WA School of Mines: Minerals, Energy and Chemical Engineering

Investigating Multispecies Biofilms on Steel Surfaces in Seawater and Biofilm Inhibition by a Novel, Multifunctional Inhibitor

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Declaration

To the best of my knowledge, this dissertation contains no previously published material, or material under consideration for publication except where due acknowledgement is assigned. This document is original and not under consideration for any award or publication elsewhere.

Signature:

Date:

18/04/2022

Dedication

This thesis is dedicated to my parents; Hayley and John Tuck who have sacrificed so much for my success, my two brothers Will and Jesse Tuck who have helped me see the light and good times, and my partner Monique Burton for her unfailing love, support and encouragement during the difficult times.

“Before this candle, my mind was a dark room. Upon striking the match, I could see no room, but instead, a universe of darkness waiting for the light”.

Abstract

Corrosion of metals is a global concern, amounting to an estimated cost of US $4 Trillion per annum. This figure considers prevention and maintenance of equipment and infrastructure from a diverse range of industrial sectors, including oil and gas, health, transportation and shipping, mining, and food processing. The metabolic activity of microorganisms is expected to contribute between 20 and 40% of total corrosion costs through a phenomenon known as microbiologically influenced corrosion (MIC). MIC depends on the development of a sessile microbial population known as a biofilm on the material surface. The biofilm phenotype is complex and organised, promoting the close association of cells known to facilitate interspecies and cell-substrate interactions. Importantly, the biofilm living arrangement also reduces chemical and physical vulnerability through the production of extracellular polymeric substances (EPS). The EPS is a structural and functional network, containing extracellular DNA (eDNA), proteins, polysaccharides, ions and other organic and inorganic material depending on the environment and contributing species. Within the EPS of a mature biofilm, cells are up to 1,000 times more tolerant to chemical treatments than their planktonic counterparts. Therefore, industries impacted by the activity of biofilms aim to target and kill biofilms at earlier, more vulnerable developmental stages. In the oil and gas industry, broad-spectrum biocides such as glutaraldehyde (GLUT) and tetrakis hydroxymethyl phosphonium sulphate (THPS) are widely applied for this purpose. Unfavourable toxicity profiles of traditional biocides gave rise to GLUT and THPS, now representing the primary line of defence against biofilms in marine oil and gas engineered systems. Although previously effective at reducing bacterial viability, GLUT and THPS are single-function compounds, and suffer from the development of tolerance acquisition by marine biofilms. Further, although current compounds have favourable toxicity profiles compared to traditional compounds, they still represent an unacceptable risk to personnel and the environment. One of the most promising of emerging strategies to tackle MIC are multifunctional organic inhibitor compounds; capable of addressing both corrosion and biofilm formation simultaneously. Organic film forming inhibitors are ideally suited for use in marine engineered systems since they can offer effective corrosion inhibition and biocidal activity with reduced toxicity. Additionally, corrosion and biofilm formation treatments are generally treated independently; thus multifunctional compounds also reduce the number of applied compounds at dosing intervals.

Importantly, application of any novel biocide can lead to biofilm tolerance acquisition. A lack of fundamental knowledge of marine biofilms has resulted in broad-spectrum treatments that invariably fail for this reason. Specifically, the early stages of biofilm formation, as well as mature biofilm species and EPS composition require further investigation to enhance biocide target specificity. Through targeted biofilm dispersal, compound application quantity, and thus costs, can be further reduced along with toxicity. The research presented in this thesis aims to contribute to the fundamental knowledge of marine biofilm formation through chapters 1 to 3. In the context of marine biofilm attachment and maturation, the research also aims to evaluate a promising novel, multifunctional corrosion inhibitor compound for biocidal efficacy, discussed in chapter 4. Ultimately, understanding biofilm development from previous chapters will lead to the enhancement of novel biocide efficacy through targeted dispersal of biofilms, evaluated and discussed in chapter 5. Each chapter is presented through published journal articles or journal articles under review.

Chapter 1 broadly and critically reviews the literature in the field and is presented in the form of two review manuscripts. The first, entitled "*Understanding natural biofilm development on steel in marine environments – a review"* assembles the current scientific understanding of biofilm developmental stages that form the foundations of modern biofilm research. This review was conducted with an emphasis on marine biofilm development on steels (Figure 1).

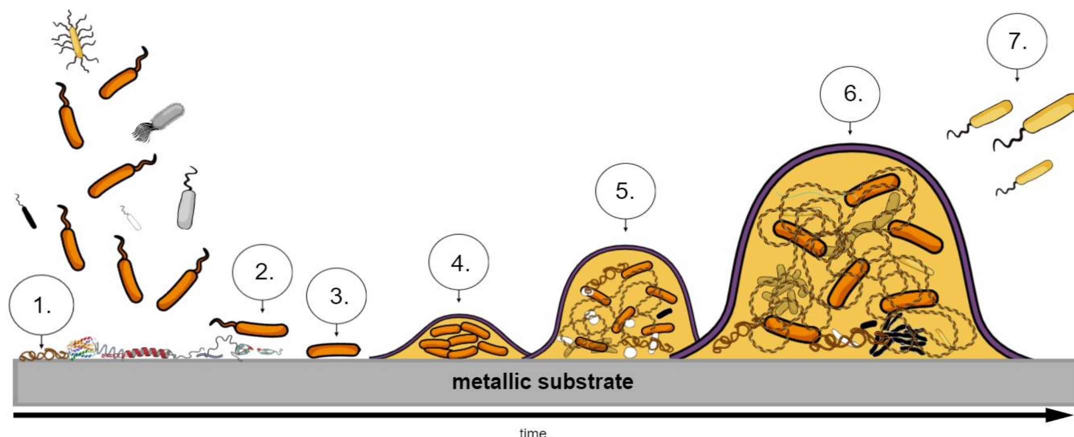

Figure 1: *Outline of biofilm development stages on metallic materials. 1) adsorption of an organic and inorganic conditioning film; 2) reversible attachment of marine bacteria; 3) irreversible adhesion involving surface acclimatisation and adaptation; 4) cellular division; 5) EPS production; 6) maturation and 7) dispersal.*

It is expressed that each stage of biofilm development is enormously complex and poorly canvased in the marine environment. Species and EPS diversity in natural biofilms are a primary cause for knowledge gaps in the field, involving interspecies interactions that are challenging to replicate *in vitro*. The manuscript; "*Understanding natural biofilm development on steel in marine environments – a review"* also discusses the emerging and developed techniques making multispecies marine biofilm studies more approachable.

Bacterial interactions are often evaluated in simplified systems that invariably lead to inconsistency in reports. Indeed, rigorous, highly controlled research has often failed to reach consensus on cell-surface interactions between metals and bacteria, the composition and role of biofilm EPS components and mechanisms that drive the developmental stages presented in Figure 1. Thus, the second review manuscript critically analyses the relevant literature to identify and discuss research inconsistencies involving multispecies biofilms. This manuscript, entitled "*Biofilms on metallic materials in seawater – a critical review*", compiles the relevant literature for this thesis concerning multispecies marine biofilms.

Chapter 2 investigates the first stages of biofilm formation under marine-simulating conditions. The adsorption of an interfacial organic conditioning film on CS is a ubiquitous process in marine environments, and typically involves proteins, amino acids, extracellular DNA (eDNA), polysaccharides, humic acids and other organic and inorganic environmental debris. The conditioning film is considered the first stage of biofilm development in chapter 2, with a central role in initial colonisation of metals. Depending on the conditioning molecule, conditioning film adsorption alters surface physico-chemical properties with direct positive or negative influences on initial bacterial contact. Two ubiquitous organic conditioning molecule types; namely eDNA and amino acids, have been previously characterised in marine conditioning films. Little is known about the adsorption properties of these molecules on steels and their unique contributions to the initial biofilm development process. Thus, chapter two explains the adsorption and effect of these molecules on the attachment of *Shewanella chilikensis* DC57, a true marine bacterial strain previously implicated in MIC. Through this research it was revealed that amino acids as conditioning molecules may also function to reduce galvanic current density and corrosion potential on CS, thereby limiting localised corrosion.

Finally, CS surfaces in marine environments are covered by an oxide film. In attachment studies outlined in chapter two, this oxide film was found to promote bacterial attachment by up to 12-fold compared to pristine metal surfaces prepared anaerobically. Bacterial attachment

in this context formed foundations for the evaluation of a novel, multifunctional inhibitor compound as a biocide. This work is described in chapter 4.

Chapter 3 considers the EPS and species composition of mature biofilms in marine conditions. It is emphasised that natural biofilms are multispecies communities, and thus chapter 3 assesses the community structure and EPS composition of a known community comprised of three bacterial isolates. Biofilms developed under marine-simulating conditions for 6 weeks were revealed to contain a structural network of eDNA. Ubiquitous biofilm polymers such as eDNA represent an ideal target for biofilm dispersal, which was further investigated in chapter 5.

Chapter 3 also combines an RNA and DNA-based sequencing approach to explore changes in population composition over the experiments. RNA-based analysis confirmed that the biofilm in marine environments is formed by populations that remain stable over time. Interestingly, the parallel DNA-based analysis indicated that the active fraction identified by RNA-based sequencing could significantly underestimate the true microbial population, which includes dormant cells with low cellular RNA. Additionally, the most active fraction of the community by relative abundance did not contribute the majority of eDNA to the matrix. The outcomes of this work are compiled in the manuscript entitled; "*Extracellular DNA is a critical structural polymer in marine multi-species biofilms*".

Chapter 4 evaluates the biocidal efficacy of a novel environmentally sustainable biocide based on a hexadecyl trimethyl ammonium cation and the *trans*-4-hydroxy-cinnamate anion. The compound, designated CTA-4OHcinn, was assessed in the context of marine bacterial attachment as previously evaluated in chapter two. The results of this research are discussed in the manuscript entitled; "*Evaluation of a novel, multi-functional inhibitor compound for prevention of biofilm formation on carbon steel in marine environments*". Biocide efficacy of at least 96.6% was observed against early stages of attachment in three marine bacterial isolates. Subsequently, mature oilfield communities were developed using samples obtained from a West-Australian oilfield under marine simulating conditions. These natural, multispecies communities were evaluated for susceptibility to CTA-4OHcinn, since treatment of biofilms at the early developmental stages is not always practical. This research is detailed in the manuscript entitled; "*Efficiency of a novel multifunctional corrosion inhibitor against biofilms developed on carbon steel*". Reports presented in this chapter confirm that the optimal corrosion inhibition concentration of CTA-4OHcinn, which is 10 mM, results in effective mitigation of biofilm development on CS. CTA-4OHcinn was also found to target the cell

membrane using a novel application of a confocal laser scanning microscope (CLSM) membrane-specific stain. Importantly, chapter four demonstrates the functionality of a novel, multifunctional corrosion inhibitor as an effective biocide. The compound has great promise for application in field scenarios.

Chapter 5 retrospectively applies information gained from previous work to improve the efficacy of CTA-4OHcinn. It is envisaged that regardless of initial potency, biocidal efficacy will be reduced by biofilm tolerance acquisition as frequently observed in response to current and traditional biocide and antibiotic compounds. Thus, through observation of recent advances in clinical biofilm treatments, and following EPS composition studies in chapter 3, chapter 5 applies reduced doses of CTA-4OHcinn to mature, multispecies biofilms and compares results obtained from the same treatment applied with a DNA degradation stage. The additional enzymatic treatment targeted eDNA in the biofilm to significantly enhance biocidal efficacy. The results of this work are detailed in the manuscript; "*Enhancing biocide efficiency: targeting extracellular DNA for marine biofilm disruption*". This research builds on previous objectives to demonstrate that enhanced target specificity can boost novel biocide efficacy without adverse environmental impacts.

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Firstly, I would like to express my appreciation and gratitude to Associate Professor Laura Machuca Suarez. In her capacity as primary supervisor, Laura has contributed significantly to the research detailed in this thesis through her wisdom, leadership, guidance and council. Thanks and acknowledgements extend to co-supervisor Professor Elizabeth Watkin, who's detailed and knowledgeable feedback was instrumental throughout the course of the project. My sincere gratitude is also expressed to co-supervisor Professor Maria Forsyth, for her guidance to all students involved in the Discovery Project and role in funding acquisition.

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List of publications included in this thesis:

This thesis is assembled by peer-reviewed publications and manuscripts currently under review, which form five chapters. Chapter 1 details the relationship between each chapter.

The research papers are listed below:

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. “Understanding natural biofilm development on steel in marine environments – a review”, in the Proceedings of the Corrosion & Prevention Conference, Paper no. 162. The Australasian Corrosion Association inc. 2020, Perth, Australia.

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. “ Marine biofilms on metallic materials – a critical review”. NPJ Materials Degradation, 2022.

B. Tuck, E. Watkin, M. Forsyth, A. Somers and L. L. Machuca. “Bacteria can utilise corrosion inhibiting amino acid conditioning film to reinitiate MIC”. *Under review in Bioelectrochemistry.*

B. Tuck, E. Watkin, M. Forsyth, A. Somers and L. Machuca Suarez. “Amino acid conditioning film enhances *Shewanella chilikensis* adhesion to metal surfaces”. *Biofouling, 2022.*

B. Tuck, S. Salgar-Chaparro, E. Watkin, M. Forsyth, A. Somers and L. L. Machuca. “Extracellular DNA is a critical structural polymer in marine multi-species biofilms”. *Under review in Proceedings of the National Academy of Sciences (PNAS).*

B. Tuck, E. Watkin, M. Forsyth, A. Somers, M. Ghorbani and L. L. Machuca. "Evaluation of a novel, multi-functional inhibitor compound for prevention of biofilm formation on carbon steel in marine environments." Nature: Scientific Reports, 2021.

B. Tuck, N. Leinecker, E. Watkin, A. Somers, M. Forsyth & L. L. Machuca. "Efficiency of a novel multifunctional corrosion inhibitor against biofilms developed on carbon steel". Frontiers in Bioengineering and Biotechnology, 2022.

B. Tuck, E. Watkin, M. Forsyth, A. Somers and L. L. Machuca. "Enhancing biocide efficiency: targeting extracellular DNA for marine biofilm disruption". *Under review in Proceedings of the National Academy of Sciences (PNAS).*

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Statement of contribution by authors

I, Benjamin Tuck, declare that planning, conceptualisation, conducting experiments, data analysis and manuscript preparation was primarily executed by myself as first author on all manuscripts comprising this thesis. Co-author contributions are outlined below with written statements provided in appendix 1.

Associate Professor Laura. L Machuca-Suarez significantly contributed to the supervision and guidance of this project, including conception of experimental methods, data analysis and critical review and feedback of all manuscripts.

Professor Elizabeth Watkin significantly contributed to the supervision and provided critical guidance in experimental design and review of all manuscripts.

Professor Maria Forsyth supervised the project and provided significant guidance, manuscript review and feedback relating to corrosion and corrosion inhibition. Maria significantly contributed to funding acquisition.

Dr. Anthony Somers contributed greatly to supervision the project and provided guidance in experimental design and all manuscript critical review. Anthony also contributed significantly to the design and synthesis of novel inhibitor compounds applied in this thesis.

Mahdi Ghorbani Contributed to the project through synthesis of novel inhibitor compounds which were transported to Curtin University for further evaluation.

Dr. Nadia Leinecker contributed significantly to the manuscript; "*A novel, multifunctional corrosion inhibitor is an effective biocide against mature multi-species biofilms*" through reactor set-up and analysis.

Dr. Silvia Juliana Salgar Chaparro Greatly helped with data processing and writing of manuscript "*Extracellular DNA is a critical structural polymer in marine multi-species biofilms*".

All research presented as part of this thesis was funded by an Australian Research Council (ARC) Discovery Project (DP) Grant and the Curtin Corrosion Centre, Curtin University, Bentley. All experiments were conducted at the Curtin Corrosion Centre and Curtin Health Innovation Research Institute (CHIRI). Confocal laser scanning microscope (CLSM) training was conducted at the University of Vienna (Universität Wien) Division of Microbial Ecology (DOME), The University of WA centre for Microscopy, Characterisation and Analysis (CMCA) and CHIRI.

Conference Presentations:

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. "Understanding natural biofilm development on steel in marine environments – a review", in the Proceedings of the Corrosion & Prevention Conference, Paper no. 162. The Australasian Corrosion Association inc. 2020, Perth, Australia.

B. Tuck & L. L. Machuca. The Australasian Corrosion Association 12th Annual Brian Cherry Awards, 2020.

B. Tuck & L. L. Machuca. **"**Ecofriendly multifunctional inhibitors against microbiologically influenced corrosion". Reservoir Microbiology Forum, 2021.

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Definitions

Anaerobic: the absence of gaseous oxygen.

Abiotic: the absence of living organisms.

Bacterial attachment: the initial, reversible stage of contact between a bacterial cell and a substrate.

Bacterial adhesion: the irreversible stage following attachment by which bacterial cells become permanently associated with the surface.

Biocide: a chemical compound that aims to reduce bacterial viability.

Biofilm: a sessile population of microorganisms living at an interface, usually associated with an aqueous phase.

Biotic: conditions that contain living organisms.

Carbon steel (CS): steel produced using carbon as the primary alloying element.

Conditioning film: a layer of adsorbed organic and inorganic material on an interface.

Corrosion: the process converting refined materials to more chemically stable forms.

Extracellular Deoxyrybonucleic acid (eDNA): deoxyribonucleic acids of genomic origin that is produced by lysis of bacteria or active excretion mechanisms.

Extracellular Polymeric Substances (EPS): self-produced polymers that form the biofilm matrix, including proteins, eDNA and polysaccharides.

Microbiologically influenced corrosion (MIC): the corrosion initiated, mediated or exacerbated by the presence and activity of microorganisms.

Microorganism: a microscopic organism such as bacteria or fungi.

Multifunctional inhibitor: a chemical capable of slowing or evading more than a single undesirable phenomenon on a substrate simultaneously; for example corrosion and biofilm formation on steels.

Under deposit corrosion: Corrosion mediated, maintained or perpetuated under deposits such as present within pipelines.

List of Abbreviations

AHS……………………... Acylated homoserine lactones

AISI………………………American Iron and Steel Institute

ASW…………………….. Artificial seawater

cAMP:……………………Cyclic adenosine monophosphate

CLSM:……………………Confocal laser scanning microscope/microscopy

CS:…………………...... Carbon steel

DNA: ……………………Deoxyribonucleic acid

EPS:………………………Extracellular polymeric substances

eDNA:……………………Environmental or extracellular deoxyribonucleic acid

HGT:……………………... Horizontal gene transfer

MEA:……………………... Multi-electrode array

MIC:………………………Microbiologically influenced corrosion

NO: ………………………Nitric oxide

PDMS:……………………Polydimethylsiloxane

PNAG:……………………Polymers of N-acetyl glucosamine

QS:………………………... Quorum sensing

SEM:………………………Scanning electron microscopy

SRB……………………….. Sulphate reducing bacteria

Chapter 1 INTRODUCTION

1.1 Literature Review

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. "Marine biofilms on metallic materials in seawater – a critical review". NPJ Materials Degradation, 2022.

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. "Understanding natural biofilm development on steel in marine environments – a review", in the Proceedings of the Corrosion & Prevention Conference, Paper no. 162. The Australasian Corrosion Association inc. 2020, Perth, Australia.

1.2 Statement of the Problem

Biofilm formation on refined materials remains a major concern in the 21st century, resulting in severe consequences for human health, transportation, food processing and oil and gas operations, among others[1-4]. Billions of dollars in annual loss, environmental impacts and loss of human life are linked directly or indirectly to biofilms. In marine environments, the establishment and development of mature biofilms on artificial surfaces threaten most infrastructure, resulting in MIC and biofouling. Carbon steel (CS), as the most broadly applied structural material in the oil and gas industry, is particularly susceptible to MIC and other forms of corrosion[5,6]. Localised damage to CS generated by microbial activity can result in catastrophic environmental and economic impacts, especially where pipelines or vessels containing crude oil are affected[6].

Although decades have transpired since the biofilm theory was first proposed, gaps in knowledge lead to inefficient treatment and management of biofilms in all affected industries including oil and gas operations. Extensive research now outlines and details the stages and mechanisms of biofilm formation; yet to date no artificial surface produced is immune to bacterial attachment as the first stage of microbial interaction with a material. In extension, MIC and biofouling continue to reduce the service life of submerged materials. The pervasiveness of MIC and biofouling reflects the urgency of improving biofilm mitigation strategies; however, gaps existing in the fundamental scientific understanding of biofilm formation must first be addressed for the success of developing treatments.

A plethora of commercially available biofilm treatments have been reported for use in and on marine infrastructure. Where mechanical treatments and coatings are not always practical (for

example inside pipelines), biocide and corrosion inhibitor compounds are often applied alongside mechanical scrubbing (for example, pigging). A critical analysis of existing research reveals a number of limitations associated with current biocidal compounds. Biocides used in seawater environments are generally toxic, and are treated separately to corrosion inhibitors, resulting in high application costs, environmental exposure and risk to operational personnel. An emerging solution to these limitations is multifunctional organic corrosion inhibitor compounds (OCIs)[7-9]. Unlike current biocides that target cell viability alone, multifunctional OCIs combine biocidal effects with corrosion inhibitory effects, ultimately to reduce dosing quantities and costs. Additionally, multifunctional OCIs can be effective with significantly lower toxicity profiles than current alternatives. Although these compounds are demonstrating great promise for biofilm and corrosion management, there is a lack of scientific evidence to support efficacy.

To develop and enhance biocides it is also important to understand the fundamental aspects of biofilm formation in greater detail. Although biofilm developmental stages universal to most environments have been well characterised, knowledge of critical aspects within each stage is not complete. The first stage of biofilm development, known as the conditioning film formation, is immediately followed by bacterial attachment and adhesion[10]. These processes have generated traction in scientific literature since the progression of a biofilm to maturity depends upon their success. Further, although conditioning film formation and bacterial attachment have been observed and characterised in marine environments, there is limited understanding of the link between the conditioning film and bacterial attachment on steel surfaces. Some organic conditioning film molecules present in the marine environment may also influence substrate corrosion. A greater understanding of the fundamental aspects of these early stages of biofilm formation is required to develop more sophisticated biofilm control measures.

Single-species biofilm investigations have provided a great deal of information about biofilm development and the effects of various biocides on bacteria. While these studies simplify data analysis and lead to more reproducible results, monocultures cannot reflect the diverse coping mechanisms exhibited by natural biofilms in response to chemical stress[11]. In the marine environment, microbial colonisation is often not controlled at the early stages of biofilm development, resulting in complex, multi-species communities that are recalcitrant to environmental stresses and treatments. Although there is evidence to suggest that EPS confers

enhanced biofilm tolerance, there is limited research addressing the EPS composition of marine biofilms formed on metal substrates.

Finally, natural biofilm tolerance acquisition is a significant concern leading to reduced biocidal efficacy[12,13]. Limited scientific reports on the multispecies biofilm EPS and species composition restrict the development of more targeted approaches to biofilm mitigation. Biocide function is typically broad-spectrum, relying on viability reduction achieved through high toxicity profiles. By understanding the composition of multispecies marine biofilms, novel inhibitor compounds can be incorporated with, or developed alongside biofilm dispersal treatments. Through targeting of specific biofilm components, application quantities and therefore toxicity and costs, can be effectively reduced.

1.3 Aim and Objectives

This project aims to enhance the fundamental understanding of early biofilm development on carbon steel in seawater environments, and identify key components of the extracellular matrix critical to the mature biofilm structure. In parallel, this investigation aims to evaluate a novel, multi-functional and organic corrosion inhibitor compound for a secondary function as a biocide. In accordance with these primary aims, specific objectives of this research are outlined as listed below:

- Critically and comprehensively review the available scientific literature surrounding biofilm developmental stages, particularly in seawater environments.

- Investigate early biofilm formation and identify key components of the conditioning film that affect CS corrosion and initial bacterial attachment to CS.

- Evaluate multi-species biofilm formation in marine environments. Identify key polymers produced by the biofilm matrix and improve the fundamental understanding of how multi-species biofilm populations change over time in marine conditions.

- Evaluate a novel organic corrosion inhibitor compound synthesised as part of the larger Australian Research Council (ARC) Discovery Project (DP), grant number 180101465. Evaluate the biocidal capacity of the compound against early attachment stages and on mature biofilms.

- Using data from previous objectives, and in anticipation of biofilm tolerance acquisition to chemical treatments, identify a targeted method to improve the efficacy of the novel, multifunctional inhibitor compound.

1.4 Connection between research topics:

Biofilm formation has been characterised according to a series of discrete stages, including conditioning film formation, initial colonisation (attachment and adhesion), development and maturation. All work from the included investigations addresses two primary aims in the context of these stages, listed below, presented in a logical order:

- To understand marine biofilm formation on steel in greater detail.
- To evaluate and improve the efficacy of a new biocide compound in the context of marine environments on steel.

Since species and EPS composition is known to vary between biofilms exposed to different environments, chapter 1 explores the scientific literature published in the context of marine biofilm development, with an emphasis on metallic materials. The chapter 1 review aims to provide a foundation for subsequent chapters and combine, expose inconsistencies and identify knowledge gaps in the relevant literature.

Mature marine biofilms on metals host diverse and recalcitrant microbial populations leading to MIC and biofouling[14,15]. Therefore, to effectively control biofilm maturation it is especially relevant to understand the preceding developmental stages. In chapter 2, knowledge gaps were addressed in relation to conditioning film formation, initial attachment and biofilm development.

The conditioning film has been characterised to reveal composition in marine environments previously[10,16]. Although DNA and amino acids have been reported as conditioning molecules[17], there is very limited understanding of how these molecules interact with metals, and the influence these molecules have on bacterial attachment. Therefore, electrochemical effects of amino acids and DNA, and the influence of these molecules on initial colonisation stages was evaluated on CS in chapter 2.

Iron oxides are also associated with survival benefits to bacteria, and therefore iron oxide surfaces were proposed as a variable affecting early colonisation of metal interfaces. This phenomenon is associated with objectives outlined for chapter 2, however the material is included in chapter 4 since the primary aim of this study was to evaluate biocidal efficacy on attachment using a novel, multifunctional compound.

Chapter 3 acknowledges that initial attachment stages involve relatively vulnerable bacterial cells that are not protected by the community or the EPS[18]. Following failed mitigation of the initial colonisation stages, biofilm maturation still progresses in treated engineered systems. As the most challenging developmental stage of the biofilm to control, chapter 3 was necessary to explore the unique elements of the mature, multispecies marine biofilm that can lead to target identification for treatment enhancement.

Forming the mature biofilm is the cellular component (primarily bacterial cells) and the extracellular component (primarily EPS). Thus, chapter 3 specifically focuses on understanding these aspects of the biofilm. The isolates used for previous studies were combined in chapter 3, forming a multi-species biofilm to evaluate shifts in biofilm community structure. In this controlled environment, the EPS composition was screened to determine the dominant matrix polymer. Chapter 3 explores the maturation of marine biofilms as a progression from the earlier stages explored in chapter 2.

Subsequently in chapter 4, a novel compound proposed as a multi-functional corrosion inhibitor was evaluated for biocidal efficacy. While limiting microbial attachment is a primary aim, biocide evaluations should also demonstrate efficacy against mature biofilms as frequently associated with industrial equipment and infrastructure failures. Inhibitor evaluation was closely related to chapters 2 and 3, since this research was conducted in the context of early and mature biofilms as presented in these chapters. The effects of CTA-4OHcinn were detailed against early colonisation of CS, where biocidal efficacy could be demonstrated regardless of initial viable cell numbers on CS surfaces. CTA-4OHcinn was subsequently evaluated against mature oilfield communities developed on CS. The effects of CTA-4OHcinn on individual cells were expressed in chapter 4 using CLSM through application of general cell membrane targeting dyes.

The final topic of this thesis builds on research outcomes from chapters 3-4. In chapter 5, biocide evaluations are combined with a targeted EPS dispersal approach that was developed based on data obtained from mature biofilm studies in chapter 3. Inhibitor doses are also applied

through an understanding of practical effectiveness obtained in chapter 4. The function of CTA-4OHcinn was significantly enhanced, indicating that DNA as a critical EPS component could be specifically targeted to enhance biocide efficacy.

1.5 Significance of the Research and Contribution

Fundamental research evaluating marine biofilm formation processes is limited. As the first stages of bacterial colonisation, conditioning film formation, bacterial attachment and bacterial adhesion represent topics of current scientific interest. By understanding these critical precursor stages, biofilm management strategies can aim to target biofilm formation before mature, chemically recalcitrant communities develop. Since MIC relies on the initial attachment of bacterial cells, which in turn rely on the adsorption of the conditioning film, further advancements to the scientific understanding of these processes holds economic and environmental significance. Research to date identifies the components in the marine conditioning film which, as a whole, promote bacterial attachment to metals. It is expressed that despite a significant research effort, still no metallic surface applied in the marine environment is immune to bacterial attachment. Without further understanding of the conditioning film components, and in particular the beneficial components for bacterial attachment, complete evasion of bacterial colonisation cannot be achieved. Chapter 2 identifies three ubiquitous marine conditioning film components that significantly promoted attachment and enhanced biofilm formation capacity of marine bacteria. This research broadens the fundamental scientific knowledge on bacterial attachment to metals exposed to marine conditions. It is also anticipated that further understanding of factors critical to the attachment process(s) can lead to the prevention of biofilm formation.

Natural marine biofilms participating in MIC are known to be heterogeneous in species and EPS composition. The superior diversity of natural biofilms compared to many laboratory simulations has led to underestimations of biofilm tolerance acquisition, a serious global economic and health concern. However, almost nothing is known about these communities on CS. Chapter 3 contributes valuable insight to more natural multispecies communities, expressing for the first time the EPS composition of marine biofilms developed on steel in marine conditions. This research reveals that extracellular DNA (eDNA) is ubiquitous in these biofilms, providing a foundation for enhancing biocide efficacy through a targeted biofilm dispersal approach. Additionally, by investigating the multispecies biofilm composition

valuable information was gained concerning eDNA origin in the biofilm and population structure. Over time, it was revealed that the active fraction of the community (as determined by RNA-based sequencing) and was not necessarily responsible for eDNA production. Dormant or less active fractions of the community were instead crucial to production of the eDNA pool. Additionally, the activity of the population and eDNA matrix remained relatively stable over several weeks of experiments. Thus, specific enabling populations of the biofilm could be targets for enhanced biofilm treatment efficacy.

Using chapters 2 and 3 as a foundation, chapter 4 explores the biocidal efficacy of a novel multifunctional inhibitor compound with low toxicity profile, designated Cetrimonium-*trans*-4-hydroxy-cinnamate (CTA-4OHcinn). By applying CTA-4OHcinn in optimal concentrations for corrosion inhibition, it was revealed that bacterial attachment and mature, multispecies oilfield communities could be effectively controlled by the compound. Importantly, this research contributes the first scientific understanding of the novel inhibitor as an effective biocide. This information will support the commercialisation of CTA-4OHcinn for real-world application, introducing it as a true multifunction inhibitor compound.

Real-world application of biocidal compounds invariably result in enhanced biofilm chemical tolerance. There is limited research evaluating approaches to enhance biocidal efficacy in a sustainable way. Chapter 5 acknowledges the importance of biocide efficacy, as well as consideration of biofilm tolerance acquisition and environmental sensibility to introduce a targeted approach to biofilm dispersal. By first targeting the ubiquitous EPS matrix polymer eDNA, biocide efficacy could be significantly increased without negative environmental impacts. This research contributes a potential mechanism for the enhancement of CTA-4OHcinn and other biocides, and more importantly identifies a significant structural weakness in marine biofilms for future treatment proposals. Finally, similarities between clinical and environmental biofilms are an important consideration (for example the EPS composition and use of virulence factors to survive environmental conditions). Research from this chapter describes a more targeted approach to biofilm control applicable to a wide range of settings besides marine infrastructure.

Together, this thesis introduces a novel and effective biocidal compound for mitigation and control of natural marine biofilms. The compound is also an effective corrosion inhibitor as determined by the broader ARC DP, with great potential for real-world application. Additionally, this research significantly enhances the understanding of critical biofilm

formation stages that lead to global concerns such as biofouling and MIC. This knowledge can be applied to other environments affected by microbial growth, including the food processing, health and agricultural industries. Lastly, this research identifies a critical structural component of an environmental biofilm matrix in unexplored industry settings, further demonstrating a target-specific approach to biofilm control that is both effective and environmentally sensitive.

1.6 Overview of the Research Design

Several methods were used in this investigation to assess bacterial attachment, biofilm formation, biocide efficiency and corrosion. Broadly, culture dependent and independent methods were employed with a special emphasis on microscopic techniques to assess bacterial viability, interfacial attachment and population dynamics. A consortium was developed using four bacterial isolates, combined or separated, and chosen based on natural source, key metabolic traits and potential or demonstrated metabolic capabilities (genotypic and phenotypic features). Bacterial attachment and biofilm formation was evaluated on carbon steel material for all experiments. Anaerobic experiments were conducted using pure nitrogen gas, and reactors were established using batch or continuous flow conditions (i.e. media and nutrient availability was supplied either periodically or continuously depending on the experimental objectives). The base test solution, a well-defined artificial seawater adapted from previous work[19], remained constant in all experiments for reproducibility. A working temperature of 30°C was also maintained across all experiments. Exposure times, nutrients and atmosphere type varied in laboratory studies depending on the objectives of these studies, and the detailed methodology and materials are outlined in each chapter. Baseline corrosion data was established using abiotic experiments for all relevant studies.

1.6.1 Microorganisms and Test Methods

- **Microorganisms:**

Bacteria exhibiting certain characteristics (i.e. metabolic, reproductive, tolerance, polymer production) were selected to form a robust, multi-species community. Hallmark features of this combined community include;

- Ability to survive and contribute to the biofilm community over time, under both anaerobic and aerobic conditions in the artificial seawater test solution;
- Ability to quickly reproduce (reach log phase within 48 hours) using the same organic carbon source;
- Capacity to be enumerated using both culture-dependant and culture-independent methods;
- Association or implication in MIC.

Additionally, unique contributions of each strain to the biofilm community were also considered, including EPS production and genetic profile. *Shewanella chilikensis* strain DC 57, a laboratory isolate of *Klebsiella pneumoniae*, *Pseudomonas balearica* strain EC 28 and a laboratory isolate of *Enterobacter aerogenes* consisted the final community 'bank'. Various combinations of these isolates were included throughout the investigation depending on the research objectives.

- **Microbiological and biochemical methods:**

Microbial enumeration was conducted using a combination of culture-dependant and culture-independent methods. Assessment of viability and attachment was conducted using the MPN 3-tube standard method and standard serial dilution followed by enumeration of colony forming units (CFUs) as described elsewhere[20]. Semi-quantitative viability assessments were assessed using confocal microscopy followed by post-image statistical analysis.

Microbial activity was assessed through quantification of adenosine triphosphate (ATP). Concentrations of ATP, ADP, and AMP were determined by luminescence after reaction with luciferin-luciferase using commercial kits (Luminultra Technologies Ltd.).

Microbial community composition: DNA and RNA was extracted from biofilm and planktonic communities and 16S ribosomal subunit next-generation sequencing (NGS) was conducted to determine relative abundance of each species in the community.

Experimental inoculations and manual cell counts: Cells were harvested in log phase for all experiments. For enumeration, a light microscope was used in conjunction with a Neubauer haemocytometer to count all experimental inoculations. In all direct comparisons (for example, experimental and control studies), identical cell volumes were used based on counted cell quantities from parent cultures. These volumes were washed by centrifugation

in ASW or PBS solution and subjected to an acclimatisation process, involved incubating in the experimental media solution for at least one hour at 30°C before inoculating into experiment reactors.

- **Microscopic analysis of attachment and biofilm formation:**

Confocal laser scanning microscopy (CLSM): Fluorescent dyes can be used to target components of the biofilm, including live or dead cells, cell components or EPS (extracellular) components. By binding to the target molecule (such as double stranded intracellular DNA), the fluorescent signal returned from fluorophores is greatly enhanced by laser excitation compared with fluorophores not bound to the target. A confocal laser scanning microscope (CLSM) can be employed to excite the fluorescent dyes and subsequently collect the emitted light. The laser used to excite the fluorescent dye is passed through a pinhole to minimise out of focus light (noise). Image processing software platforms such as IMARIS (Bitplane™) can interpret fluorescent signal and produce meaningful data from fluorescent signal. For example, multiple micrographs captured across the Z-range of the microscope can be combined and processed to form a 3D reconstruction of the surface. In this investigation, the distribution and relative quantity of live and dead cells in biofilms the EPS composition and the attachment of DNA was assessed using CLSM. The dyes used to achieve the objectives are outlined below:

- **Propidium iodide:** targets all double stranded DNA and emits a red signal. When applied with SYTO™ 9, this stain is displaced in live cells and therefore the remaining red signal corresponds with dead cells, damaged cells or extracellular DNA. Excitation/emission maxima: 493/636 nm.
- **SYTO™ 9:** targets intracellular double-stranded DNA, displacing propidium iodide. The signal obtained from SYTO™ 9 emission can be interpreted as living cells. Excitation/emission maxima: 483/501 nm.
- **DiYO™-1**: analogous to YOYO®-1, this stain is highly specific to extracellular DNA and cannot transverse the cell membrane. Excitation/emission maxima: 491/508 nm.
- **SYPRO® Orange:** stains proteins and glycoproteins as small as 6.5 kDa with high selectivity in the presence of polysaccharides and DNA, making the stain useful for targeting biofilm EPS proteins. Excitation/emission maxima: 470/570 nm.

- **Lectin-Alexa Fluor™ 633 conjugate stains:** Concanavalin A (ConA) and wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 633 stains were used simultaneously to target a-mannopyranosyl and a-glucopyranosyl residues (ConA) and sialic acid and N-acetylglucosaminyl residues (WGA). Together, these lectin stain conjugates can be used to estimate total polysaccharide content in the biofilm. Excitation/emission maxima: 632/647 nm.

Image processing and post-image analysis:

Nikon NIS-Elements software version 5.20 was used to capture micrographs and generate 3D reconstructions of biofilms using data collected from CLSM. Micrographs were captured for the purpose of surface visualisation using the entire field of view. The Nyquist function optimises the field of view for signal quantification and was applied in IMARIS (Bitplane) for (semi)quantitative analysis.

IMARIS (Bitplane) software version 9.7 and 9.8 was used to process and quantify light signal returned from target-specific stains captured during confocal microscopy. Post-image analysis provided an estimation of biofilm parameters which are useful for understanding the health, viability and composition of the biofilm architecture. Biofilm parameters including total biomass per unit area (biovolume, represented as the sum biofilm signal from all appropriate channels), live cell signal (the signal returned from SYTO™ 9 by excitation with a 489nm laser, and captured using a 525/50 nm emission filter), dead or damaged cell signal (the signal returned from propidium iodide by excitation with a 561 nm laser, and captured using a 595/50 nm emission filter) and compactness (as the sum of signal density per unit area) are used to express biofilm structure and viability in the following chapters. Finally, by quantifying total channel signals independently using methods applied for SYTO™ 9 and propidium iodide, biofilm EPS composition could be estimated as % macromolecule (eDNA, proteins and polysaccharides) contribution to the EPS matrix.

Scanning electron microscopy (SEM): Biofilm structure and architecture was assessed using Field emission scanning electron microscopy (FESEM). Two microscopes were primarily used in this investigation: the Tescan variable pressure MIRA FESEM and the Zeiss NEON FESEM. A biofilm preparation procedure was employed as previously described[21], with the following modifications:

2. To avoid shock induced by sharp temperature changes, samples taken from experiments were placed into prewarmed fixative solution and allowed to slowly cool before fixing overnight.
3. Coating was performed after dehydration stages using 9 nm of sputter coated platinum to reduce the effects of charging.

- **Substrate material surface preparation:**

Rods of grade AISI 1030 and 1020 CS were used for all experiments in this investigation, cut into coupons of dimensions 5 mm width x 12.6 mm diameter. These coupons were wet-ground with silicon carbide sandpaper in the order 80 grit, 120 grit and 320 grit before degreasing in 100% acetone and soldering a copper wire to one side. Coupons were then electrocoated using Powercron® 6000CX solution. One working surface was prepared using 120 grit sandpaper for attachment and biofilm studies. Coupons were then rinsed in 100% ethanol, dried under N_2 gas and stored under vacuum desiccation until use.

Coupons prepared as described above were used where pristine metal surfaces were applied to experiments. Where oxidised surfaces were applied in attachment studies, sterile coupons were transferred into reactors containing aerobic ASW solution until a macroscopic oxide layer had formed on the surface.

For multi electrode array (MEA) studies, a probe comprising 100 × AISI 1030 CS wires embedded in resin was wet-ground using silicon carbide sandpaper in the following order: 320g, 600g; before immersing in anaerobic 70% ethanol for 15 minutes. Coupons were then exposed to ultra violet radiation for 10 minutes each side to sterilise before applying to experiments.

1.7 Thesis Outline

Chapter 1: Introduction and literature review.

1. **B. Tuck**, E. Watkin, A. Somers, L. L Machuca. "Biofilms on metallic materials in seawater: a critical review". <u>NPJ Materials Degradation. 2022.</u>

2. **B. Tuck**, E. Watkin, A. Somers, L. L. Machuca. "UNDERSTANDING NATURAL BIOFILM DEVELOPMENT ON STEEL IN MARINE ENVIRONMENTS – A REVIEW". Corrosion & Prevention. 2020.

Chapter 2: Evaluating marine bacterial attachment to CS.

3. **B. Tuck**, E. Watkin, M. Forsyth, A. Somers and L. Machuca Suarez. "Conditioning of metal surfaces enhances *Shewanella chilikensis* adhesion". Biofouling. 2022.

4. **B. Tuck**, E. Watkin, M. Forsyth, A. Somers and L. Machuca Suarez. "Corrosion inhibition on steel by L-amino acid conditioning film is reversed by *S. chilikensis*". Under review in Bioelectrochemistry.

Chapter 3: Marine multispecies biofilm population dynamics and EPS composition.

5. B. Tuck, S. Salgar-Chaparro, E. Watkin, A. Somers, M. Forsyth and L. L. Machuca. Extracellular DNA: a critical component of marine biofilms. Manuscript ready for journal submission (Proceedings of the National Academy of Sciences).

Chapter 4: Green, multifunctional biocide evaluations.

6. **B. Tuck**, N. Leinecker, E. Watkin, A. Somers, M. Forsyth & L. L. Machuca. "Efficiency of a novel multifunctional corrosion inhibitor against biofilms developed on carbon steel". Frontiers in Bioengineering and Biotechnology. 2022

7. **B. Tuck**, E. Watkin, M. Forsyth, A. Somers, M. Ghorbani and Laura L. Machuca. "Evaluation of a novel, multi-functional inhibitor compound for prevention of biofilm formation on carbon steel in marine environments". Nature: Scientific Reports. 2021. *This paper is also relevant to chapter 1.*

Chapter 5: Enhancing biocide efficacy through targeted biofilm disruption.

8. **B. Tuck**, E. Watkin, A. Somers, M. Forsyth and L. L. Machuca. Enhancing biocide efficacy: targeting extracellular DNA for marine biofilm disruption. Manuscript ready for journal submission (Proceedings of the National Academy of Sciences).

Chapter 6: Summary, Conclusions and Future Work

Summary and conclusions

Study limitations

Future work

Appendices

1.8 References:

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Chapter 1: Introduction and literature review

Manuscript # 1 of 2

B. Tuck, E. Watkin, A. Somers, L. L. Machuca. "Marine biofilms on metallic materials in seawater – a critical review". NPJ Materials Degradation, 2022.

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REVIEW ARTICLE OPEN

A critical review of marine biofilms on metallic materials

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The formation of multi-species biofilms on marine infrastructure costs the global economy US $ billions annually, resulting in biofouling and microbiologically influenced corrosion. It is well documented that complex biofilms form on almost any submerged surface, yet there are still no truly effective and environmentally friendly treatment or prevention options available. An incomplete fundamental understanding of natural biofilm development remains a key limitation for biofilm control measures. The purpose of this review is to compile the current literature and knowledge gaps surrounding the development of multi-species biofilms in marine conditions on metals.

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INTRODUCTION

Surface colonisation by microorganisms occurs in almost all environments on earth[1]. Simulations have demonstrated that some bacterial populations can remain viable after exposure to intense radiation stress, high vacuum and temperatures simultaneously; conditions characteristic of extra-terrestrial environments[2]. The widespread abundance of bacteria in terrestrial and aquatic environments is predominantly due to the recalcitrant biofilm lifestyle; the preferred living arrangement of bacteria[3]. Biofilms are defined as aggregates of cells surrounded by self-produced extracellular polymeric substances (EPS) that develop at a phase boundary[4–6], reported to the literature by Zoebell almost 80 years ago[7]. Today biofilm establishment has been widely studied in many environmental and clinical settings[8–13]. Marine infrastructure in particular suffers from contamination and materials degradation as a result of biofilm formation, generating research incentive today.

Microbiologically influenced corrosion (MIC) is an electrochemical degradation process initiated, maintained or enhanced by microorganisms and their metabolisms, and usually includes a mixed consortium living in a biofilm[14]. Both MIC and biofouling are costly downstream effects of biofilm formation. In 2005, general corrosion was estimated to cost around $3–7 billion per annum[15,16], a figure that was revised in 2016 by the National Association of Corrosion Engineers to be closer to $2.5 trillion[17]. MIC accounts for at least 20% of these costs[18,19]. The economic impact of MIC is the product of growing equipment application in marine environments (including submerged pipelines, ship hulls and floating off-shore production facilities). As equipment ages the economic burden of MIC is expected to increase in future years. Thus, research incentive from industry has generated a wealth of information on biofilms both in natural and laboratory settings.

Biofilms in natural environments are almost always described as diverse, or having more than one species[20,21]. The flexibility and adaptability of these populations has led to difficulty in prevention and management of deleterious biofilms in marine environments, as well as in conducting reproducible research. More traditional mono-species simulations in vitro may frequently prove inadequate for the elucidation of environmental mechanisms or for replicating environmental phenomena[20–22]. Biofilms in the more complex natural state; such as on metals deployed in marine environments are also harder to treat with antibiotics and biocides, as each species may demonstrate unique tolerance features. In the marine environment biofilms form quickly and have rapid recovery times, especially compared to mono-species laboratory simulations. These phenomena are still not fully understood, despite their significance to global industry and research. While fundamental knowledge gaps remain in relation to biofilms in marine environments, treatment of MIC and biofouling continue to present a major concern for stakeholders.

Marine environments impose a unique and challenging lifestyle on biofilms, promoting the development of recalcitrant multi-species populations. Simply put, prevention of material degradation in marine environments by bacteria can be achieved by preventing biofilm formation, which occurs in a series of well-defined stages[13]. However, scientific literature so far reveals that each stage is dynamic and complex. The success of each stage is governed by a plethora of cell-substrate and cell–cell interactions, leading to difficulties in multi-species biofilm studies. Treatment of marine biofilms therefore remains a current and ongoing concern. This review critically summarises research on biofilm development in relation to metallic materials in marine environments and briefly discusses the technology that is making multi-species biofilm research more approachable[20].

BIOFILMS ON METAL SUBSTRATES

In this communication, biofilm formation on metals in marine environments is considered unique from other materials and conditions. Metal substrates in marine environments impose unique challenges that ultimately shape the community and physical structure of biofilms. A dynamic interface characterised by heterogeneous surface chemistry, for example, is an especially critical distinction between metals and other solid substrates such as polymers. This quality can confer beneficial or toxic effects on microorganisms, which enforces selective pressure on early colonisation. The change in surface physical structure, especially in aerobic conditions, can also affect early colonisation and downstream development of biofilm architecture. For example, while other solid surfaces immersed in seawater also develop multi-species communities, elemental iron and its various oxidative states available on steel provide an attractive metabolic substrate for some bacterial and archaeal populations. Thus,

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populations capable of cycling Fe (II) and Fe (III), such as iron-oxidising bacteria and iron-reducing bacteria are often found on metals in the marine environment[23]. The anaerobic environments required for sulfur cycling also promotes the activity of sulfate-reducing bacteria (SRB), leading to generation of corrosive iron sulfides[23]. It is therefore a combination of substrate and solution qualities, i.e., chemical composition of seawater and the metal, that dictate the biofilm community composition. Biofilm formation on metals is also influenced by the structure of metallic materials. For example, unlike polymer-based materials such as polyvinyl chloride, biofilms on steels are impacted by the initial rapid formation of a solid iron oxide layer. As the biofilm develops and diversifies on this layer, the population can transport nutrients through corrosion product layers to reach cells at the interface[23]. Indeed, current research indicates that iron oxide structures provide complex and highly structured microbial habitats. For example, rusticles ('rust icicles') are stalactite-like structures that form on metal surfaces in marine environments, usually at great depths. Although the latest evidence indicates an abiotic mechanism is likely responsible for their formation[24], rusticles host complex internal structures utilised or even directly produced by bacterial populations[25]. Lastly, it is known that metallic ions can also elicit toxic effects on microorganisms, and marine biofilm populations in turn can develop mechanisms to tolerate these effects. For example, heavy metals such as lead (Pb), mercury (Hg) and cadmium (Cd) are highly toxic to living organisms; however, numerous detoxification mechanisms have been reported in marine bacteria[26]. The selective influence of other metallic ions, including alloying elements on initial colonisation is discussed herein. It is proposed that unique conditions offered by metals in seawater, such as surface structure and microstructure, electrochemical properties and chemical composition provide a niche for biofilm development. These important factors provide the context for the present communication.

STAGES OF BIOFILM DEVELOPMENT

The concept that bacteria form communities at an interface is not a recent discovery. In 1683, Anthony van Leewenhoeck was the first to introduce the scientific community to bacterial communities forming dental plaque[27]. For some 300 years these observations were largely forgotten until the communities were rediscovered and made famous by Costerton in the 1980s, after first establishing the term 'biofilm' in 1978[28,29]. Before this, and as late as 1987 adhered bacterial populations were considered simple, random associations of cells[30]. Research has since expanded on the biofilm theory as a fundamental pillar of modern research in the field of microbiology. Today, research investigating biofilms on metals frequently references one or more of the following biofilm formation stages: (a) conditioning film (CF) formation; (b) reversible association with the surface (often referred to as attachment); (c) irreversible association with the surface (adhesion); (d) proliferation and biofilm growth; (e) maturation and dispersal. Such stages are discrete on most interfaces, exhibiting hallmark features that may be used to characterise the maturation of a biofilm.

Conditioning films, attachment and adhesion

Attachment and adhesion are enormously complex processes and represent key topics in current research[31–35]. Bacteriological factors, substrate characteristics and environmental conditions all govern early attachment in marine environments by influencing long-range surface interactions (including hydrophobic, electrostatic and van der Waals forces)[36]. Subsequently, physicochemical surface characteristics govern non-specific and ligand-specific interactions to promote adhesion[36]. Considering metals in seawater, the interface is characterised by a diverse mixture of organic and inorganic molecules known as the CF, which can influence early and longer-term bacterial interactions. The CF composition, together with microbiological factors, charge, microtopography, wettability, and material composition are all important when considering bacterial attachment[37,38]. Figure 1 demonstrates adhesion of *Klebsiella pneumoniae* in artificial seawater, where the heterogeneous surface characteristics of corroded CS (AISI 1030) and cellular appendages involved in adhesion are evident.

The CF is defined as a layer of adsorbed molecules on the interface of a substrate in solution[39]. For 70 years or more, the CF has been recognised for its importance in bacterial attachment[40].

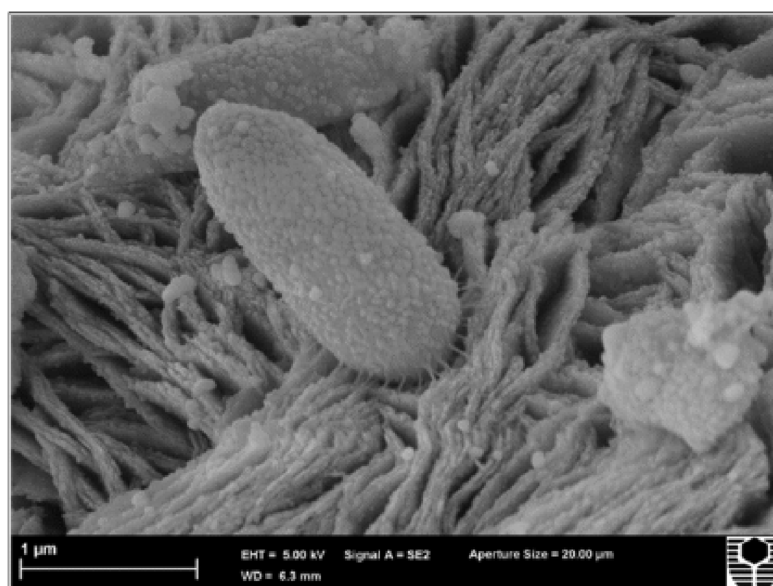

Fig. 1 Bacterial attachment to steel involving cellular appendages. Scanning electron microscopy (SEM) of *Klebsiella pneumoniae* adhesion after 24 h to CS (AISI 1030) where cellular appendages are utilised to interact iron oxides.

aureus and *P. aeruginosa* have been implicated in several recent studies. *P. aerogenosa* produces exopolysaccharides, including alginate, *PSL* and *PEL*; the latter of which was demonstrated by Jennings et al. to be pivotal to biofilm formation by crosslinking with eDNA at certain pH[78]. The authors rationalise that *PEL* may also be involved in crosslinking other polymers besides eDNA. In *S. aureus*, numerous polysaccharides are also produced, allowing attachment and biofilm formation on most surfaces it comes into contact with. Polymers of *N*-acetyl glucosamine, for example, are manufactured with the *ica* operon to produce the biofilm matrix[83]. Furthermore, López et al.[84] reviewed four biofilm-forming model bacteria and the contributions polysaccharides gave to the matrix. In marine environments, polysaccharides form a major pool of organic carbon for both planktonic and biofilm communities[85]. It is largely unknown how polysaccharides as potentially critical components of the matrix, assist in biofilm formation on submerged metals in marine environments. The variation in structure and function of polysaccharides, along with the difficulty of correctly simulating marine environments are primary reasons for this.

Proteins: Proteins also have critical roles in EPS structure and biofilm formation. For example, adhesins such as SdrC from *S. aureus* function in the development of mature biofilms by facilitating cell–cell attachment[86]. In the marine bacterium *Vibrio fischeri*, the symbiosis polysaccharide (*syp*), a gene locus encoding 18 genes, plays a central role in biofilm development and colonisation[87]. Similarly, two protein components Bap1 and RbmA were identified in *Vibrio cholerae* by Absalon et al. in 2011 and Berk et al. in 2012. These proteins have roles in the structure and spatial distribution of biofilms and are key components of the biofilm in this species[88,89]. Although *V. cholerae* is most recognised for its impact on human health, many proteins identified in bacteria associated with infection can also be involved in marine biofilm development. In 2012, Ritter et al. discovered an upregulation of genes responsible for production of biofilm proteins OmpW, OmpA, and PilF in *Pseudoalteromonas* Sp. strain D41, a marine isolate[90]. In mutant *P. aeruginosa*, a pathogenic bacterium and model biofilm former, the three proteins and associated genes were also found to impact biofilm volume and architecture[90]. The importance of proteins in biofilm composition, architecture and tolerance has led to many publications that have improved fundamental understanding of the biofilm development process. As more proteins and their roles in biofilm development are uncovered, new potential targets for mitigation of biofilms on steel substrates may be considered.

To conclude this section, there is a great deal of research covering single-species EPS composition in a variety of environments. Today, the EPS composition and function of the components in marine multi-species biofilms, especially in relation to chemical tolerance, represents a major gap in scientific research.

Communication and quorum sensing. In the developmental process (and other biofilm processes), quorum sensing (QS) facilitates mass coordination of subpopulations within biofilms. QS autoinducers involved in regulation of quorum activities (regulation of genes within subpopulations and community density[91]) are produced in mono- or multi-species systems, explaining the capacity of some biofilms to behave almost as a single organism. The importance of these molecules in development of mature biofilms was only realised within the last three decades[91], although the first report of autoinducer activity in bacterial populations was described in *V. fischeri* around 50 years ago[92]. Research has expanded rapidly since disruption of coordinated activities has been found to severely impact the ability of many isolates to form biofilms as well as metabolise and corrode steel surfaces[93,94]. A review by Bassler and Losick[95] explains the concepts of QS. There are two major autoinducer methods; namely through (1) acylated homoserine lactones, primarily used by Gram-negative bacteria, and (2) oligopeptide autoinduction, which share many similarities with Eukaryotic cell–cell communication molecules[96]. The myriad autoinducers, target receptors and affected genes, and the amount of information still absent from scientific literature makes the field an interesting and promising platform for biofilm investigations. For example, the existence and potential disruption of universal communication molecules in QS theories has broad applications. In the corrosion of carbon steels, Scarascia et al.[93] demonstrate QS signal molecules could upregulate genes involved in electron transfer, sulfate reduction and pyruvate metabolism in SRB, the main group of bacteria involved in anaerobic MIC. Downregulation of these genes was observed in the presence of QS signal suppressor molecules. The impact of QS inhibitors in biofilm mitigation is considered to be a promising potential MIC mitigation strategy[93].

