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Microorganisms associated with corrosion of structural steel in diverse atmospheres

Laura L. Machuca ^a, Robert Jeffrey ^b, Robert E. Melchers ^{b,*}

^a Curtin Corrosion Engineering Industry Centre, School of Chemical and Petroleum Engineering, Curtin University, Technology Park, Bentley, WA, 6102,

^b Centre for Infrastructure Performance and Reliability, The University of Newcastle, NSW, 2308, Australia

A B S T R A C T

the influence of atmospheric conditions on the corrosion of steel and its associated microbial community was studied. Surface analysis revealed greater localized corrosion in steel exposed to near-ocean at-mospheres with high chloride deposition compared to inland and subalpine sites. High-throughput sequencing analysis of corrosion products showed that dissimilar microbial communities and domi-nant species were deposited on steel in the different atmospheres. Close to the ocean, *Brevundimonas diminuta* were predominant whereas *Clostridium* and *Pseudomonas* species dominated for inland sites with agricultural or forestry activities. *Bacillus* and *Enterococcus* were dominant for sites close to a fer-tilizer plant and a sewage treatment plant, respectively. *Actinobacteria* species dominated at sub-alpine conditions. Results from this study indicate that microbial communities on corroding steel exposed to atmospheric conditions are the result of deposition of locally-generated aerosols. Acid-producing activity and exopolysaccharide (EPS) production was widespread and rapidly detected in microbial cultures from all the exposure sites. Sulphate-reducing bacteria were not detected in this study. These results suggest that acid production and EPS synthesis can be important mechanisms for microbial corrosion of steel under atmospheric conditions.

Keywords:

Mild steel Atmospheric corrosion Microbial deposition Microbial community
High-throughput DNA sequencing

1. Introduction

The atmospheric corrosion of structural steels continues to be a topic of interest because of the potential implications for the safety, performance and economic viability of critical infrastructure. Corrosion of steel and other surfaces in the atmosphere involves the availability of moisture either as rain, condensation through relative humidity mechanisms or deposition of sea or other water spray. Such corrosion is also influenced by atmospheric pollutants such as chlorides, NO_x and SO_x and their role on both short and long term corrosion has received much research interest for many years. Likewise, low levels of atmospheric corrosion have been associated with the lack of deposition of airborne pollutants (Evans, 1960) (de la Fuente et al., 2011).

Aerosols have been shown to play a major role in the biogeochemical processes at the air-water interface of oceans (Prospero, 2002) and are known to play a similar role in air-land surface

interfaces (Foissner, 2006; Morris and Nicot, 2014). Aerosols can transport a wide variety of inorganics, particulates of iron and other metals, and microorganisms including algae, bacteria and archaea. Typically, they are generated and transported by local wind action, thermal gradients, evaporation and dust (Prospero, 2002). Deposition to substrates such as vegetation, animals and inanimate surfaces including stones, rock and steel is commonly observed. Similarly, winds, rain, snow and mechanical abrasion can remove all or part of such deposition. In a study by Bovallius et al. (1976), the numbers of airborne bacteria and deposited bacteria were compared at four locations in Sweden. The authors found a degree of correlation between airborne and deposited bacteria with an elevated number of deposited bacteria in summer and autumn seasons. Rain and high humidity decreased the counts of deposited bacteria whereas high winds tended to increase their numbers. The authors did not investigate the microbiological species involved.

There is significant evidence that the corrosion of steels can be influenced by microbial activity, a phenomenon known as microbiologically-induced corrosion (MIC) (Melchers and Jeffrey, 2008), (Machuca et al., 2014). The main species involved in MIC have been identified as sulphate reducing bacteria (SRB) and iron-

oxidizing bacteria (Little and Lee, 2007; Chang et al., 2015) but causative microbes can vary depending on location, environment and materials. Likewise, the extent of their influence on the corrosion processes appears to be dependent on the availability of critical nutrients (Melchers, 2007, 2014; Little and Lee, 2007; Melchers, 2014). However, there is only limited evidence that MIC may be involved in atmospheric corrosion.

Santana Rodriguez et al. (2002) reported SRB on plastic test strips exposed for one month to three different sites in the coastal regions of Gran Canaria Island. The authors concluded that SRB were transported and deposited on the test strips via seawater aerosols. Santana Rodriguez and Gonzalez Gonzalez (2006) reported SRB in Green rusts corrosion products and concluded that SRB were responsible for the aggressive atmospheric corrosion observed on steel. Similarly, De Belie et al (De Belie et al., 2000), reported aggressive corrosion associated with the presence of SRB in local areas of intensive animal husbandry. In a study on atmospheric corrosion of inland Indian railways (Maruthamuthu et al., 2011) corrosion of the steel rails was attributed to toilet droppings. Using DNA analysis of corrosion products the authors identified the microbial species involved and concluded that corrosion was caused by ureolytic bacteria that produce carbonic acid and by iron/manganese-oxidizing microbes. These observations highlight the potential role of microorganisms in atmospheric corrosion.

