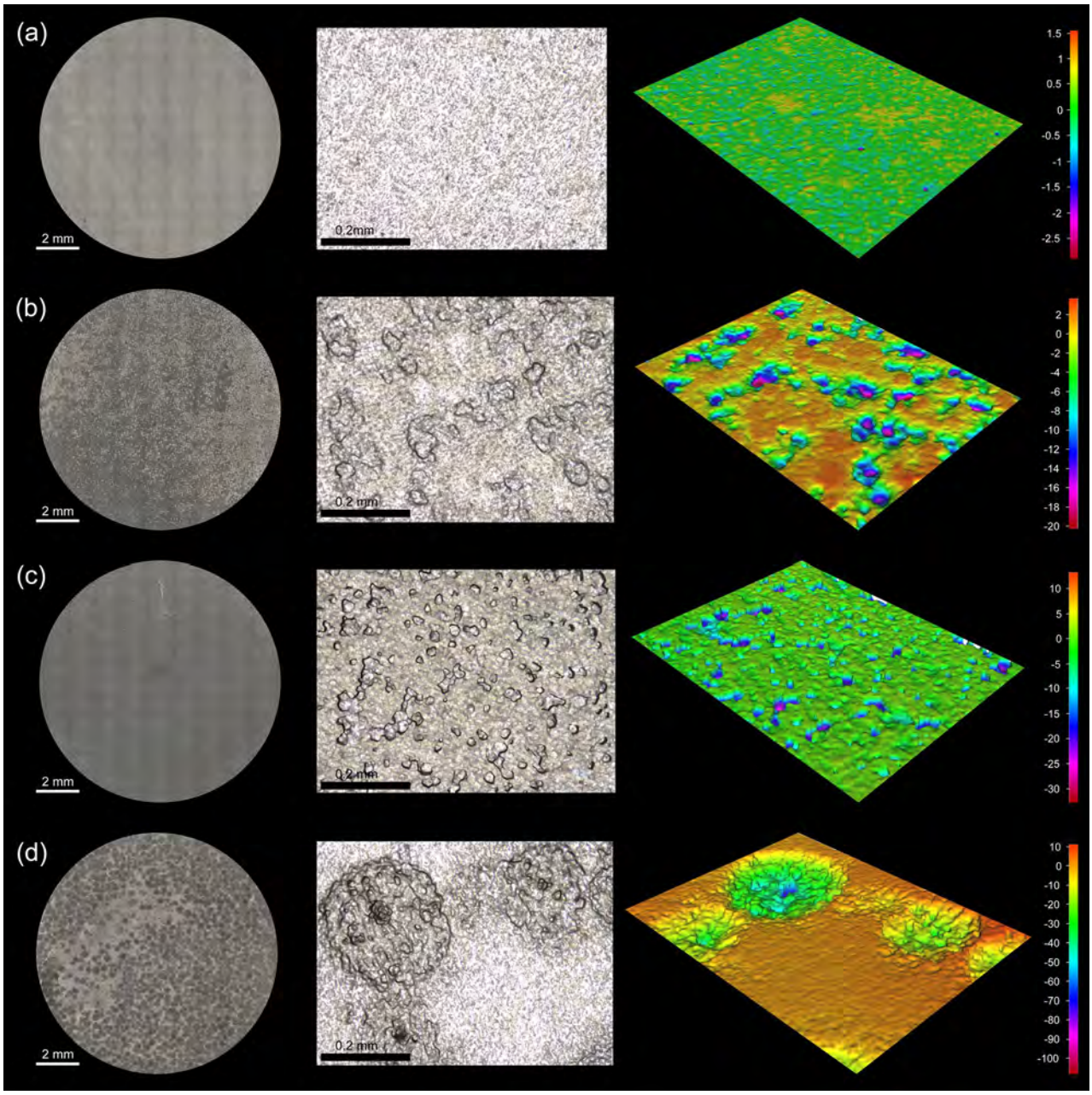

Figure 5. Microscope images of the surfaces of carbon steel coupons exposed for 21 days to (**a**) control (**b**) *P. balearica* CCC-IOB3 (**c**) *E. roggenkampii* CCC-SPP14 (**d**) *S. chilikensis* CCC-APB5. 2D image of the total surface (left image) and 3D images of 0.38 mm^2 of the coupon surface (middle and right image). Images taken with the Alicona InfiniteFocus G4 at a lens magnification of × 5 and × 20 (IFM version 3.5).

The role that bacteria played on the corrosion failure of the seal rings (root cause analysis) was not the focus of this research. This investigation aimed to characterise and isolate microorganisms from corrosion products and study their potential to instigate MIC of carbon steel. Results from this investigation indicate that the biocide treatment applied to seawater used to flood the pipe was not effective at controlling all viable microorganisms. DNA analysis of corrosion products revealed that the microbial community on the different sampled corroded seal rings was similar and dominated by marine microorganisms. Several of these microorganisms have previously been associated with hydrocarbon degradation[51–53], which may explain their thriving in the pipeline during the stagnant period. Microorganisms related to thermophilic oil environments were not detected. This suggests that MIC was mainly triggered during seawater flooding and subsequent preservation, not during normal operation conditions. The dominance of *Pseudomonas* genus in different corroded seal rings supports the assumption that microorganisms participated in the corrosion phenomenon. This genus has been found ubiquitously in diverse environments, and its ability to survive with basic minimal nutrient requirements and to tolerate harsh conditions have allowed it to persist in urban and natural settings[54]. Moreover, several *Pseudomonas* species are naturally resistant to a variety of antimicrobial substances[55–57], which help explain its dominance in the corroded

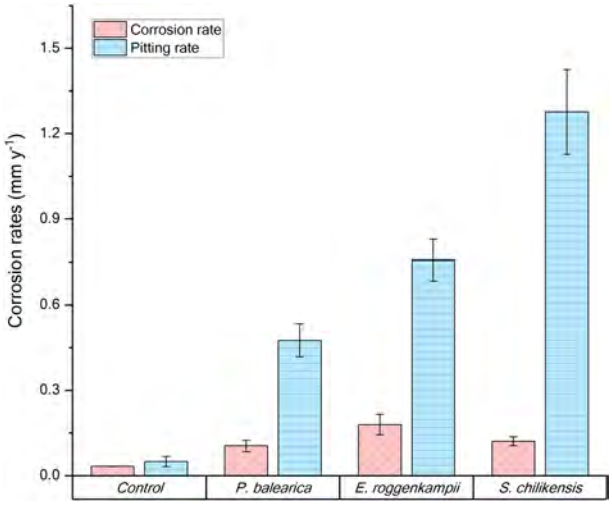

Figure 6. Corrosion rates of carbon steel after exposure to bacterial isolates for 21 days in the reactor experiment. Corrosion rate was calculated from the average weight loss of three coupons. Pitting rate was calculated from the maximum pit depth found in each replicate. Figure generated using OriginPro version 2020 (OriginLab Corporation). https://www.originlab.com/.

