Published in final edited form as:

J Mater Eng Perform. 2016 September ; 25(9): 3580–3589. doi:10.1007/s11665-016-2231-0.

Anticorrosive influence of *Acetobacter aceti* biofilms on carbon steel¹

Danielle Cook France[#]

National Institute of Standards and Technology, Boulder, CO, USA

Abstract

Microbiologically influenced corrosion (MIC) of carbon steel infrastructure is an emerging environmental and cost issue for the ethanol fuel industry, yet its examination lacks rigorous quantification of microbiological parameters that could reveal effective intervention strategies. To quantitatively characterize the effect of cell concentration on MIC of carbon steel, numbers of bacteria exposed to test coupons were systematically controlled to span four orders of magnitude throughout a seven-day test. The bacterium studied, *Acetobacter aceti*, has been found in ethanol fuel environments, and can convert ethanol to the corrosive species acetic acid. *A. aceti* biofilms formed during the test were qualitatively evaluated with fluorescence microscopy, and steel surfaces were characterized by scanning electron microscopy. During exposure, biofilms developed more quickly, and test reactor pH decreased at a faster rate, when cell exposure was higher. Resulting corrosion rates, however, were inversely proportional to cell exposure, indicating that *A. aceti* biofilms are able to protect carbon steel surfaces from corrosion. This is a novel demonstration of corrosion inhibition by an acid-producing bacterium that occurs naturally in corrosive environments. Mitigation techniques for MIC that harness the power of microbial communities have the potential to be scalable, inexpensive, and green solutions to industrial problems.

Keywords

Microbiologically influenced corrosion; *Acetobacter aceti*; ethanol; corrosion inhibition; carbon steel; biofilms

Introduction

Corrosion is a massive problem facing the industrialized world, arising as a result of material interactions with the environment. Microbes are an undeniable and often highly active element of those environments. General corrosion costs can be on the order of 3–4 % of the gross national product of an industrialized nation, including the United States¹. About half of the total cost is attributed to microbiologically influenced corrosion (MIC), meaning that

¹Contribution of NIST, an agency of the US government; not subject to copyright in the United States.

[#]Address correspondence to Danielle Cook France, danielle.france@nist.gov.

Conflict of Interest

No conflict of interest declared.

billions of dollars are associated with MIC annually². One industrial niche currently experiencing a rising incidence of MIC is that of ethanol fuel suppliers³. As a result of the relatively high affinity of ethanol for water, the ethanol fuel industry may face far more expensive corrosion issues than the traditional fuel industries from which it inherits many industrial practices, such as the use of carbon steel for storage and transportation infrastructure⁴. As ethanol fuel production and infrastructure needs expand⁵, MIC-associated costs could increase further.

Many studies have demonstrated accelerated metal corrosion rates in the presence of microbial flora,^{2b, 6} while others have shown the opposite effect: that certain microbial biofilms are able to protect surfaces from corrosion⁷. Even the same bacterial species have been implicated as corrosive or protective, depending on the study conditions⁸. Sulfate-reducing bacteria do not feature prominently in MIC inhibition studies, but iron-reducing bacteria (primarily species of *Shewanella*) have been implicated in both destructive and protective roles^{1a}. *Shewanella oneidensis* MR-1, a facultative anaerobe, protected carbon steel from corrosion through both its direct consumption of oxygen and its reduction of Fe(III) to diffusible Fe(II)⁹. Fe(II) will readily combine with oxygen in solution, thereby serving as an additional sink for oxygen and slowing oxygen-induced corrosion. The iron reducer was able to protect surfaces from corrosion even in a mixed community with sulfate reducing bacteria¹⁰. Similarly, an iron-reducing consortium isolated from a sour oil well, including close relatives of *Shewanella oneidensis*, was recently shown to lower the corrosion rate of carbon steel by a factor of five and to reduce the extent of pitting¹¹. Extensive work has been conducted on the protective effects of *Pseudomonas* spp., *Bacillus* spp., and *E. coli* strains, including a broad survey of fifteen different bacteria which revealed that better biofilm formers decreased corrosion rates most markedly compared to a control¹².

Whether a biofilm community is corrosive or protective depends on a number of factors, including but not limited to species type, nutrient availability, reduction potentials, and electron acceptors^{2c, 13}. The picture that has emerged of a protective biofilm is one that shows continuous spatial coverage and high numbers of actively respiring aerobic cells¹⁴, has specific competition with sulfate reducers (e.g., “biocompetitive exclusion” with nitrate supplementation)⁸, or actively produces antimicrobial agents against sulfate reducers¹⁵. Where biofilms and corrosion intersect, actionable understanding requires consideration of the microbial ecosystem as a whole, and the different roles that microbes can assume given the present environmental conditions.

For the particular ethanol fuel environments considered here, some sampling has already revealed relevant microbial participants. Williamson and colleagues conducted a broad survey of different fuels including E10, E85, fuel grade ethanol, and ethanol within production storage tanks^{3, 16}. *Acetobacter* and *Shewanella* species, as well as sulfate reducing bacteria and members of *Halomonas*, *Pseudomonas*, and *Corynebacterium* were identified. Notably, sequencing from DNA compared well with that from transcribed RNA, indicating that the identified communities represent prevalent and active microbes from these environments¹⁶. Specifically worth noting is that the metabolism of *Acetobacter aceti* aligns well with the sequencing metadata. *Acetobacter* and *Gluconacetobacter* were dominant in samples from tanks with pH less than five, implicating these bacteria in

acidification of the tank samples through conversion of ethanol to acetic acid. *A. aceti* is able to generate ATP from the incomplete oxidation of ethanol to acetate, and generally the acetic acid accumulates as a dead-end metabolite which can be pumped out of the cells¹⁷.