The following sections describe a study on the corrosion of steel and its associated microbial community composition after 3 months exposure to 7 different atmospheric sites including near-ocean, industrial, farming and subalpine locations. The identification of microbial communities on corroding steel has been widely applied to help understand the role of microorganisms in corrosion (Machuca et al., 2011; Li et al., 2014; Liengen et al., 2014) although most attention has been given to immersed conditions. Measuring the exact contribution of microorganisms to atmospheric corrosion is very challenging because of the logistical difficulty of setting up abiotic (sterile) controls in field studies and as such it was not the purpose of this work. The aim of the study was to gain insight into the possible mechanisms by which microorganisms can accelerate atmospheric corrosion through the identification of the microbial communities deposited on steel at the different locations. High-throughput 16S rRNA gene sequencing was used to identify the microbial communities. Microbial deposition was assessed via fluorescence microscopy and culture-dependent method was used to assess the activity of major metabolic groups associated with corrosion. Corrosion was evaluated using 3D surface profilometry.

2. Materials and methods

2.1. Test specimens and exposure sites

Mild steel coupons of 50 × 50 × 3 mm were used in this study, all cut from the same sheet of cold-rolled steel. The coupons were used in the 'as supplied' state, without polishing, to reflect normal industrial applications. Random samples were examined under an optical microscope to confirm that the surfaces were essentially identical. Atomic emission spectrometry was used to determine their composition (C 0.16, Mn 0.32, P 0.015, S 0.019, Si 0.08, Ni 0.02, Cr 0.02, Mo 0.00, V 0.003, Al 0.041, Cu 0.01, bal. Fe %wt). Prior to exposure, coupons were acid cleaned and rinsed in deionised water, alcohol and acetone, consecutively. Coupons were air dried and sterilized by spraying 70% ethanol (v/v) over the surface before deployment. In all cases the coupons were suspended vertically from a support frame using nylon cable ties that permitted free orientation. They were spaced very close together (suspended no more than 150 mm apart) on the assumption that this would have a very high likelihood that the microbial communities deposited

would be similar for each coupon.

Five coupon replicates were exposed at different test sites located in New South Wales, Australia, for three months. The sites have different environmental characteristics (Table 1). All coupons were exposed primarily during the Autumn season. After exposure, coupons were removed from the test rack using sterile gloves and placed in individual sterile plastic bags. From the five coupon replicates at each site, two coupons were processed for DNA extraction followed by 3D surface profilometry, one coupon was used for direct DAPI staining and fluorescence microscopy, one coupon used for cultivation, and one coupon for chloride deposition measurement. The average chloride deposition rates for the sites was measured using a standard technique (ASTM, 2002).

2.2. Corrosion analysis

Previous corrosion studies conducted on the same test sites have shown that corrosion mass losses (general corrosion) are not significant in these conditions and corrosion is best evaluated using surface analysis of pits. For this reason, corrosion evaluation was limited to surface examination. Coupons were cleaned in accordance with standard procedures (ASTM, 2003). Corrosion was evaluated using an optical 3D surface profilometer (IFM G4g system, Alicona Imaging) that allows measurements of pit density and pit depth.

2.3. Direct assessment of bacteria deposition on steel by DAPI

For direct evaluation of bacteria deposited onto steel, coupons from each site were stained with 4,6-diamidino-2-phenylidole (DAPI), a fluorescent nucleic acid dye, as described previously (Machuca et al., 2013). Briefly, the coupon was rinsed with sterile phosphate buffered saline (PBS) solution to remove unattached contaminants and stained with DAPI (2 µg/mL; Sigma). Coupons covered with DAPI were incubated in the dark at room temperature for 15 min. DAPI-stained coupons were examined using a BX-51 Olympus epifluorescence microscope.

2.4. Bacteria abundance and DNA extraction from coupons

Coupon replicates from each site were suspended in sterile phosphate buffer saline (PBS) containing 0.1% (w/v) Tween 20. Two coupon replicates were used for DNA extraction and 16S rRNA gene sequencing (Section 2.5) and one coupon was processed for cultivation (Section 2.6). For microbial cell detachment, vortex mixing at maximum speed was used for 2 min (Vortex Genie[®], MO BIO Laboratories, Inc.) followed by sonication in an ultrasonic bath (60 s-sonication steps). The PBS/Tween solution was replaced between sonication steps so as to avoid prolonged exposure of dislodged bacteria to sonication. Bacterial cells in suspension were harvested by microfiltration (0.2 µm pore size polycarbonate membrane filters). The retained cells were washed from the filter with phosphate buffered saline (PBS) and further processed to separate iron oxides (centrifugation 1 min, 700 × g, 4 °C). Microbial cells in the supernatant were counted using a Petroff-Hausser counting chamber and phase-contrast microscopy. Cell abundance is given as cells/cm² surface area. One-way analysis of variance (ANOVA) was conducted to examine the significance of variations in bacterial numbers as a function of exposure site at a significance level of 0.05 (calculated as statistical *p* values).