Maturation and dispersal

Dispersal may be considered the hallmark feature of a mature biofilm. Subpopulations of cells break away from the parent structure and colonise new locations in what are often highly regulated and coordinated events[97,98]. Natural dispersal eventuates as an active response to environmental stress, enabling populations of bacteria to persist in new environments when current ones become inhospitable[97]. As with other developmental processes, the onset of dispersal has been found to be a complex phenomenon involving environmental and molecular triggers. For example, while the importance of eDNA in biofilm formation and integrity is undisputed[99], research also demonstrates that eDNA can inhibit dispersal[44]. The presence of eDNA may also prevent uptake of new cells into the matrix[44]. Thus, extruded planktonic bacteria must colonise new sites. At this stage, the formation of a new biofilm relies on planktonic cell survival. Interestingly, to achieve greater survival odds the planktonic cells undergo genetic diversification when released from the parent biofilm[97]. Further information on the biology and mechanisms of dispersal can be found elsewhere[97].

Specifically pertaining to marine biofilms, dispersal is often linked to cell death. In *Pseudoalteromonas tunicata*, Δ*alpP*-mediated cell death was demonstrated by Mai-Prochnow et al. to be associated with dispersal of surviving cells. Mutant *P. tunicata* cells incapable of expressing the ΔalpP autotoxic protein; resulting in lower local cell lysis, were associated with lower dispersal rates[100]. Similarly, viral particles promote cell death (phage mediated cell lysis) and biofilm disruption that has been linked to increased dispersal[101]. In other research, Barraud et al. discovered nitric oxide to be linked to biofilm dispersal. *P. auruginosa* mutants unable to express a sole nitric reductase enzyme (ΔnirS) were unable to disperse while mutants expressing the enzyme could[98]. Coatings used in marine applications to prevent MIC and biofouling can also promote physical dispersal of biofilms before biocide application to enhance chemical effectiveness[101]. Today dispersal of marine environments is understood as a complex process that may be triggered or targeted to remove biofilms. More mechanistic research is required to fully understand the numerous approaches to dispersal, and apply this understanding to biofilm mitigation and control. In particular biofilm dispersal (as opposed to simply killing but not removing the population) from carbon and stainless steels is a high-priority challenge for marine-based industry.

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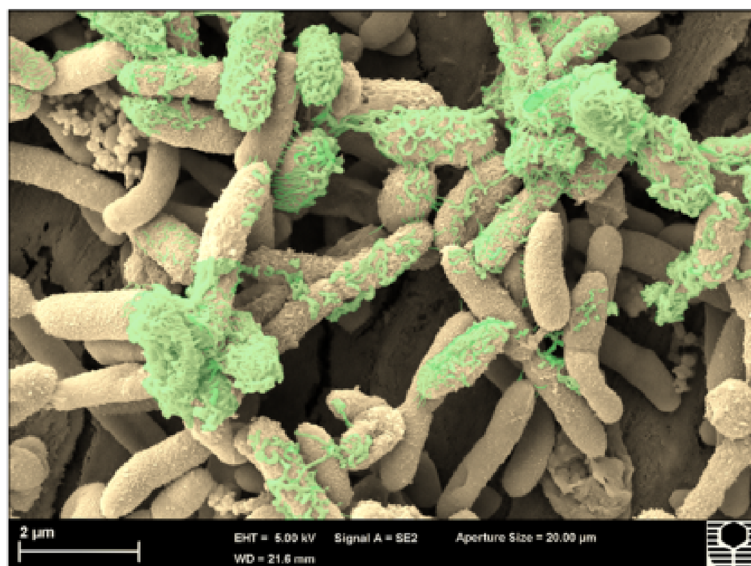

Fig. 2 SEM of a marine biofilm characterised by dense cell living arrangement and EPS. SEM micrograph of *Klebsiella pneumoniae* biofilm formation on carbon steel (AISI 1030) in artificial seawater, where structures resembling EPS (green) cover biofilm cells within 24 h.

Substrate electrochemical properties and composition. Electrical charge (substrate or cellular) and surface roughness of metallic surfaces are critical to the quantity, type and timeframe of bacterial attachment[66,67]. Alloying elements such as copper (Cu), nickel (Ni), silver (Ag), chromium (Cr), vanadium (V) and iron (Fe) influence attachment and biofilm formation of marine bacteria. However, scientific literature on the type and extent of influence fails to reach complete consensus. In a recent study involving *Halomonas titanicae*, biofilm formation was inhibited by bactericidal ions released from Ni. Compounds containing Ni also reduced attachment of the strain[38]. In a separate study from the same year, Ni was demonstrated to enhance bacterial attachment[68]. Conversely Cr, especially hexavalent Cr (VI), Ag and Cu are firmly associated with decreased bacterial attachment[69,70] while ferrous ions are frequently reported to exert positive effects on bacteria[71,72]. Further research on material composition in relation to attachment requires standard experimental methods to allow accurate comparisons. While numerous studies evaluate the initial impact of alloying elements on attachment, more research is required to understand the longer-term effects of material composition on biofilm formation.

Despite advances in reducing bacterial attachment to metals, total prevention of biofilm formation on any marine surface including metals has yet to be seen and is an area of continuing research[73]. Microbiological characteristics including adhesin expression and cell membrane organisation, as well as substrate characteristics such as material composition, surface microstructure and charge all affect attachment and adhesion of marine bacteria. Although these factors can shape the outcome of biofilm formation, early interactions of bacteria with metals remain poorly understood[66].

Proliferation and growth

For adhered cells to successfully replicate, attract community members and form robust biofilms there must be EPS production as well as communication within and between species. Proteins, eDNA, polysaccharides and other organic and inorganic molecules and ions are often identified in natural marine biofilms[74,75], providing protection for the population. Figure 2 demonstrates the compact nature of the biofilm living arrangement, characterised by EPS (depicted in green). The molecular configuration of the EPS gives rise to functions within the matrix including cell-substrate adhesion and subsequently cell–cell and cell-matrix adhesion. To date, little is known about marine multi-species biofilm EPS and its composition, its role in corrosion and its influence on biofilm tolerance.

Extracellular polymeric substances (EPS)

Extraceullar DNA: It is now well established that eDNA provides an important scaffold for the biofilm structure in many biofilms[76]. In 2002, Whichurch et al. added DNase1, a DNA degrading enzyme, to *P. aeruginosa* biofilms resulting in rapid biofilm dispersal[77,78]. In the marine environment, eDNA exists in concentrations up to $2\,\mu g\,g^{-1}$ in sediments[79] and comprises 70% or more of the total marine DNA[80]. Exactly how and why biofilms produce eDNA is currently not well understood. Evidence suggests some bacteria such as *P. aeruginosa* have a number of complex biochemical pathways involved in active manufacture and secretion of eDNA. Other reports label the dominant mode of production as cell lysis, which may be an active process. For example, Rice et al.[81] concluded that specific genes, *CidA* and *lrgA*; were involved in regulation of *S. aureus* cell lysis (i.e., *suicide genes*), which affected the number of dead cells. In cultures containing mutant copies of *CidA*, less dead cells resulted in lower attachment and weaker biofilm formation. Considering eDNA comprises a major portion of the organic carbon pool in natural marine environments, and an affinity between metal oxides and negatively charged eDNA is well established, marine-based simulations should consider what implications eDNA can have on results and conclusions of research.

Polysaccharides: Exopolysaccharides are simple sugar chains that are key to matrix formation and the establishment of a mature biofilm. The importance of polysaccharides in biofilm formation and the matrix has been known for decades[82]; after all, *glycocalyx* was an original term for the EPS. Unsurprisingly, over the last three decades the polysaccharide contribution has been a particularly important topic in biofilm research. Since then, polysaccharides have been reviewed and studied in great detail in a variety of species. Model organisms such as *Staphylococcus*

aureus and *P. aeruginosa* have been implicated in several recent studies. *P. aerogenosa* produces exopolysaccharides, including alginate, *PSL* and *PEL*; the latter of which was demonstrated by Jennings et al. to be pivotal to biofilm formation by crosslinking with eDNA at certain pH[78]. The authors rationalise that *PEL* may also be involved in crosslinking other polymers besides eDNA. In *S. aureus*, numerous polysaccharides are also produced, allowing attachment and biofilm formation on most surfaces it comes into contact with. Polymers of *N*-acetyl glucosamine, for example, are manufactured with the *ica* operon to produce the biofilm matrix[83]. Furthermore, López et al.[84] reviewed four biofilm-forming model bacteria and the contributions polysaccharides gave to the matrix. In marine environments, polysaccharides form a major pool of organic carbon for both planktonic and biofilm communities[85]. It is largely unknown how polysaccharides as potentially critical components of the matrix, assist in biofilm formation on submerged metals in marine environments. The variation in structure and function of polysaccharides, along with the difficulty of correctly simulating marine environments are primary reasons for this.

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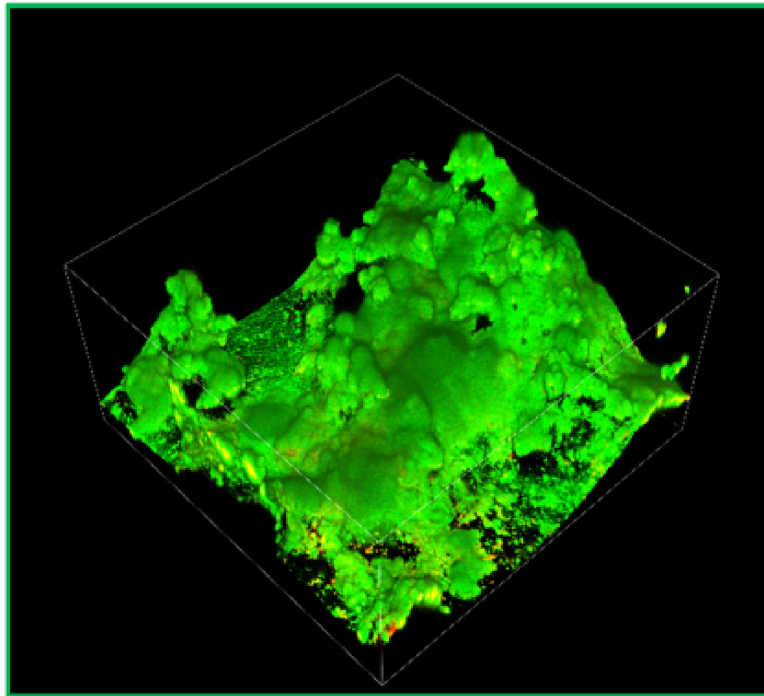

Fig. 3 Confocal laser scanning microscopy of a marine biofilm formed on steel. Confocal micrograph of a marine consortium under 20× objective demonstrating biofilm topography, surface coverage and cell viability on carbon steel (ASI 1030), where green corresponds with live cells and red corresponds with dead or damaged cells.

mutualism, respectively, and competitive behaviours specifically harm a neighbouring population or benefit the microorganism expressing the behaviour. Both can also occur simultaneously as in parasitism. These interactions are discussed by Burmølle[20] and Liu[102]. In the marine biofilm, interactions between species have been found to increase tolerance to biocidal compounds, as well as shape biofilm spatial distribution and biomass structure[102,103]. Furthermore, we now understand that specific enabling populations within the multi-species system may provide benefits to the entire population. With every species contributing uniquely to the biofilm, the cumulative capacity for tolerance is enhanced. For example, bacteria capable of producing large volumes of EPS may be supplying the bulk organic content of the matrix for other species. Horizontal gene transfer (HGT) is also a common characteristic of multi-species biofilms that contributes to physical and chemical tolerance[102]. Genes conveying chemical or environmental coping mechanisms may be exchanged between species, greatly boosting the tolerance of the entire population. Biofilms are particularly conducive to HGT, owing to high cell density[104]. This condensed living arrangement of diverse populations is frequently observed on submerged metals (Figs. 3 and 4). In order to understand and mitigate marine biofilm development, research should move towards multi-species research rather than single or dual-species. Røder et al. highlight the need for a more detailed scrutiny of microscale interactions; considering specifically how physiological parameters such as gas, pH and nutrient gradients may influence subpopulation composition and functions of these populations[103,105]. Each contributing subpopulation of a multi-species biofilm can have unique functional attributions, which Røder argues is not considered by many current techniques such as sequencing (a technique that often relies on large quantities of extracted cells for DNA input)[105]. Therefore, experimental designs should aim to balance these limitations. Confocal-based techniques, for example, (discussed below) can contribute spatial arrangement and species distribution insights, which are lost by sequencing-based techniques. It is therefore especially important to consider both qualitative and quantitative methodology when designing experiments for the evaluation of marine biofilms.

Marine biofilms and corrosion

Considerable variation exists in the scientific literature surrounding the impacts of marine biofilms on metallic materials. It is well established, for example, that microbial populations can generate corrosive conditions through either chemical mechanisms (as with chemical MIC or CMIC) or through extraction of electrons from the metal substrate (as in electrical MIC or EMIC)[106]. CMIC results from the metabolic activity of a biofilm, for example, the production of organic acids at the biofilm-metal interface[107]. CMIC mechanisms are considered less widespread than EMIC mechanisms[106], which involve the transportation of electrons either directly or indirectly from metals or other bacteria using mediators or appendages such as pili[107]. Extracellular electron transfer (EET) or interspecies electron transfer (IET) are examples of mechanisms that can occur in multi-species marine biofilms. Extensive research has explored EET by *Geobacter* and *Shewanella* spp. as model organisms in the field, including IET mechanisms[106]. Research continues to reveal direct or indirect syntrophic relationships between species, highlighting the importance of these relationships in natural communities. Indeed, IET mechanisms are considered critical to bacteria living in a variety of environments and have formed the foundations for a number of biotechnological advancements, including conversion of waste to methane gas[108]. In the field of MIC (i.e., on metallic substrates), cell-substrate and cell–cell electron transfer is poorly understood, especially in marine environments. The sum of microbial metabolic activities, cell–cell and cell-substrate interactions account for at least 20% of all corrosion costs in the oil and gas industry[109]. Biological diversity

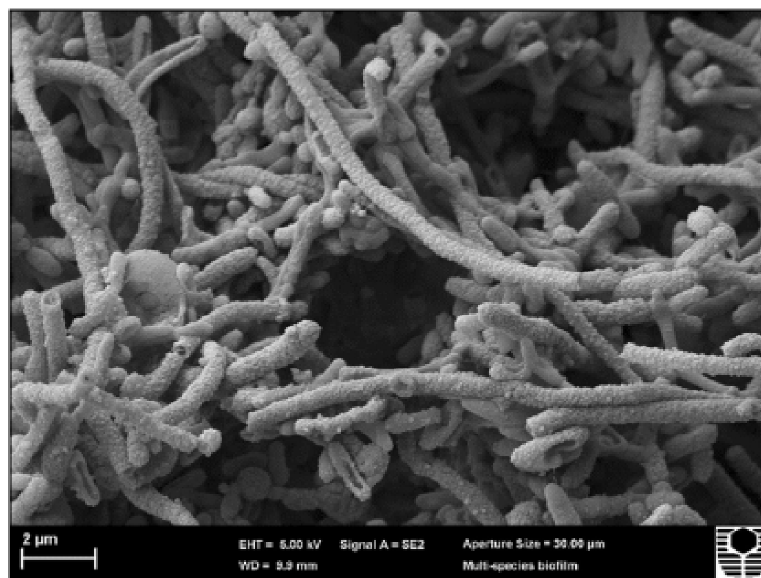

Fig. 4 SEM image of a marine consortium demonstrating close living arrangement and various cell morphologies. FESEM micrograph of a marine multi-species biofilm on carbon steel (AISI 1030) demonstrating the complexity and dense living arrangement of multi-species marine biofilms.

continues to challenge the identification and isolation of responsible MIC mechanisms, especially where other corrosion manifestations are involved[110].

Although many CMIC and EMIC mechanisms have been proposed, protection of metallic substrates from corrosion is also frequently reported by marine biofilms and EPS. In a recent study involving a strain of *Pseudoalteromonas lipolytica;* a marine bacterium, corrosion protection in seawater was afforded on steel. The isolate was found to produce a 'hybrid film', comprising both organic and inorganic material and functioning as a barrier[111]. EPS can function as a protective agent against steel corrosion either by an active chemical mechanism or by forming a passive barrier that prevents interaction with the environment (e.g., limiting O_2 contact)[112]. Research on soluble EPS, for example, has revealed that some strains such as *Bacillus cereus* produce EPS that prevents scale and corrosion simultaneously[113]. The 2019 study implicated both adsorption of the EPS and biomineralization in corrosion inhibition efficacy, which was greater than 91%[113]. Although these studies provide valuable information pertaining to biofilm on metals, most laboratory MIC simulations rely on data obtained from coupon samples. In marine environments, infrastructure comprised of metals can be many kilometres long (i.e., pipelines) with inconsistent surface conditions. While coupons used for laboratory simulations are often rapidly and uniformly covered (i.e., they represent a small surface area), the shear mass of field equipment can lead to more heterogeneous surface coverage (involving macro and microorganisms and diverse communities). Unevenly distributed (i.e., 'patchy') biofilms can therefore induce differential aeration cells on the surface of metals[112]. To tackle this problem, MIC simulations can involve a split-cell experimental design to separate two electrically connected metal substrates into a biotic and an abiotic side[112]. The major benefit of this design is its ability to prevent biofilm coverage of the entire substrate, and thus allow for simulation of heterogeneous surface coverage on larger metal structures. Laboratory simulations that explore the anticorrosion properties of biofilms should consider the practical implications of the results and the limitations of a single-cell bioreactor design.

Understanding natural marine biofilms

Data reproducibility and accuracy has been a longstanding difficulty in the analysis of multi-species simulations. Certainly, the most challenging aspect is assembling reliable and meaningful data that reproducibly supports the hypothesis. In light of this, most of the considerable data amassed on biofilm development were obtained from relatively simpler single-species simulations[20]. Today, new techniques and advances to those already established have allowed great insight into how bacterial communities interact and establish complex biofilms. In particular, advanced microscopic, spectroscopic and molecular techniques are at the forefront of multi-species biofilm research.

Visualising spatial distribution, orientation and composition of marine biofilms. Confocal laser scanning microscopy (CLSM) is one of the most frequently employed microscopic techniques for the evaluation of the 3D form of biofilms[114]. The scope, functions and operation of CLSM are described in detail elsewhere[115]. CLSM is particularly useful for evaluation of natural marine biofilms since contributing populations may be distinguished through rRNA-based probes[116], live cells may be visualised in a natural state with minimal disruption (see Fig. 3) and both qualitative and (semi) quantitative measurements are possible. Although a powerful technique, confocal micrographs can contain large quantities of data, especially where used for semi-quantitative analysis or when captured in high resolution. A major challenge for multi-species biofilm research using CLSM then becomes data processing and interpretation. In addition, the application of more complex probes for the identification of microbial subpopulations such as in catalysed reporter deposition fluorescence in situ hybridisation (CARD-FISH) can be labour intensive and expensive.

The cellular complement, EPS proteins, polysaccharide residues, eDNA and lipids may all be targeted by confocal probes[117]. Microorganisms and selected components of the matrix can also be identified using unique probes which can, for example, provide an estimation of live and dead cells (propidium iodide and Syto9™, both available commercially in kits), protein expression and even indicate membrane integrity (such as SynaptoRed™ C2 and

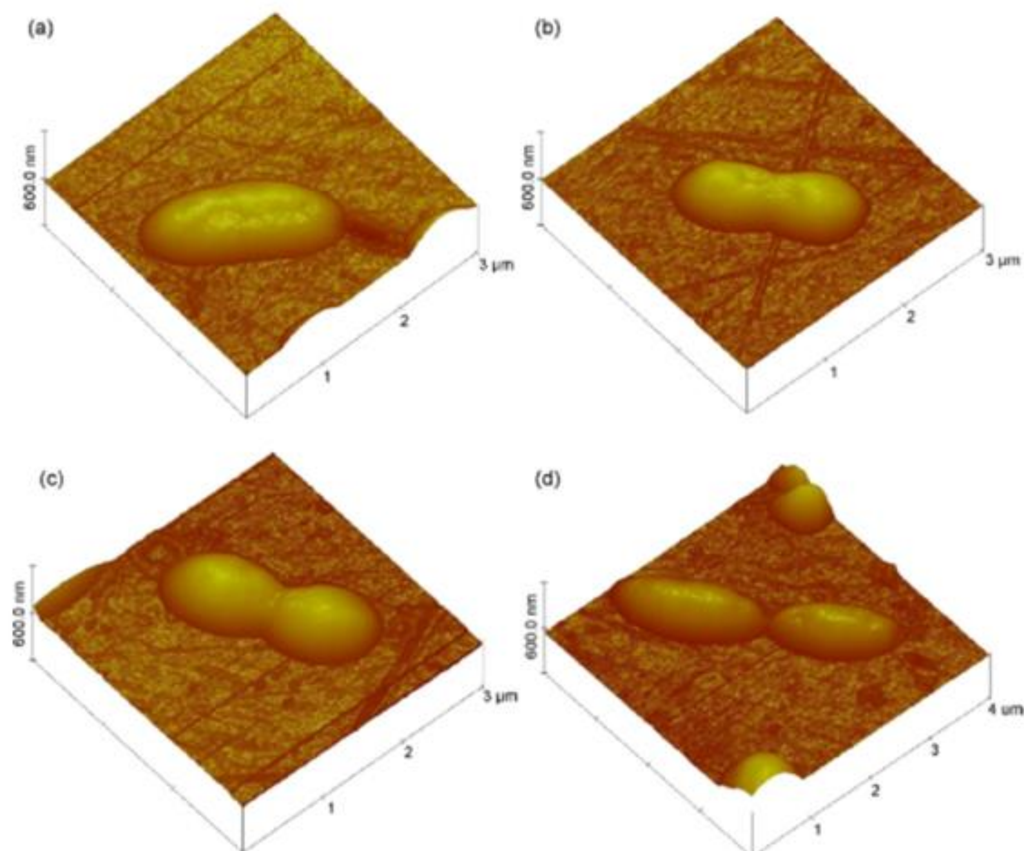

Fig. 5 AFM depicting cellular division on a steel substrate. AFM micrographs of *Pseudomonas* sp. undertaking binary fission on stainless steel where the mature cell is seen dividing and separating into daughter cells[123].

Cellbrite™ Fix stains).

To assist with post-image analysis, there are many, often free software platforms available that can provide semi-quantitative data on morphological parameters of marine biofilms, including microscale structure and heterogeneity[118] (COMSAT and ISA), biofilm coverage (PHLIP)[114] probe evaluation and direct quantification of populations within a biofilm (*daime*)[116]. *Daime* is particularly useful where FISH and various iterations are employed, as such techniques work with specific probes which are frequently used to identify subpopulations within biofilms. The selection and evaluation of the probes with software like *daime* is vital to the efficacy of the technique applied. A variety of other platforms exist that are not yet employed on marine biofilm micrographs, including *bio*Image_L that was demonstrated to identify biofilm subpopulations based on viability and metabolic activity[119]. Lastly, ImageJ (Fiji; open-source software) and IMARIS (Bitplane) software (product license required) can be used to generate parameters such as biovolume (the quantity of biofilm in a given area), compactness (the density of the biofilm based on fluorescent signal per volume) or simply fluorescent signal intensity, among other parameters[120].

Scanning electron microscopy allows a high-resolution view of biofilm architecture. The cost of this technique is in sample preparation, which includes (1) fixation of the biofilm sample for several hours using tissue fixatives such as gluteraldyde, (2) dehydration of the sample using an ethanol series, by nitrogen drying or both, and (3) sputter coating of the sample using an inert metal such as platinum or gold[121]. Figure 4 provides a view of the biofilm structure, revealing cell density and cell morphology of a multi-species marine biofilm on carbon steel. If sample preparation is performed correctly, cells and EPS hold their original profile and biofilms can appear more or less as naturally formed.

AFM provides the highest resolution of any microscopic technique available today with minimal or no sample processing. For a comprehensive review of AFM the reader is directed to Cárdenas-Pérez et al. (2018)[122]. For the investigation of multi-species marine biofilms on metals, in situ AFM is possible in the sense that submerged, living biofilms can be micrographed and various physicochemical parameters are also obtained, although this requires extensive optimisation for the given sample. In Fig. 5a, a mature *Pseudomonas* sp. cell is illustrated on stainless steel. Fig. 5b, c shows the cell dividing and finally Fig. 5d shows the separation of the cell into two complete daughter cells[123]. Probe types, physiological qualities of the fluid, atmospheric conditions, biofilm layer thickness and sample structure, among other parameters (such as microscope settings) are important considerations when attempting in situ AFM. Well-optimised applications of this technique have seen momentous advantages to the field of biofilm research, including Li et al., who demonstrated cell-substrate adhesion qualities could be measured through the use of bacterial cells as a cantilever tip[122]. An AFM tip was manufactured using living cells adhered to a cantilever, thereby directly monitoring interactions of a cell with a pyrite substrate. In a separate study, Li et al. demonstrated that EPS plays an important role in cellular adhesion to mineral surfaces using

AFM[124]. Since the first documented account of AFM in scientific literature was introduced in 1986 by Binning, Quate and Gerber[125] AFM has become a primary tool in the investigation of biofilm formation. In particular, the niche of AFM seems to be within earlier biofilm formation stages (attachment, adhesion and micro-colony formation) where the limitations of thick biological samples are much less apparent. Provided the researcher can navigate corrosion product development on metal substrates, or use a finely polished corrosion-resistant alloy, AFM can be a powerful technique for elucidating the early mechanisms of biofilm formation on metallic substrates.

As with all techniques, the limitations of microscopy must be acknowledged and the results interpreted cautiously. All microscopic techniques provide innate biases, alongside interpretation bias from the viewer. This can lead to total misrepresentation of the sample and inaccurate assumptions in any investigations. These issues have been recently discussed by Jost and Waters[126]. Where applied correctly and supplemented with alternative techniques, ideally quantitative in nature, microscopy can provide great insight to biofilm investigations.

Population dynamics and molecular functions. Much of what we know about multi-species biofilm diversity has come from the application of molecular techniques. As there is no need to culture the isolates for these techniques a more accurate idea of the community can be achieved. For example, rRNA analysis has identified many new divisions[127]. In marine systems, the majority of species are still unknown to science, leaving many yet to be discovered[127]. Molecular methods are a primary tool for elucidating species diversity; becoming central to functional diversity and distribution studies of biofilms[128]. As advances in molecular technologies continue to surge along with their use in routine microbiological investigations, next-generation sequencing technology (NGS), -omics-based techniques (meta- transcriptomics, proteomics and metabolomics) and microarray technology in particular are now becoming conventional in multi-species biofilm studies.

NGS is a contemporary, high-throughput DNA or RNA sequencing technique that has enabled a more cost-effective and time-sensitive analysis of sample types from across the life sciences. NGS techniques are particularly useful for the identification of unknown sequences; for example, in heterogeneous natural bacterial populations, and have been used to shed light on diversity and distribution within biofilms[128–130]. Omics-based techniques are also applied to understand a microbial system in more detail. For example, Beale et al. employed metabolomics and metagenomics to understand the effect of inorganic nutrients and pollutants on marine bacteria[131]. Combining the two techniques means genotypes and possible capabilities of microorganisms can be compared against actual metabolic functions. Lastly, microarrays are the technique of choice for understanding environmental responses and diversity in microbial systems on a genetic level. Microarrays already have an extensive reach into environmental and health research, which is extending into marine microbiology. To date, microarrays have been applied to evaluate anthropogenic impacts on marine bacteria[132,133], monitoring bacterioplankton communities[134], identifying pathogenic strains and pollution in seawater[135], identification of nutrient shifts in marine isolates[136], detection of marine toxins[137] and profiling marine communities[138]. Microarray technology will likely continue to advance and increase in scope of application in coming years; especially as there are no equivalent techniques for the detection of genetic markers in heterogeneous samples.

Chemical structure and function. The demand for quantification and detection of metabolites and EPS components has given spectroscopic techniques an important place in biofilm research. Research topics identifying the fundamental components of the matrix, how the matrix composition changes in response to various stimuli and influence of these stimuli on metabolisms, among many other factors, may rely on techniques such as Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption or ionisation (MALDI) or nuclear magnetic resonance (NMR)-based techniques[117].

FTIR has been used to characterise marine biofilm EPS with the aim of screening for anti-biofouling compounds[75], to determine the major saccharide components of the matrix[139] and identify harmful compounds such as heavy metals in marine biofilms[140]. MALDI has also been applied extensively in biofilm research. Most relevant to this review was work applying MALDI in conjunction with mass spectrometry to characterise EPS components in multi-species marine biofilms[141]. MALDI-typing is routinely used for the identification of single cells in diverse populations[142,143]. Techniques involving MALDI have seen a surge in advancement as microbiological identification tools, improving workforce and analysis time limitations associated with the previous techniques[144]. Lastly, NMR can be used to characterise the structure of molecules[144]. The viewing of matter and its structure is an important aspect of some biofilm studies. Xiu et al. in 2017 identified a highly motile marine isolate could be inhibited by another isolate from the same niche through motility suppression[145]. Their work employed NMR to elucidate the active compounds inhibiting motility of the isolate[145]. This work, while primarily focused on virulence suppression, represents an approach to potential multi-species biofilm interactions. NMR is a powerful tool for identifying potential molecular interactions between species vital to the establishment of robust natural biofilms. Like MALDI, the technique has also been applied to understand the biofilm composition of isolates[146], with the potential to be applied in marine biofilm research.

CONCLUDING REMARKS

Biofouling and MIC results in at least 20% of the US $2.5 trillion annual losses due to corrosion. Deleterious effects on metals by microorganisms rely on biofilm formation, which occurs in a series of discrete stages. In marine environments, problematic biofilms are characterised by species diversity that gives rise to increased chemical and environmental tolerance. Yet, natural biofilm developmental stages, composition, treatment and tolerance mechanisms are poorly understood. In the past, multi-species biofilm complexity has led to research challenges relating to reproducibility and technique limitations, which has impeded the progress of natural marine biofilm research. Although a great deal has been learned from single-species simulations, multi-species research is now possible as a result of recent technical advancements. Advanced microscopic techniques such as CLSM, for example, has been used to understand more about community distribution and interactions, while community composition and metabolic profile, for example, can be examined using molecular techniques. Research on marine biofilms on metals aims to understand complex communities in greater detail for the purpose of managing materials degradation and the associated costs.

Current research demonstrates that biofilm formation can be categorised into distinct stages in marine environments. The establishment of recalcitrant biofilm structures is governed by the EPS composition and interactions between the species that exist in natural marine biofilms. These community members provide fitness benefits such as tolerance to chemical treatments through HGT to the other members. Populations can also respond in a coordinated manner in response to quorum signals. Lastly, marine biofilms are comprised of subpopulations that can impose advantageous or deleterious effects on other members. Interactions within multi-species biofilms are therefore important to

understand in order to effectively control biofilm development on metallic surfaces.

At present, advancements in technology have permitted elaboration on some unanswered fundamental questions surrounding the control of complex marine biofilms. This review has attempted to capture research on some of the most pressing of these questions, such as how bacteria attach and adhere to metals, what contributes to the structure of biofilms, how biofilm composition affects function, how do species within a complex multi-species system interact, and lastly, what are the most promising and widely applied techniques for evaluating phenomena in multi-species biofilm research? Research focused on answering the above questions aims to achieve the ultimate goal of efficient and sustainable biofilm mitigation on metal surfaces in marine environments.

DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article.

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B.T. conducted the literature investigation and composed the draft manuscript with help from E.W., A.S. and L.L.M. All authors provided revision approved the manuscript in its current form.

COMPETING INTERESTS

The authors declare no competing interests.

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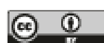

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Chapter 1: Introduction and literature review

Manuscript # 2 of 2

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UNDERSTANDING NATURAL BIOFILM DEVELOPMENT ON STEEL IN MARINE ENVIRONMENTS – A REVIEW

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SUMMARY: Surface colonisation by microorganisms is a ubiquitous process in the marine environment. The formation of a mature biofilm structure, which is described as an aggregate of attached cells surrounded by extracellular polymeric substances (EPS) is the final stage of colonisation. In this form, bacteria, fungi and archaea have been implicated in several deleterious effects on substrate surfaces; for example biofouling and subsequent microbiologically influenced corrosion (MIC). Although it is well documented that marine biofilms form on almost any submerged artificial or natural surface, there are still no truly effective and environmentally friendly treatment or prevention options available. Part of the reason biofilm growth and establishment cannot be adequately controlled is the lack of fundamental understanding of natural multi-species biofilm development. Polymicrobial systems give rise to complex synergistic and competitive behaviour, which is overlooked in single-species *in vitro* studies. So far, single-species studies have provided a great deal of information on how microorganisms assemble simple biofilms. Conversely, multi-species studies have been limited up until recently due to technique and data processing restrictions. Today, with access to advanced microscopic and molecular techniques, among others, there is greater potential for multi-species studies. It is important for the applicability of research conclusions now to move away from single-species investigations and into studies incorporating a variety of isolates which reflect marine heterogeneity. The purpose of this review is to compile the current literature and knowledge gaps surrounding multispecies biofilms, with particular focus on the development process in marine conditions on steels.

Keywords: biofilm, marine, multi-species, polymicrobial, biofouling, attachment, adhesion

1. INTRODUCTION

Surface colonisation by microorganisms occurs in almost all environments on earth[1]. Simulations have demonstrated that some bacterial populations can remain viable after exposure to intense radiation stress, high vacuum and temperatures simultaneously; conditions characteristic of extra-terrestrial environments[2]. This resilience leading to widespread abundance is predominantly due to the biofilm lifestyle; the preferred living arrangement of bacteria[3]. Biofilms are defined as aggregates of cells surrounded by self-produced extracellular polymeric substances (EPS)[4, 18] occurring at a phase boundary[17]. Charcalis' group in 1977 made the observation that biofilms in nature are almost always found in aqueous environments such as river beds, attached to surfaces. Therefore the term *biofilm* is usually specific to aqueous environments[10], because proliferation is dependent on water availability at the phase boundary. While development of biofilms is a water dependant process, tolerance of biofilms to desiccation is also well established[5, 6, 7, 53] and bacteria have been known to last weeks or more under desiccating environments[53]. Additionally, rapid recovery times are also recorded[53]. The flexibility and adaptability of attached microbial populations has led to difficulty in prevention and management of deleterious biofilms in a wide range of industrial sectors.

The phenomena of biofilm formation was first reported to the literature by Zoebell almost 80 years ago[19].Today biofilm establishment has been widely studied in many environmental and clinical settings[11, 12, 13, 14, 15, 16]. A major motivator for the explosion of research into biofilm formation is the economical and health burden associated with almost all industry

connected to an aqueous environment; including health (catheters, implants), transport (shipping, mooring chains), agriculture (marine harvesting equipment, ships), food processing (meats, surfaces in contact with food) and energy sectors (oil and gas infrastructure, nuclear power plant). All of these industries rely on steel substrates in some way which suffer from biofilm formation. Once established on a surface, biofilms can promote or cause disease, contamination, loss of efficiency and microbiologically influenced corrosion (MIC). MIC is the process of corrosion initiated, maintained or enhanced by microorganisms and usually includes a mixed-species consortium[55]. Both MIC and biofouling are particularly damaging biofilm effects. In 2005, general corrosion was estimated to cost around $3-7 Billion per annum[44,45], a figure which was revised in 2016 by the National Association of Corrosion Engineers (NACE) to be closer to $2.5 Trillion[46]. In 2018, repair and maintenance costs for general corrosion was estimated to be a $4 Trillion global concern per annum[47]. MIC may account for at least 20% of these costs[47]. The increasing cost of MIC is based on quantity of susceptible infrastructure (i.e. submerged pipelines, ship hulls, floating off-shore production facilities), which is increasing, along with infrastructure which is reaching or past decommissioning timeframes. Published literature has reached consensus that unless biofilms are managed effectively, the burden on global economy of biofilm effects such as MIC can be expected to increase in future years. This drive from industry has generated research incentive, which in turn has generated a wealth of information on the establishment of biofilms both in natural and laboratory settings. Fundamentally, research so far identifies a series of well-defined stages in the process of mature biofilm formation[16] which will be discussed herein. While this holds true regardless of the biofilm type or location, environmental conditions and species diversity have been demonstrated to affect structure and resilience[8,16,17].

Biofilms in natural environments are almost always described as diverse, or having more than one species[20,22], which has had major implications from the research perspective as well as downstream management strategies. For example, traditional mono-species simulations *in vitro* may frequently prove inadequate for the elucidation of environmental mechanisms or for replicating environmental phenomena [20,21,22]. Biofilms in the more complex natural state; such as on metals deployed in marine environments, are also harder to treat with antibiotics and biocides, as each species may demonstrate unique tolerance features. In the marine environment biofilms form quickly and have rapid regrowth times, especially compared to mono-species laboratory simulations. These observations are still not fully understood, despite their significance to global industry and research. It is a purpose of this review to summarise biofilm development and the current limitations with mono-species research, to evaluate the mechanisms mixed microbial communities employ to construct complex biofilms and briefly discuss the contemporary technology which is making multi-species biofilm research more approachable[20]. Finally, this paper will evaluate the above in the context of biofilm formation on metals in the marine environment.

1.0 Stages of biofilm development

A series of stages have been associated with bacterial community formation at an interface since the 1970's, when Bill Costerton first named these communities ***biofilms***. Before this, and as late as 1987 biofilms were depicted and perceived as simple and random associations of cells[23]. Fathers of biofilm research, particularly Costerton and his group have since expanded on the biofilm theory. Research today investigating metal-seawater interface biofilms frequently references one or more of the following biofilm formation steps: a) conditioning film (CF) formation[33,36]; b) reversible association with the surface (often referred to as attachment); c) irreversible association with the surface (adhesion); d) proliferation and biofilm growth, e) maturation and finally f)dispersal. Such stages are discrete on most interfaces, exhibiting hallmark features which may be used to characterise the maturation of a biofilm. A brief overview of the current understanding of each stage is highlighted in this review.

1.1 Conditioning films

CF formation is defined as the adsorption of ambient macro-molecules to a surface suspended in solution[25]. The importance of the CF in cellular attachment has been recognised for 70 years or more in the medical field[40]. At around the same time, in the early 1960s observations of ocean invertebrates indicated that an existing (native) organic film on sand particles favoured settlement over filtered sand[41]. Depending on location, environmental factors, bacteria present[35] and substratum type[34], the CF varies in composition and this has downstream effects on the adhering populations. Since early pioneering work identified the importance of CF formation on the establishment of complex ecosystems on natural surfaces, the molecular identity of various adsorbed films and effects on bacteria have been extensively studied[42]. Compounds frequently revealed in research as comprising part of the marine CF include proteins, humic acids, carbohydrates, aromatic amino acids and uronic acids, among others. See Jain and Bhosle for more information[42]. Besides impacts on initial attachment, the idea that population dynamics of multi-species biofilms (which may persist long after attachment and adhesion) [35] are impacted by the CF composition is also not novel. The specific cell-surface interactions between multi-species biofilm members and the conditioned substrate surface, and the roll of the CF in these interactions, is an area requiring further understanding on steel surfaces in marine environments.

Both cellular attachment[26,27,28,29] and CF formation[30,31,32] have been reported in the literature as being the first stage of biofilm formation, probably because distinguishing which process occurs first in a given system is difficult. This is particularly true for marine-based metal substrates; exposed to a fluctuating environment comprised of diverse bacterial populations and CF components. Unsurprisingly both attachment and CF formation have been described as almost immediate processes on substrates submerged in marine environments[36,37], although there is evidence to support the

claim that the CF must first form to enable cellular attachment[38, 39]. As molecules in solution begin CF formation by attaching directly to the substrate, research by Lee *et al*[39] demonstrated that bacterial attachment is more complex and may require specific surface adaptation to initiate. Lee describes a series of attachment-detachment steps within the early attachment stage, which was coupled by cyclic adenosine monophosphate (cAMP) production in the model organism *Pseudomonas aerugenosa*. Subsequently, type IV pili became more numerous, promoting adhesion, the irreversible stage. Such surface adaptations occur quickly after initial contact, and CF components may play several roles in bacterial adaptive responses.

In marine environments, the CF has been found to comprise a diverse mixture of organic and inorganic molecules. The impact of these molecules, and the surface itself, on bacterial attachment should be factored into studies evaluating early biofilm formation.

1.2 Attachment and adhesion

The many deleterious effects imposed by biofilms on industry (such as biofouling and MIC) are reliant on biofilm maturation. Attachment and subsequent adhesion are critical to maturation and therefore represent key topics in current research[48, 49, 50, 51, 52]. Although numerous published articles discuss or evaluate bacterial attachment today, the field is relatively new; less than 60 years have transpired since the first bacterial marine attachment study was published by Meadows[53]. In the 1965 paper Meadows communicates the lack of mechanistic understanding surrounding attachment tendencies. Nowadays, common research topics include investigation of molecular interactions between individual bacteria and surfaces[48] or the role of genotypes and gene expression in phenotypes facilitating attachment. It is expressed that attachment and subsequent adhesion is an incredibly complex process, reliant on a plethora of bacteriological and substratum factors. Considering only metal substrates, charge, micro-topography and wettability[142], among other factors can vary greatly which affects attachment and adhesion. Some of the most commonly addressed factors in the literature are reviewed here.

Surface interactions and compounds:

There is now increasing awareness of compounds associated with bacteria or surfaces which may promote attachment on a molecular level. Of course, genotypes and gene expression is of great importance when considering the origin of such compounds and the circumstances of their presence in natural systems. For example, adhesion molecules are expressed by virtually all bacteria[58] and are useful in attachment to the variable surfaces found in the marine environment. Molecular pathways may be involved in the production and cycling of these compounds, which are in turn effected by environmental conditions[59] (e.g. desiccation, nutrient supply).The genetic and phenotypic capabilities of the isolates in consortium govern the type and abundance of adhesins produced. The list of identified adhesins that play a role in biofilm attachment is constantly expanding, and Klemms *et al*[58] produce a review including several examples. While most adhesion studies are linked to the health industry, the same specificity to surfaces is also observed in marine ecosystems to metallic and plastic surfaces. Recent work by Chepkwony *et al*[59] demonstrated that holdfast (polar adhesins) may be synthesised from similar genes with very different outcomes. In *Caulobacterales* genera genes for holdfast production are highly conserved, although phenotypes of *Caulobacter crescentus* and *Hirschia baltica* display differences in size and composition. Interestingly, despite conserved genetics, the chemical properties and functions of the holdfast varied in the marine *H. baltica* in favour of operation at higher ionic strength. The work highlights why the genetic disposition alone is dissatisfactory for determination of function.

Bacterial attachment and adhesion are enormously adaptable processes[133]. There are also a plethora of non-specific surface interactions that may dictate attachment to a metal surface. Despite being a critical factor in determining the outcome of biofilm formation, these interactions particularly by pioneering bacteria with a surface remain poorly understood[81]. For example, the interactions of water with a surface in marine environments have been demonstrated for decades to have an important role in early attachment[80]. Specifically, surface and cell hydrophobicity can dictate the likelihood of bacterial adhesion and subsequent biofilm formation. It is also known that biofilms can alter the hydrophobicity of a substrate and quickly colonise it[79]. With this in mind, delaying or reducing biofilm formation by alteration of surface hydrophobicity has been achieved in multiple cases, however total prevention of biofilm formation on any marine surface including metals has yet to be seen[82].

Surface topography and charge:

Surface roughness and electrical charge of the bacteria and metallic surface are critical to the quantity, type and timeframe of bacterial attachment (Grasland *et al*[81] and references therein). For example, all laboratory simulations investigating attachment and adhesion of bacteria to metals should consider if strains are Gram-positive or Gram-negative, and the repulsive or attractive forces associated with the selected surface. Gram reaction describes the organisation of the cell wall, and ultimately the surface properties of these cells. CF molecules and ions in any fluidic environment also supply a charge quality to the substrate which may impact attachment tendency of the isolates under investigation.

There is a strong relationship between bacterial attachment and surface topography of a given substrate[129,130]. Attachment and tolerance of bacteria to shear stress should be favoured by rougher surfaces with a higher surface area, theoretically, by providing more landscape for bacterial interaction. In practice, literature often paints a different picture of bacterial attachment. Interestingly, attachment is a selective process that relies on bacterial dimensions relative to the surface and hydrophobicity and hydrophilicity. This phenomena has been evaluated using various strains exposed

to unique surfaces of specific dimensions crafted from polydimethylsiloxane (PDMS)[130]. Smoother surfaces were associated with 30-45% increased attachment rate. Similarly, using several strains of the model organism and food contaminant ***Escherichia coli***, Goulter-Thorsen ***et al*** found that smoother stainless steel surfaces were associated with increased attachment rates[127]. Although attachment rates may be higher on smooth surfaces in some cases, opposing findings have also been reported. Da Silva ***et al*** employed a culture of ***Streptococcus sanguis*** to surfaces of; 1) titanium as machined, 2) titanium coated with 65 µm particles of aluminium oxide (Al_2O_3) and 3) titanium coated with 250 µm particles. Cell attachment strongly correlated with increased roughness[131]. Following these observations, bacterial cells must strongly adhere and persist to build deleterious biofilms in marine environments. When shear stress was applied in work by Goulter-Thorsen ***et al***, cells on smooth surfaces were easily removed compared with rougher samples[127]. Therefore in fluid marine environments where shear stress is always a factor, higher attachment is likely to be expected to rougher surfaces. Further, '***rough topography***' is a subjective definition of surface microstructure, and can only be used in reference to other surfaces in the respective research paper. For interpretation of data and to practically reduce bacterial attachment, it is important to identify specific microstructures promoting adhesion in marine bacteria. In work by Xiao [132], marine isolates were found to favour attachment to specific 'kink sites'. The work summarises that both topography and species are important in determining attachment tendencies, which highlights the need to validate attachment studies with multiple isolates. Undeniably bacterial attachment in marine environments poses great variation from the laboratory based work described here. Understanding the complex factors involved in attachment and adhesion to metal surfaces, as well as how and why marine bacteria are selective in the process is a research area requiring further attention.

1.3 Growth and maturation

For adhered cells to successfully replicate, attract more community members and form robust biofilms there must be communication within and between species. Proteins determined by genetic expression, exDNA, polysaccharides and other organic and non-organic molecules and ions are often identified in natural marine biofilms[78]. The molecular configuration of the EPS gives rise to functions within the matrix including adhesion between cells and substrates, and subsequently cell-cell and cell-matrix adhesion. Although only proteins, exDNA and polysaccharides are discussed in this review, it is important to realise that the biofilm matrix varies greatly in composition depending on the species present. The additional complexity of multi-species marine biofilms, for example, may lead to widely different EPS components across locations.

Communication and quorum sensing:

Biofilm maturation in this communication will be defined as the development of a sessile population leading from initial attachment and adhesion, terminating at the dispersal stage (discussed separately). The distinction of dispersal as the hallmark feature of maturation views the biofilm mass as a multi-cellular organism rather than an agglomeration of single species[61], which is important in biofilm mitigation and control. In the developmental process (and other biofilm processes), for example, quorum sensing (QS) facilitates mass coordination of subpopulations within biofilms. QS autoinducers involved in regulation of quorum activities (regulation of genes within subpopulations and community density[61]) are produced in mono or multi-species systems, explaining the capacity of some biofilms to behave almost as a single organism. The importance of these molecules in development of mature biofilms has only been realised within the last three decades[61], although the first report of autoinducer activity in bacterial populations was described in ***Vibrio fischeri*** around 50 years ago[62]. The field has expanded rapidly since disruption of coordinated activities has been found to severely impact the ability of many isolates to form biofilms, metabolise and corrode steel surfaces[63, 136]. There are two major autoinducer methods; namely through (1) acylated homoserine lactones (AHS), primarily used by Gram-negative bacteria, and (2) oligopeptide autoinduction, which share many similarities with Eukaryotic cell-cell communication molecules[64]. The myriad autoinducers, target receptors and affected genes, and the amount of information still absent from literature makes the field an interesting and promising platform for biofilm investigations. A review by Bassler and Losick[65] explains the concepts of QS. In more recent work, the existence and potential disruption of universal communication molecules in QS theories has countless applications in all affected industries. In the corrosion of carbon steels for example, Scarascia ***et al***[136] demonstrate QS signal molecules could upregulate genes involved in electron transfer, sulfate reduction and pyruvate metabolism in sulfate reducing bacteria (SRB), the main group of bacteria involved in anaerobic MIC. Downregulation of these genes was observed in presence of QS signal suppressor molecules. The impact of QS inhibitors in biofilm mitigation is considered to be a promising potential MIC mitigation strategy[136].

Gene expression:

Communication by QS or other means leads to the activation or repression of genes. Such communication is vital to the biofilm formation process in many bacteria because resultant proteins have roles in EPS composition and structure. Adhesins function in the development of mature biofilms by facilitating cell-cell attachment. SdrC is an example of an adhesin protein recently identified in ***Staphylococcus aureus*** biofilms[70]. In the marine bacterium *Vibrio fischeri*, the symbiosis polysaccharide (*syp*), a gene locus encoding 18 genes, plays a central role in biofilm development and colonisation of hosts[66]. Similarly, two proteins Bap1 and RbmA were identified in *Vibrio cholerae* by Absalon ***et al*** in 2011 and Berk ***et al*** in 2012. These proteins have roles in the structure and special distribution of biofilms and are key components of the biofilm in this species[67, 68]. Although *V. cholerae* is most recognised for its impact on human health, many proteins identified in bacteria associated with infection may also be involved in biofilm development in marine

systems. In 2012, Ritter and colleagues discovered an upregulation of genes responsible for production of biofilm proteins OmpW, OmpA, and PilF in *Pseudoalteromonas* Sp. strain D41, a marine isolate. In mutant *P. aeruginosa*, a pathogenic bacterium and model biofilm former, the three proteins and associated genes were also found to impact biofilm volume and architecture[69]. The importance of proteins in biofilm composition, architecture and tolerance has led to a flood of publications which have improved fundamental understanding of the development process. As more proteins and their roles in biofilm development are uncovered, new potential targets for mitigation of biofilms on steel substrates may be considered.

Extracellular DNA:

The abbreviation *eDNA* has been frequently assigned to both *environmental DNA* and *extracellular DNA* in scientific literature[75, 76]. The authors make the distinction of the two as inclusion of DNA inside cells in the case of environmental DNA, as opposed to exclusively occurring outside the cell as with extracellular DNA. Therefore, the acronym *exDNA* will be used for the latter to avoid confusion. The roles of exDNA in establishing and maintaining biofilm structure have been investigated in depth since Whichurch and colleagues added DNase1, a DNA degrading enzyme, to *P. aeruginosa* biofilms in 2002, causing rapid biofilm dispersal[71, 72]. In the marine environment, exDNA exists in concentrations up to 2 µg g^{-1} in sediments [73] and comprises 70% or more of the total marine DNA[74]. ExDNA is repeatedly described as having important roles in structure, even from the early stages of biofilm formation[75], however its origin is still under debate. Evidence suggests some bacteria, especially *Pseudomonas aeruginosa*, have a number of complex biochemical pathways involved in active manufacture and secretion of exDNA. Other reports label the dominant mode of production as cell lysis, which may be an active process. For Example, Rice *et al*[77] concluded specific genes; *CidA* and *IrgA*, were involved in regulation of *S. aureus* cell lysis, which affected the number of dead cells. In cultures containing mutant copies of *CidA*, less dead cells resulted in lower attachment and weaker biofilm formation. Considering DNA comprises a major portion of the organic carbon pool in natural marine environments, and an affinity between metal oxides and negatively charged exDNA is common knowledge, marine-based simulations should consider what implications exDNA presence may have on results and conclusions of research.

Polysaccharides:

Exopolysaccharides are the polysaccharide complement of the matrix found exclusively outside the cell, and are comprised of simple sugar chains which are key to matrix formation and the establishment of a mature biofilm. The importance of polysaccharides in biofilm formation and the matrix has been known for decades[137]; after all, *glycocalyx* was an original term for the EPS. In a 1989 review, Christensen highlights that although vital to biofilm formation and attachment processes, almost nothing is known about natural polysaccharide composition of biofilms. The review suggests model organisms are required to understand polysaccharide contributions to the EPS[137]. Since then, polysaccharides have been reviewed and studied in great detail in a variety of species. *S. aureus* and *P. aurugenosa* are common model examples. *P. aerogenosa* for example produces several exopolysaccharides, including alginate, *PSL* and *PEL*; the latter of which was demonstrated by Jennings and colleagues to be pivotal to biofilm formation by crosslinking with DNA at certain pH[72]. The authors rationalise that *PEL* may also be involved in crosslinking other polymers besides DNA. In *S. aureus*, numerous polysaccharides are also produced, allowing attachment and biofilm formation on most surfaces it comes into contact with. Polymers of *N*-acetyl glucosamine (PNAG) for example are manufactured with the *ica* operon to produce the biofilm matrix[139]. López *et al*[140] reviewed four biofilm forming model bacteria and the contributions polysaccharides gave to the matrix. In marine environments, polysaccharides form a major pool or organic carbon for both planktonic and biofilm communities[141]. On submerged metals in marine environments characterised by nutrient flow and presence of water, attachment and biofilm formation quickly ensues. How polysaccharides in this context, as the primary component of the matrix assist in biofilm formation is still poorly understood. The inherent complexity of types and functions of polysaccharides, along with the difficulty of correctly simulating marine environments are primary reasons for this.

1.4 Dispersal

Dispersal may be considered the hallmark feature of a mature biofilm. Sub-populations of cells break away from the parent structure and colonise new locations in what are often highly regulated and coordinated events[103,105]. In this sense, and with development in general, the biofilm should be considered a single living organism, with resident cells communicating and acting in a concerted manner. The activity of dispersal eventuates as an active response to environmental stress; enabling populations of bacteria to persist in new environments when current ones become too inhospitable[103]. Dispersal may also be forced upon a biofilm by chemical or physical means. As with other developmental processes, the onset of dispersal has been found to be a complex phenomena involving many environmental and molecular triggers. For example, the production of exDNA is linked to biofilm structural integrity (see *Growth and maturation* section above). Somewhat conversely exDNA has also been demonstrated to inhibit biofilm formation and dispersal, preventing planktonic cell settling by inhibiting uptake of new cells into a biofilm[57]. The formation of a new biofilm following dispersal requires an arsenal of competitive characteristics, and planktonic cells achieve this by genetically diversifying when released from the parent biofilm[103]. Further reading on the biology and mechanisms of dispersal can be found elsewhere[103]. Specifically pertaining to marine biofilms, dispersal is often linked to cell death. In *Pseudoalteromonas tunicata*, Δ*alpP*-mediated cell death was demonstrated by Mai-Prochnow and colleagues to be associated with dispersal of surviving cells. Mutant *P. tunicata* cells incapable of expressing the ΔalpP autotoxic protein; resulting in lower local cell lysis, were associated with lower dispersal rates[104]. On a similar note, phage virus elicit cell

lysis in marine biofilms. Phage-mediated cell death has also been linked to increased dispersal[106]. This cell death may provide the physical disruption to otherwise sessile populations to promote dispersal. In other research, Barraud *et al* discovered nitric oxide (NO) to be linked to biofilm dispersal. *P. auruginosa* mutants unable to express a sole nitric reductase enzyme (ΔnirS) were unable to disperse while mutants expressing the enzyme could[105]. Coatings used in marine-based industry to prevent MIC and biofouling also can promote physical dispersal of biofilms before biocides must be employed[108]. Some years later, Barraud's group found nitric oxide releasing coatings promote dispersal with practical application for biofouling mitigation[107]. Today dispersal in marine environments is understood as a complex process that may be triggered or targeted to remove biofilms. More mechanistic research is required to fully understand the numerous approaches to dispersal, and apply this understanding to biofilm mitigation and control. In particular stripping active, mature biofilms from carbon and stainless steels is a high-priority challenge for marine-based industry.

2.0 Multispecies biofilms

The principle focus of this review is to collate information on marine biofilms on metals, characterised by species heterogeneity in all cases. While there are a wide range of behaviours described, cooperative or competitive behaviour are the two primary categories of heterogeneous biofilm interactions. Those interactions with a positive impact on one or both of the species are known as altruism and mutualism respectively, and competitive behaviours either specifically aim to harm the opposition or benefit the isolate expressing the behaviour. These interactions are discussed by Burmølle[20] and Liu[83]. When considered, any type of complex community requires interspecies interaction in order to survive. In the biofilm lifestyle, interactions between species have been found to increase tolerance to biocidal compounds, as well as shape biofilm spacial distribution and biomass structure[83, 85]. Further, we now understand that specific *enabling* populations within the multi-species system may provide benefits to the entire population. With every species contributing a unique benefit to the biofilm, the source of resilience becomes obvious; for example, strains capable of producing large volumes of EPS may be supplying the bulk organic content of the matrix for other species. Horizontal gene transfer (HGT) is also a common characteristic of multispecies biofilms which contributes to this tolerance[83]. Genes conveying chemical or environmental coping mechanisms may be exchanged between species, greatly boosting the tolerance of the entire population. Biofilms are particularly conducive to HGT, owing to high cell density[134]. This condensed living arrangement of diverse populations is frequently observed on submerged metals.