Following these microbial sequencing studies, *Acetobacter aceti* and sulfate-reducing bacteria from ethanol storage tanks were cultivated and shown to worsen the degree of stress corrosion cracking of steel over abiotic conditions¹⁸. In addition, headspace corrosion assays showed that acetic acid production by the same microbes led to severe corrosion of both copper and steel. Interestingly, however, the corrosion rate of carbon steel in solution with *A. aceti* was lower than in the headspace above the same solution, and the authors observed (possibly protective) biofilms on the surfaces of immersed steel coupons¹⁹. Results presented here show, for the first time, the potential for *A. aceti* to inhibit, rather than accelerate, corrosion of carbon steel in the presence of ethanol. While these results are not intended to demonstrate that the laboratory phenomena observed here are occurring in the natural environment, they do hold promise for further development of a protective biofilm mitigation strategy by an organism that is prevalent in natural environments, and that generates a corrosive metabolite at the same time as providing a protective barrier.

Given a simplified ecosystem with *A. aceti* as the only microbe present, the following hypothesis was tested: that the rate of corrosion of a steel coupon is a function of the number of *A. aceti* cells exposed to its surface. Quantifying numbers of cells in a growing system, particularly numbers of viable or non-viable cells within a biofilm on a surface, is neither easy nor straightforward. Every cell counting method has its limitations. For this reason, rather than exposing coupons to *A. aceti* and counting cells after corrosion has occurred, the number of cells delivered to coupons was carefully managed. In this way cellular exposure becomes an independent and controlled input variable rather than an observed quantity subject to limitations of measurement techniques. Cells were delivered by a fluidic pump system that enabled precise relative control of the microbial exposure. Corrosion rate was quantified by mass loss measurements of carbon steel coupons. The results show that high numbers of *A. aceti* delivered to a steel surface can form a biofilm that decreases corrosion rate.

Material and methods

Experimental setup

An *Acetobacter aceti* environmental isolate, termed ECT.1 (isolated from an Ethanol Containment Tank), was obtained from Chase Williamson and John Spear at the Colorado School of Mines. Details of the isolation can be found in Williamson's thesis¹⁶. The culture was maintained in a growth medium consisting of 0.5 g/L yeast extract, 0.3 g/L proteose peptone, 1 g/L NaCl, and 5 % ethanol by volume. Media components other than ethanol were mixed in ultrapure water and autoclaved for 30 minutes at 121 °C. Ethanol was filter-sterilized through a 0.2 µm PVDF membrane and added to the medium after it cooled. Batch cultures were used to seed a constant biomass bioreactor, which was continuously replenished with fresh medium as part of the fluidic delivery system used for the experiments. When needed for dilution, as described below, a water-based medium, termed ECW (Ethanol Contamination Water), was used. ECW was designed as a reasonable

approximation of the water phase that may contaminate an ethanol fuel system and consists of the following trace minerals in addition to 1 g/L NaCl and 5 % ethanol by volume: 11 $\mu\text{mol/L}$ K_2HPO_4 , 7 $\mu\text{mol/L}$ KH_2PO_4 , 6.8 $\mu\text{mol/L}$ NaCl, 11.8 $\mu\text{mol/L}$ NaNO_3 , 0.33 mmol/L MgSO_4 , 0.29 mmol/L CaSO_4 , 1.43 mmol/L NaHCO_3 , 87.6 $\mu\text{mol/L}$ $(\text{NH}_4)_2(\text{SO}_4)$, and 0.37 mmol/L NH_4Cl . This formulation was derived from reported compositions of rain water, ground water, and tap water²⁰. Minerals were dissolved in ultrapure water, and pH was adjusted to between 7.0 and 7.2 using 0.1 mol/L HCl. ECW was 0.22 μm filter-sterilized prior to use.

Control of cellular exposure to carbon steel coupons was done in a relative manner, using the fluidic system shown in Figure 1. Fluidic connections were made using Tygon R3603² tubing run through parallel channels on the same 24-channel peristaltic pump (Ismatec IPC). Vessels, including the constant biomass reactor, the four mixing tanks, and the four coupon plates, were supplied with a pumped input stream and maintained at constant volume by allowing overflow of effluent liquids into a secondary containment vessel. First, growth medium was pumped into the biomass reactor (volume = 122 mL) at a constant rate (two lines at 0.165 mL/min each), thereby continuously diluting an *A. acetii* source culture and maintaining cell concentration at a somewhat constant value. The biomass reactor was continuously aerated using an aquarium air pump. Initial inoculation of the constant biomass reactor was 40 mL of a 3-day-old batch culture combined with 40 mL of fresh growth medium. The reactor was run in constant dilution for ~ 24 hours before connecting to the coupon exposure plates, allowing time for the growth rate to balance with the dilution rate of the reactor.

From the biomass reactor, three different lines pumped cells to three different mixing tanks. Through the use of tubing of various internal diameters (where on the peristaltic pump the internal diameter determines the volumetric flow, given the same pump rotation rate for all tubing), it was possible to mix these cell inputs with ECW in set ratios relative to each other. A fourth mixing tank pulled from the second mixing tank as its cell source, thereby performing a secondary dilution. The mixing tanks were set up to cover four orders of magnitude of cell concentration. The numbers designated in Figure 1 refer to the fractional composition of each mixing tank coming from the biomass reactor; *i.e.*, the 0.985 mixing tank was composed of 98.5 % cell input from the biomass reactor and 1.5 % ECW input. Other cell fractions achieved were 0.119, 0.015, and 0.0018. This system could tolerate some variability in the absolute cell concentration in the biomass reactor, since the relative fractions delivered to each mixing tank and therefore each set of steel coupons remained constant throughout the tests. The above fractions can be multiplied by the biomass level in the reactor to obtain the biomass load delivered to each set of steel coupons at any given time.