For DNA extraction, bacterial cells in supernatant from each coupon replicate (as above) were mixed and harvested by high speed centrifugation (20 min, 13,000g, 4 °C) and the pellet used for DNA extraction using a DNA extraction kit (PowerSoil[™] DNA Isolation Kit, MO BIO Laboratories Inc.) following manufactures'

Table 1
Site descriptions, air temperatures and salt deposition rates.

Site	Location of coupons	Air temperature mean (Range) °C	Chloride deposition mg/m ² /day
Ocean beach	200 m from Pacific Ocean at Belmont Beach, close to sewage treatment facility.	18.1 (2.9–40.5)	252
Harbour mouth	2 m from edge of mouth of the Hunter River.	21.0 (6.3–48.1)	100
Coastal suburb	About 1 km from Pacific Ocean in school grounds at Belmont North	18.6 (–1–53.4)	16–30
Fertilizer plant	About 1 km from Pacific Ocean and 100 m from Hunter River and adjacent to an enclosed facility where chemicals are stored and blended for production of fertilizers	21.3 (2.4–46.1)	40
Dairy farm	Inland and located between milking holding pens and a local sewage treatment facility.	18.7 (1.1–53.5)	0–56
Turf farm	Inland site with periodic application of high nitrogen fertilizers to produce turf.	22.6 (–1.7–50.5)	6–44
Sub-alpine	Located inland on a sheep and cattle-grazing property on south-eastern edge of the Northern Tablelands, approx 425 km north of Sydney and about 1000 m elevation.	11.2 (–8.4–39.3)	13

instructions. Nanodrop spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) and 1% agarose gels were used for quantification and qualification of DNA extracts.

2.5. Microbial community composition by high-throughput 16SrRNA gene sequencing

The microbial community on steel at the different atmospheric sites was identified by high-throughput sequencing (MiSeq System - Illumina) [42] using the primer pair of 341F/806R that targeted the variable region V3-V4 of the 16S rRNA gene. PCR amplification and sequencing was performed by the Australian Genome Research Facility. Briefly, PCR amplicons were generated using the primers and PCR conditions, as follows: 341F (CCTAYGGGRBGCASCAG) at 94 °C and 30 s and at 50 °C for 60 s and also 806R (GGAC-TACNNGGGTATCTAAT) for 29 cycles at 72 °C and 60 s. AmpliTaq Gold 360 mastermix (Life Technologies, Australia) was used for the primary PCR. The primer set employed should amplify 16S rRNA genes from a broad range of archaeal and bacterial groups (Bates et al., 2011). A secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were measured by fluorometry (Invitrogen Picogreen) and normalised. An equimolar pool was then created and quantified by qPCR (KAPA) followed by sequencing on the Illumina MiSeq with 2 × 300bp Paired End Chemistry.

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2014). Primers were trimmed using Seqtk (version 1.0). Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010) USEARCH (version 8.0.1623) (Edgar 2010, Edgar et al., 2011) and UPARSE software (Edgar 2013). Using USEARCH tools, sequences were quality filtered and full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered, followed by chimera filtered using “rdp_gold” database as reference. To obtain the number of reads in each OTU, the reads were mapped back to OTUs with a minimum identity of 97%. Taxonomy was assigned using the Greengenes database (Version 13_8, Aug 2013) (DeSantis et al., 2006). The results are presented as percentage of sequencing reads for the identified OTUs in each sample.

2.6. Acid production and sulphate reduction capability

Based on previous studies, two of the potential microbial metabolic activities associated with atmospheric corrosion are

acid-production and sulphate reduction. To evaluate the ability of the culturable microorganisms to carry out acid-production and sulphate-reduction, bacterial suspensions (Section 2.4) were inoculated in Phenol Red dextrose (PRD) medium and anaerobic Postgate Medium B (NACE, 2004). In the PRD medium, a colour change (pH indicator) accompanying turbidity gives a positive result for acid production. For Postgate Medium B, vials that turn black (iron sulphide precipitation due to SRB activity) were scored positive. Salinity of the media was adjusted as required. Bacteria were incubated at 30 °C for 28 days.

3. Results

3.1. Corrosion observations

Localized corrosion was observed in all coupons at the different exposure sites. Corrosion was evaluated by pit depth and density of pitting per unit area (Table 2). Due to the wide variation in pit depths, from newly formed to mature pits, only the depth of the 8 deepest pits is reported as well as the maximum pit depth found for each exposure condition. The results show that the most severe pitting occurred at the sites subjected to higher chloride deposition (cf. Table 1) as well as at the Fertilizer plant. Some examples of the deepest pits observed on coupons from four different locations are shown in Fig. 1. It can also be seen that despite widely different exposure conditions, at the local level the individual pits are very similar in shape characteristics.

