MICROBIOLOGICALLY INFLUENCED CORROSION OF CARBON STEEL
CAUSED BY A SULFATE REDUCING BACTERIUM

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MICROBIOLOGICALLY INFLUENCED CORROSION OF CARBON STEEL
CAUSED BY A SULFATE REDUCING BACTERIUM

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ABSTRACT

Sulfate reducing bacteria (SRB) are common culprits of microbiologically influenced corrosion (MIC) that has been reported to cost $138 billion annually in the United States. Most literature reported the study results of SRB-induced corrosion when organic nutrients were provided to bacteria. But SRB-metabolizable organic substrates are not always available in the field conditions. There is a clear need to identify how SRB can induce pitting under the condition of long term starvation of organic substrates. The goals of this work are to elucidate the corrosion mechanisms of organic starving SRB on carbon steel (C1010) and propose possible MIC mitigation approaches. The specific objectives are: (1) monitoring MIC by correlating results of electrochemical impedance spectroscopy (EIS) and potential difference (PD) with measurements of bioactivities, biofilm and corrosion deposits; (2) development of a faster methodology for pit characterization to significantly reduce characterization time; (3) explication of the survival and corrosion mechanisms of a well-known SRB, *D. vulgaris* under long term starvation; and (4) development of possible MIC mitigation approaches. Coupons and sulfate-reducing biofilms were examined by confocal laser scanning microscopy (CLSM), infinite focus microscopy (IFM), scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS). Bioactivities in the liquid phase were measured by high-performance liquid chromatography (HPLC) and various biological assays. Metal-biofilm interfacial layer evolution was monitored by EIS and PD. Our results supported
the following conclusions: (1) EIS may be used for online monitoring of biofilm and corrosion product evolution, but the signals are complicated and require more systematic studies to improve understanding and ensure correct interpretation. Under certain conditions, pitting occurrence might be detectable from inner layer porous resistance and PD profiles. (2) Empirical correlations that enable fast estimation of maximum and average pit depths and pitted area percentage from the standard 3D surface parameters obtainable with IFM were established. (3) *D. vulgaris*, a common H₂-utilizing SRB can survive on carbon steel up to 46 days under organic starvation by coupling direct electron uptake from steel surface with sulfate reduction. Direct cell attachment to metal is essential for this survival mechanism and the resultant pitting corrosion. (4) Future mitigation approach should target at preventing direct cell attachment or killing all the cells of biofilm including innermost layer of cells. KH₂PO₄ was demonstrated to be a promising MIC inhibitor by significant reduction of sessile SRB number and inhibition of pitting and intergranular corrosion.
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1.1. Background and significance

Microbiologically influenced corrosion (MIC) was corrosion affected by presence and activities of microorganisms [1]. MIC assisted and caused corrosion failures in many industries, i.e. oil, gasoline, food, nuclear and power generation industries [1, 2]. The direct or indirect costs of associated corrosion damage kept increasing. A recent Federal Highway Agency (FHWA) report documented that corrosion damages cost the United States $276 billion in 1998 (3.1% of the 1998 U.S. gross domestic product [GDP]) [1, 3], and MIC accounted for 20-50% of the total cost of corrosion damage [4]. Iron is low cost metallic material that has been widely used in the world. Iron is the basic component of steel. Because of the broad application of steel, demand of steel kept increasing. The world production of raw steel increased from 904 to 1640 million tons from year 2002 to 2015 [5, 6]. Comparing with steel alloy with better corrosion resistance (i.e. stainless steel), carbon steel is the most widely used for large-scale applications (i.e. transmission pipeline and oil reservoir), because of its low cost. However, carbon steel is more corrosion susceptible. Corrosion of transmission pipelines is one of the largest risks that the Oil and Gas industry faces today for both onshore and subsea assets. In US alone,
there are $4.8 \times 10^5$ miles of transmission pipelines and 60% of them were old [3]. According to a federal study conducted by NACE in 2003, corrosion of transmission pipelines in the United States accounts for $8.6$ billion in costs annually [7] and MIC is related with $2$ billion annually in the oil and gas industry [3].

Dissimilatory sulfate reducing bacteria (SRB), the main cause of MIC in oilfield systems [8-10], are widespread in both seawater and freshwater [11]. In the oil production field, water flooding was carried out as a secondary recovery process. Meanwhile, the water injected provided a proper environment for SRB proliferation [10]. The common SRB species found in seawater were *Desulfovibrio alaskensis*, *Desulfovibrio salexigens*, *Desulfonema limicola* and those found in freshwater were *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans* and *Desulfotomaculum nigrificans*. They thrive under anaerobic condition using sulfate as the electron acceptor. *Desulfovibrio desulfuricans* ATCC 27774 and *Desulfobulbus propionicus* can also use nitrate as the electron acceptor, reducing nitrate to nitrite and then to ammonia [12-14]. Some can tolerate certain levels of oxygen in natural environments, for example, *D. vulgaris* can tolerate and even grow with 3 h of aeration through the growth medium, if no H$_2$S was present. If H$_2$S was present, *D. vulgaris* were all alive after 6 mins of aeration, whereas they were all dead after 3 h of aeration [15]. SRB, such as *desulfovibrio*, *desulfobacter* and *desulfotomacula*, can grow by fermenting various organics to H$_2$, acetate and CO$_2$. They obtain additional energy from H$_2$ oxidation, coupled with sulfate reduction and sulfide production, and some SRB (i.e. *Desulfotomaculum acetoxidans* and *Desulfobacter postgatei*) can grow by oxidizing acetate [16-19]. Biogenic sulfide may induce sulfide stress cracking (SSC) or hydrogen induced cracking (HIC) [20, 21], but
the most frequently encountered form of SRB-induced corrosion was pitting corrosion, which was the root of many disastrous corrosion failures [22, 23]. Pitting corrosion induced by SRB activities is more difficult to detect and predict, due to the patchy nature of biofilm [24] and stochastic nature of pitting [25, 26]. Pits might also be concealed by biofilm and corrosion deposits. Thus, there is an urgent need to elucidate MIC mechanism and then develop effective mitigation approaches.

1.2. Long-term goal and specific objectives

The long-term goal of this research is to inhibit SRB-induced corrosion, so as to minimize the cost in MIC damage. The most effective prevention and mitigation approach can be proposed and applied only if the mechanism of SRB-induced corrosion can be clarified. The research targets at elucidation of SRB-induced corrosion mechanism by using multidisciplinary approaches (i.e. microbiological methods, material science tools as well as electrochemical techniques).

As shown in Figure 1.1, several specific objectives need to be achieved in order to determine the mechanism and thus develop effective mitigation approach. For elucidation of SRB corrosion mechanism, the following specific objectives were identified. (1) To prove or disprove that conventional \( \text{H}_2 \)-utilizing SRB can develop the mechanism of direct uptake of electrons from steel for sulfate reduction for their survival under long term starvation. (2) To develop a fast pitting-characterization method in order to allow quantitative study of failure risk and pitting pattern of carbon steel pitting corrosion caused by starving SRB. (3) To elucidate long term interfacial layer evolution for MIC
online monitoring, establishments of correlations between electrochemical response (impedance spectroscopy and potential difference) and bioactivities and corrosion deposits were necessary.

According to the clarified mechanism, possible MIC mitigation approaches can be developed. Phosphate is commonly used as corrosion inhibitor. In this study, the effects of KH$_2$PO$_4$ in culture media on SRB-induced corrosion were investigated.

Figure 1.1. Scope, objectives and significance of the dissertation.
1.3. Structure and scope of dissertation

The dissertation was divided into four parts based on the specific objectives in 1.2.2 (Figure 1.1): (1) Mechanism of SRB-induced corrosion on carbon steel; (2) Improved method for fast pitting-characterization to evaluate failure risk and pitting pattern; (3) Correlating electrochemical measurement, bioactivities and corrosion product for elucidating interfacial layer evolution; (4) According to MIC mechanism, possible mitigation approaches were proposed and demonstrated.

A short overview of the problem statement, significance and research objectives is described in Chapter I.

Chapter II introduces the literature background of mechanisms of SRB-induced pitting and the possible mitigation approaches. Also described in Chapter II are the current available methods for pit characterization.

Chapter III includes a summary of all the material, microorganisms, methods and techniques used in this work.

Chapter IV describes the experimental results that indicate the capability of a conventional H₂-utilizing organotrophic SRB in directly utilizing the electrons from steel surface to help their long term survival under organic-starvation.

Chapter V describes the quantification of pitting corrosion induced by long-term organic-starving SRB. Effects of surface roughness are also discussed.

Chapter VI describes an improved method for pit characterization, which allowed fast, reliable quantification of pitted area percentage, average and maximum pit depths. Two types of diagrams are proposed: (1) pitting pattern (four-phase) diagram, which
describes four types of pitting patterns and (2) failure risk diagram, which provides an approach for risk evaluation.

Chapter VII describes the results of a study that compares anaerobic carbon steel corrosion by 3.5% NaCl solution versus by a sulfate reducing bacterium. Application of fast pitting-parameter-quantitation method (Chapter VI) to chloride and SRB-induced pitting was validated. Surface roughness effects and pit morphologies were quantified in all the systems.

Chapter VIII reports the results of studies on interfacial-layer evolution on carbon steel in abiotic and SRB systems. EIS results were correlated with SRB activities, biofilm and corrosion deposits.

Chapter XI describes the results of a study that demonstrates inhibition of SRB-induced corrosion on carbon steel by KH$_2$PO$_4$, which might be used as a MIC inhibition approach.

Chapter X summarizes all the conclusions achieved from this work and the recommendations.
CHAPTER II

OVERVIEW OF THE LITERATURE

2.1. Sulfate reducing bacteria

Dissimilatory sulfate reducing bacteria (SRB) were anaerobes that reduce sulfate to sulfide for respiration. In other words, they breathe sulfate instead of oxygen. Most SRB can also reduce other chemicals (i.e. thiosulfate, sulfite and nitrate) [27].

2.1.1. Metabolism of D. vulgaris

Many typical SRB species have similar metabolism, which is closely related with MIC. Desulfovibrio vulgaris is a model SRB species that commonly present in the natural environment, who is important for sulfur cycle [28]. Based on the work done by Tang, et al. [29], the metabolic pathway of D. vulgaris was summarized in Figure 2.1. The first metabolic pathway was pyruvate fermentation to acetate, as shown in Equation (2.1) and the second metabolic pathway was production of succinic acid from pyruvate and CO$_2$, as shown in Equation (2.2). Pyruvate was mainly going through two metabolic pathways. (1) Pyruvate was converted to acetyl-CoA, while generating NADH and CO$_2$. NADH can be used for sulfate reduction. As reported by Tang, et al. [29], around 85% of acetyl-CoA
was converted to acetate and \( \text{H}_2 \) and only very small portion of that (around 0.7%) was going through tricarboxylic acid cycle (TCA cycle). Thus the ATP generated was mainly coming from pyruvate fermentation to acetate and \( \text{H}_2 \). The \( \text{H}_2 \) produced can be coupled with sulfate reduction and generate ATP. The TCA cycle is incomplete, since citrate was converted through several intermediates to \( \alpha - \text{ketoglutarate} \) as an end product. During this process, NADH was produced for sulfate reduction and ATP production. (2) Pyruvate can also react with \( \text{CO}_2 \) and NADH by malic enzyme to produce malate [30, 31]. Malate was then converted to fumarate and then succinate, as shown in Equation (2.2). Sulfate reduction to \( \text{H}_2\text{S} \) was coupled with either \( \text{H}_2 \) gas (Equation [2.3]) or NADH (Equation [2.4]) [29, 32].

Figure 2.1. Metabolism pathway of \textit{D. vulgaris} ATCC 7757 with electron donors. Solid arrows indicate active reaction; dashed dot ( -- • ) arrows indicate presence of
corresponding gene, but the reaction didn’t happen; “×” means lacking of corresponding gene for the reaction [33]; long dashed (——) arrow indicates possible source of NADH.

\[
\begin{align*}
\text{CH}_3\text{COCOOH} + \text{H}_2\text{O} & \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + \text{H}_2 \tag{2.1} \\
\text{CH}_3\text{COCOOH} + \text{CO}_2 + 4 \text{ NADH} & \rightarrow \text{Succinic acid} + \text{H}_2\text{O} + 4 \text{ NAD}^+ + 4e^- \tag{2.2} \\
4 \text{ H}_2 + \text{H}^+ + \text{SO}_4^{2-} & \rightarrow \text{HS}^- + 4 \text{ H}_2\text{O} \tag{2.3} \\
4 \text{ NADH} + 5\text{ H}^+ + \text{SO}_4^{2-} & \rightarrow \text{HS}^- + 4 \text{ H}_2\text{O} + 4 \text{ NAD}^+ \tag{2.4}
\end{align*}
\]

2.1.2. SRB biofilm and corrosion

Biofilm development on surfaces includes several steps. Organic substances and inorganic ions deposit onto the metal and form a thin film. Bacteria randomly attach onto the surface, consume the nutrients and develop into biofilm [22, 34]. Depending on the condition, some cells in biofilm might detach and re-attach. This procedure changes the properties of metal solution interface [22]. Meanwhile, corrosion is happening upon submerging into the liquid. Metal dissolution and corrosion products formation further complicate the interpretation of the mechanism. Biofilm and corrosion products affect each other synergistically. Corrosion products might block or assist electron transfer for cell respiration and growth, while biofilm metabolic products might change the corrosion products.

In natural environment, aerobic biofilm initially dominant, since certain amount of oxygen usually present. When the oxygen was consumed by aerobic bacteria, the outer layer of biofilm still had access to oxygen. The inner layer of biofilm was under anaerobic condition, which is an optimum environment for anaerobic growth. Sulfate
reducing biofilm usually formed in the inner layer in the absence of oxygen [22, 34, 35]. Microbial activities thus created many types of gradients (i.e. oxygen, pH and potential), which providing many sites for localized corrosion [24].

2.2. Mechanism of SRB-induced corrosion

MIC is usually a complex process that involves several mechanisms. In order to elucidate complicated MIC mechanisms in natural environment, simplified mechanism that directly related with pure SRB species is critical to investigate. Currently, the most accepted mechanism of SRB-induced corrosion can be categorized into two main theories. Firstly, the corrosion was closely related with chemical effects of H$_2$S. Although the cathodic depolarization theory (CDT) used to be well-accepted, more and more people questioned the validity of this theory. CDT proposed that iron can serve as a hydrogen source for SRB culture. While atomic hydrogen was consumed by SRB, the abiotic corrosion reaction (Fe + 2H$^+$ → Fe$^{2+}$ + H$_2$) was accelerated [36]. Under this scenario, the close association of *Desulfovibrio vulgaris* with the CS coupon may have been a reflection of the enhanced mass transfer of H$_2$ to cells as has been observed in syntrophic cocultures [37]. However, there are limitations of this theory. Stott [38] pointed out that the hydrogenase enzyme from *Desulfovibrio desulfuricans*, strain Hildenborough could only reversibly work on molecular hydrogen [39], instead of using atomic hydrogen to depolarize the cathode [40]. Furthermore, several kinetic studies showed that scavenging of H$_2$ did not accelerate Fe$^0$ oxidation [41, 42]. The acceleration of cathodic reaction was explained by the chemical reaction of H$_2$S with Fe (Fe + H$_2$S → FeS + H$_2$) [24, 43, 44].
This reaction rate is faster than the abiotic reaction and thus increases the cathodic reaction rate. When FeS film was formed during this reaction, FeS film itself may to protect the underlying metal from exposure to the corroding environment [45]. However, it can be functioned as a cathode to iron surface, when the film was partially destroyed [46]. The liquid medium in most cases of SRB-induced corrosion in the field contained certain amount of oxygen. Higher corrosion rates associated with SRB were observed in the field, compared with laboratory results of corrosion caused by SRB under the anoxic condition [47]. The faster corrosion could be attributed to environmental perturbation such as occasional availability of low levels of oxygen [47], which cause non-uniform oxidation of the iron sulfide film and, thus, generate anodes and nearby cathodes to accelerate localized corrosion [48]. According to all the theories mentioned above, the biologic role of bacteria seems to be minimized, while the chemical effects have been emphasized.

Recently, on the other hand, some more interesting findings have been published to propose a new SRB-induced corrosion mechanism that directly related with biological activities of SRB. Dinh et al. [49] isolated a SRB strain that can use metallic iron directly as the sole energy source, as opposed to being an indirect energy source via, e.g., hydrogen generation. Enning et al. [50] demonstrated that the SRB strain isolated by Dinh et al. caused faster and more extensive corrosion than the H₂ utilizing SRB. When closely examining their published results (particularly Figure 4 [c] of reference 50), we noticed that the H₂ utilizing SRB tended to induce micro pits, while the directly iron-utilizing SRB caused severe weight loss and macro pits. Sherar et al. [51] observed that the cells of a SRB isolated originally from an oil well formed a nanowire structure and
attached firmly on a CS surface under the organic substrate limited condition. They hypothesized that the nanowire structure can be an approach for the SRB to uptake either electrons or molecular hydrogen directly from the steel surface. Similarly, it has been suggested that (semi)conductive corrosion products may facilitate transfer of electrons from steel-Fe$^0$ to cells [50]. Cord-Ruwisch et al. [52] reported that much more sulfide was produced when a SRB culture was incubated with steel wool than the control without steel wool. Gu [53] proposed a mechanism that the electrons generated by iron oxidation can be used at the cathode for sulfate reduction. Furthermore, they showed that addition of electron mediators such as FAD and riboflavin in the medium could accelerate the SRB-induced pitting corrosion [54]. All the above literature reports suggest the possibility of direct electron uptake from the metal surface by at least some SRB species. However, there is no experimental evidence to prove the capability of iron oxidation mechanism by conventional H$_2$ utilizing SRB.

Based on the discussion above, there is clear need to clarify (a) whether the conventional H$_2$-utilizing SRB can also gradually develop the iron oxidation mechanism to survive under long term starvation; (b) whether direct attachment is a requirement for SRB to survive and induce corrosion. For practical purposes it is of critical importance to verify and understand the abilities of SRB to survive under long-term starvation of organic substrates (electron donors) and to continue causing the potentially disastrous pitting corrosion.
2.3. Pitting characterization method

The hallmark of corrosion caused by SRB is pitting corrosion [22, 47], which was the root of many disastrous corrosion failures [8, 9, 55]. Pitting is more difficult to predict and detect due to its stochastic nature [25, 26], so it is important to analyze laboratory or field samples to characterize pitting pattern, pitting parameters and thus elucidate critical factors. Maximum pit depth is among the most important factors to be determined [56], because the deepest pit would likely be the failure-initiating point when a load was applied to the steel [57, 58]. Although important, the maximum pit depth alone does not complete the characterization of pitting corrosion. It has been reported that the number of cracks increased, if pit density was higher [59]. Also, it has been experimentally proved that defects such as pits can lead to several crack nucleations [60]. Thus, pit density and pit distribution are also important. For example, two surfaces have similar maximum pit depths and similar average pit depths; but, one has only one deep pit while the other has many deep pits with similar depths. Thus, when applied with the same load, the first surface has a much lower number of crack initiation points than the second surface does [60]. Clearly, it is also important to characterize how the pits with different depths distribute.

There are many limitations in current pit characterization methods. Electrochemical techniques are good methods for online evaluation of pitting [61-65], but still lacking of quantitative information of pitting parameters. Although microscopic methods are also popular, they usually required manual identification, counting and measuring the depth of each individual pit. When the studied surface area is large and
severely pitted, this manual process is very tedious. The maximum pit depth determined by manual method may be questionable, because it is easy to miss one pit out of thousands of deep pits. Thus, this characterization method might be operator dependent. Few commercial equipment-dependent software specifically designed for pit characterization are available [66]. Unfortunately, such software is not available for many other equipment that were commonly used by corrosion professionals. For example, infinite focus microscopy (IFM) is a popular technique for pit characterization [67-71] based on focus variation technology [72]. This technique can be worked on steeply sloped surfaces [72], whereas other equipment cannot (i.e. white light interferometry). However, characterization of pitting using IFM requires tedious manual identification, counting and measurement of each individual pit. Thus, a fast and reliable pitting characterization method is in urgent need.