From each of the four mixing tanks, solutions were delivered through an inlet in the side wall of a plastic petri dish containing a number of carbon steel coupons. Coupons were

²Commercial equipment, instruments, or materials are identified only in order to adequately specify certain procedures. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.

arranged radially in an 85 mm diameter round plastic petri dish with randomized distances from the flow inlet. Fifteen to twenty-four coupons were exposed in each dish, allowing for at least triplicate samples to be removed after each measurement time interval (for final time points, up to five coupons were removed). Inlet flow impinged from a hole in the side of the petri dish at a flow rate of 0.2 mL/min. Outlet flow from the petri dishes was by overflow into the surrounding secondary container. Since all four dishes were housed in the same secondary container, the atmospheric exposure of each dish (i.e., oxygen supply to the surface of the solution) can be taken to be the same.

Circular coupons were punched from a roll of low carbon steel shim stock (grade 1008 – 1010, conforming to ASTM standard A109, McMaster Carr). Elemental composition of these steel grades is the following, by weight percentage: 0.08 % – 0.13 % C, 0.30 % – 0.60 % Mn, 0.04 % P, 0.05 % S, with the balance being Fe. Coupon thickness was $25.4 \mu\text{m} \pm 2.54 \mu\text{m}$, and diameter was approximately 6.5 mm. Coupons were cleaned by sonication in acetone for five minutes, followed by three rinses in ultrapure water and one rinse in 200 proof ethanol. After air drying, each coupon was weighed for its initial mass and stored under vacuum until needed for testing.

The presence of grain structure in the carbon steel shim stock was verified by polishing and etching a cleaned, unexposed coupon. The coupon was polished through a sequence of 1200 grit sand paper, 2400 grit sand paper, 3 μm diamond polish, and 1 μm paper. It was then etched in a 2 % nital bath for 60 seconds with light agitation, rinsed with water and ethanol, and blown dry. Optical light images were collected at a magnification of 50X. The grain structure can be seen in Figure 2. A ferritic microstructure is exhibited.

Data collection

Throughout the experiment, both pH and optical density (at 600 nm) were monitored in the constant biomass reactor and in each coupon dish. pH was measured with a refillable saturated KCl electrode linked to a benchtop meter. The instrument was calibrated daily with buffers at pH 1.68, 4, 7, and 10. Relative accuracy of the instrument is 0.002 pH units, reproducibility is ± 0.001 pH units, and measurements were taken with a resolution of 0.01 pH units. Optical density measurements were taken at 600 nm on a UV-visible spectrophotometer. A few seconds were allowed at each measurement point for the reading to stabilize before recording, and recordings were taken with a resolution of 0.001 absorbance units. At each time point, three coupons were removed from each petri dish and subjected to the following analysis:

1. Live/dead cell assay: Excess liquid was blotted from the underside of the coupon, and 30 μL of dye solution were immediately applied to the surface. Dye solution was made up of 1 g/L NaCl containing 5 $\mu\text{mol/L}$ SYTO9 and 30 $\mu\text{mol/L}$ propidium iodide (from Live/Dead BacLight Bacterial Viability Kit L7012, Molecular Probes®). After a five minute incubation, the coupon was inverted onto a coverslip and imaged at 30X with an inverted microscope and cooled CCD camera. GFP and TRITC filter sets were used to visualize the SYTO9 (“live”) and propidium iodide

(“dead”) cells, respectively. Images were false-colored and merged in RS Image software and calibrated using ImageJ software²¹.

2. Mass measurement: After fluorescence imaging, coupons were allowed to air dry, and a mass measurement was taken on a balance with 0.01 mg readability. This measurement includes corrosion products.
3. Cleaning: Coupons were immersed in ultrapure water and sonicated for five minutes to remove corrosion products. This relatively gentle cleaning procedure was selected with regards to the thinness of the coupons and visual observations that visible rust-colored corrosion products were removed in sonication. They were then rinsed in 100 % ethanol and allowed to air dry.
4. Mass measurement: A final mass measurement was taken after cleaning, to indicate the final mass of the coupon after removal of corrosion products. Coupons were stored under vacuum until examined by SEM.
5. Scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDS): Cleaned coupons were mounted on carbon tape for imaging but no further specimen preparation was performed. Images were collected on a JEOL JSM-6100 SEM equipped with a Princeton Gamma-Tech Prism 2000 EDS detector. Accelerating voltage was 15 kV. Working distance was 30 mm for 12X images, 12 mm for 500X and 3000X images, and 12 mm for EDS collection.

At various stages, photomicrographs of some coupons were obtained using a stereo microscope and digital camera.

Two separate cellular exposure tests were run, each lasting for one week. At the close of the first test, cells were collected by centrifugation from a 15 mL sample taken from the bioreactor. Following mechanical lysis the sample was boiled at 100 °C for five minutes in 0.1 % Triton X. Direct PCR of the 16S rRNA gene used primers 27F²² and 805R²³. The amplified DNA sample was sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core. Sequence results were compared to the Ribosomal Database Project²⁴ and the amplified DNA was confirmed to come from *A. aceti*, with a seqmatch score of $S_{ab} = 0.989$ to the *A. aceti* type strain

Analysis

The *A. aceti* cell exposure of any given coupon was calculated from optical density measurements taken from the biomass reactor in the following manner: Cell exposure = (area under OD_{600nm} versus time curve) × cell fraction (i.e., [0.0018, 0.015, 0.119, or 0.985]) × exposure time × flow rate. This yields a number for cell exposure in units of absorbance units × mL. Since optical density is proportional to the cell concentration (cells per milliliter), this cell exposure number is proportional to the total number of cells exposed to the coupon surface over the course of the week-long test.