3.2. Bacterial deposition on coupons (DAPI fluorescence microscopy)

The presence of microorganisms on the steel coupons was confirmed for all the exposure sites (Fig. 2). DAPI-stained coupons reflect microbial cells of different size and shape with some

Table 2
Coupon corrosion measurement results.

Site	Average depth of 8 deepest pits (µm)	Maximum pit depth (µm)	Pit density
Ocean beach	260	296	very high
Harbour mouth	100	174	high
Coastal suburb	115	144	high
Fertilizer plant	125	131	medium
Dairy farm	108	113	medium
Turf farm	115	141	low
Sub-alpine	37	49	low

Fig. 1. 3D optical surface images of steel exposed to test sites for 3 months. Black arrows indicate the deepest pits found on the coupons. (a) Ocean Beach (pit depth: 296 µm); (b) Coastal suburb (pit depth 144 µm); Harbour mouth (pit depth: 174 µm) and (d) Fertilizer plant (pit depth: 131 µm).

distinctive cell morphologies apparent for particular exposure sites. For instance, coupons exposed to the Fertilizer plant were colonized predominantly by long rod-shaped bacteria (Fig. 2a-b) whereas coupons exposed to the Sub-alpine site show extensive occurrence of long-branching filamentous structures (hypha-like structures) over the surface (Fig. 2c-d). Structures compatible with algae were observed on steel in all the test sites (Fig. 2f).

3.3. Bacterial abundance by optical microscopy

The total number of bacteria deposited on steel at the different exposure sites are as follows (cells/cm² x10⁶): Ocean beach 1.07, Harbour mouth 2.14, Coastal suburb 1.85, Fertilizer plant 1.64, Dairy farm 2.42, Turf farm 2.07, Sub-alpine 2.99. Although total cell numbers are clearly of similar magnitude for all sites, the highest microbial density was observed for coupons exposed to the Sub-alpine site followed by the Dairy farm, Harbour mouth and the Turf farm, subsequently. Lower cell numbers were observed for steel at near-coastal sites and the Fertilizer plant.

3.4. Microbial community on corroding steel by high-throughput 16SrRNA gene sequencing

Microbial communities on the steel coupons exposed to the seven different atmospheres were identified by high-throughput 16S rRNA gene sequencing. DNA extracted from coupons was sequenced using primers targeting bacterial and archaeal 16S rRNA genes. The sequences obtained were grouped into operational taxonomic units (OTUs) and classified against the Greengenes data base, thereby providing genus/species-level resolution of communities. The major microbial species identified on the steel coupons at each exposure site are summarized in Table 3, together with their percentage of relative abundance within the microbial community.

For steel coupons exposed at the Ocean beach site, the microbial community was dominated by an unclassified member of the family Moraxellaceae (66.33%) and by *Enterococcus* (33.56%). *Shewanella algae* and *Propionibacterium* were identified as minor

members of this community. In contrast, the coupons at the marine site at the Harbour mouth were predominantly colonized by *Brevundimonas diminuta* (53.75%). Other major genera identified included *Actinomyces*, *Granulicatella* and *Bacillus*. Minor species identified include *Veillonella dispar*, *Veillonella parvula* and species from the genera *Porphyromonas* and *Fusibacter*. At the Coastal suburb site, the steel coupons were colonized predominantly by *Brevundimonas diminuta* (81.29%) followed by species of the genus *Coprococcus* (13.65%). Minor populations identified included *Trichococcus*, *Vibrio*, *Pseudomonas*, *Bacillus* and *Actinomyces*. These differences are not as unexpected as might at first seem, since the sites are at different distances from the coast and there are local topographical features (e.g. headlands at the harbour mouth) that affect, for example, wind direction and strengths. This heterogeneity is reflected also in the chloride deposition rates for these three sites (Table 1).

Steel coupons exposed at the Fertilizer plant were colonized predominantly by *Bacillus*, representing about 99.55% of the community. Other microbial species including *Microbacterium*, *Paenibacillus lentimorbus*, *Vibrio*, *Bradyrhizobium* and *Brevundimonas diminuta* also were identified but represented less than 1% of the total community.

Away from the coastal region, the microbial community at the Dairy farm was dominated by *Pseudomonas* (39.20%) and an unclassified member of the family Enterobacteriaceae (30.03%). Other populations included *Erwinia dispersa* (11.89%), *Bacillus thermoamylovorans* (11.44%) and *Stenotrophomonas* (2.27%). Minor bacteria within the community included *Paenibacillus*, *Brevundimonas diminuta* and *Bacillus*.