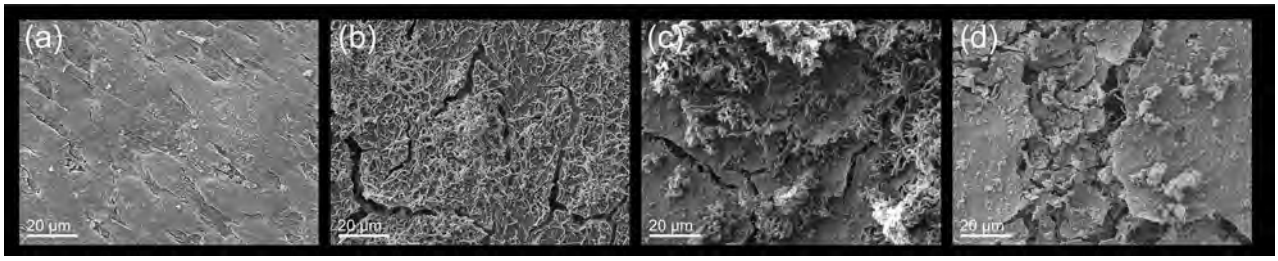

Figure 7. FESEM views of the biofilms/corrosion products formed over carbon steel coupons. (**a**) Control (**b**) *P. balearica* CCC-IOB3 (**c**) *E. roggenkampii* CCC-SPP14 (**d**) *S. chilikensis* CCC-APB5. Images taken with a Zeiss Neon Dual-Beam FESEM.

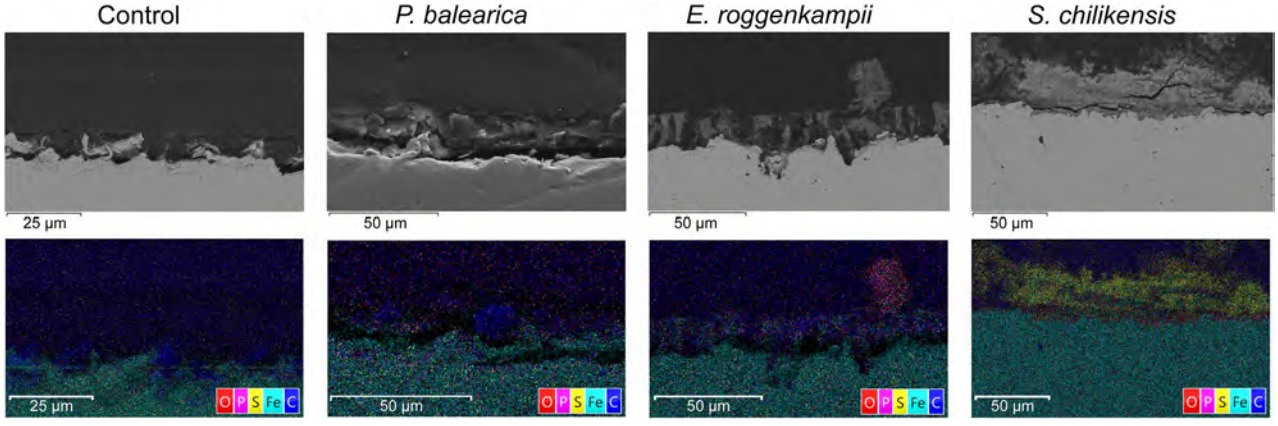

Figure 8. FESEM image and EDS-elemental mapping of cross-sectioned coupons exposed to the control and three bacterial species *P. balearica* CCC-IOB3, *E. roggenkampii* CCC-SPP14, and *S. chilikensis* CCC-APB5. Images generated using AZtec version 3.0. https://nano.oxinst.com/products/aztec/.

seal rings despite the biocide treatment applied to the seawater. Although DNA-based next-generation sequencing (NGS) analysis cannot discriminate between live and dead bacteria, the recovery and cultivation of several bacterial species from corrosion products prove that corrosion products hosted living microorganisms.

In this investigation, nine bacterial species were isolated from the failed seal rings and identified with the 16S rRNA gene sequence. Microorganisms can be classified in different microbial groups, including sulphide producing prokaryotes, acid-producing bacteria, iron-oxidising bacteria, iron-reducing bacteria, nitrate reducing

bacteria. Some of the isolates can be classified in two or more of these groups, which show the versatility and complexity of the microbial population living in the corroded steel. Corrosion of carbon steel by isolated bacteria was also evaluated in this study. Results demonstrated that all bacterial isolates were able to catalyse corrosion reactions under anaerobic conditions. Surface and corrosion analyses revealed that microorganisms increased general corrosion rates and pitting rates of carbon steel. Different isolates triggered different corrosion rates and patterns of attack (Figs. 3, 4). In the short term corrosion experiments, coupons exposed to *E. roggenkampii* CCC-SPP14 exhibited the highest corrosion rate while coupons exposed to *P. aeruginosa* CCC-IOB1 showed the highest pitting rate.