In the case of abiotic control tests, where cellular exposure is not applicable, hydrogen ion exposure was calculated instead. Hydrogen ion exposure was calculated from pH measurements in the coupon dishes of the *A. aceti*-containing test, in a similar manner to the cell exposure calculation; i.e., H^+ exposure = (area under $[H^+]$ versus time curve) $\times$ exposure time. This yields a number for hydrogen ion exposure in units of (mole/liter) $\times$ hour which was then used as a basis for comparing biotic and abiotic corrosion rates.

Corrosion rate, in millimeters per year, was calculated as rate = $W / (ATD)$, where W is mass loss, T is exposure time, A is coupon surface area (33.2 mm²), and D is coupon density (7.85 g/cm³), according to ASTM standard G31-72.

Control experiments

Control coupons were exposed to the same fractions of growth medium as described above, but in the absence of bacteria. Both flowing and static tests were conducted, with hydrogen ion exposures and total fluid volumes matched to the *A. aceti* tests; i.e., the pH values for the test solutions were chosen such that the integrated H^+ exposure over the course of the test was equivalent to that measured in the bulk fluid (i.e., above and not below the biofilm) of the flowing tests with bacteria. Low pH values for the test solutions were then achieved through the addition of 1 mol/L acetic acid to the 0.0018, 0.015, 0.119, and 0.985 growth medium/ECW blends. Flowing control test solutions also included 0.1 % glutaraldehyde to inhibit bacterial contamination. Glutaraldehyde has been shown not to affect steel corrosion at concentrations significantly higher than 0.1 %²⁶. The control test flow setup was simpler than the *A. aceti* test since media fractions could be pre-mixed in bulk for flow directly into the coupon exposure dishes.

For the static control test, coupons were immersed in 100 % ethanol for a few minutes before placement into test vessels, as a sterilization technique. In order to match the hydrogen ion exposure (in terms of (mole/liter) $\times$ hour) to the *A. aceti* test with a minimal amount of handling, three solutions were mixed for each media fraction, each with a different pH. Table 1 shows pH values of the solutions mixed and the time that coupons were exposed to each. These values integrate to similar hydrogen ion exposure values as the flowing tests for each cell/media fraction.

Results

Periodic sampling of microbial environments in the cellular exposure tests revealed behavior consistent with expectations of *A. aceti* growth and metabolism. Laboratory batch cultures of the ECT.1 isolate in the described medium, grown at room temperature and shaking at 60–100 rotations per minute, show a doubling time in logarithmic growth of approximately 5.8 hours (data not shown). The duration of the logarithmic growth phase is approximately 48 hours at this growth rate. In the constant biomass reactor, both pH and cell density were maintained at relatively constant levels (pH $\approx$ 4, OD_{600nm} $\approx$ 0.01) by continuous dilution with new growth medium. Given the bioreactor dilution rate of 0.33 mL/min, the *A. aceti* source culture maintained in the bioreactor was growing with a doubling time of approximately 4.3 hours. Figure 3 shows pH and OD_{600nm} measurements taken from the bioreactor throughout the experiment.

As the *A. aceti* culture was diluted and delivered to the metal test coupons, cells began to attach to the coupon surfaces, establishing adherent biofilms that began to drop the pH of the dish solution. Figure 4 shows plots of the pH of each dish over time. The dishes receiving lower cell fractions remained at a pH above 7.5 (corresponding to the pH of the ECW/growth medium mix without cells) for a few days before the pH decreased, while pH in the dishes receiving higher cell fractions decreased by more than one pH unit within hours. After a week, the pH reached values less than 4 in all four dishes.

Fluorescence micrographs of live/dead stained biofilms on coupon surfaces throughout the course of the experiment showed earlier and more comprehensive surface coverage of the coupons with higher cell fractions. Figure 5 shows the time course of biofilm development on coupons with the highest cell exposure at the end of the 171 hour *A. aceti* test. Figure 6 shows representative live/dead fluorescence images taken at the end of the week-long exposure, when the lowest cell fraction (0.0018) coupons showed intermittent, spotted coverage with primarily live cells, and the highest cell fraction (0.985) coupons showed a continuous biofilm with a mix of live and dead cells.

Visual observations of the coupons showed development of typical rust-colored corrosion products, although the coverage of this corrosion product was considerably heavier on the low cell fraction coupons. High cell fraction coupons remained almost free of rust-colored corrosion product throughout the test while in solution. Upon drying, some corrosion products were apparent on the high cell fraction coupons. Figure 7 shows three replicate coupons taken from each of the four cell fraction dishes after 146 hours of exposure. Coupons were allowed to dry in air without removal of corrosion products. Coupon appearance is consistent across replicates and noticeably dependent on cell exposure.

After cleaning to remove corrosion product, coupons were weighed, and mass loss was used to calculate the corrosion rate of each coupon under the various cell exposure conditions. Data from the two separate tests, along with the control tests, are compiled in Table 2. In Figure 8 the data for the *A. aceti* tests are plotted to show the dependence of corrosion rate on the number of cells exposed to a coupon surface. Higher cell exposures resulted in lower corrosion rates. A 1000-fold increase in cell exposure resulted in an approximately 10-fold reduction in corrosion rate. A logarithmic fit to the data shows a strong inverse dependence of corrosion rate (y) on cell exposure (x): $y = -0.024\ln(x) + 0.1245$ with a correlation coefficient of $R^2 = 0.88$.