In several previous studies, intergranular corrosion (IGC) was observed on the steel or alloy incubated with SRB. Lee et al. [73] observed that IGC was associated with loose FeS precipitation. Ringas et al. [74] reported that both pitting and IGC occurred when stainless steel was exposed to SRB culture. Gouda et al. [75] documented that severe IGC manifested inside the pits on a nickel-copper alloy after exposure to SRB. Geesey et al. [76] proved that SRB significantly decreased elemental iron composition relative to Ni at the grain boundaries on stainless steel. Zhang and Frankel [77] demonstrated that pitting and IGC transition can happen at critical potential. However, the relationship between the pitting corrosion and IGC was poorly understood, especially when SRB activities were involved. In our study, we attempted to correlate the SRB activity with the pitting corrosion and to elucidate the cause and effect of IGC. The aim
was to further the understanding of the mechanisms and/or factors that contribute significantly to the pitting corrosion.

2.4. MIC monitoring

Current literatures on MIC either focused on characterization of the corroded metal surface with or without biological metabolism information [70, 78, 79] or solely relied on electrochemical techniques [80-82]. For the former, limited samples can be drawn, due to the requirement of sterile experiment. Online monitoring approaches are thus more important to develop. For the latter, EIS and electrochemical noise are popular MIC electrochemical monitoring approaches [1]. The bottleneck of these approaches is the failure to correlate microbiology to corrosion [1]. Techniques that assist real-time monitoring of biofilm-metal interface are essential, since sessile SRB that directly responsible for MIC, are more important to study than planktonic SRB [53, 83]. EIS is a powerful technique that can elucidate evolution of biofilm-metal interface[84]. However, the correlations between biological metabolism and electrochemical signals are limited in the current literature to allow EIS to monitor MIC reliably. Halim et al. [85] compared EIS and open circuit potential results with scanning electron microscopy (SEM) images to study the layer formation of SRB, nitrate reducing bacteria (NRB) and their mixture on carbon steel. Xu et al. [86] compared EIS Bode plots with SEM images of corrosion products of carbon steel under disbonded coating in the presence and absence of Desulfovibrio desulfuricans. Further studies to correlate biological activities of sessile SRB and corrosion are necessary in order to develop EIS real time monitoring approach.
2.5. MIC mitigation and challenges

Currently, both inspection and mitigation approaches were developed for solving internal corrosion of pipelines problems, but there is no efficient solution. The main challenge to provide solution to internal corrosion was that the complex corrosion mechanisms including too many factors. MIC is the most uncertain factor, because of ever-changing biological activities. SRB were the main culprit of MIC. The commonly used 3 pipeline inspection approaches were very expensive and might not be able to detect localized corrosion (pitting). The pipeline inspectors usually used “find it and fix it” philosophy. Although this approach reduced cost in short term, it incurred tremendous cost in longer term [7]. Pigging technology is also used inside pipelines for various purposes, i.e. cleaning, inspection and corrosion inhibition [87]. Cleaning and scraping pigs are used for removing corrosion deposits, dirt and biofilm, while adding corrosion inhibitors. Inline inspection pigs are particularly useful for visually detecting pits, dents and ovality [87]. However, these intelligent pigs are very expensive. Many narrow pipes cannot be pigged. Another approach to control MIC was addition of inhibiting biocide [88-91]. However, biocides commonly used were highly toxic, so novel environment-friendly methods need to be developed to inhibit or prevent MIC [24]. Nitrate treatment has been proposed as a green strategy to control the damaging effects of SRB-induced corrosion based on five mechanisms [92]. Nitrate could enhance growth of heterotrophic nitrate reducing bacteria to compete with SRB for organic electron donors [93-95]. However, the effectiveness of this approach has been inconsistent in field settings [10, 96]. The ability of many SRB to use nitrate as the electron acceptor likely also contributed to the limited effectiveness of nitrate treatment [12, 13]. More effective
environment-friendly methods need to be developed. In order to propose effective prevention and mitigation approaches, mechanism of SRB-induced corrosion is of great importance to understand.

Phosphate is one type of corrosion inhibitor based on the mechanism that it can stabilize the rust layer as binuclear phosphate-metal complexes on metal surfaces [97-99]. Phosphate solution passivates pits with oxygen to form a layer mixed with iron oxide and iron phosphate [100, 101]. Under anaerobic condition, iron phosphate (green rust) was suggested to form as a physical barrier that inhibited pitting, when mild steel was submerged in Na$_2$HPO$_4$ solution [102-104]. The possible composition of green rust was Vivanite [Fe$_3$(PO$_4$)$_2$.8H$_2$O] [104, 105]. Although vivanite is not as protective as iron oxides, it is still less corrosion prone than soluble complex [104]. Thus, phosphate is a promising MIC inhibitor, whereas very limited literatures reported effects of phosphate on MIC. Volkland et al. [106] showed that vivanite layer caused by bioactivities of Rhodococcus sp. strain C125 and Pseudomonas putida mt2 inhibited corrosion of mild steel in term of weight loss. Effects of phosphate on SRB-induced pitting are of interest to study.

Biosurfactants attract more and more attention in recent years. The advantages of biosurfactants over chemical surfactants or biocide include biodegradable, highly effective, low-toxic, better foaming properties and better stability at extreme pH, salinity and temperature [107]. The most promising biosurfactant is rhamnolipid, because it has the lowest minimum surface tension among biosurfactants [108]. Based on the trend summarized by Muller et. al. [109] according to ISI Web of Science (Thomson Reuters,
2011), the number of worldwide publications for biosurfactants and rhamnolipids increased exponentially from year 2000 to the end of 2011. Rhamnolipids are glycolipids containing L-rhamnose and β-hydroxy fatty acids moieties [110]. There are four common structures of rhamnolipids (i.e. Rha-C10-C10, Rha-C10, Rha-Rha-C10-C10 and Rha-Rha-C10) produced by *P. aeruginosa* [111]. The individual rhamnolipid is able to reduce the surface tension of water from 72 mN/m to 25±30 mN/m at concentrations of 10±200 mg/l [108]. Rhamnolipids have low critical micelle concentration (CMC). 5 mg/L of CMC value was reported for the di-rhamnolipid with 2 hydrocarbon chains and 40 mg/L of CMC value was reported for the mono-rhamnolipid with 2 hydrocarbon chains [112]. Also, rhamnolipids have many potential applications in enhanced oil recovery [113], removing spilled oil [114], bioremediation, the cosmetic industry [115], fungicide [107] and healing burn wounds [116]. The reported rhamnolipid properties, i.e., anti-bacteria [117], prevention of bacterial attachment [118, 119] and biofilm disruption [120], provide a new potential application on MIC control in oil pipeline. The application of rhamnolipids in oil and gas industry can be more meaningful, when combined with the applications of both MIC control and tertiary oil recovery. To date, no study has been done on effects of rhamnolipids on SRB growth, biofilm disruption or inhibition of MIC.

MIC control by beneficial biofilm (secreting antimicrobials or not) is also promising [121]. Beneficial biofilm inhibited corrosion based on the following three mechanisms. (1) Depletion of cathodic agents by bacteria metabolism (i.e. consumption of oxygen); Removal of oxygen by biofilm mainly inhibited uniform corrosion under aerobic condition [122]. Pitting might still happen, due to differential aeration cell [24]. (2) Formation of passive layer by biofilm and corrosion products. Passive layer formation
can be assisted by biofilm. For example, *Pseudomonas cichorii* was able to form iron oxide/iron phosphate layer inside biofilm matrix and thus inhibited corrosion of mild steel in phosphate buffered basal salt solution under aerobic condition [123]. However, this mechanism does not work well under anaerobic condition, especially when SRB flourished (3). Microorganisms secreting antimicrobials that inhibited growth of corrosion causing bacteria or secreting corrosion inhibitors that repress corrosion [121]. Genetically engineered *Bacillus subtilis* biofilms producing antimicrobials indolicidin, bactenecin, and probactenecin were reported to be applied in situ for corrosion inhibition by inhibiting growth of SRB. Biofilm matrix assisted maintaining relatively high concentration of antimicrobials by preventing diffusion from inner biofilm to bulk fluid [124]. Other naturally antimicrobials secreting biofilms *Bacillus brevis* were also reported to inhibit SRB attachment, growth and MIC [125]. As discussed above, rhamnolipids producing *P. aeruginosa* and other non-pathogenic rhamnolipid producing strains of *Pseudomonas* (i.e. *P. chlororaphis*) [126] were also promising in in-situ application of biofilm in MIC inhibition. No report has investigated effectiveness of this approach, yet.
CHAPTER III

MATERIALS AND METHODS

3.1. Microorganisms and medium

*Desulfovibrio vulgaris* (American Type Culture Collection 7757) was cultured in the Postgate medium C [27]. The medium contained 6 g sodium pyruvate (C$_3$H$_3$NaO$_3$), 4.5 g sodium sulfate (Na$_2$SO$_4$), 1 g yeast extract, 1 g ammonium chloride (NH$_4$Cl), 0.5 g monopotassium phosphate (KH$_2$PO$_4$), 0.3 g sodium citrate dihydrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O), 0.06 g magnesium sulfate heptahydrate (MgSO$_4$·7H$_2$O), 0.04 g calcium chloride hexahydrate (CaCl$_2$·6H$_2$O), and 0.004 g ferrous sulfate heptahydrate (FeSO$_4$·7H$_2$O) per 1L deionized water, and the pH was adjusted to 7.0 by addition of 1 M NaOH solution. Anaerobic culture techniques were used throughout the cultivation, including the use of serum bottles and syringes pre-purged by N$_2$ gas. The medium was transferred into serum bottles (Supelco, Milwaukee, WI), which were then sealed with butyl rubber stoppers and crimped with aluminum seals. N$_2$ was bubbled through the medium for at least 20 min, while the bottles were heated till boiling, to remove O$_2$ from the medium. Medium was autoclaved at 121 °C for 20 min. Similarly, O$_2$ was removed from a cysteine stock solution (2 g/L) by bubbling with N$_2$ and was subsequently sterilized by autoclaving. The cysteine stock solution was added to the medium to achieve a final concentration of 0.002
g/L prior to the inoculation of SRB cells. Stock culture of *D. vulgaris* was revived and added (5% v/v) to the anaerobic Postgate medium C. Cultures were grown at 25 °C for 26 h to reach the late exponential growth phase or early stationary phase (optical density of 0.51 ±0.03; cell density of $1.4 \times 10^9$ cells/mL) and then used as the inoculum. After 26 h of growth, the cultures had an optical density (OD) at 600 nm of 0.51 ±0.03 and cell number was around $1.4 \times 10^9$ cells/mL. All the operation procedures were done under anaerobic and sterile conditions.

3.2. Experimental setup and equipment

Customized reactors, each holding 4 CS coupons, were used for the study. The reactors used were 600 mL glass beakers closed with rubber stoppers (Figure 3.1 (a)) or specially designed reactors (Figure 3.1 (c)). The coupons (Figure 3.1 (b)) were hung vertically, using plastic tie-wraps, on stainless steel hooks that were anchored to the rubber stopper. The coupons were completely submerged in the medium. The reactors and the medium were sterilized by autoclaving at 121 °C for 30 min. All the tubing, fitting and connections were made of glass, teflon or stainless steel to prevent oxygen permeation.

To satisfy the requirement of sterile and anaerobic condition, the reactors were tested with a 0.001 g/L resazurin solution to ensure anoxic condition under the experimental operation. The resazurin solution turned from blue to bright pink under the nitrogen bubbling for 30 min. The color of resazurin turned into colorless after the cysteine solution was added. The test confirmed the adequacy of maintaining anoxic
condition with the systems and procedures used in the corrosion study. On the other hand, dissolved oxygen (DO) concentration was measured and monitored by a DO micro-sensor (PreSens Precision Sensing GmbH) attached inside the wall of the reactor. To measure the DO concentration, an optical fiber tip connected with an oxygen meter (Fibox 3-trace v3, PreSens Precision sensing GmbH) was attached on the micro-sensor outside of the reactor. The DO concentration was then monitored recorded by the DO sensor connected with a computer.

Figure 3.1. Experimental setup of (a) and (c) specially designed anaerobic reactors. (c) CS coupons were submerged inside the liquid of (a) and (c).

3.3. Methods and techniques

Multi-disciplinary techniques and analytical methods were deployed for measurement of liquid broth behavior, characterization of biofilm, monitoring of metal corrosion and metal-biofilm interface layer evolution.
3.3.1. Planktonic cell concentration measurements

For determination of cell concentration in the cell-containing systems, the solids collected were washed once with deionized water and then resuspended in a known amount of deionized water and measured for optical density at 600 nm (OD\textsubscript{600}) using a UV-Vis spectrophotometer (Model 1601, Shimadzu Corp., Canby, OR). The suspension was diluted to have OD\textsubscript{600} within the linear range (< 0.6). Cell concentration was also determined by measuring the intracellular protein concentration. The following procedure was used: The washed solids were treated with 0.2 N NaOH at 95°C for 20 min, to lyse the cells. The released intracellular proteins were quantified by the standard Bradford method [127] using a diagnostic kit (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories, Hercules, CA) and measuring the reacted sample for absorbance at 595 nm.

It was desirable to know the cell dry-weight concentrations (g/L) and the cell number concentrations (number per ml) in the reactors. Experiments were therefore also made to establish the calibration curves for converting OD\textsubscript{600} to these two concentrations. For cell number concentration, the suspended cells were counted using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). For dry weight concentration, the washed cell suspension (containing the cells collected from 5 ml broth in the reactor) was transferred into a (pre-weighed) aluminum dish and dried in an oven set at 50°C for at least 3 days till constant weight. A Pioneer analytical balance (Model PA214, Ohaus, Parsippany, NJ) was used for weighing.
3.3.2. Nutrients and cell metabolites analysis

Carbon source pyruvate as well as cell metabolites acetate can be measured by a Shimadzu LC-10A high-performance liquid chromatography (HPLC) system (Shimadzu Corp.; Columbia, MD) equipped with a Supelco Supelcogel H column (Sigma-Aldrich Corp., St. Louis, MO) and UV-Vis detector (SPD-10A). $H_3PO_4$ (0.1%) was used as the mobile phase at 0.14 mL/min. Sulfate concentration was measured by ion exchange chromatography with conductivity detection using a Dionex DX 100 system fitted with an AS-14 column (Dionex Corp., Sunnyvale, CA). Samples for measurement of sulfide were preserved with equal volume of a zinc acetate solution (100 g/L). ZnS was separated from the fluid by centrifugation, solids were resuspended in deionized water, and sulfide was measured using the methylene blue assay [128].

3.3.3. Carbon steel coupon preparation

The CS coupons (UNS G10100) used for the low phosphate systems (0.5 g/L $KH_2PO_4$) were rectangular (25 × 11 × 0.3 mm, Q-Lab). One side of the CS coupons was polished following the polishing procedure described below. The unpolished side was marked with number by scratching the coupon surface using stainless steel tweezers. For deployment in high phosphate medium systems, CS coupons (Metal Samples Company, Munford, AL) with LAP finish on one side and 120 GRIT on the other side were custom made from the company. The coupons had dimensions 20.3 × 10.2 × 1.6 mm, with 5.1 mm diameter centered hole 5.7 mm away from the end. Coupons were cleaned in a
sonicating acetone bath for 20 min, followed by sonication in ethanol. For sterilization coupons were soaked in pure ethanol for 2 days at room temperature, and sonicated during the periods of 0 - 2 h and 46 - 48 h to remove gas bubbles on the coupon surface. Gas bubbles, if not removed, could prevent complete wetting of coupon surfaces by ethanol and compromise the effectiveness of sterilization.

CS coupons were polished with SiC papers (240/P280, 600/P1200, 800/P1500, 1200/P2500, 8” diameter, Carbimet 2, Buehler Company) using a grinding and polishing machine (Model DAP-7, Struers, Inc.) at 200 rpm. A sand paper (240/P280) was secured on the machine, and then the acrylic holder was pressed down to polish the coupons with the sand paper for 5 min. During the polishing process, water was added to rinse and cool the coupons and the sand paper. The process was repeated three times, each with a higher grit (finer) sand paper, i.e., 600/P1200, 800/P1500, and then 1200/P2500, respectively. The polished coupons were immediately rinsed with deionized water to remove particles and then sonicated, first in ethanol and then in DI water, each for 5 min. The cleaned coupons were dried under a stream of nitrogen. Cleaned coupons were observed under infinite focus microscope (IFM, Alicona imaging GmbH) and only those without clear pits or defects were used in the corrosion study.

3.3.4. Biofilm characterization

Confocal laser scanning microscopy (CLSM, Zeiss laser scanning system; LSM 510) was used to characterize biofilm structure. Sessile cells on the coupons or planktonic
cells in the medium were characterized by first staining the cells with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies Corp.; Grand Island, NY), followed by visualization in 2 dimensions or 3 dimensions using a CLSM with 488 nm and 559 nm excitation wavelengths by sequential mode. Live cells were visualized based on green fluorescence when observed under the CLSM, while dead cells exhibited red fluorescence and partially damaged cells appeared yellow. A Zeiss LSM 510 CLSM (Carl Zeiss AG; Jena, Germany) was used for the systems with low phosphate medium (0.5 g/L KH₂PO₄). An Olympus FV1000 CLSM (Olympus America Inc., Center Valley, PA) was used for the systems with high phosphate medium (10 g/L KH₂PO₄).

Number of sessile SRB was quantified according to the average of 4 sets of biofilm CLSM images at each exposure time by using ImageJ (National Institutes of Health, Bethesda, MD). For biofilms with multiple cell layers, the cell numbers from all layers were summed to give the total sessile SRB number on the coupon. Percentage of viable cells was calculated based on the ImageJ-counted results.

The surface structure of biofilm was also examined by a scanning electron microscope (SEM) equipped with an Energy Dispersive X-ray Spectrometer (Hitachi EDS, Quantax 70). EDS will be utilized for surface composition evaluation. The coupons were removed inside an anoxic glove bag (Coy Laboratory Products) containing 97.5% N₂ and 2.5% H₂. An anoxic 2% (w/w) glutaraldehyde solution was utilized to fix the SRB biofilm. Coupons with biofilm were submerged in the glutaraldehyde solution at 4 °C for 1 day. For dehydration the coupons were gently rinsed with ethanol-water solutions with increasing ethanol concentrations (0%, 20%, 50%, 75% and, finally, 100%). The coupons
were then placed in a desiccator for 1 day at room temperature. All the above procedures were done in the anoxic glove box to avoid post-sampling oxidation. The fixed biofilms on coupons were observed under the SEM.

3.3.5. Coupon surface characterization

The coupons from the biotic systems and the abiotic controls, were cleaned following the American Society for Testing and Materials (ASTM) G1-03 standard chemical cleaning procedure for iron and steel (See C.3.1 in Table A1.1 of ASTM G1-03) [129]. Afterwards, coupons were observed under IFM, SEM and EDS, again.

3.3.6. Statistical analysis

The statistical significance testing was carried out to compare difference of the sessile SRB (live or dead) cell number, uniform and pitting corrosion rates between different exposure time (3, 21 and 43 days) [130]. The p values of the corresponding parameters were calculated by Minitab (Minitab Inc., USA).

3.3.7. Weight loss measurement

All coupons were weighed before the corrosion study. After the corrosion tests, every coupon after cleaning by the method described in 3.3.5 was weighed twice.
Average weight loss of four coupons at each sampling time was calculated based on the eight measurements. Corrosion rate was calculated from the weight loss measurements according to the ASTM standard method (Equation [3.1]) [131], i.e.,

\[
\text{Corrosion rate (micrometers per year, } \mu\text{m/y}) = \frac{3650 \ W}{(d \ a \ t)} \quad (3.1)
\]

where

\[
W = \text{weight loss, mg},
\]

\[
d = \text{density of the metal, g/cm}^3,
\]

\[
a = \text{exposed area of the coupon, cm}^2, \text{ and}
\]

\[
t = \text{time, day}.
\]

3.3.8. Characterization of pitting according to individually identified pits

The 3D and surface pit morphology, pit distribution, density, average and maximum pit depths and average surface roughness were measured by IFM. The coupon that suffered the most severe pitting corrosion in each system was chosen for the surface roughness quantification. All the other pit parameters were obtained from all the coupons collected from each reactor. The entire coupon surface, approximately 270 mm$^2$ on each side, was first scanned to locate deep pit clusters. Many areas did not have pits and only those with pits were scanned and measured. The deepest pit from each scanned area (around 0.85 mm$^2$) was identified and the depth recorded. Then, these depths from several scanned areas were compared. Finally, the depth of the deepest pit from the whole
side of coupon surface was reported as the maximum pit depth. At least 50% of the pits were measured for pit depth and length, and included in pit number counting for determination of the pit density.