At the rural agriculture Turf farm site, three major genera were identified, including *Clostridium* (60.21%), *Enterococcus* (30.80%) and *Rummeliibacillus* (8.54%). Minor populations within the community included *Vibrio* and several unclassified members of the families Bacillaceae, Lachnospiraceae and Enterobacteriaceae.

For steel coupons exposed at the Sub-alpine site, several microbial populations were identified, including *Rhodococcus* (64.36%) as the predominant species, an unclassified member of the order Actinomycetales (16.61%), *Paenibacillus lentimorbus* (9.82%),

Fig. 2. Fluorescence microscopy images of DAPI-stained coupons after 3 months exposure to test conditions: (a–b) Fertilizer Plant; white arrow points out characteristic long rod-shaped bacteria, commonly observed on the surface; (c–d) Sub-alpine; red arrows highlight long filamentous structures compatible with hyphae commonly found on the surface; white arrows indicate bacteria cells; (e) Turf Farm and (f) Dairy Farm; diverse bacteria cells were observed on Turf farm and dairy farm and no distinctive morphologies were found. Yellow dashed arrow highlight structures compatible with algae observed in all exposure sites. Coupons exposed to Coastal Suburb, Ocean Beach and Harbour Mouth exhibited similar patterns to (e) and (f) (not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Stenotrophomonas (2.33%) and an unclassified member of the family Promicromonosporaceae (1.92%). Several other minor populations were identified but represented only between 0.1 and 1% of the community.

Diversity profiling at the phylum level is shown in Fig. 3. The majority of the bacteria deposited on the steel coupons at the different exposure sites belong to the phylum Proteobacteria and Firmicutes, except for the Sub-alpine exposure site which was colonized by Actinobacteria. Proteobacteria were dominant in near coastal areas, however the Turf farm and the Fertilizer plant, both exposed to fertilizers, were dominated by Firmicutes.

A representation of microbial diversity can be given by the Shannon Wiener Index (Xi et al., 2015). For the sites considered herein the results are shown in Fig. 4. These indicate that microbial diversity is the highest on steel coupons exposed at the Dairy farm, followed by the Harbour mouth site. Interestingly, microbial diversity at the rural Sub-alpine site was found to be higher than diversity at sites such as the Turf farm, Ocean beach and Coastal suburb sites. Microbial diversity was lowest at the Fertilizer plant.

3.5. Acid production/sulphate reduction capabilities of deposited bacteria

Acid production was detected in PRD bacterial cultures from all the exposure sites within 24–48 h of incubation. Interestingly, strong production of exopolysaccharides (EPS) was also detected in PRD cultures, an activity that is not typically observed in PRD cultures. Sulphate-reduction activity was not detected for any of the exposure sites investigated.

4. Discussion

The potential for microorganisms to be involved in corrosion processes has long been recognized (Little and Lee, 2007). Most attention has been given to immersion environments, with freshwater conditions and associated MIC mechanisms differentiated from those for seawaters. Relatively little attention has been given thus far to the potential for microbial activity to influence the atmospheric corrosion of steels and other metals. However,

Table 3

16S rDNA targeted metagenomics analysis of microorganisms deposited on steel at the different exposure sites showing the percentage of sequencing reads (relative abundance) for identified OTUs in each sample: (a) Ocean beach; (b) Harbour mouth; (c) Coastal suburb; (d) Fertilizer plant; (e) Dairy farm; (f) Turf farm and (g) Subalpine.