Scanning electron microscopy of the cleaned coupon surfaces also revealed different corrosion morphologies according to cell exposure level. As shown in Figure 9, coupon surfaces with low cell exposure and higher corrosion rate showed noticeable pitting. In contrast, no pitting was observed on the 0.985 cell fraction coupon images.

As a control, the effects of high acetic acid concentrations without the influence of biological factors were examined in both flowing and static control tests. In contrast to the results seen with cells present, these tests showed a relatively constant corrosion rate across the range of hydrogen ion exposures tested. Figure 10 compares the results of the *A. aceti* tests and the abiotic controls on the same hydrogen ion exposure axis. After grouping results

by media fraction and presence/absence of cells, a folded F test was used to test equality of the group variances. Variances were found to be equal between the *A. aceti* and control groups at each media fraction, allowing the use of two-sided equal variance t tests to determine whether the same group means were significantly different. The t tests showed that the presence or absence of *A. aceti* did not significantly affect the mean corrosion rate at a media fraction of 0.0018, where the number of cells delivered to steel coupon surfaces was very low. For all other media fractions, the t tests showed significant differences ($p < 0.005$) between the mean corrosion rates with and without cells: [media fraction, p-value] = [0.0018, $p = 0.3934$; 0.015, $p = 0.0021$; 0.119, $p < 0.001$; 0.985, $p < 0.001$].

Discussion

Taken together, the results shown here indicate that an *A. aceti* biofilm is able to protect a carbon steel surface from corrosion even while producing a corrosive species like acetic acid. By varying the number of cells delivered to steel surfaces, the time scales of biofilm development and acetic acid production were varied over orders of magnitude. Higher delivered cell numbers led to more biofilm coverage in shorter times, and lower pH due to acetic acid production. Corrosion rate showed a strong inverse dependence on cell exposure.

A priori expectations might be that lower pH would lead to an increase in corrosion rate, as has been shown in abiotic steel corrosion tests²⁷, and in tests contrasting the presence versus absence of *A. aceti* and biologically-produced acetic acid¹⁸. Sowards *et al.* demonstrated a sharp increase in stress corrosion cracking in the presence of *A. aceti*, relative to abiotic controls that were close to neutral pH with no exogenous acetic acid present. For tests in the presence of *A. aceti*, cells were introduced to the steel at relatively high numbers (approximately 10^7 cells / milliliter) in solutions that were already at low pH (3.3 – 3.6) due to produced acetic acid¹⁸. These test conditions may not have allowed the formation of a protective biofilm before the steel was exposed to the highly corrosive test solution. In contrast, in the *A. aceti* tests described here, test conditions allowed biofilms to form on the steel concurrently with acetic acid production, such that there was an opportunity for a protective biofilm to form before test solutions reached very low pH. Rather than an increase in corrosion rate for low pH (and high cell count) test solutions, a decrease in corrosion rate was seen. Control tests were designed to isolate the effect of cells as much as possible, by lowering control pH using acetic acid at concentrations matched to those seen in experiments containing cells. Control tests confirmed that reductions in corrosion rate were due to the presence of cells and not other differences in media composition. Within the control tests, however, the corrosion rate remained relatively constant across different levels of hydrogen ion exposure (and different media fractions), when it might have been expected to increase with higher H^+ exposure. Some cloudiness was observed in the high media fraction dish of the flowing control test by the end of a week. It was suspected (although not determined) that this cloudiness resulted from precipitation of some media components and not any significant bacterial growth. No biofilms or cloudiness were visible on the control test coupons themselves. The effect of different media compositions alone on corrosion rate was tested (*i.e.*, 0.985, 0.119, 0.115, and 0.0018 ratios of media to ECW without any added acetic acid to drop pH; data not shown) and no significant correlation between media fraction and corrosion rate was seen after 143 hours of exposure. The decrease in corrosion

rate seen in these experiments is therefore attributable to active formation of protective biofilms on the surface, and not to other experimental variables.

A few mechanisms have already been proposed to explain MIC inhibition, including neutralization of corrosive substances (e.g., oxygen consumption within the biofilm) and provision of a physical protective barrier (e.g., extracellular polysaccharides) preventing contact of corrosive substances with the surface^{13b, 14b}. The first mechanism, oxygen consumption, is likely to be at play in this study. *A. aceti* is an obligate aerobe that can couple incomplete oxidation of ethanol to oxidative phosphorylation, thereby consuming oxygen and depleting it from the medium. The oxygen profile within an actively respiring biofilm can create anaerobic conditions within one hundred micrometers of an aerobic interface²⁸. It is hypothesized that *A. aceti* biofilms may protect the steel surface from corrosion by oxygen, through consumption of oxygen through the depth of the film. This hypothesis would need to be confirmed by measurements with a dissolved oxygen probe, which were beyond the scope of this study.

The presence of a diffusion-inhibiting protective barrier, formed by cells and secreted extracellular polysaccharides, is also likely to be a factor. However, such a barrier could act in two opposing ways: it could either limit the concentration of acetic acid reaching the metal interface, or increase it by hindering its dispersion into the bulk fluid. Since pH measurements were taken from the bulk fluid phase overlying the biofilm in the test dishes, it is not known what the pH within the biofilm or at the metal surface may have been. Imaging of pH-sensitive dyes or nanoparticles²⁹ entrained in the biofilm matrix and near the metal surface would help to elucidate major source points of acetic acid within the biofilm and any pH gradients that arise as a result. The biofilm may also provide a physical barrier that stabilizes protective iron oxides formed at the metal surface; this is another hypothesis that can be tested with future work involving imaging and elemental analysis of the metal-biofilm interface.