3.3.9. Selection of 3D Functional Parameters

Roughness parameters were generated by IFM according to EN ISO 4287 [132] and DIN_4776-1990 [133]. Among many roughness parameters [134], the following were selected to be used in this work:

- $S_k$: Height of the core material or kernel (subscript “k”; μm)
- $S_v$: Maximum depth of the valley (subscript “v”) below the core material (μm)
- $S_{vk}$: Mean depth of valleys below the core material (μm)
- $V_{vv}$: Valley void (subscript “vv”) volume of the surface (ml/m$^2$), and
- $S_{dr}$: Developed interfacial area ratio,

$$S_{dr} = \frac{\text{actual surface area} - \text{projected surface area}}{\text{projected surface area}}.$$

Defined by the Abbott Firestone curve [133], $S_k$ corresponds to the random surface roughness excluding significant peaks and valleys. $S_v$, the maximum valley depth, as confirmed by experimental results in the later Results and Discussion section, correlates very well with the commonly reported maximum pit depth, differing in principle only by the $S_k$ value, which is included in the determination of maximum pit depth but not in $S_v$. This difference is discussed further for $S_{vk}$ as follows. $S_{vk}$ and $V_{vv}$,
the mean depth and void volume of the valleys, are parameters obviously affected by pitting. $S_{vk}$ differs from the average pit depth in several aspects. First, pit depth is measured from the pit mouth to the bottom. In the IFM generated roughness parameters, the upper portion near pit mouth is considered part of the core material. In this sense, $S_{vk}$ alone gives a value closer to the pit depth than $S_{vk}$ alone does. Second, the coupon surface has non-pitted areas interspersed among the pits. Our experience showed that when the surface contains only micro pits (pit depth < approximately 9 μm), $S_{vk}$ is more or less the value averaged over the whole surface (in one scanned image), including both the pitted and non-pitted areas; thus, surface roughness affects the reported $S_{vk}$ value significantly for the mildly pitted or non-pitted surfaces. As the pitting extent increases, the surface roughness effect on $S_{vk}$ calculation decreases and the reported $S_{vk}$ value is based increasingly more on the pitted area. On the other hand, the manually calculated “average pit depth” is the average depth of only real pits identified and measured individually from the bottom tip of the pit to the pit mouth (excluding all non-pitted areas). Accordingly, in a scanned area of only one or few deep pits, the average pit depth is large while the $S_{vk}$ value does not increase much; only if there are numerous pits in the scanned area, the $S_{vk}$ value would show a clearer increase. The $S_{vk}$ value is therefore affected by both pit density and pit depths, including both deep pits and micro pits in the scanned area. And, the $S_{vk}$ value is expected to be significantly smaller than the average pit depth, particularly when the pit density is low. Furthermore, if the surface has no pit, the IFM software still separates the surface into three parts: peak, core, and valley, and reports a $S_{vk}$ value.
3.3.10. Electrochemical techniques.

Impedance spectroscopy is a useful tool for characterizing metal, biofilm, corrosion products and medium interfaces [135]. It is also an effective method for online monitoring of uniform corrosion rate [136-138]. Impedance as well as potential difference between the CS coupons and counter electrode can be measured by a New Research Grade Potentiostat/Galvanostat/EIS (Model: SP-200). pH of the cell free supernatant will be measured by a pH meter (Model 420A). With the information of potential and pH, the possible corrosion products can be estimated according to Pourbaix diagram [139]. This will be further confirmed by SEM-EDS scanning of the coupons that exposed to SRB for certain period of time. On the other hand, we can determine evolution of the layers (corrosion products or biofilm) present on CS coupons by impedance spectroscopy. Phase angle diagram demonstrates the following information: (1) the number of semicircles showed up indicates the number of layers present on CS coupons; (2) the position of the layers can be estimated by the rule that the outer layer on the coupons corresponding to the semicircle at high frequency, the middle layer corresponding to the semicircle at middle frequency and the inner layer (the layer directly attach on the coupon surface) corresponding to the semicircle at low frequency; (3) assuming each layer functioned as a parallel plate capacitor, the thickness of the layer can be estimated by the formula: \( d = \frac{\varepsilon A}{C} \) (where \( d \)= thickness of the layer; \( C \)=capacitance in Farads; \( \varepsilon \)=absolute permittivity of dielectric; \( A \)=Area of layer overlap in square meter). This part need to combine SEM/EDS results as well as corrosion products estimation according to Pourbaix diagram so as to target at certain chemical species. An impedance plane plot describes the limitation process and qualitatively indicates the trend of uniform
corrosion rate. The radius of semicircle is inversely proportional to corrosion rate, so if the radius is higher, the corrosion rate is lower. Furthermore, with the tool of impedance, instantaneous corrosion rate can be quantified. Uniform corrosion rate can be calculated by Faraday’s law and localized (pitting) corrosion was monitored by transmission line approach.
CHAPTER IV

MECHANISM OF CARBON STEEL CORROSION INDUCED BY DESULFOVIBRIO VULGARIS

4.1. Introduction

Microbiologically influenced corrosion (MIC) has received much attention because of the increasing recognition of the costs associated with corrosion damage. According to a Federal Highway Agency (FHWA) report, corrosion damages cost the United States $276 billion in 1998 [11], and MIC was related to half of the total cost of corrosion damage [4]. Iron, the major component of steel, is the most widely used but least expensive metal in the world. The world production of raw steel increased from 904 to 1500 million tons from year 2002 to 2012 [5, 140]. Carbon steel (CS) is the most economically feasible form of iron for large-scale applications (e.g. oil and gas industry), comparing with other more corrosion resistant alloy (e.g. stainless steel). However, CS is a corrosion prone material. According to a federal study in 2003, the corrosion cost for transmission pipelines can reach 8.6 billion annually [7].

Sulfate reducing bacteria (SRB) are generally considered as one of the most troublesome groups of bacteria that cause MIC, especially of steel [22]. Conventionally, SRB is known to oxidize a variety of organic compounds or H$_2$ by reduction of sulfate or
other partially oxidized inorganic sulfur species to sulfide [16, 29]. Although the biological consumption of $H_2$ (produced by water-metal reactions) has been commonly used to explain the SRB-induced pitting [24, 36, 38], more studies have demonstrated that the SRB-induced corrosion is closely related with the chemical effects of $H_2S$ [43]. The biogenic sulfide can cause pitting corrosion, which is the most frequently encountered form of SRB-induced corrosion and the root of many disastrous corrosion failures [22, 23]. The sulfide associated corrosion may induce sulfide stress cracking (SSC) or hydrogen induced cracking (HIC) and can lead to serious corrosion failures [20, 21].

SRB with the organotrophic, $H_2$-utilizing metabolic activities are rather ubiquitous in natural environments and have been studied extensively [16, 141]. All the SRB-involved MIC mechanisms proposed in the past, including the well known cathodic depolarization theory [36], are based on the metabolic activities of these common SRB. More recently it has been suggested that SRB may directly oxidize $Fe^0$ on steel surfaces (without formation of $H_2$ intermediates) coupled with sulfate reduction [49, 50, 53]. And, special SRB strains have been recently isolated, which have such unique lithotrophic ability of using metallic iron as the electron donor for sulfate reduction [43]. Nonetheless, these new isolates were not the common type of SRB and the lithotrophic metabolism was considered to be limited to this new group of SRB [43]. There was no previous experimental evidence that illustrated the capability of direct iron oxidation by the more widespread, conventionally known (organotrophic, $H_2$-utilizing) SRB.

In the current study, *Desulfovibrio vulgaris* (ATCC# 7757), a common organotrophic SRB, was examined for ability of long-term survival under starvation of
organic electron donor but in the presence of carbon steel (CS) coupons. While there had been many studies examining the influence of SRB on pitting corrosion, they were conducted under conditions with abundant organic electron donor [81, 142, 143]. However, consumable organics to SRB may not be constantly available in corroding environments. Seawater, for example, contains only 0.5–0.8 mg/L organic carbon [144]. In the context of corrosion, steel itself represents a potential electron donor to support SRB metabolism under organic electron donor-limited conditions [53, 145]. The objectives of the study were therefore to examine whether this common SRB would die after the exhaustion of organic electron donor (pyruvate, in this case) or transition to survive with iron as the electron donor, and to evaluate the potential change of CS corrosion during the long-term organics-starving condition. For the latter objective, some results of pitting corrosion characterization including the maximum and average pit depths, pit density and 3D pit morphology, and the effects of different surface roughness levels have been reported elsewhere [70]. The focus of this current work was to closely examine the survival behaviors of this common (organotrophic, H$_2$-utilizing) SRB in the long-term (57 d) organics-starving but CS present environment. The biological persistence, activity, and biofilm morphology of *D. vulgaris* on CS under such conditions were observed by microscopic approaches.

4.2. Materials and Methods

The materials and methods used for elucidation of SRB corrosion mechanism were described in the following sub-sections.
4.2.1. Culture conditions and incubation setup

*D. vulgaris* (ATCC# 7757) was provided by Dr. Tingyue Gu at Ohio University (Athens, OH, USA) and was routinely cultivated on Postgate medium C [16], containing sodium pyruvate (6 g/L), Na$_2$SO$_4$ (4.5 g/L), yeast extract (1 g/L), NH$_4$Cl 1 (g/L), KH$_2$PO$_4$ (0.5 g/L), Na$_3$C$_3$H$_5$O(COO)$_3$·2H$_2$O (0.3 g/L), MgSO$_4$·7H$_2$O (0.06 g/L), CaCl$_2$·6H$_2$O 0.04 g, and FeSO$_4$·7H$_2$O (0.004 g/L). The pH of the medium was adjusted to 7.0 by addition of NaOH. Resazurine (1 mg/L) was included in the medium as an E$_h$ indicator. O$_2$ was removed from the medium in serum bottles by bubbling with N$_2$. The serum bottles were sealed with butyl rubber stoppers, and the N$_2$ headspace was subsequently replaced with 80:20 N$_2$:CO$_2$. Afterwards, medium was sterilized by autoclaving, and then cysteine (150 mg/L) was added as a reductant.

Cultures were grown at 25 °C for 26 h to reach the late exponential growth phase or early stationary phase and then used as the inoculum (5%) for experiments in which *D. vulgaris* was cultivated in the absence and presence of CS coupons. To establish correlations between optical density at 600 nm (OD$_{600}$) and cell number concentration, duplicate samples were taken from the cultures diluted to different values of OD$_{600}$ and the cell number in each sample was counted using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) on an Olympus light microscope coupled with a DP71 digital camera (Olympus Imaging America, Center Valley, PA). OD$_{600}$ was measured with 1 cm pathlength using a UV-Vis spectrophotometer (Model 1601, Shimadzu Corp., Canby, OR). After 26 h of growth, the cultures had an OD$_{600}$ of 0.51 ± 0.04. At this OD, cell number was about $1.4 \times 10^9$ (±5%) cells/mL.
Batch culture studies in triplicate were carried out at 37 °C to establish the profiles of cell growth, substrate consumption and metabolite production by *D. vulgaris*. The inoculum and medium were prepared as described in the previous section. Inoculum (4 mL) was added to each 100 mL crimp top serum bottle containing 80 mL medium. CS coupons were not included in this study. Periodic samples were taken for analyses using N₂flushed sterile syringes and analyses were conducted as described in the next section.

Cultivation of *D. vulgaris* in the presence of CS coupons was conducted in a similar fashion with ten systems in four sets of experiments. In three of the systems *D. vulgaris* was grown in Postgate medium C with 0.5 g/L KH₂PO₄ (low phosphate medium) in 600 mL glass Griffin beakers. These beakers were modified to remove pour spouts and sealed with rubber stoppers (size 14, Cole-Parmer, Vernon Hills, IL) to which stainless steel hooks were attached. With plastic tie wraps, CS coupons were hung vertically without direct contact with the metal hooks, and immersed in the medium. Metal hooks were maintained above the medium to avoid unrelated corrosion. In the other seven systems the medium used had 10 g/L KH₂PO₄ (high phosphate medium) to minimize pH change along the experiments. Two of these systems, other than the use of high phosphate medium, were cultured in exactly the same way as those described above for the low-phosphate cultures. Two other high-phosphate systems were made with coupons sealed inside 40 g/L agar (0.5-1 mm thick) and laid on the reactor bottom. The agar sealing was designed to limit direct cell contact with coupon so that the effect of cell-metal attachment on long-term cell survival could be more clearly assessed. The final three cultures with high phosphate medium were carried out in serum bottles (instead of Griffin reactors) with vertically hung coupons. In two of the systems, one side of the
coupons was covered with agar: one on the polished side, the other on the unpolished side. The third system was the control without agar coating. Note that to prevent O\textsubscript{2} intrusion into the reactors made from Griffin beakers, humidified, filter-sterilized (0.2 \(\mu\)m) N\textsubscript{2} was constantly bubbled through the medium (around 0.7 L/h) and the sampling ports and tubing for N\textsubscript{2} purge were made of stainless steel 304 (McMaster Car, Aurora, OH). Constant N\textsubscript{2} bubbling was not done for the serum bottle cultures (only the medium was purged with N\textsubscript{2} for 1 h prior to \textit{D. vulgaris} inoculation). Without constant N\textsubscript{2} purge, significantly more FeS precipitated in serum bottle cultures. Thus the last 3 high phosphate systems offered the opportunity of observing potential effects of FeS precipitation layer on \textit{D. vulgaris} survival and CS pitting.

To prepare CS coupons (C1010; Q-Lab, Westlake, OH) for deployment in the systems with low phosphate medium, the coupons were polished on one side [70], and numbers were scratched on the unpolished sides of coupons to aid in identification during weight loss analysis (described below). The coupons had dimensions 25 \(\times\) 11 \(\times\) 0.3 mm, with holes at one end, through which plastic tie-wraps were used to hang the coupons vertically on stainless steel hooks anchored to the rubber stopper. For deployment in high phosphate medium systems, CS coupons (Metal Samples Company, Munford, AL) with LAP finish on one side and 120 GRIT on the other side were custom made from the company. The coupons had dimensions 20.3 \(\times\) 10.2 \(\times\) 1.6 mm, with 5.1 mm diameter centered hole 5.7 mm away from the end. Coupons were cleaned in a sonicating acetone bath for 20 min, followed by sonication in ethanol. Sterilization of the coupons was carried out by soaking in 100\% ethanol for 2 days at room temperature. Four coupons were deployed in each reactor. Medium was inoculated with \textit{D. vulgaris} as described
above and incubated at 25°C. Uninoculated reactors in duplicate or triplicate were included in every set of experiments to serve as negative controls. Liquid samples and coupons were taken periodically from the reactors and analyzed as described below.

4.2.2. Analytical techniques

Planktonic cell biomass in cultures was evaluated based on optical density, protein concentration, cellular dry mass and confocal laser scanning microscopy (CLSM). For determination of OD$_{600}$, samples recovered from incubations were washed with deionized water, resuspended in water, and OD$_{600}$ was measured using a Shimadzu Model 1601 spectrophotometer (Shimadzu Corp., Canby, OR). The suspension was diluted to achieve an OD$_{600}$ within the linear range (<0.6) of the instrument. In the corrosion study with steel coupons, the cell concentration was also determined by measuring the intracellular protein concentration, which minimized the interference from non-cell solids. To measure intracellular protein concentrations, washed solids were treated with 0.2 N NaOH at 95°C for 20 min, to lyse the cells, and protein concentration was quantified by the standard Bradford method [127] using a commercially available kit (Bio-Rad Protein Assay II kit, Bio-Rad Laboratories, Hercules, CA). To determine cell dry mass concentration, a washed cell suspension (containing the cells collected from 5 mL broth in the reactor) was transferred into a (pre-weighed) aluminum dish and dried in an oven set at 50°C for at least 3 days till constant weight and the remaining mass was determined. A Pioneer analytical balance (Model PA214, Ohaus, Parsippany, NJ) was used for weighing. Planktonic cells were also quantified microscopically by first staining the cells with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies
Corp.; Grand Island, NY), followed by visualization in 2 dimensions using a CLSM with 488 nm and 559 nm excitation wavelengths. Live cells were visualized based on green fluorescence when observed under the CLSM, while dead cells exhibited red fluorescence and partially damaged cells appeared yellow. A Zeiss LSM 510 CLSM (Carl Zeiss AG; Jena, Germany) was used for the earlier experiments with low phosphate medium. An Olympus FV1000 CLSM (Olympus America Inc., Center Valley, PA) was used for all samples from the high phosphate medium systems.

The pH of samples was measured (Model 420A meter, Orion, Watsonville, CA) immediately upon removal from the reactors. In preparation for evaluation of aqueous chemistry of cultures, solids were separated from fluids by centrifugation and dissolved constituents in the supernatant were quantified. Pyruvate, acetate, and succinate concentrations were measured with a Shimadzu LC-10A high-performance liquid chromatography (HPLC) system (Shimadzu Corp.; Columbia, MD) equipped with a Supelco Supelcogel H column (Sigma-Aldrich Corp., St. Louis, MO) and UV-Vis detector (SPD-10A). H₃PO₄ (0.1%) was used as the mobile phase at 0.14 mL/min. Sulfate concentration was measured by ion exchange chromatography with conductivity detection using a Dionex DX 100 system fitted with an AS-14 column (Dionex Corp., Sunnyvale, CA). Samples for measurement of sulfide were preserved with equal volume of a zinc acetate solution (100 g/L) and sulfide was quantified. ZnS was separated from the fluid by centrifugation, solids were resuspended in deionized water, and sulfide was measured using the methylene blue assay [128]. The measured results for all the liquid samples were averaged from 4 to 6 measurements, 2 for each system.
4.2.3. Biofilm and CS coupon characterization

Coupons were removed from reactor vessels and processed for subsequent analyses in an anaerobic glove bag (Coy Laboratory Products, Grass Lake, MI) with 97.5% N₂ and 2.5% H₂. Many of the procedures described below were also performed inside the glove box whenever interference of atmospheric O₂ with post-sampling evaluation might be a concern. Digital pictures of the CS coupons were taken right after the coupons were removed from the reactor. *D. vulgaris* biofilms on CS coupons were stained and visualized as described above, except that the CLSM was operated in 3-dimensional scanning mode. Five to ten sets of CLSM images were taken for each CS coupon. Number of sessile SRB was quantified according to the average of 4 sets of biofilm CLSM images at each exposure time by using ImageJ (National Institutes of Health, Bethesda, MD). For biofilms with multiple cell layers, the cell numbers from all layers were summed to give the total sessile SRB number on the coupon. The statistical significance testing was carried out to compare difference of the sessile SRB (live or dead) cell number between different exposure time (3, 21 and 43 days) [130]. The *p* value of the sessile SRB cell number was calculated by Minitab (Minitab Inc., USA). Percentage of viable cells was calculated based on the ImageJ-counted results. Coupons that were recovered from incubations (with or without the SRB biofilm) were examined by using a scanning electron microscope (SEM, TM-3000 Tabletop SEM, Hitachi High Technologies America, Dallas, TX). To fix biofilms, coupons were immersed in anoxic glutaraldehyde solution (2% w/w) at 4 °C for 1 day, dehydrated in an ethanol series (0%, 20%, 50%, 75% and 100%), and placed in a desiccator for 1 day at room temperature. All the above procedures were performed in the anaerobic glove box. The fixed biofilms on
coupons were first observed using the SEM. All coupons were subsequently cleaned following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (C.3.1 in Table A1.1 of ASTM G1-03) [129], and examined again by SEM. All coupons were weighed before they were placed in the reactors. For each system (SRB or the control), four coupons were collected from one reactor at a sampling time. Every coupon (after being characterized for other purposes as described earlier) was cleaned according to the ASTM G1-03 C.3.1 [129] and then weighed twice. Average weight loss at each sampling time was calculated based on the eight measurements.

4.3. Results and Discussion

In this section, metabolism of *D. vulgaris* was studied in the presence and absence of carbon steel using various microbiological assays. Organic-starving SRB corrosion mechanism was also discussed.

4.3.1. Batch culture study of *D. vulgaris*

To examine the dynamics of *D. vulgaris* growth and metabolism in the absence of CS, pyruvate, sulfate consumption, and acetate, succinate accumulation during growth in Postgate medium C were evaluated in serum bottles without N₂ bubbling. During the first 24 h, pyruvate was completely consumed, and 17 mM acetate and 29 mM succinate were produced concurrently with cell growth and reduction of approximately 12 mM sulfate (Figures 4.1 [a], [c] and [d]). Acetate accumulation is attributable to conversion of pyruvate to acetate, CO₂, and H₂ via acetyl-CoA. While it is unclear whether succinate
accumulation was via oxidative or reductive arms of the TCA cycle [33], the fact that further succinate metabolism was not observed after cell growth stopped suggests that succinate accumulation was not completely related to assimilatory metabolism. After complete pyruvate depletion, no further acetate, succinate, or sulfate metabolism was observed and both cell protein and dry mass decreased (Figure 4.1), suggesting that cell death was occurring. Diminished cell activity and cell death were confirmed microscopically by live-dead staining; few cells were actively metabolizing after 43 days of incubation (Table 4.1).