In order to understand natural systems and the development of environmental biofilms, research is moving towards multi-species research rather than single or dual-species. For further reading, comprehensive reviews by Burmølle *et al*[20] and subsequently Røder *et al*[21] address multi-species biofilm research trends at the time. Both reviews emphasise the absence of literature surrounding natural biofilms and the requirement for further understanding of development and interactions within these more complex systems. In 2020, Røder *et al*[84] produced a comprehensive review of current multi-species biofilm investigation trends and provides insight on research considerations and techniques. An important note on multispecies biofilms highlighted by Røder and colleagues is the need for a more detailed scrutiny of interactions on the microscale; considering specifically how physiological parameters such as gas, pH and nutrient gradients may influence subpopulation composition and functions of these populations[84,85]. Each contributing subpopulation of a multi-species biofilm can have unique functional attributions, which Røder argues is not considered by many current techniques such as sequencing (which often relies on large quantities of extracted cells for DNA)[84]. Therefore, experimental designs should aim to balance these limitations. Confocal based techniques for example (discussed below) can contribute spacial organisation and species distribution, which is lost by sequencing based techniques.

2.1 Evaluating multispecies biofilms

Data reproducibility and accuracy has been a longstanding difficulty in the analysis of multi-species simulations. Certainly the most challenging aspect is assembling reliable and meaningful data that reproducibly supports the hypothesis when microorganisms can quickly adapt and change to new circumstances. In light of this, most of the considerable data amassed on biofilm development was obtained from inherently simpler single-species simulations[20]. Today, new techniques and advances to those already established have allowed great insight into how bacterial communities interact and establish complex biofilms. Microscopic, spectroscopic and molecular techniques are on the forefront of multi-species biofilm research and are reviewed here.

Microscopy:

Confocal laser scanning microscopy (CLSM) is one of the most frequently employed microscopic techniques for evaluation of biofilms. CLSM works at its most basic level through emission of a laser, which passes through a pinhole to reduce unfocused light. The pinhole ensures a direct beam of high intensity light is used for capturing a micrograph, offering many benefits to conventional microscopy. For example, the light source is often powerful enough to penetrate thick biofilm samples, especially when probes such as fluorescent dyes are employed on the sample. The use of probes and a high power light source enables X, Y and Z mapping of specifically targeted components of biofilm matrix (such as cells or lipids). The scope, functions and operation of CLSM are described in detail elsewhere[86]. CLSM is particularly useful for evaluation of natural marine biofilms since contributing populations may be distinguished through rRNA-based probes[89], live cells may be visualised in their natural form with minimal disruption (see *Figure 1*) and both qualitative and quantitative measurements are possible. Although a powerful technique, confocal micrographs can contain large quantities of data, especially in quantitative assays, which can be difficult to manipulate. A major challenge for multi-species biofilm research using confocal microscopy then becomes data processing and interpretation. Additionally,

application of more complex probes for identification of microbial subpopulations such as in CARD-FISH (catalysed reporter deposition fluorescence ***in situ*** hybridisation) can be labour intensive and expensive.

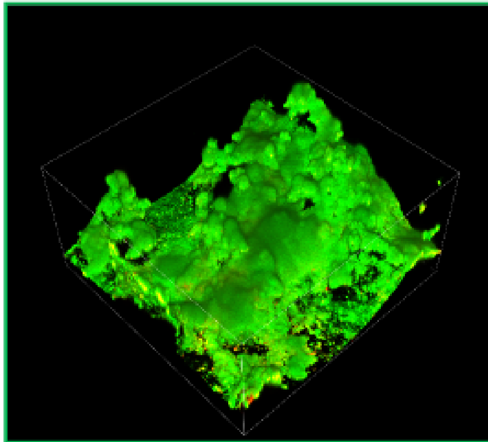

Figure 1 Confocal micrograph of marine consortium under 20x objective demonstrating biofilm topography, surface coverage and cell viability on carbon steel (ASI 1030), where green corresponds with live cells and red corresponds with dead cells.

Despite these limitations, confocal microscopy remains a prime technique for evaluating the three-dimensional form of biofilms[87]. Additionally the cellular complement, EPS proteins, polysaccharides, exDNA and lipids may all be targeted by confocal probes and micrographed[56]. Microorganisms and selected components of the matrix can also be identified using unique probes which can, for example, provide an estimation of live and dead cells (propidium iodide and Syto9™, both available commercially in kits), protein expression and even indicate membrane integrity (such as SynaptoRed™ C2).

To assist with analysis where required, there are many and often free software platforms available. There have been a variety of image processing platforms available for some decades which can provide data on morphological parameters of marine biofilms; including microscale structure and heterogeneity[88] (COMSAT and ISA), biofilm coverage (PHLIP)[87] probe evaluation and direct quantification of populations within a biofilm (*daime*)[89]. *Daime* is particularly useful where fluorescence *in situ* hybridisation (FISH) and various iterations are employed, as such techniques work with specific probes which are frequently used to identify subpopulations within biofilms. The selection and evaluation of the probes with software like *daime* is vital to the efficacy of the technique applied. A variety of other platforms exist which are not yet employed on marine biofilm micrographs; including *bio*Image_L which was demonstrated to identify biofilm subpopulations based on viability and metabolic activity[90].

As with confocal microscopy, epifluorescence microscopy is well-established and relies on the use of probes for emission of light and micrograph acquisition. Probes for confocal microscopy can often be used in epifluorescence microscopy where light is the illumination source. Instead of lasers emitting at specific wavelengths, light passes through a filter to achieve a pre-determined wavelength. The relative simplicity has seen widespread use of this technique in simpler sample types and quantitative enumeration of biofilm cells is also possible[91], however three-dimensional image acquisition is not a feature of this technique. Standard light-based illumination cannot penetrate thick biofilm samples and so there is limited application in complex biofilm studies.

Scanning Electron Microscopy (SEM) allows mainly qualitative analysis of biofilm architecture. High resolution microscopy is possible because the traditional illumination source, light, is exchanged for an electron beam. The cost of this technique is in the sample preparation stages, which include;1) fixation of the biofilm sample for several hours using tissue fixatives like gluteraldyde, 2) dehydration of the sample using an ethanol series, by nitrogen drying or both, and 3) sputter coating of the sample using an inert metal such as platinum or gold[92]. The extensive sample preparation for SEM is a result of chamber vacuum, although there are variable pressure scanning electron microscopes available which allow some samples to be imaged almost *in situ,* with minimal sample preparation. For biofilm imaging even on metals, both variable pressure and high vacuum systems may suffer from conductivity issues leading to charging of the sample and poor microscopy. SEM still remains one of the highest resolution techniques available today, and the application of the technique itself is far simpler than CLSM or atomic force microscopy (AFM). '***A picture paints a thousand words***', in this case, where SEM brings a unique and high quality interpretation to any biofilm study. ***Figure 2*** demonstrates the biofilm coverage and structure, cell density and cell morphology of a marine isolate biofilm on carbon steel. If sample preparation is performed correctly, cells and EPS hold their original profile and biofilms can appear more or less as naturally formed.

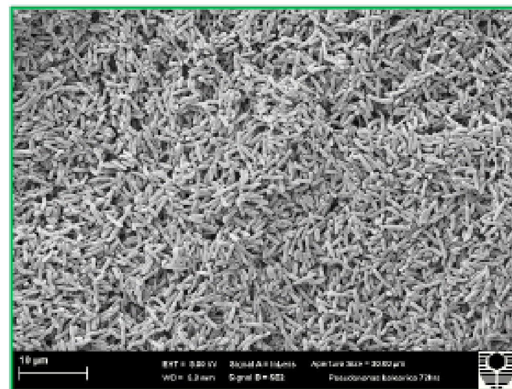

Figure 2 FESEM micrograph of marine isolate *Pseudomonas balearica* strain EC28 on carbon steel (AISI 1030)

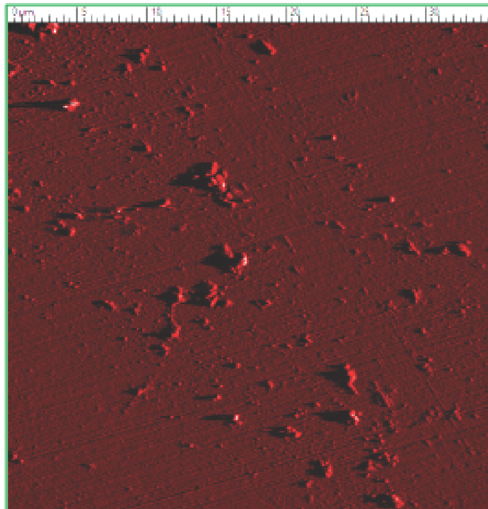

Figure 3 in situ height sensor AFM micrograph of carbon steel (AISI 1030) in ASW solution exposed to marine isolate *S. chilikensis* strain DC57.

Surpassing the rigorous sample preparation seen in SEM, atomic force microscopy (AFM) provides the highest resolution of any microscopic technique available today with minimal or no sample processing. AFM works through the raster scanning of a cantilever possessing a microscopic tip across the sample. The cantilever is targeted by a laser, which refracts off the cantilever as it moves. The refracting laser movements are proportional to the cantilever movements, which are collected by a photosensor and processed using computer software. The resulting image displays topographic qualities of the sample as with a traditional microscope, based on the laser movements. The technique is also capable of mapping many nano-mechanical properties of a sample including Young's Modulus (E), adhesion, dissipation and elacticity. For a recent comprehensive review of AFM the reader is directed to Cárdenas-Pérez *et al*, 2018[93]. For the investigation of multi-species marine biofilms on metals, *in situ* AFM is very possible in the sense that submerged, living biofilms may be micrographed (see *Figure 3*) and various physicochemical parameters also obtained; although this requires extensive optimisation for the given sample. In *Figure 3*, a low resolution micrograph, carbon steel is constantly corroding under biofilm physiological conditions. A 1 µm diamond finish polish was employed and the sample submerged under nitrogen-flushed artificial seawater (ASW) comprising washed *Shewanella chilikensis* cells.

Probe types, physiological qualities of the fluid, atmospheric conditions, biofilm layer thickness and sample structure, among other parameters (such as microscope settings) are important considerations when attempting *in situ* AFM. Well-optimised applications of this technique have seen momentous advantages to the field of biofilm research, including Li *et al*, who demonstrated cell-substrate adhesion qualities could be measured through the use of bacterial cells as a cantilever tip[93]. A novel AFM tip was manufactured using living cells adhered to a cantilever, thereby directly monitoring interactions of a cell with a pyrite substrate. In a separate study, Li *et al* demonstrated using AFM, that EPS plays an important role in cellular adhesion to mineral surfaces [94]. Since the first documented account of AFM in scientific literature was introduced in 1986 by Binning, Quate and Gerber[95] AFM has become a primary tool in the investigation of biofilm formation. In particular, the niche of AFM seems to be within those earlier stages (attachment, adhesion and micro-colony formation) where the limitations of thick biological samples are much less apparent. Provided the researcher can navigate corrosion product development on a chosen metal, or use a corrosion-resistant alloy, AFM can be a powerful technique for elucidating the early mechanisms of biofilm formation on metallic substrates.

Like all techniques, the limitations of microscopy must be acknowledged and the results interpreted cautiously. All microscopic techniques provide unique biases, combined with interpretation bias from the viewer. This can lead to total misrepresentation of the sample and inaccurate assumptions in any investigations. These issues have been recently discussed by Jost & Waters[96]. Where applied correctly and supplemented with alternative techniques, ideally quantitative in nature, microscopy can provide great insight to biofilm investigations.

Molecular techniques:

Much of what we know about multi-species biofilm diversity has come from the application of molecular techniques. Traditionally, microorganisms were cultivated on plates or broth which presented many inconsistencies. Researchers quickly found that plate counts could never be matched to counts from samples, which became known as the Great Plate Count Anomaly[135]. Why was it that plated microorganisms could never match numbers directly counted from samples? Also, why did so many species fail to reproduce in nutrient rich conditions presented by the petri dish? Indeed, we now understand that natural environments host diverse populations by meeting complex nutrient, atmosphere, water, temperature and pH requirements of these fastidious microorganisms[99] which is often not practical in a laboratory setting. Uncultivatable microorganisms by this respect are not uncultivatable, but yet-to-be cultivated[99]. Unrestricted by the traditional culturing prerequisites like many other techniques (for example quantification by plate count or mean probable number [MPN] or identification through biochemical testing), rRNA analysis for example has identified many new divisions which are reviewed by Lewis *et al*[99]. In marine systems especially, the vast bulk of species so far unknown to science is staggeringly high[99]. At this stage, how many species actually remain uncultured is unknown. Since this plethora of new species became apparent to science, molecular methods are now central to functional diversity and distribution studies of any biofilm system[98]. As advances in molecular technologies continue to surge, and with this their use in routine microbiological investigations, -omics based techniques (meta- transcriptomics, proteomics and metabolomics) Next Generation Sequencing technology (NGS) and Micro-array technology in particular are now conventional in multi-species biofilm studies.

NGS is a contemporary, high-throughput DNA or RNA sequencing technique which has enabled more cost effective and time sensitive analysis of sample types from across the life sciences. There are four main types of NGS: cyclic reversible termination, sequencing by ligation, pyrosequencing and real-time sequencing which are reviewed by

Metzker[101]. NGS techniques are particularly useful for the identification of unknown sequences; for example in heterogeneous natural bacterial populations, and have been used to shed light on diversity and distribution within biofilms[97,98,102]. Although the technology has been available for the past 15 years, the structure of biofilms even in everyday environments like the household remains elusive, and NGS can be used to fill this gap[102]. Although powerful in quantitative enumeration and identification of unique sequences, extraction of usable RNA or even DNA directly from metal surfaces remains a major challenge as iron can interfere with the efficiency of extraction protocols. As has been the trend over the past decade, preparation of DNA and RNA will likely become a more streamlined process - further consolidating NGS and other molecular techniques as primary tools in multi-species biofilm investigations.

Omics-based techniques hold a very prominent position in marine based biofilm studies. The genetic complement of a system ([meta] genomics), what is transcribed ([meta] transcriptomics) and the resulting protein expression (proteomics) especially can provide a great deal of information on the functions of a population on a molecular level. In some cases multiple omics-based techniques may be applied to understand a microbial system in more detail. For example Beale *et al* employed metabolomics and metagenomics to understand the effect of inorganic nutrients and pollutants on marine bacteria[107]. The beauty of combining the two means genotypes and possible capabilities of microorganisms can be compared against actual metabolic functions. The researcher can learn useful information about a biofilm system such as which genes are active and how gene expression changes under different circumstances, such as in response to toxins.

The adaptation of bacterial communities to environmental stimuli is of great importance to the management and prevention of deleterious biofilms, understanding the health of marine systems and routine monitoring practices. Microarrays are the technique of choice for understanding environmental responses and diversity in microbial systems on a genetic level. A microarray or ***DNA chip*** is a slide comprising potentially thousands of dots of known location, each with a unique known sequence or gene, which become probes for DNA or RNA mixtures. Recent advances have seen the development of dense microarrays with many thousands of 'spots', or with multiple probes per spot (many probes one spot or MPOS-microarrays). The bound probes identify gene expression, or transciptome properties of a sample. For further information on MPOS-microarrays see Kostina *et al*[116]. Microarrays already have an extensive reach into environmental and health research, which is extending into marine microbiology. To date, microarrays have been applied to understanding anthropogenic impacts on marine bacteria[109,110], monitoring bacterioplankton communities[111], identifying pathogenic strains and pollution in seawater[112], identification of nutrient shifts in marine isolates[113], detection of marine toxins[114] and profiling marine communities[115]. Microarray technology will likely continue to advance and increase in scope of application in coming years; especially as there are no equivalent techniques for the detection of genetic markers in heterogeneous samples.

Chemical structure and function:

The demand for quantification and detection of metabolites and EPS components has given spectroscopic techniques an important place in biofilm research. Research topics identifying the fundamental components of the matrix, how the matrix composition changes in response to various stimuli and influence of these stimuli on metabolisms, among many others, may rely on techniques such as Fourier transform infrared spectroscopy (FTIR), matrix assisted laser desorption or ionisation (MALDI) or nuclear magnetic resonance (NMR) based techniques[58].

The use of FTIR is widespread for the evaluation of components within the matrix. EPS are obtained through development of a biofilm at the desired level of maturation, homogenised and the EPS extracted. IR spectra are then obtained from the sample and a spectrum is produced. From this spectrum, presence of EPS components (for example polysaccharides) may be deduced. In work by Muthusamy Ashok and colleagues, a single-species marine biofilm was grown using a flow reactor system and EPS extracted and chemically evaluated. FTIR was employed to determine the major saccharide components of the matrix, which were glucose and galactose[117]. Sometimes harmful compounds can accumulate in biofilms giving them forensic value, and FTIR is useful for locating these compounds. For example, cadmium has been detected in heavy metal tolerant marine biofilms[118]. In this article, attenuated total reflection was used in conjunction with FTIR (ATR-FTIR), a technique capable of reducing the sample preparation and in doing so damage of the sample. They successfully identified a functional group within the biofilm which was able to bind Cd. Further, upregulation and downregulation of Cd resistance genes were associated with pH and salinity in the marine biofilm forming bacteria. FTIR has also been used to characterise marine biofilm EPS with the aim of screening for anti-biofouling compounds[119].

Matrix assisted laser desorption/ionization time of flight spectrometry (MALDI-TOF) is a spectrometry-based technique which has traditionally relied on laser destruction of a sample and identification and analysis of the components based on particle time of flight (the larger the molecule, the shorter its flight time). The technique has been applied extensively in biofilm research. Most relevant to this review was work applying MALDI in conjunction with mass spectrometry (MALDI-MS) to characterise EPS components in multi-species marine biofilms, first described in 2011[120]. Hasan *et al* therein describe the optimal conditions for the technique on such samples. There are many iterations and extensions of the MALDI technique which has considerably diversified the capability. MALDI-ID (MALDI-TOF MS) for example is used routinely for identification of single cells in a population[121,122]. MALDI-typing is an iteration with potential for typing bacteria in diverse populations[122,123]. Techniques based on MALDI have seen a surge in advancement as microbiological identification tools and reduced the application of previous time and workforce intensive techniques[123].

While the significance of this lies mostly in the health industry for diagnostic turnaround, MALDI diversify and improve the efficiency of research in environmental biofilms.

Nuclear magnetic resonance (NMR) is the epitome of techniques characterising structure of molecules[123]. The technique relies on the fact that the isotopes of some atoms (for example hydrogen, carbon, phosphorus and nitrogen) have a magnetic dipole at the atom nucleus, with multiple identical energy levels which can be split when they are placed in a magnetic field. The energy gap which results is specific to the magnetic and electronic environment surrounding the atom (or nucleus) and thus acts as a fingerprint which reveals sample structure. Details on the technique and NMR applications are outlined by Mopper *et al*[23]. The viewing of matter and its structure is an important aspect to some biofilm studies. Xiu *et al* in 2017 identified a highly motile marine isolate could be inhibited by another isolate from the same niche through motility suppression. Their work employed NMR to elucidate the active compounds inhibiting motility of the isolate[125]. This work, while primarily focused on virulence suppression, represents an approach to potential multi-species biofilm interactions. NMR is a powerful tool for identifying potential molecular interactions between species vital to establishment of robust natural biofilms. Similar to MALDI, the technique has also been applied to understand the biofilm composition of isolates[126], with the potential to be applied in marine biofilm research.

3.0 Concluding remarks

Multispecies biofilms in the marine environment exhibit great complexity. Certainly, there is also a great deal that has been uncovered regarding multi-species biofilms in various natural environments using advanced techniques such as contemporary molecular technology and advanced microscopy. Knowledge we have obtained on biofilm formation has facilitated mitigation and control measures in health, energy, transportation, agriculture and construction industries, and will continue to do so into the future. Biofilm formation in these industrial sectors leads to deleterious effects such as MIC, contamination and biofouling on infrastructure, machinery and manufactured products. The attachment and biofilm formation processes as occurring in marine environments to steel surfaces has received a great deal of attention these last decades due to these negative impacts. Through this corporate and scientific interest, we have found that bacteria attach to surfaces preferentially, using a variety of EPS components. General and surface-specific proteins, polysaccharides and exDNA are all implicated in this process. Single cells may also initiate attachment and biofilm formation on steels based on surface microstructure, charge and composition, a process which is controlled and preferential. Cellular appendages and characteristics may also be involved in attachment to metals; such as surface charge, pilus and flagellum presence and hydrophobicity/hydrophilicity properties of the membrane. From this enormously complex and still poorly understood process in marine settings, biofilms are known to quickly develop placing the topic at the forefront of MIC research. Finally, information gained from other industrial sectors such as health is often very applicable to prediction of phenomena (for example, attachment) occurring in marine-based industry.

Research has also concluded that biofilm formation after attachment is divided into several distinct stages, which holds true for most metal substrates. The establishment of strong, mature biofilm structures is reliant on EPS composition and interactions between the many species that exist in natural marine biofilms. These community members provide fitness benefits such as tolerance to chemical treatments through horizontal gene transfer (HGT) to the other members. There are a variety of such tolerances to adverse conditions which have been identified in more complex systems. Interactions within multi-species biofilms are therefore vital for current research to understand in order to effectively control biofilm development on metallic surfaces. Such research frequently addresses the recurrent tolerance of environmental biofilms to biocidal compounds. Increasing environmental awareness and global regulation stringency has also led to banning and reduced dosing of effective but toxic treatment options. The multi-pronged problem of addressing marine biofilms in an economically, environmentally and production-sustainable manner should be, if not already, the main goal of research in the field. At present, advancements in technology have permitted elaboration on some of many unanswered questions surrounding the fundamentals and control of complex marine biofilms. This review has attempted to identify some of the most pressing of these questions; such as ***how and why bacteria attach and adhere to various surfaces, what contributes to the structure of biofilms, how does biofilm composition affect its function; especially in terms of attachment and establishment of a mature structure***, and lastly, ***how do species within a complex multi-species system interact and how does this affect the overall structure and function of the biofilm?*** Most, if not all of the research herein in some way pertains to mitigation and control of biofilms which is still not reliably achievable in the vast majority of natural systems, costing billions of dollars annually. Some of the most promising mitigation methods looking to the future of multi-species biofilm control on steels based on this review are considered to be multi-functional biocides and CIs, quorum sensing suppressors and phage virus treatment, all of which bypass the fundamental ideas of previously employed biocide treatment options (for example dosing with heavy-metal based toxins). These more traditional options, while effective, present unacceptable risk given current knowledge. Research focused on answering the above questions is required to achieve the ultimate goal of efficient and sustainable biofilm mitigation on metal surfaces, where required, but also to harness and employ the many capabilities of bacterial biofilms in industry and society today.

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AUTHOR DETAILS

Benjamin Tuck is a PhD student at Curtin Corrosion Centre, and a member of Dr. Laura Machuca Suarez' team of researchers investigating the interaction of microorganisms with metal surfaces. Ben's work involves assessment of green, multi-function corrosion inhibitors on multi-species systems and evaluation of the attachment, adhesion and development of biofilms to subsea surfaces.

Dr. Laura Machuca Suarez is a senior research fellow at Curtin Corrosion Centre, where here research interests are applied in the field of microbiologically influenced corrosion (MIC). Dr. Laura leads a team of MIC researchers engaged in investigating and mitigating the effects of bacteria on corrosion and deterioration of metallic equipment, vessels and pipelines, which cost the industry billions of dollars annually.

Chapter 2: EVALUATING MARINE BACTERIAL ATTACHMENT TO CS

Chapter 2: Evaluating marine bacterial attachment to CS

Manuscript # 1 of 2

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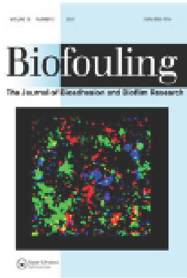

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Conditioning of metal surfaces enhances *Shewanella chilikensis* adhesion

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ABSTRACT

Microbiologically influenced corrosion and biofouling of steels depend on the adsorption of a conditioning film and subsequent attachment of bacteria. Extracellular deoxyribonucleic acid (eDNA) and amino acids are biologically critical nutrient sources and are ubiquitous in marine environments. However, little is known about their role as conditioning film molecules in early biofilm formation on metallic surfaces. The present study evaluated the capacity for eDNA and amino acids to form a conditioning film on carbon steel (CS), and subsequently, the influence of these conditioning films on bacterial attachment using a marine bacterial strain. Conditioning films of eDNA or amino acids were formed on CS through physical adsorption. Biochemical and microscopic analysis of eDNA conditioning, amino acid conditioning and control CS surfaces demonstrated that organic conditioning surfaces promoted bacterial attachment. The results highlight the importance of conditioning the surface in initial bacterial attachment to steel.

KEYWORDS

Microbiologically influenced corrosion; conditioning film; eDNA; amino acids; biofouling; attachment; adhesion; biofilm

Introduction

Corrosion costs an estimated $4 Trillion USD per annum in repairs, maintenance and prevention globally (Li et al. 2018). Of this cost, microbiologically influenced corrosion (MIC) is expected to contribute around 20% (Little and Lee 2007; Skovhus et al. 2017). Indeed, assessments of corrosion failures record localised MIC figures greater than 10 mm y^{-1} (Machuca Suarez and Polomka 2018) making MIC one of the most costly and unpredictable forms of corrosion. MIC is the accelerated corrosion caused, initiated or maintained by microorganisms and their metabolic products and is dependent on microorganisms living in a biofilm or sessile community (Moura et al. 2013; Albahri et al. 2021). Once a biofilm develops at a metallic interface, the subsequent corrosion process is characterised by the species and metabolic diversity within that community (Al-Abbas et al. 2012). Biofilms in nature are complex and dynamic, and therefore it is ideal to prevent MIC at early developmental stages where microorganisms are much more susceptible to treatments (Olson et al. 2002).

Establishment of biofilms begins with attachment of microorganisms to the metal surface (Javed et al. 2016). A plethora of scientific literature has investigated the various mechanisms of bacterial attachment with the aim of ultimately preventing it (Bazaka et al. 2011; Pandit et al. 2020). One critical process identified in the attachment and adhesion stages is the formation of an organic film at the substratum interface. The importance of organic molecules in the attachment of living things to solid surfaces was first acknowledged in the scientific literature almost 60 years ago (Crisp and Meadows 1963). The study uncovered a tendency for macroscopic organisms such as barnacle larvae to preferentially adhere to surfaces conditioning or 'bathed' in environmentally-derived organic compounds. These compounds almost immediately adsorb to surfaces exposed to seawater (Bhagwat et al. 2021). Locally available organic and inorganic components that form an interfacial film, usually in aqueous environments, have been referred to as the conditioning film. Since its discovery, the development and role of the conditioning film has been investigated on various surfaces including ceramics (Siboni et al. 2007), synthetic polymers (Bakker et al. 2004), glass (Garg et al. 2009) and steels (Compère et al. 2001). The composition of the conditioning film has been described as environment- (Schneider et al. 1994) and substratum-dependant (Garg et al. 2009), although common constituents

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include proteins, polysaccharides, humic acids, glycoproteins, lipids, nucleic acids and amino acids (Garg et al. 2009). Importantly, the presence and composition of the conditioning film can influence the attachment of organisms, especially bacteria (Hwang et al. 2012). For example, Bakker et al. (2003) demonstrated that organic carbon in marine environments could adsorb onto glass and the work concludes that the number of adhered bacteria was proportional to the amount of adsorbed organic carbon to those surfaces.

Since the early studies investigating the effect of a general conditioning film, limited studies have evaluated the role of its individual components. The impact of an alginate (polysaccharide) conditioning film has been detailed using *Pseudomonas aeruginosa* as a model microorganism (de Kerchove and Elimelech 2007). Similarly, biofilm exopolymeric substances have been extracted from *Bacillus subtilis* and the isolate's attachment to those surfaces evaluated (Omoike and Chorover 2006). Research by Hohmann et al. (2017) also identified the proteins involved in the conditioning film and biofilm formation process. These studies provided insight into the importance of conditioning film polysaccharides and proteins for bacterial attachment, but did not explore the role of other important interfacially adsorbed organic carbon sources or their relationship with metals.

The conditioning film invariably forms on materials in most aqueous environments, and the research reported here investigated this layer in marine-simulating conditions. Proteins are a combination of amino acids that form peptide chains, both of which are ubiquitous in marine environments and have long been associated with the marine conditioning film (Taylor et al. 1997). Amino acids are also chemotactic (Yang et al. 2015). Interestingly, chemotactic behaviour elicited by amino acids does not necessarily correspond to the usability of this organic carbon source by bacteria, indicating that bacterial attraction is not limited by the need to consume organic carbon alone (Yang et al. 2015). Extracellular DNA (eDNA) has also been identified as a common biofilm and conditioning film component on glass surfaces (Vilain et al. 2009) and represents an ubiquitous source of organic carbon in marine environments (Nagler et al. 2018). Extracellular DNA can also be involved in the transfer of survival traits within the biofilm through horizontal gene transfer (Abe et al. 2020). Bacteria in contact with eDNA at the interface may therefore receive additional survival benefits leading to competitive advantages.

Based on survival benefits afforded by amino acids and eDNA in the conditioning film, these molecules are likely to influence microbial behaviour in the early stages of biofilm formation. However, eDNA and amino acids do not always confer a positive effect on biofilm formation. For the later, isomerism is critical to the nature of the influence on bacteria (Cava et al. 2011). For example, in the *L*-configuration, amino acids are exceptional carbon sources (Jørgensen et al. 1993). On the contrary, amino acids in the *D*-configuration are rare in nature and have been applied as biofilm dispersal agents (Cava et al. 2011). Structural similarity in this case is not linked to function. It remains unclear if amino acid mixtures can form a conditioning film on steels, and what effect these molecules have on surface attachment.

Like amino acids, eDNA is not always associated with positive effects on bacterial processes. Although eDNA is a source of organic carbon and hosts survival benefits to bacteria, salmon eDNA was recently demonstrated to repel bacteria (Pingle et al. 2018). These authors found a negative correlation between the attachment of *P. aeruginosa* and the presence of salmon eDNA adsorbed on silicon wafers. This study limited bias by other factors involved in attachment and elegantly evaluated the effect of eDNA on attachment. However, bacterial attachment to steels in natural environments is inherently more complex. For example, carbon steel produces iron oxides in marine environments which can promote adsorption of organic compounds and bacterial attachment (Shenoy et al. 2020; Tuck et al. 2021). In more complex scenarios where surface physico-chemical properties and solution ionic strength are considered, eDNA has been found to promote bacterial attachment. For example, Regina et al. (2014) established a positive correlation between attachment of bacteria to hydrophobic surfaces and the presence of eDNA at the interface. In separate work, removal of DNA from cultures of a Gram-positive isolate was demonstrated to impede initial attachment (Das et al. 2010). Increased attachment of Gram-positive bacteria was found to be associated with the generation of favourable acid-base interactions afforded by DNA at the interface (Das et al. 2010).

It is well established that charge, among other surface physio-chemical factors, can also influence bacterial attachment (Regina et al. 2014). Gottenbos et al. (1999) for example measured the attachment of *P. aeruginosa* to positively and negatively charged poly(-methacrylate) surfaces. Early attachment was twice as fast to the positively charged surface. Since DNA and

P. aeruginosa cells carry a net negative charge, repulsive forces apply between them (Gottenbos et al. 1999). Similarly, charge afforded by iron oxides can influence attachment (Shenoy et al. 2020). However effects of the marine environment must also be considered. For example iron oxide nanoparticles (Fe_3O_4) can attract eDNA in seawater (a net negative charge) only since salts reduce the effects of charge repulsion (Liu and Liu 2014). Therefore factors influencing bacterial attachment to eDNA-adsorbed metals in marine environments are complex. This complexity is expected to give rise to very different surface qualities compared with glass or silicon based substrata.

A greater understanding of the effect of these organic conditioning film components can lead to development of monitoring tools for early detection of biofilms in aquatic systems (Boualam et al. 2002). Further, by generating a link between conditioning film components and biofilm development, the risk of MIC may be better understood and managed since MIC depends on biofilm formation to progress (de Carvalho 2018).

The present research aimed to address knowledge gaps surrounding the potential for amino acids and DNA to adsorb onto carbon steel (CS) and develop a conditioning film that influences bacterial attachment. *Shewanella chilikensis* strain DC57 was selected to evaluate the effect of the conditioning film on bacterial attachment based on previous implications in MIC of carbon steel (Salgar-Chaparro, Castillo-Villamizar et al. 2020; Salgar-Chaparro, Darwin et al. 2020). Confocal microscopy and electrochemical analysis were used to assess adsorption of eDNA and amino acids onto CS. Both conditioning film types were then independently assessed for their impact on *S. chilikensis* DC57 attachment to CS in seawater.

Materials and methods

Carbon steel coupon preparation and experimental design

CS rods (grade AISI 1030) were cut into coupons of 5 mm thickness and 12.6 mm diameter. Coupons were electrocoated with a protective epoxy (PowerCron™ 6000CX, PPG industrial coatings) and a single working surface was exposed to the test solution. The working surface was wet ground using silicon carbide paper to a 120 grit finish. Grit size for surface preparation was selected to provide a stable base to anchor bacteria and the conditioning film. The surface finish also promoted stable oxide layer formation that could withstand movement of the reactor fluid without disruption. The coupons were then degreased with acetone, washed with absolute ethanol and dried under nitrogen gas. This freshly wet-ground surface represented the working surface for all amino acid based experiments. For eDNA experiments, wet-ground surfaces were pre-oxidised as described elsewhere to form a uniform iron oxide film (Tuck et al. 2021). The multi-electrode array (MEA) was wet-ground to a finer 600 grit finish to facilitate acquisition of accurate galvanic current measurements.

Reactors were filled with 50 mL of artificial seawater (ASW) medium, pH 7.4, as described elsewhere (Eguchi et al. 1996) with calcium chloride the only alteration in composition (from 1.5 to 0.1 g l^{-1}, to limit precipitation). Each reactor contained four identical coupons at the base with the working surface face-up, fully immersed in reactor solution. To establish and evaluate the amino acid conditioning film, ASW was supplemented with 5 mM casamino acids (Bacto™). This mixture comes from the hydrolysis of casein and contains mainly L-isomers of all amino acids except tryptophan. For eDNA experiments, 40 µg mL^{-1} of salmon DNA (Sigma-Aldrich) was supplemented to ASW. Conditioning film molecules were injected to reactors 30 min prior to inoculation with *S. chilikensis* cells to allow the molecules to adsorb to the working surface. Finally, the reactors placed on gentle agitation (50 rpm) to promote active interaction with the surface as opposed to passive settling of bacterial cells.

Bacterial strain

S. chilikensis strain DC57 was previously sequenced and characterised (Salgar-Chaparro, Castillo-Villamizar et al. 2020). The strain was originally isolated from a West-Australia floating oil production facility, where it was implicated in MIC of seal rings (Salgar-Chaparro, Darwin et al. 2020). The isolate was cultivated anaerobically at 30 °C in ASW as described above, supplemented with 5 mM casamino acids, 5 mM sodium pyruvate, 5 mM glucose and 15 mM ammonium nitrate. Anaerobic conditions were maintained by preparing 50 mL serum vials with 30 mL of ASW medium and flushing with pure N_2 gas, before inoculating with *S. chilikensis* DC57. Growth was monitored under these nutrient-rich conditions to determine the log phase of the isolate (data not included). For attachment experiments, log phase *S. chilikensis* DC57 cultures were washed several times (to remove any remnants of culture media components) and resuspended in fresh phosphate-buffered saline (PBS, Sigma,

pH 7.4). The washed cells were acclimatised by incubating at 30 °C for 1 h before inoculating into reactors. Acclimatisation was performed to allow recovery from shock induced by the centrifugation process. Centrifugation involves compacting cells to form a pellet; which can lead to shear force against the cell membrane, ultimately affecting experiments sensitive to cell membrane changes (Peterson et al. 2012).

Evaluation of amino acid and eDNA adsorption to CS via electrochemical analysis

Adsorption of amino acids on CS was evaluated using electrochemical analysis, since electrochemical changes were expected to occur in response to organic molecule adsorption. Galvanic current and corrosion potential changes were monitored in real time using a MEA (CPE systems Pty Ltd) that provides temporal and spatial information about the interface (Tan 2011).

For amino acid adsorption, electrochemical experiments using the MEA system were conducted at 30 °C with and without 10 mM Bacto™ casamino acids. A custom designed reactor was employed to hold the MEA with the working surface orientated face-up, where temperature was controlled using a submersed heater element. The sensor consisted of 100 API X65 pipeline steel electrodes (0.0595 cm^2 each) electrically insulated and tightly packed with a total surface area of 5.95 cm^2. Local galvanic current density and corrosion potential measurements were obtained immediately after MEA reactor set-up (i.e. directly after probe immersion in reactor solution) as described elsewhere (Tan et al. 2011; Tan 2012). It was considered important to capture electrochemical data as soon as possible from the point of probe immersion in solution since conditioning molecules have the capacity to adsorb within minutes of surface exposure (Lorite et al. 2011). The resulting electrochemical changes were then used to predict molecule adsorption by comparing to controls.

Electrochemical tests were conducted using eDNA. However, no significant changes to surface electrochemistry were observed (see supplementary Figure 1). Therefore, direct visualisation of eDNA using fluorescent dyes and spectrophotometry were used for the evaluation of eDNA adsorption to CS.

Evaluation of DNA adsorption to CS via spectrophotometry and confocal microscopy

Pure salmon DNA (Sigma-Aldrich) was applied at 40 µg mL^{-1} to reactor solution as the eDNA source. The adsorption of eDNA on pre-oxidised coupons was evaluated using a Qubit fluorimeter and DNA high sensitivity reagent kit in three scenarios: (1) pre-oxidised coupon in ASW containing eDNA, (2) DNA-glassware adsorption control which consisted of the same experimental design without a coupon to ensure no DNA adsorbed to glassware, and (3) a freshly wet-ground coupon in ASW. The concentration of eDNA in solution was then monitored at the time of addition and after 24 h to confirm adsorption on surfaces.

Confocal laser scanning microscopy (CLSM) was used to directly visualise the eDNA on the surface of coupons. Propidium iodide, a DNA specific stain, was applied to pre-oxidised coupons before and after DNA exposure. The stain was applied for 10 min before gently rinsing in phosphate buffered saline (PBS) to remove excess stain. For all confocal experiments a Nikon A1+ confocal microscope and the latest version of Nikon Elements software was used. Stained samples were micrographed using a 489 and 561 nm laser and a 500–550 and 570–620 nm emission filter with a pinhole radius of 1 AU. A 20× objective was used for all confocal microscopy.

Bacterial attachment to conditioned and control CS

CLSM was also used to visualise attachment of *S. chilikensis* DC57 to control and conditioned surfaces at 24 h. After incubation for 6 and 24 h with *S. chilikensis* DC57, the control and pre-conditioned surfaces were lightly rinsed in PBS (Sigma, pH 7.4) and stained for 10 min using propidium iodide and Syto9™ (Filmtracer™ LIVE/DEAD™ Biofilm Viability kit, ThermoFisher). Syto9™ stains intact cells green and propidium iodide is membrane impermeable staining dead and membrane compromised cells as well as binding to eDNA. Thus, this stain was able to confirm both eDNA and *S. chilikensis* DC57 presence at the interface simultaneously. Microscope and aquisition settings remained the same as detailed above.

SEM confirming bacterial attachment to CS

SEM was conducted to visualise attachment of the isolate with and without a conditioning film. Coupons were also micrographed after exposure to the control ASW and ASW with conditioning molecules to compare attachment of *S. chilikensis* DC57 to these substrata. Coupons were preserved for 22 h at 4 °C in 2.5% glutaraldehyde before drying under

nitrogen gas overnight. Dried samples were sputter coated with platinum and stored under desiccation. SEM micrographs were captured using a Neon field emission SEM. A combination of in-lense and secondary electron detectors was employed at an EHT of 5 kV and an aperture of 30 µm.

ATP quantification assays

Attachment of *S. chilikensis* DC57 was quantified by measuring total adenosine 5′-triphosphate (tATP) on coupons after exposure for 6 h and 24 h to the bacterial cells. Coupons were extracted from reactors with care and placed into Luminultra™ Quench-Gone Organic Modified Kit UltraLyse™ tubes. To enhance the lysis process, the suspensions were vortexed to remove the bulk cells and sonicated for 2 min. The remainder of the assay was conducted as specified in the manufacturer's instructions. Triplicate experiments included technical triplicates for each measurement for all scenarios evaluated.

Quantification of viable sessile bacteria through colony forming units (CFUs)

Quantification of sessile bacteria from the conditioned and control surfaces was conducted to support ATP analysis. Specifically, the experiment was repeated as outlined above, however at 6 and 24 h coupons were transferred to tubes containing 5 mL of ASW. The tubes containing coupons were then sonicated for 10 s followed by 15 s on ice, for a total of 2 min to remove attached cells. The tubes were then vortexed for 30 s to remove any residual cells. CFU counts were then performed according to standards for the drop plate method as described previously (da Silva et al. 2019). All CFU plates were prepared using ASW identical to reactor solution, with the addition of 15 g l^{-1} of bacteriological agar. CFU experiments were conducted aerobically in triplicates for each condition (i.e. for each control and for each conditioning molecule) and incubated for 48 h at 30° C (until visible colonies were observed).

Statistical analysis

A one-way ANOVA statistical analysis was conducted using PAST (V4.83) software (Hammer et al. 2001) on all ATP assay technical triplicates to confirm the significance of differences between conditioned and control surfaces. Results returning a P-value of ≤ 0.05 were considered statistically significant.

Results

Amino acid adsorption to carbon steel

The corrosion potential and galvanic currents were measured on CS after 20 min (Figure 1A and B) and 1 h (Figure 1C and D) to assess the adsorption of amino acids and the formation of a conditioning film. Galvanic current measurements were collected from surfaces conditioned with DNA, however, no significant reduction in current values was observed (Supplementary Figure 1). Comparatively low galvanic currents in amino acid conditioned CS surfaces were observed (A) compared with the controls (B) after immersion for 20 min in reactor solution. A similar trend was observed after immersion for 1 h (C and D). The corrosion potential data also demonstrated a stabilising effect by amino acids (Figure 2). Figure 2A shows greater potential value homogeneity in surfaces conditioned with amino acids, reflecting a lower surface reactivity. Conversely, the control surface showed more heterogeneous electrochemical behaviour with more positive zones (Figure 2B). These results indicate an interaction of amino acids with CS within 20 min.

DNA adsorption to CS

The results of eDNA quantification in the solution compared with the controls are presented in Supplementary Figure 2. A lower quantity of solution eDNA was detected in reactors with pre-oxidised coupons after 24 h, indicating adsorption of eDNA to the surface. Supplementary Figure 2 demonstrates that the eDNA was almost completely absent from the solution after 24 h when exposed to a pre-oxidised coupon, whereas controls for eDNA binding to glassware with no coupon (control 1) and a control for eDNA binding to wet-ground coupon (control 2) demonstrated no reduction in eDNA in the solution over 24 h. Extracellular DNA attachment was also detected using confocal microscopy (Figure 3). The strong red signal in eDNA conditioned surfaces indicates the presence of DNA whereas the controls show limited red signal, indicating little to no eDNA on the surface.

Confocal analysis of *S. chilikensis* DC57 attachment

Control and conditioned surfaces were visualised using CLSM and SEM. In Figure 4, CLSM micrographs depict *S. chilikensis* DC57 attachment after

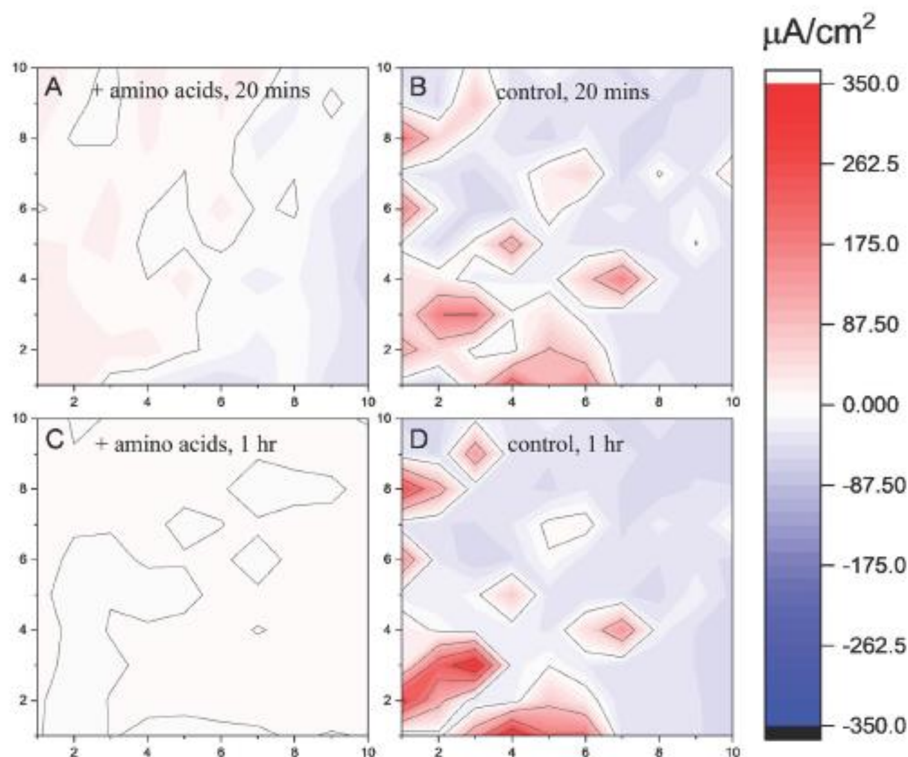

Figure 1. Galvanic current heat maps from MEA surface exposed to aerobic ASW with 10 mM amino acids (A, C) and ASW containing no additional supplements (B, D). The first measurement was taken after exposure to the test solution for 20 min (A, B) and the second measurement taken after exposure for 1 h (C, D).

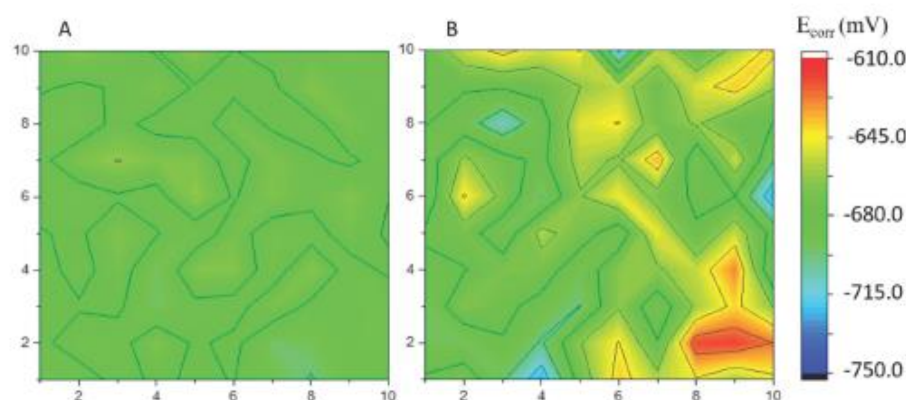

Figure 2. Corrosion potential heat maps from MEA surfaces exposed to aerobic ASW with 10 mM amino acids (A) and ASW containing no additional supplements (B). Measurements were taken after exposure to the test solution for 10 min.

exposure for 24 h to the conditioned and control surfaces. The pre-oxidised control (no DNA conditioning film) is dominated by a 'green' signal indicating the attachment of live cells, with a minor 'red' signal (indicating eDNA or dead cells; Figure 4A). In the presence of an eDNA conditioning film the 'red' signal is greatly enhanced (Figure 4B). As with Figure 3, some red signal is still expected in all CLSM, which is likely to be the result of the presence of dead cells or eDNA of endogenous origin.

Amino acid conditioned surfaces are displayed in Figure 4C and D). In the absence of an amino acid

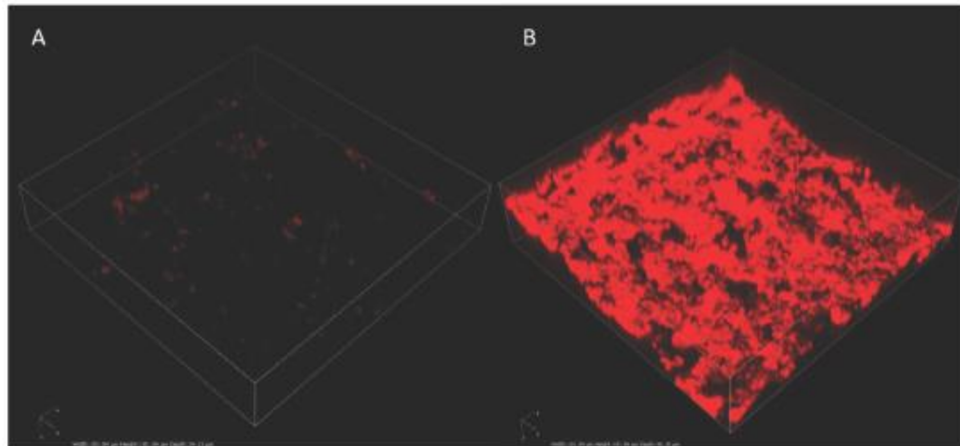

Figure 3. CLSM of eDNA adsorption to the surface of pre-oxidised CS (AISI 1030) coupon surfaces stained with propidium iodide after 24 h: (A) control (no eDNA conditioning film) and (B) eDNA conditioning film.

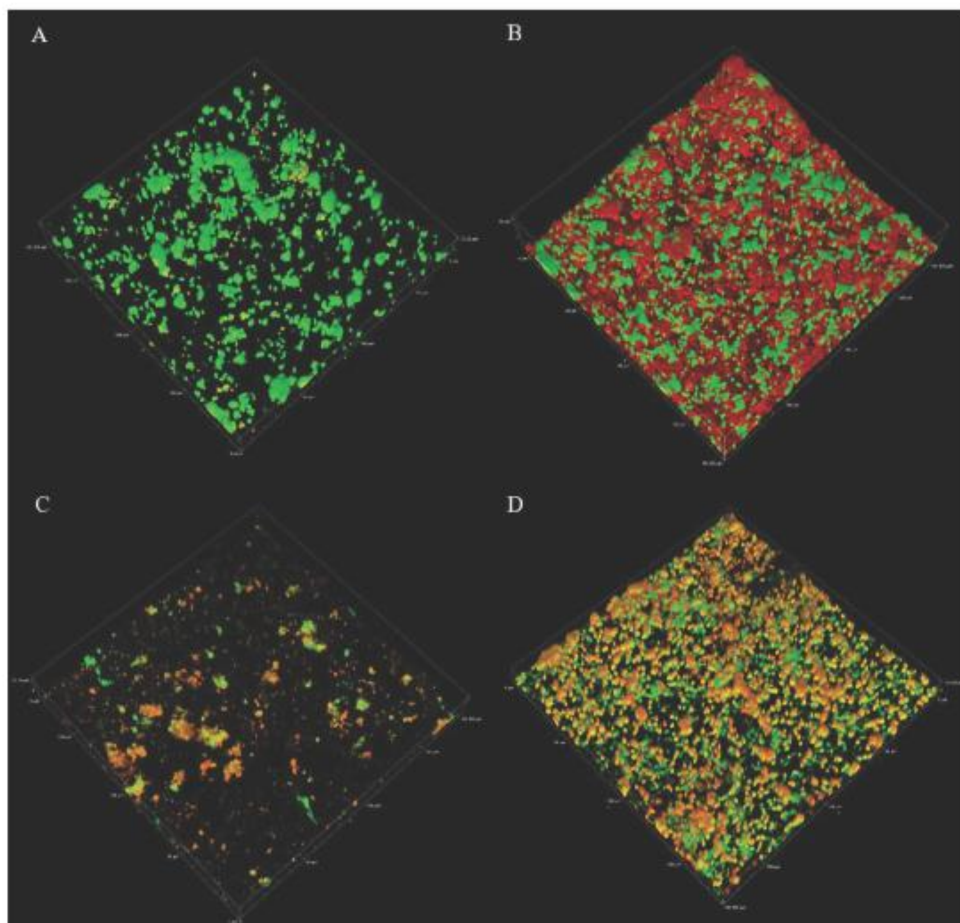

Figure 4. CLSM (20×) of CS (AISI 1030) coupon surfaces after exposure to conditioning film components and *S. chilikensis* DC57 for 24 h. (A) pre-oxidised control surface exposed to bacterial cells only, (B) pre-oxidised surface with a DNA conditioning film and exposed to bacterial cells, (C) wet-ground control surface exposed to bacterial cells only and (D) wet-ground surface with an amino acid conditioning film exposed to bacterial cells. Yellow and orange depicts colocalisation of live and dead cells, or live cells and eDNA.

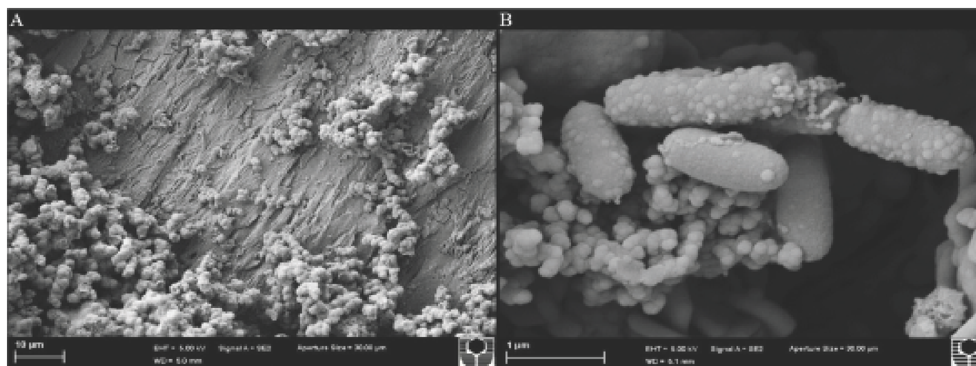

Figure 5. SEM images of the surface of pre-oxidised CS (AISI 1030) coupons after exposure to *S. chilikensis* DC57 for 6 h: (A) control and (B) eDNA conditioning film. Structures resembling bacterial cells were scattered across the eDNA conditioned surface.

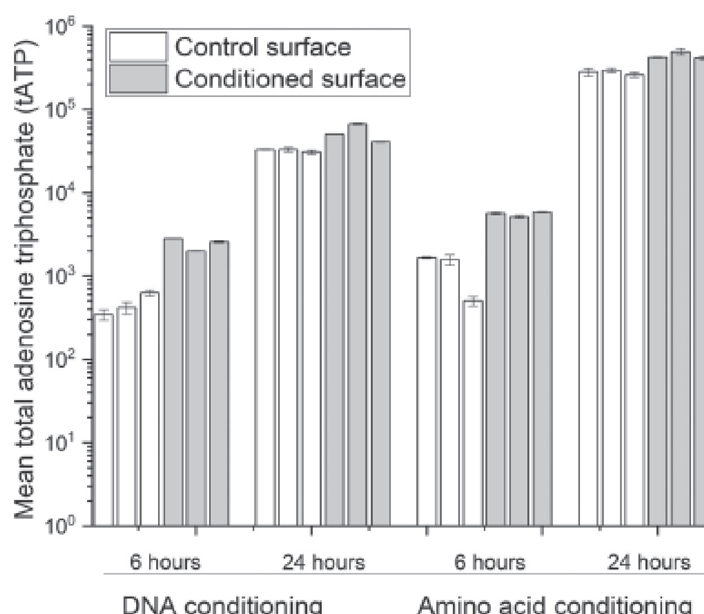

Figure 6. Total ATP recovered from CS (AISI 1030) coupon surfaces exposed to *S. chilikensis* DC57 for 6 and 24 h. The results are expressed as biological triplicates, where each experiment is represented by technical triplicates. Error bars depict the standard deviation of technical triplicate data points.

conditioning film there is a reduced 'green' signal (Figure 4C) compared with the wet ground surfaces in the presence of the amino acid conditioning film (Figure 4D) indicating less attachment of bacterial cells to the surface. Locations with 'red' and 'green' signals in close proximity are depicted in yellow and orange, which can be explained by co-localisation of live bacterial cells and endogenous eDNA or dead cells.

Through visualisation with SEM, structures resembling bacterial cells were not observed on the pre-oxidised control (Figure 5A). Conversely, cell-like structures were observed on the eDNA conditioned surfaces (Figure 5B).

Quantification of S. chilikensis DC57 attachment to CS

After incubation for 6 h with *S. chilikensis* DC57 CS coupons with an amino acid conditioning surface had at least a three-fold greater attached cell energy (intracellular ATP) when compared with the wet-ground control coupons (Figure 6). An overall increase in attached cell energy was observed for both amino acid conditioned and wet-ground control surfaces after 24 h. However, the difference between the two surfaces was less significant than at 6 h (Figure 6). *S. chilikensis* DC57 attachment between conditioned and

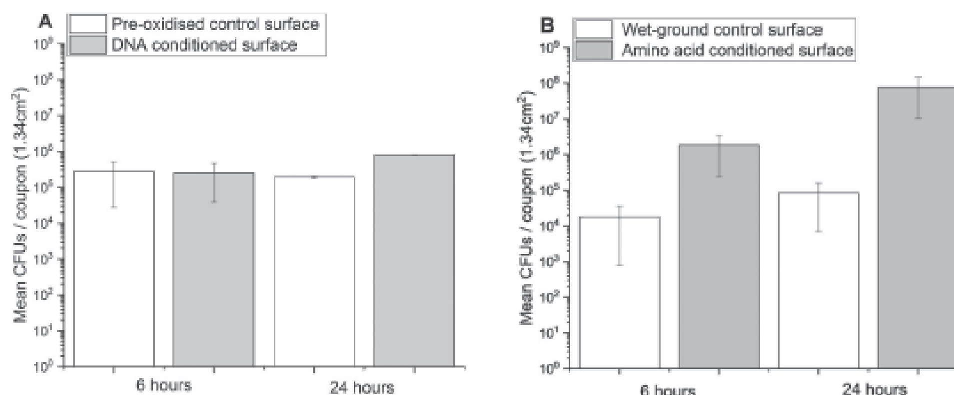

Figure 7. CFUs recovered from CS (AISI 1030) coupon surfaces exposed to *S. chilikensis* DC57 where (A) compares pre-oxidised control and eDNA conditioned surfaces and (B) compares wet-ground control and amino acid conditioned surfaces at 6 and 24 h. The results are expressed as the means of technical triplicates, with error bars presented as standard deviation of these data.

control surfaces was found to be statistically significant ($p \leq 0.05$).