Many complicating factors must be addressed before corrosion-inhibiting biofilms are ready for field deployment. Not least of these are an understanding of biofilm coverage on the macroscale (both area and thickness), which has been described as stochastic⁸, and an understanding of competition and population dynamics among the microbial participants of a protective biofilm. However, when compared to other corrosion control strategies (e.g., protective coatings, corrosion inhibitors, oxidizing and non-oxidizing biocides, anodic and cathodic protection, or corrosion resistant metals and alloys), protective biofilms provide many potential advantages. If implemented correctly, these “material probiotics” could protect infrastructure as well as the environment while providing a cost savings over other methods.

Acknowledgments

The author would like to thank Jeff Sowards and Elisabeth Mansfield for establishing support for this work at NIST, and for helpful planning discussions. In addition, Jeff Sowards, Teresa Kirschling, William Cordell, Emma Schwartz, and Jolene Splett provided assistance in the laboratory or with data analysis. John Spear and Chase Williamson of the Colorado School of Mines provided insights and the *A. aceti* environmental isolate. The author was funded by a National Research Council Research Associateship at NIST. The University of Colorado Cancer

Center DNA Sequencing and Analysis Core is supported by a NIH/NCI Cancer Center Core Support Grant (P30 CA046934).

References

1. (a) Javaherdashti, R. Microbiologically Influenced Corrosion: An Engineering Insight. Springer-Verlag; London: 2008. (b) National Research Council, C. o. A. C. E. Assessment of Corrosion Education. The National Academies; Washington, DC: 2009. p. 12
2. (a) Koch, GH.; Brongers, MPH.; Thompson, NG.; Virmani, YP. U. S. D. o. Transportation. 2002. Corrosion costs and preventive strategies in the United States. (b) Little, B.; Lee, J. Microbiologically Influenced Corrosion. John Wiley & Sons; Hoboken, New Jersey: 2007. (c) Lin J, Ballim R. Biocorrosion control: Current strategies and promising alternatives. African Journal of Biotechnology. 2012; 11(91):15736–15747.
3. Jain L, Williamson C, Bhola SM, Bhola R, Spear JR, Mishra B, Olson DL, Kane RD. Microbiological and electrochemical evaluation of corrosion and microbiologically influenced corrosion of steel in ethanol fuel environments. Corrosion 2010. 2010
4. (a) Kane, RD.; Maldonado, JG.; Klein, LJ. Corrosion 2004. NACE International; 2004. Stress corrosion cracking in fuel ethanol: a newly recognized phenomenon. (b) Jain S. Ethanol-water phase separation. What it is -- and what you can do. PEI Journal 2010, (Fourth Quarter 2010). :48–54.(c) Bhola SM, Bhola R, Jain L, Mishra B, Olson DL. Corrosion behavior of mild carbon steel in ethanolic solutions. JMEPEG. 2011; 20:409–416.
5. Renewable Fuels Association, B. D. Falling Walls and Rising Tides: 2014 Ethanol Industry Outlook. Renewable Fuels Association; Washington, DC; St Louis, MO; Omaha, NE: 2014.
6. (a) Peng CG, Park JK. Principal factors affecting microbiologically influenced corrosion of carbon steel. Corrosion Science. 1994; 50(9):669–675.(b) Almahamedh HH, Spear JR, Olson DL, Williamson C, Mishra B. Identification of microorganisms and their effects on corrosion of carbon steel pipelines. Corrosion 2011. 2011
7. (a) Morikawa M. Beneficial biofilm formation by industrial bacteria. Bacillus subtilis and related species Journal of bioscience and bioengineering. 2006; 101(1):1–8. [PubMed: 16503283] (b) Ghafari MD, Bahrami A, Rasooli I, Arabian D, Ghafari F. Bacterial exopolymeric inhibition of carbon steel corrosion. International Biodeterioration & Biodegradation. 2013; 80:29–33.
8. Little B, Lee J, Ray R. A review of 'green' strategies to prevent or mitigate microbiologically influenced corrosion. Biofouling. 2007; 23(1–2):87–97. [PubMed: 17453733]
9. (a) Dubiel M, Hsu CH, Chien CC, Mansfeld F, Newman DK. Microbial iron respiration can protect steel from corrosion. Applied and Environmental Microbiology. 2002; 68(3):1440–1445. [PubMed: 11872499] (b) Lee AK, Newman DK. Microbial iron respiration: impacts on corrosion processes. Applied microbiology and biotechnology. 2003; 62(2–3):134–9. [PubMed: 12734693]
10. Lee AK, Buehler MG, Newman DK. Influence of a dual-species biofilm on the corrosion of mild steel. Corrosion Science. 2006; 48(1):165–178.
11. AlAbbas FM, Bhola SM, Spear JR, Olson DL, Mishra B. The shielding effect of wild type iron reducing bacterial flora on the corrosion of linepipe steel. Engineering Failure Analysis. 2013; 33:222–235.
12. (a) Jayaraman A, Cheng ET, Earthman JC, Wood TK. Importance of biofilm formation for corrosion inhibition of SAE 1018 steel by axenic aerobic biofilms. Journal of Industrial Microbiology & Biotechnology. 1997; 18:396–401. [PubMed: 9248069] (b) Jayaraman A, Earthman JC, Wood TK. Corrosion inhibition by aerobic biofilms on SAE 1018 steel. Applied microbiology and biotechnology. 1997; 47:62–68.(c) Jayaraman A, Sun AK, Wood TK. Characterization of axenic Pseudomonas fragi and Escherichia coli biofilms that inhibit corrosion of SAE 1018 steel. Journal of Applied Microbiology. 1998; 84:485–492. [PubMed: 9633647] (d) Ismail KM, Gehrig T, Jayaraman A, Wood TK, Trandem K, Arps PJ, Earthman JC. Corrosion control of mild steel by aerobic bacteria under continuous flow conditions. Corrosion. 2002; 58(5): 417–423.
13. (a) Little B, Ray R. A perspective on corrosion inhibition by biofilms. Corrosion. 2002; 58(5):424–428.(b) Zuo R. Biofilms: strategies for metal corrosion inhibition employing microorganisms. Applied microbiology and biotechnology. 2007; 76(6):1245–53. [PubMed: 17701408] (c) Videla