Three isolates (*P. balearica*, *E. roggenkampii*, and *S. chilikensis*) associated with different microbial groups (iron-oxidising bacteria, acid-producing bacteria and iron-reducing bacteria, respectively) were further studied under semi-batch conditions for longer exposure time. The behaviour of *P. balearica* CCC-IOB3 isolate was different for short term and long term experiments. Carbon steel exposed to this isolate in serum bottles under batch conditions (short term) experienced corrosion inhibition; however, the isolate induced corrosion of carbon steel in the longer-term reactor study where semi-batch conditions allowed the replenishment of test solution. These results provided evidence that corrosion inhibition by bacteria is not necessarily a characteristic of specific microorganisms but instead, it is likely the result of experimental conditions. Contradictory corrosive behaviour of bacteria has been previously reported. Miller et al.[58] showed that depending on biofilm distribution, *Shewanella oneidensis* MR-1 could inhibit or enhance corrosion of steel. When steel was completely covered by the biofilm, the bacteria inhibited corrosion, likely due to O_2 scavenging, whereas when steel was partially covered, corrosion was accelerated in the uncovered area. MIC inhibition has also been indicated for some species of the *Pseudomonas* genus[59,60]. Studies suggest that biopassivation of the steel is influenced by the production of extracellular polymeric substance (EPS) and biofilm structure that prevent corrosive species in the bulk solution from reaching the metal surface. Nonetheless, the formation of a uniform biofilm layer does not always prevent corrosion. It has also been demonstrated that biofilms can promote corrosion by the creation of differential concentration cells, alteration of anion ratios, generation of corrosive substances, and inactivation of corrosion inhibitors[61].

Proposed MIC mechanisms triggered by selected isolates. Microbial corrosion has predominantly been associated with localised corrosion; however, most often, MIC results in a combination of general and pitting corrosion. General corrosion is the uniform oxidation of the metal across its surface resulting in mass loss from the metal, whereas, pitting corrosion is localised, and although it may result in relatively minor mass loss, it typically results in wall penetration and loss of containment thus significantly reducing the service life of industry assets[62]. Differences in the deterioration pattern and corrosion measurements suggest variations in the corrosion mechanisms promoted by each isolate. *E. roggenkampii* stimulated significant general corrosion while *S. chilikensis* resulted in the highest localised corrosion and the most severe pitting attack.

Shewanella species are well known for their metabolic versatility to utilise a variety of electron acceptors, which include nitrate, nitrite, thiosulphate, elemental sulphur, iron (III), Manganese (III), fumarate, among others[58,63–65]. Additionally, *Shewanella* can use the metal as electron donor by secreting electron shuttles such as riboflavins, or by producing conductive filaments (nanowires)[66]. The broad metabolic capabilities of this genus suggest that these microorganisms can induce or accelerate corrosion through different mechanisms including EET-MIC (extracellular electron transfer MIC) and CMIC (chemical MIC)[62]. The expression of various metabolic pathways depends on the nutrients availability and environmental conditions. For example, in the present investigation, the test solution only contained thiosulphate and nitrate as electron acceptors which limited the metabolism of *S. chilikensis* CCC-APB5 to the anaerobic respiration of these molecules. However, after initiation of the corrosion process on the metal surface, the iron oxides layers produced could have also been used by the isolate as electron acceptors during the degradation of organic compounds. Likewise, the isolate could have induced corrosion by using the carbon steel as electron donor for the reduction of the thiosulphate, nitrate, and iron oxides. The genome sequence of *S. chilikensis* (GenBank accession number CP045857) confirmed that the isolate has the genetic machinery to conduct all these activities. A dual system for nitrate reduction (NAP, NAR), a system for metal reduction (MTR), genes involved in thiosulphate reduction (*phsA*, *glpE*), genes for riboflavin biosynthesis (*ribF*), and genes related to nanowires (prepilin peptidases and biogenesis proteins – Msh complex) were detected in its genome. *S. chilikensis* was the only isolate that produced sulphide and blackening of the test solution during the experimental period. The use of thiosulphate for anaerobic respiration was confirmed with the X-ray maps of the corrosion products that revealed the presence of iron and sulphur elements along the cross-sectional profile. During thiosulphate reduction, hydrogen sulphide is generated and in contact with ferrous iron precipitates as iron sulphide. Apart from sulphur related metabolism, *S. chilikensis* also expressed nitrate metabolism, which was determined by the presence of nitrite in the test solution after microbial growth and its absence in the control test. Hence, *S. chilikensis* could have contributed to MIC via three mechanisms, the dissolution of protective iron oxides, the production of corrosive metabolites (nitrite and H_2S), and the uptake of electrons from the metal surface. It is important to highlight that *Shewanella* spp. are usually classified as iron-reducing bacteria (IRB); however, in this study, *S. chilikensis* reduced thiosulphate and nitrate, which again, demonstrates the versatility of these microbes.

E. roggenkampii CCC-SPP14 isolate belongs to the *Enterobactereacea* family, which is mostly comprised of acid-producing and nitrate reducing bacteria[67]. Some species of the *Enterobacter* genus have also been classified as iron-oxidising bacteria with the ability to cause MIC of carbon steel[68]. The capability of *E. roggenkampii* isolate to perform carbohydrate fermentation, iron oxidation and nitrate reduction was also confirmed through the genome sequencing (GenBank accession number JAACJF000000000). The pH decrease observed in the test solution suggested that *E. roggenkampii* was using the fermentation pathways and producing acids during its

metabolic activities. The highest general corrosion observed in the coupons exposed to this isolate support the acid corrosion mechanism[46,69]. Although more than 90% of MIC occurs as pitting corrosion, it has been reported that the stimulation of acid corrosion by fermenting microorganisms promotes general corrosion[46]. Similar to the *S. chilikensis* experiment, nitrite was also detected in the test solution after microbial growth, indicating that the species reduced nitrate. Accumulation of nitrites could have induced the creation of the pits observed on the metal surface[70]. Based on the above, *E. roggenkampii* was likely to contribute to MIC via production of corrosive metabolites such as acidic species and nitrites.