![Figure 4.1](image-url)

Figure 4.1. Profiles of (a) cell growth (intracellular protein (◆), cell dry weight (○)), (b) pH (■) change, (c) nutrient (pyruvate (▲), sulfate (◇)) consumption, and (d) metabolite (acetate (△), succinate (□)) generation in batch culture of *D. vulgaris* ATCC 7757 in
Postgate medium C with 0.5 g/L KH$_2$PO$_4$, made in serum bottles in the absence of metal coupons.

Table 4.1. Viability of sessile and planktonic *D. vulgaris* cells at different days of incubation in Postgate medium C with low and high phosphate observed in various systems examined in this study

<table>
<thead>
<tr>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>With coupon incubation</th>
<th>Agar coating on coupon</th>
<th>Time (day)</th>
<th>Viability (%)$^+$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sessile cell</td>
<td>Planktonic cell</td>
<td></td>
</tr>
<tr>
<td>Low (0.5)</td>
<td>Yes</td>
<td>No</td>
<td>3</td>
<td>44 ± 8</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td>37 ± 5</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>43</td>
<td>65 ± 16</td>
<td>4 ± 1</td>
<td></td>
</tr>
<tr>
<td>High (10)</td>
<td>Yes</td>
<td>No</td>
<td>7</td>
<td>94 ± 6*</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td>92 ± 7</td>
<td>46 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>46</td>
<td>89 ± 3</td>
<td>5 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>57</td>
<td>85 ± 13*</td>
<td>0.4 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>21</td>
<td>100 ± 0</td>
<td>30 ± 15</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>46</td>
<td>92 ± 5</td>
<td>6 ± 4</td>
<td></td>
</tr>
<tr>
<td>Low (0.5)</td>
<td>No</td>
<td>No</td>
<td>43</td>
<td>--</td>
<td>0.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>High (10)</td>
<td></td>
<td></td>
<td>57</td>
<td>--</td>
<td>0.6 ± 1.0*</td>
<td></td>
</tr>
</tbody>
</table>

-- Values not available

* Made in serum bottles without N$_2$ bubbling; all others in modified Griffin reactors with constant N$_2$ bubbling

$^+$ Cell viability was estimated according to average cell counts of live, dead or partially damaged cells in 4 sets of confocal images
4.3.2. Batch corrosion studies of *D. vulgaris* incubated with CS

Cell growth and metabolism in incubations containing CS coupons in low phosphate medium exhibited patterns of planktonic cell growth, pyruvate consumption, and acetate and succinate accumulation similar to coupon-free cultures (Figures 4.2 [a] and [b]). A larger fraction of pyruvate was converted to acetate (Figure 4.2 [b]), but presumably due to removal of H$_2$ and H$_2$S by constant N$_2$ bubbling in these systems, no sulfate consumption could be detected and only 0.3 mM sulfide was found in the cultures. H$_2$S stripping also caused the sulfide concentration to decrease between 21 and 43 days, from 0.24 (±0.01) to 0.01 (±0.004) mM. Cell metabolites in high phosphate cultures made in reactors with constant N$_2$ bubbling, with or without agar coating of the CS coupons, also showed similar patterns. These results suggested that in these systems the initial cell growth was predominantly supported by substrate level phosphorylation and that sulfate reducing activity was diminished by H$_2$ removal as the cultures were continuously purged with N$_2$ [146]. No further cell growth (as indicated by cell dry mass and protein concentration) or organotrophic metabolism (as indicated by the constant acetate and succinate concentrations) was observed after 3 days (Figures 4.2 [a] and [b]).

Optical density measurements (Figure 4.3 [a]) also showed that the planktonic cells, after pyruvate depletion, would die and lyse faster in the high phosphate medium than in low phosphate medium, presumably due to high osmotic pressure of the high phosphate medium used. Agar sealing/coating of CS coupons, on the other hand, had no appreciable effects on planktonic cell growth and death profiles. Similar to the finding in coupon-free cultures, microscopic examination of cells using live-dead staining showed that few of the planktonic cells remained viable, though a slightly higher percentage of viable cells
was observed in the coupon-containing cultures than in the coupon-free cultures (Table 4.1). In conclusion, *D. vulgaris* experienced organic electron donor limitation between day 3 and day 43. After 3 days, planktonic cells started dying faster in the medium with 10 g/L KH$_2$PO$_4$ than in the medium with 0.5 g/L KH$_2$PO$_4$.

Throughout the experiments no cells were detected in the cell-free control systems with either high or low phosphate medium, when examined periodically by optical microscopy or UV/Vis spectrophotometry (Figure 4.3 [a]). pH increased in the control and SRB systems with low phosphate medium (Figure 4.3 [b]) whereas pH was roughly stable in the high phosphate systems. With low phosphate medium, the pH of the SRB-containing systems increased and then stabilized around 8.2-8.5 after 3 days but pH of the abiotic control increased rapidly to 8.8 by day 7, and further increased slowly to more than 9.2 in the rest of the experiment. As the pH increase in abiotic control matched the trend of cumulative weight loss (Figure 4.3 [c]), abiotic corrosion was likely accompanied by Fe(OH)$_2$ formation (see Equation [4.1] in Section 4.3). As for the effect of *D. vulgaris* metabolism on pH, pH decreased in the batch culture made in serum bottles where the systems did not have metal coupons and were not bubbled with N$_2$, as shown in Figure 4.1 (b). Therefore, the pH increase (from 6.13 to 8.33) observed in the low phosphate SRB systems was associated with CS corrosion and/or removal of H$_2$, CO$_2$, and sulfide due to the constant N$_2$ bubbling [147, 148].
Figure 4.2. Concentration profiles of (a) cell dry weight (○), intracellular protein (◆), sulfide (●) and (b) acetate (△), pyruvate (▲), succinate (---) and sulfate (◇) observed in long-term batch culture of *D. vulgaris* incubated in the presence of CS coupons in Postgate medium C with 0.5 g/L of KH$_2$PO$_4$, made in reactors with constant N$_2$ bubbling.
Figure 4.3. Effects of low and high phosphate media (with 0.5 vs. 10 g/L KH$_2$PO$_4$) on (a) *D. vulgaris* cell concentration, (b) pH and (c) CS coupon weight loss, shown with coupon-containing systems made in reactors with constant N$_2$ bubbling. Low phosphate systems are shown with solid lines; high phosphate systems with dashed lines. Cell-containing systems are shown with filled symbols; cell-free controls with unfilled symbols. Compared in (a) are the cell concentration profiles, represented by optical density at 600 nm, for the low phosphate (▲) and high phosphate (---▲---) systems (coupons in the latter were agar coated; uncoated coupons showed similar profile). OD profiles of the uninoculated systems: low phosphate (△) and high phosphate (---△---), are also shown to indicate no growth of other micorbial contaminants; the residual OD readings were from medium turbidity and precipitates. Compared in (b) are the pH profiles for systems with *D. vulgaris*: low phosphate (●), high phosphate (---●---), and uninoculated systems: low phosphate (○), high phosphate (---○---). It is clear that pH increased less in high phosphate systems and that pH increased more in uninoculated systems. Compared in (c) are the profiles of cumulative weight loss of coupons (without agar coating, except one specified otherwise) for systems with *D. vulgaris*: low phosphate (■), high phosphate (---■---), high phosphate with agar-coated coupons (---◇---), and uninoculated systems: low phosphate (□), high phosphate (---□---). Weight loss corrosion was highest in the uninoculated high-phosphate medium.
4.3.2.1. Biofilm characterization

Dynamics of *D. vulgaris* biofilm formation on CS surfaces was characterized by CLSM using live-dead staining. Details are described below, as example, for low phosphate systems with N₂ bubbling. Biofilms examined were on CS coupons incubated for 3, 21, and 43 days. After 3 days biofilm was formed with non-uniform coverage of coupon surface (Figure 4.4 [a]), giving a high standard deviation for sessile cell numbers at different positions examined (Table 4.2 [a]). The sessile cell viability was 44 ± 9 % (Table 4.1), with random distribution of live and dead cells (Figure 4.4 [a]). This viability distribution might be caused by the limited diffusion of pyruvate to support the organotrophic metabolism of cells in the inner layers. At day 21 *D. vulgaris* biofilm covered the CS surface more uniformly (Figure 4.4 [b]), giving a smaller standard deviation of total sessile cell number than at day 3 (Table 4.2 [a]). Total sessile cell number, per cm², also increased from (2.3 ± 0.7) × 10⁷ at day 3 to (3.5 ± 0.2) × 10⁷ at day 21 (Table 4.1, p = 0.012). Significantly more cells became attached on CS coupons after the organic electron donor was depleted, but the live cell numbers were similar at days 3 and 21 (p = 0.301); accordingly, the sessile cell viability was lower at day 21 (37 ± 5 %). More importantly, the biofilm structure at day 21 showed a pattern of live and dead cell distribution in which cells in outer layers of biofilm were dead, while those in direct contact with the coupon surface were mostly alive (Figure 4.4 [b]). This distribution pattern was the opposite of that observed at day 3. It indicated the important role played by the coupon in maintaining long-term survival of *D. vulgaris* cells after pyruvate depletion. At day 43 pyruvate had been depleted for 40 days. There was considerably less coupon coverage by cells (Figure 4.4 [c]) and the thickness of remaining biofilm
decreased to approximately 3 or 6 μm (Figures 4.3 [c] and [d]). The viable and total sessile cell numbers per cm$^2$ decreased to $(6.1 \pm 3.1) \times 10^6$ and $(9.3 \pm 6.2) \times 10^6$, respectively. The standard deviations were the highest at this time, indicating uneven biofilm distribution on the coupon. Despite the large standard deviations, the p-values for comparison of live and total sessile SRB numbers between days 21 and 43 were small (0.009 and 0.000), so the decreases in sessile cell numbers during this period were statistically significant. On the other hand, the sessile cell viability reached the highest value of $65 \pm 16\%$ at day 43; therefore, the cells detached from CS were mostly dead cells (Table 4.1). It is unclear if this sloughing of dead cells caused the slightly higher amount of live planktonic cells in CS-containing cultures than in the CS-free cultures (Table 4.1), with the components released from lysed cells supporting survival of a very small planktonic population. Examination of biofilms by SEM revealed similar dynamics of cell-CS association, with substantial decreases in surface coverage and biofilm thickness between 21 and 43 days, (Figures 4.5 [a] and [b] vs. [c] and [d]). At day 43 the coupons had only one thin layer of cells (Figures 4.5 [c] and [d]). The presence of thin cell layers in large areas of coupon surface supported the CLSM images that the attachment of cells to coupon enhanced survival after depletion of organic electron donor. Despite the minimal survival of planktonic cells in the cultures after 43 days ($4 \pm 1\%$), the majority of sessile cells remained alive. The even lower planktonic cell viability in the CS-free system ($0.7 \pm 0.5\%$) further confirmed the beneficial effects of CS on SRB survival (Table 4.1).
Figure 4.4. CLSM 3D images of *D. vulgaris* biofilm on CS coupons incubated in Postgate medium C with 0.5 g/L of KH$_2$PO$_4$ in modified Griffin beakers with constant N$_2$ bubbling after (a) 3 days, (b) 21 days, and (c) and (d) 43 days with thin and thickest biofilms. The bottom layer of the 3D image corresponds to the carbon steel surface. Cells were stained by FilmTracer™ LIVE/DEAD® Biofilm Viability Kit immediately after the coupons were collected from the reactors. Red color indicates dead cells, green color indicates live cells and yellow color indicates partially damaged cells. The 3D images were acquired by overlaying and aligning six horizontal optical thin sections.
Figure 4.5. SEM images of CS coupons incubated with *D. vulgaris* culture in low phosphate Postgate medium C for (a) and (b) 21 days, (c) and (d) 43 days, respectively. Images were taken before cleaning with Clarke’s solution. The biofilm of multilayered cells of short rod shape can be most clearly seen in (a). The uneven biofilm coverage on CS coupon is shown in (b) where the thicker biofilms appear black as indicated by the arrows. A more magnified image of the thick biofilm is shown in (a). Images (c) and (d) show the biofilm morphology near the corroding pits, which are the dark sunken areas in the images. As more clearly shown in (c), cells are accumulated on coupon surface around the pit, and a few cells (c) or cell aggregates (d) can be seen inside the pits.

4.3.2.1.1. Effect of high phosphate medium.

Similar general phenomena to those described above were observed in the experiments with high phosphate medium and constant N₂ bubbling and with CS coupons not being sealed or coated by agar. Several differences were noticed and they were all attributable to the faster cell lysis due to high osmotic pressure of the high phosphate
medium. For planktonic cells this effect was apparent with the faster decline of planktonic cell population in the high phosphate systems after pyruvate was exhausted (Figure 4.3 [a]). For sessile cells, this effect influenced biofilm structure in two aspects. Firstly, up to 21 days there were significantly fewer attached cells in the high phosphate systems (Tables 4.2 [a] and [b]). Secondly, while the biofilm in low phosphate systems had several outer layers of dead cells and an inner layer of live cells (Figure 4.4 [b]), the sessile cells in high phosphate systems were present as a scattered thin layer of mostly live cells during the entire exposure time (Figures 4.6 [d] and 4.7 [b]) and the sessile cell number remained rather constant (Tables 4.2 [a] and [b], p = 0.213 for comparison between days 21 and 43). As dead cells would lyse rapidly, the sessile cell viability in the high phosphate systems was very high: At day 7 most of the sessile cells were alive and only a few were partially damaged (Figure 4.6 [b]), giving a high viability of 94 ± 6% (Table 4.1), whereas the viability of low phosphate systems already decreased to 44 ± 9% at day 3. At day 21 sessile cell viability in high phosphate systems remained high at 92 ± 7% while the viability in low phosphate systems was still low at 37 ± 5%. The dead sessile cells in low phosphate systems eventually lysed or detached after 43 days and the biofilm structures (Figures 4.3 [a] and 4.4 [c]) and sessile cell numbers (Table 4.2 [a]) became similar between the high and low phosphate systems, although the viability was still higher in the high phosphate systems (89 ± 4%) than in the low phosphate systems (65 ± 16%). The sustained viability in the high phosphate systems further confirmed that the sessile cells directly attached on CS survived whereas the planktonic cells did not (Table 4.1 and Figure 4.3 [a]).
Table 4.2. (a) Numbers of live, dead and total sessile *D. vulgaris* cells on CS coupons incubated for different days in various systems examined in this study and (b) p-values calculated by Minitab for statistical significance when these sessile cell numbers are compared for effects of different incubation time, KH$_2$PO$_4$ concentrations, and extents of FeS formation due to use of different reactors

(a)

<table>
<thead>
<tr>
<th>Reactor</th>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>Time (day)</th>
<th>Live</th>
<th>Dead</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Griffin, negligible FeS on coupon</td>
<td>Low (0.5)</td>
<td>3</td>
<td>(1.0 ± 0.5) × 10^7</td>
<td>(1.3 ± 0.3) × 10^7</td>
<td>(2.3 ± 0.7) × 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>(1.3 ± 0.2) × 10^7</td>
<td>(2.2 ± 0.3) × 10^7</td>
<td>(3.5 ± 0.2) × 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>(6.1 ± 3.1) × 10^6</td>
<td>(3.3 ± 3.2) × 10^6</td>
<td>(9.3 ± 6.2) × 10^6</td>
</tr>
<tr>
<td></td>
<td>High (10)</td>
<td>21</td>
<td>(8.1 ± 2.4) × 10^6</td>
<td>(9.1 ± 8.2) × 10^5</td>
<td>(9.0 ± 3.1) × 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46</td>
<td>(5.9 ± 0.9) × 10^6</td>
<td>(7.7 ± 3.4) × 10^5</td>
<td>(6.7 ± 1.2) × 10^6</td>
</tr>
<tr>
<td>Serum bottle, clear FeS on coupon</td>
<td>High (10)</td>
<td>7</td>
<td>(2.5 ± 0.5) × 10^6</td>
<td>(1.9 ± 2.0) × 10^5</td>
<td>(3.0 ± 0.4) × 10^6</td>
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<td></td>
<td>57</td>
<td>(1.7 ± 0.9) × 10^6</td>
<td>(3.1 ± 2.6) × 10^5</td>
<td>(2.0 ± 1.0) × 10^6</td>
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(b)

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<th>Factors for comparison</th>
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<th>Time (day)</th>
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<td></td>
<td></td>
<td>Live</td>
<td>Dead</td>
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<tr>
<td>Incubation time</td>
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<td>3 vs. 21</td>
<td>0.301</td>
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<td>3 vs. 43</td>
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<td>0.004</td>
<td>0.026</td>
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<tr>
<td></td>
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<td>21 vs. 43</td>
<td>0.009</td>
<td>0.000</td>
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<tr>
<td></td>
<td>10</td>
<td>21 vs. 46</td>
<td>0.134</td>
<td>0.763</td>
<td>0.213</td>
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<tr>
<td>FeS</td>
<td>10</td>
<td>46 vs. 57*</td>
<td>0.001</td>
<td>0.072</td>
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<td>KH$_2$PO$_4$ concentration</td>
<td>0.5 vs. 10</td>
<td>21</td>
<td>0.020</td>
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<tr>
<td></td>
<td>43 or 46</td>
<td>0.931</td>
<td>0.178</td>
<td>0.439</td>
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<tr>
<td></td>
<td>3 or 7*</td>
<td>0.043</td>
<td>0.000</td>
<td>0.004</td>
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<tr>
<td></td>
<td>43 or 57*</td>
<td>0.037</td>
<td>0.119</td>
<td>0.060</td>
<td></td>
</tr>
</tbody>
</table>

* Made in serum bottles without N$_2$ bubbling; all others in modified Griffin reactors with constant N$_2$ bubbling
(a) 7 d, on agar surface

(b) 7 d, on CS without agar coating

(c) 46 d, on agar surface

(d) 46 d, on CS underneath the agar

(e) 46 d, in agar layer
Figure 4.6. CLSM 3D images showing effects of agar coating of CS coupon on *D. vulgaris* biofilm structure and viability in Postgate medium C with 10 g/L of KH$_2$PO$_4$. In (a) and (b) *D. vulgaris* was incubated in serum bottles for 7 days (with still low FeS formation) with (a) agar coated and (b) uncoated CS coupons. As shown in (a), many cells were already dead (red) or damaged (yellow), due to organic electron donor starvation, in the biofilm formed on the outer (medium facing) surface of agar coating while in (b) all the cells attached on CS surface were alive. Images (c) – (e) are all for agar coated coupons incubated for 46 days with *D. vulgaris* in modified Griffin beakers under constant N$_2$ bubbling. The sketch in (e3) was for indication only, not in scale. Cells were stained by the same test kit as in Figure 4.4.

4.3.2.1.2. Effect of FeS crust formation

For *D. vulgaris* to survive on CS by coupled iron oxidation and sulfate reduction, the starving cells must be able to get electrons directly from the metal, which would likely require direct contact of cells with CS or other electron conductor to allow the electron transport. FeS mineral crust was reported to be able to conduct electrons for sulfate reduction of SRB [50]. To evaluate the effect of FeS crust formation on survival of *D. vulgaris*, some experiments were conducted in serum bottles without constant N$_2$ bubbling. Note that in the previously described experiments, the constant N$_2$ bubbling stripped H$_2$S, and FeS formation was minimal (Figure 4.7 [a]). The experiment with serum bottles used the high phosphate Postgate medium C and lasted for 57 days. Black FeS crust formed on the entire surface of CS coupons collected at the end of experiment, which had a strong rotten egg smell (Figure 4.7 [b]). In contrast to the more or less flat (Figure 4.7 [c]) or continuous layer of biofilm (Figure 4.4 [b]) on coupon surface in the systems without FeS crust, cells were found to be dispersed in different layers of FeS crust, with some as aggregates (Figure 4.7 [d]). As shown by the side view of cell aggregates in Figure 4.7 (d), cells inside the aggregate were dead while those on the outer layer of aggregate were mostly alive. This distribution suggested that the outer layer of cells could obtain electrons conducted through FeS crust to survive but the cells inside
could not. As in the previously described systems, sessile cell viability, \((85 \pm 13)\%\), was again much higher than the planktonic cell viability, \((0.4 \pm 0.2)\%\) (Table 4.1). However, the viable sessile cell number after 57 days in the system with FeS crust formation, i.e., \((1.7 \pm 0.9) \times 10^6\) per cm\(^2\), was significantly lower than those in the systems without serious FeS precipitation (in either low or high phosphate medium) after 43 or 46 days, i.e., \((6.1 \pm 3.1) \times 10^6\) and \((5.9 \pm 0.9) \times 10^6\) per cm\(^2\) \((p = 0.037\) and 0.001, respectively) (Table 4.2 (a) and (b)). Also, more dead cells were observed inside the FeS crust (Figure 4.7 [d]), than on the CS surface with negligible FeS (Figure 4.7 [c]). It is hypothesized that these differences are attributable to lower availability of sulfate (terminal electron acceptor) to \(D. vulgaris\) cells due to diffusion limitation of sulfate through the rather thick (up to \(~45\ mu m\) FeS crust. Nevertheless, these results indicated that FeS was able to transfer electrons from CS to \(D. vulgaris\) [50] to help their survival.