Genus/Species identification (Phylum)	Ocean Beach	Harbour mouth	Coastal suburb	Fertilizer plant	Dairy farm	Turf farm	Sub-alpine
Relative abundance (%)							
Actinomyces (Actinobacteria)		15.42	0.01				
Unclassified Dermabacteraceae (Actinobacteria)							1.09
Microbacterium (Actinobacteria)				0.29			
Unclassified Micrococcaceae (Actinobacteria)							0.01
Rhodococcus (Actinobacteria)							64.36
Unclassified Promicromonosporaceae (Actinobacteria)							1.92
Unclassified Actinomycetales (Actinobacteria)							16.61
Propionibacterium (Actinobacteria)	0.04	0.21			0.05		0.12
Porphyromonas (Bacteroidetes)		0.03					
Unclassified Chitinophagaceae (Bacteroidetes)			1.36				
Unclassified Bacillaceae (Firmicutes)		1.05				0.01	
Unclassified Bacillaceae (Firmicutes)							
Bacillus (Firmicutes)		12.47	0.01	99.55	0.01		
Bacillus thermoamylovorans (Firmicutes)					11.44		
Paenibacillus (Firmicutes)					0.98		
Paenibacillus lentimorbus (Firmicutes)				0.04			9.82
Unclassified Planococcaceae (Firmicutes)				0.01			
Rummeliibacillus (Firmicutes)						8.54	
Unclassified Gemellaceae (Firmicutes)		2.84					
Granulicatella (Firmicutes)		13.48					
Trichococcus (Firmicutes)			0.21				
Unclassified Enterococcaceae (Firmicutes)	0.01				0.04		
Enterococcus (Firmicutes)	33.56				30.80		
Streptococcus (Firmicutes)							
Unclassified Clostridiaceae (Firmicutes)							0.01
Clostridium (Firmicutes)						60.21	
Unclassified Lachnospiraceae (Firmicutes)						0.01	
Coprococcus (Firmicutes)			13.65				
Unclassified Ruminococcaceae (Firmicutes)				0.01			
Veillonella dispar (Firmicutes)		0.46					
Veillonella parvula (Firmicutes)		0.10					
Fusibacter (Firmicutes)		0.01					
unclassified Caulobacteraceae (Proteobacteria)		0.16	0.39				
Brevundimonas diminuta (Proteobacteria)		53.75	81.29	0.01	0.01	0.01	
Bradyrhizobium (Proteobacteria)				0.01			
Pigmentiphaga (Proteobacteria)							0.55
Acidithiobacillus albertensis (Proteobacteria)			0.01				
Shewanella algae (Proteobacteria)	0.01						
Unclassified Enterobacteriaceae (Proteobacteria)					30.03	0.01	
Unclassified Enterobacteriaceae (Proteobacteria)							
Erwinia dispersa (Proteobacteria)					11.89		
Unclassified Moraxellaceae (Proteobacteria)	66.33				0.01		
Pseudomonas (Proteobacteria)			0.01		39.20		
Vibrio (Proteobacteria)			0.01	0.01		0.01	0.02
Stenotrophomonas (Proteobacteria)					2.27		2.33

Fig. 3. Diversity profiling at the Phylum level for identified microorganisms deposited on steel exposed to the different test sites.

microorganisms are known to be present in the atmosphere as bio-aerosols and to deposit on surfaces (Round, 1981). Because this raises the possibility of MIC in atmospheric conditions, this research aimed to identify the microbial communities deposited on steel surfaces at different atmospheric conditions and thereby gain better understanding of the potential for MIC mechanisms for steel in the atmosphere. Defining and measuring the exact contribution of microorganisms to atmospheric corrosion through field studies is very challenging because of the logistical difficulty of setting up abiotic (sterile) controls.

Results from this study demonstrate that deposition of airborne microbes on steel readily takes place in atmospheric conditions and that the immediate surrounding environment plays a significant role in the microbial community formed on the corroding steel surfaces. This phenomenon is likely to be related to the action of local winds transporting and depositing locally-generated bio-aerosols onto metal surfaces (Bovallius et al., 1978) and to have parallels with observations for particulates such as salt particles (Morris and Nicot, 2014).

Corrosion of steel as measured by 3D surface profilometry was greater in locations with high chloride deposition, i.e. in coastal or near-coastal atmospheres. The least amount of corrosion was observed in the Sub-alpine atmosphere. These results are in agreement with previous studies on atmospheric corrosion and confirm the significance of chloride deposition on atmospheric corrosion of steel. As evident from Figs. 3 and 4, the dominant bacterial groups varied as a function of exposure conditions, however the identified microbes appeared to exhibit similar metabolic capabilities (as inferred by phylogeny and indicated by culture growth tests). A degree of correlation was observed between the species identified and results from microscopy and microbial growth tests.

Although DNA testing for algae was not carried out, microscope observations (Fig. 2) highlight the widespread deposition of algae onto steel at the different locations. This should not be surprising since, as noted, aerial transport of algae is a well-known phenomenon (Round, 1981). Algae have been widely identified in biofilms (Little and Lee, 2007) and it is likely that they, biofilms and bacteria all contribute to the creation of differential aeration cells on metal surfaces (Machuca et al., 2014). However, their individual and collective potential contribution to MIC in atmospheric environments remains an area for further investigation.

A detailed discussion on the microorganisms deposited at the

different atmospheric conditions is given below.

4.1. Coastal or near-coastal atmospheres

The Ocean beach site has been used for many years as an extremely aggressive marine atmospheric corrosion test site subjected to marine aerosols and high levels of salt deposition (Table 1). Based on observations by others (Santana Rodriguez et al., 2002) on the predominance of SRB on corroding steel exposed to marine atmospheres for one month, it was expected that similar microbes would be detected at the Ocean beach site. However, in the present investigation SRB were not identified. The observed dominant populations in this study (Family Moraxellaceae and Genus *Enterococcus*), are not known to be directly involved in the corrosion of steels. However, *Enterococcus* is a known lactic acid producer. The detection of acid production by culture growth tests is consistent with the reported capabilities of this genus. The unexpected observation of these species at this site may be the result of relatively recent changes at the corrosion test site. The corrosion racks were located some considerable distance from what was for many years a sewage treatment facility. However, recent expansion of the sewage treatment facilities has led to some filtration units and activated sludge tanks now being only some 20 m away from these racks. These observations suggest there was a stronger effect of aerosols from the sewage treatment plant on the microbial community deposited on steel at this location compared with those transmitted by marine aerosols.

