Corrosion Science 83 (2014) 189–197

Contents lists available at ScienceDirect

Corrosion Science

journal homepage: www.elsevier.com/locate/corsci

Marine rust tubercles harbour iron corroding archaea and sulphate reducing bacteria

K.M. Usher [a,*], A.H. Kaksonen [a], I.D. MacLeod [b]

[a] Commonwealth Scientific and Industrial Research Organization (CSIRO), Land and Water, Underwood Ave., Floreat, Western Australia 6014, Australia
[b] Western Australian Maritime Museum, Fremantle, Western Australia 6160, Australia

ARTICLE INFO

Article history:
Received 28 November 2013
Accepted 6 February 2014
Available online 12 February 2014

Keywords:
A. Carbon steel
B. SEM
C. Microbial corrosion
C. Rust

ABSTRACT

Marine corrosion has significant economic impacts globally. Marine rust on carbon steel in Western Australia was investigated to determine the importance of various microorganisms in corrosion. Microorganisms were imaged, identified and enumerated by pyrosequencing. The base of tubercles was anaerobic. Pyrosequencing demonstrated the presence of diverse bacteria and archaea. However, the dominant group were methanogenic archaea, representing 53.5% of all sequences. One methanogenic species, *Methanococcus maripaludis*, comprised 31% of sequences, and can significantly increase corrosion rates by extracting electrons directly from steel. Methanogenic archaea may be significant contributors to marine corrosion of carbon steel.

1. Introduction

Accelerated low water corrosion is a global problem involving high rates of corrosion on steel structures in the ocean at around the low tide region of the steel [1]. Where this phenomenon occurs the rate of metal loss is greatly increased, leading to extensive repairs and maintenance of structures [2,3] which is often accompanied by costly downtime. It is generally accepted that microorganisms play an important role in accelerated low water corrosion through microbially influenced corrosion (MIC) [4], and any wet metal surface will form a biofilm that is potentially corrosive for susceptible steel [5].

Sulphate reducing bacteria (SRB) are a diverse group of autotrophic and heterotrophic microorganisms that are ubiquitous in the environment [6]. They are believed to play an important role in corrosion of steel [7–10] in both marine and terrestrial environments, and are typically the focus of corrosion research. SRB are anaerobic and obtain energy by coupling the oxidation of hydrogen (H_2) or organic matter and dissimilatory reduction of sulphate (SO_4^{2-}), producing hydrogen sulphide (H_2S) [6,11], which is a corrosive gas [12]. Many SRB can also reduce other inorganic sulphur compounds, including sulphite, thiosulphate, and elemental sulphur [6]. Sulphur reducing bacteria and archaea reduce elemental sulphur rather than sulphate, and also produce H_2S [13].

It is increasingly being recognised that the presence of methanogenic archaea is associated with increased corrosion rates of steel [14–16] in different environments. Archaea are single celled prokaryotes that belong to a separate domain to bacteria [17]. Their biochemistry differs to bacteria, permitting some species to live in extreme environments. Methanogenic archaea are anaerobes that can utilise H_2 or organic compounds to produce methane. Many are autotrophic (self feeding), and thus do not rely on organic carbon for energy or as a carbon source.

Understanding the phylogenetic diversity of microorganims in natural environments has been labour intensive and costly, typically utilising multiple rounds of polymerase chain reactions (PCR) and purifications together with methods such as cloning or denaturing gradient gel electrophoresis, followed by sequencing of individual bands or clones within a sample. Pyrosequencing is a recent advance in sequencing technology that bypasses many of these steps, together with the biases that these steps introduce [18]. It relies on detecting the release of pyrophosphate when nucleotides are added during the synthesis of complementary DNA strands [19] in a process that is fundamentally different to the Sanger method of sequencing. Pyrosequencing is used to evaluate the diversity of microorganisms in complex communities without first separating the DNA fragments originating from various species. The ability to rapidly detect large numbers of different species, including rare species [20,21], together with enumeration

* Corresponding author. Address: Commonwealth Scientific and Industrial Research Organization (CSIRO), Land and Water, Private Bag #5, Wembley, Western Australia 6913, Australia. Tel.: +61 8 9333 6270; fax: +61 8 9333 6211.
E-mail address: Kayley.Usher@csiro.au (K.M. Usher).

http://dx.doi.org/10.1016/j.corsci.2014.02.014
0010-938X/

of the relative abundance of taxa within communities [22–24] has revolutionised research into microbiological ecology. Microbial diversity in many environments has been found to be orders of magnitude higher than previously thought [18].

We believe the present study is the first to use pyrosequencing to identify and enumerate both bacteria and archaea in established marine corrosion tubercles. Our aim was to determine the importance of the various metabolic groups of bacteria and archaea present in marine corrosion. Archaea may be important in accelerating corrosion, yet they are rarely identified or enumerated in corrosion studies. More complete information on microorganisms that increase corrosion may lead to the development better diagnostic tests for susceptible or corroding infrastructure, and to more effectively manage infrastructure to avoid corrosion problems.