![Figure 4.7. CLSM 3D images showing effects of FeS crust formation on \(D. vulgaris\) biofilm structure and viability. \(D. vulgaris\) was incubated with CS (without agar coating) in high phosphate Postgate medium C. In image (a), coupon was incubated for 57 days in a serum bottle and a thick FeS crust formed on the coupon (more than 45 \(\mu m\) at some places). Cells were distributed at different heights in the crust. Most cells were alive but many were dead. A cut view of a cell aggregate is provided to show the dead cells inside.](image-url)
In image (b), coupon was incubated for 46 days in a modified Griffin beaker with constant N₂ bubbling and negligible FeS formation. Cell viability was clearly higher than that in (a) and cells were attached on the relatively flat coupon surface. Cells were stained by the same test kit as in Figure 4.4.

4.3.2.1.3. Effect of agar coating

To further confirm the necessity of direct attachment for cell survival, experiments were done with agar-sealed CS coupons. Coupon samples were removed for examination at days 7, 21 and 46. At some locations, presumably due to heterogeneous agar density, cells were able to penetrate into certain areas of agar layer and even reach the coupon surface (Figure 4.6 [e]). Even at day 7 the cells on the agar surface (exposed to liquid medium) were mostly dead (Figure 4.6 [a]). Migrating cells that were trapped in the agar (without reaching the coupon surface) were either partially damaged or dead whereas the cells attached on CS surface were alive (Figure 4.6 [e]). Viability comparison between planktonic and sessile cells is summarized in Table 4.1. All the cells that penetrated through the agar and attached on CS coupons survived after 21 days and the viability remained high at 92 ± 5% after 46 days; on the other hand, viability of planktonic cells was only 30 ± 15% at day 21 and decreased further to 6 ± 4% at day 46.

The results obtained from all experimental systems indicated that D. vulgaris cells could survive through long absence of organic electron donors provided that the cells were directly attached to CS or in contact with conductive FeS crust on CS.

4.3.2.2. Corrosive activities of D. vulgaris

The long-term cell survival on CS suggested that the steel could serve as an electron donor to support cell maintenance [49, 50, 53]. This iron oxidation could drive
the SRB-induced corrosion [43]. The extent of CS corrosion was therefore evaluated by mass loss analysis and the coupon surfaces were examined by SEM and CLSM. For the systems with low phosphate medium, during the first 3 days, weight loss of the CS coupons incubated in *D. vulgaris* cultures with pyruvate present (0.20 ± 0.03 mg/cm² per day, Figure 4.3 [c]) occurred at a greater rate and to a greater extent than that of coupons incubated in cell-free medium (0.06 ± 0.02 mg/cm² per day, Figure 4.3 [c]). On the other hand, weight loss between day 3 and day 21 was higher in uninoculated medium than in *D. vulgaris* cultures without pyruvate (Figure 4.3 [c]), suggesting that the non-growing biofilm on CS was protective against weight loss. As more biofilm sloughed off the CS surface after day 21, weight loss during day 21 to day 43 became comparable in *D. vulgaris*-containing incubations and in the uninoculated medium (Figure 4.3 [c]). The results suggested that SRB biofilm had a protective effect against uniform corrosion of CS coupons. This protective biofilm effect could also be seen with agar coated and uncoated coupons in the high phosphate systems (Figure 4.3 [c]). The weight loss was clearly higher for the agar coated coupons where the biofilm coverage was restricted by the agar layer.

No pits were found on the coupons recovered from the cell-free incubations (not shown), but coupons recovered from *D. vulgaris* cultures exhibited severe pitting (Figures 4.5 [d] and 4.6 [d]). Pitting corrosion is a more serious threat to metal integrity than uniform corrosion [23, 57]. For the coupons collected at day 43 from the systems with low phosphate medium, pits had either few cells (Figure 4.5 [c]) or very dense SRB aggregates/biofilms inside (Figure 4.5 [d]), while many cells distributed around the pits. The observations suggested that pitting corrosion was associated with the activities of
attached *D. vulgaris* cells. This association was further concluded from the results obtained with the agar-coated coupons. As described earlier, the cells penetrated through the agar only in certain areas. When carefully observed by CLSM, pits were found in all the areas with direct cell attachment on coupon and no pits were found in areas without cell attachment. The attached cells were mostly distributed around the coupon surface outside the pits but some cells were seen inside the pits (Figures 4.5 [d] and 4.6 [d]). Almost all the cells near the pits were alive (Table 4.1 and Figure 4.6 [d]).

Some more results for pit morphology, distribution, depth, density, and statistical surface roughness information obtained by SEM and IFM are available elsewhere [70]. With surviving *D. vulgaris* cells on CS, a maximum pit depth of 46 µm was reached in 21 days and pit density increased from day 3 (1.76 × 10^5/m^2) to day 43 (7.42 × 10^5/m^2) [70]. Pitting corrosion sustained during the prolonged absence of organic electron donors.

4.3.3. Survival and corrosion mechanism of *D. vulgaris* under organic starvation

The results showed that long term survival of *D. vulgaris* without organic electron donors could be enhanced by direct cell attachment on CS surface. Metabolism of the surviving cells led to extensive pitting corrosion of CS. Anaerobic MIC used to be commonly explained by the cathodic depolarization theory (CDT). According to this theory, H\textsubscript{2}-consuming microorganisms deplete cathodic H\textsubscript{2} or atomic hydrogen by, e.g., sulfate reduction (Equations [4.1] and [4.2]). This microbial activity allows further cathodic H\textsubscript{2} production and electron “extraction” from iron (Fe\textsubscript{0}) [36, 149]. However, the role of microbial consumption of cathodic H\textsubscript{2} in MIC has been questioned. Stott [38] pointed out that the hydrogenase enzyme from *Desulfovibrio desulfuricans* could only
reversibly work on H\textsubscript{2} [39], not on atomic hydrogen for cathodic depolarization [38]. Several kinetic studies further showed that scavenging of H\textsubscript{2} did not accelerate Fe\textsuperscript{0} oxidation [41, 42]. Another theory explained the accelerated corrosion by the chemical reaction of Fe\textsuperscript{0} with H\textsubscript{2}S (Equation 4.3) [24, 43, 44]. Under this scenario, close association of *D. vulgaris* with CS would have enhanced the mass transfer of H\textsubscript{2} from metal surface to cells for driving H\textsubscript{2}S production by sulfate reduction, as has been observed in syntrophic cocultures [37]. This mechanism, however, would not require direct contact of cells with CS. In the systems with constant N\textsubscript{2} bubbling used in the current study, the stripping of H\textsubscript{2} and H\textsubscript{2}S would have seriously affected cell survival and reduced the corrosion. Also, the FeS crust formed in the systems made in serum bottles would have significantly hindered both H\textsubscript{2} production from the metal underneath [45] and the H\textsubscript{2} diffusion to reach the encrusted cells. Pitting corrosion and cell survival would have been essentially stopped. These hypotheses, therefore, do not adequately explain the long term cell survival and pitting corrosion consistently observed in this study with various systems.

\[
\text{Fe} + 2\text{H}_2\text{O} \rightarrow \text{Fe(OH)}_2 + \text{H}_2 \quad (4.1)
\]
\[
4 \text{H}_2 + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4 \text{H}_2\text{O} \quad (4.2)
\]
\[
\text{Fe} + \text{H}_2\text{S} \rightarrow \text{FeS} + \text{H}_2 \quad (4.3)
\]

Alternatively, as shown in Figure 4.8, the enhanced viability of cells directly attached to CS or in contact with conductive FeS layer might be better explained if *D. vulgaris* could accept electrons directly (or via FeS) from Fe\textsuperscript{0} oxidation for sulfate reduction, with the following overall reaction (Equation 4.4) [53, 150, 151]:

\[
4 \text{Fe} + 9 \text{H}^+ + \text{SO}_4^{2-} \rightarrow \text{HS}^- + 4 \text{H}_2\text{O} + 4 \text{Fe}^{2+} \quad (4.4)
\]
The mechanisms by which *D. vulgaris* may utilize Fe\(^0\) as an electron donor remain unclear, but others have also shown that the special SRB isolated/enriched using steel-Fe\(^0\) as the sole electron donor could oxidize Fe\(^0\) at greater rates and to greater extents than those explainable exclusively by microbial consumption of cathodic H\(_2\) [49, 50]. These results also suggested that the Fe\(^0\) oxidation might have been facilitated by direct microbial metabolism of Fe\(^0\). Structures putatively identified as conductive microbial nanowires [152] were observed in MIC-associated SRB cultures [153]. These nanowires may facilitate the direct transfer of electrons from Fe\(^0\) to the attached cells. Similarly, it has been suggested that (semi)conductive corrosion products may facilitate transfer of electrons to cells [50]. It appears that certain SRB strains are adapted to “H\(_2\)-independent” oxidation of Fe\(^0\) [49, 50], but it is unknown whether the strain of *D. vulgaris* used in this current study is capable of this form of metabolism. Also, by SEM examination we did not find nanowire-like structures associated with the cells attached on CS coupons. Nevertheless, the results of this study clearly showed that the exploitation of Fe\(^0\) as an electron donor would be enhanced by the direct attachment of the H\(_2\) utilizing SRB to the CS surface, with and without the formed FeS film.

Thermodynamically, the iron oxidation mechanism can be explained as following. One SRB-involved cathodic reaction was the sulfide production from sulfate reduction (Equation [4.5]), as shown in Figure 4.2. According to the confocal images shown in Figures 4.4, 4.6 and 4.7, carbon steel has beneficial effects to *D. vulgaris* survival. Hence, iron oxidation to ferrous or ferric ions (Equations [4.6] and [4.7]) were considered as two possible anodic reactions, which corresponded to the full reactions as shown in Equations (4.4) and (4.8). The Gibbs free energy change (ΔG) at different time for both sets of
coupled cathodic-anodic reaction were calculated to estimate whether the reaction was thermodynamically favorable. ΔG estimation was based on Equations (4.9) and (4.10). Average sulfate and sulfide concentration shown in Figure 4.2 were used in the calculation. Hydrogen ion concentration was converted from measured pH shown in Figure 4.3. (b). Ferrous and ferric concentration were measured by ferrozine assay, as shown in Table 4.3. The summary of Gibbs free energy for Equations (4.4) and (4.8) was presented in Table 4.4. All the values of Gibbs free energy were negative for Equation (4.4), which indicated that iron oxidation to ferrous ion was thermodynamically favorable. However, all the values of ΔG for Equation (4.8) were positive, which indicated the reaction was not spontaneous. For the long-term stagnant systems with SRB, H₂ might contribute to cell survival, if it was not completely purged out by N₂, but special live cell distribution especially showed in the confocal picture Figure 4.4 (b) proved that iron oxidation should be the main reaction to maintain the cell survival. In conclusion, the most possible reaction happened to help SRB survive was iron oxidation to ferrous ion coupled with sulfate reduction, shown in Equation (4.4). By this mechanism corrosive SRB biofilms can persist in organic carbon-poor, corrosive environments. This long-term survival mechanism can explain the prevalence of SRB in biofilm samples associated with corroding metals containing iron.

\[
\text{Cathode: } 8\text{H}^+ + \text{SO}_4^{2-} + 8\text{e}^- \rightarrow \text{S}^{2-} + 4\text{H}_2\text{O} \quad (4.5)
\]

\[
\text{Possible anode 1: } \text{Fe} \rightarrow \text{Fe}^{2+} + 2\text{e}^- \quad (4.6)
\]

\[
\text{Possible anode 2: } \text{Fe} \rightarrow \text{Fe}^{3+} + 3\text{e}^- \quad (4.7)
\]

\[
\text{Possible full reaction: } \frac{8}{3}\text{Fe} + 8\text{H}^+ + \text{SO}_4^{2-} \rightarrow \text{S}^{2-} + 4\text{H}_2\text{O} + \frac{8}{3}\text{Fe}^{3+} \quad (4.8)
\]
\[ \Delta E = \Delta E^\circ - \frac{RT}{nF} \ln Q \]  

\[ \Delta G = -nF \Delta E \]

where  
- \( R \) = the universal gas constant (8.3145 J/mol K),  
- \( T \) = temperature in K,  
- \( F \) = Faraday’s constant, (96,500 C mol\(^{-1}\)),  
- \( n \) = number of moles of electrons exchanged between the redox and oxidation reaction.  
- \( Q \) = the reaction coefficient and for a reaction of the form. If the redox reaction is  
  \[ aA + bB \rightarrow cC + dD, \]  
  \( Q \) is given by \( Q = [C]^c[D]^d / ([A]^a[B]^b] \)  
  \[ \Delta E^\circ = \Delta E^\circ \text{ (cathode)} - E^\circ \text{ (anode)} \]  
  = standard potential at the cathode – standard potential at the anode

Table 4.3. Ferrous and ferric concentration in the liquid bulk solution of with SRB systems.

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<td>c(Fe(^{2+}), mM)</td>
<td>0.045 ± 0.016</td>
<td>0.089 ± 0.011</td>
<td>0.018 ± 0.003</td>
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<td>c(Fe(^{3+}), mM)</td>
<td>0.076 ± 0.034</td>
<td>0.086 ± 0.010</td>
<td>0.028 ± 0.008</td>
<td>0.066 ± 0.004</td>
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Table 4.4. Estimation of Gibbs free energy change with time for reactions shown in Equations. (4.4) and (4.8) in with SRB systems.

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<td>( \Delta G ) (Equation 4.4) (kJ)</td>
<td>-400.4</td>
<td>-319.7</td>
<td>-326.6</td>
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<td>( \Delta G ) (Equation 4.8) (kJ)</td>
<td>224.9</td>
<td>309.4</td>
<td>309.8</td>
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4.4. Conclusions

According to the results of CLSM, SEM, cell metabolism analysis and viability comparison, the following conclusions can be drawn:

(1) In the absence of organic electron donors, the sulfate-reducing bacterium *D. vulgaris* could survive for a long period, up to 55 days, by attaching directly on CS surface or in the FeS crust formed on CS. Attached cells were mostly distributed around or inside the pits. Results also suggested that FeS crust could transfer electrons from oxidizing CS surface to the *D. vulgaris* cells embedded in the FeS crust to help their long term survival.
(2) Medium with 10 g/L KH₂PO₄ buffer caused faster lysis of both planktonic and sessile *D. vulgaris* cells, presumably due to high osmotic pressure. Accordingly, in the high phosphate medium the biofilm on CS was only found at scattered locations and had mostly one layer of cells, in contrast to the thick biofilm observed on CS in the low phosphate medium.

(3) For agar-coated CS, pitting corrosion occurred only at a few locations where *D. vulgaris* cells succeeded in migrating through agar coating and attaching directly on CS. After 46 days the viability of CS-attaching cells remained high at (92 ± 5)% while the planktonic cell viability was only (6 ± 4)%. The results proved that the pitting corrosion was directly associated with the cell attachment and that the cell attachment on CS was essential to the long-term cell survival.

(4) While causing serious pitting corrosion, *D. vulgaris* biofilm was protective against uniform corrosion in terms of weight loss.

The results from all of the experiments examined under different conditions, i.e., with high or low phosphate medium, with agar coated or uncoated CS coupons, and with or without FeS crust formation, consistently showed that long term survival of *D. vulgaris* in the absence of organic electron donor was supported by direct cell attachment on CS, with or without FeS crust. Exploitation of electrons from Fe⁰ by the cells for sulfate reduction was essential for long term cell survival. This mechanism explained the persistence of *D. vulgaris* and the sustained pitting corrosion in steel-containing environment.
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CHAPTER V

CHARACTERIZING PITTING CORROSION CAUSED BY A LONG-TERM STARVING SULFATE-REDUCING BACTERIUM SURVIVING ON CARBON STEEL AND EFFECTS OF SURFACE ROUGHNESS

5.1. Introduction

Sulfate reducing bacteria (SRB) are widespread in natural environment. They usually attach to substrate surface and form biofilm [1, 143]. They can utilize sulfate as the terminal electron acceptor and various organic nutrients as electron donors [27]. SRB are well known for attacking CS in the form of pitting corrosion [22, 47]. The damaging effects caused by pitting are typically much more severe than those by uniform corrosion. Pitting leads to perforation or crack initiation and, accordingly, has been the root to many corrosion failures [8, 9, 57, 154]. Pitting corrosion is much more difficult to detect in the field than the uniform corrosion, because the pits are often micron-sized and are concealed under the corrosion products and/or biofilm. Pitting has also been considered to be of stochastic nature [25, 26]; thus, it is harder to ascertain the absence of pits or to

---

predict the maximum pit depth in the whole facility by examining only selected samples or sections. Corrosion pits induced by SRB were reported to cause catastrophic problems, especially in the oil and gas industry [8, 9]. More studies are necessary to help understand the pitting corrosion and address the difficulties in detection of pits and estimation of pitting progression.

Most literature reports on the SRB-involved corrosion had organic energy sources (electron donors) in the media. However, corroding environments can lack rich supply of organic nutrients. For example, seawater contains only 0.5-0.8 g/L organic carbon (C) and most of these organics are not SRB-consumable [144]. Even if organic energy sources are available, the cells in deeper layers of the biofilm can still be starved due to consumption of the inward-diffusing organics by cells in the outer layer [1]. Whether SRB can survive and cause pitting under such starvation is an important topic. For example, a nitrate treatment strategy has been proposed to inhibit the SRB-induced corrosion according to five mechanisms [155]. One of the mechanisms is that nitrate enhances the activities of nitrate reducing bacteria, which can compete with SRB for carbon nutrients [93, 94]. However, in some cases, the treatment was ineffective and unpredictable in the field [96, 156]. The question remained if the SRB induced corrosion can be prevented by periodic elimination of carbon nutrients or if SRB can still cause pitting corrosion in the absence of organic energy source.

Current literature about the SRB induced corrosion under conditions without organic energy sources is rather limited and incomplete. Cord-Ruwisch et al. [52] reported that much more sulfide was produced when a SRB culture was incubated with steel wool than the control without steel wool. According to the cathodic depolarization
theory (CDT) [36], they proposed that iron can serve as a hydrogen source for that SRB culture. CDT has been revisited by many researchers. Stott [38] pointed out that the hydrogenase enzyme from Desulfovibrio desulfuricans, strain Hildenborough could only reversibly work on molecular hydrogen [39], instead of using atomic hydrogen to depolarize the cathode. Dinh et al. [49] isolated a SRB strain that can use metallic iron directly as the sole energy source, as opposed to being an indirect energy source via, e.g., hydrogen generation. Enning et al. [50] demonstrated that the SRB strain isolated by Dinh et al. caused faster and more extensive corrosion than the H\textsubscript{2} utilizing SRB. When closely examining their published results (particularly Figure 4 [c] of reference 50), we noticed that the H\textsubscript{2} utilizing SRB tended to induce micro pits, while the directly iron-utilizing SRB caused severe weight loss and macro pits. Sherar et al. [51] observed that the cells of a SRB isolated originally from an oil well formed a nanowire structure and attached firmly on a CS surface under the organic substrate limited condition. They hypothesized that the nanowire structure can be an approach for the SRB to uptake either electrons or molecular hydrogen directly from the steel surface. Gu [53] proposed a mechanism that the electrons generated by iron oxidation can be used at the cathode for sulfate reduction. Xu and Gu [157] later showed with experiments that both weight loss and maximum pit depth caused by a SRB were higher during a 7-day period of organic substrate starvation, as compared with those under the non-starving condition. Furthermore, they showed that addition of electron mediators such as flavin adenine dinucleotide (FAD) and riboflavin in the medium could accelerate the SRB-induced pitting corrosion [54]. All the above literature reports suggest the possibility of direct electron uptake from the metal surface by at least some SRB species.
In a parallel study made in our laboratory [83], the SRB *Desulfovibrio vulgaris* ATCC 7757 was maintained for 40 days in a medium exhausted with organic energy source but in the presence of CS coupons. It was found that only the layer of cells directly attached on the coupons survived the long starvation. In addition, by counting the sessile cells it was revealed that more cells became attached on the coupon surface after the organic energy source was consumed out. The results strongly suggested this SRB strain relies on the attachment to steel surface for survival through the starvation. Melchers [158] reported that much higher number of cells were counted on the rust layers than the bulk seawater, which also indicated that bacteria colonized surface as a survival strategy. *D. vulgaris* is ubiquitous in natural environment and is a model SRB species for MIC studies [27, 29, 141]. However, most corrosion-oriented studies reported weight loss and/or used images of pits to indicate SRB’s ability to induce pitting corrosion [148, 159, 160], while the metabolism/physiology-oriented studies on SRB in the environments of no organic energy source lacked the characterization of pitting corrosion [49, 50]. These studies did not provide complete quantitative information about the pits, such as the maximum pit depth, pit density, and 3-dimensional pit morphology and distribution. Maximum pit depth is among the most important factors to be determined [56], because the deepest pit would likely be the failure-initiating point when a load was applied to the steel [57, 58]. A systematic study to characterize corrosion pits caused by SRB under the condition of limited organic energy sources is very useful to our understanding of the SRB-induced corrosion.