Total ATP results from eDNA conditioned surfaces and the controls demonstrated a similar trend at 6 and 24 h (Figure 6) which was also found to be statistically significant ($p \leq 0.05$). After 6 h, eDNA conditioned surfaces hosted cell energy values at least three times greater than oxidised control surfaces, and again after 24 h the eDNA conditioned surfaces hosted < 50% more ATP than the controls. In all ATP experiments, attachment was favoured by conditioned surfaces with the greatest impact on early attachment (6 h).

The calculation of CFUs on the coupon surface further confirmed that amino acid conditioned surfaces attracted more cells than eDNA conditioned surfaces (Figure 7). While there were similar initial numbers of *S. chilikensis* DC57, the amino acid conditioned surface was overwhelmingly favoured for attachment compared with the eDNA conditioned surfaces and the control surfaces. Interestingly, viable cell attachment to eDNA conditioned surfaces was not significantly higher than the oxidised control surface, and attachment to the oxidised surface decreased after 24 h. Attachment was found to be preferential in the order amino acid conditioned > eDNA conditioned > pre-oxidised control > wet-ground control.

Discussion

This research aimed to determine whether conditioning films on carbon steel surfaces could be generated by a mix of amino acids or DNA and to understand the influence of this organic conditioning film on bacterial attachment to CS in marine-simulating conditions. In marine environments conditioning films are comprised of both organic and inorganic molecules which attach to CS from the surrounding seawater (Jain and Bhosle 2009). Of these molecules, both amino acids (Bhosle et al. 2005) and eDNA (Bakker et al. 2004) are ubiquitous.

Initially the ability of these compounds to form a conditioning film on CS was evaluated. For eDNA interactions with the pre-oxidised surface, the phosphate backbone of nucleic acids is expected to be important. In a study by Liu and Liu (2014) fluorescently labelled nucleic acids were able to bind iron oxides (Fe_3O_4) through phosphate (see Figure 8), where quenching of the fluorescent signal on nucleic acids was used to indicate iron oxide adsorption. Similarly, research has indicated that small peptides and amino acids form part of the conditioning film under aqueous environments (Das and Reches 2015). Specifically histidine, aspartate, glutamine and tyrosine can provide metal binding sites in proteins (Yamauchi et al. 2002). Amine and carboxylate groups are mainly responsible for this phenomenon (Yamauchi et al. 2002; Pušnik et al. 2016). Serine, threonine, glutamine and glutamic acid adsorption to steel surfaces have also been evaluated, indicating a capacity as corrosion inhibitors (Amin et al. 2012; Zhu et al. 2019). Based on the current literature and known interactions of eDNA and amino acids with CS (AISI 1030), it was hypothesised that eDNA would require an iron oxide-based CS interface for adsorption and amino acids would interact with CS without requiring corrosion products (i.e. a freshly wet-ground surface). Two surfaces were therefore established for conditioning film formation: pre-oxidised and freshly wet-ground CS. Attachment of both

eDNA and amino acids to wet-ground surfaces was evaluated, and demonstrated that only amino acids were able to adsorb on wet-ground CS. A layer of amino acids formed on CS when applied overnight in a stagnant system, therefore electrochemical experiments were conducted to confirm the interaction of this layer with wet-ground surfaces. Since CS corrodes quickly in aerobic ASW (Refait et al. 2020), and amino acids have been applied as corrosion inhibitors (Zhao et al. 2014), galvanic current and corrosion potential measurements were an ideal platform for evaluating the formation of a conditioning film by amino acids.

Heat maps of galvanic current distribution showed reduced anodic values in the presence of amino acids compared with the controls, indicative of reduced corrosion. These values continued to reduce compared to the controls with time (Figure 1). Corrosion potential measurements also indicated that an amino acid conditioning film had a stabilising effect on the corrosion potential. Although it is likely that amino acids were preventing corrosion on CS, this phenomenon has been previously investigated and was outside the scope of this work. Importantly for this investigation, galvanic current and corrosion potential data confirmed the formation of a conditioning film almost immediately upon immersion.

The attachment of eDNA to CS surfaces was confirmed by confocal microscopy and fluorimetry. Propidium iodide was applied to control and conditioned surfaces, which showed an abundant red signal across the entire eDNA treated samples, while the controls showed background levels of auto-fluorescence, suggesting that an eDNA conditioning film had formed on the pre-oxidised CS (Figure 4). Based on a previous Raman spectroscopic analysis of the pre-oxidised surface, the final conditioned surface for this study comprised aggregations of hematite and magnetite bound to DNA in experimental replicates (Tuck et al. 2021). Finally, CLSM attachment studies for *S. chilikensis* DC57 revealed that on wet-ground control surfaces, DNA adsorption had not occurred. Extracellular DNA from other potential sources such as *S. chilikensis* DC57 cell lysis or active excretion was therefore negligible. The application of propidium iodide showed background levels of red signal (Figure 4A). Along with live cells, there was a strong signal from propidium iodide generated by the pre-oxidised surface (Figure 4B) indicating eDNA presence at the interface.

In support of the CLSM data, a fluorimetric analysis was conducted to confirm the binding of DNA to the pre-oxidised surface. A control reactor consisting of clean glassware and ASW without supplementation produced no precipitation of eDNA or binding to reactor glass within 24 h. Subsequently, with the addition of a freshly wet-ground CS coupon, eDNA again remained suspended in solution as expected, since the proposed interaction between eDNA and CS involves corrosion products rather than elemental iron. However, when a pre-oxidised coupon was placed in the test solution a sharp reduction in eDNA concentration within the solution was observed (Supplementary Figure 2). Therefore by deduction, the only remaining source of binding sites was the coupon. Based on direct observations with confocal microscopy and indirect DNA binding assays, suspended eDNA was considered to have bound to the iron oxide surface. This evidence confirms the presence and rapid formation of an eDNA conditioning film on pre-oxidised surfaces.

Confirmation of attachment of *S. chilikensis* DC57 to CS surfaces was achieved using SEM (Supplementary Figure 3). Large numbers of cells had colonised the surface after 24 h, indicating the isolate would be suitable as a model for subsequent bacterial attachment studies. Further evidence of attachment was not required since this was confirmed by ATP and CFU quantification.

Once adsorbed to metal structures, it remains unclear how amino acids and eDNA will affect bacterial attachment. Both organic compounds provide numerous benefits to bacteria, and were therefore hypothesised to elicit a positive effect on attachment. DNA for example acts as a platform for horizontal gene transfer (Draghi and Turner 2006), which can boost chemical and environmental tolerance through the incorporation of new genes (Ibáñez de Aldecoa et al. 2017). Amino acids have been demonstrated to be effective chemotactic agents, actively attracting motile bacteria (Yang et al. 2015). Further, both amino acids and eDNA represent organic carbon sources utilised by bacterial metabolisms (Jørgensen et al. 1993). Based on this research, it was hypothesised in the present study that these molecules at the CS interface would result in enhanced surface colonisation compared with surfaces not pre-conditioned with these molecules.

To evaluate the attachment of bacteria to CS surfaces, 50 mL reactors containing ASW were inoculated with *S. chilikensis* DC 57. The isolate was previously implicated in MIC (Salgar-Chaparro, Castillo-Villamizar et al. 2020), is a facultative anaerobic and halotolerant, therefore providing an ideal model for

bacterial attachment studies. *S. chilikensis* DC57 was previously shown to attach to pre-oxidised surface within 24 h (Tuck et al. 2021), with 24 h attachment to wet-ground surfaces observed here (Supplementary Figure 3).

Bacterial attachment to the surfaces was quantified by tATP, a universal energy molecule in nature that that was previously used to estimate cell number (Dexter et al. 2003). It was demonstrated that *S. chilikensis* DC57 overwhelmingly favours an organic conditioned surface compared with non-conditioned controls (Figures 6 and 7), regardless of the organic molecule type or sampling time. To ensure cellular division was not responsible for higher attachment rates to organic conditioned surfaces after 24 h, experiments were repeated during the lag phase of washed and acclimatised *S. chilikensis* DC 57 (incubation for 6 h). Attachment to organic conditioned surfaces was statistically more significant than the 24 h data, suggesting that an active attraction mechanism was involved rather than attachment followed by cellular division, which occurred in response to the presence of an organic carbon source.

Importantly, tATP is not a direct measure of cell number, but a quantification of cellular energy. While greater tATP values are associated with higher cell numbers, the results also indicated that attached cells on conditioned surfaces have greater available energy than those on the control surfaces. Based on these results, conditioning film molecules may facilitate migration to the surface as well as attachment to it.

CFUs extracted from the coupon surface are a direct method of quantifying viable attached cells. As observed in tATP assays, viable cell attachment in particular favoured the amino acid conditioned surface. Surfaces preconditioned with amino acids attracted at least one order of magnitude higher attachment than any other surface evaluated (Figure 7). Cellular division undoubtedly played a role in the high numbers attached to these surfaces after 24 h. However, again much greater attachment rates were also observed on the amino acid conditioned surface after only 6 h. For 6 h experiments, a chemotactic mechanism is thought to be responsible for increased attachment. Indeed, 50 years ago *Escherichia coli* was found to display chemotactic behaviour towards amino acids (Mesibov and Adler 1972). Although chemotaxis in some bacterial species has been established in relation to amino acids, the link between amino acids as conditioning film molecules and attachment has so far not been evaluated to the best of the authors' knowledge. The present research

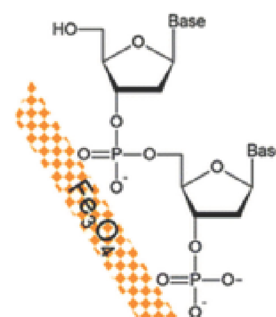

Figure 8. Two nucleotides in a single stranded DNA molecule where the PO_4^- interacts with magnetite (Fe_3O_4), a common oxidation product of CS. This diagram was adapted from Liu and Liu (2014).

reveals that amino acids not only form a conditioning film on metals but the conditioning film also confers chemotactic behaviour in *S. chilikensis* DC57.

In the present study, CFUs also revealed differences between viable attached cells and surface tATP. While tATP results indicated much greater available energy on eDNA conditioned surfaces compared with the controls, no significant difference was found between attached *S. chilikensis* DC57 numbers to oxidised controls and eDNA conditioned coupons after 6 h (Figure 7). Most bacteria carry a net negative charge due to carboxyl and amine groups present on the cell wall as well as teichoic acids (Natarajan 2018), and the phosphate 'backbone' structure confers the negative charge carried by eDNA (Figure 8). In this respect, more negatively charged eDNA conditioned surfaces should introduce repulsive forces to attaching bacteria. However, repulsion was not observed in any results, indicating that attachment to eDNA conditioned surfaces is governed by a variety of factors. For example, survival benefits including organic carbon enrichment by eDNA were also expected to enhance attachment to eDNA conditioned surfaces. Yet, CFU calculations indicated no significant increase in cellular attachment compared with oxidised controls at 6 h. While viable cell attachment may not have increased in this scenario, the energy available to these cells was greater according to the tATP assays. ATP is critical to many cellular processes, including EPS production, cellular division and biofilm formation in general. Indeed, greater available ATP after 24 h on eDNA conditioned surfaces (compared with the controls) suggested that eDNA does have a positive influence on early biofilm development even if cell numbers are not significantly affected.

Interestingly, CFUs recovered at 6 and 24 h from the pre-oxidised surface (control) were similar. Further, while 24 h oxidised and wet-ground controls showed similar quantities of attached cells, approximately 10 fold fewer cells had attached to the wet-ground surface at 6 h. The results indicate that the available *S. chilikensis* DC57 cells had already attached to pre-oxidised surfaces within 6 h, and that the strain favours iron oxides compared with the pristine metal surface. Previous research is able to confirm that other members of the genus *Shewanella* irreversibly attach to iron oxides within just two hours (Roberts et al. 2006). These interactions are greatly governed by *c*-type cytotochromes (*c*-Cyts). OmcA and MtrC for example are terminal reductases associated with *Shewanella* species that bind iron (hydr)oxides for use in respiration. In a recent study, Δ*omcA*, Δ*mtrC*, and Δ*omcA* Δ*mtrC* mutants demonstrated the important short and long term roles in governing *S. odeniensis* MR-1 cell attachment to goethite. In the present study *c*-Cyts are expected to influence early attachment, explaining the rapid colonisation of this strain to iron oxides. Importantly, a bias towards oxidised control surfaces as observed in the present work could affect results presented in this communication. Higher attachment to oxidised controls was observed compared with the wet-ground controls, which was reported in previous research (Tuck et al. 2021). It is also well established that attachment of other *Shewanella* sp. is positively influenced by iron oxides (Glasauer et al. 2001). Therefore iron oxides are likely to act as a sufficient conditioning film for *Shewanella* sp. alone. Future work aims to use other strains to elucidate how attraction to iron oxides by *S. chilikensis* DC57 influences attachment rates to eDNA conditioned surfaces.

In the present study, the results indicated that conditioning film molecule type adsorbed on CS surfaces influenced early attachment stages (i.e. the numbers of attached cells present at the interface within 24 h). Since bacterial cell numbers produced by cellular division follow an exponential function under favourable growth conditions, it was expected that initial attached cell quantity would influence the biofilm development rate. Indeed, the conditioning film is considered the foundation for biofilm development, directly supplementing cell nutrient requirements (Kumar and Anand 1998). Alteration in the surface chemistry and provision of nutrients is thought to enhance downstream biofilm production (Kumar and Anand 1998; Lorite et al. 2011). For example, in a study that assessed the factors affecting attachment of *X. fastidiosa*, the important role of a conditioning film was revealed, where chemical surface changes were heavily influential over the growth of the biofilm (Lorite et al. 2011). Additionally, while bacterial attachment is known to be influenced by surface roughness presented by the inorganic surface, the importance of roughness in the presence of the organic conditioning film is less significant. Gubner and Beech (2000) evaluated surface roughness in the presence of the conditioning film, finding its effects to be negligible on the metal surface. Subsequently, research by Lorite et al. (2011) supported this hypothesis, further extending the scientific understanding of factors influencing attachment in the presence of conditioning molecules. The results of this study indicated that bacterial attachment was significantly affected by the conditioning film molecules and their functional groups, not surface roughness (Lorite et al. 2011). In the present study, chemotaxis and bacterial mechanisms were expected to play an important role in colonisation of conditioned surfaces.

S. chilikensis DC57 displayed preferential attachment to surfaces pre-conditioned with amino acids, suggesting that amino acids adsorbed at the interface of metals may be chemotactic. For eDNA conditioned surfaces, a similar trend was observed (Figure 5). SEM revealed clumps of cell-like structures attached to pre-oxidised surfaces conditioned with eDNA, but not in the absence of an eDNA conditioning film. While these results support the hypothesis that DNA conditioning improves bacterial attachment, the technique is localised and subjective by nature. Although no cells were seen on this attempt, it is likely that a few attached cells were not found on the surface or they were removed in the sample processing stages. Such bias is absent in tATP and CFU assays which utilised the entire coupon surface. SEM remained useful for observing the morphology of attached bacteria in the present research, where *S. chilikensis* DC57 attached in close proximity to other cells rather than evenly across the eDNA conditioned surface.

Unlike SEM studies, cells were detected on control surfaces in CLSM experiments, indicating that cells still attach to surfaces free of environmental carbon. Stains used for confocal microscopy allow for specific targeting of bacterial cells, and at much lower resolution than SEM. Therefore, CLSM is the preferred technique for visualising bacterial cells among the detritus present on the interface (e.g. precipitates or EPS), detecting fluorescent signal across large areas of the coupon surface. CLSM demonstrated that after 24 h surfaces conditioned with amino acid promoted

the greatest attachment to CS, supporting the tATP, CFU and SEM observations. However, a live cell signal between pre-oxidised and DNA conditioned surfaces was similar (Figure 4A and B). Finally, the confocal results also indicated more attachment occurred on all surfaces compared with the wet-ground amino acid control (Figure 4).

In summary, the results indicated that conditioning films adsorb to metals in marine environments, and that amino acids and eDNA are important organic molecules in the conditioning film that can influence attachment behaviour in bacteria. Amino acids in particular were found to promote attachment in the early stages of biofilm formation (6 h and 24 h). Evidence in this communication indicates that organic molecules can influence biofilm formation to different degrees, and further work is required to characterise the effects of other known conditioning molecules ubiquitous in the marine environment. Future research should aim to establish a link between environmental conditioning molecules in engineered systems (i.e. pipelines) and mature biofilm developmental stages. Specifically, predictions of biofilm viability, thickness, EPS composition and species diversity are parameters associated with MIC that may be better predicted and managed by monitoring these natural organic carbon sources.

Conclusions

The present research demonstrated conditioning film formation on carbon steel by DNA and amino acids, and subsequently provided evidence for enhanced attachment to conditioned surfaces. Amino acid and eDNA conditioning film formation was evaluated in marine simulating conditions on carbon steel. Attachment of a marine bacterial isolate was subsequently assessed on pre-conditioned and control surfaces to determine the effects of these molecules on early biofilm formation. The results demonstrated that both molecules formed an organic conditioning film at the interface of CS. Interestingly, the film formed by amino acids impacted CS electrochemical behaviour. Amino acids also promoted a significant increase in available cellular energy in the form of ATP for attached cells. Microscopic observations and CFU calculations demonstrated that increased ATP values correlated with greater attached cells to amino acid conditioned surfaces. Although the bacterial strain used in this study and the eDNA conditioning film both carry a net negative charge, attachment of bacteria to eDNA conditioned surfaces was comparable to the controls, indicating that repulsive forces are overcome by other factors such as chemotaxis. Of all the surfaces evaluated, attachment was most preferential towards the amino acid conditioned surface. Understanding the importance of ubiquitous marine organic compounds on bacterial attachment can lead to the development of more advanced biofilm monitoring tools for biofouling and MIC management.

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No potential conflict of interest was reported by the authors.

Author contributions statement

BT, LM and EW designed the experiments, BT conducted the research and composed the draft manuscript. EW, AS, MF and LM provided revision and feedback. AS, MF and LM secured funding for the research.

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Chapter 2: Evaluating marine bacterial attachment to CS

Manuscript #2 of 2

B. Tuck, E. Watkin, M. Forsyth, A. Somers and L. Machuca Suarez. "Corrosion inhibition on steel by L-amino acid conditioning film is reversed by *S. chilikensis*". Under review in Bioelectrochemistry.

Corrosion inhibition on steel by amino acid conditioning film is reversed by *Shewanella chilikensis*

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Keywords: Microbiologically Influenced Corrosion, Biofouling, attachment, adhesion, conditioning film

Highlights:

- A multi-electrode array system was used to evaluate corrosion in the presence of organic carbon sources
- Amino acids confer protective properties to carbon steel in aerobic seawater
- A marine bacterial strain isolated from corroding equipment was found to break passivation and re-initiate corrosive conditions in the presence of the amino acids.

Abstract:

In the marine environment, organic compounds adsorb to substrates and form a layer known as the conditioning film, which is considered a critical stage in the biofilm formation process. Understanding the influences of organic conditioning molecules on corrosion processes is important in experimental design, especially in the laboratory where biofilms are grown *in vitro* in order to simulate environmental MIC. In the present study, electrochemical effects of organic conditioning films on carbon steel (CS) corrosion were evaluated in aerobic seawater solution using a multi-electrode array (MEA) system. MEA experiments mapped local galvanic currents and corrosion potential of organic conditioned and non-conditioned surfaces. The results demonstrated that compared to controls, galvanic currents were significantly reduced with the application of 10 mM casamino acids, corresponding to lower corrosion rates. The correlation between galvanic cell development and higher localised corrosion was confirmed through 3D surface profilometry. Although effective at limiting corrosion on CS, the casamino acid conditioned surface was subsequently found to hold reduced corrosion protection after the addition of a marine bacterial strain. This study highlights the importance of considering the metabolic activity of bacteria in the presence of organic conditioning films, which can promote MIC.

1.0 Introduction:

Recent estimates predict combined global costs of corrosion prevention and maintenance to be around USD 4T annually[22]. Between 20 and 50% of the total corrosion costs are attributed to microbiologically influenced corrosion (MIC)[23]; the corrosion processes initiated, perpetuated or maintained by microorganisms and their metabolisms[7,24,25]. In marine environments, microorganisms are ubiquitous, forming recalcitrant sessile populations known as biofilms that mediate the mechanisms of MIC.

Importantly for the initiation of MIC , bacteria must first attach to and colonise a surface[26]. Numerous studies have attempted to prevent or limit bacterial attachment to submersed materials through alteration of the surface microstructure directly (including self-cleaning surfaces and immobilised polymer functionalised surfaces)[27,28], through the application of anti-fouling compounds in coatings[29-31], or where possible by dosing with biocides[7,32], however to date no artificial or natural surface is immune to microbial colonisation[33,34]. To effectively prevent bacterial attachment to artificial surfaces, many unanswered questions remain relating to the interactions between bacteria and the substrate.

The spontaneous adsorption of organic and inorganic molecules to submersed surfaces forms a layer known as the conditioning film[16,35]. Development of the conditioning film has been explored extensively in the food processing[36-38] and medical fields[10,37,39,40], where the deleterious impacts of microbial attachment have direct consequences to human health. Although research surrounding the conditioning film is focused on health-related fields, the effects of interfacially adsorbed organic molecules on bacterial attachment appear to be universal[10,39]. In marine environments, for example, it was recognised over 60 years ago that organic conditioning molecules positively influence macro and microorganism interactions with inorganic surfaces[40]. Today, attachment of bacteria in diverse environments has been demonstrated to rely on physico-chemical changes to the interface afforded by the conditioning film[35]. Although considered critical to biofilm formation, the process of adsorption and the downstream implications of organic film components on microbial attachment remains unclear[35,41].

The composition of the conditioning film in marine conditions has been previously reported on glass[41], ceramics[42], polyurethane[41] and steels[43]. These reports indicate that seawater results in adsorption of a heterogeneous layer comprising proteaceous material, carbohydrates, humic acids, nucleic acids and other ambient organic molecules[10, 41-43]. Amino acids are the basic monomers of proteins, a primary constituent of the marine conditioning film on steels[44,45]. Additionally, amino acids are well established as critical nutrients in seawater; accounting for up to 50% of the total organic carbon pool and up to 100% of the nitrogen source[46]. The abundance of amino acids in marine ecosystems emphasises the need to consider their effects on bacterial attachment, and on submerged metals susceptible to MIC.

Tyrosine, tryptophan, arginine, methionine, serine and cysteine have been demonstrated to inhibit corrosion mechanisms at the interface of carbon steel (CS)[47,48]. While *L*-amino acids have demonstrated

corrosion inhibition, they also represent ideal organic carbon sources to drive bacterial metabolisms[49]. Amino acids can exist as optical enantiomers: the *D* (biologically rare), and *L* (biologically common) forms[50]. Although chemically identical, optical enantiomers are mirror images of each other, differing only in spatial orientation[50]. Chemically identical enantiomers may therefore have very different functions. Antimicrobial or surfactant properties can be afforded by uncommon *D*-amino acids since they mimic the structure, but not necessarily the function of common *L*-amino acids. The crucial difference between amino acid enantiomer atomic spatial orientations has led to the successful incorporation of *D*-amino acids in biocides, resulting in increased biocidal efficiency[51]. *L*-amino acids on the other hand are utilised by bacteria in metabolic processes, in signalling pathways and in the construction of cellular proteins. They are also shown to be chemotactic even if not consumed by bacteria[52]. Indeed, *L*-amino acids are biologically critical; a factor that must be considered when evaluating *L*-amino acids as corrosion inhibitors, since increased bacterial activity can potentially result in MIC or biofouling[53,54].

Understanding the role of the conditioning film as the first stage of biofilm formation on CS surfaces has major incentives[42]. The superior durability, weldability, affordability and availability make CS a widespread material with extensive global uses[55,56], even in marine conditions that invariably result in component failure by corrosion[57,58]. CS is particularly susceptible to MIC, yet still selected after weighing the cost of the material against expected life[59]. Simply, by extending the life expectancy of CS the application costs may be reduced. Extensive scientific research exists today to support this concept, ultimately aiming to reduce or eliminate the threat of pervasive corrosion types such as MIC on these surfaces[4,30,60-62]. Amino acids are emerging as a promising solution to CS corrosion when applied as inhibitors since they are cost effective, non-toxic and environmentally safe. Although *D*-amino acids are proving to be effective and environmentally safe corrosion inhibitors and biocides, many studies do not specify the important difference between chiral variations or investigate unspecified or *L*-conformation amino acids. In milk protein hydrolysates, as in most natural settings, amino acids are most abundant as L-enantiomers[63].

Electrochemically integrated multi-electrode arrays (MEA) have been used to investigate heterogeneous corrosion processes of steel surfaces[64]. In recent years, a MEA designated the wire beam electrode (WBE) has proven an effective tool in research fields such as MIC and under deposit corrosion (UDC), coatings, and corrosion inhibition, amongst others[65-69]. Using traditional electrochemical techniques such as linear polarisation resistance (LPR) electrical resistance (ER), electrochemical impedance spectroscopy (EIS), and microscopic techniques (atomic force microscopy; AFM and *in situ* confocal laser scanning microscopy; CLSM) paired with mass-loss analysis can provide a great deal of information about corrosion processes on various metallic surfaces[70,71], but can fail to compensate for non-uniform conditions that lead to heterogeneous electrochemical process, such as pitting corrosion and crevice corrosion[64]. In the present study, a MEA was employed to map the surface electrochemistry

of carbon steel in the presence of amino acids. It was hypothesised that amino acid molecules would adhere to the surface and afford some degree of protection against corrosion over the relatively short-term. Simultaneously, the amino acids were expected to function as a conditioning film on the steel surface, therefore enhancing attachment of the marine isolate. The MEA was used to evaluate the spatiotemporal electrochemical changes on the surface and therefore shed light into corrosion mechanisms in presence of amino acids and bacteria.

Although amino acids inhibit corrosion of CS in aerobic conditions, it is suggested that the presence of amino acids will result in favourable conditions for attachment of bacteria at the interface, and thus propagation of MIC. Additionally, researchers should be cognizant of electrochemical effects imposed by media components such as organic carbon sources that are required to generate enhanced environmental simulations. In the use of amino acids as corrosion inhibitors, research should pay particular attention to their metabolic usability (i.e. chiral variations) by bacteria. This study emphasises the influence of amino acid conditioning films in the corrosion of steels in marine environments.

2.0 Materials and Methods:

Conditioning film formation:

Conditioning film electrochemical effects on CS were screened using three organic carbon sources: extracellular DNA (pure salmon DNA, Sigma), sodium pyruvate and casamino acids. For the former two, no significant changes to galvanic current density or corrosion potential measurements was observed compared to controls (see *Supplementary Information*). Conversely an amino acid conditioning film produced immediate changes to surface electrochemistry, and was therefore selected for further investigation. The amino acid conditioning film was produced using 10 mM sterile dissolved Bacto™ casamino acids. Casamino acids are produced by casein hydrolysis, and contain all amino acids except tryptophan as well as iron and inorganic salts. Milk protein acid hydrolysis results in the formation of amino acids as *D* and *L* forms, with the latter the most abundant in the mixture[63].

Amino acids were added to the reactor media at time zero before recording electrochemical data. The ability of amino acids to form a film at the interface of CS was evaluated using electrochemical techniques.

Shewanella chilikensis **DC57 culture preparation:**

Pure *S. chilikensis* DC57 cultures were grown in ASW solution as used in reactor experiments, with the addition of 3 g/L glucose and 3 g/L sodium pyruvate at 30°C. For biotic experiments, cells were counted using a Neubauer counting chamber and washed twice in PBS (Sigma-Aldrich, pH 7.4). The washed

cells were then incubated in ASW solution at 30°C for 1 hour to acclimatise before adding to reactor experiments in a concentration of 1 x 10^6 cells per mL of ASW solution.

Experimental Reactor and multi-electrode array experiments:

A major advantage of the MEA compared with traditional electrochemical techniques is the ability to separate the 'wires' in the array to monitor electrochemical changes on each independent electrode. The technique therefore allows for monitoring of local electrochemical changes that may not affect the entire electrode[64]. Further, galvanic currents and corrosion potentials can be mapped immediately and continuously, providing spatial and temporal information on localised corrosion events[72]. In the present study, a custom designed reactor by Curtin University accommodated the multi-electrode array in temperature-controlled artificial seawater (ASW) solution as described elsewhere (Figure 1)[19]. To prevent salt precipitation, calcium content was reduced to 0.1 g/L. Counter and reference electrodes are not necessary for the MEA method and were not included in these experiments, as explained elsewhere[72].

Atmospheric oxygen was pumped into the reactor at all times through a two-way inlet/outlet system mounted to the reactor lid. The array consisted of one-hundred (100) working electrodes embedded in resin and wet-ground to a 600g sandpaper finish before affixing to the Teflon base. The roughness of 1030 CS wet-ground to this finish is discussed elsewhere[58]. ASW was used in 2 L volumes for all experiments. This solution was either used as prepared (controls) or supplemented with 10 mM Bacto™ casamino acids. Corrosion potential measurements were taken through a multiplexer and 16 bit Analogue-to-Digital Converter (ADC) with MEA Measurement Instrument software using a script as previously described[73]. Galvanic current measurements were collected. The experiments were conducted in duplicate to confirm results.

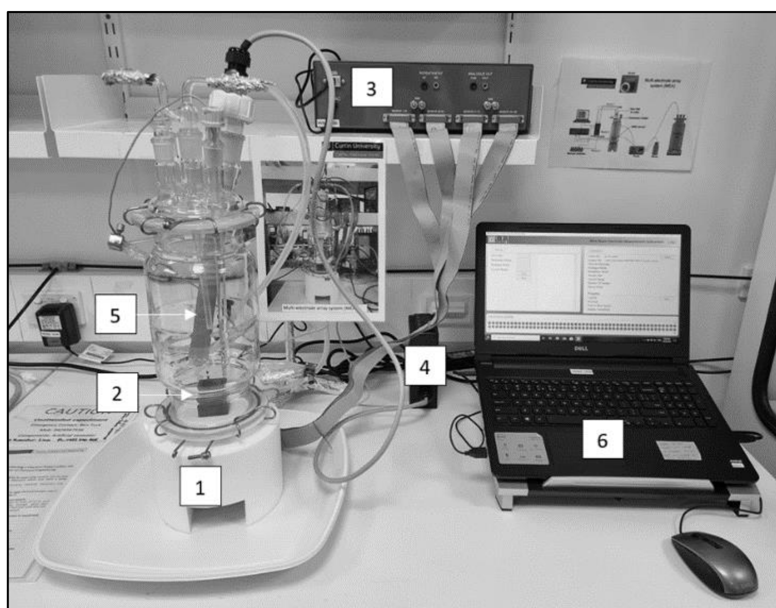

Figure 1: Multi-electrode array setup, comprising; 1) Teflon base attached to modified glass reactor, 2) MEA consisting of 100 electrodes embedded in resin, 3) multiplexer/16 bit Analogue-to-Digital Converter (ADC), 4) thermostat attached to 5) glass rod with heater and thermocouple probe, 6) computer (galvanic current distribution mapping).

3D Profilometry of CS surfaces:

A Solarius SolarScan 150NP 3D Inspection System with single point white light chromatic and optical sensors was used for 3D analysis of the CS MEA sensor using a method as previously described[73].

Confirming interfacial attachment to CS with scanning electron microscopy (SEM):

CS coupons of grade AISI 1030 were immersed in artificial seawater (ASW) solution with washed and acclimatised *S. chilikensis* DC57 cells for 24 hours. Coupons were removed, washed in PBS (Sigma, pH 7.4) and dried overnight in nitrogen before fixing for 22 hours in 2.5% glutaraldehyde solution. Dry coupons were sputter coated with platinum before microscopy. A Neon field emission scanning electron microscope was used for all SEM with a secondary electron detector. Micrographs were captured using a beam intensity of 5 kV, an aperture size of 30 µm and a working distance of 5 mm.

3.0 Results:

Galvanic current and corrosion potential measurements:

MEA experiments were performed with three carbon sources; including DNA, sodium pyruvate and Bacto™ casamino acids over 18 hours to determine the effects of these carbon sources on CS passive layer formation under aerobic conditions. Aerobic ASW solution was used for control measurements. Figure 2 shows galvanic current density heat maps from the MEA array after 18 hours of exposure for aerobic ASW supplemented with 10 mM casamino acids (A) and control experiments with

unsupplemented ASW (B). Development of galvanic cells was observed in controls after 18 hours but not in the casamino acid conditioned surface (A).

During 18 hours of exposure, peak measurements obtained from across the array were also plotted over 5 time intervals. Galvanic currents taken from experiments supplemented with casamino acids remained stable and close to 0 compared to controls (Figure 3). This trend is clear in both Figure 2 and Figure 3, where current values obtained from controls consistently deviated further from 0 $\mu A/cm^2$ compared to amino acid supplemented experiments.

Initial peak current density measurements (< 6 hours) in controls were unstable compared to measurements taken after 6 hours, and although a trend towards more stable galvanic currents was observed, peak currents from casamino acid supplemented experiments remained closer to 0 for the duration of the exposure time in both replicates (Figure 3). Experiments including DNA and sodium pyruvate did not affect peak galvanic current density on CS, leading to similar performance to the control (Supplementary Figure 1) and were therefore not included in subsequent experiments.

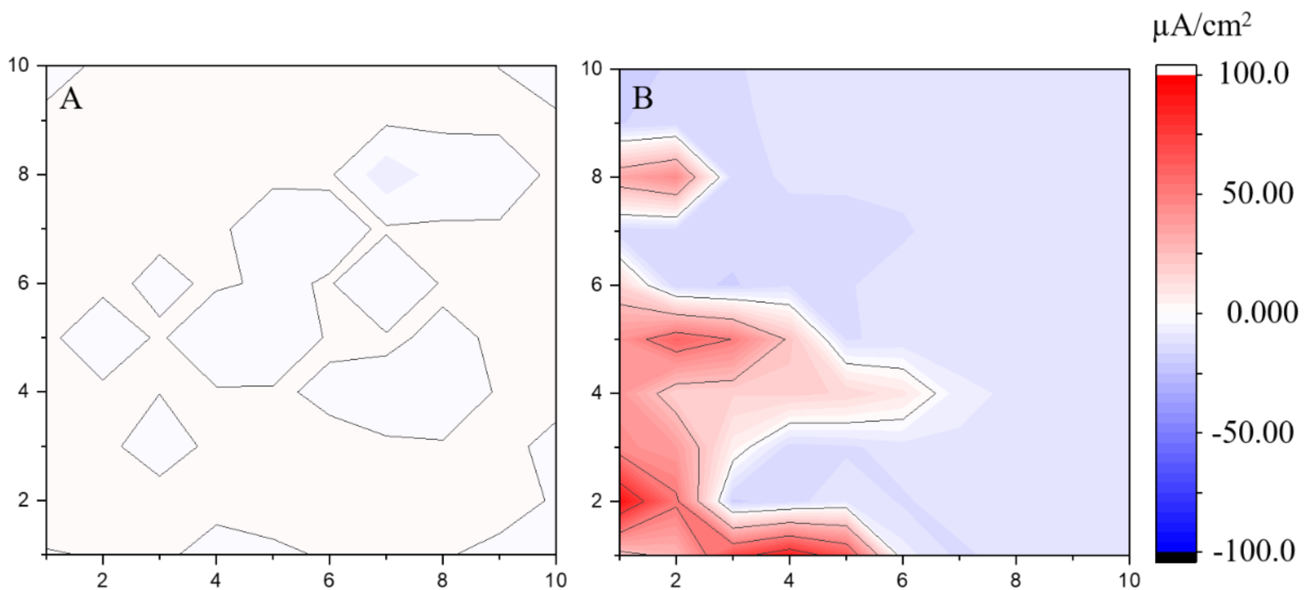

Figure 2: Heat maps depicting galvanic currents on the MEA array after 18 hours of exposure to the casamino acid supplemented ASW (A) and control (unsupplemented) ASW (B).

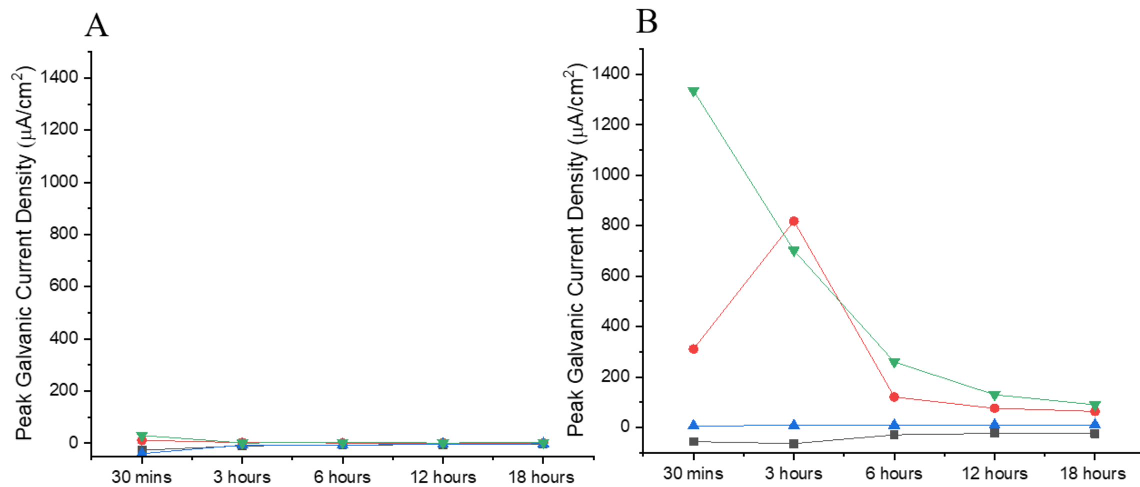

Figure 3: A) Peak galvanic current density measurements taken from the MEA array at 5 time intervals in casamino acid supplemented and B) in control (unsupplemented) ASW. Red and grey represent measurements taken for experimental replicate 1, green and blue represent experimental replicate 2.

Figure 4: Heat maps depicting corrosion potential measurements taken after; A) 15 minutes of exposure to control (unsupplemented) ASW, B) 18 hours of exposure to control ASW, C) 15 minutes of exposure to casamino acid supplemented ASW and D) 18 hours of exposure to casamino acid supplemented ASW.

It is expected that over the area of a uniformly corroding surface galvanic currents and corrosion potential measurements will remain uniformly distributed. Therefore, experiments were also conducted to obtain corrosion potential data on the MEA array after exposure to casamino acid supplemented and control (unsupplemented) ASW. Figure 4 (A) supports electrochemical heterogeneity observed in control galvanic current measurements (Figure 2, B). Areas of more negative corrosion potential after 18 hours (Figure 4, B) corresponded to areas of more positive galvanic currents (Figure 2, B). Corrosion potential measurements obtained from the MEA array exposed to amino acid supplemented ASW remained around – 660 mV to -690 mV for the duration of the 18 hours. These measurements were more stable than controls, which ranged from around -620 mV to -710 mV. These more uniform corrosion potential measurements are indicative of low, uniform corrosion on the MEA array exposed to casamino acid supplemented ASW.

Confirming corrosion events with 3D profilometry:

To confirm how current density and corrosion potential trends translated to expected corrosion events, 3D profilometry was conducted on the array after the experiments. Reconstructions of the electrodes exposed to casamino acid supplemented and control ASW were produced (Figure 5 A, B respectively). Results of the profilometry analysis indicate that the relative galvanic current heterogeneity observed in controls (Figure 2; B) compared to the amino acid conditioned surface (Figure 2, A) and more negative corrosion potential measurements in controls (Figure 4, B) was linked to higher localised corrosion events on these surfaces. Areas of pitting observed in 3D reconstructions match to regions of higher galvanic current measurements and more negative corrosion potential measurements in these control experiments. The 3D reconstructions of casamino acid conditioned surfaces show that amino acid conditioning film was able to effectively limit galvanic cell formation preventing localised corrosion events.

After the effect of the amino acid conditioning film was confirmed on 1030 CS in aerobic conditions, experiments were conducted over a 3.5 day (84 hour) timeframe to ensure the cathodic passivation afforded by casamino acids remained over the relatively longer-term. These results (Figure 6) indicated that galvanic cell development was suppressed compared to controls over the duration of exposure as seen in Figure 2. The results from the 3.5 day biotic experiments supplemented with amino acids (Figure 6 A-D) compared to abiotic experiments (Figure 6 E-F). Identical experimental parameters were used for the biotic experiments, with the addition of twice-washed, pre-acclimatised *S. chilikensis* DC57 cells at the start of the experiment. Galvanic current and corrosion potential was again monitored over 3.5 days. Peak galvanic current density results of duplicate experiments are seen on Figure 6. These

measurements indicate that surface corrosion remained low and uniform until around 42 hours of exposure to bacterial cells.

Both biotic and abiotic control experiments provided comparable galvanic current measurements at 42 hours, and the galvanic currents associated with low, uniform corrosion persisted for at least the first 42 hours in both conditions. This was consistent with conditioning film presence reducing corrosive galvanic current cell formation at these interfaces.

After 42 hours, galvanic current measurements began to deviate from 0 in biotic experiments compared to abiotic controls. At 54 hours biotic surfaces indicate higher corrosion compared with controls (Figure 7 B). While heat maps in Figure 7 depict clear anodic and cathodic areas, the peak currents are much lower than the previous CS control and the areas are not as localised.

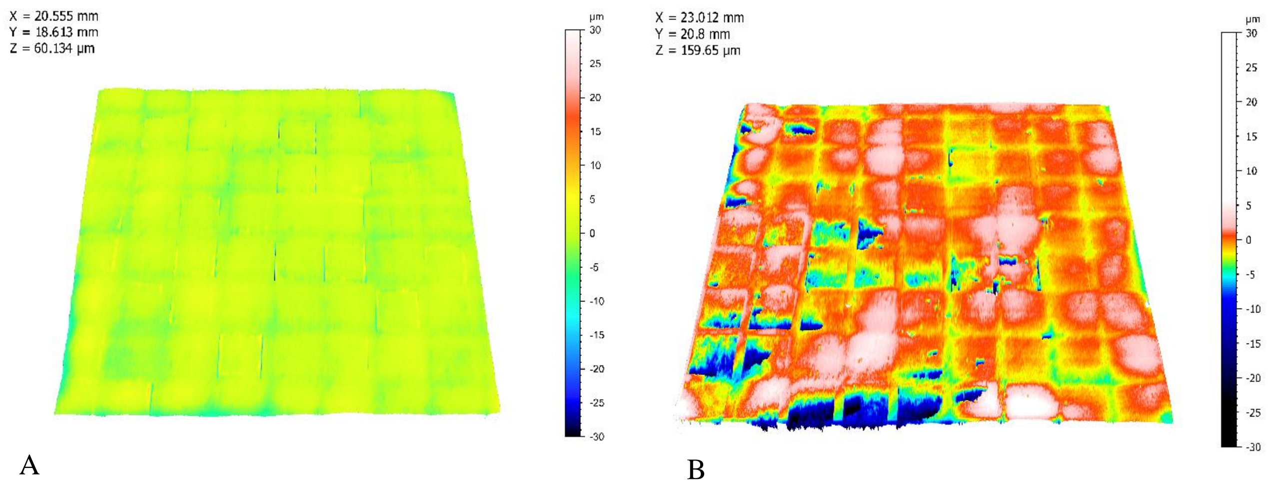

Figure 5: Solarius SolarMap 3D reconstruction of the MEA array exposed to ASW supplemented with amino acids (a) and ASW control (b).

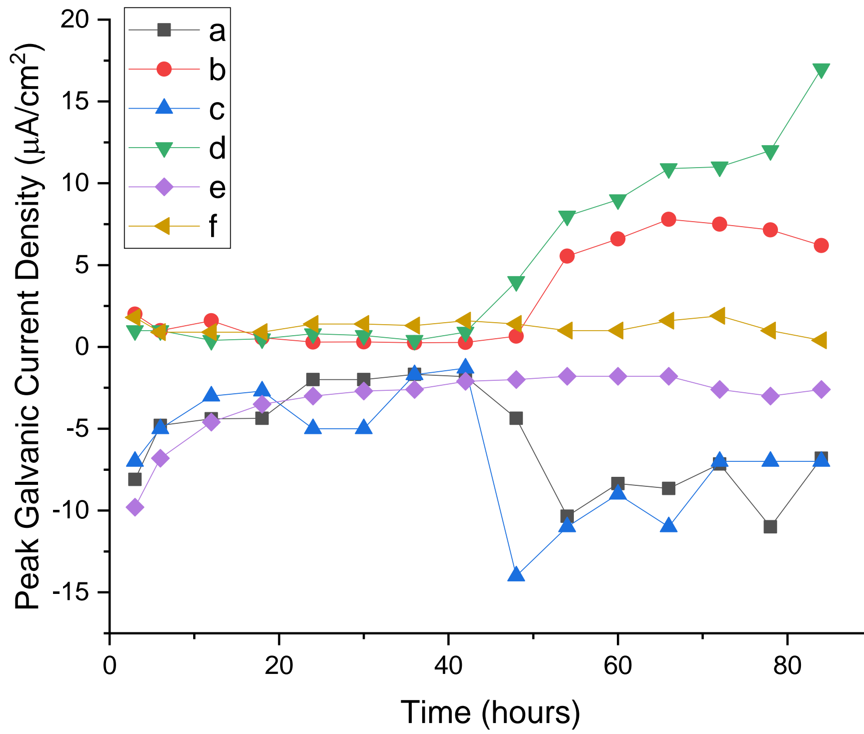

Figure 6: Representative abiotic amino acid conditioned peak anodic and cathodic galvanic current measurements over a 3.5 day time period inoculated with S. chilikensis cells (A-D) compared to control measurements (E,F). Measurements were taken every 6 hours.

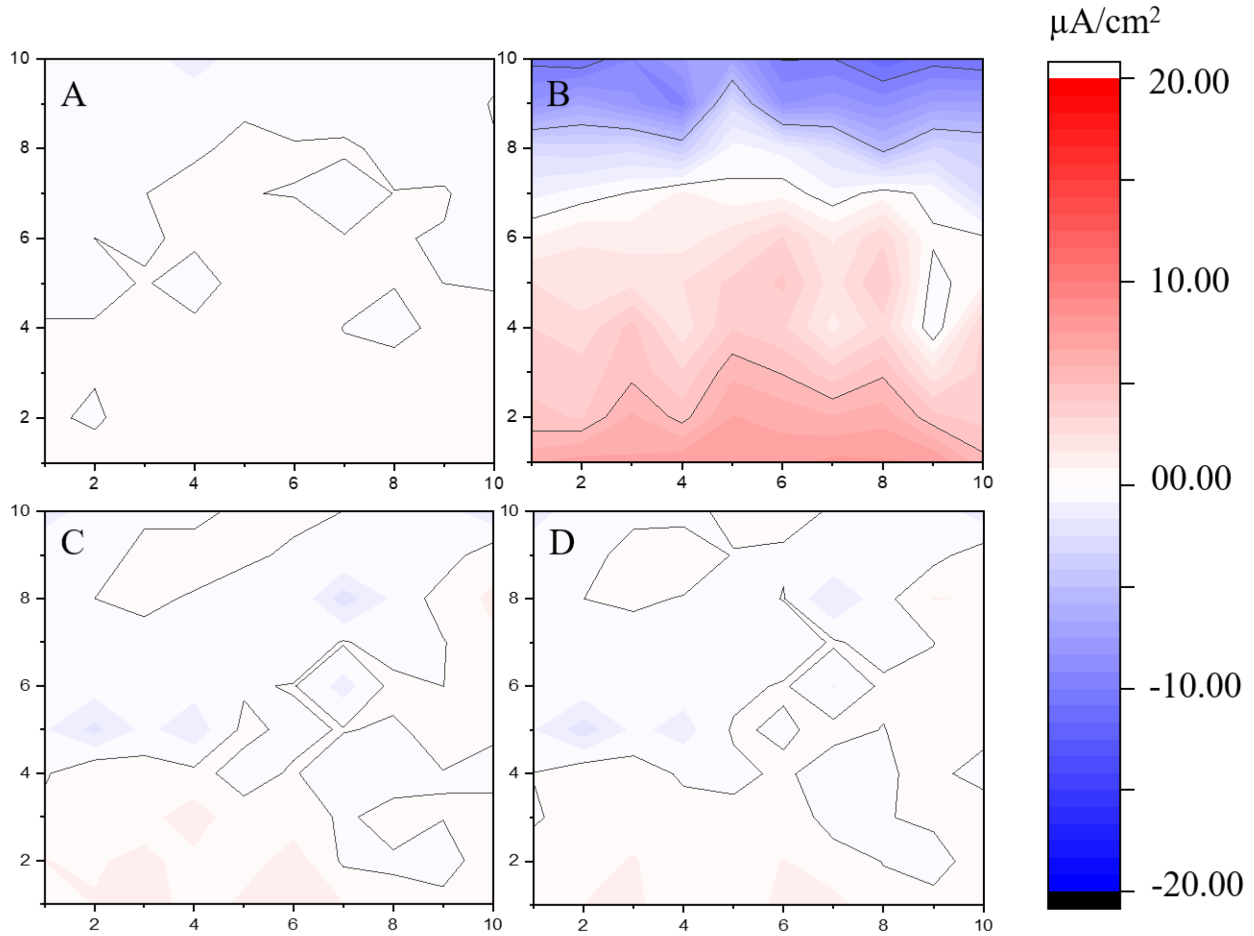

Figure 7: Galvanic current distribution maps from biotic experiment (A, B) and abiotic controls (C, D). Measurements were plotted from 42 hours (A, C) and again at 54 hours of exposure to test solution (B, D).

Confirmation of bacterial attachment to CS with SEM:

Scanning electron microscopy revealed structures resembling *S. chilikensis* cells attached to wet-ground 1030 carbon steel substrates, in agreement with previous findings[8]. The isolate was able to attach to CS substrates (Figure 8) and was therefore considered an appropriate model for attachment studies.

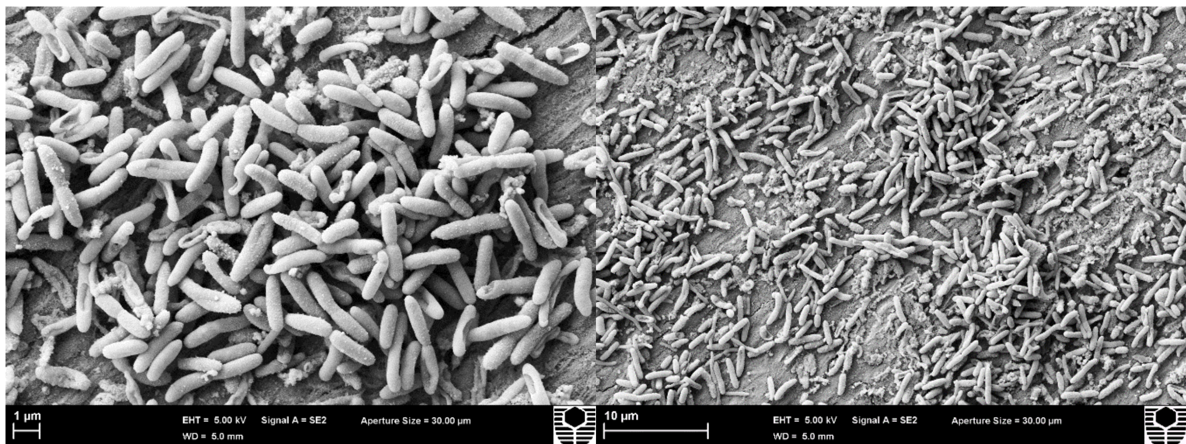

Figure 8: Representative SEM micrographs of wet-ground 1030 CS coupons with structures resembling bacterial cells abundant across the surface.

4.0 Discussion:

Microbiologically influenced corrosion (MIC) is a downstream effect of bacterial colonisation in marine environments and contributes an estimated 20-50% of the $4T USD global corrosion cost[22,25]. The various MIC mechanisms are complex from a diagnostic and prognostic standpoint, although they depend on the initial colonisation of bacteria at the interface[74]. Referring to the well-characterised biofilm formation stages[75], development of a conditioning layer at the interface of submersed materials is considered an initial crucial stage of microbial colonisation[35]. Characterisations of adsorbed interfacial organic carbon in marine conditions have revealed that proteins, carbohydrates, nucleic acids and other organic and inorganic debris will rapidly adsorb to most surfaces, including steels, forming the conditioning film[10,41]. Proteinaceous materials including amino acids are among the first of these compounds to attach to steel surfaces in marine conditions[42]. Interactions of total dissolved organic carbon (DOC) with metal surfaces influences the surface physico-chemical and electro-chemical properties of the substrate, however limited research has evaluated what components of the conditioning film are responsible for bacterial attachment and surface changes[41].

For respiration, bacteria require an electron donor and electron accepter[76]. To evaluate MIC, bacterial proliferation and metabolic reactions are often accelerated through addition of organic carbon sources to media such as glucose, sodium pyruvate and casamino acids[77]. These accelerated scenarios are more manageable *in vitro* compared to relatively nutrient-deprived environmental conditions, which can take much longer to produce similar results. Although widely utilised in MIC studies in culture media or in reactor solutions as the *L* (most biologically common)-configuration[78-82], the impact of *L*-amino acids and other organic carbon sources on the surface of steels is not well documented. Although both *L* and *D*-configuration amino acids have been proposed as corrosion inhibitors, consideration of microbiological responses to isomeric forms is important, i.e. in non-sterile environments, using metabolically favourable amino acid forms could result in propagation of microbial activity. The aim of the present research is to investigate the formation of an organic conditioning film on CS, in particular by amino acids, and the subsequent effect of this layer on the development of localised corrosion events in abiotic and biotic conditions.

Screening for conditioning film molecules that affect surface electrochemistry was first conducted on sodium pyruvate, nucleic acids and amino acids. Screening involved exposure of the multi-electrode array system (Figure 1) to aerobic ASW solution containing the organic carbon source. Sodium

pyruvate is crucial to many aerobic and anaerobic respiration processes and is a common in the natural environment as the protonated pyruvic acid[83]. Pyruvate is also widely utilised as an organic carbon source in MIC studies[81,84] and can comprise part of the biofilm matrix[85,86]. Nucleic acids are seldom applied as organic carbon sources in MIC studies but represent a ubiquitous conditioning film component in marine environments[41,42]. Lastly, amino acids are also ubiquitous in marine environments[87,88] and have been detected as part of the normal conditioning film composition[89,90]. Bacto™ casamino acids are a source of all amino acids except tryptophan, and are also commonly used in MIC studies as an amino acid source. Results from galvanic current changes induced by sodium pyruvate and DNA are included in Supplementary Figure 1. With similar current density values to controls (Figure 2, B), changes induced by these carbon sources were not considered significant and were not further investigated. Conversely, screening involving casamino acids revealed a galvanic current reduction which was observed for the duration of screening experiments (heat map in Figure 2 and peak values in Figure 3 A). Therefore, the casamino acid conditioning film was further investigated in the present study.

To evaluate the effect of casamino acids on CS, the MEA set-up was used to characterise heterogeneous electrochemical patterns across an array of 100 electrodes as previously described[72,91]. In conditioning film experiments, 10 mM sterile casamino acids were completely dissolved in ASW solution and added to the MEA reactor at T_0 to simulate the amino acid film on CS. Results are expressed as a locally corrosive short-term control (18 hour) for organic molecule comparison (Figure 2 B, Figure 3 B, Figure 4 C, D).

The results indicate that addition of casamino acids to ASW media has an immediate and lasting effect on the electrochemistry of CS. Galvanic currents (Figure 2, A; Figure 3, A) and corrosion potentials (Figure 4 C, D) in MEA experiments exposed to ASW containing casamino acids were indicative of corrosion inhibition for the short term. The relatively longer-term results, depicted in Figure 7, D, demonstrate a similar trend. These results were supported by 3D profilometry observations (Figure 5). Experiments so far provided evidence for amino acid corrosion protection on CS against a highly corrosive control.

Any substance added in small concentrations to form a cohesive layer that limits corrosion is known as an inhibitor[92]. Amino acids contain heteroatoms (including N, S, P, O) and some functional groups that are typical attributes of the most effective organic corrosion inhibitors (OCIs)[92]. Some amino acids have been documented as inhibitors of CS corrosion, for example glutamic acid[92], L-methionine[93] and L-histidine [94]. Although considered the most promising environmentally safe inhibitor compounds for their accessibility, low toxicity and cost[93,95], the L-enantiomer of these compounds is a fundamental metabolic substrate for bacteria. It is also established that motile bacteria may exert a targeted effort to locate these compounds in natural environments, even if not utilised for metabolic consumption[96].