The *Pseudomonas* genus has been reported several times to be involved in corrosion processes[38,71–74]. Species that belong to this genus are pioneer colonisers in biofilm formation[60]. It has been reported that the metal cation binding by EPS increases the ionisation of metals, leading to the accumulation of metal ions that change the electrochemical properties of the metal surface and promote corrosion[75]. Moreover, *Pseudomonas* species produce electron mediators such as pyocyanin and phenazine-1-carboxamide (PCM) that facilitate electron transfer between cells and metals, and accelerate corrosion rates by EET-MIC mechanism[76,77]. Most of the previous investigations of the corrosion abilities by *Pseudomonas* species have been carried out simulating aerobic environments[38,71,73,74]. Therefore, the primary corrosion mechanism described in the literature for *Pseudomonas* spp. is the formation of differential aeration cells. Some studies have also reported corrosion under anaerobic conditions and the described mechanism is iron oxidation coupled to nitrate reduction[72,78]. *P. balearica* CCC-IOB3 was isolated in IOB medium and precipitation of iron as a result of iron oxidation was observed in colonies grown on solid medium, which only contained nitrate as an electron acceptor. The presence of genes involved in iron metabolism and nitrate reduction was confirmed through genome sequencing (GenBank accession number CP045858)[79]. Different to the IOB medium, the test solution used for the corrosion evaluation had different nutrients (acetate, glucose, casamino acids), which the isolate could have used as electron donors for a heterotrophic nitrate reduction. Nonetheless, the metal surface (Fe^0) or ferrous iron (Fe^{2+}) released from the corrosion process could have also been used by this isolate as electron donors for nitrate reduction. High precipitation of iron oxides over the surface of the coupon was not observed in the FESEM analysis, suggesting that iron was not the main electron donor in the metabolism. Hence, the most likely MIC mechanism that promoted pitting of carbon steel in the presence of this isolate was the production of nitrite via nitrate reduction. Nevertheless, EET-MIC and iron oxidation cannot be disregarded entirely in this study.

The production of nitrite during anaerobic respiration was shared by the three isolates as a possible corrosion mechanism. Although nitrite is known as a corrosion inhibitor, it can also induce pitting corrosion when it is not available in a sufficient concentration that can fully passivate the steel[80]. It has also been reported that nitrite increases the ionic conductivity of the bulk fluid, which accelerates the corrosion rate[70]. Accumulation of nitrite is the most expected corrosion mechanism of nitrate reducing bacteria; however, other mechanisms induced by this microbial group have been proposed including the formation of concentration cells, the consumption of the cathodic H_2 (cathodic depolarisation), and the direct microbial metabolism of Fe(0)[62]. Confirmation of all possible corrosion mechanisms triggered by the bacteria isolated in this study requires further investigation, including their behaviour under different environmental conditions and their effect on other steels.

Conclusion

An investigation of corroded seal rings showed that the rings were inhabited by a microbial community dominated by members of the following genera *Pseudomonas*, *Martelella, Marinomonas, Shewanella, Alcanivorax,* and *Halomonas*, which have been associated with hydrocarbon degradation. In all sampled seal rings, *Pseudomonas* was the most abundant genus, which supported the hypothesis that microorganisms participated in the corrosion of the seal rings. Additionally, nine different microbial species were isolated from corrosion products and their ability to induce corrosion of carbon steel demonstrated in the laboratory. Differences in the surface deterioration patterns among isolates indicated variations in the corrosion reactions and mechanisms promoted by each isolate. Three isolates were further investigated to understand their effects on corrosion. Differences in the corrosion rates were observed among the isolates; *S. chilikensis* triggered the most severe pitting on carbon steel, followed by *E. roggenkampii*, and *P. balearica*. Corrosion mechanisms of the selected microorganisms were suggested based on their metabolic capabilities, which included the production of corrosive metabolites: organic acids, nitrites and H_2S.

Methods

Sample collection and microbiological characterisation. *Sampling.* Samples were collected from corroded seal rings (made of 17–4 PH martensitic precipitation-hardening stainless steel) that failed during hydrostatic testing in the piping system of a FPSO facility located on the Australian North West Shelf. Corrosion products had a black/dark brown colour, containing both thick and thin solids. Corrosion products and sessile microorganisms attached to the deposits from three different corroded seal rings were scrapped and collected in sterile containers. Samples were transported at 4 °C to a research facility (2 days after collection) for microbiological analysis. Upon arrival, samples for microbial growth were processed immediately, and samples for DNA sequencing were stored at − 20 °C until extractions were conducted (maximum 2 weeks after collection).

Microbial composition analysis by 16S rRNA sequencing of DNA extracted from corrosion products of failed seal rings. The composition of the microbial communities on the failed seal rings was determined using Illumina NGS of the 16S rRNA gene hypervariable region V3–V4. For this, DNA was extracted from corrosion products using the DNeasy PowerSoil Kit (Qiagen), following the manufacturer's instructions. DNA concentration was quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies), and PCR reaction was carried out using the prokaryotic universal primers 341F (5′ CCTAYGGGRBGCASCAG 3′) and 806R (5′ GGA

CTACNNGGGTATCTAAT 3′)[81], which have been previously applied for identification of microbial communities in corrosion products and oil production facilities[82,83]. Library preparation and sequencing were conducted by the Australian Genome Research Facility (AGRF), as described elsewhere[83]. Downstream analysis of the sequences was completed with QIIME2 version 2019.4[84]. The q2-dada2 plugin[85] was applied for denoising and q2-feature-table plugin for the removal of singletons and low-frequency sequences (< 10 reads). Subsequently, taxonomy was assigned to each amplicon sequence variant (ASV) using the q2-feature-classifier plugin[86] and q2-classify-consensus-blast plugin[87]. SILVA database version 132[88] was used for the taxonomic classification. Microbial composition was visualised in bar charts created with the ggplot2 package version 3.1.0[89] employing R version 3.4.3[90]. Only taxonomic orders with relative abundances equal or greater to 1% in at least one sample were included in the graph.

Bacterial isolation, identification and phylogenetic analysis. *Enrichment and isolation.* Corrosion products were inoculated into several culture media to maximise the recovery of cultivable populations. Culture media targeted the growth of SPP, APB and IOB. The culture media for the recovery of IOB (iron oxidising bacteria media) and APB (phenol red dextrose broth) were prepared according to the guidelines and composition described in the standard test method NACE TM0194[91]. Composition of the culture medium used for the recovery of SPP is described elsewhere[92]. All culture media were prepared anaerobically and dispensed in Hungate tubes (15 mL capacity). In an anaerobic chamber filled with N_2–CO_2–H_2 (75:10:5) gas mixture, 1 g of each sample was inoculated in the Hungate tubes containing 10 mL of growth medium. Tubes were incubated in dark for 28 days at 40 °C without shaking. After the incubation period, cultures that showed positive growth were subcultured onto solid media using the anaerobic chamber. Solid medium was prepared by adding 15 g L^{-1} of agar–agar (Sigma-Aldrich) to the same composition used to make the broths. Plate cultivation resulted in the growth of colonies with different morphologies; only one colony representative of each morphology type was selected for purification and further study. Purification was achieved using the streak plate method. All inoculated plates were incubated under anaerobic conditions by placing them in anaerobic jars with AnaeroGen sachets (Oxoid, Thermo Fisher Scientific). Single colonies were transferred to liquid medium, and microscopic observation in a phase contrast microscope (Nikon Eclipse Ci-L) was used to confirm consistent cell morphology of isolates.