Roughness has been known to affect microbe-influenced corrosion, mainly on two aspects: the number of bacterial cells attached to the metal surface [161] and the
degree of pitting corrosion [162-164]. For the former, surface roughness was reported to significantly affect bacterial attachment if the roughness was beyond 3.3 μm [161, 165]. For the latter, Sasaki and Burstein [163] demonstrated that the pitting potential is higher for smoother surfaces. Burstein and Pistorius [164] reported that a smoother surface has fewer sites that can be activated into metastable pit growth. Hong and Nagumo [162] further confirmed that metastable pit growth tended to occur on rougher surfaces by the alternating current (AC) impedance method. All the literatures indicated that smoother surfaces had lower tendency for metastable pit growth in chloride solutions. Nevertheless, pit characterization is rare for studying the effects of roughness on SRB-induced corrosion.

In this study, effects of the availability of organic energy source and the surface roughness on pitting corrosion were investigated by following the corrosion of CS coupons placed in the *D. vulgaris* culture over a 43-day period. The corrosion was examined and compared for the initial short period (2-3 days) when the organic energy source (pyruvate) was still available and for the following 40-day starvation. Corrosion of the coupons in abiotic controls was also followed. To study the effects of surface roughness, the two sides of every coupon were treated differently: one side was carefully polished to be similar to a mirror; the other side was left unpolished. The coupon samples removed from test systems at different exposure times were characterized for maximum pit depth, pit density as well as average surface roughness. Pit penetration rate (i.e., the rate of increase in the maximum pit depth) and uniform corrosion rate were calculated.
5.2. Materials and Methods

The methods used in this study were to characterize liquid culture metabolism, pitting corrosion of carbon steel induced by *D. vulgaris* starved of metabolizable organic nutrients.

5.2.1. Culture, medium and inoculum preparation

*Desulfovibrio vulgaris* (American Type Culture Collection 7757) was grown in the Postgate medium C [27]. The medium contained, per L deionized (DI) water, sodium pyruvate (C$_3$H$_3$NaO$_3$) 6 g, sodium sulfate (Na$_2$SO$_4$) 4.5 g, yeast extract 1 g, ammonium chloride (NH$_4$Cl) 1 g, monopotassium phosphate (KH$_2$PO$_4$) 0.5 g, sodium citrate dihydrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O) 0.3 g, magnesium sulfate heptahydrate (MgSO$_4$·7H$_2$O) 0.06 g, calcium chloride hexahydrate (CaCl$_2$·6H$_2$O) 0.04 g and ferrous sulfate heptahydrate (FeSO$_4$·7H$_2$O) 0.004 g. The pH was adjusted to 7.0 by addition of 1 M NaOH. Anoxic culture techniques were used throughout the cultivation, including the use of serum bottles and syringes pre-purged by N$_2$ gas. The medium was added to serum bottles (Supelco). The serum bottles were closed with butyl rubber stoppers and crimped with aluminum seals. To exclude O$_2$, N$_2$ was bubbled through the medium for at least 20 min while the bottles were heated at around 100 °C. The medium was then sterilized by autoclaving at 121 °C for 20 min. A cysteine stock solution (2 g/L) was similarly treated by N$_2$ and autoclaved separately. The cysteine stock solution (oxygen scavenger) was added to the medium prior to inoculation of SRB cells to give a final cysteine concentration of 0.002 g/L.
Stock culture of *D. vulgaris* was revived and added at 5% (v/v) to the anoxic Postgate medium C. The culture was grown at 25 °C for 26 h to reach the late exponential-growth phase or early stationary phase (optical density of 0.51 ± 0.03; cell density of $1.4 \times 10^9$ cells/mL) and then used as the inoculum for corrosion experiments. All the operation procedures were done under anoxic and sterile conditions.

5.2.2. Carbon steel coupon preparation

The CS coupons (UNS G10100 (1)) used were rectangular (25 × 11 × 0.3 mm, Q-Lab). One side of the coupons was polished following the polishing procedure described below. The unpolished side was marked with number by scratching the coupon surface using stainless steel tweezers. For sterilization coupons were soaked in pure ethanol (CH$_3$CH$_2$OH) for 2 days at room temperature, and sonicated during the periods of 0 - 2 h and 46 - 48 h to remove gas bubbles on the coupon surface. Gas bubbles, if not removed, could prevent complete wetting of coupon surfaces by ethanol and compromise the effectiveness of sterilization.

CS coupons were polished with SiC sand papers (240/P280, 600/P1200, 800/P1500, 1200/P2500, 8” diameter, Carbimet 2, Buehler Company) using a grinding and polishing machine (Model DAP-7, Struers, Inc.) at 200 rpm. A sand paper (240/P280) was secured on the machine, and then the acrylic holder was pressed down to polish the coupons with the sand paper for 5 min. During the polishing process, water was added to rinse and cool the coupons and the sand paper. The process was repeated three times, each with a higher grit (finer) sand paper, i.e., 600/P1200, 800/P1500, and then
1200/P2500, respectively. The polished coupons were immediately rinsed with DI water to remove particles and then sonicated, first in ethanol and then in DI water, each for 5 min. The cleaned coupons were dried under a stream of nitrogen. Cleaned coupons were observed under infinite focus microscope (IFM, Alicona imaging GmbH) and only those without clear pits or defects were used in the corrosion study.

5.2.3. Corrosion study

Six containers, each holding 4 CS coupons, were used for the study. Three of the containers were inoculated with SRB cells; the other three containers were the corresponding abiotic controls (with sterilized medium only). On Day 3, 21 and 43 one SRB-inoculated container and one container with sterile medium were removed (sacrificed) from the study. The coupons and liquid broth samples were taken for analyses. This design was used to satisfy the requirement of sterile and anaerobic condition. The containers used were 600 mL glass beakers closed with rubber stoppers. The coupons were hung vertically, using plastic tie-wraps, on stainless steel hooks that were anchored to the rubber stopper. The coupons were completely submerged in the medium. The reactors and the medium were sterilized by autoclaving at 121 °C for 30 min.

To exclude the effects of Cl⁻ and iron sulfide deposits, the medium used had low chloride concentration (19.2 mM, as compared to 446 mM in seawater) and 0.3 g/L sodium citrate dihydrate to chelate Fe²⁺. Humidified and filter-sterilized (0.2 µm) nitrogen gas was bubbled through the medium for 1 h. Medium was then reduced by
addition of the anoxic sterilized cysteine stock solution. Afterwards, SRB cells were inoculated to three of the systems. All the tubing and fittings for gas bubbling and sampling were made of stainless steel (Type 316 [UNS S31600]) to avoid oxygen penetration. Nitrogen was continuously bubbled through the medium to maintain anoxic conditions (including the generation of a slightly positive pressure inside the containers to avoid infiltration of ambient air). The experiment lasted for 43 days. Samples taken periodically from both SRB and control systems were visually examined for contamination using an optical microscope (Olympus BX60) equipped with a digital camera (Olympus DP71).

The reactors were tested with a 0.001 g/L resazurin solution to ensure anoxic condition under the experimental operation. The resazurin solution turned from blue to bright pink under the nitrogen bubbling for 30 min. The color of resazurin turned into colorless after the cysteine solution was added. The test confirmed the adequacy of maintaining anoxic condition with the systems and procedures used in the corrosion study.

5.2.4. Analytical techniques

Liquid samples taken from the reactors were measured for optical density and pyruvate and acetate concentrations. For these analyses each liquid sample was measured twice and the results shown in this paper were the averages for samples taken from 1, 2 or 3 parallel reactors, depending on the number of reactors remaining after one being sacrificed for corrosion examination of the coupons at one sampling time. The samples were first centrifuged to separate the cells from the medium. The supernatants collected were analyzed for pyruvate and acetate concentrations, using a Shimadzu LC-10A high-
performance liquid chromatography (HPLC, Shimadzu Corp., Kyoto, Japan) system equipped with an ultraviolet-visible (UV-Vis) detector (SPD-10A). The mobile phase used was 0.1% phosphoric acid (H₃PO₄) at a flow rate of 0.14 mL/min; the column was a Supelcogel H column (25 cm x 4.6 mm, Sigma-Aldrich, St. Louis, MO). To indicate the cell concentration, optical density was measured at 600 nm (OD₆₀₀) by a UV-Vis spectrophotometer (Model 1601, Shimadzu Corp., Kyoto, Japan). Cell pellets collected from centrifugation were washed with DI water first and then diluted with known amounts of deionized water to adjust OD₆₀₀ to the linear range of < 0.6.

The coupon surface with attached SRB biofilm was examined under a scanning electron microscope (TM-3000 Tabletop SEM, Hitachi High Technologies America, Dallas, TX). The coupons were removed inside an anoxic glove box (Coy Laboratory Products, Grass Lake, MI) containing 97.5% N₂ and 2.5% H₂. An anoxic 2% (w/w) glutaraldehyde solution was utilized to fix the SRB biofilm. Coupons were submerged in the glutaraldehyde solution at 4 °C for 1 day. For dehydration the coupons were gently rinsed with ethanol-water solutions with increasing ethanol concentrations (0%, 20%, 50%, 75% and, finally, 100%). The coupons were then placed in a desiccator for 1 day at room temperature. All the above procedures were done in the anoxic glove box to avoid post-sampling oxidation. The fixed biofilms on coupons were observed under the SEM. Afterwards, the coupons, and those from the abiotic controls, were cleaned following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (See C.3.1 in Table A1.1 of ASTM G1-03) [129].

Corrosion rate was calculated from the weight loss measurements according to the ASTM standard method (Equation [5.1]) [131], i.e.,
Corrosion rate (micrometers per year, μm/y) = \( \frac{3650 W}{(da t)} \) \hspace{1cm} (5.1)

where

\( W \) = weight loss, mg,

\( d \) = density of the metal, g/cm\(^3\),

\( a \) = exposed area of the coupon, cm\(^2\), and

\( t \) = time, day.

All coupons were weighed before the corrosion study. The coupons were weighed again after being removed from the containers and cleaned [129]. For each system (SRB or control), four coupons were collected from one container at a sampling time and every coupon (after being characterized for other purposes) was weighed twice. Average of the eight measurements was used to determine the weight loss at that sampling time.

The 3D and surface pit morphology, pit distribution, depth and density, and average surface roughness were measured by IFM. The coupon that suffered the most severe pitting corrosion in each system was chosen for the surface roughness quantification. All the other pit parameters were obtained from all 4 coupons collected from each reactor. The entire coupon surface, approximately 270 mm\(^2\) on each side, was first scanned to locate deep pit clusters. Many areas did not have pits and only those with pits were scanned and measured. The deepest pit from each scanned area (around 0.85 mm\(^2\)) was identified and the depth recorded. Then, these depths from several scanned areas were compared. Finally, the depth of the deepest pit from the whole side of coupon surface was reported as the maximum pit depth. At least 50% of the pits were measured for pit depth and length, and included in pit number counting for determination of the pit density.
Maximum pit penetration (MPP) rates, i.e., the increasing rates of maximum pit depth, were also determined (and given in the unit of μm/y) for three periods: 0-3, 0-21 and 0-43 days. These penetration rates were compared for both the polished and unpolished sides of the coupons.

Roughness parameters were calculated according to EN ISO 4287 [132] and DIN 4776-1990 [133]. 124 images were scanned on each side of the coupon for quantification of surface roughness. Each image scanned covered an area of about 0.85 mm$^2$. So, the 124 images together represented about 40% (107 mm$^2$) of the surface area on one side of the CS coupons. Both the areas with and without pits were scanned for roughness quantification. The area marked with number avoided during roughness characterization. Areal surface roughness parameters are reported in this paper, according to the functional parameters proposed by Stout et al. [134]: $S_{pk}$ - mean height of the peaks (thus, the subscript “p”) above the core material (μm), $S_k$ - height of the core material or “kernel” (thus, the subscript “k”) (μm), $S_{vk}$ - mean depth of the valleys (thus, the subscript “v”) below the core material (μm), and $V_{vv}$ – Volume of the valley voids (μL/m$^2$). For these parameters, “S” corresponds to surface depth parameters and “V” corresponds to the volume parameter.

5.3. Results and Discussion

In this paper, uniform corrosion and pitting corrosion caused by *D. vulgaris* were compared. Effects of carbon steel surface roughness were discussed. Pit morphology and distribution were also described.
5.3.1. Growth, survival and metabolism of *D. vulgaris*

In this study, the carbon substrate, pyruvate, was supplied only in the initial medium. As shown in Figure 5.1 for the SRB system, the OD$_{600}$ increased from 0.03 to 0.97 during the first 2 days, then fluctuated around 0.88 (± 0.09) till Day 6, and then decreased to 0.67 and 0.72 at Day 21 and Day 43, respectively. The profile of OD$_{600}$ demonstrated that cells grew actively during the first 2 days but the planktonic population declined by about 30% afterwards. The cells stopped growing at about the same time as pyruvate was exhausted completely (Figure 5.1). Note that pyruvate consumption was accompanied by acetate formation; at Day 2, 36.3 mM of acetate was produced. The acetate concentration maintained essentially constant afterwards, confirming that the *D. vulgaris* cells did not catabolize acetate as carbon substrate. There was no evidence, from the HPLC analysis, that any other organic nutrients had served as the substrate for maintaining the survival of cells after pyruvate was depleted. The above findings are consistent with the known metabolism of *D. vulgaris* in fermenting pyruvate to acetate and H$_2$ [27, 166, 167]. For comparison, the OD$_{600}$ profile, essentially zero throughout the study, was also shown for the abiotic control in Figure 5.1.
Figure 5.1. Profiles of cell concentration (by optical density at 600 nm) for the *D. vulgaris* system (●) and abiotic control (□) and profiles of pyruvate (▲) and acetate (◇) concentrations observed in the *D. vulgaris* system.

5.3.2. Surface morphology of original untreated coupon and the coupons exposed to sterile medium (Control)

For each coupon used in the corrosion study, one side was polished while the other side was not. The surface morphology of both sides for a representative coupon, prior to exposure to any medium, is shown in Figures 5.2 (a) and (b). The polished side was smooth while the unpolished side had some features. The average roughness information is given in Table 5.1. The $S_{pk}$ values were $0.29 \pm 0.08 \mu m$ and $0.55 \pm 0.33 \mu m$ for the polished and unpolished sides, respectively. Both the average and standard deviation values were much reduced by polishing. Note that the standard deviation indicates the differences in the $S_{pk}$ values among multiple scanned areas. To definitively conclude whether pitting corrosion occurred in the sterile medium, entire surfaces of all the CS coupons in all three control systems were scanned by IFM. Figure 5.2 (c) and (d)
show the representative coupon surfaces, on the polished and unpolished sides, after 43 days exposure to the sterile medium. No pits were found on either the polished or unpolished sides in all the coupons removed from sterile control systems.

![SEM images of carbon steel coupons](image)

Figure 5.2. (a, b) SEM images of carbon steel coupons after cleaning before contacting with medium or SRB cells and (c, d) 3D IFM images of coupons after immersion in abiotic medium for 43 days, for both polished (a, c) and unpolished sides (b, d); no apparent pitting by the abiotic medium.
5.3.3. Uniform and pitting corrosion rates caused by SRB

The MPP rates were high in the SRB systems, compared with the uniform corrosion rates, as summarized in Table 5.1. Also given in Table 5.1 are the p values for comparison of the average MPP rate and half of the uniform corrosion rate, at Day 3, 21 and 43, respectively, and on both the polished and unpolished sides. All the p values were far lower than 0.05, confirming that the differences between the uniform corrosion rates and the pitting corrosion rates were statistically significant [130]. Note that because the uniform corrosion rates were calculated from the weight loss with contributions from both the polished and unpolished sides, half of the uniform corrosion rate roughly represented the weight loss of one side. Weight loss from the edge surface was ignored, because the area of the edge surface was only 3.8% of the entire coupon surface area.

Comparisons were made between MPP rates on either the polished or unpolished side and half of the uniform corrosion rate. During the first 3 days, when pyruvate was available, the MPP rates were 3285 µm/y on the polished side and 4502 µm/y on the unpolished side. MPP rates on both sides were at least 72 times higher than half of the uniform corrosion rate calculated from weight loss (45.6 µm/y). After pyruvate was depleted, during 0-21 days, the MPP rates decreased, giving rates of 800 µm/y on the polished side and 713 µm/y on the unpolished side. The half of weight loss-based corrosion rate decreased even more markedly, down to 5.0 µm/y, during this period. Therefore, the average MPP rates were at least 142 times higher than half of the weight loss rate. After 21 days, the MPP rates further decreased, giving rates of 365 µm/y on the polished side and 275 µm/y on the unpolished side during 0-43 days. These rates were still at least 26 times higher than the half of uniform corrosion rate (10.3 µm/y). In
summary, the coupons exposed to the SRB culture showed mild weight loss and the biofilm coverage seemed to protect the underneath coupon surfaces from uniform corrosion; on the other hand, the SRB induced significantly higher pitting corrosion locally.
Table 5.1. Summary of corrosion characterization results for polished and unpolished sides of carbon steel coupons, before and after exposure to sterile medium and *D. vulgaris* culture for 3, 21 and 43 days

<table>
<thead>
<tr>
<th>System Parameter</th>
<th>With SRB</th>
<th></th>
<th>Abiotic control</th>
<th></th>
<th>As received coupon after polishing and cleaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure time (day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3</strong></td>
<td><strong>21</strong></td>
<td><strong>43</strong></td>
<td><strong>P</strong></td>
<td><strong>U</strong></td>
<td><strong>P</strong></td>
</tr>
<tr>
<td><strong>Pit density (m$^{-2}$)</strong></td>
<td>$1.76 \times 10^5$</td>
<td>$1.10 \times 10^6$</td>
<td>$4.78 \times 10^5$</td>
<td>$3.00 \times 10^7$</td>
<td>$7.42 \times 10^5$</td>
</tr>
<tr>
<td><strong>p value (pit density of different sides)</strong></td>
<td>0.002</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maximum pit depth (µm)</strong></td>
<td>27</td>
<td>37</td>
<td>46</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>$S_{pk}$ (µm)</td>
<td>$0.33 \pm 0.11$</td>
<td>$0.64 \pm 0.74$</td>
<td>$0.55 \pm 0.60$</td>
<td>$0.79 \pm 0.65$</td>
<td>$0.56 \pm 0.64$</td>
</tr>
<tr>
<td>$S_{vk}$ (µm)</td>
<td>$0.45 \pm 0.42$</td>
<td>$0.79 \pm 0.77$</td>
<td>$0.91 \pm 1.43$</td>
<td>$1.88 \pm 1.65$</td>
<td>$0.95 \pm 1.92$</td>
</tr>
<tr>
<td>$S_{hk}$ (µm)</td>
<td>$0.62 \pm 0.05$</td>
<td>$0.76 \pm 0.12$</td>
<td>$0.59 \pm 0.08$</td>
<td>$0.87 \pm 0.27$</td>
<td>$0.86 \pm 0.19$</td>
</tr>
<tr>
<td>$S_{h} + S_{pk} + S_{vk}$ (µm)</td>
<td>$1.37 \pm 0.63$</td>
<td>$2.18 \pm 1.52$</td>
<td>$2.05 \pm 2.07$</td>
<td>$3.54 \pm 2.46$</td>
<td>$2.37 \pm 2.69$</td>
</tr>
<tr>
<td>$V_{v}$ (µL/m²)</td>
<td>$37 \pm 23$</td>
<td>$69 \pm 55$</td>
<td>$70 \pm 91$</td>
<td>$146 \pm 125$</td>
<td>$79 \pm 130$</td>
</tr>
<tr>
<td>Uniform corrosion rate (µm / y)$^*$</td>
<td>$91.2 \pm 15.2$</td>
<td>$9.9 \pm 0.8$</td>
<td>$20.6 \pm 7.6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum pit penetration rate (µm / y)$^*$</td>
<td>3285</td>
<td>4502</td>
<td>800</td>
<td>713</td>
<td>365</td>
</tr>
<tr>
<td>p value (Corrosion rate)$^*$</td>
<td>0.003</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2. The p values for comparison of uniform corrosion rates or pit densities observed on carbon steel coupons incubated with the *D. vulgaris* culture for different exposure times (i.e., 3, 21 and 43 days)

<table>
<thead>
<tr>
<th></th>
<th>3d vs. 21d</th>
<th>21d vs. 43d</th>
<th>3d vs. 43d</th>
</tr>
</thead>
<tbody>
<tr>
<td>p value for uniform</td>
<td>0.000</td>
<td>0.016</td>
<td>0.000</td>
</tr>
<tr>
<td>corrosion rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value for pit density on polished side</td>
<td>0.003</td>
<td>0.035</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value for pit density on unpolished side</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Also, the p values for comparison of the uniform corrosion rates at different exposure times (i.e., 3, 21 and 43 days) were all lower than 0.05, as shown in Table 5.2. The uniform corrosion rates at different exposure times were thus confirmed to be statistically different.