Therefore, when applied in non-sterile environments, bacteria may be able to use the proteinaceous conditioning film to enhance colonisation, eventually leading to bio-deterioration. When applied in sterile environments where bacteria can multiply, amino acids may increase the likelihood of contamination. Therefore, attachment of a marine isolate and the effects on surface electrochemistry was subsequently assessed. It was hypothesised that amino acids would drive bacterial metabolisms and prevent adsorbed molecules from reducing localised corrosion. For these experiments, a longer-term control was established using abiotic ASW supplemented with casamino acids (an extension of experiments conducted above). Peak galvanic current measurements confirmed that galvanic currents remained stable in abiotic conditions (Figure 6; E-F).

Before evaluating surface electrochemical behaviour with the addition of microorganisms, attachment of a marine isolate *Shewanella chilikensis strain DC57*, isolated from oilfield samples, was first confirmed using SEM. Coupons consisting of the same AISI 1030 CS as used in the MEA array were exposed to *S. chilikensis* and imaged after 24 hours of incubation. The affinity of the cells for this surface is highlighted in Figure 8, forming a multi-layer biofilm in some areas which was consistent with previous findings[97].

To investigate how *S. chilikensis* would interact with the amino acid conditioned array surface, pre-conditioned and washed cells were first employed in unsupplemented ASW. The results of this control can be seen in Supplementary Figure 2. Galvanic current measurements in Supplementary Figure 2 were similar to those observed in previous abiotic controls (Figure 3, B Figure 5, B). Therefore, the addition of bacteria was considered to have no significant effect on surface electrochemistry without organic carbon. This was likely because corrosive environments were already established, and cells were not attracted to this surface[97]. Without organic carbon sources, cell replication is limited and therefore these experiments contained relatively low cell numbers compared to biotic experiments with casamino acids.

Since casamino acids are employed as carbon sources and have been demonstrated to initiate chemotactic behaviour in motile bacteria, it was suggested that corrosion inhibition afforded by casamino acids would not remain after the addition of the isolate. *S. chilikensis* cells in experiments supplemented with amino acids demonstrated the ability to metabolise and change galvanic currents on the surface of CS in line with the hypothesis (Figure 6, A-D). Although not as significant as corrosive galvanic current magnitude seen in controls, incorporation of the isolate lead to the re-establishment of corrosive conditions on the array (Figure 6, Figure 7 B). *S. chilikensis* cells were inoculated atT_0, however the effects of bacteria on surface electrochemistry were not observed until after 42 hours. The results indicate that amino acids can provide good corrosion protection of CS, although this protection is partially lost by the activity of bacteria. Partial loss of corrosion protection likely coincided with metabolism of interfacially adsorbed casamino acids.

Previous research highlights a specific order to biofilm formation, starting with the adsorption of a conditioning film followed subsequently by bacterial attachment[41]. It has been proposed that microfouling events that lead to MIC and biofouling are in fact mediated by the initial attachment of dissolved organic carbon (DOC)[43]. Although it is now clear that the marine conditioning film is comprised of a complex and environment-dependant mixture of organic carbon sources[98], proteinaceous material is the first known DOC source to adsorb to steels[41,43]. In fact, early research on conditioning film composition described the layer as "essentially protein"[43]. Marine environments are often deprived of organic and inorganic nutrients[99,100], especially those easily metabolised by heterotrophs like proteins. Competition between populations is high for such nutrient sources, thus making interfacially adsorbed proteinaceous material an attractive metabolic substrate. Little is known about the effects proteinaceous material can have on steel in seawater, and how bacteria interact with this layer. The present study aimed to enhance the understanding of how proteinaceous material and other carbon sources can influence the corrosion of carbon steel, and subsequently demonstrate how bacteria can utilise the conditioning film to change surface electrochemistry. Evidence suggests that casamino acids, comprising monomers and small polymers of proteinaceous material, forms a conditioning film within minutes of carbon steel submersion which is consistent with previous research. This research also links the adsorption of proteinaceous material with suppression of galvanic cell development, although the presence of bacteria could begin to alter CS electrochemistry again to more corrosive conditions. Future research should explore the effects of conditioning molecules over the longer term, and how specific organic nutrients can influence attachment of microorganisms and subsequent biofilm formation. Further knowledge pertaining to the roles of specific organic carbon sources in the conditioning film can assist early warning and monitoring tools for MIC and biofouling on marine infrastructure.

5.0 Conclusions:

A number of amino acids have been proposed as corrosion inhibitors. While *D*-amino acids are uncommon in nature and can have toxic effects on cells, metabolic usability can promote bacterial replication and confer chemotactic behaviour in some species. In the present study the results demonstrate that galvanic current density measurements taken from a multi-electrode array in the presence of a cocktail of biologically beneficial amino acids are significantly reduced in aerobic, abiotic conditions compared to controls. Current density and corrosion potential measurements were assessed using profilometry to evaluate localised corrosion patterns. Results of this study highlight the ability of amino acids to adsorb to CS and act to reduce localised corrosion in marine environments. The study subsequently aimed to assess the effect of microorganisms on corrosion inhibition afforded by metabolically usable amino acids. The addition of microorganisms to corrosive, organic carbon free experiments demonstrated no significant changes to surface electrochemistry compared to controls. However, on amino acid conditioned surfaces bacteria were able to change galvanic currents and corrosion potential, reflecting more corrosive conditions. This study demonstrates that amino

acids can form a conditioning film on CS, and this film can effectively reduce localised corrosion. In biotic conditions, this protection is reduced by the presence of a marine bacterium. Future research should evaluate the long-term effects on microbial attachment and biofilm formation caused by organic conditioning film components.

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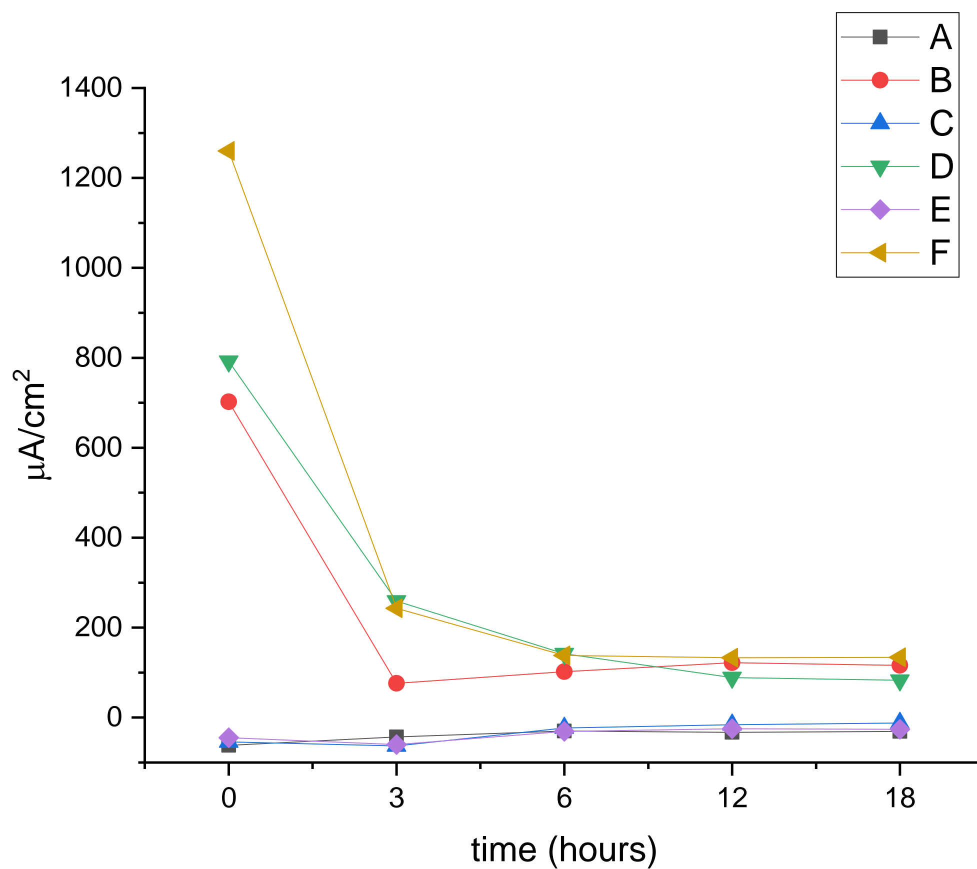
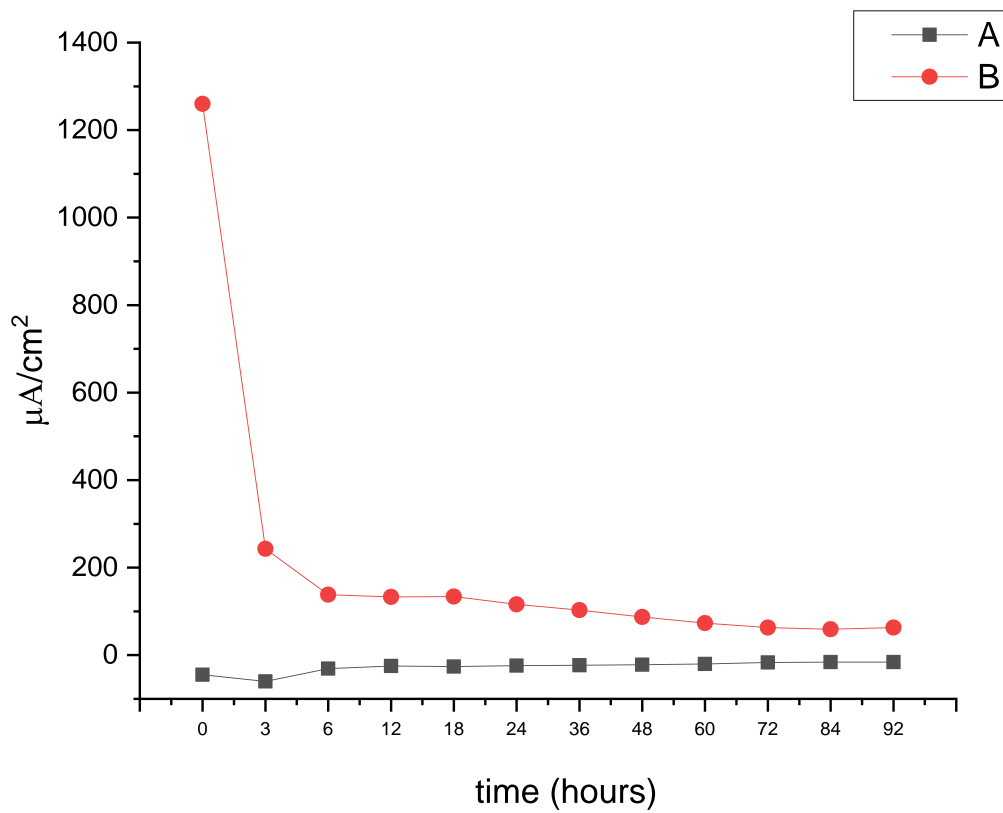
Conflicts of interest:

The authors declare no competing financial or other conflicts of interests.

7.0 Appendices



Supplementary figure 1: Peak cathodic and anodic current values for ASW supplemented with 1 mg/L DNA (A, B respectively), 10 mM sodium pyruvate (C, D) and S. chilikensis DC57 with no organic carbon source (E, F).



Supplementary figure 2: MEA peak cathodic (A) and anodic (B) current values from MEA exposed to S.chilikensis DC57 cells in ASW without organic carbon supplementation.

Chapter 3: MULTISPECIES MARINE BIOFILM POPULATION DYNAMICS AND COMPOSITION

Chapter 3: Marine multispecies biofilm population dynamics and EPS composition.

Manuscript # 1 of 1

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Extracellular DNA: a critical component in marine biofilms

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Abstract:

Multispecies biofilms represent a pervasive threat to marine based industry, resulting in US $ billions in annual losses through biofouling and microbiologically influenced corrosion (MIC). Effective, specific and environmentally sensible control of marine biofilms is dependent on the scientific understanding of the biofilm composition. In particular, extracellular polymeric substances (EPS) play multiples roles towards influencing biocide tolerance within the biofilm population. Biocides represent a primary line of defence against biofilms on marine infrastructure, and also face toxicity challenges as environmental awareness increases. A lack of fundamental understanding of the species and EPS in marine biofilms remains a bottleneck for the development of effective, target-specific biocides with lower environmental impact. In the present study, marine biofilms were developed on steel with three bacterial isolates to evaluate the composition of the EPS and population dynamics with time. Confocal laser scanning microscopy, scanning electron microscopy and fluorimetry revealed that extracellular DNA (eDNA) was a critical structural component of the multispecies biofilm. Under marine conditions presented in this study, eDNA can represent >90% of the biofilm EPS. Thus, eDNA is a promising target for the enhancement of biocide effectiveness against industrial biofilms. Further, population analysis indicated that all three strains were active members of the biofilm community, and the results indicate that biofilms in marine conditions can be stable, well-defined communities.

1. Introduction

Biofilms are a pervasive threat to marine infrastructure. The impacts of biofilm formation on metals and other materials manifest as biofouling, contamination and microbiologically influenced corrosion (MIC)[101-104]. MIC alone can be expected to contribute 20-30% of all global corrosion costs, amounting to a conservative $30-50 Billion per annum[105,106]. Biofouling and MIC are not well understood or effectively controlled in the marine environment, leading to application of toxic, broad spectrum chemical treatments (biocides). Biocides represent a primary line of defence against biofilms on marine infrastructure.

To remain effective against adaptive microbial populations, constant chemical treatment revision and optimization is required. However, the fundamental understanding of natural marine biofilm composition remains a bottleneck for biocide efficacy improvement. Species diversity represents a contemporary challenge for natural biofilm research. Multispecies biofilms host complex behaviour which single-species simulations fail to reproduce[107]. Competition and synergistic relationships within the community for example can help shape

the molecular and species composition of the biofilm[108,109]. Scientific literature published on the extracellular and cellular composition of multi-species biofilms on metallic surfaces is limited, leading to the absence of environmentally sensible, effective and targeted biocide options. Understanding fundamental aspects of biofilm formation will assist the management of deleterious biofilms into the future, especially as greener treatment options are becoming more desirable[2].

The EPS provides resident cells with a physical and chemical barrier reported to enhance biocide tolerance by as much as 1,000 times compared to planktonic counterparts[110,111]. Extracellular polymeric substances (EPS) are produced by bacterial cells to form the biofilm matrix[112]. The EPS composition has been screened across a variety of terrestrial and non-terrestrial environments, revealing polysaccharides, proteins, nucleic acids and lipids as major constituents[113]. In marine multi-species biofilms developed on metal surfaces, the range and abundance of EPS components is relatively unexplored. In other aqueous environments, the EPS is considered primarily polysaccharides and proteins[114-117]; however significant gaps in understanding are still evidenced by inadequate biofilm control measures. Specifically pertaining to engineered systems in seawater, the EPS can also interact with iron and pose a direct MIC risk[58]. Since EPS provides many functions linked to survival of the biofilm; including substrate attachment, protection and horizontal gene transfer, understanding the EPS composition is critical for a targeted approach to biofilm mitigation[118].

Extracellular DNA (eDNA) is a biofilm matrix component (as opposed to genomic DNA; located intracellularly) gaining considerable traction in recent years due to its important structural role[119]. Over the past two decades, eDNA degrading enzymes have been associated with biofilm dispersal[120,121], thus establishing nucleic acids as critical matrix polymers. Although a plethora of research identifies and describes the role of eDNA in the context of clinical biofilms[119,122,123], research relating to environmental biofilm EPS is limited. In this communication, marine biofilms developed on steel are hypothesised to produce eDNA and share a similar dependence on eDNA for structural integrity.

Although further research is required to catalogue the EPS composition in marine biofilms, EPS production is closely associated with population dynamics. Even in single-species biofilms, genetic variants are exploited for upregulated EPS production pathways to promote the survival of the greater biofilm[124]. Similarly in dual species biofilms, EPS quantity is strongly influenced by interactions between species[125]. Since changes in multi-species biofilm populations are likely to influence the biofilm tolerance profile, EPS research should be supplemented by information on the contributing biofilm species.

The present research aimed to progress the understanding of marine multi-species biofilms on metal surfaces by: A) Investigating the prevalence of major EPS components; in particular eDNA, in marine biofilms, B) characterising population dynamics and their association with eDNA synthesis and composition, and C) outline the importance of eDNA in the process of biofilm formation on steel. To address research objectives, marine microorganisms were allowed to form biofilms on carbon steel (CS) over a period of 6 weeks. Over the course of the study genomic and eDNA were extracted and characterised based on 16S rRNA amplicon sequencing from the biofilms to understand how community structure fluctuated over time and to discriminate key eDNA producers within the biofilm. Considering that DNA-based analysis cannot discriminate between active and inactive species, RNA-based analyses (amplicon sequencing of 16S rRNA transcripts) was conducted to assess the link between active members in the community and eDNA production. Finally, confocal laser scanning microscopy (CLSM), adenosine triphosphate (ATP) qualification and viability assays (culture based) were also included at each sampling period to confirm viability of the population.

2. Materials and Methods

Microbial isolates:

Experiments were conducted using three marine bacterial strains with demonstrated ability to form biofilms on CS[8]. Before inoculation into experimental reactors, *Shewanella chilikensis* DC57[126], *Pseudomonas balearica* EC28[127] and a laboratory strain of *Klebsiella pneumoniae* were grown anaerobically in liquid-phase cultures using ASW media (Supplementary Table 1) supplemented with Bacto™ casamino acids (3 g/L *w/v*), sodium pyruvate (3 g/L *w/v*), D (+) glucose (3 g/L *w/v*) and ammonium nitrate (NH_4NO_3; 3 g/L *w/v*). Strains in liquid-phase cultures were counted using a Neubauer haemocytometer chamber and inoculated into reactors as previously described[8], using 10^5 cells/mL from each pure culture.

Sample preparation and surface finish:

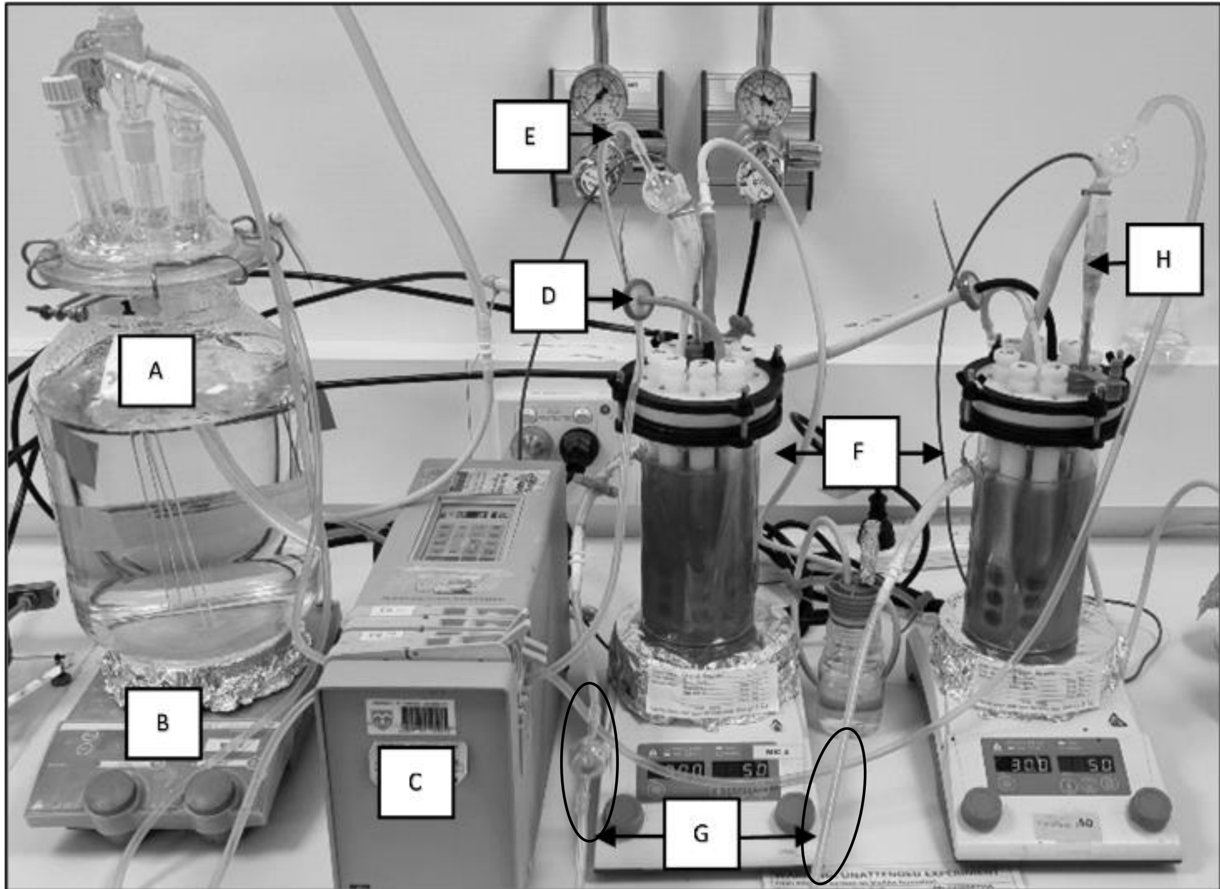
Coupons of 5 mm thickness with a working surface of 1.27 cm^2 were cut from CS rods (AISI 1030). Coupons were prepared by wet-grinding with successively finer grit finish, in the order of 80, 120 and 320 (SiC grit paper), before electrocoating with Powercron® 600 CX solution. The final working surface was freshly wet-ground to a 120 grit finish. The coupons were washed in 100% pure ethanol, dried under nitrogen gas and irradiated with ultraviolet (UV) light for 10 minutes each side to sterilise. Coupons were inserted into Center for Disease Control (CDC) reactor rods using a biosafety cabinet (aseptic conditions), UV irradiating the coupons again after manual manipulation.

Experimental setup:

Biofilms were developed in CDC reactors over 6 weeks as experimental replicates (Figure 1). ASW media was used in all bioreactor experiments as previously described[19], with the following modifications (Supplementary Table 1): solution 1 addition of glucose (0.9 g/L *w/v*), sodium pyruvate (0.55 g/L *w/v*), Bacto™ casamino acids (1.5 g/L *w/v*) to $CaCl_2$ (0.1 g/L *w/v*), NH_4NO_3 (1.2 g/L w/v). Experimental reactors were established simultaneously and flushed with pure nitrogen gas before inserting rods containing UV-treated coupons. Once anaerobic conditions were established, reactors were directly inoculated with equal cell numbers of each bacterial strain. Anaerobic conditions were selected in this investigation to remove bias associated with corrosion product formation and detachment. The reactor solution was then maintained for the experiment duration at 30ºC under a constant nitrogen flow (pure nitrogen gas, 90 ml/min) and agitation at 50 rpm. A reservoir containing reactor solution was connected to a peristaltic pump, calibrated to flush 30% of the reactor solution every 7 days. To allow the population to establish a biofilm, reactors remained under batch conditions until day 3 after

inoculation (when turbidity was observed). Continuous flow conditions ensured a constant, limited nutrient availability. Sampling was conducted after 2, 4 and 6 weeks.

Figure 1: CDC reactor experimental set-up: A) feeding cell with fresh reactor solution, B) hot plate set to 30°C and 50 rpm for CDC reactors, C) pump for continuous flow replacing reactor solution by 30% weekly, D) reactor gas inlet with 0.2 µm filter, E) media inlet with air lock to prevent feeding cell contamination, F) CDC reactor duplicate experiments, G) reactor solution



outlet for continuous flow (with air locks), and H) thermocouple probe.

Confocal laser scanning microscopy (CLSM):

CLSM was used to identify biofilm EPS components, directly monitor eDNA presence over the experiment and visualise the viability of the biofilm. All CLSM analyses were conducted on a Nikon A1+ confocal microscope equipped with a 20 x dry objective lens, using version 5.20 of Nikon NIS Elements software. Coupons were removed from reactors and lightly rinsed in phosphate buffered saline (PBS; Sigma, pH 7.4) before staining.

Biofilm EPS components were targeted using the following stain and stain-lectin conjugate concentrations, optimised for biofilm samples: proteins were targeted using Sypro® Orange (Thermo Fisher) in a 5X concentration. DiYO™-1 (AAT Bioquest inc.) was used to visualise eDNA at a working concentration of 5 µM. Total polysaccharides were captured using Wheat Germ Agglutinin (WGA)-Alexa Fluor™ 633 conjugate (Thermo Fisher) and Concanavalin A (ConA)-Alexa Fluor™ 633 conjugates (Thermo Fisher) at 50 µg/mL and 100 µg/mL working concentrations respectively. WGA and ConA conjugates were applied simultaneously to bind sialic acid and N-acetylglucosaminyl residues (WGA) as well as α-mannopyranosyl and α-

glucopyranosyl residues (ConA). Stains were combined in Ultrapure milliQ water and applied to coupon surfaces for at least 10 minutes before rinsing lightly in PBS (Sigma, pH 7.4). Coupons were then transferred into a purpose-built dish for all CLSM (ibidi®, Germany). The dish contained a central hole of radius 10 mm, covered by a glass coverslip. This design preserved the biofilm architecture by preventing compression of the sample.

After confirming the relative abundance of eDNA in biofilm EPS, eDNA was stained independently on coupons using DiYO™-1 (AAT Bioquest). The stain was applied for 10 minutes at a working concentration of 5 µM before gently rinsing again in PBS (Sigma, pH 7.4) for eDNA visualisation. Independent CLSM analysis with DiYO-1 provided technical replicates to confirm eDNA presence over 6 weeks in experimental replicates, while confirming the absence of signal bleed-through in EPS staining protocols. Micrographs were captured sequentially using a 489.3 nm laser and a 500-550 nm emission filter. All microscope and software settings remained uniform between sampling times and micrographs, with the following exception: EPS micrographs were captured at a smaller resolution (512x512) to minimise cell death and changes to EPS as a result of longer acquisition time.

Scanning electron microscopy (SEM):

SEM sample preparation was conducted as previously described[8,128]. Briefly, coupons were removed from reactors and lightly rinsed in PBS (Sigma, pH 7.4) before fixing in 2.5% glutaraldehyde solution for 22 hours at 4ºC. Fixed biofilms were dried overnight under pure nitrogen gas and sputter coated with 9 nm of platinum before imaging on a Tescan MIRA variable pressure field emission scanning electron microscope (VP-FESEM).

eDNA extraction and quantification*:*

eDNA was quantified in the biofilm matrix using a Qubit fluorimeter and HS reagent kit (Thermo Fisher). Coupons with biofilms were removed from reactors and lightly rinsed in PBS (Sigma, pH 7.4) before transferring to tubes containing 2 mL of fresh PBS (Sigma, pH 7.4). To extract eDNA from biofilms, a basic digestion and filtration protocol was conducted. All cells and debris were removed from coupons using a non-lytic sonication procedure. Briefly, tubes containing coupons were vortexed for 30 seconds and sonicated for 10 seconds followed by 15 seconds on ice, repeating for 7 cycles. Large particles were then removed from the sample by centrifugation at 15,000 xg for 5 minutes, and the supernatant was filtered using a Sartorius Minisart® 0.2 µm pore polyethersulfone (PES) membrane filter. Fluorimetry was conducted directly on the filtered volume. This procedure was repeated for the reactor planktonic samples, without the detachment (homogenisation) stage.

Total DNA (genomic and eDNA) extraction

Total DNA from planktonic and biofilm communities was extracted from duplicate experiments at 2, 4 and 6 weeks. Total biofilm and planktonic DNA samples contained genomic DNA and eDNA, therefore referred to as total biofilm DNA and total solution DNA respectively throughout this communication. Total DNA was extracted using a DNeasy® PowerSoil® Pro DNA extraction kit (Qiagen) following the manufacturer's instructions. DNA from biofilm samples was extracted from the pellets after centrifugation at 15,000 x *g* for 5 minutes described above. DNA from planktonic cells was extracted from pellets after centrifugation of 5 mL of the test solution at 15,000 x *g* for 5 minutes.

RNA extraction

RNA from biofilm communities was extracted from biological replicate experiments at 2, 4 and 6 weeks to identify active populations within the biofilm over time. RNA was extracted using

the RNeasy® PowerBiofilm® kit (Qiagen), as recommended by the manufacturer. Subsequently, RNA was treated with DNase using Turbo DNA-free kit (Invitrogen) to remove the remaining DNA. A PCR targeting the 16S rRNA gene was performed to verify the complete removal of DNA. Afterwards, RNA was purified and converted to cDNA using a SuperScript IV first-strand synthesis system (Invitrogen).

16S rRNA sequencing and data analyses

eDNA and total DNA extracted from biofilms and solution, and cDNA synthetised from RNA extracted from biofilms were used as a template to generate amplicons of the V3-V4 gene region of the bacterial 16S rRNA gene for the estimation of the relative abundance of each isolate in the community. PCR was conducted using the primers 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5'GGACTACNNGGGTATCTAAT 3')[128]. PCR amplicons were sequenced on an Illumina MiSeq instrument with a V3 (600 cycles) kit (Illumina).

Resulting sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME2) software pipeline (QIIME2 v. 2020.11)[129]. Raw reads were visually inspected with the demux plugin and quality filtered with DADA2 pipeline (--p-trunc-len-f = 280 and --p-trunc-len-r" =220)[130]. The DADA2 plugin was also used to denoise and obtain representative amplicon sequence variants (ASV). Representative sequences and their abundances were extracted by feature-table plugin[131] and taxonomically classified using the Naïve Bayesian classifier against the SILVA database v.138[132].

In order to visualize the multivariate dispersion of the community composition based on the DNA source and the microbial community at each sampling period, a nonmetric multidimensional scaling (NMDS) analysis was conducted based on the weighted UniFrac distance matrix[133]. The NMDS was performed in Rstudio (v1.3.1093)[134] using the "vegan" R package[135]. Microbial taxa were fit into the ordination by using the envfit function and their significance assessed under 999 permutations. Correlation between the NMDS and the relative abundance of the microbial taxa was considered significant if p-value < 0.05.

A linear discriminant analysis (LDA) effect size (LEfSe)[136] was applied to identify the specific bacterial taxa significantly associated with DNA source ('eDNA' or 'total biofilm DNA') or with the microbial community ('biofilm' or 'planktonic'). For LEfSe, Kruskal–Wallis and pairwise Wilcoxon tests were performed, followed by LDA to assess the effect size of each differentially abundant taxon. A p-value of < 0.05 was considered significant for both statistical methods. The threshold for the logarithmic discriminant analysis (LDA) score was set to 3.

Colony forming unit (CFU) quantification:

CFUs were extracted from coupons in 10 mL PBS (Sigma, pH 7.4) using the non-lytic sonication and vortex procedure described above. CFU plates were prepared using ASW solution (Supplementary Table 1) with 3 g/L *w/v* Bacto™ casamino acids, 3 g/L *w/v* sodium pyruvate, 3 g/L *w/v* D (+) glucose and 15 g/L *w/v* bacteriological agar and (Sigma). Ammonium nitrate (NH_4NO_3) was excluded since agar plates were prepared and cultivated in aerobic conditions where electron acceptor supplementation was not required. The drop plate method was then used to prepare and quantify CFUs according to existing standards[20].

Adenylate energy charge (AEC) analysis:

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) adenylates are critical for the metabolism of all cells[137,138]. Hydrolysis

of phosphate in ATP forms the more energy depleted ADP and AMP, releasing energy for use by cellular processes. The AXP assay kit takes advantage of cellular dependence on these molecules for a rapid, sensitive estimation of biofilm energy charge[137]. In the present research, AXP assays were conducted according to the manufacturer's instructions, with an additional sonication and vortex stage as detailed above to facilitate detachment and lysis. The suspension was then processed through the AXP assay kit and Quench-Gone Organic Modified (QGO-M) ATP assay kit (LuminUltra Technologies, Ltd).

3. Results:

Identification of eDNA as a major structural polymer in the biofilm:

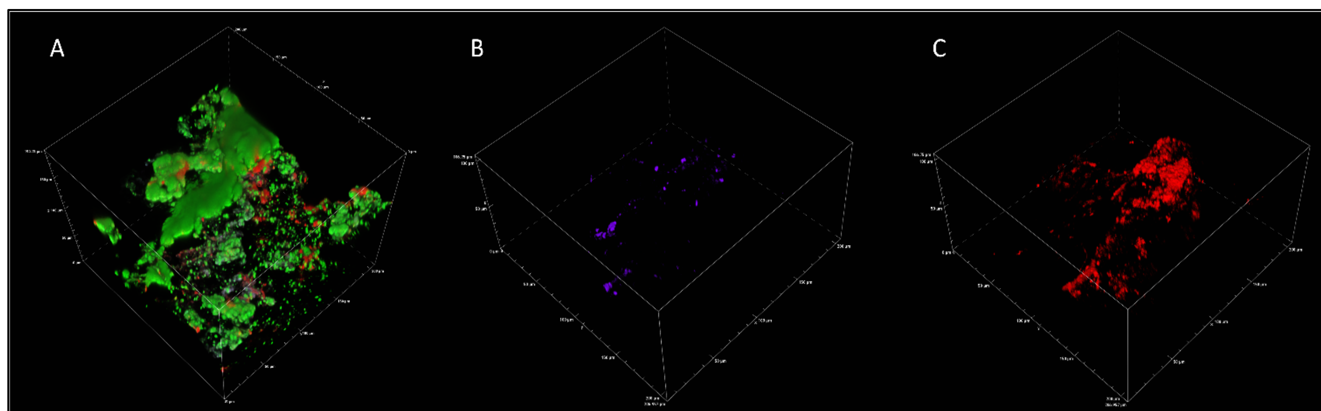
To determine the composition of the EPS, biofilm components were targeted with specific stains and imaged using confocal laser scanning microscopy with eDNA presenting the greatest signal (Figure 2 A,B and C).

IMARIS (Bitplane) statistical analysis was conducted to determine the relative contribution of each macromolecule to the biofilm. After 2 weeks, eDNA contributed >90% to the total biofilm composition in experimental replicate 1 with proteins and polysaccharides contributing <10% combined (Figure 2D). A similar trend was observed in experimental replicate 2 (Supplementary Figure 1). Although some variation exists between % contribution of macromolecules, a dominant eDNA signal was consistent.

Biofilm eDNA was micrographed separately in experimental replicates across 2, 4 and 6 weeks to confirm the abundance of this macromolecule over the relatively longer term. The results of the CLSM analysis in Figure 3 (A-F) represent experimental replicate 1 (A-C) and experimental replicate 2 (D-F). The abundance of eDNA in biofilm samples collected over 6 weeks was comparable between all results as indicated by green fluorescence.

Scanning electron microscopy was conducted to understand the physical morphology of the biofilms after 2, 4 and 6 weeks. Structures resembling bacterial cells were observed at all time periods (examples indicated by arrows). The structure of the EPS, especially at earlier sampling periods (2-4 weeks) resembled a fibrous net-like appearance resembling eDNA. Two-week-old biofilms contain cell-like structures surrounded by an abundance of EPS (Figure 4).

Microscopic data was supported by direct quantification of free-floating DNA in the biofilm and planktonic population. At all time points the biofilm contained more eDNA than the solution with the greatest amount of eDNA observed at 4 weeks; with 190 ng/mL and 130 ng/mL observed in the biofilm and solution respectively (Supplementary Figure 2).



D

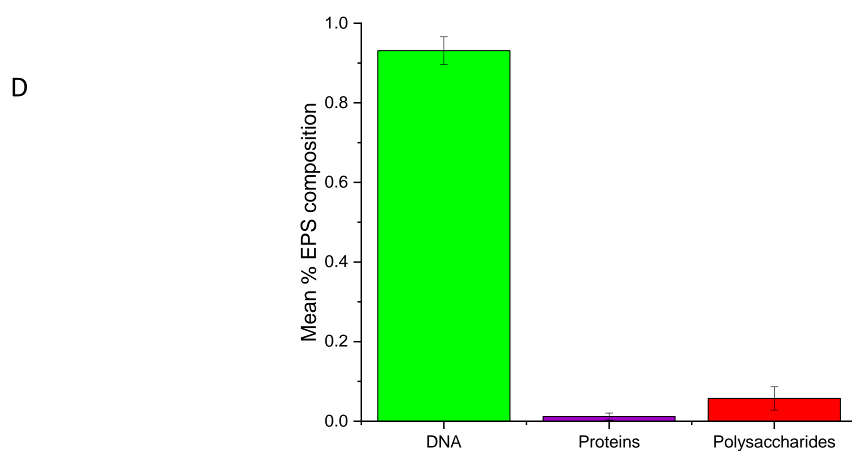


Figure 2: A-C) Confocal micrographs of early stage biofilms (2 weeks) depicting the EPS composition, where A) all channels combined, B) protein targeting channel, and C) polysaccharides targeting channel. D) IMARIS (Bitplane) analysis depicting the average % contributions of eDNA, proteins and polysaccharides to the matrix of biofilms from experimental replicates. Error bars represent the standard deviation of triplicate micrographs.

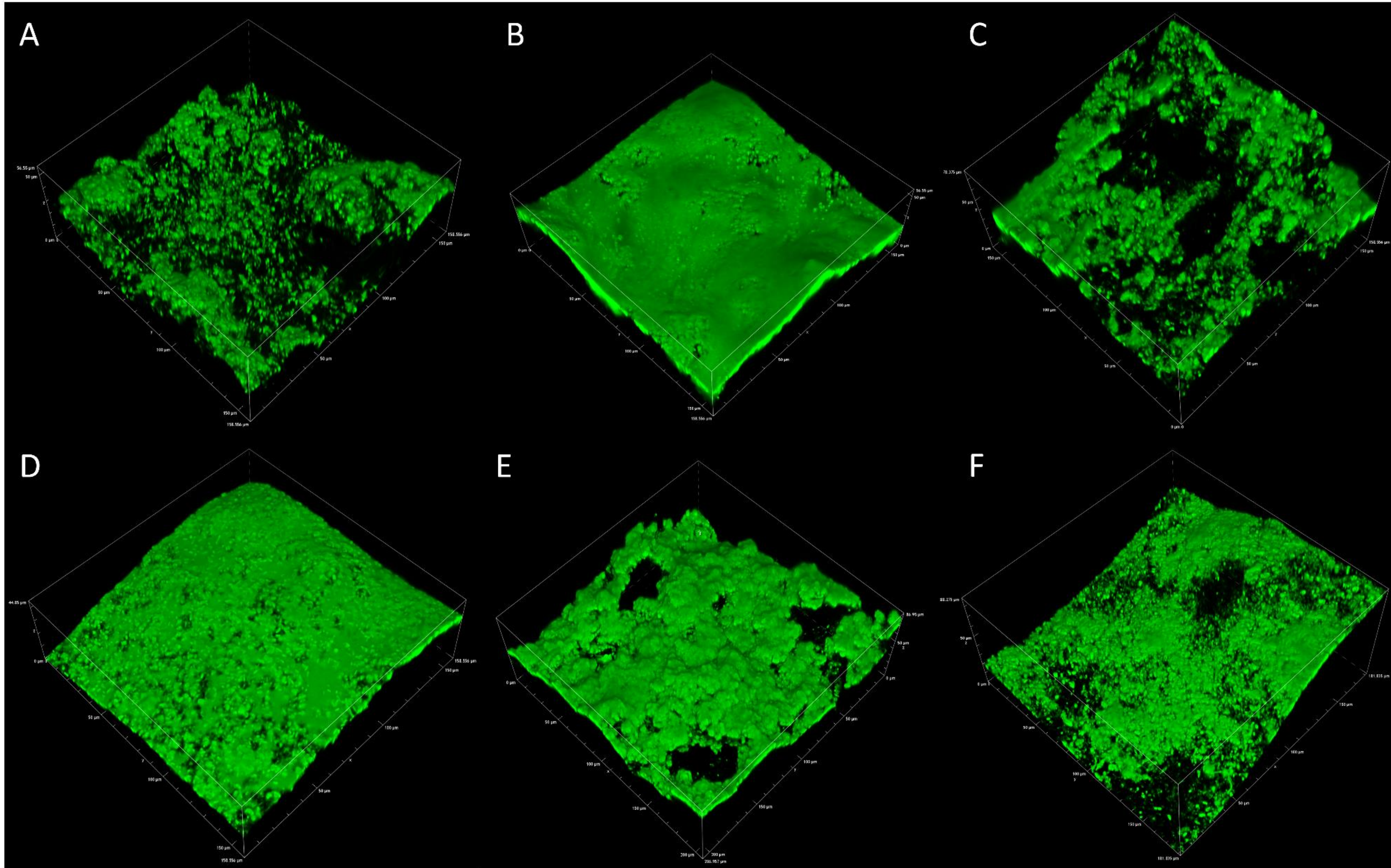


Figure 3: Representative CLSM of eDNA in multispecies biofilms after 2, 4 and 6 weeks for experimental replicate 1 (A-C) and experimental replicate 2 (D-F).

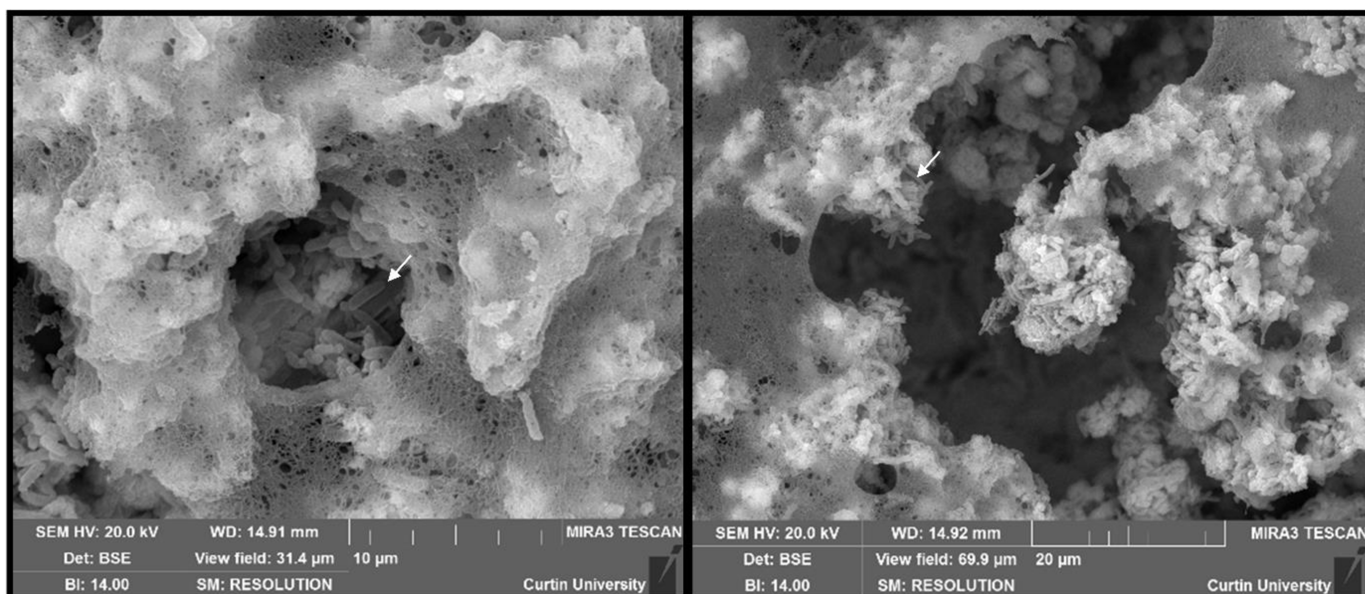


Figure 4: *Scanning electron micrographs captured from 2-week-old biofilms showing a net-like structure within EPS. Structures resembling bacterial cells are also evident in the samples.*

To assess the viability of the biofilm across the 6-week sampling period, live and dead CLSM assays, CFU quantification and AXP assays were conducted. Viability was maintained for the duration of the experiment, although it appeared to decrease with sampling time. CLSM analysis revealed large, mushroom-like structures developed over 2 weeks (A) gradually reduced to thin homogeneous biofilms by six weeks (C). Although CLSM indicated a reduced viability trend with time, live cells (green fluorescence) were still detected in all micrographs for the duration of the experiment. Viability was quantified to reveal a similar trend (gradual viability reduction). CFU counts revealed that the 2-week sampling period produced the greatest number of viable cells from coupons (Figure 7 A), which corresponded to biofilm activity as indicated by AXP analysis (Figure 7 B). Both techniques indicted a gradual decline in viability over 6 weeks.

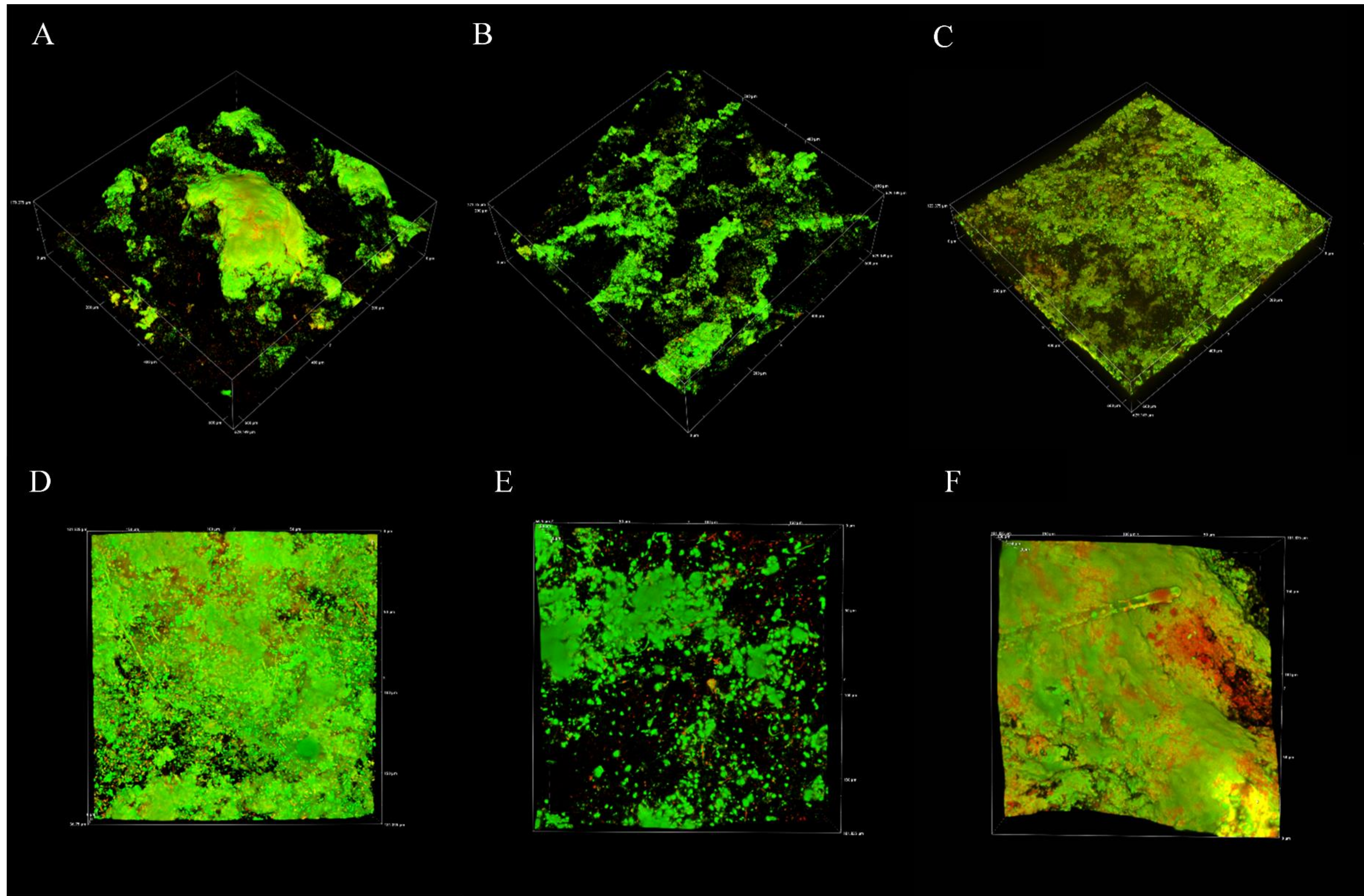


Figure 6: Live (green) and dead (red) staining of biofilms at 2, 4 and 6 weeks. Images were obtained with CLSM. A-C are larger micrographs (600 x 600 um). E-G represent the same surface captured using the Nyquist function of the Nikon Elements software for increased resolution.

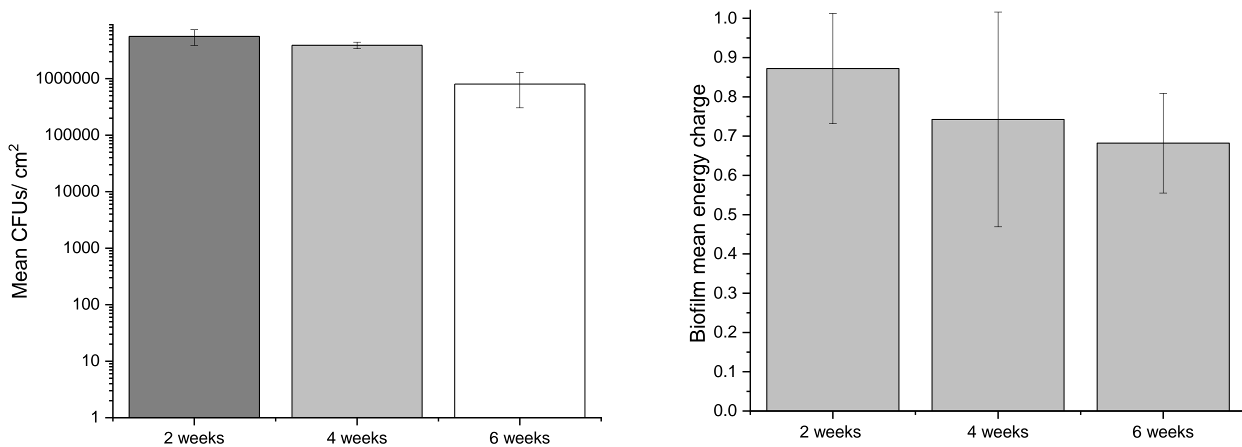


Figure 7: Mean CFUs (left), and the pooled energy charge(right) of biofilms at 2, 4 and 6 weeks. Error bars represent the standard deviation of 2 experimental and 3 technical replicates.

Community composition (DNA based sequencing)

Differences in the microbial community composition between the eDNA fraction and the total DNA in both biofilm (sessile) and reactor solution were observed at all sampling points (Figure 8). A higher relative abundance of *K. pneumoniae* in total biofilm DNA was clearly evident, with similar contributions by the other two strains. After separation of eDNA from the biofilm, DNA sequencing revealed that eDNA primarily belonged to *P. balearica* and *S. chilikensis*. This trend was also observed in the solution, revealing again that *K. pneumoniae* eDNA contributed relatively less to the free eDNA pool than the other two strains. Additionally, the solution eDNA pool was significantly enriched by the *S. chilikensis* strain as indicated by Supplementary Figure 3. Statistically significant biomarkers among groups were also determined for use in the NMDS ordination analysis (Figure 9).

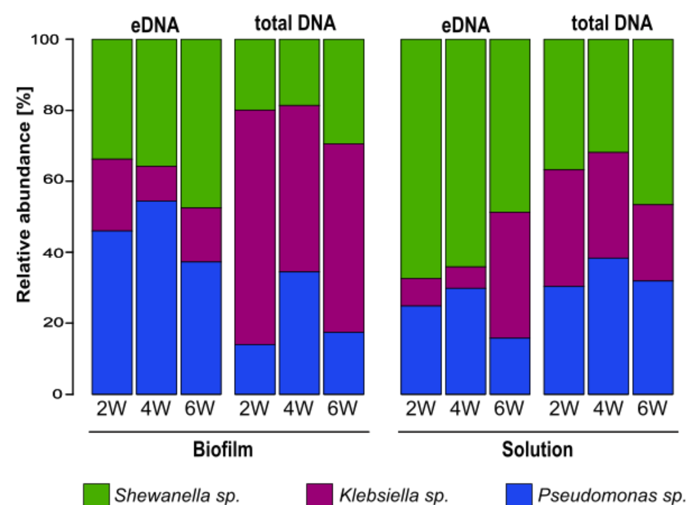


Figure 8. Mean relative abundance of biofilm and planktonic microbial taxa: eDNA: extracellular DNA. 2W: 2 weeks exposure; 4W: 4 weeks exposure; 6W: 6 weeks exposure. Data is the average of two biological replicates.

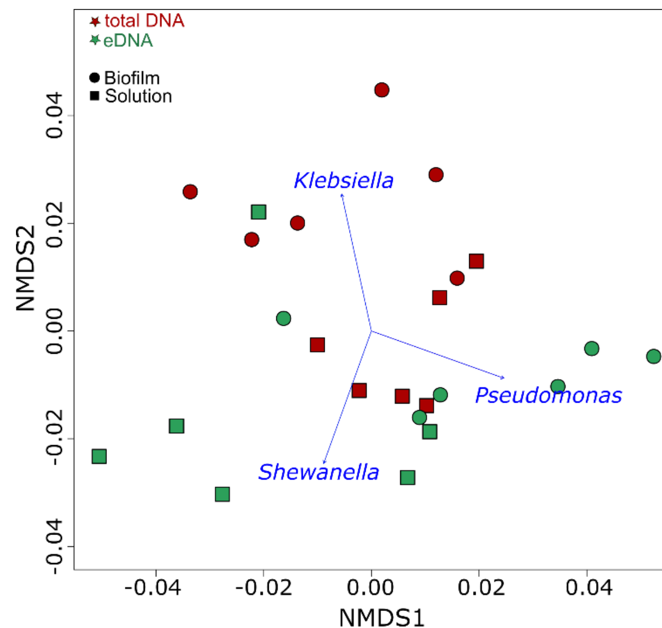


Figure 9. NMDS of the microbial communities at each sampling period. Microbial taxa significantly correlated (p = 0.001) to microbial community structure are indicated by blue arrows.

Biofilm community composition of active microorganisms

RNA-based sequencing profiles revealed that *Pseudomonas balearica* was the most active microorganism in the biofilm along the experimental period (Figure 10). A reduction on the relative abundance of *K. pneumoniae* and an increase on the relative abundance of the *S. chilikensis* was observed after 2 weeks of biofilm growth. Complementary DNA-based and RNA-based profiling indicates that the high relative abundance of the *Klebsiella* genus in the total biofilm DNA fraction at all sampling periods was mainly related to dormant or inactive cells.

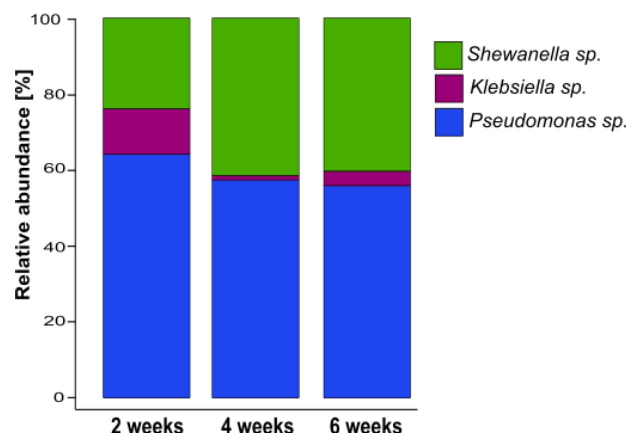


Figure 10. 16S rRNA based microbial community composition of biofilms showing the mean relative abundances of each microbial taxa. Data is derived from the average abundance of two technical and two experimental replicates.

4. Discussion

CLSM analysis targeted proteins, polysaccharides and eDNA presence in the biofilm for visual representation and semi-quantitative analysis. All macromolecules identified in this analysis have been associated with EPS in previous communications[139-141]. Although eDNA is often reported in biofilms, especially from clinical isolates, it is often not the primary EPS component[142,143]. Polysaccharides and proteins are more frequently identified, and are believed to comprise the bulk of the EPS in most environments[115,144-146]. CLSM results and post-image analysis results (Figure 2 and Supplementary Figure 1) indicate that eDNA comprised the majority of the EPS under marine simulating conditions presented in this study (>90% and >60% in replicates 1 and 2 respectively). This finding was supported by a separate CLSM analysis (Figure 3), which demonstrated an abundant fluorescence by the eDNA-specific stain DiYO-1™ in experimental replicates at 2, 4 and 6 weeks. Scanning electron microscopy (SEM) revealed a fibrous, net-like appearance in biofilms where eDNA was detected (Figure 4). Similar EPS structures have been reported in staphylococcus biofilms, where mesh structures were also associated with eDNA[147]. To support these microscopic observations consistent with eDNA presence in the biofilm matrix, DNA quantification by fluorometric analysis was conducted on biofilm and solution samples across a 6-week period (Supplementary Figure 2). Fluorimetry results of biological replicates revealed the presence of eDNA peaked in the EPS and reactor solution at four weeks, remaining above 2-week values by the end of the experiments.