HA, Herrera LK. Understanding microbial inhibition of corrosion. A comprehensive overview. *International Biodeterioration & Biodegradation*. 2009; 63(7):896–900.

14. (a) Jayaraman A, Cheng ET, Earthman JC, Wood TK. Axenic aerobic biofilms inhibit corrosion of SAE 1018 steel through oxygen depletion. *Applied microbiology and biotechnology*. 1997; 48:11–17. [PubMed: 9274042] (b) Potekhina JS, Sherisheva NG, Povetkina LP, Pospelov AP, Rakitina TA, Warnecke F, Gottschalk G. Role of microorganisms in corrosion inhibition of metals in aquatic habitats. *Applied microbiology and biotechnology*. 1999; 52:639–646. (c) Zuo R, Kus E, Mansfeld F, Wood TK. The importance of live biofilms in corrosion protection. *Corrosion Science*. 2005; 47(2):279–287. (d) Lewandowski, Z.; Beyenal, H. Mechanisms of microbially influenced corrosion. In: Flemming, H-C.; Murthy, PS.; Venkatesan, R.; Cooksey, KE., editors. *Marine and Industrial Biofouling*. Vol. 4. Springer; 2008. p. 35–64.
15. Zuo R, Wood TK. Inhibiting mild steel corrosion from sulfate-reducing and iron-oxidizing bacteria using gramicidin-S-producing biofilms. *Applied microbiology and biotechnology*. 2004; 65(6): 747–53. [PubMed: 15278311]
16. Williamson, C. An investigation of microbial diversity and microbiologically influenced corrosion in automotive fuel environments. Colorado School of Mines; Golden, CO: 2012.
17. (a) Matsushita K, Inoue T, Adachi O, Toyama H. *Acetobacter acetii* possesses a proton motive force-dependent efflux system for acetic acid. *Journal of Bacteriology*. 2005; 187(13):4346–4352. [PubMed: 15968043] (b) Sakurai K, Arai H, Ishii M, Igarashi Y. Transcriptome response to different carbon sources in *Acetobacter acetii*. *Microbiology*. 2011; 157:899–910. [PubMed: 21081762]
18. Sowards, JW.; Weeks, TD.; McColskey, JD.; Fekete, JR.; Williamson, C.; Jain, L. Effect of ethanol fuel and microbiologically influenced corrosion on the fatigue crack growth behavior of pipeline steels. DoD Corrosion Conference 2011; La Quinta, CA: NACE International; 2011.
19. Sowards JW, Mansfield E. Corrosion of copper and steel alloys in a simulated underground storage-tank sump environment containing acid-producing bacteria. *Corrosion Science*. 2014; 87:460–471.
20. (a) Reipa V, Almeida J, Cole KD. Long-term monitoring of biofilm growth and disinfection using a quartz crystal microbalance and reflectance measurements. *Journal of Microbiological Methods*. 2006; 66(3):449–459. [PubMed: 16580080] (b) de Roda Husman AM, Lodder WJ, Rutjes SA, Schijven JF, Teunis PFM. Long term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Applied and Environmental Microbiology*. 2009; 75(4):1050–1057. [PubMed: 19074604]
21. Rasband, WS. ImageJ. National Institutes of Health; Bethesda, Maryland, U.S.A: 1997–2014.
22. Lane, DJ. 16S/23S rRNA sequencing. In: Stackebrandt, E.; Goodfellow, M., editors. *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons; New York, NY: 1991. p. 115–175.
23. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng*. 2005; 89:670–679. [PubMed: 15696537]
24. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*. 2014; 41(Database issue):D633–D642.
25. International, A. ASTM Standard G31-72, 2004, Standard Practice for Laboratory Immersion Corrosion Testing of Metals. West Conshohocken, PA: 2004.
26. (a) Dow Chemical Company, T. *International Hatchery Practice Magazine*. 2005. Glutaraldehyde: An effective broad spectrum biocide; p. 10 (b) Walsh SE, Maillard J-Y, Russell AD. *Ortho-phthalaldehyde*: a possible alternative to glutaraldehyde for high-level disinfection. *Journal of Applied Microbiology*. 1999; 86:1039–1046. [PubMed: 10389251]
27. Lou X, Singh PM. Role of water, acetic acid and chloride on corrosion and pitting behaviour of carbon steel in fuel-grade ethanol. *Corrosion Science*. 2010; 52:2303–2315.
28. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G. Biofilms, the customized microniche. *Journal of Bacteriology*. 1994; 176(8):2137–2142. [PubMed: 8157581]
29. (a) Hidalgo G, Burns A, Herz E, Hay AG, Houston PL, Wiesner U, Lion LW. Functional tomographic fluorescence imaging of pH microenvironments in microbial biofilms by use of silica

nanoparticle sensors. *Applied and Environmental Microbiology*. 2009; 75(23):7426–7435. [PubMed: 19801466] (b) Schlafer S, Raarup MK, Meyer RL, Sutherland DS, Dige I, Nyengaard JR, Nyvad B. pH landscapes in a novel five-species model of early dental biofilm. *PLoS ONE*. 2011; 6(9):1–11.