5.3.4. Effects of surface roughness on pitting corrosion

The two most important parameters for quantifying pitting corrosion are maximum pit depth and pit density [56]. As shown in Table 5.1, the overall trends for maximum pit depth observed on the polished and unpolished coupon surfaces were
similar. For the polished side, the maximum pit depth increased rapidly from 0 to 27 µm during the first 3 days, when the organic electron donor was available. After the organic electron donor had been depleted, the maximum pit depth increased (less rapidly) to 46 µm at 21 days, and then stayed about the same (43 µm) at 43 days. Due to the uniform corrosion described above, the thickness of the coupon decreased by about 5 to 6 µm. Without this effect, the maximum pit depth at 43 days would have been higher than 43 µm.

While the maximum pit depths on the polished and unpolished sides were rather similar, the initial surface roughness had very significant effects on the pit density, as shown in Table 5.1. Throughout the incubation, the pit density on the polished side of coupons was significantly lower than that on the unpolished side (p << 0.05 for comparison on Day 3, 21 and 43, Table 5.1). During the first 3 days, the pit density was low with $1.8 \times 10^5$ pits/m$^2$ on the polished side and $1.1 \times 10^6$ pits/m$^2$ on the unpolished side. Between Day 3 and Day 21, there was a sharp increase in pit density on the unpolished side (from $1.1 \times 10^6$ to $3.0 \times 10^7$ pits/m$^2$), whereas the pit density increase on the polished side was slow (from $1.8 \times 10^5$ to $4.8 \times 10^5$ pits/m$^2$). Between Day 21 and Day 43, the pit density on the polished side continued to increase slowly to $7.4 \times 10^5$ pits/m$^2$, while the pit density for the unpolished side appeared to decrease from $3.0 \times 10^7$ to $2.1 \times 10^7$ pits/m$^2$.

The p values for the changes of pit density at different exposure times were lower than 0.05 on both the polished and unpolished sides, indicating that the differences in pit density at different exposure times were statistically significant (Table 5.2). Nevertheless, the pitting was characterized with different coupons collected from different reactors at
different sampling days, due to the requirement of anoxic sterile condition. There are some uncertainties in comparison of these values.

The roughness parameters obtained by the IFM scanning are summarized in Table 5.1, including the values of mean peak height ($S_{pk}$), mean valley depth ($S_{vk}$), height of core material ($S_{k}$), sum of the three ($S_{pk} + S_{vk} + S_{k}$), and the valley void volume ($V_{vv}$). The standard deviations given in Table 5.1 for these parameters correspond to the extent of variation in the average values among different scanned areas. The $S_{vk}$ and $V_{vv}$ values focus more on the “valleys” of roughness and, intuitively, may provide more information on the pits. The ($S_{pk} + S_{vk} + S_{k}$) value more or less indicates the mean depth of surface roughness, which can be decreased by uniform surface corrosion and increased by pitting. For the polished side of the coupon, essentially all average and standard deviation values of all roughness parameters increased with incubation time, even after the organic energy source had been exhausted. The increasing averages indicated more pitting and increasing roughness of the originally smooth surface; the increasing standard deviations correlated well with the finding of deep pit clusters only in certain scanned areas, i.e., higher heterogeneity in pit distribution and/or pitting progression. For the unpolished side of coupon, the general trends were similar up to 21 days. However, from Day 21 to Day 43, the trends reversed; for example, the average $S_{vk}$ decreased from 1.88 μm to 1.04 μm, the standard deviation of $S_{vk}$ decreased from 1.65 μm to 0.90 μm, the average $V_{vv}$ decreased from 146 μL/m$^2$ to 96 μL/m$^2$, and the standard deviation of $V_{vv}$ decreased from 125 μL/m$^2$ to 77 μL/m$^2$. These decreases did not appear on the polished side. Note that at this stage the unpolished side was almost completely covered with pits and the pit density was significantly larger than the pit density on the polished side. Under such conditions the
corrosion of so many pits simultaneously could cause a more pronounced thinning effect, similar to uniform corrosion, and lead to the apparent decrease of surface roughness. The following conclusions are clear statistically: (1) pit initiation was faster and the pitting corrosion was significantly more severe on the rougher unpolished surface, and (2) pitting continued during the long period of starvation from organic energy source.

5.3.5. Pit morphology and distribution

The entire coupon surfaces were scanned by IFM to locate deep pits. Shown in Figures 5.3-5.5 are the results around the deepest pits on the polished side after exposure to the SRB culture for 3, 21 and 43 days, respectively. The results shown in Figures 5.6 and 5.7 are, respectively, for the area around the deepest pit and another pitted area on the unpolished side of a coupon incubated with SRB for 21 days. Few pits were evident on the polished side of a coupon that had been incubated with D. vulgaris for 3 days (Figure 5.3), although the maximum pit depth had reached 27 µm (Figure 5.3 [c]). In this case, the deepest pit exhibited an elliptical morphology and two pits appeared to have coalesced, as shown in Figures 5.3 (a) and (b). After 21 days of incubation with SRB, more pits showed up on the polished side and the deepest one was approximately 46 µm deep (Figure 5.4). After 43 days, when the cells had already been starved for 40 days, pits on the polished side of coupons were larger (the volume of corroded material was larger), as shown in Figure 5.5. On the unpolished side, the pit density was much higher than that observed on the polished side, but the size of the pits was generally much smaller (Figures 5.6 and 5.7).
Pits were not evenly distributed on the coupon surface. Figure 5.8 shows the pit distribution on the polished side of coupons that had been incubated with SRB for 43 days; the white circles are the areas with clusters of pits. Especially for the polished side, pits only developed in certain areas, mostly along the coupon edges. Although pits appeared in much more areas on the unpolished side, they also formed pit clusters (Figure 5.6 [a]). One set of pit clusters was enlarged in Figure 5.8 (b) that marked with circles and arrows. Intergranular corrosion as well as pits were observed in the enlarged image.
Figure 5.3. (a) Top view, (b) side view of 3D pit morphology, and (c) 2D pit morphology of the polished side of a carbon steel coupon that had been incubated with *D. vulgaris* for 3 days. The pit marked with the white line was the deepest pit found on the entire coupon surface.

Figure 5.4. (a) Top view, (b) side view of 3D pit morphology, and (c) 2D pit morphology of the polished side of a carbon steel coupon that had been incubated with *D. vulgaris* for 21 days. The pit marked with the black line (on the edge of this scanned area) was the deepest pit found on the entire coupon surface.
Figure 5.5. (a) Top view, (b) side view of 3D pit morphology, and (c) 2D pit morphology of the polished side of a carbon steel coupon that had been incubated with *D. vulgaris* for 43 days. The pit marked with the white line was the deepest pit found on the entire coupon surface.
Figure 5.6. (a) Top view, (b) side view of 3D pit morphology, and (c) 2D pit morphology of the unpolished side of a carbon steel coupon that had been incubated with *D. vulgaris* for 21 days. The pit marked with the white line was the deepest pit found on the entire coupon surface.
Figure 5.7. (a) Top view, (b) side view of 3D pit morphology, and (c) 2D pit morphology of the unpolished side of a carbon steel coupon that had been incubated with *D. vulgaris* for 21 days. The pit marked with the white line was the deepest pit found on the entire coupon surface.
Figure 5.8. SEM images of the polished side of carbon steel coupons that had been exposed to *D. vulgaris* culture for 43 days. Each image scanned here represented around 16% of the polished side of coupon surface. White circles indicate the locations of pit clusters. White arrows and circles indicate enlargement of corresponding circled area.

(a) 43 d SRB — area 1

(b) 43 d SRB — area 2

(a) 21 d SRB — ×800 or ×5000

(b) 43 d SRB — ×5000

No clear SRB biofilm coverage
Figure 5.9. SEM pictures of carbon steel coupons incubated with *D. vulgaris* culture for (a) 21 days and (b, c and d) 43 days, respectively. SRB biofilm morphology on carbon steel surface or inside pits was shown.

5.4. Discussion

The results showed that the *D. vulgaris* culture caused significantly higher pit penetration rates than uniform corrosion rates (Table 5.1) on CS coupons, confirming that weight loss does not represent pitting corrosion well. Changes of maximum pit depth, pit density and surface roughness parameters (such as $S_{vk}$, $V_{vv}$ and $S_{pk} + S_{vk} + S_k$) provide more valuable information for characterizing the pitting corrosion.

Since the p values for comparison of uniform corrosion rates at different exposure times were all lower than 0.05, the changes in the uniform corrosion rate were statistically significant. The uniform corrosion rate was the highest during the first 3 days and then decreased significantly during 3 to 21 days. As shown in Figure 5.9 (a), the biofilm on the coupons removed at 21 days was very dense and the biofilm covered a higher portion of the coupon surface compared with that at 43 days (Figure 5.9 [b]). The decrease in uniform corrosion rate might be attributed to the protective effect of biofilm
against the uniform corrosion. During 0 to 43 days, the uniform corrosion rate increased a little, as compared with that between Day 0 to 21. During the period after 21 days, sloughing of the SRB biofilm occurred. As shown in Figure 5.9 (b), most of the dense SRB biofilm observed on Day 21 detached (although the dense SRB biofilm could still be seen inside the pits, as shown in Figures 5.9 (c) and (d)). The sloughing of the biofilm may explain why the uniform corrosion rate increased during the period from 21 to 43 days. Therefore, the observed changes in uniform corrosion rate, i.e., both the substantial decrease after Day 3 and the slight increase after Day 21, supported the potential protection effect of biofilm against the uniform corrosion.

Throughout the incubation of CS coupons with the SRB, MPP rates were proved statistically to be significantly higher than half of the uniform corrosion rates (Table 5.1). One thing to note is that the MPP rate was calculated with the measured maximum pit depth at each sampling time, without considering the thinning effect of uniform corrosion. In other words, the thickness of the whole coupon decreased due to the uniform corrosion, which offset the depth increase by the pit growth. For example, the weight loss rate for the period of 21 to 43 days was higher than that of the period from 3 to 21 days. So, the actual maximum penetration rate from 21 to 43 days should be slightly higher than the calculated values given.

According to the MPP rates, pitting of the coupons continued even after the organic energy source had been exhausted for up to 40 days and the SRB cells attaching to the coupon surfaces survived the long starvation. The maximum pit depth reached 41-46 μm by 21 days but did not further increase afterwards. One possible reason for the diminished increase in pit depth after 21 days was the mass transfer limitation within pits.
Literatures reported that a solid salt film (i.e., FeSO₄ for steel in sulfate containing solution) formed inside the pit can decrease pit growth rate by mass transfer limitation [154, 168]. Similarly, as shown in Figures 5.9 (c) and (d), the pits were filled with the SRB cells. Pits were likely formed because the biofilm did not uniformly cover the coupon surface, as shown in Figure 5.9 (a), and the iron dissolution (coupled with sulfate reduction) occurred only in localized areas. If the dense SRB biofilm or some corrosion products in the pits prevented sulfate from diffusing to the depths near the bottom of the pit, localized corrosion in the deep pits would cease. It should be noted that citrate was included in our medium to chelate Fe²⁺. Also, constant N₂ purging was employed to ensure anaerobic condition but it caused sulfide stripping too. The chelation and N₂ purging could thus prevent, delay and/or reduce the formation of corrosion product deposits (e.g. FeS) and thus minimize the passivation of growing pits. These might be responsible for the much deeper pits (40+ µm) observed in our study than, for example, the pits (≤ 10 µm) observed by Xu and Gu [157] in their study with the same SRB strain. Future studies in medium without citrate and/or with less or no N₂ purging are warranted to clarify their effects.

According to the results of this study (Table 5.1), the initial surface roughness plays a major role in the SRB-induced pitting corrosion. Pit density increased continuously on the polished side, whereas it initially increased from Day 0 to 21 and then decreased from Day 21 to 43 (Table 5.1). (As shown in Table 5.2, the corresponding p values were all far lower than 0.05, confirming that the pit density changes were statistically significant.) This latter decrease in pit density on the unpolished side of the coupon might be partially explained by the uniform corrosion that removed the very
shallow and small pits formed previously. In other words, some shallow, initial pits were formed during 3 to 21 days and some of them might have disappeared because of the uniform corrosion that occurred after 21 days. (This assumption was supported by the IFM scanning results described in the next paragraph.) Most importantly, significantly higher (up to 60x) pit densities were found on the rougher surfaces. The finding is consistent with the reports from several research groups [162-164]. They demonstrated that pitting potential is lower for rougher surface [162, 163]. The sites that can be activated to metastable pitting were fewer on a polished surface than on a rougher surface [164]. On the other hand, the maximum pit depths observed in this study were similar on the polished and unpolished sides on Day 21 and Day 43. This finding suggested that polishing the surface alone might not be sufficient for preventing MIC pitting.

The pit morphology and distribution shown with the IFM 3D images (Figures 5.3 to 5.7) is consistent with the pit density results reported in Table 5.1: much more micro pits were seen on the unpolished side (Figures 5.6 and 5.7). On Day 3, just after the exhaustion of pyruvate, only few pits were evident on the polished side, although most of the pits were already rather deep (Figure 5.3). This was probably because the polished side has only few sites that can be activated to metastable pitting [164]. At this time, most of the metal surface, covered with biofilm, did not have observable pits but, once the pitting was initiated in the few places, the pits grew deep rapidly. As shown in Figures 5.4 and 5.5, from Day 21 to Day 43, increasingly more pits and deeper pits appeared on the polished surface. On the unpolished side, a much larger number of micro pits were apparent after 21 days of incubation (Figures 5.6 and 5.7) and some pits were very deep (Figure 5.6 [b]). As for the pit size/diameter, pits can become wider not only by the
enlargement of individual pits but also by the coalescence of two or several growing pits (Figures 5.3-5.7).

Most of the pit clusters were found near the coupon edges (Figure 5.8). The following are two possible reasons: (1) the coupon roughness is higher near the edges than the center; the lower pitting potential renders the edge areas more prone to pit initiation. (2) Near the edges, SRB can attach on both the top and side surfaces, giving potentially higher SRB cell concentrations and corrosive activities. Others have observed that bacteria tend to attach more on the weld than on the base metal, because the weld is rougher, more porous and provides more sites for bacteria colonization [169-171]. This observed edge effect deserves further investigation. If the deepest pit clusters always appear near the edges or welds, reducing their numbers and focusing pit investigation on these areas can be effective for pitting control and monitoring.

To our knowledge, this is the first report that follows and characterizes the pitting corrosion of CS by a long-term starving SRB. Extensive IFM scanning and laborious data analysis were performed in this study to identify the deepest pits, obtain their morphology, recognize the pit distribution, and quantify the pit density. The systematic pit characterization has provided useful information for our understanding of the SRB-induced pitting corrosion. The characterization methods can be used in future studies to further our understanding of pitting corrosion. One useful study is to follow the corrosion when pyruvate is made periodically available, at different intervals and concentrations, to the SRB culture. This condition may be more similar to the field environments. Results obtained would also help answer if the stopped increase in maximum pit depth observed after 21 days in the current study was caused by the long-term absence of organic
nutrients or the effect of pit depth itself (i.e., slower pitting in deep pits) or the combination of both. Several other studies are also ongoing to improve our understanding of the complex corrosion phenomena and guide the development of mitigation approach for the SRB-induced pitting corrosion.

5.5. Conclusions

The SRB *D. vulgaris* caused statistically significant pitting corrosion even long after the consumable organic substrate was exhausted. Comparatively, the uniform corrosion was very mild, with statistically significantly lower rates than the pitting corrosion rates. During the long starvation the SRB cells survived by attaching to the coupon surface and corroding the metal into localized pits. Roughness played an important role in the pitting corrosion. The pit densities on the rough unpolished coupon surfaces were statistically significantly higher than those on the polished surfaces. In addition, the pits on the polished surface occurred mostly along the coupon edges. These pits also appeared in clusters and widened in size with time. On the other hand, pits spread in more areas on the unpolished surface, and the average pit size was smaller than that on the polished surface. Maximum pit depths were however similar on the polished and unpolished surfaces, reaching about 41-46 µm in 21 days.
CHAPTER VI

METHOD FOR FAST QUANTIFICATION OF PITTING USING 3D SURFACE PARAMETERS GENERATED WITH INFINITE FOCUS MICROSCOPE

6.1. Introduction

Sulfate reducing bacteria (SRB) can cause pitting corrosion of steel, which can lead to stress corrosion cracking or perforation [57, 154, 172] and create serious problems particularly to the oil and gas industry [8, 9, 173]. Pitting corrosion is difficult to predict because of its stochastic nature [25, 26]. The pits are also hard to detect in the field because they are small and usually covered by corrosion products and/or biofilm.

For pitting corrosion, maximum pit depth, average pit depth and pit density (i.e., number of pits per unit area) are the important pit parameters to determine [56, 59]. Maximum pit depth is the most important because the deepest pit is likely the initiation point of cracking [57, 58]. Currently pitting corrosion is characterized by microscopic or electrochemical methods. Electrochemical methods are powerful for online assessment of pitting [61-65], but absolute, quantitative correlations between electrochemical signals and the aforementioned pit parameters are still missing. Microscopic methods are

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commonly used to obtain these pit parameters, but this approach generally involves manual pit identification, counting, and individual depth assessment. This manual process is very tedious, especially on large surfaces. Faster microscopic methods for pit characterization are desirable.

Equipment-dependent software designed for identifying pits is available. For example, the white light interferometry (WLI) based 3-dimensional (3D) optical microscopy, fitted with specific software, has been used to identify pits and calculate the pit depth and density [66]. However, such software is not available for many other instruments used by corrosion professionals and WLI does not work on sloped surfaces [174, 175]. Infinite focus microscopy (IFM) is an important technique based on focus variation technology [72]. IFM is commonly used by corrosion researchers for pit characterization [67-71]. Because the 3D IFM image is constructed by compiling a number of focused 2D images, reliable measurements can be obtained for all types of surfaces, including steeply sloped ones [72]. Unfortunately, the use of IFM for pit characterization requires the same manual identification and measurement of each individual pit, as mentioned earlier.

Because of these limitations, maximum pit depths are only scarcely reported for the SRB-induced corrosion [157] and there are no comprehensive reports on the pit density measured experimentally. Most reports have included only weight loss data and/or microscopic images of pits [148, 159]. Only in modeling work has the assumed or predicted statistical information been reported for average and maximum pit depths [150, 176-179]. The maximum pit depth determined also may not be the real maximum because it is easy to miss one pit out of hundreds or even thousands of deep pits from, for
example, the 2D view images obtained by IFM. The authors’ own experience also shows the pitting characterization to be very operator dependent. There is a clear need for faster and more reproducible methods for pit characterization.