The production of eDNA was evaluated on marine multi-species biofilms developed under oligotrophic conditions (low organic nutrient supply). CLSM (live/dead) assays, CFU quantification and AXP analysis were conducted to ensure EPS and community analysis was performed on living (viable) biofilms. CLSM live/dead results (Figure 6) demonstrate that biofilm viability was maintained for the duration of the experiments, although biofilm architecture and live cells appeared to reduce with time. CFUs and adenylates AMP, ADP and ATP were recovered and quantified from biofilm coupons to quantitatively estimate changes to biofilm viability, revealing a similar trend across experimental replicates. Biofilm viability as determined by CFU diminished over the exposure period, consistent with CLSM findings (Figure 7). This was also supported by AXP analysis (Figure 7), with available biofilm energy also reducing over the experiment. This behaviour is typical of oligotrophic conditions, which cause stress to biofilm cells[148].

Origin of eDNA and community structure

To understand the dynamics of the sessile and planktonic populations, and the origin of eDNA, DNA and RNA-based (16S rRNA gene and transcripts) sequencing was conducted. Results demonstrate that *Pseudomonas balearica* EC28, a laboratory strain of *Klebsiella pneumoniae* and *Shewanella chilikensis* DC57 constructed a reproducible multi-species biofilm in marine simulating conditions over 6 weeks (Figure 8). RNA-based sequencing and total biofilm DNA (genomic and eDNA) sequencing were expected to yield similar diversity profiles. However, relative abundance based on total biofilm DNA differed from expected population contributions

predicted by RNA-based sequencing. Thus, cell number was not indicative of species activity, indicating that more significant contributions to the matrix may come from less abundant community members. To support this hypothesis, an eDNA fraction was also sequenced (Figure 8). Results of this analysis indicate that activity was more indicative of eDNA contribution to the biofilm as opposed to cell number, which can include dormant or inactive cells.

The DNA-based sequencing analysis revealed unexpected differences between total biofilm DNA and eDNA samples as well as between the sessile and planktonic populations. For instance, the relative abundance of each strain varied between biofilm and solution samples (Figure 8). While *Klebsiella pneumoniae* dominated the total biofilm DNA as indicated by LefSe analysis (Supplementary Figure 3), the abundance of *K. pneumoniae* in the eDNA fraction was significantly lower than the abundance of other strains. Therefore, *K. pneumoniae* contributed relatively less to the biofilm eDNA while contributing a large number of inactive or dormant cells to the population. This hypothesis is supported by RNA sequencing results, revealing *K. pneumoniae* genus was the least active.

In the planktonic population, LefSe analysis revealed a statistically significant enrichment of *Shewanella* (Supplementary Figure 3). Although T_0 inoculations comprised relatively equal volumes of each community member, the final community composition established over time (probably in response to system parameters such as interspecies interactions, temperature, attachment substrate, shear stress and atmospheric conditions). Results of this investigation also imply that the planktonic community does not necessarily reflect the composition of the biofilm community. This was expected, since sessile and planktonic cell phenotypes can vary greatly, and bacteria colonise surfaces at various rates. Finally, bacterial eDNA contributions are likely to be active (excreted or produced by active cell lysis) or passive (produced by normal cell death) based on relative contributions by each strain. While a single strain can dominate the biofilm cellular complement, the results indicate that eDNA contribution can be produced predominantly by other strains in either the biofilm or surrounding solution.

The biofilm RNA-based sequencing revealed community similarities at 2, 4 and 6 weeks (Figure 10). Relative biofilm contribution in terms of activity for the duration of the experiments was *Pseudomonas balearica* > *Shewanella* > *Klebsiella*. While DNA-based sequencing results demonstrated some variation in the biofilm and planktonic cellular and eDNA contribution, RNA-based sequencing indicated a more stable biofilm structure. Indeed, RNA-based relative abundance is expected to be associated with the active fraction of the community. As expected, the stability of RNA-based compared to DNA-based diversity profiling results imply that eDNA persisted in reactor solution longer than exogenous RNA. While RNA has been recovered from simulations up to 13 hours after release to the environment, DNA can persist for years, and is generally expected to degrade at a slower rate due to a more stable double helix structure compared to single-stranded RNA. In the present research, similarities between RNA analysis results (between replicates and at different sampling times) revealed that microorganisms in the local environment do not form a random community. Instead, each strain contributed to the community composition in a relatively reproducible way with a unique level of stable activity.

The role of eDNA in biofilms:

In clinical and single-species biofilms, eDNA has been reported in the EPS. For example, while polysaccharides and proteins were present, the EPS composition of *Pseudomonas aeruginosa* biofilms was reported to be primarily eDNA[149]. Indeed, the structural role of eDNA in *P. aeruginosa* biofilms is well characterised with distinct production pathways. *P. aeruginosa* can actively excrete eDNA or generate it through autolysis triggered by quorum sensing

events[150]. Sacrificing healthy cells for contribution of eDNA to the matrix indicated that eDNA plays a critical role in the matrix. In this species, eDNA forms a scaffold that provides structural stability[150-152]. In the present research, CLSM targeted the spatial distribution of eDNA, indicating a similar structural role in the biofilm under marine simulating conditions.

Interestingly eDNA in the *Pseudomonas balearica* is also known to contribute to tolerance of the biofilm to antimicrobials[152]. Wen-chi *et al* associated exogenously supplemented eDNA to *P. aeruginosa* biofilms with tolerance to aminoglycosides[153]. Therefore, eDNA from the surrounding environment is incorporated into the biofilm to support structural integrity and enhance tolerance to chemical treatments. In the present study, *P. balearica* EC28 was included in the multi-species community. The strain was recently implicated in an MIC failure of an oil production facility in Western Australia[4]. After isolation and sequencing of the strain in previous work[127], attachment to steel was evaluated using SEM. In Supplementary Figure 4, a pure culture of *P. balearica* EC28 was grown on CS over 24 hours, revealing 100% surface coverage by the strain and net-like structures resembling an eDNA network. Microscopic screening of the strain indicated that *P. balearica EC28* may be central to biofilm eDNA contribution. Further analysis is required to identify the genetic pathways employed by this strain in either apoptotic or active excretion mechanisms. Although biofilm eDNA sequencing supports the hypothesis that *Pseudomonas* eDNA dominated the matrix, the results also imply that eDNA is contributed by *S. chilikensis* DC57 and *K. pneumoniae*.

As with *Pseudomonas* sp., eDNA plays an important role in biofilms of *K. pneumoniae*. Recently, Liu *et al* report a novel biofilm structure designated the 'R-biofilm' in *K. pneumoniae*, formed by breaks to double-stranded DNA[154]. The ring-like structures contained proteins and eDNA. Importantly, the R-biofilm was implicated as the more protective phenotype against adverse conditions such as chemical treatment. *K. pneumoniae* biofilms are characterised to a lesser extent compared to *P. aeruginosa*, however the significance of further research in this area related mainly to clinical impacts.

Like *P. balearica* EC28, *S. chilikensis* DC57 is a true marine strain. The strain was also cultivated and sequenced in separate work after identification in an MIC related equipment failure[4,126]. Almost nothing is known about the EPS composition of *S. chilikensis* biofilms, however eDNA sequencing results demonstrate that eDNA is contributed by this strain to the multispecies community.

5. Conclusions

To effectively manage marine biofilms with reduced environmental impact, a greater understanding of the EPS and community composition is required. The present research aimed to enhance the understanding of natural biofilm EPS by identifying the dominant structural matrix component under marine simulating conditions. Subsequently, through DNA and RNA-based sequencing analysis, this communication aimed to underpin changes to population dynamics over time and assess the origin of eDNA in marine biofilms. Microscopic analysis, post-image analysis and direct quantification of extracellular DNA (eDNA) suggest that eDNA is the most abundant and structurally important molecule in marine multispecies biofilms. Sequencing of eDNA, total biofilm DNA and RNA-based sequencing revealed that all originally inoculated bacterial strains contributed to the biofilm composition. The biofilm structure declined over time under limited, consistent metabolic supplementation, although cell viability and eDNA remained throughout the experiment. Interestingly the active fraction determined by RNA-based sequencing revealed that relatively stable communities form on

carbon steel over 6 weeks of exposure. Lastly, no correlation was found between activity within the biofilm and DNA presence in the system.

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Conflicts of interest statement:

The authors declare no financial or non-financial conflicts of interest.

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SUPPLEMENTARY INFORMATION

Extracellular DNA a critical structural polymer in marine multi-species biofilms

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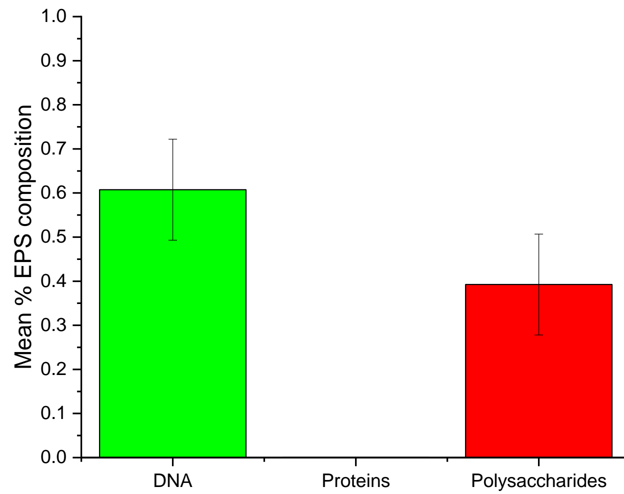
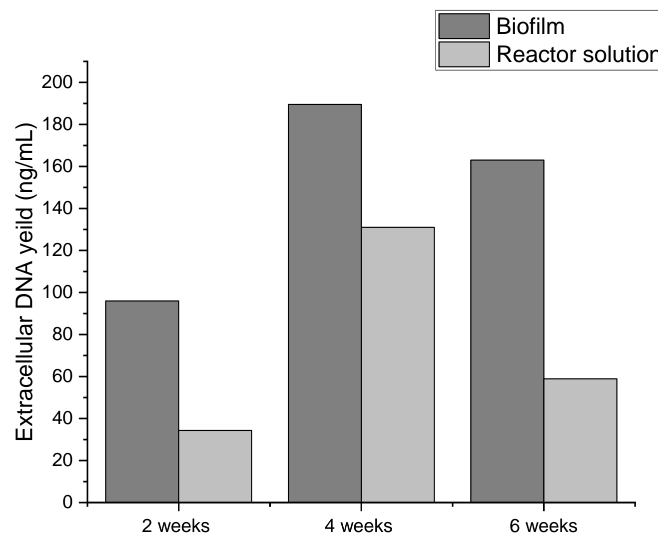
Table 1: Artificial seawater solution	
Solution 1	g/L
Sodium Chloride (NaCl)	24.0
Sodium Sulfate (Na_2SO_4)	4.00
Potassium Chloride (KCl)	0.68
Potassium Bromide (KBr)	0.10
Boric Acid (H_3BO_3)	0.025
Sodium Fluoride (NaF)	0.002
Magnesium Chloride, hydrated ($MgCl_2$*6H_2O)	10.8
Calcium Chloride ($CaCl_2$)	0.10
Strontium Chloride ($SrCl_2$)	0.024
Bacto™ Casamino Acids	1.50
Sodium Pyruvate	0.55
Glucose	0.90
Ammonium Nitrate (NH_4NO_3)	1.20
Deionized water	968 mL
Solution 2	
Sodium Dihydrogen Phosphate (NaH_2PO_4)	0.04
Deionized water	10 mL
Solution 3	
Sodium Hydrogen Carbonate ($NaHCO_3$)	0.20
Deionized water	10 mL
Solution 4	
Ammonium Chloride (NH_4Cl)	0.50
Deionized water	10 mL
Solution 5	
Wolfe's Vitamins Solution*	1 mL
Trace Elements Solution*	1 mL
*Separate solution prepared directly as outlined in previous work[19]	

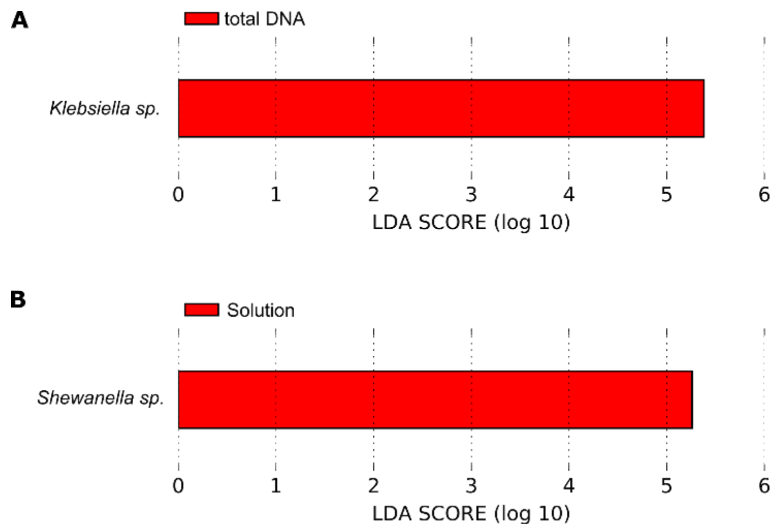
Supplementary Table 1: ASW reactor solution composition used for all experiments. All reagents were AR grade.



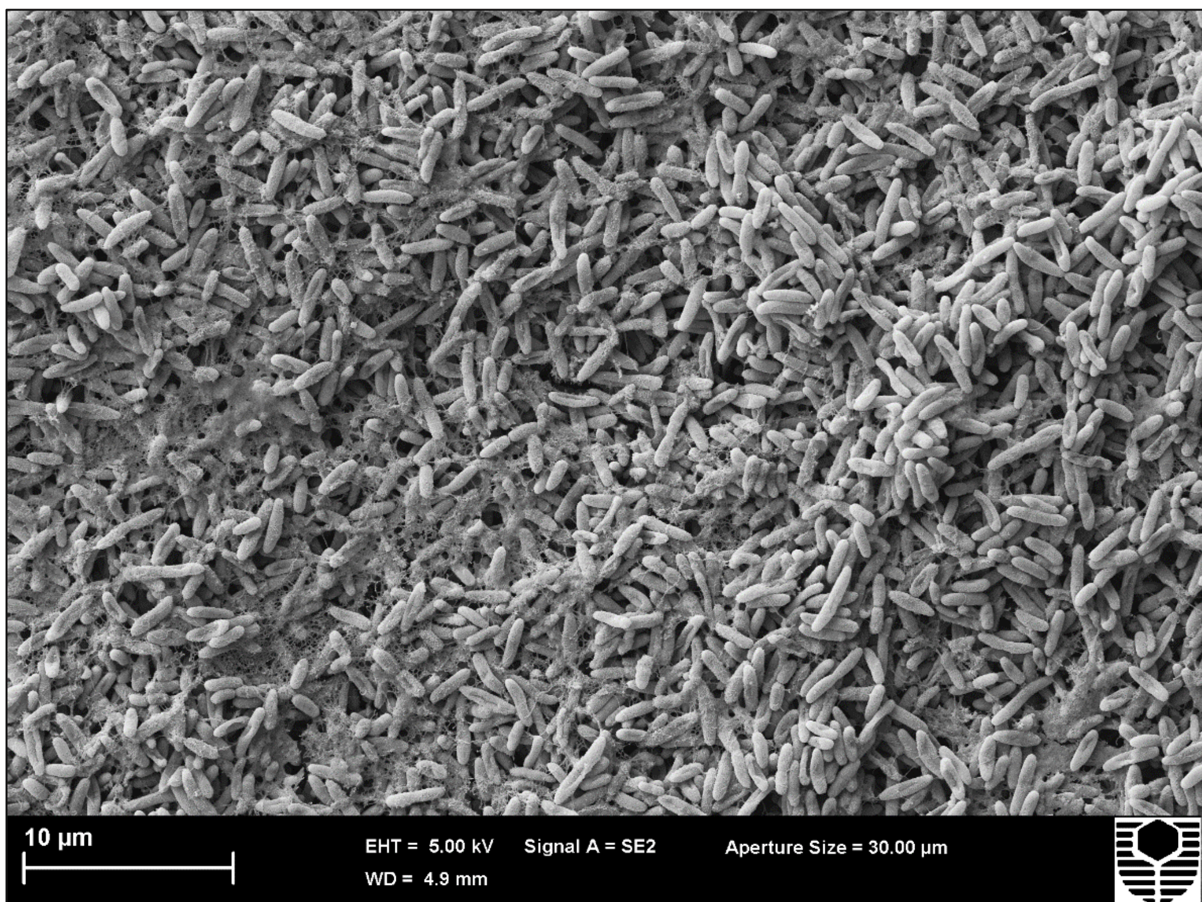
Supplementary Figure 1: The mean percent composition of eDNA, proteins and polysaccharides in the biofilm EPS matrix of reactor 2 at 2 weeks. Error bars represent the standard deviation of triplicate 20 x micrographs.



Supplementary Figure 2: Qubit quantification of eDNA from biofilms and reactor solution at 2, 4 and 6 weeks. Measurements were taken using a pooled sample from experimental replicates.



Supplementary Figure 3. Linear discriminant analysis effect size analysis LEfSe plot of taxonomic biomarkers. (A) Microbial genera significantly associated with DNA source. (B) Microbial genera significantly associated with microbial cell. Horizontal bars represent the effect size for each significantly associated taxon. The length of the bar represents the log10 transformed LDA score, indicated by vertical dotted lines.



Supplementary Figure 4: SEM of *Pseudomonas balearica* EC28 biofilm grown on CS over 24 hours. Surface coverage was 100%, and the strain revealed a fibrous, net-like EPS structure resembling eDNA.

Chapter 4: EVALUATION OF A NOVEL, ENVIORNMNETALLY SAFE AND MULTIFUNCTIONAL CORROSION INHIBITOR AS A BIOCIDE

Chapter 4: Green, multifunctional biocide evaluations.

Manuscript # 1 of 2

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Efficiency of a Novel Multifunctional Corrosion Inhibitor Against Biofilms Developed on Carbon Steel

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In natural environments, populations of microorganisms rapidly colonise surfaces forming biofilms. These sessile communities comprise a variety of species which contribute to biofouling and microbiologically influenced corrosion (MIC), especially on metals. Species heterogeneity in natural systems confers higher tolerance to adverse conditions such as biocide treatment compared with single species laboratory simulations. Effective chemical treatments to combat recalcitrant biofilms are often dangerous to apply; both to operators and the environment, and face international embargoes. Today, there is a drive to exchange current toxic and environmentally hazardous biocides with less harmful compounds. One effective method of achieving this goal is to generate multi-functional compounds capable of tackling corrosion and biofilm formation simultaneously, thus reducing the number of compounds in dosing procedures. In a previous study, a novel corrosion inhibitor demonstrated biocidal effects against three marine isolates during the early stages of biofilm formation. The compound; CTA-4OHcinn, holds great promise as a multi-functional inhibitor, however its effect on complex, multi-species biofilms remains unknown. Here we evaluate CTA-4OHcinn biocidal capacity against multi-species biofilms developed from oilfield samples. Mature biofilms were developed and treated with 10 mM CTA-4OHcinn for 4 h. The effects of the compound were assessed using mean probable number (MPN), adenosine triphosphate (ATP) quantification, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Results demonstrate that CTA-4OHcinn significantly reduces the viability of mature biofilms, supporting previous demonstrations on the secondary function of CTA-4OHcinn as a biocide. CLSM results further indicate that CTA-4OHcinn targets the cell membrane resulting in lysis. This finding complements the established corrosion inhibition function of CTA-4OHcinn, indicating the compound is a true multi-functional organic corrosion inhibitor.

Keywords: biofilm, biofouling, corrosion inhibitor, microbiologically influenced, biocide

1 INTRODUCTION

Microorganisms are ubiquitous in the marine environment as free-floating planktonic cells (Ista et al., 2004; Van Tol et al., 2017). Planktonic microorganisms contact submersed surfaces, where they attach and form diverse communities surrounded by extracellular polymeric substances (EPS) (Kumar et al., 2011; El-Kirat-Chatel et al., 2017). EPS typically consists of nucleic acids, polysaccharides, proteins and other organic and inorganic substances (Saravanan and Jayachandran, 2008; Flemming and Wingender, 2010; Kavita et al., 2014). This biofilm phenotype is the preferred lifestyle for most bacteria, owing to the numerous benefits afforded by the community and the EPS (Kumar et al., 2011). For example, the EPS can provide protection from desiccation, chemical treatment, and mechanical stress (Dieltjens et al., 2020). Among other benefits, the biofilm lifestyle also provides a source of nutrition and a reservoir for antimicrobial or biocide tolerance (Schlafer and Meyer, 2017). The mature biofilm population with EPS can be as much as 1,000 times more tolerant to chemical treatments compared to the free-floating planktonic phenotype (Dieltjens et al., 2020). Unlike planktonic cells, metabolic functions of mature biofilms are known to impact the base substrate resulting in serious economic, environmental and health impacts.

In the marine environment, a plethora of recent studies have investigated the attachment and biofilm formation of bacteria to engineered systems, since progression of the biofilm results in biofouling and microbiologically influenced corrosion (MIC) (Tuck et al., 2020; Albahri et al., 2021; Catubig et al., 2021; Qian et al., 2021; Tran et al., 2021). MIC is particularly costly, expected to contribute around 20–50% of the total $4T USD global corrosion costs (Little and Lee, 2007; Li et al., 2018). The initial biofilm stages involving attachment and adhesion are considered critical to biofouling and MIC; therefore attracting considerable attention in recent years (Javed et al., 2015; Javed et al., 2016; Caruso, 2020). If biofilm formation can be limited at these early stages, deleterious impacts of biofilm formation may be evaded. Although research from the last two decades has greatly enhanced the scientific understanding of initial biofilm formation, no natural or artificial surface can totally prevent bacterial attachment (Vanithakumari et al., 2018; Tuck et al., 2020). In reality, a mature biofilm will develop on any substrate exposed to the marine environment if effective treatments are not regularly applied.

Biocides are a primary tool for controlling bacterial attachment and mature biofilms in marine environments. As biofilms develop tolerance to chemical treatment, the use of potent and potentially harmful biocides in engineered systems such as pipelines is widespread (Machuca Suarez et al., 2019). Glutaraldehyde (GLUT) for example is the most widely used commercial biocide (Kjellerup et al., 2009; Simões et al., 2011). GLUT has been an effective antimicrobial, although some reports suggest that this efficiency is reduced against multi-species biofilms (Simões et al., 2011). Although not corrosive, GLUT is not a corrosion inhibitor and is not effective at limiting attachment of bacteria (Simões et al., 2011). While mature biofilms are important targets, biocides should also control attachment of bacteria in order to prevent recovery of the population. Unlike single-function compounds such as GLUT, multi-functional compounds can inhibit corrosion as well as limit biofilm formation (Tuck et al., 2021). The primary aim of multi-functional compounds is to reduce the amount or number of compounds used in dosing protocols. If corrosion and biofilm effects can be mitigated effectively with multi-functional compounds, there are major economic advantages, time can be saved at dosing intervals and human or environmental toxicity can be reduced.

In a previous communication, a novel multi-functional organic corrosion inhibitor (OCI) compound, CTA-4OHcinn, was evaluated for its effectiveness at reducing the initial stages of biofilm formation (Tuck et al., 2021). This study demonstrated the effective reduction of the initial stages of biofilm formation (within 24 h of exposure) by three marine isolates. Although CTA-4OHcinn demonstrated promise as a corrosion inhibitor and biocide, the efficacy of this compound when applied to natural biofilms remains unknown. The present research aims to assess CTA-4OHcinn as a chemical treatment for mature, multi-species biofilms and expand the scientific understanding of this compound.

Based on a previous study, it was hypothesized that 10 mM CTA-4OHcinn would be effective at controlling multi-species biofilms (Tuck et al., 2021). To evaluate this hypothesis, biofilms were developed using an environmental consortium obtained from a West-Australian oilfield and grown on carbon steel (CS) over a 2 week period in marine-simulating conditions. These biofilms were then exposed to 10 mM CTA-4OHcinn for 4 h. Cell quantities calculated using the mean probable number (MPN) method, total adenosine triphosphate (tATP) quantification, confocal laser scanning microscopy (CLSM), post-image analysis and SEM was used to evaluate the effects of the compound on multi-species biofilms. In a novel application of the stain Cellbrite™ Fix 555, the ability of CTA-4OHcinn to cause lysis by targeting the cell membrane of a bacterial isolate was assessed.

2 MATERIALS AND METHODS

2.1 Microbial Consortium

Produced water samples collected from a West-Australian oilfield were used to recover microorganisms. Aliquots of 100 ml produced water was filtered using a sterile 0.2 µm pore size membrane filter (Whatman®). Each filter was then placed in anaerobic SPP culture media as previously described (Salgar-Chaparro et al., 2020b) and incubated at 40°C. Subsequently, enrichment cultures were developed using media selective for sulphate producing prokaryotes (SPP), methanogenic bacteria and archaea (MET), acid producing bacteria (APB) and iron reducing bacteria (IRB). The microbial consortium for inoculation of reactors was established by pooling samples of the different microbial enrichment cultures. Enrichment samples were washed in phosphate-buffered saline (PBS, Sigma, pH 7.4) to remove wastes. Cells from each enrichment culture were then enumerated using a Neubauer haemocytometer and added in

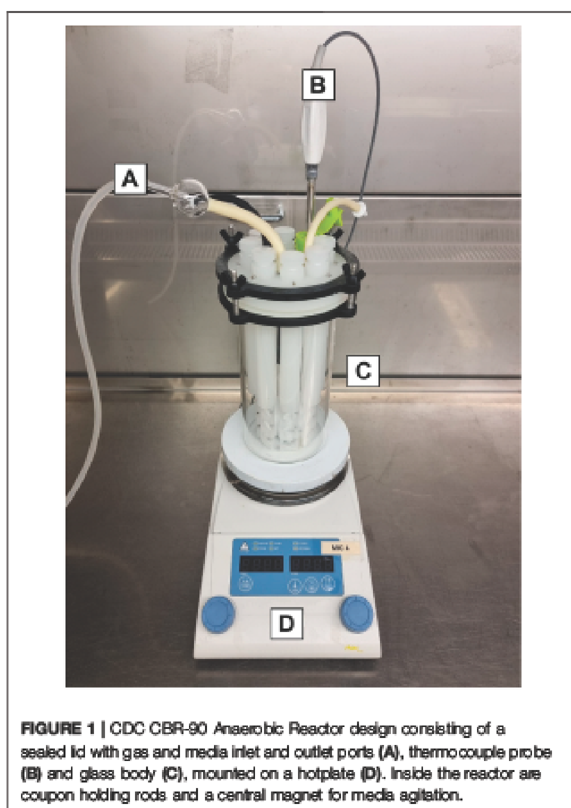


FIGURE 1 | CDC CBR-90 Anaerobic Reactor design consisting of a sealed lid with gas and media inlet and outlet ports **(A)**, thermocouple probe **(B)** and glass body **(C)**, mounted on a hotplate **(D)**. Inside the reactor are coupon holding rods and a central magnet for media agitation.

equal numbers to a final pooled inoculum. The consortium enrichment cultures were characterized, revealing *Shewanella* as the most abundant genus (52–67%). *Thermovirga* sp., *Caminicella* sp., *Pseudomonas* sp., *Paramaldivibacter* sp., *Proteiniphilum* sp. were also detected. For CLSM analysis of cell membrane disruption, a laboratory strain of *Klebsiella pneumoniae* was used as a model. Cells were cultivated in log phase for 24 h, suspended in phosphate-buffered saline (PBS, Sigma, pH 7.4) and washed twice by centrifugation at 12,000 x g for 2 min per cycle.

2.2 Reactor Setup and Test Conditions

A Center for Disease Control (CDC) CBR-90 Anaerobic Biofilm Reactor® (**Figure 1**) was established in a biological safety cabinet (sterile conditions) to grow biofilms on carbon steel (AISI 1030) coupons. The coupons were placed into rods, irradiated for 10 min under UV radiation and then inserted into the reactor. The reactor gas atmosphere was established with a ratio of 20/80 CO_2/N_2 respectively (1 bar). Sterile test solution was pumped into the reactor after 1 h of deoxygenation with 20/80 CO_2/N_2 gas. Solutions and gas were sterilised by micro-filtration (0.2 µm, Whatman®). The reactor was placed over a stirring hot plate to maintain a temperature of 40°C with gentle agitation (50 rpm) throughout the experiment. After the temperature was established, the reactor was inoculated with the pooled microbial consortia at a concentration of 1×10^7 cells/mL. To allow surface colonization, continuous flow was delayed for 36 h. After the stagnant period elapsed, continuous flow was maintained by a reservoir reactor attached to a peristaltic pump that exchanged 30% of the test solution every 24 h. These conditions ensured microbial activity was maintained throughout the experiment. Control biofilm development was assessed before CTA-4OHcinn treatment.

2.3 Carbon Steel Coupons

All experiments were conducted using a substrate comprised of a commercial grade of carbon steel (CS, AISI 1020). Cold finished round CS1020 bars of 12.6 mm diameter were cut into coupons 5 mm thick, before electro-coating with a protective epoxy (Powercron 6000CX, PPG Industrial coatings). Coupons were wet-ground with successively finer silicon-carbide abrasive paper in the order: 80, 120, 320, and 600 grit, to expose one side to the test solution. Each coupon had a final exposed area of 1.27 cm^2. The ground specimens were washed with ultra-pure deionised (DI) water (18.2 $M\Omega.cm^2$, Milli-Q), degreased with acetone, washed with ethanol, and dried with nitrogen gas. Before applying to experiments, coupons were sterilised using ultraviolet (UV) radiation for 15 min on each side.

2.4 Test Solution

Produced seawater used in the present study (water injected into the process stream to improve product recovery in oil and gas operations (Yeung et al., 2011)) was collected from a West-Australian oil refinery where MIC was suspected. Produced water was supplemented with (final concentrations): 2 mM sodium thiosulphate (Na2S2O3), 6.1 mM sodium acetate (C2H3NaO2), 7.4 mM sodium formate (HCOONa), 4.7 mM sodium DL-lactate (CH3CH(OH)COONa), 4.5 mM sodium pyruvate, 5 mM glucose, 1 mM bacto™ casamino acids, 71.4 mM sodium bicarbonate (NaHCO3), 10 ml wolfe's vitamin solution and 990 ml produced water. The final pH of the test solution was 7.2–7.5.

2.5 Biocide Treatment

Biocide treatment was applied after 2 weeks of biofilm growth. After 2 weeks, coupons yielded mature, complex biofilm structures with visible EPS. Before the biocide injection, the CDC reactor was drained and rinsed with a fresh deoxygenated test solution to eliminate waste products or dead cells. Control samples were extracted to perform microbiological and microscopic analyses before the biocide injection. The reactor was again drained and finally replenished with a fresh deoxygenated test solution containing 10 mM of CTA-4OHcinn. After 4 h of exposure, the remaining samples were collected to assess the effect of the biocide on the biofilm viability and structure.

2.6 Microbiological Analyses

Coupons were rinsed with sterile PBS (Sigma, pH 7.4) and immersed in sterile tubes with 10 ml of anaerobic PBS solution before and after biocide injection. Seven sonication cycles (10 s ON followed by 20 s OFF) were performed to

detach microorganisms from the coupons. Samples were immersed in ice during OFF cycles to prevent heat damage. The remaining suspension was then used to conduct the microbial analysis (MPN and ATP) described below.

2.6.1 Mean Probable Number (MPN) Cell Quantity Estimations

MPNs were determined as described in the Microbiological Examination Methods of Food and Water: A Laboratory Manual (da Silva et al., 2019). Briefly, a 1 ml cell suspension from each coupon was inoculated into 9 ml of anaerobic SPP culture medium serially diluted 10-fold 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 media turbidity and the colour of the culture media, according to a standard protocol outlined elsewhere (da Silva et al., 2019). Positive vials were confirmed by phase-contrast microscopy using a Nikon Eclipse Ci-L. The microbial concentration was determined using the three-tube standard table for MPN quantification assays outlined in the standard (da Silva et al., 2019).

2.6.2 Adenosine Triphosphate (ATP) Assays

Total biofilm ATP was quantified on coupons before and after exposure to CTA-4OHcinn. Experiments were conducted using the Quench-Gone Organic Modified test kit (QGO–M™; Luminultra Technologies Ltd.) according to the manufacturer's instructions, with the exception of a sonication step as described above during the 5 min Lumilyse™ incubation. ATP measurements were obtained using the PhotonMaster™ Luminometer (Luminultra Technologies Ltd.), and ATP content was calculated from the measured luminescence by comparison with a standard.

2.6.3 Statistical Analysis

A one-way ANOVA statistical analysis was conducted on MPN and ATP triplicate data points using PAST (V4.03) (Hammer et al., 2001) to determine if differences between MPN and ATP data before and after treatment were significantly different. A one-way ANOVA statistical analysis on CLSM micrographs was also conducted to verify that fluorescent signal obtained from CTA-4OHcinn treated samples was significantly greater than untreated samples. Results were considered significant upon returning a p-value ≤ 0.05.

2.7 Scanning Electron Microscopy

Coupons from before and after biocide exposure were removed from reactor rods and lightly rinsed in PBS at 30°C (Sigma, pH 7.4) before fixation in 2.5% glutaraldehyde for 22 h at 4°C. Fixed coupons were placed under nitrogen gas overnight to completely dry before sputter coating with 9 nm of platinum. This procedure was developed to minimise the effects of charging caused by non-conductive biofilm-covered surfaces. Microscopy was conducted using a Neon field emission scanning electron microscope equipped with a secondary electron detector. An emission voltage of 5 kV, aperture of 30 μm and working distance of 6 mm (adjusted to 5.8 and 5.9 mm during focusing) was used to image surfaces before and after exposure to CTA-4OHcinn.

2.8 Confocal Laser Scanning Microscopy

CLSM was conducted on a Nikon A1+ confocal microscope equipped with the latest version of Nikon Elements software and a 20 x dry objective lens. Coupons from before and after biocide exposure were stained according to the manufacturer's instructions. Briefly, coupons were immersed for 10 min in the Syto9™ and propidium iodide (PI) mixture (Invitrogen™ Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit) and lightly rinsed in PBS (Sigma, pH 7.4). Micrographs were captured sequentially using 489.3 and 561 nm lasers and 500–550 nm and 570–620 nm emission filters respectively. Spectral bleed-through was reduced by acquiring z-stack images in separate tracks for emission and excitation paths. Two random locations were sampled from each coupon using the Nyquist function for semi-quantitative analysis. The low magnification (20 x) was selected to capture a larger sample size of 182 μm^2. A third micrograph was captured to represent the coupon surface through 3D reconstruction (20 x full field of view, measuring 600 × 600 μm). All micrographs were captured using the same microscope and software settings for uniformity.

To visualise the effect of CTA-4OHcinn on bacterial membranes, Cellbrite™ Fix 555 (Biotium) was used in a 10 x concentration to stain *K. pneumonia* cells. Cells from the same culture were stained (controls) or combined with 10 mM CTA-4OHcinn and stained, before transferring 10 μL to a glass slide. Since Cellbrite™ Fix 555 stains do not require washing, microscopy was performed after 1 h of exposure to the stain or stain and CTA-4OHcinn mixture. A glass cover slip of 0.17 mm thickness was placed over the droplet and sealed using clear epoxy resin. Microscopy was conducted using a transmission detector and 561 nm laser with a 595/50 emission filter. A 100 x oil immersion objective was used to visualise individual cells, maintaining microscope settings between control and experimental samples. Triplicate micrographs were then captured across random areas of untreated control and treated samples for post-image analysis. The micrographs are also presented with combined and separate signals to depict the cell morphology and membrane damage (indicated by fluorescence intensity and distribution) under control and treated scenarios.

2.8.1 Post-image Analysis

Replicate Nyquist micrographs from each surface were captured randomly and used to estimate biofilm parameters including % live and % dead cells, relative biomass (expressed as mean signal intensity) and compactness. Z-stacks were converted and loaded into the latest version of IMARIS (Bitplane) software (9.7.2), generating a series of statistics used to calculate the following parameters: compactness (a measure of signal density per area sampled), % live cells on each surface, % dead cells on each surface and relative mean signal intensity for each channel on each surface (red and green, or live and dead).

Micrographs from Cellbrite Fix 555 stained *K. pneumoniae* were also processed using IMARIS (Bitplane) software to quantify

signal from membrane compromised cells. A comparison of triplicate micrographs captured after 1 h was made by comparing the untreated control against a sample treated with 10 mM CTA-4OHcinn. Membrane compromised cells were discriminated using background subtraction, eliminating signal from live cells leaving only signal from membrane compromised cells for quantification. Triplicate micrographs from untreated control and treated samples were compared using the same analysis method and the same threshold values.

3 RESULTS AND DISCUSSION

Biofilms in marine environments are responsible for biofouling and MIC, and are managed in engineered systems such as pipelines using mechanical cleaning and biocides (Okoro, 2015). Some of the most effective and widely used biocides in engineered systems and healthcare are associated with toxic effects to people (Smith and Wang, 2006) and the environment (Leung, 2001; Emmanuel et al., 2005; Sano et al., 2005), and are usually dosed in parallel with other compounds such as corrosion inhibitors (Zhong et al., 2020). The choice of corrosion inhibitor in a given system is dependent on a number of other factors; including but not limited to flow rate of pipelines, produced water composition, H_2S and CO_2 content and suspected corrosion mechanisms (Mesquita and Marchebois, 2020). Thus, a variety of organic and inorganic compounds have been developed to address corrosion concerns in oil and gas operations.

In the context of expanding limitations imposed by environmental guidelines, "greener" options are gaining increasing traction in the scientific literature (Chugh et al., 2020). Organic plant extracts for example can be affordable and effective at low concentrations, i.e. ginger extract at 20 ppm can achieve corrosion inhibition efficiency of ~80% on CS (Chugh et al., 2020). Similarly multifunctional organic compounds (such as those based on QACs) are a promising new strategy to combat MIC and other corrosion types with reduced cost, toxicity and environmental impact (Zhong et al., 2020). Along with reduced compound toxicity, corrosion inhibitors with biocidal properties such as CTA-4OHcinn eliminate the need for these functions to be addressed by individual compounds, thus reducing exposure to personnel and dosing costs (Zhong et al., 2020).

Previous research evaluated the corrosion inhibition efficacy of CTA-4OHcinn on CS (AISI 1030) applied at 5, 7.5, and 10 mM, finding 95, 95, and 96% corrosion inhibition efficiency respectively after 30 min (Ghorbani et al., 2020). The results of corrosion inhibition efficiency experiments indicate that CTA-4OHcinn shows great promise as a corrosion inhibitor. Based on the concentrations applied in this study, 10 mM was applied in a subsequent study to evaluate the effect of CTA-4OHcinn on early biofilm formation (Tuck et al., 2021). This earlier study evaluated the attachment and colonization of three isolates exposed to the compound using CDC reactors. The results indicated that CTA-4OHcinn reduced bacterial viability by between 96 and 100% on wet-ground and oxidised CS substrates (Tuck et al., 2021). Although these results support biocidal efficacy of CTA-4OHcinn, a greater understanding of the susceptibility of mature biofilms is required before practical application of CTA-4OHcinn can be considered.

Natural biofilms are characterized by species diversity, giving rise to complex and poorly understood synergistic and competitive interactions (Guillonneau et al., 2018). Higher abundance of species in natural biofilms is also associated with greater tolerance to biocidal compounds, although limited work explores the mechanisms governing this tolerance (Sanchez-Vizuete et al., 2015). The present study investigates the efficacy of CTA-4OHcinn in the context of developed biofilms grown using multi-species oilfield samples to better reflect recalcitrant environmental populations. *Shewanella* sp, *Paramaledivibacter* sp. and *Pseudomonas* sp., previously identified marine taxa, represented the dominant species of the consortium. Although little is known about *Paramaledivibacter* sp., the *Shewanella* and *Pseudomonas* genus are frequently associated with MIC of steels in marine environments (De Windt et al., 2003; Yuan et al., 2007; Machuca Suarez et al., 2016; Salgar-Chaparro et al., 2020a; Wurzler et al., 2020).

The effect of 4 h of exposure to CTA-4OHcinn on cell viability and biofilm metabolic energy of 2-week-old biofilms were evaluated using mean probable numbers (MPN) and total adenosine triphosphate (tATP) (**Figure 2**). Results from both assays suggest that at T_0 (before dosing with CTA-4OHcinn), biofilms were healthy. A statistically significant reduction in both viable cells and tATP was observed after exposure to CTA-4OHcinn. Regardless of the number of cells initially detected in healthy biofilms, MPN and tATP measurements indicated that most, but not all cells were killed and removed by CTA-4OHcinn.

Confocal laser scanning microscopy (CLSM) and post-image analysis indicates that almost all biofilm was killed and removed by the biocide (**Figure 3**). CLSM dyes bind to DNA and can distinguish live (Syto9™- green signal) and dead or damaged (propidium iodide-red signal) cells through membrane permeability. Once bound to the target, fluorescent signal is greatly enhanced. Post-image analysis can provide a 3D reconstruction of the surface based on the fluorescent signal returned. The spatial distribution and quantity of live and dead/damaged cells can then be visualized or represented numerically through quantitation of biofilm parameters. In the present study Bitplane (IMARIS) software was selected for this purpose. According to this analysis, biofilm compactness was reduced 10 fold in the presence of CTA-4OHcinn and biofilm composition changed from a mean of 85–1% live cells after the addition of CTA-4OHcinn. The greatly reduced mean combined signal intensity from both channels after exposure indicates that CTA-4OHcinn may work as a surfactant. Combined intensity values were reduced 12 fold (**Figure 3**), implying a significant reduction in surface adhered biofilm. This data supports MPN and ATP calculations, indicating that the biocide was effective at reducing viability as well as biomass of the biofilm.

CLSM 3D reconstructions of the biofilm were also generated to compare biofilm architecture and distribution of live and dead cells (**Figure 4**). Biofilm CLSM reconstructions show a strong green signal (live cells) in untreated biofilms (**Figure 4**),

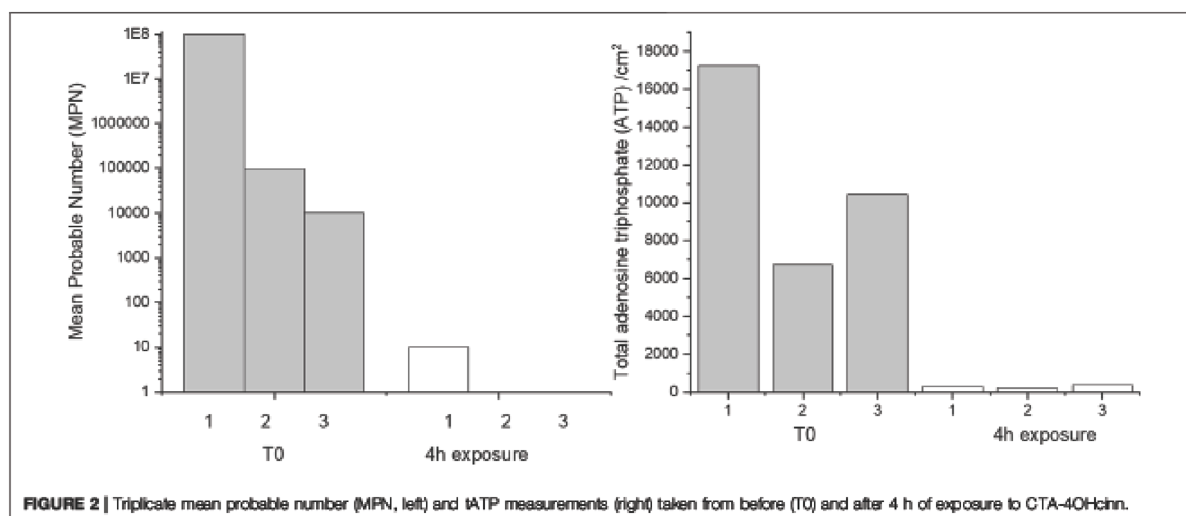
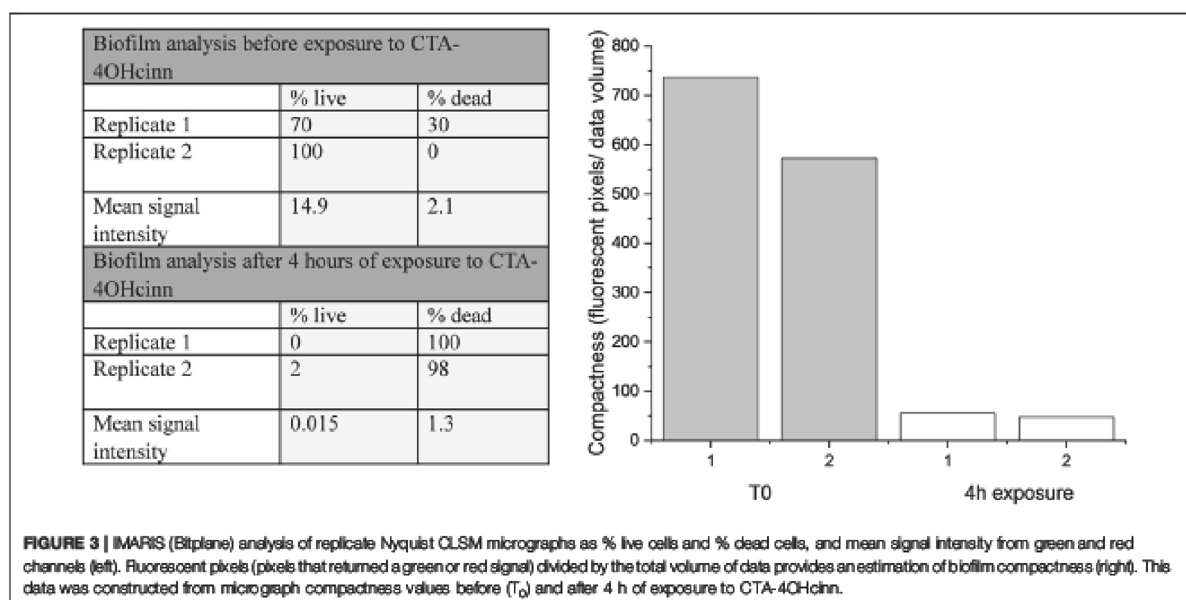


FIGURE 2 | Triplicate mean probable number (MPN, left) and tATP measurements (right) taken from before (T0) and after 4 h of exposure to CTA-4OHcinn.



Biofilm analysis before exposure to CTA-4OHcinn		
	% live	% dead
Replicate 1	70	30
Replicate 2	100	0
Mean signal intensity	14.9	2.1
Biofilm analysis after 4 hours of exposure to CTA-4OHcinn		
	% live	% dead
Replicate 1	0	100
Replicate 2	2	98
Mean signal intensity	0.015	1.3

FIGURE 3 | IMARIS (Bitplane) analysis of replicate Nyquist CLSM micrographs as % live cells and % dead cells, and mean signal intensity from green and red channels (left). Fluorescent pixels (pixels that returned a green or red signal) divided by the total volume of data provides an estimation of biofilm compactness (right). This data was constructed from micrograph compactness values before (T_0) and after 4 h of exposure to CTA-4OHcinn.

compared to treated biofilms where almost no green signal was observed (**Figure 4**). **Figure 4B** also demonstrates low combined signal from both channels with little biofilm architecture remaining after treatment. For lethal biocides, lysis is expected to change the cell signal to red, leaving the biofilm architecture relatively undamaged. This result was achieved in a confocal study where ethanol treated and untreated multi-species biofilms were compared. Ethanol treatment resulted in cell lysis, indicated by a red signal, while untreated biofilms remained mostly viable as indicated by a green signal (Samarian et al., 2014). The results from that study demonstrate that when simply killed but not removed, propidium iodide fluorescence reveals the biofilm architecture in red. CLSM results in the present communication indicate that CTA-4OHcinn functions as a biocide as supported by viability assays, and also as a dispersal agent as indicated by significantly reduced biofilm architecture at the interface.

Scanning electron microscopy (SEM) provided a visual comparison between the biofilm at T_0 and after treatment with CTA-4OHcinn. **Figure 5A** shows a complex, multi-

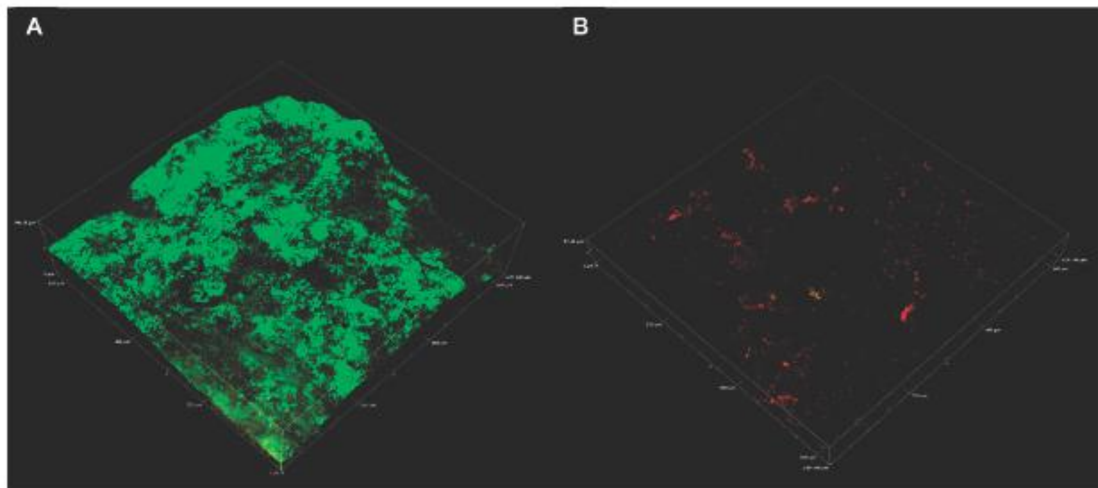


FIGURE 4 | Representative confocal micrographs captured using a 20 x objective depicting biofilms on CS. Micrographs were captured on coupons with 2 week old biofilms **(A)** and on 2 week old biofilms exposed to CTA-4OHcinn for 4 h **(B)**. Green indicates live cells and red indicates dead or damaged cells.

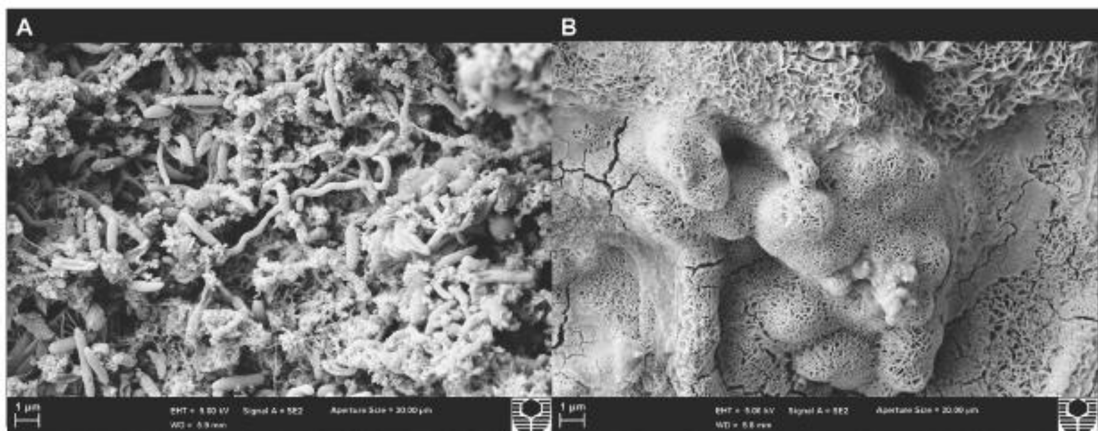


FIGURE 5 | Scanning electron micrographs of a coupon surface captured before **(A)** and after 4 h exposure to CTA-4OHcinn **(B)**. Surface A shows structures resembling bacterial cells not observed on surface B.

species biofilm with structures resembling various morphologies on the surface at T_0. The absence of these structures after exposure (B) indicates successful lysis and removal of cells.

The present study found CTA-4OHcinn to be effective at reducing the viability and architecture of mature, multi-species marine biofilms under reactor conditions. While the results are in agreement with a previous study (Tuck et al., 2021), comparisons of CTA-4OHcinn performance, especially compared to results from established biocidal compounds (such as glutaraldehyde or tetrakis (hydroxymethyl)phosphonium sulphate, are still required. CTA-4OHcinn functions simultaneously as a corrosion inhibitor and biocide, and thus scientifically valid comparisons are difficult to achieve. Future research aims to compare CTA-4OHcinn with commercial biocides in consideration of multiple functions.

Environmental toxicity is also critical to consider when introducing novel biocides for application in marine environments. QACs demonstrate low LD_{50} values when compared against commercial biocides such as glutaraldehyde (Seter et al., 2013). In the present study, CTA-4OHcinn is considered environmentally sensible as a QAC, with a recent study confirming low LD_{50} values and adsorption properties

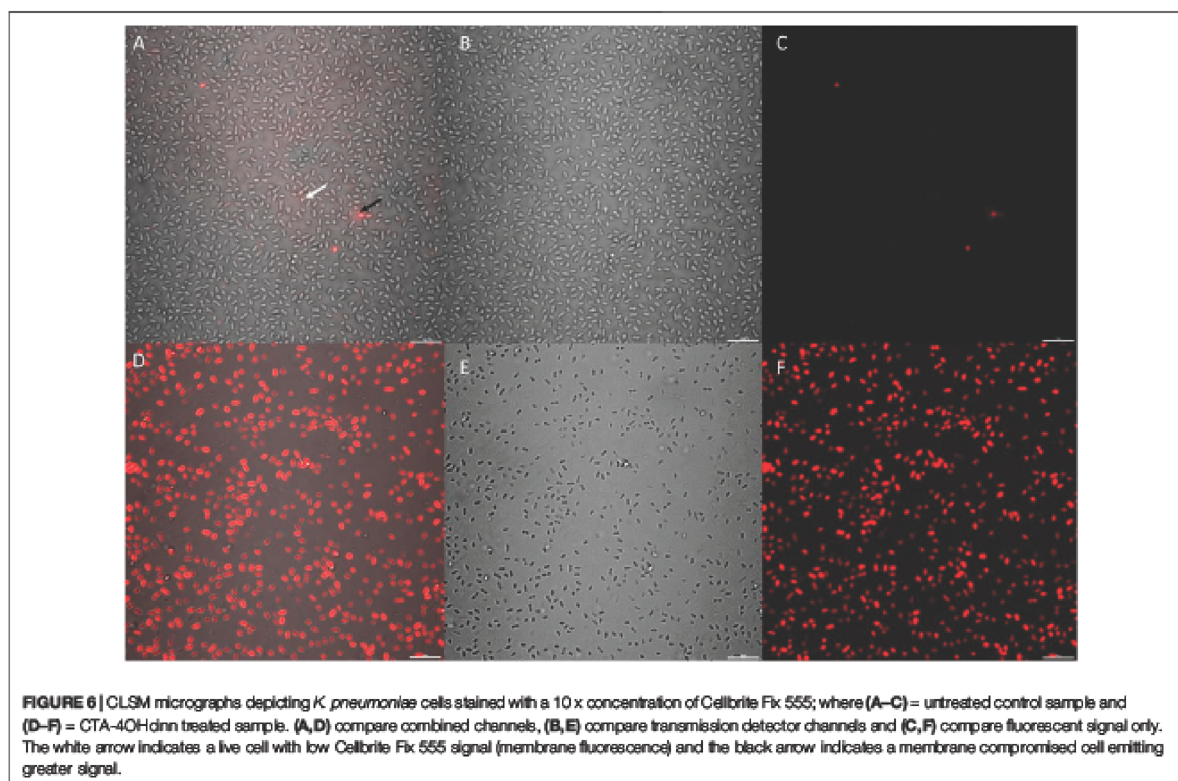


FIGURE 6 | CLSM micrographs depicting *K. pneumoniae* cells stained with a 10 x concentration of Cellbrite Fix 555; where **(A–C)** = untreated control sample and **(D–F)** = CTA-4OHcinn treated sample. **(A,D)** compare combined channels, **(B,E)** compare transmission detector channels and **(C,F)** compare fluorescent signal only. The white arrow indicates a live cell with low Cellbrite Fix 555 signal (membrane fluorescence) and the black arrow indicates a membrane compromised cell emitting greater signal.

against human keratinocyte (skin) and duodenum (intestinal) cell lines (Catubig et al., 2021). The communication found the CTA-4OHcinn toxicity profile to be comparable to cetrimonium bromide; a safe and widely applied additive in cosmetic products and disinfectants (Catubig et al., 2021). Additionally, the multifunctional nature of CTA-4OHcinn eliminates the requirement to treat biofilm formation and corrosion with separate compounds.

As a novel compound, the biocidal mechanism for CTA-4OHcinn remains unknown. Since the cetrimonium cation is associated with biocidal activity through cell membrane disruption, it was proposed that CTA-4OHcinn will exert similar effects (Ittoop et al., 2015). To determine if the membrane-disruptive function of the cetrimonium cation was maintained in CTA-4OHcinn, healthy cultures of *K. pneumoniae* were treated with CTA-4OHcinn and compared to untreated controls by staining with Cellbrite™ Fix 555.