16S rRNA gene sequencing and identification of closest related species. Molecular identification of isolates was based on 16S rRNA gene sequencing. A 5 mL aliquot of a pure culture in an exponential growth phase was centrifuged at 15,000×*g* for 5 min to harvest cells from each isolate. Genomic DNA was extracted with DNeasy PowerSoil Kit (Qiagen), as described by the manufacturer. For PCR amplification, the universal bacterial primers 27F (5′ AGAGTTTGGATCCTGGCTCAG 3′)[93] and 1492R (5′ CGGTTACCTTGTTACGACTT 3′)[94] were employed. The reaction mixture was prepared according to the BIOTAQ DNA polymerase protocol (Bioline), with a final volume of 25 µL per reaction. Amplification was conducted in a T100 Thermal Cycler (Biorad) by the following steps: initial denaturation at 95 °C for 5 min, 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega), and Sanger sequencing was conducted by AGRF. Sequences of 16S rRNA genes were compared with sequences stored in the NCBI using the standard nucleotide basic local alignment search tool (BLASTn)[95].

Phylogenetic analysis. Phylogenetic and similarity analyses were conducted using the Molecular Evolutionary Genetics Analysis version 10 (MEGAX)[96]. ClustalW function was used for alignment of the 16S rRNA gene sequences found in this study and other organisms related retrieved from the NCBI. Alignment gaps were treated as missing data. The phylogenetic tree was constructed by the Neighbour-Joining method[97], and 1,000 bootstrap replications were carried out to validate internal branches[98]. The paired similarity and pairwise distance were calculated using the transversion/transition weighting ($R = s/V$) and the Kimura-2-parameter model[99].

Corrosion experiments. *Screening corrosion test of isolates.* To evaluate their corrosive potential, all isolates were grown separately under anaerobic conditions using serum bottles (100 mL capacity) containing a metal sample. The metal used in this study was commercial 1,030 carbon steel, which is commonly used in the oil and gas industry. This material had the following elemental composition (weight %): C (0.43–0.5), Mn (0.6–0.9), Si (0.15–0.35), S (0.01–0.35), P (0–0.035), Cr (0–0.40), and Fe (balance). Square coupons (1 × 1 × 0.3 cm) with an exposed area of 1 cm^2 were prepared for the analysis. First, coupons were electro-coated with a protective epoxy (Powercron 6000CX, PPG Industrial coatings). Then, one side of the coupon was wet ground (deionised water) using silicon carbide papers of 80, 120, 320, and 600 grit, consecutively. The polished specimens were washed with ethanol, dried with nitrogen gas and stored in a desiccator until the test. Before the test, coupons were sterilised with ultraviolet (UV) radiation for 15 min each side before immersion in the test solution. One coupon was immersed in each serum bottle and placed in the bottom at a horizontal position. Serum bottles were filled with 90 mL of test solution that had been prepared anaerobically by sparging a gas mixture of N_2–CO_2 (80:20). Supplemented artificial seawater was used as test solution, which had the following composition: 35 g L^{-1} of sea salts (Millipore), 20 mM of Na-lactate, 20 mM Na-acetate, 20 mM Na-formate, 10 mM glucose, 1.3 g L^{-1} bacto casamino acids (BD), 20 mM NH_4NO_3, 10 mM Na-thiosulphate, 1 mM iron (III) citrate, 1 mM iron (II) sulphate heptahydrate, and 1 mM Na-sulphide nonahydrate. The pH of the test solution was adjusted to 7.3 with sodium bicarbonate. Serum bottles were inoculated with 1 mL at an appropriate dilution of overnight cultures of the isolated bacteria to obtain a final concentration of 1×10^6 cells mL^{-1} in each bottle. All isolates were evaluated independently and in triplicate by inoculation of the same culture in three serum bottles. Cultures were incubated

for 7 days at 40 °C and shaking of 100 rpm. At the completion of the immersion period, coupons were removed and the surface examined for corrosion using light optical microscopy. Coupons in three serum bottles without the addition of bacteria were exposed to the same conditions to discriminate abiotic corrosion.

Additional corrosion experiments for selected isolates. Three isolates were selected and studied for a longer period of time (21 days) using glass reactors (150 mL capacity) under anaerobic conditions. Carbon steel round coupons with same elemental composition described earlier, and an exposed area of 1.27 cm^2 were used for this experiment. A total of four metal coupons were immersed in each reactor. These metal coupons were prepared as described in "Screening corrosion test of isolates" section. Supplemented artificial seawater was used as test solution, which had the following composition: 35 g L^{-1} of sea salts (Millipore), 20 mM of Na-lactate, 20 mM Na-acetate, 30 mM Na-formate, 10 mM glucose, 1.3 g L^{-1} mM bacto casamino acids (BD), 15 mM NH_4NO_3, 8 mM Na-thiosulphate, 0.007 mM iron (II) chloride tetrahydrate, 0.007 mM manganese (II) chloride tetrahydrate, and 59 mM Na-bicarbonate. The pH of the test solution was 7.3 ± 0.2. Anaerobic conditions were maintained by continuous sparging of the test solution with a filter sterilised gas mixture of N_2–CO_2 (80:20) at a flow rate of 20 mL min^{-1}. Each reactor was inoculated with one of the selected isolates to a final concentration of 1×10^7 cells mL^{-1}. The temperature was controlled at 40 °C ± 1 °C with an IKA RCT digital hotplate and a thermocouple. To maintain active growth of isolates during coupons exposure, the test reactors were operated under semi-batch conditions, by replenishing 70% of the test solution with fresh test solution every 3 days. After 21 days, the presence of sulphide and nitrites were determined using the sulphide methylene blue method 8,131 (Hach) and nitrate/nitrite semi-quantitative test strips (Quantofix). Coupons were analysed as described in the following section. A sterile control without the addition of bacteria was set up under the same conditions to discriminate abiotic corrosion.