2. Methods

2.1. Sample collection

Ten corrosion tubercles were removed from 70-year old carbon steel railway lines forming a World War II slipway that were heavily encrusted in corrosion tubercles at an average depth of 0.5 m in the sea at Fremantle harbour, Western Australia in March (autumn) 2013. All equipment and reagents used were sterile. Each tubercle was placed into a separate 50 mL centrifuge tube with seawater for transport. Sub-samples of 4 larger tubercles were placed in 2.5% glutaraldehyde in 2X phosphate buffered saline (PBS, Sigma, pH 7.4 containing 0.28 M NaCl and 0.005 M KCl, pH 7.4) and stored at 4 °C for electron microscopy imaging. The remainder of the samples were washed in PBS buffer then stored at −80 °C for sequencing.

2.2. Scanning electron microscopy

Following preservation in 2.5% glutaraldehyde samples were washed in 2X PBS then placed in 1% osmium tetroxide (in PBS) for 15 min, followed by another PBS buffer wash. This was followed by an ethanol series, 50%, 70%, 90% and 100% ethanol for 15 min each. Samples were then critical point dried using CO_2. On completion samples were coated in 3 nm thick platinum for imaging.

Scanning electron microscopy (SEM) imaging was performed on a Zeiss 1555 VP-FESEM. An in-lens detector was used for imaging with a 30 mm aperture, accelerating voltage of 3 kV and a working distance of 4–5 mm. Energy-dispersive X-ray spectroscopy (EDS) distribution maps and point spectra were constructed using AZtec TruMap software, which applies background and overlap corrections to map data to provide artefact-free element distribution images. EDS was performed at 15 kV, a working distance of 10 mm and an aperture of 60 mm. Point spectra were taken from 8 sites on 3 tubercles.

2.3. Dissolved oxygen concentrations

Dissolved oxygen concentrations were measured in sea water at the time of sampling, and from three tubercles 1–2 mm under the surface of the tubercle, and at the base of the tubercle next to the steel, using fibre-optic oxygen sensors with a robust tip (430 μm) and optical isolation (Pyroscience, Germany), following 2 point calibration. Pyroscience employs "Redflash technology", using fibre-optics to deliver red light excitation and detection in the near infrared with luminescent oxygen indicators. These probes are not affected by dissolved chloride or other ionic species. Data was automatically adjusted for temperature using a temperature probe attached to the instrument. Data was downloaded using Firesting O_2 meter and software (Pyroscience, Germany) and logged on a laptop.

2.4. Temperature, pH and redox

Water temperature was measured using a Firesting probe and software (Pyroscience, Germany) and logged on a laptop at the time of sampling. Solution pH and redox potentials were taken at the same time using a flowing seawater $Ag/AgCl_{sea}$ reference electrode in a waterproof meter box after calibration against a standard double junction Ag/AgCl reference electrode. This had been calibrated in the laboratory using the reference voltage at a platinum working electrode in Quinhydrone saturated solution at pH 4.

2.5. Pyrosequencing

DNA was extracted from eight tubercles using a PowerMax Soil DNA Isolation kit (Mo Bio laboratories, Inc.) following manufacturer's instructions, with DNA from samples kept separate throughout the sequencing process. DNA quality was assessed using a Nanodrop ND-1000 spectrophotometer. DNA was stabilised using DNA Stable Plus (Biometrica) as per manufacturer's instructions, and sent at ambient temperature to Molecular Research (MrDNA, Texas) for PCR and 454 sequencing of 16S rRNA genes. Bacterial 16S rRNA genes were sequenced using universal Eubacterial primers 27F (AGRGTTTGATCMTGGCTCAG) and 530R

Table 1
Breakdown of numbers of microorganisms sequenced on marine tubercles. Top: major microbial metabolic groups present in tubercles with percentage of total sequences (bacteria and archaea) for each group. Only bacterial species representing 1% or more of total bacterial sequences are shown. Middle: major archaeal metabolic groups present in tubercles with percentage of all archaeal sequencing reads and total reads. Bottom: major bacterial metabolic groups present in tubercles with percentage of sequencing reads for bacterial species and total reads. Only bacterial species representing 1% or more of total bacterial sequencing reads are shown.

All Microorganisms	Number	% Total microbes	
Methanococcus maripaludis	55,164	31.28	
Other methanogens	39,379	22.33	
Sulphate reducing microbes	14,679	8.32	
Sulphur oxidising microbes	3243	1.84	
Ammonia oxidising microbes	3084	1.75	
N fixing microbes	6739	3.82	
Other	8607	4.88	
H_2 producing microbes	24,013	13.62	
Archaea: metabolic group	**Number**	**% Total microbes**	**% Archaea**
Methanogens	94,543	53.61	96.61
Ammonia oxidisers	3084	1.75	3.15
Sulphate reducers	5	0.00	0.01
Sulphur oxidisers	227	0.13	0.23
Other	34	0.02	0.03
Bacteria: metabolic group			
H_2 producing heterotrophic	24,013	13.62	30.60
Sulphate reducing	14,674	8.32	18.70
Sulphur oxidising	3016	1.71	3.80
Nitrogen fixing	6739	3.82	8.60
Other (unknown etc.)	8573	4.86	10.90