High magnification CLSM post-image analysis comparisons required a small, dense and uniform cell sample. Variations in multi-species consortium samples can skew downstream analysis results, and thus a single species culture was preferential. *K. pneumoniae* was selected for these experiments based on rapid reproduction in enriched seawater and large, easily visible cell morphology under 100 x magnification. Additionally, the isolate endured washing stages with minimal damage to cell membranes, as evidenced by controls (**Figure 6A**).

Cellbrite™ Fix 555 was selected as a general membrane stain to visualize damage caused by CTA-4OHcinn under CLSM. Cellbrite™ Fix 555 is an amine-reactive fluorescent dye that emits a low red signal when in contact with membrane proteins. Due to covalent binding of the stain to amines, fluorescent signal is greatly enhanced in cells with membrane damage since intracellular proteins contain abundant binding sites. Therefore, Cellbrite™ Fix stains represent an ideal tool to visualize cell membrane disruption in bacteria. In post-image analysis, fluorescent signal was quantified only from intracellular fluorescence to eliminate live cells from this analysis (low signal intensities not corresponding to cell damage).

K. pneumoniae cells were treated with CTA-4OHcinn for 1 hour and compared to the same cultures without treatment (**Figure 6**). Cell samples were denser in untreated controls compared to cells treated with CTA-4OHcinn (**Figures 6B,E**). Since experimental replicates cannot control for exact cell numbers, micrographs for post-image analysis were captured favoring more cells in the field of view for untreated samples. Bias introduced by sample size was eliminated in this way to ensure significance was maintained.

Lastly, the mean sum of fluorescent signal returned from triplicate control micrographs was 2.4E7, compared to 2.5E8 returned from treated samples. Thus, a 10 fold average increase in fluorescent signal intensity in CTA-4OHcinn treated samples was observed (**Figures 6A,C,D,F**). The

comparison between treated and untreated samples by post-image analysis revealed a statistically significant difference ($p = \leq 0.002$), confirming that CTA-4OHcinn acts on the cell membrane to cause cell lysis.

This study provides the first evidence that application of a novel organic corrosion inhibitor, CTA-4OHcinn, functions as a biocide and is effective against multi-species biofilms developed on CS. Further, the investigation suggests that CTA-4OHcinn biocidal mechanism is membrane disruption. In similar QACs, the positive charge exerts an electrostatic interaction with negatively charged bacterial membranes (Seter et al., 2013). After initial adsorption, the quaternary ammonium cation moiety is able to diffuse through the cell wall (Seter et al., 2013). The downstream effects of the cetrimonium cation permeation are phospholipid bilayer destabilization resulting in cell death (Schachter, 2013; Iñiguez-Moreno et al., 2018). Additionally, the cation could result in cell lysis through its quenching action against ATP synthase, ultimately resulting in energy deprivation (Schachter, 2013). It is also hypothesized that the *trans*-4OHcinn anion is capable of contributing to the antimicrobial activity of the cation, although the interactions involved, and synergism between the cation and anion in the present study remains unknown and is a topic under further investigation.

4 CONCLUSION

Demand for environmentally friendly and cost-effective biocides has never been higher. By developing corrosion inhibitors with biocidal properties, the number of chemicals used at dosing intervals can be reduced along with costs and environmental impact. For multi-functional compounds to be attractive for use in industrial systems such as pipelines, they must be effective at inhibiting both mature biofilms and bacterial attachment. In a previous study, a novel, multi-functional organic corrosion inhibitor designated CTA-4OHcinn was found to have anti-corrosive properties and biocidal properties against bacterial attachment and early biofilm formation. In light of these studies, the present research aimed to understand the effect of this compound on more mature, multi-species biofilms developed using a West-Australian oilfield consortium. The application of 10 mM CTA-4OHcinn was recently found to be optimal for corrosion inhibition. In this study, 4 h of treatment with 10 mM CTA-4OHcinn significantly reduced biofilms as demonstrated by reduced cell numbers and total adenosine triphosphate (tATP) values. Biofilm dispersal qualities in CTA-4OHcinn were also observed. Confocal micrographs and post-image analysis indicate that biofilm compactness, live cell quantity and cell viability was significantly reduced after biocide application; with few live or dead cells remaining on the treated surface. Under scanning electron microscopy, structures resembling a complex, multi-species biofilm were present in healthy control biofilms but were not present in the same biofilms after CTA-4OHcinn treatment. Lastly, CTA-4OHcinn was found to act on the cell membrane of bacterial cells. This study introduces CTA-4OHcinn as an effective biocide for use against multi-species biofilms. The compound is among the first of its kind to be found effective at simultaneously mitigating corrosion as well as limiting early and mature biofilm formation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BT conducted the experiments and procured the draft manuscript. NL significantly contributed to the laboratory work and writing of the manuscript. AS and MF designed the novel multi-functional inhibitor concept and together with LM designed the overall research goals. LM supervised the research, contributed to manuscript drafting and revision. EW provided technical supervision and analysis advice. All authors contributed to discussion of research and manuscript revision.

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Chapter 4: Green, multifunctional biocide evaluations.

Manuscript # 2 of 2

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Evaluation of a novel, multi-functional inhibitor compound for prevention of biofilm formation on carbon steel in marine environments

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Chemical biocides remain the most effective mitigation strategy against microbiologically influenced corrosion (MIC), one of the costliest and most pervasive forms of corrosion in industry. However, toxicity and environmental concerns associated with these compounds are encouraging the development of more environmentally friendly MIC inhibitors. In this study, we evaluated the antimicrobial effect of a novel, multi-functional organic corrosion inhibitor (OCI) compound, cetrimonium trans-4-hydroxy-cinnamate (CTA-4OHcinn). Attachment of three bacterial strains, *Shewanella chilikensis*, *Pseudomonas balearica and Klebsiella pneumoniae* was evaluated on wet-ground (120 grit finish) and pre-oxidised carbon steel surfaces (AISI 1030), in the presence and absence of the new OCI compound. Our study revealed that all strains preferentially attached to pre-oxidised surfaces as indicated by confocal laser scanning microscopy, scanning electron microscopy and standard colony forming unit (CFU) quantification assays. The inhibitor compound at 10 mM demonstrated 100% reduction in *S. chilikensis* attachment independent of initial surface condition, while the other two strains were reduced by at least 99.7% of the original viable cell number. Our results demonstrate that CTA-4OHcinn is biocidal active and has promise as a multifunctional, environmentally sound MIC inhibitor for industrial applications.

Microbiologically influenced corrosion (MIC) is an electrochemical process leading to dissolution of a substratum, which is initiated, facilitated or maintained by microorganisms and their metabolisms[1–4]. MIC accounts for at least 20% of the total global corrosion cost [5–7] and including mitigation measures this figure is over \$2.5 trillion annually[8]. Even if microorganisms are not directly corroding a substrate, biofouling is also a major concern leading to decreased efficiency of equipment such as heat exchangers and transport vessels[1]. The various mechanisms of MIC are governed by biofilm formation, which occurs when planktonic microorganisms colonise a surface, becoming sessile[9]. The term *biofilm* specifically pertains to aggregates of microorganisms suspended in a matrix comprised of extracellular polymeric substances (EPS) including but not limited to extracellular DNA (exDNA), proteins, carbohydrates and other trapped organic and inorganic compounds[10]. The difficulty in controlling MIC and biofouling is due in part to the flexibility and ubiquity of microorganisms in almost all environments on Earth[11–13]. Such widespread success is owed to the ability of biofilms to provide nutrients, protection and facilitate interspecies interactions, often giving rise to enhanced chemical and physical tolerance of microbial populations. To develop biofilm communities, planktonic cells must first undergo a series of attachment and adhesion stages[14]. Therefore, understanding microbial attachment and adhesion is of particular interest to MIC research.

Attachment of planktonic cells is the first reversible stage of biofilm development, eventually resulting in adhesion; the permanent irreversible association of a cell with the surface. These initial interactions with the surface are complex, specific and active processes, and there are still gaps in understanding of these interactions. In particular, limited work specifically evaluates bacterial attachment to steel surfaces. We know that steel grades

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have been associated with different rates of early attachment in some microorganisms; for example, Javed et al. point out that *Escherichia coli* attachment to carbon steel (CS) may be impacted by cell and substrate characteristics, including CS grade and pearlite content[15]. The authors concluded that *E. coli* exhibited preferential attachment to pearlite-free locations. In later attachment (>60 min), ferrous ions have been associated with enhanced attachment to CS[16], indicating attachment is a dynamic process affected by environmental conditions. Likewise, the presence of dissolved iron and precipitated iron oxides appear to have an effect on bacterial populations. Appenzeller et al. revealed iron presence provides growth advantages to *Escherichia coli* in drinking water[17]. Similarly, *Shewanella oneidensis* was found to respond to iron oxide surfaces in anaerobic conditions by a two to five times increased adhesion energy for electron transfer respiration[18]. On the same note, Kim et al. found attachment of *Enterococcus faecalis* was favoured at locations on activated carbon impregnated with iron oxides[19]. Although the affiliation of various bacterial strains with iron oxides has been established for decades, this phenomenon has not yet been investigated in carbon steel and associated iron corrosion products formed in seawater. Understanding the connection between metal oxides and microbial attachment to steel in seawater is a primary goal in understanding biofilm formation and its mitigation in marine structures.

Biocides and corrosion inhibitors (CI) are a primary method for controlling bacterial proliferation and corrosion in marine infrastructure. Biocides frequently employed by industry today include glutaraldehyde, tetrakis hydroxymethyl phosphonium sulphate (THPS), organo-sulphur compounds and quaternary ammonium compounds (QUATS), which often have derivatives or additives to improve biocidal function. More information can be found in a recent review by Suarez et al.[20]. Although effective, such compounds are toxic and environmentally hazardous leading to increased global stringency governing their application[21,22]. On the other hand, application of CIs alone without also controlling bacteria in the system may still lead to MIC once the CI has been degraded or depleted via biotic or abiotic mechanisms. Increasing regulations and awareness of the natural environment have resulted in a surge of interest in development and manufacture of novel environmentally sustainable compounds[21,23]. Likewise, the need to apply both biocides and CIs has resulted in efforts to combine the two compounds into a single multi-functional inhibitor compound. A prime example is the green biocidal organic corrosion inhibitor (OCI) *cetrimonium nalidixate*, a combination of cetrimonium bromide and nalidixic acid[21]. Seter and colleagues reported enhanced solubility and antimicrobial capacity of the multi-functional compound when compared to the individual compounds. The mechanism of corrosion inhibition is suggested as suppression of the anodic reaction by nalidixate, which is strengthened by the presence of cetrimonium. On the other hand, the two compounds used to produce cetrimonium nalidixate are antimicrobials employed in the pharmaceutical industry. Based on enhanced functional range and increased environmental sensitivity, multifunctional OCIs show great promise for the future of biocides and corrosion inhibition[24].

For the present study, a novel multi-functional OCI was synthesised as described elsewhere[25]. The compound CTA-4OHcinn, is a quaternary ammonium carboxylate, which combines the organic *trans*-4-hydroxy-cinnamate anion with hexadecyl trimethyl ammonium cation. An established corrosion inhibition capacity exists for the organic anion through the formation of wormlike micelles, which attach to the CS substrate, forming a bilayer at the interface[25]. Additionally, the cation is a known antimicrobial surfactant[21]. Combined, it is suggested that this compound forms a layer on the CS substrate exhibiting effective corrosion inhibition while also inhibiting microbial attachment. Since CTA-4OHcinn has previously demonstrated effective corrosion inhibition, the purpose of this study is to evaluate the capacity of this OCI to reduce attachment of three bacterial isolates, therefore preventing biofilm formation. *Shewanella chilikensis, Pseudomonas balearica* and *Klebsiella pneumoniae* were selected for this investigation based on genetic propensity for attachment to an iron substrate (CS), potential for tolerance to harsh chemical treatment, synthesis of extracellular polymeric substances (EPS), occurrence in natural and industry settings (marine environments) and ability to thrive under marine-simulating laboratory conditions (determined experimentally, growth curve data not presented). Microbial attachment and its inhibition was evaluated in two different surface conditions: CS with pre-oxidised surface finish (covered with iron oxides) and CS with freshly ground surface finish.

Methods

Microbial isolates. Three strains were employed for this study; *Shewanella chilikensis* DC57, *Pseudomonas balearica* EC28 and a laboratory strain of *Klebsiella pneumoniae. S. chilikensis* DC57[26] *and P. balearica* EC28[27] were recently isolated from corroded steel in an industrial facility. Bacteria were grown in pure culture and harvested during log phase at between 48 and 96 h of incubation. The media for cell cultivation was an artificial seawater (ASW)[28] with the addition of Bacto™ casamino acids (3 g/L w/v), sodium pyruvate (3 g/L w/v), D+glucose (3 g/L w/v) and ammonium nitrate (3 g/L w/v). Cell counts were performed manually using a Neubauer haemocytometer. Cells were then washed three times in ASW at 12,000 rpm. Each pellet was resuspended in ASW and allowed to incubate for one hour at 30 °C before direct introduction into reactor media. The final cell number used for all reactors was 10^6 cells/mL of each isolate.

Synthesis of CTA-4OHcinn. A stirring solution of p-Coumaric acid (50 mmol, 8.2 g) in ethanol (75 mL) was slowly added to a solution of sodium hydroxide (50 mmol, 2 g) in ethanol (50 ml) at room temperature. After addition, the reaction was allowed to stir for two hours. The solution was then filtered and the product dried to collect the intermediate salt, sodium cinnamate. In the second step, sodium cinnamate (33.5 mmol, 6.23 g) was dissolved in an amount of water, then slowly added to a stirring solution of silver nitrite (33.5 mmol, 5.70 g) in water. After completion of the reaction, the solution was allowed to stir for a further hour in dark conditions (covered by aluminium foil). The product was filtered off and added to 50 mL of methanol. The provided solution was added slowly to a stirring solution of cetrimonium bromide (22.35 mmol, 8.15 g) in methanol (50 mml). The reaction was carried out at 80 °C in dark conditions for a week. Finally, AgBr and any unreacted

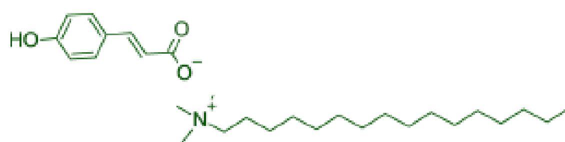

Figure 1. The molecular structure of CTA-4OHcinn used in this study.

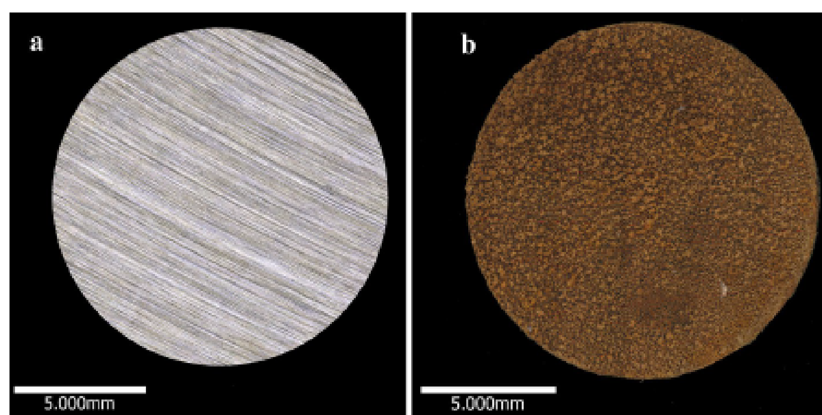

Figure 2. Micrographs of the entire wet-ground surface (**a**) and oxidised surface (**b**).

silver cinnamate were firstly filtered off by filter paper and later by syringe filter. The methanol was evaporated through a rotary evaporator and later dried for 48 h in vacuo to get CTA-4OHcinn. The structure of the final product, CTA-4OHcinn is shown in Fig. 1. ^{1}H NMR (400 MHz, CD_3OD); δ 7.39 (d, J = 15.1 Hz, –CH=CH–, 1H), 7.40 (d, J = 9.5 Hz, ring, –HC=CH 2H), 6.8 (d, J = 8.63 Hz, ring, 2H), 6.34 (d, J = 16.82 Hz, HC=CH, 1H), 3.33 (m, NCH_2, 2H), 3.11 (s, $N(CH_3)_3$, 9H), 1.77 (m, NCH_2CH_2, 2H), 1.30 (m, CH_2, 26H), 0.91 (t, J = 6.57 Hz, CH_2CH_3, 3H) ppm. ^{13}C NMR (100 MHz, CD_3OD); δ 174 (COO), 158.6 (ring carbon attached to OH), 140.6 (ring C), 128.9 (ring C, C–C(OH)–C), 127.1 (C=CH–COO), 121.5 (C=C–COO), 115.2 (ring C), 66.49 (NCH_2), 52.1 ($N(CH_3)_3$), 32 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 28.8 (CH_2), 25.9 (CH_2), 22.5 (CH_2), 22.3 (CH_2), 13.1 (CH_3) ppm. ES^+ m/z 285.3 ($((CH_3)_3\ N(C_{16}H_{33})^+$), ES^- m/z 163.0 (*trans*-4-hydroxy-cinnmate)$^-$. Anal. Calculated for $C_{28}H_{50}N_1O_{3.5}$; C, 73.64; H, 11.04: N, 3.06. Found: C, 73.74; H, 10.71: N, 2.66.

Material characterization. High Resolution Mass Spectra (HRMS) were obtained on an Agilent 1200 series HPLC system, and the sample of CTA-4OHcinn was sent to The Campbell Microanalytical Laboratory, New Zealand for elemental analysis. The results of this analysis can be viewed elsewhere[25].

Sample preparation and surface finish. CS coupons (AISI 1030) of surface area 1.34 cm² were wet-ground to 120 grit finish (SiC grit paper), washed in deionized water, degreased using absolute ethanol and dried under nitrogen gas. Coupons were UV irradiated for at least 10 min each side before fixing to a rod and transferring into a Centre for Disease Control (CDC) reactor. Coupons prepared by this method served directly as wet-ground surface finish coupons. These coupons were not visually corroded (Fig. 2a). To generate the oxidised surface finish, wet-ground coupons were transferred to a reactor with sterile ASW at 30 °C and agitated at 50 rpm for at least 12 h at atmospheric pressure of air to establish an iron oxide film. After oxidation had occurred, the reactor was then flushed with nitrogen for one hour (Fig. 2b). At this time, freshly wet-ground coupons were also introduced to the same reactor. This procedure (both surfaces evaluated using the same reactor) was performed to ensure the same population of cells was evaluated, to limit bias associated with cell counting and ensure no favourable attachment occurred as a result of the experimental design. Coupons representing oxidised surfaces were placed on opposite sides of the reactor to avoid influence of oxides on wet-ground surfaces. The oxidised and wet-ground surfaces represented the working surface for inhibitor evaluation.

Experimental setup. Experiments were conducted using CDC reactors over a 24 h period spanning from inoculation of microorganisms to coupon sampling. Anaerobic conditions were generated by bubbling with pure nitrogen gas at atmospheric pressure. Deoxygenated ASW adapted from Eguchi et al.[28] pH 7.4 was used as test solution for all experiments, with the only modification being the reduction in calcium chloride concentration from 1.5 to 0.1 g/L in order to limit precipitation. No additional supplements were added to the test solution. Passive attachment (settling) of molecules and bacterial cells was prevented by suspending the coupons verti-

cally. The test solution was maintained under constant agitation at 50 rpm using an inbuilt magnetic stirrer and a temperature of 30 °C was generated using a hotplate. Fluid motion allowed non-motile bacteria (*K. pneumoniae*) access to the metal surfaces and ensured iron oxide layers were firmly associated with the surface before evaluating attachment. CTA-4OHcinn was added to the reactors 24 h before inoculation with the bacterial isolates to allow surface interaction before addition of microorganisms. CTA-4OHcinn was completely dissolved in ASW at 50 °C with agitation for 1 h. The solution was added to the reactors at a 10 mM concentration and gently agitated. The reactors were then inoculated with the bacterial isolates and incubated for 24 h after which time the coupons were removed and gently washed in phosphate buffered saline (PBS: Sigma, pH 7.4). Washed coupons were placed into 10 mL of PBS for CFU analysis.

Confocal laser scanning microscopy (CLSM). A Nikon A1+ confocal microscope was used for all CLSM analysis. Syto9™ and propidium iodide (PI) were purchased through Invitrogen™ as the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit. All experiments were conducted using a pinhole radius of 1.2 AU. Stains were mixed in phosphate buffered saline (PBS; pH 7.4) and coupons incubated in 200 µL of the stain mixture for 10 min before removing excess stain and inverting on a dish with central depression of radius 1 cm and covered by a glass cover slip (ibidi®, Germany). Controls were conducted to eliminate the possibility of auto-fluorescence (Supplementary Fig. 1). All micrographs were generated with a 20× objective for uniformity and to capture a large surface area of each coupon (600 µm²). Images were captured sequentially using a 489.3 nm laser and a 500–550 emission filter for Syto9™ and 561 nm laser with a 570–620 nm emission range for propidium iodide. Signal bleed-through between channels was reduced by acquiring z-stacks using separate tracks for emission and excitation paths. The area acquired also remained constant for all confocal microscopy.

Post-image analysis was conducted using IMARIS (Bitplane) software. The average ratio of live to dead cells and biofilm compactness was calculated for the oxidised surfaces using a representative micrograph from each microorganism before and after CTA-4OHcinn application, providing triplicate results for the surface evaluated. Post-image analysis was also performed on wet-ground surfaces before and after CTA-4OHcinn application (data not shown). The parameters evaluated here provide information on the disruption to cell density (compactness) and disruption to cell membranes associated with the compound application[29].

Scanning electron microscopy (SEM). Coupons were lightly rinsed in pre-warmed PBS (30 °C, Sigma, pH 7.4) before fixing them in a 2.5% glutaraldehyde solution and dehydrating in an ethanol series as described elsewhere[30]. Coupons were then incubated for 22 h at 4 °C, removed and dried under nitrogen before coating with 9 nm of platinum. Coupons were micrographed using a Tescan MIRA-3 variable-pressure field emission scanning electron microscope at an emission voltage starting at 5 kV. *K. pneumoniae* attached to wet-ground surfaces were imaged at 8 kV to minimise charging.

Cell quantification. Colony forming units (CFUs) were quantified for coupon surface area following existing standards[31]. Nutrient agar plates containing ASW supplemented with Bacto™ casamino acids (3 g/L w/v), glucose (3 g/L w/v), sodium pyruvate (3 g/L w/v) and ammonium nitrate (3 g/L w/v) were first evaluated for rapid growth of all isolates in aerobic conditions (countable colonies after 2 days). Coupons were removed from reactors and gently rinsed with PBS (Sigma, pH 7.4) before transferring to centrifuge tubes containing 10 mL fresh PBS. Cells were stripped from the surface by sonication as described elsewhere[30]. Plates were incubated at 30 °C for 2 days until visible colonies appeared. To confirm CFU/mL, most probable number (MPN) calculations were performed as described elsewhere[31].

Statistical analysis. A one-way ANOVA statistical analysis was conducted on triplicate CFU calculations using PAST (V4.83)[32] to determine if microbial attachment rates to oxidised and wet ground surfaces was significantly different. Results returning a p-value ≤ 0.05 was considered significant.

Oxidised surface analysis. Raman spectroscopy measurements to determine the oxide layer composition formed on oxidised carbon steel surfaces were performed using an Alpha300RA+ confocal Raman microscope (WITec GmbH, Ulm, Germany) equipped with a 20×/0.4 NA objective (Zeiss, Germany) and a an excitation laser with a wavelength of l = 532 nm. Single spectra were acquired at 50 ms integration times and averaged over 100 accumulations. Triplicate coupons were prepared as described previously (see Sect. "Sample preparation and surface finish") with the exception of using a finer wet-ground finish for this analysis (600 grit).

Results

Effect of surface finish on bacterial attachment. Oxidised and wet-ground surfaces were evaluated as substrates for attachment of the three bacterial strains. Results of quantification of sessile microorganisms as CFUs are presented in Fig. 3. All bacterial strains attached to oxidised surfaces in significantly higher numbers than wet-ground surfaces ($p = 0.002$). On average, over 4.16×10^6 CFUs were measured for *S. chilikensis* on the oxidised surface, while 1.11×10^6 CFU of *S. chilikensis* were obtained from the wet-ground surface. For *K. pneumoniae*, 1.46×10^6 viable cells attached to oxidised surfaces as opposed to 1.26×10^5 CFUs for the wet-ground surface. A similar trend was observed for *P. balearica* (5.36×10^6 CFUs for wet ground surface compared with 6.33×10^5 CFUs). Under conditions provided in the present work, oxidised surfaces were more favourable for bacterial attachment. However, the degree of difference between those surfaces as expressed in CFUs varied depending on the isolate. *S. chilikensis* viable cells attached just 3.7 times greater to oxidised surfaces than a wet-ground surface, while *K. pneumoniae* cells were 11.5 times more likely to attach to an oxidised surface. *P.*

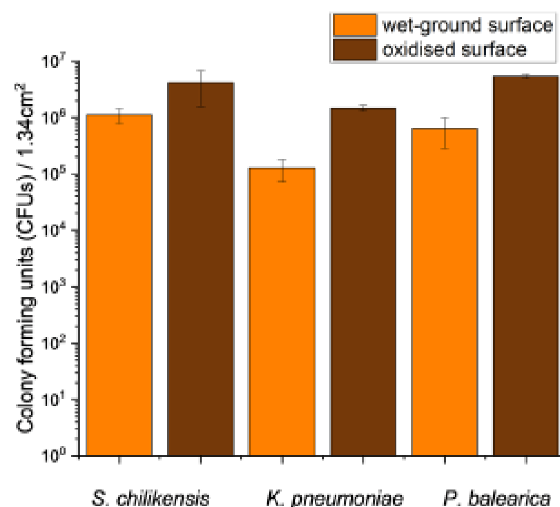

Figure 3. Direct cell quantification performed on oxidised surfaces and wet-ground surfaces after 24 h of exposure. Results are based on triplicate surface counts for each microorganism and surface type, and are expressed as CFUs per 1.34 cm^2. Error bars represent the standard deviation of triplicates.

balearica cells exhibited an 8.5 times greater affinity to the oxidised surface. Lastly, *P. balearica* and *S. chilikensis* attached to both surfaces in greater numbers than *K. pneumoniae*.

CLSM generated a series of z-stacks for each surface, representing a surface area of 600 μm^2. Figure 4a–c shows oxidised surfaces and attached *S. chilikensis, K. pneumoniae* and *P. balearica* cells, respectively. The technique was also applied to wet-ground surfaces seen in Fig. 4d–f. CLSM confirmed trends observed in CFU calculations; the bacteria evaluated in this study are more likely to attach to oxidised CS compared to wet-ground CS surfaces in seawater solution.

SEM was conducted to directly observe bacterial attachment to wet-ground and oxidised surfaces. Figure 5a–c shows oxidised surfaces exposed to *S. chilikensis, K. pneumoniae* and *P. balearica,* respectively. Micrographs in Fig. 5d–f represent attachment of these strains to wet-ground surfaces. The micrographs clearly demonstrate the heterogeneous surface of the oxidised coupons, which corresponds to greater bacterial attachment. Conversely, the wet-ground surfaces hosted smoother topography with less bacterial attachment. In agreement with previous results, SEM results indicate that bacteria preferentially attached to the oxidised surface over the wet-ground surface.

Effect of CTA-4OHcinn on bacterial attachment. Cell quantification, CLSM and SEM were employed to evaluate the effect of the inhibitor on the attachment of the isolates to oxidised and wet-ground surfaces. For all inhibitor analysis, a concentration of 10 mM of CTA-4OHcinn was evaluated. Results of quantification of the isolates attached to oxidised and wet-ground surfaces in the presence of 10 mM of CTA-4OHcinn are provided in Table 1. CFU quantification was represented by the mean count of three replicate surfaces. In the presence of CTA-4OHcinn, bacteria experienced a significant reduction in cell attachment to the oxidised surface, i.e., 100% reduction for *S. chilikensis*, 99.7% for *K. pneumoniae* and 99.8% for *P. balearica*. The rates of cell attachment on wet-ground surface were reduced by 100%, 96.6% and 98.9% respectively (See Supplementary Fig. 2). Although similar reductions to attachment were observed between the two surface types, oxidised surfaces had initially higher numbers of attached cells. Although oxidised and wet-ground surfaces demonstrated marked difference in attached cells before biocide exposure, attached *K. pneumonia* and *P. balearica* to these surfaces were reduced after exposure to CTA-4OHcinn to $<1.17\times10^4$ CFUs per 1.34 cm^2. Therefore, an initially larger quantity of attached cells did not necessary result in higher viable cell number after CTA-4OHcinn exposure.

Reduced cell numbers in the presence of 10 mM CTA-4OHcinn were supported by CLSM observations and post-image analysis (Fig. 6). Although live/dead cell ratios remained similar, especially for *P. balearica* (Table 2), compactness of all biofilms (cell density) was strongly impacted by the application of the compound. Reductions to compactness values were 56 x, 18,000× and 11× for *S. chilikensis, K. pneumoniae* and *P. balearica* respectively. In micrograph 3D reconstructions, decreased SYTO9™ (*live* signal) was observed for all strains and surface finishes, returning signal intensity to levels similar to abiotic controls (See Supplementary Fig. 1). Increased PI (*dead* signal) was also observed, especially for *P. balearica* cells on oxidised surfaces. All results indicate a reduction in cell attachment, cell membrane integrity and cell viability, regardless of the isolate or surface finish.

SEM microscopy supported the data generated by CLSM and cell quantification. On all wet-ground surfaces, SEM analysis indicated total inhibition of bacterial attachment after biocidal application (Supplementary Fig. 3). *S. chilikensis* on wet-ground surfaces exposed to test solution without CTA-4OHcinn (see Fig. 5d), had low numbers of attached cells, whereas no cell attachment was observed on wet-ground surfaces for the other isolates (representative micrographs *e* and *f*) even though CFUs were extracted from those samples. While SEM images

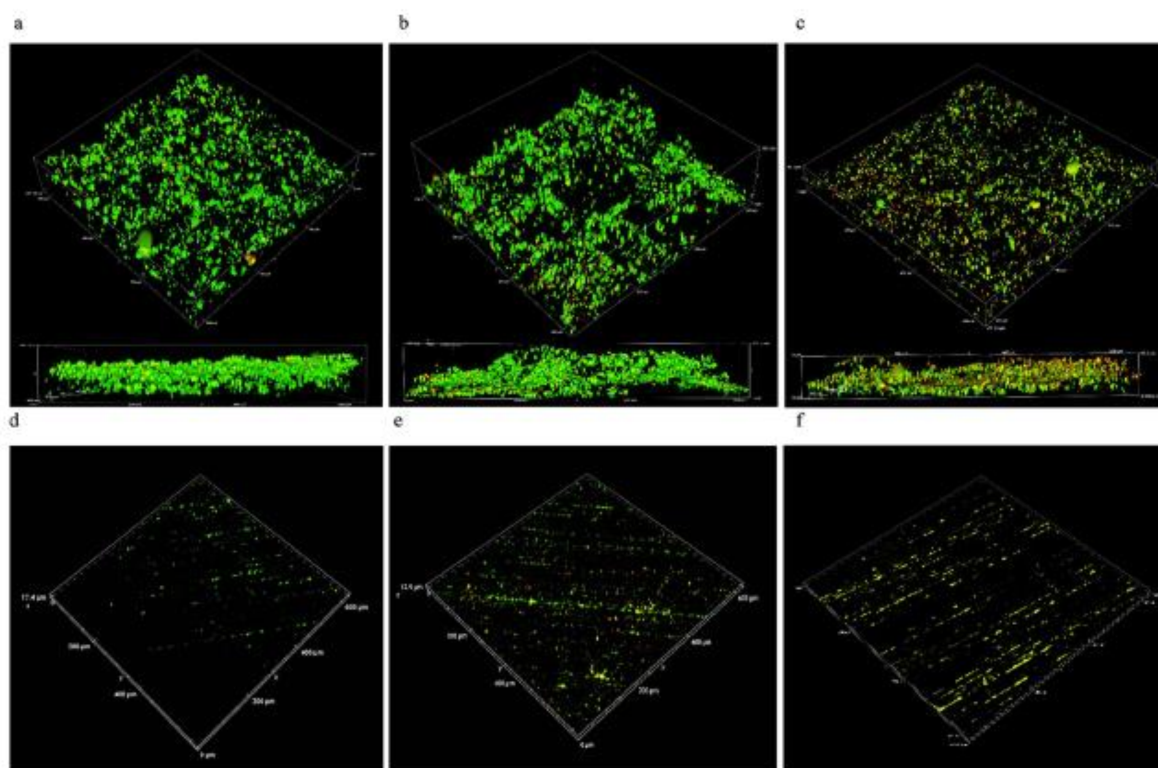

Figure 4. LIVE/DEAD confocal micrographs of attached cells on oxidised surfaces compared to wet-ground surfaces using a ×20 objective. Micrographs (**a–c**) represent *S. chilikensis*, *K. pneumoniae* and *P. balearica* attached to oxidised surfaces respectively. Micrographs (**d–f**) represent the isolates attached to wet-ground surfaces.

are localised by nature, the images shown represented the surface and highlight the limited cell attachment on the wet ground samples. Conversely, oxidised surfaces were associated with aggregation of cells attached to the oxides (see Fig. 5a–c). Upon exposure to CTA-4OHcinn, very few *P. balearica* cells were observed on the oxidised surface (Supplementary Fig. 3f). This is in agreement with confocal micrographs (Supplementary Fig. 2c), highlighting the survival of some *P. balearica* cells in the presence of CTA-4OHcinn.

The results from CFU counting, CLSM and SEM indicate that CTA-4OHcinn was effective at reducing bacterial viability and cell attachment on both wet-ground and oxidised surfaces of carbon steel.

Surface oxide analysis. Spectra obtained from random locations across three coupons contained peaks at wavelengths 212, 273 and 384 rel. 1/cm (Supplementary Fig. 4), likely corresponding to bands produced by goethite and hematite[33,34].

Discussion

Increased environmental awareness and regulation stringency has forced marine industrial processes, particularly oil and gas operations, to switch to greener biocide alternatives. In a recent study by Ghorbani et al.[25], the authors described the novel OCI designated CTA-4OHcinn (Fig. 1) a compound expected to act both as a biocide and corrosion inhibitor simultaneously. The compound demonstrated corrosion inhibition efficiencies on CS (AISI 1030) when applied at 5, 7.5 and 10 mM of 95%, 95%, and 96% after 30 min[25]. The effective inhibition capacity is predicted to be the result of *trans*-4-hydroxy cinnamate or cetrimonium ions, or both, interacting with the surface and suppressing anodic cell development[25]. Specifically, through NMR diffusion studies and electrochemical methods the authors predicted cylindrical micelle formation of the compounds on the fluid/CS interface, leading to bilayer film formation as the mechanism of corrosion inhibition. Since optimal corrosion prevention was previously observed at a concentration of 10 mM, this concentration was evaluated for antimicrobial capacity in the present study.

Along with CI capacity, film forming OCIs such as CTA-4OHcinn may also function as an effective biocide. CTA contains a quaternary ammonium functional group (QAC), which have been frequently employed as disinfectants in the health sector for decades[35]. Major reductions in cell viability and attachment is expected after exposure to CTA compounds, supported by previous reports on organic film-forming QACs[35,36].

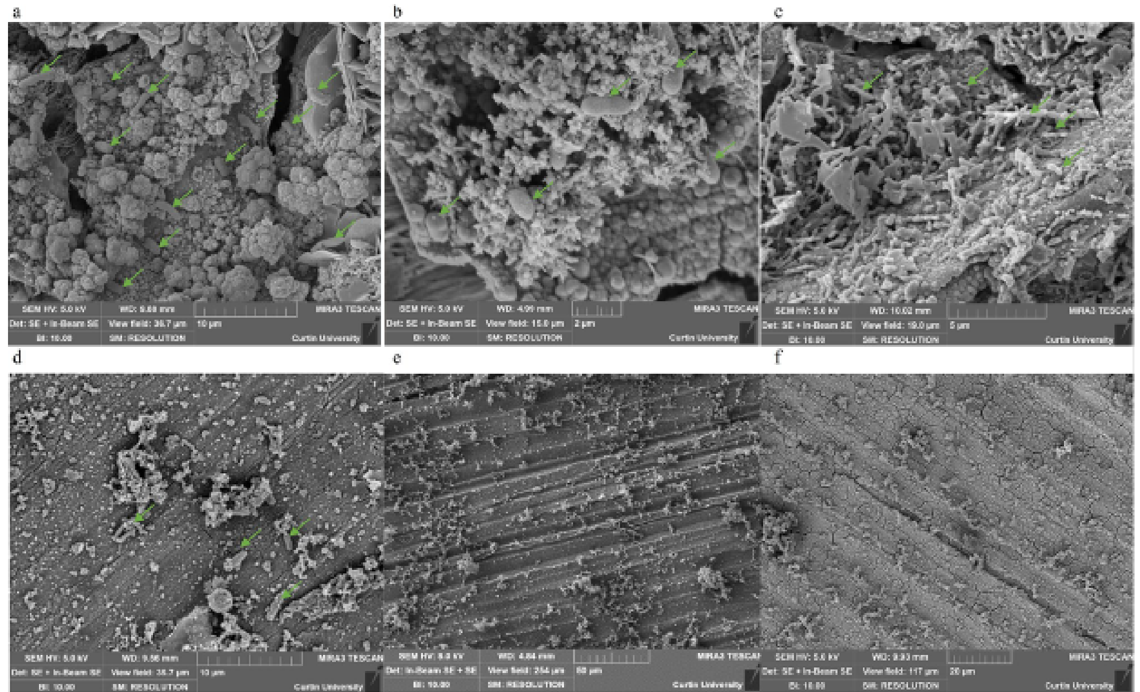

Figure 5. Representative SEM micrographs of oxidised and wet-ground control surfaces. Arrows indicate structures resembling bacterial cells. Micrographs (**a–c**) represent *S. chilikensis*, *K. pneumoniae* and *P. balearica* attached to oxidised surfaces respectively. Micrographs (**d–f**) represent the isolates attached to wet-ground surfaces.

	Isolate	Without CTA4OHcinn	With CTA-4OHcinn	% reduction
Oxidised surface	*S. chilikensis*	4.17×10^{6}	0.00	100
	K. pneumoniae	1.46×10^{6}	4.33×10^{3}	99.7
	P. balearica	5.37×10^{6}	1.17×10^{4}	99.8
Wet-ground surface	*S. chilikensis*	1.11×10^{6}	0.00	100
	K. pneumoniae	1.27×10^{5}	4.33×10^{3}	96.6
	P. balearica	6.33×10^{5}	6.67×10^{3}	99.0

Table 1. Cell numbers attached to oxidised and wet-ground surfaces before and after exposure to 10 mM of CTA-4OHcinn for each isolate. Original values for surface colonisation are taken from Fig. 3.

On the contrary, it has also been suggested that utilising QACs on metal surfaces can promote bacterial attachment. Work by Mousavi et al.[37] suggests increased attachment rates observed in *Pseudomonas putida* to steel surfaces coated with QACs was the result of alteration of the surface microstructure to a more porous texture, providing a viable landscape for attachment of the isolate. Acquisition of tolerance mechanisms to QACs is also of increasing concern, which may lead to enhanced attachment[37]. QACs still present low LD_{50} values compared with many other available alternatives (e.g., glutaraldehyde)[21] and are therefore safer and more desirable from an environmental and holistic approach if effectiveness is enhanced. In a recent study, LD_{50} values of CTA-4OHcinn in human keratinocyte (skin) and duodenum (intestinal) cell lines were compared against cetrimonium bromide (CTA-Br), a well-established and safe additive to disinfectants and cosmetics[38]. Results of this study conclude lower cytotoxicity in CTA-4OHcinn-exposed cell lines compared with CTA-Br, as well as a lower absorption rate into the cell layer[38]. QACs such as CTA-4OHcinn with biocide-enhancing additives therefore represent promising MIC mitigating compounds moving into a more environmentally sensible future.

In addition to lower relative toxicity compared to similar compounds and other biocide classes, the incorporation of certain compounds has been demonstrated to act synergistically to enhance the biocidal effectiveness of QACs, or introduce new functions. Seter et al. described the OCI cetrimonium nalidixate, with corrosion inhibition properties, which could also limit biofilm formation at the interface of carbon steel[21]. In the present study, we assessed the novel OCI CTA-4OHcinn consisting of a cetrimonium cation paired to an organic carboxylic

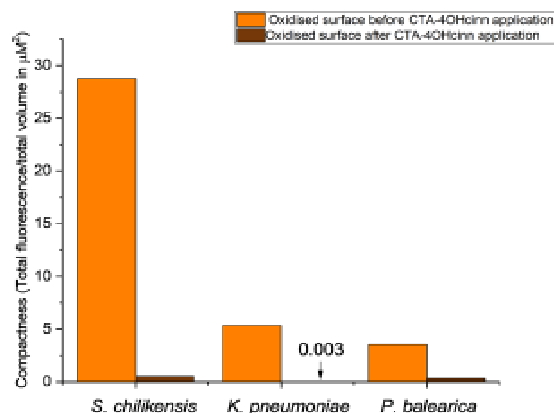

Figure 6. Biofilm compactness of oxidised surfaces expressed as total fluorescence/total volume in µM³ of substrate surface area.

	S. chilikensis		*K. pneumoniae*		*P. balearica*	
	Before	After	Before	After	Before	After
Live cells	82%	66%	88%	55%	75%	76%
Dead cells	18%	34%	12%	45%	15%	14%
Live cell ratio	41:50F	33:50	22:25	11:20	3:4	19:25

Table 2. Live and dead cell fluorescence intensity with live and dead cell ratio for oxidised surfaces before and after application of CTA-4OHcinn.

acid, 4-OH cinnamate. The incorporation of 4-OH cinnamate to the QAC is hypothesised to enhance its biocidal capacity, adding a function normally requiring separate treatment. Although ongoing, it is speculated that bacterial attachment is limited by a synergistic action; that is, the cetrimonium cation provides rapid surface protection bringing 4OHcin with it, where it can disrupt cell membrane integrity[39]. In the present work, the increased PI signal in confocal micrograph post-image analysis after compound application supports this mechanism.

To evaluate biocidal capacity, studies still frequently include the calculation of minimum inhibitory concentration on planktonic cells, despite the well-established difference between planktonic MIC and minimum biofilm elimination concentrations (MBEC)[40]. Additionally, the vast majority of microorganisms are found living at an interface in biofilms[12]. In this phenotype (unlike planktonic lifestyles) microorganisms can begin to initiate MIC or biofouling. It is therefore necessary to evaluate the efficiency of biocides to prevent attachment, adhesion and biofilm maturation of native bacteria to the metal surface. Additionally, attachment and adhesion of bacteria may be highly variable in marine conditions owing to the diversity of attachment phenotypes, surface properties and fluid content, among other factors[41]. In the present study, bacterial attachment was selected for evaluation of biocidal activity since both biofilm formation[15] and MIC[42] rely on initial colonisation of the surface.

Surface oxidation was considered an important parameter when evaluating attachment and biocide activity. It is now well established for example that bacteria gain a range of metabolic benefits from the presence of surface ions such as FeII⁺ and FeIII⁺[43], usually present as iron oxides on CS. When present in the environment (such as pipeline fluids), bacteria may be initially attracted to a metal surface[44], a phenomena evaluated in the present work using a pristine CS control compared against a pre-corroded CS surface. Based on the metabolic benefits afforded by FeII⁺ and FeIII⁺ and enhanced surface roughness, it is hypothesised that bacteria preferentially adhere to oxidised surfaces, and that CTA-4OHcinn will impede bacterial attachment to both surfaces.

Hence, the secondary aim of this study was to underpin any differences in bacterial attachment to these surfaces in the presence and absence of the CTA-4OHcinn. The context of this study is marine infrastructure where both MIC and biofouling are prevalent concerns. Therefore three native marine bacteria were selected to evaluate the compound. These isolates were selected based on the following considerations; (a) presence or expression of genes that may promote adhesion to CS, (b) exhibiting diversity in morphology, (c) proliferate under marine-simulating conditions in a reasonable timeframe and (d) express facultative respiration. Lastly, the authors recognise that MIC is a complex problem that may be driven by a variety of bacterial species[8]. The selection of strains employed in this study therefore attempts to capture genetic and phenotypic differences in the selected strains.

EPS, especially viscous mucous expressed by members of the *Klebsiella* genus assist in attachment, chemical (antimicrobial treatment) protection and tolerance to environmental stressors such as heat, host cytokine

responses and desiccation. *K. pneumoniae* was selected for this study for its ability to thrive in marine conditions, metabolic traits and diverse range of virulence factors; especially a thick mucus capsule, which were expected to provide inhibitor protection and enhanced attachment over less prolific EPS producers.

Shewanella are known for their strong affiliation with metallic surfaces. *S. odeniensis* for example is attracted to iron oxide surfaces where it expresses proteins such as cytochromes to reduce FeIII+. *Shewanella chilikensis* strain DC57 was isolated from a floating production storage and offloading unit (FPSO) where it was thought to be involved in MIC[26,45]. Very little has been revealed about *S chilikensis* since its recent discovery by Sucharita et al. in the Chilika lagoon, India[46]. Work by Sucharita et al. [46] at the time of original isolation demonstrated tolerance of NaCl up to 8%, and based on its recent isolation from corroded industry equipment exposed to seawater (FPSO), *S. chilikensis* DC57 was expected to thrive in marine simulating conditions used in this study. *S. chilikensis* is a facultative anaerobe. Metabolic traits and genes associated with metal reduction present in the genome of this isolate, native location and previous findings of the isolate[26,45], were selective criteria resulting in use of *S. chilikensis* DC57 for this study. The attachment and inhibition of this isolate on metallic substrates have not been investigated so far.

Pseudomonas are frequently seen in the marine environment and nosocomial infections, especially model biofilm forming strain *P. aeruginosa* which has been described as one of the most clinically significant opportunistic pathogens[47,48]. *Pseudomonas* spp. can have a diverse range of metabolisms[49] and virulence factors including mixtures of secreted biosurfactants[48] providing them renowned chemical treatment tolerance. Studies have also revealed that the presence of metallic ions may also facilitate metabolic functions in some *Pseudomonas*[50]. Like *S. chilikensis*, limited research evaluates *P. balearica* despite being proposed as a new species 25 years ago[51]. The *Pseudomonas* strain used in this work, *P. balearica* EC28, was also isolated from an FPSO and expected to contribute to MIC[27]. This facultative anaerobic isolate can tolerate up to 8.5% NaCl and is considered a true marine isolate[49], meeting the criteria for isolate selection in this study.

The primary aim of the present study was to assess the biocidal capacity of the new OCI CTA-4OHcinn. In this study attachment to pre-oxidised and wet-ground CS surfaces was evaluated for these three isolates in marine simulating conditions, providing control data for the evaluation of CTA-4OHcinn. Attachment rates were compared after a 24 h timeframe with and without exposure to 10 mM CTA-4OHcinn. This timeframe was selected to; (a) demonstrate the rapid capacity of marine bacteria to colonise metal surfaces, (b) distinguish the immediate differences in attachment tendency of the isolates to the different surfaces and (c) investigate the capacity of CTA-4OHcinn to prevent rapid attachment to these surfaces. Quantifying and visualising attached cells directly through a range of techniques (including CFU quantification, SEM and CLSM) provides a more detailed representation of biofilm forming units as opposed to planktonic cells which do not represent the more recalcitrant sessile counterparts likely to cause corrosion. Membrane integrity can be used as a measure of cell viability, however the tendency of PI to enter cells with compromised membranes can represent bias (for example, by entering the cytoplasm of viable cells undergoing membrane repair[52]). Therefore attachment was evaluated under constant agitation to ensure cell attachment was an active process, with cells damaged only due to action of the inhibitor.

Results indicate that attachment of marine bacteria to CS surfaces is effectively controlled by CTA-4OHcinn. Confocal micrographs of the isolates that had not been exposed to the compound showed a strong SYTO9™ (live) signal (82% average signal contribution) with PI (dead) contributing an average of 18% of the total signal. Some cell death is expected during initial colonisation of a surface, especially in *Pseudomonas* strains. Early attachment of *Pseudomonas* is likely to incorporate active cell lysis, a process responsible for excretion of compounds that promote attachment including extracellular DNA (exDNA)[53]. In the presence of CTA-4OHcinn, the signal from SYTO9™ was reduced to an average of 66%. For the *Shewanella* and *Klebsiella* strains on both surfaces, significant reductions in compactness and live:dead cell ratio were observed. The third isolate, *P. balearica*, indicated a 98% reduction in compactness with no significant change to the live:dead ratio in remaining cells. The signal from both stains was minimal after exposure, suggesting most non-viable or membrane-compromised bacteria did not remain attached to the surfaces. Although live and dead cell ratio was not heavily affected by the application of CTA-4OHcinn, compactness values demonstrated that the reservoir of viable cells on the surface is significantly reduced. A sharp reduction in compactness is in accordance with expectations, since the media was under constant agitation and cells not actively attaching to the surface would be removed by the stirring effect. In *Pseudomonas*, initial attachment to oxidised CS was also relatively uniform. A higher signal with PI was recorded in *Pseudomonas* on oxidised surfaces compared to other strains, supporting evidence that *Pseudomonas* strains actively lyse cells or secrete exDNA to condition a surface. DNA excreted during early attachment of *Pseudomonas* strains has a known affinity to iron oxides[54].

Bacteria with membranes damaged by CTA-4OHcinn that are still viable may also be generated from the PI stain, adhered irreversibly before exposure to the OCI. CFU results expressing the reduction of cells after dosing (Fig. 3) confirmed that attachment of viable cells was controlled effectively by presence of 10 mM CTA-4OHcinn. Results from SEM further support the hypothesis that regardless of the isolate or surface employed, bacterial attachment is effectively controlled. However, bacterial attachment was not completely prevented by application of CTA-4OHcinn.

The study also hypothesised that bacteria prefer to adhere to pre-oxidised CS compared to wet-ground CS. Unsurprisingly when comparing oxidised and wet-ground surfaces in this study, all isolates overwhelmingly favoured attachment to the oxidised surface. Oxidised surfaces also demonstrated a greater biofilm compactness compared to wet-ground surfaces (Fig. 6a–c, Supplementary Fig. 2a–c). To better characterise this experimental surface, prepared oxidised coupons were analysed using confocal Raman spectroscopy. Peaks likely to correspond with common oxides haematite and goethite were observed (see Supplementary Fig. 4)[33,34], however, due to high background fluorescence an intra-red laser is suggested for further work to determine the exact nature of the sample.

There are many factors that may influence attachment of individual strains to a surface, a topic which has been subject to extensive research. A review of such factors is provided by Goulter et al., including surface roughness and hydrophobicity[55]. Surface roughness of the oxidised surfaces employed for the present study can be seen in Fig. 2b. Typical surface structures deviated between − 5 and 65 µm from the reference point in oxidised surfaces compared to the freshly wet-ground surface, which deviated between −6 and 6 µm. Additionally metal ions FeII+ and FeIII+, probably present on the pre-oxidised surface as iron oxides hematite and magnetite, provide metabolic advantages to many marine isolates. In the present work, a source of organic carbon was not provided in the test solution. FeII+ and FeIII+ could have promoted selective attachment based on an ability for chemolithotrophic metabolism, a well-established phenomenon in *Shewanella* strains[18]. In *Klebsiella* and *Pseudomonas*, research has demonstrated that addition of iron to cultures results in faster replication and formation of more robust biofilms[56,57].

Direct cell quantification of wet-ground and oxidised surfaces was conducted to confirm viability observed in confocal microscopy and attachment observed through SEM. Numbers of viable cells initially attached to surfaces (in the absence of CTA-4OHcinn) confirmed that all isolates preferentially attached to oxidised surfaces (Fig. 3). For all strains, attachment to oxidised surfaces was present in numbers from 10^6 to 10^7 CFUs/1.34 cm^2, demonstrating a large number of viable cells were present on the surface. Wet-ground surfaces hosted lower cell numbers than oxidised surfaces (10^5 and 10^6 cells/1.34 cm^2). This was in accordance with confocal microscopy. SEM was conducted to directly examine the surface and the associations of cells with the distinctive surfaces, confirming increased attachment of the isolates to oxidised surfaces (Fig. 5).

Conclusion

Bacterial attachment to carbon steels is a pervasive problem to oil and gas operations, resulting in biofouling and microbiologically influenced corrosion (MIC). To effectively control bacterial attachment, environmentally hazardous biocides are currently still employed in marine environments. As these compounds face international embargos, there is a drive to develop more sustainable and environmentally sensible biocide alternatives. This study introduces the novel, dual-action organic corrosion inhibitor (OCI) cetrimonium trans-4OH cinnamide (CTA-4OHcinn), which was evaluated for biocidal capacity against three bacterial isolates native to marine environments and previously implicated in MIC. Attachment of these isolates to 1030 carbon steel wet-ground and pre-corroded substrates was also evaluated and compared to determine the impact of iron oxide films on early bacterial colonisation. All isolates demonstrated preferential and enhanced attachment to carbon steel with an oxidised surface finish compared to the freshly wet-ground surfaces under the experimental conditions described. Results of direct viable cell quantification (CFU quantification), confocal microscopy, post-image analysis and scanning electron microscopy indicated that attachment of bacteria to these surfaces can be effectively controlled by the new OCI CTA-4OHcinn. The compound was applied at a 10 mM concentration, which was found to be optimal for corrosion inhibition in previous work. Based on these dual functions, this study provides the first evidence that CTA-4OHcinn has potential for use as a multi-functional OCI, and that biocidal function is not impacted by initial viable bacterial numbers and biofilm compactness on pre-oxidised carbon steel surfaces.

Data availability

The data supporting this study is available from the corresponding author under request.

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Author contributions

B.T. and L.M. designed the experiments, B.T. conducted the experiments under supervision of L.M. and E.W. The novel inhibitor compound was manufactured by M.G. under the supervision of A.S. and M.F. Section "Synthesis of CTA-4OHcinn" was written by MG. Research funding was obtained by M.F., A.S. and L.M. Lastly the manuscript was compiled by B.T. and L.M. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

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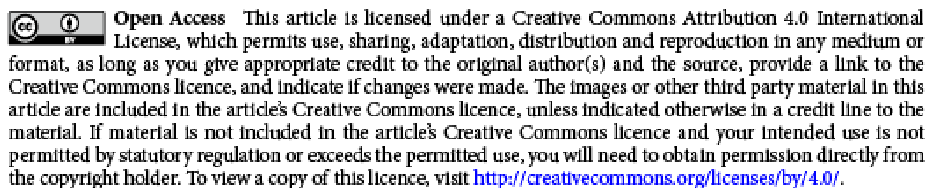

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Chapter 5: ENHANCING BIOCIDE EFFICACY THROUGH TARGETED BIOFILM DISRUPTION

Chapter 5: Enhancing biocide efficacy through targeted biofilm disruption.

Manuscript # 1 of 1

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Enhancing biocide efficacy: targeting extracellular DNA for marine biofilm disruption

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Abstract

Annually, US $4 trillion is lost to corrosion through maintenance and prevention, of which 20-40% is attributed to the metabolic activity of microorganisms. The biofilm lifestyle is the root cause of deleterious effects generated by marine microorganisms, which manifest as biofouling and microbiologically influenced corrosion (MIC). Insufficient scientific literature explores the cellular and extracellular composition of marine biofilms, leading to an absence of targeted biofilm mitigation strategies. Drive also exists from industry and research to develop and apply environmentally sensible chemical treatments (biocides). Recently, extracellular DNA (eDNA) was implicated as a critical structural polymer in marine biofilms. A novel, multi-functional biocide was also introduced to manage corrosion and biofilm formation. The compound, cetrimonium *trans*-4-hydroxy-cinnamate (CTA-4OHcinn), demonstrates great promise as a biocide and organic corrosion inhibitor with a low toxicity profile. To anticipate biofilm tolerance acquisition to chemical treatments and reduce application quantities of CTA-4OHcinn, the present research investigated eDNA as a target for biofilm dispersal and the resulting enhancement of CTA-4OHcinn. Results indicate that mature biofilm viability can be reduced by 2-fold using 1 mM CTA-4OHcinn. This could be enhanced by a further log reduction in viability by the incorporation of an eDNA degradation stage. The results indicate that endonuclease targeting of the matrix allows greater penetration of CTA-4OHcinn and also disperses biofilm architecture from the interface of steels.

Significance Statement

Natural biofilms are diverse microbial populations encased in a polymeric matrix. The matrix, comprised mainly of extracellular DNA (eDNA), polysaccharides and proteins, confers physical and chemical tolerance to the microbial community. In this phenotype, microorganisms in marine environments participate in biofouling and microbiologically influenced corrosion (MIC) and are recalcitrant to chemical treatments. MIC is a globally significant concern, estimated to cost the economy US $1 trillion in prevention and maintenance alone. Although biocides are a primary defence against marine biofilms, they pose an unacceptable environmental threat and biofilms develop tolerance to the compounds over

time. By incorporating enzymatic dispersal of biofilms to enhance biocide target specificity and efficacy, environmental impact and biocide concentrations can be reduced.