Analytical methods. *Corrosion measurements.* Corrosion measurements were carried out to determine corrosion rates and pitting on the metal surface. For this, three coupons from each test were cleaned by immersion in Clarke's solution and sonication for 30 s, followed by washing steps with deionised water and ethanol. Cleaning cycle was repeated until a constant weight was measured with an electronic balance. Corrosion rates were estimated from weight loss, as described in the ASTM G1 standard[100]. Pitting analysis was conducted with a 3D optical profilometer (Alicona InfiniteFocus G4) using the instrument's software (IFM version 3.5). Pitting rate was calculated using the maximum pit depth found in each test, as described in the NACE SP-0775 standard practice[44].

Microbial quantification. Direct cell counts of planktonic (bulk solution) bacteria were performed periodically using Neubauer counting chamber and a phase-contrast microscope (Nikon Eclipse Ci-L). After 21 days of exposure, the number of bacteria attached to the metals (sessile) was determined by the qPCR method. Bacteria were detached from the coupons by vortexing and sonication in 10 mL of sterile 1 × PBS (Sigma-Aldrich) as described elsewhere[101]. Detached cells were harvested by centrifugation at 15,000×*g* for 5 min. The pellet obtained after centrifugation was used for DNA extraction and subsequent qPCR analysis. Genomic DNA was extracted using DNeasy PowerBiofilm Kit (Qiagen), as described by the manufacturer. The number of total bacteria was estimated by quantifying the number of copies of the *rpoB* gene, which is a single-copy gene[102]. PCR reaction was conducted as described elsewhere[83,103]. All analyses were performed in triplicate.

Biofilm imaging and surface analysis. One coupon from each reactor was examined using FESEM to visualise the morphology of the biofilm. The analysis was executed using a Neon Dual-Beam field emission scanning electron microscope (Zeiss). For the analysis, biofilms were prepared as described elsewhere[104].

Additionally, a cross-sectional analysis of the corrosion products and biofilms was performed using the FESEM coupled with energy-dispersive X-ray spectroscopy (EDS). Coupons for this analysis were dried under nitrogen flow for 5 days. Then, coupons were mounted in Epofix Resin under vacuum (0.1 bar), followed by dry-polishing to 1 µm diamond finish to reveal the cross-sectional profile[105]. Cross-sectioned samples were coated with a platinum layer (5 nm thick) and observed in the microscope for elemental composition analysis. AZtec version 3.0 (Oxford Instruments NanoAnalysis) was used for FESEM/EDS data analysis.

Statistical analysis. Corrosion rates and measurements were performed in triplicate, and results are presented as the mean ± standard deviation. Kruskal–Wallis analysis was implemented to test if there were statistically significant differences in the corrosion rates between biotic and abiotic tests. All statistical analyses were performed with PAST version 3.25[106] software. The statistical significance for all analyses was accepted at $p < 0.05$ (significance level).

Sequence data deposition. The nucleotide sequences of the NGS data reported in this study were deposited in the NCBI under the bioproject number PRJNA613253. The 16S rRNA gene sequences of the isolated bacteria were deposited in the GenBank with the accession numbers MT215169, MT215170, MT215171, MT215172, MT215173, MT215174, MT215175, MT215176, and MT215177.

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Author contributions

SS-C and LM conceived the study. AD executed the sampling. SS-C performed the bacterial isolation and identification with support of AK. SS-C conducted the experiments, carried out the analysis and prepared the manuscript with the contribution of LM, AK, AD.

Competing interest

The authors declare no competing interests.

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GENOME SEQUENCES

Complete Genome Sequence of *Pseudomonas balearica* Strain EC28, an Iron-Oxidizing Bacterium Isolated from Corroded Steel

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ABSTRACT *Pseudomonas balearica* strain EC28 is an iron-oxidizing bacterium isolated from corroded steel at a floating production storage and offloading facility in Australia. Here, we report its complete genome sequence, which comprises 4,642,566 bp with a GC content of 64.43%. The genome harbors 4,164 predicted protein-encoding genes.

Iron-oxidizing bacteria (IOB) derive energy from the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron, resulting in the formation of dense iron oxide deposits that in contact with metals can promote under-deposit corrosion (1, 2). Several investigations have reported *Pseudomonas* spp. to be involved in microbiologically influenced corrosion (MIC) of different steels in industrial facilities and marine habitats (3–5). However, to our knowledge, the species *Pseudomonas balearica* has not been associated with MIC before. *Pseudomonas balearica* strain EC28 was isolated from corroded steel at a floating production storage and offloading (FPSO) facility in Australia. First, 1 g of corrosion products was scraped from the corroded steel and inoculated into 9 ml of anaerobic culture medium for IOB (6). The tube was incubated in darkness at 40°C for 28 days. Changes in the medium color and iron precipitation were observed after the incubation period. The culture was plated onto solid medium, which had the same IOB culture medium composition with the addition of 15 g/liter agar-agar. Plates were incubated under anaerobic conditions using anaerobic jars with AnaeroGen sachets (Oxoid, Thermo Fisher Scientific). Single colonies were subcultured and purified using the streak plate method. Pure colonies of strain EC28 showed iron precipitation, confirming its ability to oxidize ferrous iron.

Genomic DNA of *P. balearica* strain EC28 was extracted using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The isolated DNA was used to generate Illumina shotgun paired-end sequencing libraries. Sequencing was performed employing the MiSeq system and the MiSeq reagent kit v3 (600 cycles) as recommended by the manufacturer (Illumina, San Diego, CA, USA). In addition, DNA was prepared following the 1D genomic DNA by ligation (SQK-LSK109) protocol. Sequencing was conducted with a MinION device (Oxford Nanopore, Oxford, UK). End repair was performed using NEBNext formalin-fixed, paraffin-embedded (FFPE) repair mix (New England Biolabs, Ipswich, MA, USA). The library for Nanopore sequencing was loaded onto a SpotON flow cell Mk I (R9.4). The raw reads were quality filtered with fastp v0.19.4 (7). High-quality reads (1,717,952 short reads and 23,249 long reads) were hybrid *de novo* assembled using the Unicycler assembler v0.4.7 (8). Unicycler reported one circular chromosome of 4,642,566 bp (64.43% GC content, 124-fold coverage) and the absence of extrachromosomal elements. Bandage v0.8.1 (9) was

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used for visual validation of the assembly. Default parameters were used for all software unless otherwise specified.