The Harbour mouth site, located very close to the mouth of the Hunter River, is also expected to have much seawater aerosol deposition from the immediately adjacent Pacific Ocean. As noted, for the steel coupons exposed at this site *Brevundimonas diminuta*, from the Alphaproteobacteria, was the dominant species. This species was until recently classified as *Pseudomonas diminuta* and physiologically related to *Paracoccus denitrificans*, which has been considered to play a role in marine microbiological corrosion (Little and Lee, 2007). They are classified as strict aerobes. Alphaproteobacteria have been commonly identified in seawaters. For example, for North Atlantic seawater it was observed that 40–50% of the population belonged to Alphaproteobacteria (Agogu et al., 2011), the proportion depending on water depth (Friedline et al., 2012). Elifantz et al. (2013) reported Alphaproteobacteria as the most dominant polygenetic group in Mediterranean seawater at 26–48% of the microbial community. Other dominant group of

Fig. 4. Microbial diversity as a function of exposure site as measured by the Shannon Wiener Index.

microorganisms identified at the Harbour mouth site are the Firmicutes with the dominant species *Granulicatalla*. These species are commonly associated with soils and with human infections (Cargill et al., 2012) which may indicate there was an important contribution from aerosols generated from inland sources to the microbial community deposited on steel at this location. Neither Agogu et al. (2011) nor Elifantz et al. (2013) observed Firmicutes in seawater. However, the identification of the genus *Actinomyces* at the Harbour mouth is consistent with Elifantz et al. (2013) who observed 8–35% *Actinomyces* in Mediterranean seawater. *Actinomyces* are also often associated with bacterial activity in soils.

The Coastal suburb site, although further away from the Pacific Ocean, is subjected to local sea mists and moist saline films on surfaces and to considerable aerosol deposition. In this site, *Brevundimonas diminuta* (Alphaproteobacteria) and *Coprococcus* (Firmicutes) constituted the dominant species. *Coprococcus* are classified as facultative anaerobic bacteria able to produce fatty acids such as acetic acid and butyric acid through fermentation of hydrocarbons and often associated with human faeces (Holdeman and Moore, 1974). Acid production was confirmed for these microbes via culture growth tests. Sulphate-reducing bacteria were not identified in this site which is in agreement with results from the other coastal sites investigated. As for the Harbour mouth site, results from this test site suggest that aerosols generated from inland sources has a stronger influence on the microbial community developed on steel at these locations compared to marine aerosols. As earlier, although these are all sites in the coastal zone, they are at different distances from the ocean and potentially influenced in microclimate by local topographical features and anthropological influences. The potential effects of climate or seasons can be expected to be similar for all sites since the experiments were run in parallel.

It can be speculated that the absence of SRB in these sites, as opposed to findings from other coastal sites at different geographical locations, can be associated with differences in the layers of corrosion products formed on steel at the different locations. The thickness of the corrosion product layer has been shown to have an effect on the microbial community formed on corroding steel (Melchers and Jeffrey, 2008; Lanneluc et al., 2015). When the layers of corrosion products are thin, the corrosion rate is mainly controlled by the reduction of oxygen which can easily diffuse through the corrosion layers. Under these conditions, the activity of strict anaerobes such as SRB would be limited. Conversely, aerobes and facultative bacteria would be predominant. Anaerobic bacteria seem to flourish only when corrosion layers thicken and oxygen diffusion through the layers becomes restricted. Differences in structure and thickness of corrosion product layers in coastal environments at different geographical locations can be attributed to differences in the corrosivity of the environments and/or dissimilar exposure times for steel.

4.2. Near-industrial atmosphere

The Fertilizer plant site is located 1 km from Pacific Ocean and thus was considered likely to be exposed to seawater aerosols (Table 1). However, since this test site is adjacent to a facility where fertilizer chemicals are stored and processed, it was of particular interest to include it as part of this study. Coupons racks were specifically located approx. 10 m from the plant entry road that is used by large trucks conveying fertilizers into and out of the plant. Thus, this site does not correspond to a natural environment but it is a highly artificial one created by industrial activity.

Species associated with seawater aerosols were not detected. Instead the predominant species identified belongs to the Firmicutes and, within this group, to the genus *Bacillus*. This result

correlates well with microscopy analysis (Fig. 2) that showed extensive deposition of long rod-shaped bacteria in steel at this location. The predominance of *Bacillus* is usually associated with soil and aquatic environments, being active in moderate temperature and circum-neutral conditions. Species from this genus are known to produce fatty acids (Xu and Cote, 2003). This is also in agreement with the detection of acid-production in bacterial cultures from this site. Small traces of Proteobacteria and Actinobacteria were detected. These results again suggest that microbial composition on steel in this location was mainly influenced by aerosols from fertilizers and transporting trucks and not by seawater aerosols. Although the dominant species (genus *Bacillus*) are not commonly considered to contribute to corrosion of steels, they are known to be acid producers and EPS synthesizers which can potentially contribute to corrosion (Vlamakis et al., 2013). *Bacillus* species have also been described as iron-reducing bacteria (Emde et al., 1992).