Main text

Introduction

Microorganisms generate globally significant health and economic impacts due to biofilm formation. Biofilms can be described as complex microbial communities living at a solid/liquid interface embedded in protective polymeric substances. By forming on steels, biofilms invariably result in microbiologically influenced corrosion (MIC) or biofouling, especially in marine environments. Marine biofilms alone contribute between 20 and 40% of all corrosion prevention and maintenance costs[155,156], which is estimated at around US $4T per annum[22]. In most natural environments the biofilm is the preferred living arrangement of microorganisms, offering up to 1000 times greater chemical tolerance to bacterial cells[157,158]. Chemical tolerance in these populations is ultimately the result of species diversity and extracellular polymeric substances (EPS), especially in the marine environment. The EPS is a self-produced matrix comprised mainly of polysaccharides, extracellular DNA (eDNA) and proteins [159-161]. The molecular composition of the matrix is critical to the structure and integrity, as well as function of biofilms.

Although EPS is important for cell adhesion/cohesion, horizontal gene transfer, metabolism and interspecies interactions, the composition of the EPS in marine environments is not well described. Conversely, the composition of single and multi-species biofilms in clinical settings has been extensively studied over the past two decades, leading to notable breakthroughs in fundamental understanding and treatment of clinically significant biofilms. For example, using a DNA degrading enzyme, it was demonstrated that dispersal of *Pseudomonas aeruginosa* biofilms could be achieved.[121] Since this sensitivity to eDNA dispersal was first revealed, a number of publications have further demonstrated the structural role of eDNA in biofilms from clinical strains; including *Staphylococcus* sp.[162], *Enterococcus* sp.[163], *Pseudomonas* sp.[164], *Burkholderia* sp.[165] and others[166]. Based on the importance of eDNA in most clinically relevant biofilms, it was hypothesized that multi-species marine biofilms developed on metals would also rely on the structural stability afforded by eDNA.

In the previous investigation of this chapter, multi-species biofilms were developed over six weeks in marine simulating conditions and evaluated for EPS composition. In agreement with the hypothesis and previous reports, eDNA was more abundant in the matrix than other macromolecules frequently identified in the EPS. The eDNA pool was also relatively stable over six weeks, indicating a long-term involvement in biofilm architecture. In the present study, marine biofilm eDNA was targeted for degradation to enhance the efficacy of a novel biocide compound.

Biocides are the primary defence against biofilms in marine environments. Engineered systems such as pipelines are susceptible to biofouling and MIC and can be difficult to access for mechanical scrubbing (pigging) and inspection. Equipment and infrastructure must therefore rely on chemical dosing to ensure material longevity and functionality. Current biocide compounds are toxic and environmentally hazardous, and thus face growing global scrutiny[167]. However, replacement of biocides with contemporary environmentally sensitive options is a challenging task. Novel greener compounds must also be effective and economically viable. Of developing alternatives, multi-functional organic inhibitor compounds are a promising strategy[8,168,169]. With multiple functions (for example, corrosion inhibition and biocidal capacity), the number of compounds applied, and thus dosing costs, can be reduced. Recently, a 10 mM concentration of a novel, green corrosion inhibitor exhibited 96% corrosion inhibition efficiency on CS (AISI 1030) after 30 minutes of exposure[9]. The compound, CTA-4OHcinn, contains a hexadecyl trimethylammonium cation and a trans-4-hydroxy-cinnamate anion[9]. Quaternary ammonium salts are broadly applied as antimicrobial surfactants[7,9,170,171], with low toxicity when

combined with the *trans*-4-hydroxy-cinnamate anion. Indeed, recent research demonstrates that the CTA-4OHcinn toxicity profile is reduced compared to cetrimonium bromide (CetBr), a common and safe antimicrobial additive in cosmetic products[172]. Established as an effective corrosion inhibitor and biocide[8,9,32], CTA-4OHcinn is a promising candidate for real-world applications.

The history of chemical treatment application has demonstrated that biofilms develop tolerance to biocides[173]. Glutaraldehyde (GLUT) is applied on a global scale to mitigate MIC, yet it faces efficacy challenges as a result of microbial tolerance acquisition. *Escherichia coli* for example utilizes overexpression of aldehyde reductases to impede GLUT activity[174]. Reports of single species biofilms capable of tolerating harsh chemical treatments are concerning, especially as natural biofilms contain complex communities that are known to develop tolerance at a faster rate. Thus, the present study anticipates tolerance acquisition to chemical treatments, with the aim of enhancing CTA-4OHcinn efficacy by targeting the EPS matrix.

Based on previous research identifying eDNA as a critical structural polymer in marine multispecies biofilms, the present research aimed to develop a method to evaluate enhancement of CTA-4OHcinn function. Mature, multispecies marine biofilms were developed and compared against biofilms treated with a low dose of CTA-4OHcinn. Subsequently, the same treatment was applied with an additional eDNA degradation stage. The results reveal for the first time in marine conditions that targeted biofilm dispersal approaches can enhance multifunctional biocide efficacy.

Results

Biofilm controls:

Microscopic techniques were applied to assess the biofilm architecture and structure before treatment application. After two weeks of development, confocal laser scanning microscopy (CLSM) revealed a complex biofilm structure that was dominated by living cells (Figure 1A, C). A Bitplane (IMARIS) post-image analysis of control surfaces indicated a mean biovolume between 200 and 300 µM^3(Figure 2A). Between 65 and 75% of control biofilms were composed of living cells (green signal), with dead or damaged cells occupying 25-35% of the biovolume (Figure 2B).

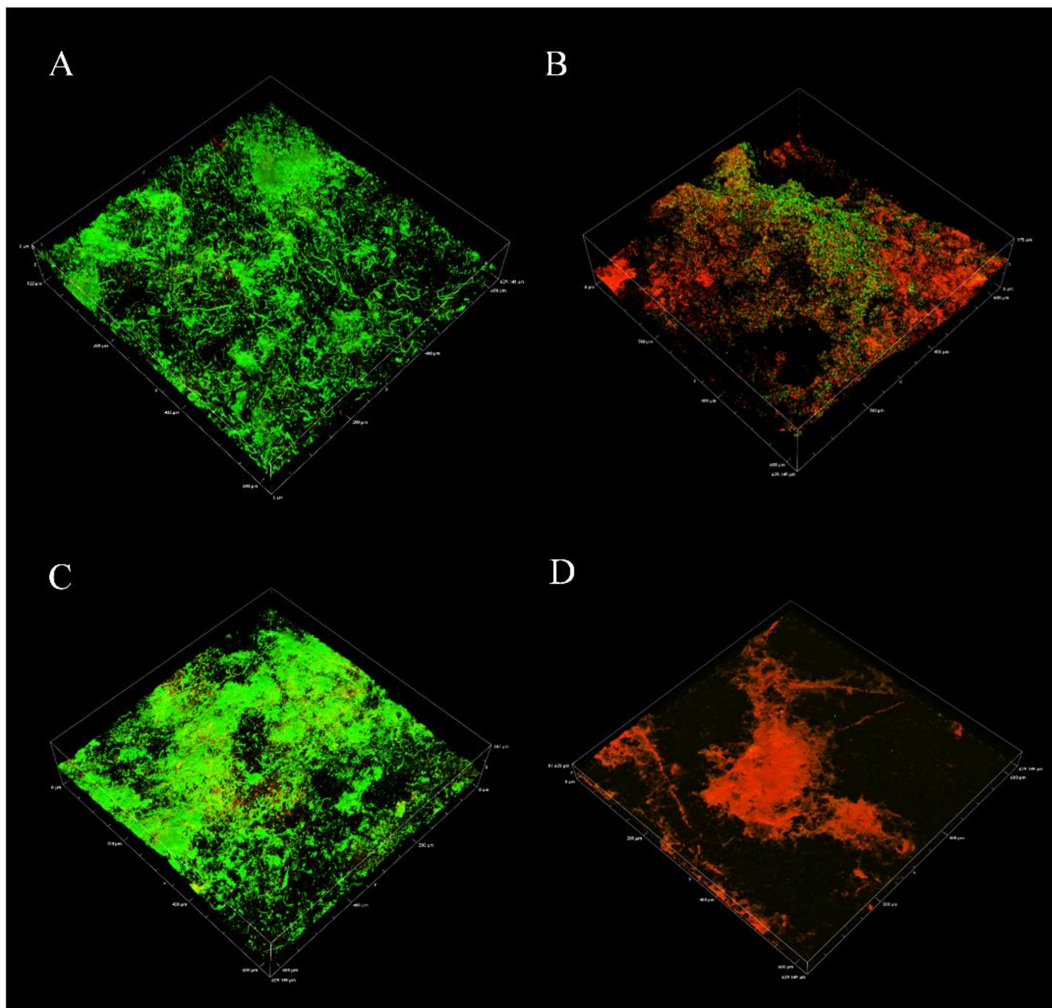

Figure 1: Confocal laser scanning micrographs showing live and dead cell in control biofilms compared to treatments, where: A, C = control biofilm, B = CTA-4OHcinn treated biofilm, and D = dual CTA-4OHcinn and DNase treated biofilm. Control micrographs (A, C) were captured from separate experiments before their respective treatments. Live cells are indicated by green and dead or damaged cells are indicated by red.

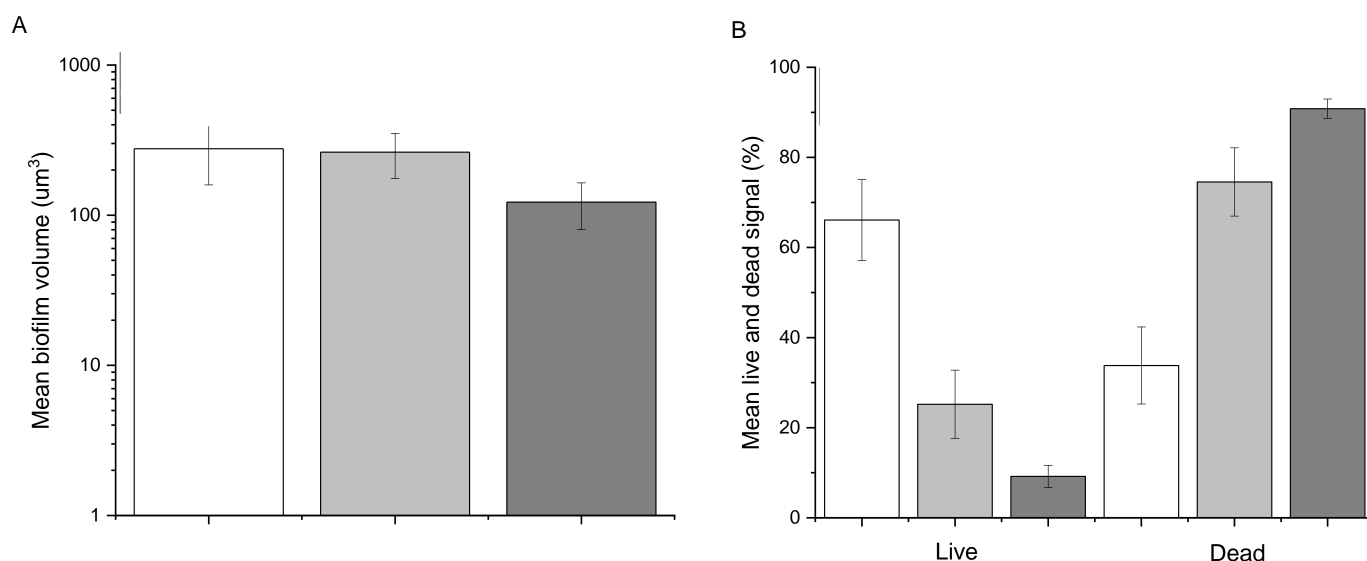

Figure 2: Mean biovolume as an average of 5 micrographs randomly captured across the coupon surface (A) and mean % live and dead cells from these micrographs (B) where the control biofilms (□), are compared against CTA-4OHcinn treated biofilms (■) and dual CTA-4OHcinn and DNase treated biofilms (■). Error bars represent the standard deviation of the data.

Scanning electron microscopy (SEM) revealed the biofilm architecture, cell morphology and living arrangement in controls (Figure 3 A, B). Micrographs captured of a large surface area on control coupons (A) indicated a complex, mature structure with dense cell arrangements and EPS. Through observation at higher resolution, various structures resembling cell morphologies were apparent (B), including single cells and long bacilli chains. Importantly, the dense living arrangement of cells within control biofilms was evident (Figure 3 B).

CLSM and SEM observations were supported by viability assays (Figure 4A), indicating that 1×10^8 viable cells could be extracted per cm^2 of coupon surface area in experimental (biological) replicates. Measurements of tATP are proportional to viability and were conducted to confirm CFU measurements (Figure 4B). In control experiments biofilm tATP extracted from coupons between 60,000 and 80,000 units/ cm^2, further confirming the viability of control biofilms.

Inhibitor treatments:

Treated biofilms demonstrated significantly different biofilm morphology and viability from controls. Both CTA-4OHcinn treated (B) and CTA-4OHcinn + DNase treated (D) biofilms had reduced 'green' signal (corresponding to live cells). 'Red' signal, corresponding to dead or damaged cells, was increased in all post-treatment CLSM.

Biofilm viability was not completely eliminated after 4 hours of exposure to 1 mM CTA-4OHcinn alone (Figure 1 B). This was confirmed by tATP assays (data not shown). When biofilms were exposed to both CTA-4OHcinn and DNase only 'red' signal was detected in 3D reconstructions (Figure 1 D), indicating enhanced degradation of biofilm architecture in the presence of an endonuclease.

A post-image analysis of CLSM micrographs indicated that after CTA-4OHcinn treatment, biofilm architecture remained relatively intact compared to controls (Figure 2 A). However, viability was reduced by at least 40% (Figure 2B). In biofilms exposed to the dual treatment, biofilm structure was significantly reduced, as indicted by CLSM and post-image analysis (Figure 1 D, Figure 2A). Although

3D reconstructions revealed little or no 'green' signal after CTA-4OHcinn + DNase treatments, viability remained in some areas as indicated by Figure 2B. A mean viability of 8-10% was detected, with dead or damaged cells representing 90% or more of the biofilm structure.

To support CLSM analysis, SEM was also conducted on coupons after 4 hours of CTA-4OHcinn or dual CTA-4OHcinn and DNase treatments. Reduced biofilm density was observed in all treated samples compared to controls (Figure 3). Density of the biofilm living arrangement was also reduced (Figure 3D, F) compared to controls (Figure 3B). Lastly, cell arrangements were reduced from long chains to single bacilli. Although this observation could be the result of high stress, the authors recognize that these samples come from multi-species biological replicate experiments, where some morphological variation can be expected.

Finally, biofilm viability was assessed after treatment. All viability assays indicated a significant reduction in viability was induced by either treatment with CTA-4OHcinn or the dual CTA-4OHcinn and DNase treatment. In Figure 4A, a reduction in viability of two orders of magnitude (from 1×10^7 to 1×10^5) was observed after CTA-4OHcinn treatment. Microscopic results indicate that DNase assisted biofilm dispersal, allowing CTA-4OHcinn enhanced access, leading to greater efficacy. In CFU assays, a further log reduction in biofilm viability after exposure to the dual CTA-4OHcinn and DNase treatment was observed compared to the CTA-4OHcinn-only treatment, supporting microscopic analysis. Biofilm tATP (Figure 4B) demonstrated a similar trend, with a statistically significant reduction of viability compared to controls in both treatments. Finally, dual CTA-4OHcinn and DNase treated samples yielded less tATP than the CTA-4OHcinn-only treatment, which was statistically significant.

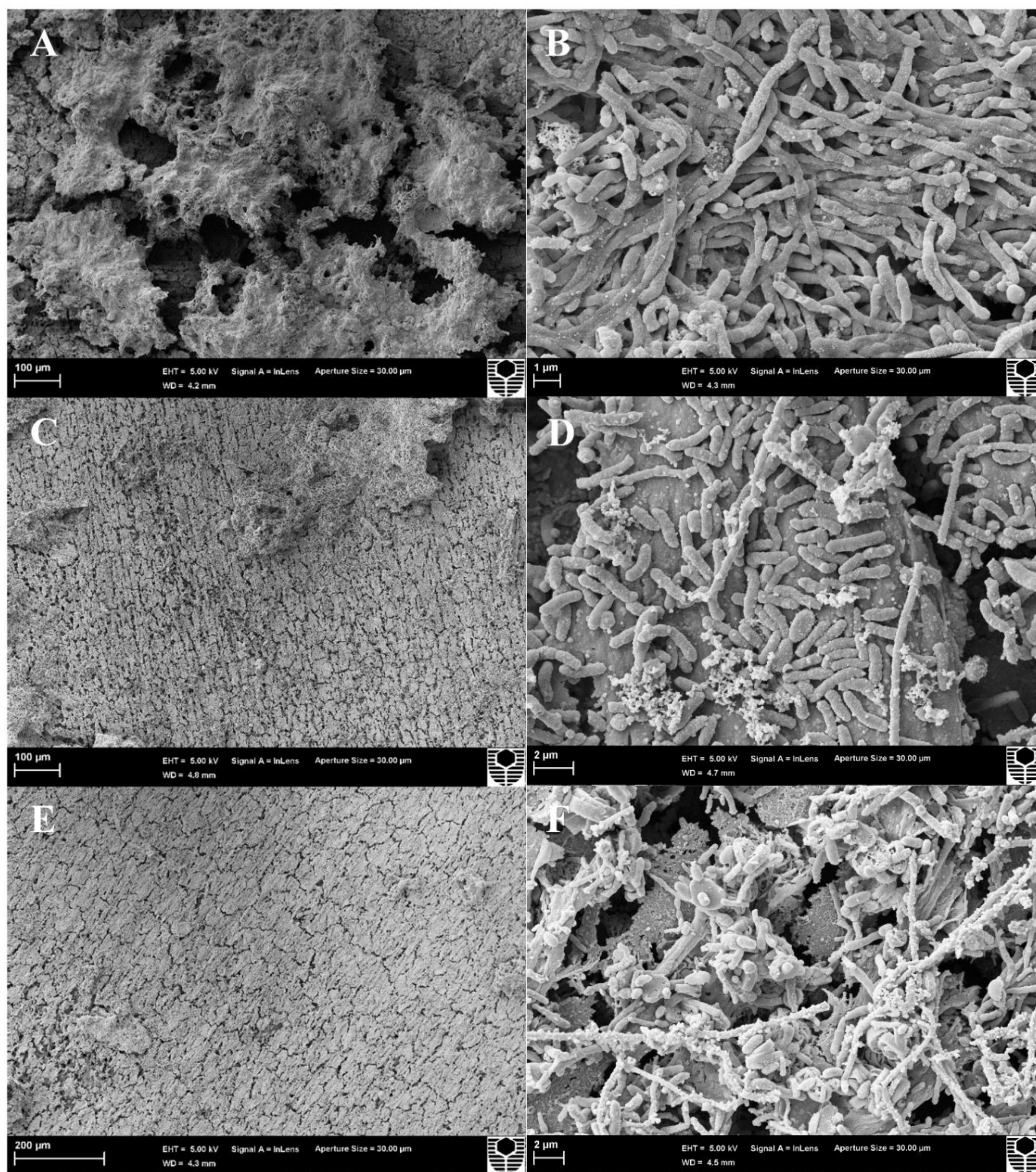

Figure 3: Scanning electron microscopy of biofilms before treatment (A, B), after treatment with 1 mM CTA-4OHcinn (C, D) and after dual treatment with 1 mM CTA-4OHcinn and DNase (E, F). Micrographs on the left of the figure depict the biofilm under low magnification to show a larger surface area, and micrographs on the left depict the cell arrangement within the biofilm.

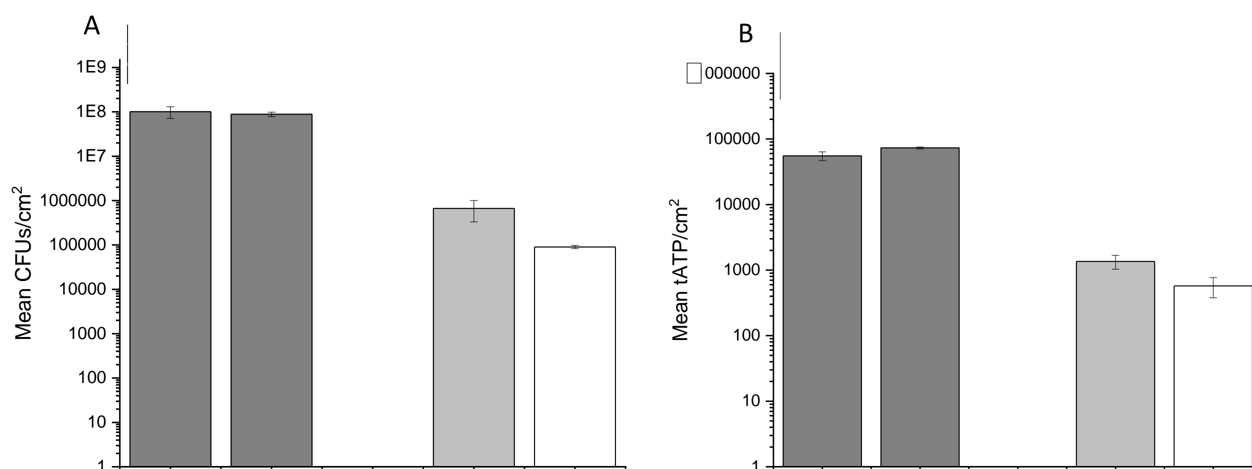

Figure 4: Biofilm viability assays colony forming units (CFUs) (A) total adenosine triphosphate (tATP) (B) assays of control ( ), 1 mM CTA-4OHcinn ( ) and 1 mM etc ( ). Each bar represents measurements taken from triplicate biofilms with two technical replicates. Error bars represent the standard deviation of this data.

Discussion

Regardless of efficacy when first introduced into an engineered marine system, research indicates that multi-species biofilms develop chemical tolerance to almost any compound[175,12]. It is now critical to anticipate bacterial tolerance acquisition for enhanced long-term efficacy of biocidal compounds. By incorporating biofilm dispersal into dosing procedures, cells protected in EPS become directly exposed to chemical treatments. This approach has been successfully applied in the health industry[176] and is now considered for application in other industries affected by biofilm formation[177]. Guided by recent research implicating eDNA in marine multispecies biofilms developed on steel, it was hypothesized that eDNA degradation could enhance the function of a novel, multi-functional biocide compound.

Research involving clinically significant biofilms has so far pioneered the incorporation of dispersal mechanisms for viability reduction. Advancements in the understanding of clinically significant biofilms have developed rapidly, however these communities are exposed to very different conditions compared to marine biofilms implicated in biofouling and MIC. In 2015, Okshevsky and colleagues identified eDNA as a target for biofilm disruption leading to enhancement of antibiotic efficacy[178], which was supported by a plethora of subsequent clinically-related studies[179-181]. Other promising dispersal targets to consider are emerging besides eDNA, such as extracellular proteins[182]. Research indicates that proteins have diverse roles within the biofilm, including facilitation of cell-cell cohesion and adhesion to surfaces[183], as well as cross-linking to eDNA structures[184]. While a combined enzymatic cocktail approach to dispersal seems logical based on the available literature, enzyme compatibility and target biofilm composition should also be considered. A recent study investigating antibiotic enhancement through EPS targeting revealed that tobramycin efficacy could be enhanced by application of either dispersin B (an enzyme that degrades 6-N-acetyl-d-glucosamine; PNAG) or DNase[181]. Interestingly a combination of these approaches was significantly less effective at enhancing the antibiotic treatment. Thus, co-treatment compromised enzyme activity. In the present study, a single enzymatic treatment was therefore selected to target mature biofilm dispersal. Since eDNA is an established ‘mortar’ in biofilms from other environments and was identified as a primary EPS component in previous research, biofilm eDNA was targeted in the present study using an endonuclease enzyme.

Biofilm composition and dispersal in native marine strains is scarcely investigated in the scientific literature. In one 2021 publication, use of Peptide A (a 14-mer peptide structure resembling the Equinatoxin II protein) as a biofilm dispersal agent enhanced the efficacy of the biocide tetrakis hydroxymethyl phosphonium sulphate (THPS)[185]. Additionally, the aggressive MIC of a *Desulfovibrio* strain was reduced by up to 83% with Peptide A application[185]. The study supports biofilm dispersal mechanisms as viable options for targeted biofilm mitigation, however the methods described do not consider the composition of the biofilm EPS.

In parallel with a biofilm dispersant, a novel, multifunctional biocide was applied at reduced concentrations to evaluate biofilm disruption. The multifunctional compound, CTA-4OHcinn is an effective corrosion inhibitor and biocide as demonstrated in previous reports[8,9]. CTA-4OHcinn was found to reduce bacterial viability during attachment stages by at least 99.7% at the recommended concentration (10 mM)[8]. Thus, the working concentration was reduced in the present research to 1 mM, and treatment time was optimized to preserve enough viability for treatment comparison.

Microscopic results confirmed that a complex biofilm had formed on CS after 2 weeks under the marine-simulating conditions evaluated. CLSM and post-image analysis further imply that biofilm parameters between experimental replicates were very similar (live and dead or damaged cells, biovolume; Figure 1 and 3). SEM revealed a compact living arrangement and various cell morphologies, which is consistent with previous reports on multispecies communities[186,187]. Densely packed cell arrangements in the natural community promotes beneficial behaviour such as communication by quorum sensing, metabolic cooperation and horizontal gene transfer[187], which positively influences biofilm chemical tolerance. Compact, mature structures were also implied by CFU assays and tATP measurements. In the present research, these mature multi-species biofilm communities were considered baseline controls for biocidal treatment and treatment enhancement experiments.

After treatment with 1 mM CTA-4OHcinn, significantly reduced biofilm architecture and viability was observed. In CLSM results, a greater 'red' (dead) signal was recorded in z-stacks captured after treatment application (Figure 1) which was generally observed across replicates as indicated by post-image analysis (Figure 2). As previously expressed, greater 'red' signal correlates with higher numbers of dead or membrane compromised cells[188]. SEM revealed a general reduction in biofilm material (Figure 3 C) as well as a reduction in density (Figure 3 D). This was expected, since CTA-4OHcinn contains the established antimicrobial hexadecyl trimethylammonium cation. The compound is expected to act on cell membranes to cause lysis based on research conducted on similar quaternary ammonium surfactants[32,189]. In CTA-4OHcinn, the quaternary ammonium cation is paired with the *trans*-4-hydroxy cinnamate anion to offer corrosion protection[9]. Thus, CTA-4OHcinn delivers at least one additional function along with reduced toxicity compared to CetBr.

Compared to controls and the CTA-4OHcinn-only treatment, the dual CTA-4OHcinn and DNase-1 treatment resulted in significantly reduced biofilm viability. Microscopic results demonstrated a decrease in biofilm architecture and cell density (Figure 1, 3) which was supported by quantitative assays (Figure 4). Importantly, the results of dual treatments compared to controls indicate a significant structural role of eDNA in marine biofilms. As seen in previous studies in terrestrial environments, the present study demonstrates that eDNA can be considered a 'building block' of biofilms that can be targeted by endonucleases to disperse marine biofilms.

DNase-1 is an endonuclease that cleaves the phosphodiester backbone structure in double stranded DNA to produce smaller fragments[190]. Since DNase-1 is widely available, has a similar functional temperature range to bacterial growth conditions (30-40ºC) and shows promise as a dispersal agent in clinical biofilms, the enzyme was selected for dispersal of biofilms in the present investigation. DNase-1 enzymes are also able to continue functioning after reaction catalysis and are environmentally benign and easy to apply. Despite these benefits, limitations to large scale applications of enzymatic biocide enhancement still exist. Most importantly, mammalian endonucleases can be expensive to produce in

large quantities. However, bacterial extracellular nucleases represent a practical solution to economic concerns[178]. Bacterial cells divide rapidly and can be cultivated affordably. Further, DNase-1 was applied in the present communication directly to ASW solution. Although the test solution contains critical Ca^{2+} and Mg^{2+} ions for DNase activity[190], bacteria also require these ions for growth and development processes[191]. Without presence of both divalent ions the activity of DNase-1 is known to be reduced[190], thus locations depleted of fresh seawater with high microbial activity will pose a challenge to DNase-1 treatment. Here, we treated biofilms emersed in phosphate buffered saline (PBS), which was not supplemented with Ca^{2+} or Mg^{2+} ions, yet significant biofilm reductions were still observed with enzymatic treatment. Future investigations aim to further enhance enzymatic biofilm treatment efficacy through exploration of the optimal conditions for DNase-1 activity against biofilms in marine conditions.

The results of this study confirm that complex marine biofilms formed on CS after 2 weeks. Application of CTA-4OHcinn in 10 times reduced concentrations from the optimal recommended dose still demonstrated adverse effects on these biofilms, which was further enhanced by incorporation of an eDNA degrading stage. Incorporation of enzymes to assist with biofilm dispersal is a promising strategy for the effective and environmentally sensible control of pervasive marine biofilms.

Materials and Methods

Microorganisms:

A consortium comprising *Shewanella chilikensis* strain DC57, *Pseudomonas balearica* strain EC28 and a laboratory strain of *Klebsiella pneumoniae* was employed in this research based on growth characteristics as described in previous work[8]. *S. chilikensis* DC57 and *P. balearica* EC28 were recently recovered from corroded steel in a marine industrial facility where MIC was implicated[4]. Pure bacterial strains were cultivated in artificial seawater (ASW) as previously described[19,192] supplemented with Bacto™ casamino acids (10 mM), sodium pyruvate (10 mM), D (+) glucose (10 mM) and ammonium nitrate ($NH_4NO_3$30 mM). After 24 - 48 hours of incubation at 30ºC, the cultures were harvested in log phase and manually counted using a Neubauer haemocytometer before washing twice in ASW at 12,000 rpm. Cell pellets were resuspended in ASW before inoculation into reactor media. The final cell number used for all reactors was 1×10^5 cells/mL of each isolate.

Sample preparation and surface finish:

CS coupons (AISI 1030) were prepared as previously described. Briefly, coupons were cut to produce a working surface of 1.34 cm^2 and electrocoated using Powercron® 600CX solution. The working surface was then wet-ground using 120g SiC sandpaper, rinsed in 100% ethanol and dried under nitrogen gas. Before and after fixing to reactor rods, coupons were irradiated with ultraviolet light (UV) for at least 10 min on each side.

Experimental setup:

Replicate Centre for Disease Control (CDC) bioreactors were used to develop biofilms on CS coupons over 2 weeks (Figure 5). Anaerobic conditions were generated by constant pure N_2 gas injection. A solution temperature of 30ºC and gentle agitation was maintained using a stirring hotplate set to 50 rpm. Continuous nutrient replenishment was achieved using a 5 L reservoir cell connected to a peristaltic pump. The pump was calibrated to replace 30% of the reactor solution in each reactor every 24 hours. ASW solution as described elsewhere[19,192] was used for the experiments with the following supplementation: 10 mM Bacto™ casamino acids, 10 mM sodium pyruvate, 10 mM D (+) glucose and 30 mM ammonium nitrate (NH_4NO_3). Nutrient concentrations were established to allow rapid progression of biofilm development without promoting transition of the population to the planktonic lifestyle. Sampling was conducted after 2 weeks of CS exposure to the consortium.

Figure 5: CDC reactor setup; with continuous flow for nutrient replenishment, maintained using an external reservoir attached to a peristaltic pump, replacing 30% of the reactor solution every 24 hours. Stirring hotplates were used to generate a constant temperature of 30ºC and agitation at 50 rpm. Reactors were constantly flushed with pure N_2 gas to maintain anaerobic conditions.

Control assessment and inhibitor dosing:

After 2 weeks of biofilm development, coupons were extracted for control CLSM, SEM, CFU and ATP measurements. Subsequently, clean, sterile bioreactors containing phosphate buffered saline (PBS, Sigma, pH7.4) were used to apply treatments. In one reactor, PBS containing 1 mM of completely dissolved CTA-4OHcinn was applied. The remaining reactor contained the same solution with the addition of DNase-1 (100 ug/mL, Sigma). The reactor lids containing rods and remaining coupons were placed into fresh PBS (Sigma, pH 7.4) and then into the treatment reactors. Coupons were exposed to treatments for 4 hours before transferring again into reactors containing fresh PBS (Sigma, pH 7.4). Finally, coupons were removed from rods for further processing and analysis.

Confocal laser scanning microscopy (CLSM) and post-image analysis:

A Nikon A1+ confocal laser scanning microscope equipped with a 20 × dry objective lens was used to assess cell viability. The Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit (propidium iodide and Syto9™) was purchased through Invitrogen™ and mixed in ultrapure deionized water. The stain mixture was applied to coupons in 200 uL aliquots and incubated for 10 minutes before rinsing the coupon gently in PBS (Sigma, pH 7.4). Coupons were inverted and placed into a purpose-built dish with a central depression of radius 1 cm covered by a glass coverslip, to preserve biofilm architecture (ibidi®, Germany). Sequential micrographs were obtained using a 489.3 nm and 500-550 nm emission filter (Syto9™ imaging) and 561 nm laser with a 570-620 nm emission range (propidium iodide imaging). Separate tracks for emission and excitation paths were used in z-stack acquisition to minimize signal bleed-through. All experiments were conducted using the same microscope settings.

Micrographs were captured for visual representation of the control and treated samples, where the latest version of Nikon Elements software was applied for 3D reconstruction. Triplicate micrographs (z-stacks) were also captured from each surface and applied to calculate biovolume (um^3), % live biofilm and % dead biofilm composition using Bitplane (IMARIS) software.

Scanning electron microscopy (SEM):

SEM was conducted using a Zeiss Neon field emission scanning electron microscope. Biofilm samples were rinsed and fixed as previously discussed[21]. Briefly, samples were rinsed gently in PBS (Sigma, pH 7.4) and incubated at 4ºC for 22 hours in a 2.5% glutaraldehyde fixative solution. To minimize cell

shrinkage, a temperature ramping stage was introduced to the fixation procedure. PBS and fixative solutions were prewarmed to 30ºC, and biofilms in pre-warmed fixative solution were allowed to gradually cool to room temperature before placing at 4ºC. Coupons were then removed from the fixative solution and dried under pure N_2 gas for 12 hours before sputter coating with 9 nm of platinum. An emission voltage of 5 kV was applied to the sample at a working distance starting at 4.5 mm. Microscopy was conducted using an in-lens secondary electron detector and an aperture size of 30 µm.

Standard biofilm colony forming unit (CFU) enumeration:

CFUs were extracted and quantified from control and treated coupons following existing standards[193]. To strip the biofilm from the coupon and homogenize the sample, coupons were placed into a tube containing 10 mL sterile PBS (Sigma, pH 7.4), vortexed for 30 seconds and sonicated for 10 seconds on followed by 15 seconds on ice for a total of 2 minutes[21]. Agar plates containing the same nutrient composition as the reactor solution were produced using 15 g/L bacteriological agar. Plates were incubated at 30ºC for 2 days until visible colonies had appeared before counting.

Biofilm adenosine triphosphate (ATP) assays:

ATP is a nucleoside triphosphate involved in energy cycling for all life[194]. As the primary source of energy in bacterial cells, ATP is a prime biomarker for assessing biofilm viability. In the present research ATP was quantified from biofilms using the luciferase enzyme, which reacts with ATP to produce light correlating linearly with the ATP quantity in solution[194]. Total biofilm ATP was quantified using a Luminultra Quench-Gone Organic Modified ATP Test Kit and a Luminometer™ spectrophotometer. Samples were prepared in tubes containing PBS (Sigma, pH 7.4) as described above. A standard calibration was performed before measurements were obtained.

Conclusions:

Multispecies marine biofilms were developed on carbon steel over two weeks as a platform for evaluating a targeted approach to biofilm mitigation. Marine biofilms were found to rely on structural extracellular DNA (eDNA) in a previous study, targeted for dispersal in the present communication using an endonuclease (DNase-1). By applying an enzymatic biofilm dispersal stage, it was hypothesized that efficacy of a novel multi-functional biocide compound could be significantly enhanced. The compound, CTA-4OHcinn, is an environmentally safe corrosion inhibitor and biocide with an optimal working concentration of 10 mM. To evaluate the hypothesis, CTA-4OHcinn was applied in a 10 times lower concentration (1 mM) to treat biofilms, resulting in a reduction in viability by two orders of magnitude. However, biofilm architecture was largely preserved as indicated by microscopic analysis, and although significantly reduced, CFUs and biofilm energy measurements indicated persistence of viable bacteria after treatment. After the same treatment was applied with simultaneous nuclease digestion, microscopic observation indicated a reduction in biofilm architecture. CFU and biofilm energy values also indicated a significant reduction in bacterial viability compared to treatment with CTA-4OHcinn alone. The results reveal that biofilm eDNA degradation is a promising, environmentally sensible method for biocide enhancement in field scenarios.

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Chapter 6: SUMMARY, CONCLUSIONS AND FUTURE WORK

6.1 Summary and conclusions

The formation of multispecies biofilm communities on steel applied in marine environments is considered the root cause of biofouling and MIC. Engineered marine systems deployed by oil and gas operations, especially the internal pipeline surface, depend on biocidal compounds to limit biofilm progression. Rising microbial tolerance to existing biocides as well as high toxicity and environmental impact have generated drive from industry and academia to develop more effective, targeted chemical treatments. However, knowledge gaps pertaining to the development, structure and composition of marine biofilms remain. Advancing the fundamental understanding of natural biofilms, as well as biofilm treatment options is critical for an economical and more sustainable approach moving into the future. The aim of this investigation was to enhance the fundamental understanding of marine multispecies biofilms, while evaluating the biocidal function of a novel multifunctional inhibitor compound. Finally, the investigation aimed to employ information gained over the course of the project to identify a mechanism for biocidal efficacy improvement.

The first objective of this thesis was to compile research in the area in the form of review manuscripts, which is included in chapter 1. Research was further refined to form a succinct overview of relevant topics, with a particular emphasis on critical analysis of contradictory research outcomes and knowledge gaps. A refined manuscript published in 2022 is also presented to capture additional information gained since the initial review conducted between the years 2018 and 2020.

Guided by existing literature and knowledge gaps identified in chapter 1, the second objective of this thesis aimed to enhance the fundamental understanding of initial bacterial colonisation on carbon steel as the first stage of biofilm formation. Initial colonisation encompasses the adsorption of the conditioning film, followed by initial attachment of bacterial cells, eventually leading to irreversible adhesion. By successfully limiting or evading these critical biofilm developmental stages, it is possible to prevent biofilm formation. At present, knowledge of the conditioning film components and how they affect bacterial attachment and adhesion remains unclear. It has been established that in marine conditions, a diverse mixture of organic and inorganic molecules is attracted to steels, and

overall, this is reported to elicit a positive response in bacteria. By understanding which common conditioning molecules attach to steels and their impacts on bacterial attachment, prevention of biofilm formation can become more effective and target-specific. Therefore, the first study evaluated the propensity of two ubiquitous organic molecule types; namely DNA and amino acids, to form a conditioning film on carbon steel. Both molecules have been identified in the marine conditioning film in previous reports. The study found that, in agreement with existing research, DNA and amino acids interacted with carbon steel surfaces to form a conditioning film. Subsequently, we evaluated the capacity of each molecule to influence the bacterial attachment process. Using a marine bacterial isolate, biofilm surface coverage was assessed in parallel with bacterial intracellular energy and viability when exposed to either the DNA or amino acid conditioned surface. The results found no significant impact on attached cell number, as assessed with viability assays, imposed by a DNA conditioning film. However, the cells that were adsorbed to the DNA conditioned surface yielded significantly higher intracellular energy values, which are associated with a faster rate of biofilm development. The amino acid conditioned surface yielded significantly higher viable cell numbers as well as intracellular energy levels, demonstrating enhanced surface attachment.

The research so far demonstrated that biofilm formation can be influenced from the very first stages of biofilm development depending on the conditioning molecule type. Since early biofilm formation could be influenced by particular conditioning molecules, it was subsequently proposed that surface electrochemistry, and thus localised corrosion, could also be influenced by conditioning film composition.

Organic molecules present in the marine ecosystem were screened in a subsequent study for their effect on surface electrochemical patterns by monitoring local galvanic current density in real-time. In these studies, DNA and sodium pyruvate were found to have no effect on surface electrochemical behaviour. However, introduction of amino acids to aerobic seawater significantly reduced localised corrosion events. This study provided the first insights regarding the effects of common conditioning film molecules on carbon steel corrosion. Further, amino acids are commonplace as organic nutrient supplements in MIC simulations. It is envisaged that understanding of conditioning molecule effects on steel will lead to new corrosion inhibition strategies, while providing valuable knowledge to researchers hoping to evaluate MIC in laboratory scenarios.

Chapter 3 acknowledges the biofilm phenotype as the root cause of MIC and biofouling. In the marine environment, mature biofilms are characterised by species diversity, leading to treatment challenges. Multispecies marine biofilms are surrounded by EPS, affording cells protection from exogenous pressures such as heat, desiccation, predation, and importantly chemical treatments (biocide dosing). In chapter 3, biofilms were developed over the relatively long term (6 weeks) using three bacterial isolates for composition, viability and structure assessments. The composition and role of the EPS, and in particular in marine biofilms, remains largely uncanvassed. Despite its central role in the generation of deleterious effects observed in steels across a variety of industrial sectors, more research is required to identify key components of the matrix. By understanding the composition of the biofilm components, greater target specificity can be achieved in biocide development, leading to enhanced efficacy. In a world of increasing environmental awareness, enhanced specificity is also important for toxicity reduction. Therefore, chapter 3 aimed to identify important components of the biofilm that may be targeted for dispersal of the structure. By employing a combination of microscopic, culture-based and biochemical assays, chapter 3 identified significant enrichments of eDNA in the biofilm at 2, 4 and 6 weeks of the investigation. The results indicated that eDNA may play an important role in biofilm structure, which was expanded on practically in chapter 5.

In parallel with viability and EPS analysis, DNA and RNA-based sequencing analysis was conducted in chapter 3 using the same biofilm development strategy. This analysis was conducted to shed light on the cellular component of the biofilm, revealing important characteristics of the biofilm community. Interestingly, RNA-based sequencing revealed that the three bacterial isolates formed a stable community that contributed similar levels of activity to the total biofilm over the course of the study. Analysis of genomic and extracellular DNA from the same system revealed that species contributing more RNA in the biofilm (and therefore likely to be more active) can have a minor role in biofilm eDNA production compared to more dormant biofilm members.

In summary for chapter 3, eDNA was identified as an important EPS component in biofilms developed in steel in seawater. The origin of eDNA in this study challenges evidence that active members produce the bulk of the EPS; instead implicating more dormant members of the community in eDNA manufacture.

Fundamental research as part of this thesis was conducted during the synthesis of a novel, environmentally sustainable corrosion inhibitor through Deakin University, Melbourne, as part of the larger Australian Research Council (ARC) Discovery Project (DP). The compound, cetrimonium *trans*-4OH-cinnamate (CTA-4OHcinn), is a quaternary ammonium cation paired with the *trans*-4OH-cinnamate anion, was found to afford competitive corrosion protection on carbon steel. Additionally, it was hypothesised that the quaternary ammonium cation would function as a biocide. Chapter 4 of this thesis related to the evaluation of this promising corrosion inhibitor as a multifunctional compound. Specifically, chapter 4 assessed the capacity of CTA-4OHcinn as a biocide.

The first manuscript included in chapter 4 employed CTA-4OHcinn against three bacterial strains in marine conditions and evaluated their propensity to attach to steel compared to controls. The results of this study were promising, reducing attachment by between 96.6% and 100%. Ideally, biofilm formation is intercepted at these early stages, and under these conditions CTA-4OHcinn was found to be an effective biocide using microscopic, culture-based and biochemical assays. However, biofilms in marine environments are typically dense living arrangements and treatments are not always applied at the early stages of biofilm formation. The second manuscript in chapter 4 therefore explored the capacity of CTA-4OHcinn to tackle mature, oilfield biofilms developed in seawater. Using similar techniques, chapter 4 revealed that CTA-4OHcinn could also reduce mature biofilms by as much as 100%. With the addition of a novel application of a membrane-targeting CLSM stain, this chapter also identifies the cell membrane as the primary target for CTA-4OHcinn-induced lysis.

In summary, chapter 4 provides a substantial body of evidence to support the efficacy of CTA-4OHcinn as a biocide, both against early attachment stages and mature biofilms. Thus CTA-4OHcinn can be considered a true multifunctional inhibitor compound, with great promise for real-world application.

Finally, chapter 5 builds on preceding objectives outlined in this thesis to tackle mature biofilms more effectively using targeted dispersal. Additionally, the chapter aimed to achieve an environmentally sustainable method for effective dispersal leading to enhancement of biocide function. The studies in chapter 5 were conducted in the context of seawater conditions, using CTA-4OHcinn as the biocide.

After development of mature biofilms as described in chapter 3, a ten-times reduced concentration of CTA-4OHcinn was applied in parallel with a biofilm dispersal stage. A DNA degrading enzyme, DNase-1, was selected as a biofilm dispersal agent based on results obtained in chapter 3.

After treatment with CTA-4OHcinn, a 2-fold reduction in attached cell viability was observed. Viable bacterial cells remained in biofilms, which was important for observing the effect of biofilm dispersal. By dispersing the biofilm during biocide dosing, it was anticipated that CTA-4OHcinn diffusion across the biofilm would be more effective, and therefore cell lysis would be enhanced.

With the application of DNase-1 to the same reduced concentration of CTA-4OHcinn, a 30% increase in biocide efficacy was observed based on microscopic observation of the biofilm and quantitative analysis of viable cells. The results of chapter 5 demonstrate that dosing of biofilms using a novel biocide compound can be significantly enhanced using greener methods. Indeed, non-specific dispersal agents have been previously employed in marine environments. The research from chapter 5 introduces an enzymatic dispersal stage to biocide dosing not previously evaluated in marine conditions, or against marine multispecies biofilms. The enzyme used in chapter 5 is an effective biofilm dispersant and is both widely available and environmentally sustainable. Therefore, this effective and novel approach to targeted biofilm mitigation in seawater is driven by consideration for the environment, as well as the need for a practical and effective solution to biofilm formation.

6.2 Study limitations:

The complexity of MIC in engineered systems by marine multispecies biofilms has represented a significant challenge for all investigations in the field. By generating simplified laboratory simulations of natural MIC phenomena, the capacity of microbial communities can be underestimated. However, incorporating natural communities in simulations also leads to limited reproducibility. Further, untangling mechanisms behind outcomes such as MIC generated by natural communities is a complex and often impossible task. Thus, to further develop the scientific understanding of biofilm development, use of complex natural biofilms is not always sensible.

In chapter 2, a gram-negative bacterial strain was employed to evaluate corrosion re-initiation in the presence of a conditioning film. This microorganism was selected to represent key traits widespread in MIC causative strains; such as membrane arrangement, environmental origin and previous implication in MIC. However, gram-negative bacteria are not the only microorganisms involved in MIC. Fungi and archaea, as well as gram-positive bacteria may also be associated with deleterious effects on metallic surfaces in marine environments. While this research attempts to understand the phenomenon of bacterial and conditioning film adsorption to CS, these other populations should be investigated to confirm their role in the initial colonisation of metallic surfaces in marine environments. Challenges in isolation and cultivation, especially of archaea, must first be overcome in order to explore their contribution to biofilm formation. Since initial colonisation stages involve single cells rather than communities, and reproducibility is enhanced by using the methods applied in chapter 2, single strains were still the preferred platform for attachment studies. In subsequent chapters, a multispecies community was employed to overcome the limitations imposed on research by single-species simulations.

Interpretation of laboratory results to real-world efficacy is a challenge in the development and commercialisation of biocidal compounds. Biofilm mitigation should ideally target early attachment stages where bacteria are most vulnerable, however mature biofilms rapidly develop in marine environments. The research described in this thesis attempted to overcome limitations associated with evaluation of a single biofilm developmental stage by investigating CTA-4OHcinn efficacy against ideal and worst-case scenarios: initial colonisation (attachment and adhesion) and mature, multispecies biofilms respectively. In the context of heterogeneous environmental samples extracted from marine oil and gas infrastructure, evaluations of CTA-4OHcinn captured species diversity, interspecies interactions and the EPS barrier. Future investigations should also consider additional chemical tolerance offered to the biofilm by deposits, which can lead to under-deposit corrosion (UDC) in the presence of microorganisms. By incorporating coupons embedded in deposits, the experimental design can better reflect the internal environment of pipelines and provide a more accurate depiction of CTA-4OHcinn efficacy under such conditions.

6.3 Future work:

Bacterial attachment and adhesion were demonstrated to vary significantly depending on the type of conditioning molecule present at the interface of CS. Specifically, either viable cell number (colony forming units) or intracellular energy reserves (adenosine triphosphate abundance) directly impacted by the conditioning film are in turn likely to influence long term biofilm formation. Research in this thesis identifies knowledge gaps pertaining to the composition of the mature biofilm EPS as well as the marine conditioning film. Importantly, all stages of biofilm formation (from conditioning film to maturation) are connected; for example, the progression of biofilm growth relies on successful irreversible adhesion. In this sense, canvassing the conditioning film in marine conditions can lead to a deeper understanding of mature biofilm EPS and biofilm development. As part of future work, studies should aim to understand the connection between specific conditioning molecules and the mature biofilm. Since species composition and diversity in particular are major drivers of MIC and biofouling, it is essential to understand how the initial foundations of the biofilm (attached cells and conditioning molecules) influence the mature community. The applications of this knowledge are broad and significant. For example, by understanding the association between molecular chemistry of the conditioning film in a given environment and the microbial species or capabilities in a system, community composition could be altered or selected to favour substrate preservation. Microbial community engineering would see momentous advantages to human health, food processing, agriculture and oil and gas industries, among others. Additionally in the biofilm phenotype, chemical MIC by production of waste products, or electrical MIC through intercellular and cell-substrate transfer of electrons can progress. It is unclear how organic conditioning molecules impact these processes that represent global concerns for stakeholders. By understanding the early stages of biofilm formation and the factors affecting maturation in greater detail, novel and improved biofilm treatment options can follow.

Evaluation of novel, multifunctional inhibitor compounds for biocidal efficacy was an objective of this thesis. Corrosion inhibition studies conducted through the broader Australian Research Council (ARC) Discovery Project (DP) collaboration revealed that a novel compound synthesised through the project (designated CTA-4OHcinn) demonstrated great promise as a corrosion inhibitor. Studies outlined in this thesis subsequently assess

CTA-4OHcinn for its efficacy against early and mature biofilm formation. However, other environmentally sustainable, potentially multifunctional compounds were also developed over the course of the project. For real-world application as corrosion inhibitors, biocides or multifunctional compounds, further research is required to understand the functional capacity of these compounds. Specifically, studies designed to evaluate biocidal efficacy of CTA-4OHcinn against early and mature biofilms should be replicated on other candidate compounds and compared in the context of CTA-4OHcinn results. After providing a baseline for multifunctionality and biocidal capacity, the range of functional application should then be assessed in terms of environmental conditions. Temperature, salinity, pH and solvent chemistry are all important factors to consider for the evaluation of novel compounds for real-world application in industrial settings.

A methodology for biocidal evaluation of CTA-4OHcinn, generated through the present project, was developed and described in the thesis outcomes. Subsequent research can now apply these methods to evaluate other compounds, however comparisons against biocides already commercialised and practically applied can add further perspective to the research. Glutaraldehyde (GLUT), as the most widely applied biocide in oil and gas industry today around the world, is the product of choice for baseline comparisons. Thus, future work aims to incorporate the biocide GLUT into existing biocidal evaluation assays to be used as an efficacy baseline comparison for CTA-4OHcinn and other novel compounds. When referring to baseline, many important parameters exist for the release of a novel to an engineered system exposed to the marine environment. Toxicity is one factor evaluated as part of the broader ARC DP project on human cell lines and fish. These studies should be repeated against other candidate compounds. While the compounds are specifically intended to be environmentally sustainable exact degradation time in environmental conditions (environmental persistence) should also be established for each compound. Finally, all compounds applied in engineered systems must be compatible with existing chemicals (for example, corrosion inhibitors must function with hydrocarbons present in pipelines). Thus, future investigations should encompass the intended system chemistry in the experimental design.

Since the scope of the project is time-limited, screening of multi-species biofilm composition and treatment effects could be expanded in future work. It is acknowledged that biofilms form under different situations within the marine environment, which extends to the various engineered marine systems (for example, the internal pipeline environment

can change depending on the contents of the pipeline). Therefore, as part of future work, samples from various field locations should be collected and biofilms developed under conditions reflecting the original sampling location, as previously discussed for inhibitor evaluations. The primary aim of these experiments would be to determine the dexterity of CTA-4OHcinn and other compounds to tackle biofilms of different origin, including under deposit biofilms (microbial populations that thrive in the protection of deposits built up inside pipelines) which are notoriously recalcitrant to chemical treatments. Subsequently, evaluation of the biofilm EPS composition under the various conditions imposed by each field scenario would lead to an enhanced understanding of potential biofilm targets. As a following stage of future research, the complementary dispersal treatment can be expanded depending on the results of biofilm composition studies. Specifically, other macromolecules (such as proteins or polysaccharides) can represent biofilm dispersal targets to enhance CTA-4OHcinn efficacy further or in various environmental conditions.

A deeper understanding of the EPS and species composition in marine biofilms as provided through the present research is applicable to all biocides. Through specific degradation or dispersal (targeting) of biofilm components, costs can be reduced at dosing intervals along with environmental impact, regardless of the type of biocide employed. i.e., the more efficient a given biocide, the lower the impact on the surrounding environment and the more economically viable the product becomes. Further, localised attack on extracellular biofilm structures such as eDNA can provide a solution to biocide tolerance acquisition. In parallel with dual treatments, biocide multifunctionality can further reduce the need for large quantities of biocide. A dual treatment approach, paired with use of a multifunctional biocide such as CTA-4OHcinn can offer an effective, greener solution for environmental biofilm control. However, transition from a single compound as primarily used by industry today to the multifaceted approach described here requires an intricate understanding of molecular interactions. For future work, interaction between CTA-4OHcinn and dispersants such as the enzymatic treatment described in chapter 5 should be explored. Although research from the present communication indicates that CTA-4OHcinn and DNase-1 work together well in simulated conditions, there are real opportunities for improvement of biofilm dispersal, biocide function or both. For example, enzymes require specific conditions to function at optimal capacity. Since DNase-1 was isolated from bacterial cells or mammalian cells with an optimal temperature of 37ºC. All experiments were conducted at 30ºC in the present communication for uniformity.

Additionally, DNase-1 requires the presence of specific ions to function optimally. By supplementing DNase-1 with Mg^{2+} and Ca^{2+} ions required for DNA hydrolysis, function could be further enhanced. Although the solvent applied in this research (ASW) originally contained Mg^{2+} and Ca^{2+}, these ions are metabolically valuable to many bacteria and were likely depleted in the test solution. Thus, the functional range of dispersants, especially enzymes, should be mapped in future research. This research should include other parameters not mentioned so far, including salinity and compatibility with the biocide itself.

Finally, besides compatibility of the system chemistry, synergistic interactions can significantly boost functional capacity while reducing costs and environmental impact. As discussed, synergy between the hexadecyl trimethyl ammonium cation and the *trans*-4-hydroxy-cinnamate anion in CTA-4OHcinn is an important topic of future research. Other potential synergisms that require exploration and development include those between biocides and dispersal agents (or other co-treatments). Interestingly, research detailed in chapter 4 indicates that CTA-4OHcinn may have a dispersal effect without the inclusion of a separate biofilm dispersal stage. Thus, synergistic interactions between CTA-4OHcinn and DNase-1 in chapter 5 may be partially responsible for the efficacy observed in these experiments. Greater dispersal qualities lead to greater biocide penetration, which again leads to economic and environmental benefits. By understanding synergistic interactions between CTA-4OHcinn and DNase-1, or any biocide and biofilm dispersant treated in parallel, optimisation and refinement can lead to enhanced efficacy.

In conclusion, there is great promise for dual treatments such as CTA-4OHcinn paired with localised biofilm dispersants for engineered systems moving towards an economically and environmentally sensitive future. However, development of advanced, target-specific treatments requires further understanding of the mature marine biofilm, as well as the conditioning film as the foundation of biofilm development. In this thesis, a repository of relevant literature in the field is provided, fundamental aspects of the conditioning film and the mature biofilm are evaluated, a significant body of evidence is introduced for the functional efficacy of a novel, multifunctional biocide compound and finally a practical solution is proposed to help combat biocide tolerance acquisition and environmental toxicity observed in existing biocides.

APPENDICES

APPENDIX 1

Written statement of contribution by publication co-authors

To whom it may concern,

I, Benjamin Tuck, contributed to the synthesis of original research entitled "**Understanding natural biofilm development on steel in marine environments – a review**", including research, data interpretation and manuscript compilation.

Candidate Signature:

I, as co-author of the specified manuscript, endorse the contribution made by the candidate and declare the candidate's statement is accurate.

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To whom it may concern,

I, Benjamin Tuck, contributed to the synthesis of original research entitled **"Corrosion inhibition on steel by amino acid conditioning film is reversed by *Shewanella chilikensis*"**, including research, data interpretation and manuscript compilation.

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I, Benjamin Tuck, contributed to the synthesis of original research entitled "**Extracellular DNA is a critical structural polymer in marine multi-species biofilms**", including research, data interpretation and manuscript compilation.

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APPENDIX 2

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