The genome was annotated based on the NCBI Prokaryotic Genome Annotation Pipeline (10) v4.10. A total of 4,350 genes, including 4,164 protein-encoding genes with predicted functions, were detected. Moreover, 110 genes coding for hypothetical proteins, 60 tRNA genes, 12 rRNA genes, and 4 noncoding RNA (ncRNA) genes are also present. The average nucleotide identity (ANI) between EC28 and 27 publicly available genomes from different *Pseudomonas* species was determined with the Python module for average nucleotide identity analyses (pyANI) v0.2.7 (11). The ANI value above the threshold range (95 to 96%) of species delineation (12) with the genome of *P. balearica* strain DSM 6083 (GenBank accession number NZ_CP007511) indicates that strain EC28 belongs to the same species.

The genome of *P. balearica* strain EC28 contains a number of genes involved in iron metabolism, including iron uptake regulators, iron transporters, iron reductases, iron-binding proteins, and *c*-type cytochromes. Genes for complete dissimilatory nitrate reduction were also detected in the genome. The genome sequence provided here is expected to broaden our knowledge regarding corrosion processes initiated or accelerated by anaerobic nitrate-dependent iron oxidizers.

Data availability. The genome sequence of *Pseudomonas balearica* strain EC28 was submitted to GenBank under the accession number CP045858. The associated BioProject and BioSample accession numbers are PRJNA587695 and SAMN13198556, respectively. The raw reads have been deposited in the NCBI SRA database under the accession numbers SRR11483657 and SRR11483658.

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GENOME SEQUENCES

Draft Genome Sequence of *Enterobacter roggenkampii* Strain OS53, Isolated from Corroded Pipework at an Offshore Oil Production Facility

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ABSTRACT Here, we report the genome sequence of *Enterobacter roggenkampii* strain OS53, isolated from corroded pipework at an offshore oil production facility. The draft genome sequence comprises 6 contigs and contains 5,194,507 bp with an average GC content of 55.90%.

Enterobacter roggenkampii is a facultative, anaerobic, rod-shaped, Gram-negative bacterium that belongs to the family *Enterobacteriaceae*. Most *Enterobacter* spp. can produce organic acids from their metabolic activities (1). Acid-producing microorganisms promote microbiologically influenced corrosion by local acidification (2, 3). *E. roggenkampii* strain OS53 was isolated from corrosion products formed on corroded pipework at an Australian offshore oil production facility. Tubes containing sterile culture medium for sulfide-producing prokaryotes (SPP) (4) were inoculated with corrosion products and incubated at 40°C under anaerobic conditions. OS53 was isolated using a streaking technique with plates prepared with the same SPP medium composition and 15 g/liter agar-agar. Plates were incubated in anaerobic jars with AnaeroGen sachets (Oxoid). Individual colonies were restreaked onto SPP agar until they were axenic, as determined by microscopy.

Single colonies were transferred to SPP broth and grown overnight at 40°C for DNA extraction with a DNeasy PowerSoil kit (Qiagen). Extracted DNA was used for both Illumina and Nanopore sequencing. The Illumina library was prepared with the Nextera XT DNA sample preparation kit, and paired-end sequencing was performed using the MiSeq reagent kit v3 (600 cycles) and the MiSeq instrument as described by the manufacturer (Illumina, San Diego, CA, USA). The library for Nanopore sequencing was prepared using the one-dimensional (1D) genomic DNA sequencing protocol (SQK-LSK109) without any size selection. The library was loaded on a SpotON flow cell Mk I (R9.4) and sequenced with a MinION device (Oxford Nanopore), and reads were base called using Albacore v2.3.1. After quality filtering using fastp v0.19.4 (5), totals of 1,077,509 long reads (Nanopore) with an average length of 2,769 bp and 2,630,600 short reads (Illumina) with an average length of 281 bp were used for the assembly. Sequences were assembled *de novo* using a hybrid assembly strategy with Unicycler v0.4.7 (6). The assembly comprised 6 contigs in total. The largest contig length was 4,916,357 bp and covered 94.6% of the total assembled genome sequences. The draft genome sequence is 5,194,507 bp long with an average GC content of 55.90% and coverage of 147-fold. The assembly was visualized and validated using Bandage v0.8.1 (7). Default parameters were used for all software tools unless otherwise noted.

Genome annotation with the NCBI Prokaryotic Genome Annotation Pipeline v4.10 (8) predicted 5,067 genes, including 4,859 protein-coding genes with predicted

Citation Salgar-Chaparro SJ, Castillo-Villamizar G, Poehlein A, Daniel R, Machuca LL. 2020. Draft genome sequence of *Enterobacter roggenkampii* strain OS53, isolated from corroded pipework at an offshore oil production facility. Microbiol Resour Announc 9:e00583-20. https://doi.org/10.1128/MRA.00583-20.

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functions, 91 genes coding for hypothetical proteins, and 86 tRNA, 25 rRNA, and 6 noncoding RNA (ncRNA) genes. Average nucleotide identity (ANI) was calculated using pyANI v0.2.7 (9). It was found that OS53 is closely related to *E. roggenkampii* strain DSM 16690 (GenBank accession no. CP017184) with an ANI value of 98.62%.

The metabolic pathway identification was carried out using the KEGG Automated Annotation Server (KAAS) (10). The analysis revealed that the genome possesses an entire set of genes for glycolysis, tricarboxylic acid cycle, pentose phosphate, and fatty acid biosynthesis and degradation. Other genes potentially involved in corrosion reactions were also detected.

Data availability. This genome sequence was submitted to GenBank under accession no. JAACJF000000000. The raw reads have been deposited in the NCBI SRA database under accession no. SRR11492388 and SRR11492389.

ACKNOWLEDGMENTS

S.J.S.-C. thanks Woodside Energy Ltd. for the financial resources to assist with this work via a postgraduate scholarship. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We thank Mechthild Bömeke and Melanie Heinemann for technical support.

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GENOME SEQUENCES

Complete Genome Sequence of *Shewanella chilikensis* Strain DC57, Isolated from Corroded Seal Rings at a Floating Oil Production System in Australia

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ABSTRACT Here, we describe the genome of *Shewanella chilikensis* strain DC57, a facultatively anaerobic bacterium isolated from corroded seal rings at a floating oil production system in Australia. The genome of strain DC57 has a size of 4.91 Mbp and harbors 4,178 predicted protein-encoding genes.