4.3. Farming atmospheres

The Dairy farm site, located between holding pens in a Dairy facility and a local sewage treatment plant, showed the highest species diversity of all sites. Bacterial abundance on the steel coupons also was very high in this site. The dominant species corresponded to the genus *Pseudomonas*, an unclassified Enterobacteriaceae and *Erwinia dispersa*. The latter is a recognized species of soft-rot bacteria spread by aerial dispersion presumably by birds and insects (Harrison and Brewer, 1982). The Enterobacteriaceae are mostly facultative, acid producers and nitrate reducers, widely found in the gut flora of humans and animals, in soils and in water and on some plants (Begon, 2006). *Bacillus thermoamylovorans* was also a dominant species within this microbial community. *B. thermoamylovorans* have been previously isolated from dairy farms, including raw milk, straw or milking equipment (Coorevits et al., 2011). This species has been described as facultative anaerobes that grow optimally at 40–50 °C and produce a variety of fatty acids from fermentation. These results are also in agreement with culture growth tests for this site which detected strong microbial acid production. It appears evident that the presence of these bacteria on the coupon surfaces was the result of deposition of locally-generated aerosols.

At the Turf farm, the steel coupons contained almost entirely Firmicutes as the dominant group including the genera *Clostridium*, *Enterococcus* and *Rummeliibacillus*. These microorganisms can produce fatty acids such as acetic acid and butyric acid through fermentation. *Clostridium* is a relatively common anaerobe found in soils, water and decomposing plant and animal matter and in the human gut (Minton and Clarke, 2013; Cato and Stackebrandt, 2013). Clostridiaceae strains have been found in rust layers of corroding steel and have been shown to have iron-reducing capabilities (Duan et al., 2008) (Emde et al., 1992). Acid production via microbial growth tests was also confirmed for microbes recovered from this location.

4.4. Sub-alpine atmosphere

At the remote Sub-alpine site, the microbial community on steel mainly contained Actinobacteria, a small amount of Firmicutes and some Gammaproteobacteria. The long-branching filamentous structures deposited on steel observed via microscopy, are very likely to correspond to Actinobacteria hyphae. *Rhodococcus* was the predominant genus in the Actinobacteria group. The members of this genus usually are associated with soils and water occurring as part of the natural environment. Cold deserts have been shown to harbour Actinobacteria as one of the major phylogenetic groups.

Results from this study showed that microorganisms recovered from corroding steel at the different sites have the common ability to produce acids and EPS through their metabolism. This suggests that microbial acid production and EPS synthesis can be important MIC mechanisms for steel under atmospheric conditions. The possible contribution of acid-producing bacteria (APB) to atmospheric (and marine) corrosion has not previously been recognized. To date, APB have been associated only with corrosion in oil production systems (Gu and Galicia, 2012). Acidic microbial metabolites include organic acids such as volatile fatty acids (VFA), e.g. lactic acid and acetic acid, which are known to accelerate corrosion of iron via cathodic reduction of protons. Within patchy biofilms, local acidity can lead to concentration cells and pitting corrosion. Areas underneath biofilms containing acid-producing bacteria have been reported to have pH as low as 2.0 caused by the protons released by the organic acids (Dexter and Chandrasekaran, 2000). Free VFAs such as acetic acid can continuously supply protons that are consumed by corrosion (Xua et al., 2016). The role of microbial EPS on corrosion has been reviewed by Beech et al. (2005). Microbial EPS has been described as a complex matrix containing proteins, polysaccharides, lipids and nucleic acids. It not only confers structural support to biofilms on surfaces but it also provides a matrix for nutrient concentration and a barrier for protection against harsh environments. Previous studies have shown that EPS can accelerate corrosion by trapping metal ions that catalyse cathodic reactions and by enhancing the formation of concentration cells. The importance of EPS in microbial adhesion to surfaces is widely recognized.

5. Conclusions

sites have common acid-producing and EPS synthesising capabilities. Sulphate-reducing bacteria were not detected in any of the sites. This suggests that microbial acid production and EPS synthesis can be important MIC mechanisms for steel under atmospheric conditions. The possible contribution of acid-producing bacteria (APB) to atmospheric corrosion has not previously been recognized.

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Further reading

NCBI sequence Read Archive database: for submission of the original paired Illumina Miseq reads: **SRA accession:** SRP074858 Temporary Submission ID: SUB1509383 Release date: 2016-05-11