NIST Author Manuscript

NIST Author Manuscript

NIST Author Manuscript

Figure 1.

Schematic of cell growth and delivery system. All tubing lines ran through the same peristaltic pump, with various inner diameters (indicated by line thickness) enabling different volumetric flow rates. Growth medium was supplied to a constant biomass reactor to keep the *A. acetii* source culture at a somewhat constant concentration. The source culture was then diluted with a water medium (ECW - see text) in four different ratios in four different mixing tanks (labeled 0.985, 0.119, 0.115, and 0.0018). The lowest relative cell concentration, 0.0018, required a secondary dilution from the 0.119 mixing tank. Cells at these four different concentrations were then delivered across the surface of steel coupons contained in open petri dishes ("coupon exposure plates"). All vessels, except the growth and water media sources, were open to secondary containment to allow overflow of any excess fluid.

Figure 2.
Optical micrograph showing grain structure of carbon steel foil used for coupons.

Figure 3. Measurements taken from constant biomass reactor during exposure test. (▲) pH; (○) optical density at 600 nm, which is proportional to the cell concentration.

Figure 4.

Measurements of pH taken in coupon exposure dishes with different fractional *A. aceti* cell exposures over the course of a week-long test. Cell fractions are represented by (□) 0.0018 (●) 0.015 (△) 0.119 and (■) 0.985.

Figure 5.

Fluorescence micrographs of cells associated with coupon surfaces at various time points during exposure to the 0.985 cell fraction. Cells with intact membranes appear green via SYTO9 dye; cells with compromised membranes appear red via propidium iodide. Time points shown are (a) 3 (b) 26 (c) 50 (d) 75 (e) 146 and (f) 171 hours after start of exposure.

Figure 6.

Fluorescence micrographs of cells associated with coupon surfaces after one week of exposure. Cells with intact membranes appear green via SYTO9 dye; cells with compromised membranes appear red via propidium iodide. Cell fractions are (a) 0.0018 (b) 0.015 (c) 0.119 and (d) 0.985.

Figure 7.

Coupons with associated corrosion products following 146 hours of exposure to *A. aceti*.

Each column shows three replicate coupons exposed to the following cell fractions, from left to right: 0.0018, 0.015, 0.119, 0.985.

Figure 8.

Corrosion rate versus *Acetobacter aceti* cell exposure after one week. Circles represent data taken in the 171 hour test with *A. aceti* while squares represent data taken in the 166 hour test (as shown in Table 2). Error bars indicate 95 % confidence intervals for the average corrosion rate measured under each exposure condition.

Figure 9. Scanning electron micrographs of coupon surfaces after one week of *A. aceti* exposure and subsequent cleaning. Arrows indicate pits. Cell fractions are (top) 0.0018 and (bottom) 0.985.

Figure 10.

Corrosion rate versus hydrogen ion exposure after one week. Average measurements from tests run with delivered *A. acetii* are marked by open symbols and abiotic control tests are marked by solid symbols. Media fraction of the test is indicated by symbol shape: Δ = 0.0018; $\diamond$ = 0.015; $\circ$ = 0.119; $\square$ = 0.985. Horizontal error bars indicate the range of H⁺ exposure values for each media fraction over two independent experiments. Vertical error bars indicate 95 % confidence intervals on the average corrosion rate for each media fraction.

Table 1

pH values for solutions used to expose carbon steel coupons in a static control test.

Medium Fraction	0 to 77 hours	77 to 168 hours	168 to 172 hours
0.0018	8.1	4.8	3.9
0.015	6.0	4.5	3.8
0.119	4.8	4.2	3.6
0.985	4.4	3.7	3.4

Table 2

Raw mass loss (ML, in mg) and calculated corrosion rates (CR, in mm/year) for data depicted in Figures 8 and 10. Exposure times are listed for each independent test.

Medium Fraction	with <i>A. aceti</i> 171 hours		with <i>A. aceti</i> 166 hours		control 172 hours		control 166.33 hours	
	ML	CR	ML	CR	ML	CR	ML	CR
0.0018	1.05	0.206	0.83	0.168	1.17	0.23	1.05	0.209
	0.94	0.184	0.84	0.169	0.91	0.177	1.13	0.225
	1.08	0.212	1.03	0.209	1.17	0.229	0.9	0.18
0.015					0.9	0.175	1.02	0.203
							1.05	0.209
							0.89	0.177
0.119	0.73	0.144			0.89	0.175	0.84	0.167
	0.63	0.124			0.76	0.149	0.84	0.168
	0.7	0.137			0.84	0.164	0.88	0.175
0.985					0.72	0.141	0.85	0.17
							0.81	0.161
							0.84	0.167
0.119	0.46	0.09	0.3	0.06	1.31	0.255	0.86	0.172
	0.46	0.09	0.23	0.047	1.18	0.23	0.71	0.141
	0.36	0.071	0.3	0.06	1.24	0.243	0.67	0.133
0.985					1.06	0.208	0.82	0.163
							0.74	0.147
							0.95	0.189
0.119	0.48	0.094	0.14	0.028	0.83	0.162	0.86	0.172
	0.49	0.096	0.003	0.001	0.81	0.158	0.71	0.141
	0.18	0.035	0.1	0.02	0.74	0.144	0.67	0.133
0.985					0.67	0.131	0.82	0.163
							0.74	0.147
							0.95	0.189