Shewanella chilikensis is a facultatively anaerobic, Gram-negative, and rod-shaped bacterium (1). Members of the genus *Shewanella* have been reported to be associated with microbiologically influenced corrosion (2–6). *Shewanella* spp. have the ability to use a variety of electron acceptors, including nitrate, thiosulphate, and iron oxides (7), which indicates that these microorganisms can cause corrosion by different mechanisms.

S. chilikensis strain DC57 was isolated from corroded seal rings at a floating oil production system located in waters on the North West Shelf of Western Australia. Corrosion products were collected and inoculated in anaerobic phenol red broth medium (8). After positive growth in tubes incubated at 40°C, the culture was plated onto phenol red agar and incubated in anaerobic jars with AnaeroGen sachets (Oxoid). DC57 was purified using the streaking method until an axenic culture was obtained, as determined by microscopy. Single colonies were transferred to phenol red broth medium, and DNA was extracted using the DNeasy PowerSoil kit (Qiagen). Extracted DNA was sequenced with a combination of sequencing platforms. For Illumina sequencing, the library was prepared with the Nextera XT DNA sample preparation kit, and paired-end reads were generated on the MiSeq platform using the MiSeq reagent kit v3-600, as recommended by the manufacturer (Illumina, San Diego, CA, USA). For Nanopore sequencing, genomic DNA was prepared using the ligation sequencing kit 1D (SQK-LSK109) without any size selection. Sequencing was performed with the MinION Mk1B device and a SpotON flow cell R9.4, as recommended by the manufacturer (Oxford Nanopore Technologies, Oxford, UK). Base calling was performed using Albacore v2.3.1. Quality filtering of the reads was performed with fastp v0.19.4 (9), which resulted in 3,370,098 short reads (Illumina) with an average length of 245 bp, and 654,567 long reads (Nanopore) with an average length of 1,813 bp. A hybrid assembly strategy using Unicycler v0.4.7 (10) was applied to perform a *de novo* genome reconstruction, with overlap removal, circularization, and rotation. The assembly was validated with Bandage v0.8.1 (11). Default parameters were used for all software unless otherwise specified.

The complete genome of DC57 comprises a single circular chromosome (4,910,425 bp) with an overall GC content of 52.35% and 162-fold coverage. Annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v4.10

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(12), which predicted 4,434 genes, including 104 tRNA genes, 25 rRNA genes, 4 noncoding RNA genes, 4,178 genes encoding proteins with predicted functions, and 123 genes encoding hypothetical proteins. Classification was performed by calculating the average nucleotide identity (ANI) with the Python module for ANI analyses (pyANI) v0.2.7 (13). This analysis revealed that DC57 is closely related to *S. chilikensis* strain JC5 (GenBank accession number NZ_NIJM00000000.1) with an ANI value of 98.86%.

The genome analysis revealed the presence of the metal reduction pathway (MTR), two pathways for nitrate reduction (NAP and NAR), and genes for thiosulfate reduction (*phsA* and *glpE*), which could be related to the corrosive potential of the strain.

Data availability. The genome sequence of *Shewanella chilikensis* strain DC57 was submitted to GenBank under accession number CP045857. The raw reads were deposited in the NCBI SRA database under accession numbers SRR11492373 and SRR11492374.

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We thank Mechthild Bömeke and Melanie Heinemann for technical support.

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Appendix 7

Written statements from co-authors of the publications

To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled **"Effect of sample storage conditions on the molecular assessment of MIC"**.

_____
Signature of Candidate

I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Laura L. Machuca Suarez

_____ _____
Full Name of Co-author 1 Signature of Co-author 1

To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled **"Complementary DNA/RNA-Based Profiling: Characterization of Corrosive Microbial Communities and Their Functional Profiles in an Oil Production Facility"**.

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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Laura L. Machuca Suarez

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Full Name of Co-author 1 Signature of Co-author 1

To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Investigating the effect of temperature in the community structure of an oilfield microbial consortium, and its impact on corrosion of carbon steel**".

[redacted]
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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms**".

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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Nutrient Level Determines Biofilm Characteristics and Subsequent Impact on Microbial Corrosion and Biocide Effectiveness**".

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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Carbon Steel Corrosion by Bacteria from Failed Seal Rings at an Offshore Facility**".

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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Laura L. Machuca Suarez

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Full Name of Co-author 1	Signature of Co-author 1

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Anna Kaksonen

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Complete genome sequence of *Pseudomonas balearica* strain EC28, an iron-oxidizing bacterium isolated from corroded steel**".

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Genome Sequence of *Enterobacter roggenkampii* Strain OS53, Isolated from Corroded Pipework at an Offshore Production Facility**".

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I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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To Whom It My Concern,

I, Silvia Juliana Salgar Chaparro, contributed by conducting the research, interpreting the obtained data and writing up contents reported in the publication entitled "**Complete Genome Sequence of *Shewanella chilikensis* Strain DC57, Isolated from Corroded Seal Rings at a Floating Production System in Australia**".

_____
Signature of Candidate

I, as Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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Appendix 8

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Microbiologically influenced corrosion as a function of environmental conditions: A laboratory study using oilfield multispecies biofilms

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Complete Genome Sequence of *Pseudomonas balearica* Strain EC28, an Iron-Oxidizing Bacterium Isolated from Corroded Steel

Silvia J. Salgar-Chaparro, Genis Castillo-Villamizar, Anja Poehlein, Rolf Daniel, Laura L. Machuca

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Genome Sequences

Draft Genome Sequence of *Enterobacter roggenkampii* Strain OS53, Isolated from Corroded Pipework at an Offshore Oil Production Facility

Silvia J. Salgar-Chaparro, Genis Castillo-Villamizar, Anja Poehlein, Rolf Daniel, Laura L. Machuca

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Genome Sequences

Complete Genome Sequence of *Shewanella chilikensis* Strain DC57, Isolated from Corroded Seal Rings at a Floating Oil Production System in Australia

Silvia J. Salgar-Chaparro, Genis Castillo-Villamizar, Anja Poehlein, Rolf Daniel, Laura L. Machuca

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