For SRB-induced corrosion, the conventional thinking is that if there are no organic energy sources available, the cells would die after a certain period and the corrosion associated with the biological sulfate reduction and sulfide production could be minimized. However, an isolated marine SRB strain was found to use metallic iron as a sole electron donor [49, 50]. More recently, a study conducted showed that a common SRB Desulfovibrio vulgaris (D. vulgaris) can survive without an organic energy source on carbon steel (CS) coupons for at least 41 d [83]. In another work, it was shown that the organics-starving SRB significantly enhanced pitting corrosion and increased the pit density on the CS coupons [70, 180]. In this study, to further characterize the pitting corrosion caused by the starving SRB, a new four-phase diagram was developed using the information reproducibly obtainable by IFM. As described in detail later, the four-phase diagram was constructed using the percentage of pitted area (x-axis) and average pit depth (y-axis) information estimated from IFM-generated standard 3D surface parameters [134, 181]. These parameters can also be obtained by other types of equipment, such as the 3D profilometers. The fast extraction of pitted area percentage and average pit depth information was, in turn, achieved by establishing correlations using the data obtained from a large number of pits, each individually identified, counted, and measured for pit depth and width (size). These correlations enable fast, reproducible, and quantitative descriptions of the pitting characteristics and the four-phase diagram allows easy categorization of the pitting patterns and potential risk. An IFM-generated
parameter was also shown to allow reliable determination of the maximum pit depth. Using the methods mentioned, characteristics of the CS pitting caused by the organics-starving *D. vulgaris* were more clearly revealed. These characteristics are described and discussed in this work in addition to the development of the new methods for quantitative pitting characterization.

6.2. Materials and Methods

The main technique deployed in this study for pit method development was to characterize large number of pits using infinite focus microscope. Other necessary materials and methods for conducting the experiment were also described below.

6.2.1. Culture, medium and inoculum preparation

The bacteria strain used in this study was *D. vulgaris* ATCC 7757 (1). Postgate medium C [27], an aqueous electrolyte with essential nutrients for SRB growth, was used for cell incubation. The SRB culture was maintained and inoculum-prepared under anaerobic and sterile conditions in serum bottles (Supelco). Detailed procedures are available elsewhere [70].

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(1) American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110.
6.2.2. CS coupon preparation

Rectangular (25 mm × 11 mm × 0.3 mm) CS coupons (UNS G10100)\(^{(2)}\) were used. One side of the coupons was unpolished and, for identification, was marked using stainless steel tweezers. The other side was polished with SiC sand papers (600, 800, and 1200 grit, 8 in [20.32 cm] diameter, Carbimet 2, Buehler Company) using a grinding and polishing machine (Model DAP-7, Struers, Inc.) at a speed of 200 rpm. The process was repeated three times, each with a finer sand paper. More detailed descriptions about the grinding procedure and the initial surface roughness on the polished and unpolished sides before the SRB exposure are available elsewhere [70]. Coupons were first washed with acetone and then sterilized by soaking in ethanol for 2 d at room temperature, with sonication during the periods of 0 h to 2 h and 46 h to 48 h to remove gas bubbles on the coupon surface.

6.2.3. Corrosion study with anaerobic \(D.\ vulgaris\) culture

The medium (electrolyte) used in all systems was Postgate medium C. Six stagnant systems were used in the study, three of which had SRB cells. The other three were cell-free abiotic controls. For sterilization, all of the system setups and the medium were autoclaved for 30 min at 121°C. The medium and, later, the inoculated culture were bubbled with humidified and filter-sterilized (0.2 μm) nitrogen to maintain the anaerobic conditions throughout the experiments. The medium contained 6 g/L pyruvate, which

\(^{(2)}\) UNS numbers are listed in \textit{Metals and Alloys in the Unified Numbering System}, published by the Society of Automotive Engineers (SAE International) and cosponsored by ASTM International.
was shown to support cell growth only for the first 2 d [83]. In the SRB-inoculated (biotic) reactors, the bacterial cells would grow as individual cells in the medium and as biofilm on the coupons during the first 2 d; afterward, the cells became starved of an organic energy source. In each reactor, four coupons were vertically hung and completely submerged in the medium. All four coupons from the biotic reactor and the coupons from the abiotic control were collected at one sampling time, i.e., 3, 7, 21, or 43 d. Each coupon was subjected to one or more specific measurements or characterizations, as described in the following section.

6.2.4. Analytical techniques

Before the corrosion experiments, coupons were checked and selected by observing them under an IFM to ensure the absence of any defects or pits. Coupon samples collected from the corrosion experiments were first cleaned for 40 min with Clarke’s solution, following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (C.3.1 in Table A1.1 of ASTM G1-03) [129], and then subjected to IFM examination. The surface pit morphology, distribution, depth, density, and statistical roughness information were obtained by the IFM examination. Out of the four coupons collected at each sampling time, the coupon that showed the most severe pitting was selected for thorough pit characterization, so as to evaluate the highest risk of pitting corrosion. The IFM images had a lateral resolution of 0.4 μm and a vertical resolution of 0.2 μm. The entire coupon surface, with an approximate area of 270 mm² on each side, was skimmed with a 2D-IFM view to identify all possible candidates for the deepest pit. Pitted areas were scanned and documented with IFM (the areas of no or very low pitting
were deemphasized when pitted areas were selected). These pits were individually examined with IFM to select the deepest one and determine the maximum pit depth on the coupon surface. Then, at least 50% of the pits were characterized to allow determination of average pit depths and sizes (widths) and the pit numbers tallied to determine the pit density. On the other hand, 124 IFM scans were obtained for each coupon side under examination. The 124 images were scanned following the location of the surface, including the pitted and the nonpitted areas. Each scan covered an area of approximately 0.85 mm$^2$; in total, the 124 scans covered 40% (107 mm$^2$) of the entire surface on the coupon side. Each scan generated: (1) a 2D image including the side profile view for pit depth measurement, (2) a 3D image with different views (top, side, and angle) for pit density and pitted area percentage measurements, and (3) the surface roughness parameters. For each scan, the pit number was tallied to determine the pit density and almost all of the pits were characterized to determine the average pit depth and size (width).

To determine the pitted area percentage, the top views of IFM 3D images were processed by using the ImageJ software. The 3D images were first converted into grayscale. The pitted areas in these images appeared darker than the nonpitted areas. By manually adjusting the contrast in the built-in threshold adjustment function, the darker color could be converted into black and the lighter color into white. The contrast was adjusted until the black pitted areas in the converted image matched the pitted areas shown in the corresponding IFM 3D images. The pitted area percentage measured by this method and the maximum and average pit depths and the pit density determined as described earlier were used to establish the correlations with certain grouped parameters.
from IFM 3D morphological analysis, as described later. These corrosion parameters, determined for all of the 124 scans, which included at least 50% of all of the pits on the surface, were considered when estimating the average parameters for the entire coupon side under examination.

The regression analysis for calculation of $p$ and $R^2$ values for Equations (6.1) through (6.3) and $S_{dr}/V_{vv}$ (developed interfacial area ratio/pit void volume) of pitted versus nonpitted areas was performed by using the Minitab 16 software.

6.3. Development of the Fast Pitting Characterization Methods

This section describes the necessary steps for gathering useful information for fast pitting characterization methods.

6.3.1. Useful operations for obtaining good-quality Infinite focus microscopy 3D parameters

Several steps reported in the equipment manual and standard book [134] were very important to follow in order to generate reproducible and reasonable results. These steps are summarized in the following sections.

6.3.1.1. Reference plane adjusting

The original 3D images generated by the IFM can be slightly tilted, which can affect the roughness results significantly. For each image, it is important to adjust the reference plane to match the sample surface using the built-in robust method [134]. This
method automatically adjusts the reference plane to exclude significant outliers, such as the pits in the samples of this study. For pitted samples, omitting this simple step would cause the reference plane to be set at a level below the actual surface, resulting in erroneous analysis.

6.3.1.2. 3D surface filtering and form removal

3D surface filtering is an important step to eliminate the effects of sample shape (form). The original 3D surface profile constructed by IFM (termed “primary” profile) contained both short wavelength (high frequency) and long wavelength (low frequency) components. The short wavelength component usually reveals the surface roughness, while the long wavelength component corresponds to more gradual changes of the surface (the form). To eliminate the form effect, the “roughness” profile should be extracted for each scan and the cutoff wavelength Lc manually selected. The Lc selection can be guided by the color preview of surface filtering. If the Lc selected is too large, the entire surface shows a gradual color change, which is caused by the form (sample shape) of the surface. At this Lc, the pit depth measurement is higher than the actual value because it was interfered by the form of the coupon. As the Lc is lowered, the entire surface eventually shows a similar color (depth), except for the pitted areas. If the Lc is lowered too much, the entire surface continues to appear in a similar color but the measured pit depth is lower than the actual pit depth. Thus, the proper Lc is the maximum Lc value at which the entire surface (excluding the pits) has a similar color, i.e., when the form effect first disappears. The Lc values selected in this study were approximately 200 μm to 350 μm.
The surface filtering technique works well only for flat surfaces. If the sample is not flat, it may be possible to use the proposed method after applying the built-in form removal function that is available in many software products (e.g., IF-MeasureSuite Version 4.1, Alicona). This function helps to minimize the effect of shape, i.e., flat, cylindrical, and spherical.

6.3.2. Selection of 3D Functional Parameters

Roughness parameters were generated by IFM according to ISO 4287 [132], ISO 13565-2:1996 [181], and DIN 4776-1990 [133]. Twenty-four roughness parameters [134] were considered and tested for the purposes of this study. The following parameters were found to be most useful (most of their physical meanings except $S_{dr}$ are illustrated in Figure 6.1):

- $S_k$: Height of the core material or kernel (subscript “k”; μm)
- $S_v$: Maximum depth of the valleys (subscript “v”) below the core material (μm)
- $S_{vk}$: Mean depth of the valleys below the core material (μm)
- $V_{vv}$: Void volume of the valleys on the surface (mL/m$^2$), and
- $S_{dr}$: Developed interfacial area ratio (%),

$$= \frac{(\text{actual surface area} – \text{projected surface area})}{(\text{projected surface area})} \times 100.$$  

Defined by the Abbott Firestone curve [133], $S_k$ corresponds to the random surface roughness (the kernel part in Figure 6.1) excluding significant peaks and valleys (pits, for this work). $S_v$ is the maximum valley depth, shown by the double arrow in Figure 6.1 (a). As confirmed by experimental results (see the Results and Discussion section), $S_v$ correlates very well with the commonly reported maximum pit depth for
pitted samples. In principle, $S_v$ and maximum pit depth differ only by the $S_k$ value, which is included in the determination of maximum pit depth but not in $S_v$. This difference is discussed further for $S_{vk}$ as follows. $S_{vk}$ and $V_{vv}$, the mean depth and void volume of the valleys, are parameters clearly affected by pitting. $S_{vk}$ differs from the average pit depth in several aspects. First, pit depth is measured from the pit mouth to the bottom. In the IFM-generated roughness parameters, the upper portion near pit mouth is considered part of the core material. In this sense, $S_{vk} + S_k$ gives a value closer to the pit depth than $S_{vk}$ alone does, as shown in Figure 6.1 (a). Second, the coupon surface has nonpitted areas interspersed among the pits. In this study, it was found that when the scanned area contains only shallow micropits (pit depth < ~9 μm), $S_{vk}$ is essentially the valley depth value averaged over both the pitted and nonpitted areas (with only random roughness) on the scanned surface (shown in Figure 6.1 [b]). In such cases, this averaging effect causes the surface roughness to affect the reported $S_{vk}$ value significantly. As the pitting extent increases, the reported $S_{vk}$ value is based increasingly more on the pitted area (Figure 6.1 [a]) and the surface roughness effect on $S_{vk}$ decreases. The $S_{vk}$ value is therefore affected not only by the pit depth but also, secondarily, by the pit density because both can affect the level of interference associated with the averaging over nonpitted areas. Accordingly, it was observed that in a scanned area of only few pits, the $S_{vk}$ value did not increase much; only if there were numerous pits in the scanned area did the $S_{vk}$ value show a clearer increase. On the other hand, the manually determined “average pit depth” is calculated with only real pits identified and measured individually, never involving any nonpitted areas. Thus, the $S_{vk}$ value is expected to be smaller than the manually determined average pit depth, particularly when the pit depths and pit density are low. It
should also be noted that, if the surface has no pits, the IFM software still separates the surface into three parts: the peak, core, and valley, and reports a $S_{vk}$ value, as shown in Figure 6.1 (b).

![Diagram of surface parameters](image)

Figure 6.1. Schematic diagrams of standard 3D surface parameters on the side view of (a) pitted and (b) nonpitted or mildly pitted areas according to DIN 4776 [133]. The double arrow in the deepest pit of (a) indicates $S_v$. $S_{vk}$ is calculated according to multiple measurements in each valley, as shown by the stripes.

6.3.3. Pit characterization using derived parameter groups

This section describes empirical correlations between average pit depth, percentage of pitted area and 3D surface derived parameter groups. Four-phase diagram was developed based on the correlations.

6.3.3.1 Average pit depth

As mentioned earlier, $S_{dr} (%) = (\text{actual surface area} - \text{projected surface area})/(\text{projected surface area}) \times 100$. For a perfectly smooth and flat surface, the actual surface area is the projected surface area (e.g., in a 2D top-view image), i.e., $S_{dr} = 0$. The
actual surface area (and thus $S_{dr}$) increases with presence of any nonflat, nonsmooth features. Accordingly, the $S_{dr}$ value would increase with increasing pitting of the surface because of the additional pit “wall” surface generated. However, the $S_{dr}$ value does not differentiate increased surface areas as a result of having deeper pits (pit depth effect), larger pits (pit size effect), or more pits (pit density effect). For example, the same $S_{dr}$ value can result from one deep narrow pit, one broad shallow pit, or several pits with depths and sizes in between the two extremes. In this example, the broad shallow pit would have a much larger pit void volume ($V_{vv}$) than the deep narrow pit. Accordingly, it was thought that by normalizing $S_{dr}$ by $V_{vv}$, the resultant $S_{dr}/V_{vv}$ value might correlate well with the average pit depth ($S_{dr}/V_{vv}$ signifies the increased surface area associated with a unit volume of voids/pits generated by the pitting corrosion). This hypothesis was proven valid, as shown by the results presented later in the Results and Discussion section.

6.3.3.2. Percentage of pitted area

As described earlier, $S_{vk}$ depends on both pit depth and pit density. $S_{vk}$ is high only when both average pit depth and pitted area percentage are high, corresponding roughly with (pitted area percentage $\times$ average pit depth). Since average pit depth was found to correlate with $S_{dr}/V_{vv}$, it was thought that the pitted area percentage might correlate with the derived group [$S_{vk}/(S_{dr}/V_{vv})$, i.e., $S_{vk} : V_{vv} / S_{dr}$]. This hypothesis was also proven workable, as shown by the results presented later in the Results and Discussion section.
6.3.3.3. Four-phase diagram

Based on the two correlations, average pit depth and pitted area percentage were selected to establish a four-phase diagram to characterize the pattern and extent of pitting. The y-axis of the diagram is the average pit depth obtained from the $S_{dr}/V_{vv}$ value, while the x-axis is the pitted area percentage obtained from the $S_{vk}V_{vv}/S_{dr}$ value. The four phases were then defined according to the different corrosion patterns and extents observed with the SRB-corroded CS coupons investigated in this study. If a surface only contains few micropits, it has low values for both the average pit depth and pitted area percentage, which is assigned as Phase I. If a surface has many micropits, average pit depth is low but the pitted area percentage is higher, which is assigned as Phase II. If a surface has few deep pits, average pit depth is high but the pitted area percentage remains relatively small, which is Phase III. A Phase III is already dangerous, because the maximum pit depth can be high. The most dangerous situation is Phase IV, where clusters of deep pits have formed. In Phase IV, the average pit depth and pitted area percentage are both high. Based on the four-phase diagram, the pattern and severity of pitting corrosion is better characterized for predicting/estimating the risk level.

6.4. Results and Discussion

Pitting parameters obtained from measurements of each individual pit were compared with those generated from improved pitting characterization methods. Four-phase diagram was also developed to categorize pitting pattern and extent. Scan area effects were also discussed.
6.4.1. Pitting characteristics: maximum pit depth and average pit depth, density and width, determined from individually identified and measured pits

CS coupons collected at 3, 21, and 43 d from the experimental systems were examined. No pit was found on the coupons from the abiotic controls. On the other hand, on the coupons incubated with the SRB (Table 6.1), pits grew rapidly in the first 3 d to maximum depths of 27 μm and 37 μm on the polished and unpolished sides, respectively. On the polished side, the maximum pit depth further increased, to 46 μm on day 21 and 43 μm on day 43; on the unpolished side, the maximum pit depth mostly stopped increasing after the first 3 d (41 μm on day 21 and 30 μm on day 43). The measured pit density, in number of pits/m², was $1.76 \times 10^5$ (polished) and $1.10 \times 10^6$ (unpolished) on day 3, $4.78 \times 10^5$ (polished) and $3.00 \times 10^7$ (unpolished) on day 21, and $7.40 \times 10^5$ (polished) and $2.10 \times 10^7$ pits/m² on day 43. These pit density and maximum pit depth results were previously reported [70]. Clearly, there were far more pits on the rough unpolished surface. Also, while the maximum pit depth appeared to increase most rapidly in the first few days, pitting corrosion continued to worsen in the form of increasing pit density. Neither maximum pit depth nor pit density alone provided a complete picture of the pitting. In addition to this maximum pit depth and pit density information, the average pit depths and widths on these coupons were more recently measured; the values are given in Table 6.1. These values were averaged from all of the values individually determined for each pit (by using IFM); at least 50% of the pits identified on each coupon surface were measured to obtain these averages. Significant time and effort were spent on these tasks. Because there were pits of widely varying dimensions on the surface, the standard deviations associated with the average pit depth and width were very high and
did not show any statistically meaningful trends to improve characterization of the severity of pitting corrosion. The four-phase diagram approach developed in this work can offer a more meaningful representation of the distribution of pitting extent and pattern on the surface, as described with the experimental results (Section 6.4.3).

Table 6.1. Pitting properties obtained by individually identifying, counting, and measuring pit depth and width on polished and unpolished sides of carbon steel coupons before and after incubation with D. vulgaris culture in Postgate Medium C for 3, 21, and 43 d\(^{(A)}\).

<table>
<thead>
<tr>
<th>Surface treatment</th>
<th>Time (day)</th>
<th>Pit density (\text{m}^{-2})</th>
<th>Maximum pit depth (µm)</th>
<th>Average pit depth (µm)</th>
<th>Average pit width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polished or Unpolished</td>
<td>As received coupon after cleaning</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polished</td>
<td>3</td>
<td>(1.76 \times 10^5)</td>
<td>27</td>
<td>11 ± 7</td>
<td>80 ± 42</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>(4.78 \times 10^5)</td>
<td>46</td>
<td>13 ± 9</td>
<td>100 ± 27</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(7.42 \times 10^5)</td>
<td>43</td>
<td>16 ± 8</td>
<td>94 ± 23</td>
</tr>
<tr>
<td>Unpolished</td>
<td>3</td>
<td>(1.10 \times 10^6)</td>
<td>37</td>
<td>10 ± 8</td>
<td>67 ± 20</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>(3.00 \times 10^7)</td>
<td>41</td>
<td>7 ± 6</td>
<td>55 ± 17</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>(2.08 \times 10^7)</td>
<td>30</td>
<td>8 ± 4</td>
<td>65 ± 12</td>
</tr>
</tbody>
</table>

\(^{(A)}\)Note: Before being used in the study, the coupons had all been confirmed by IFM to be free of pits on both the polished and unpolished sides.

6.4.2. Correlations developed for obtaining pitting characteristics from Infinite focus microscopy-generated parameters

The pitting characteristics determined painstakingly from individually identified pits, as reported in the previous section, were used to develop correlations that allow
extraction of these characteristics from IFM-generated 3D surface parameters, according to the principle described previously in the Pit Characterization section. As shown in Figure 6.2 (a) and Table 6.2 (a), the maximum pit depth (µm) correlated linearly with \( S_v + S_k \) (µm). The initial regression equation generated was: maximum pit depth = \(-0.44 + 0.98 \times (S_v + S_k)\), with a p-value of 0.42 (> 0.05) for the constant term and a p-value of 0.00 for the linear term. The statistically insignificant constant term was therefore dropped and the regression was performed again with only the statistically significant linear term (Table 6.2[b]). The final correlation equation, with \( R^2 = 0.96 \), is:

\[
\text{Maximum pit depth} = 0.96 \times (S_v + S_k)
\]  

(6.1)

The average pit depth (µm) is shown in Figure 6.2 (b) to correlate linearly with \( S_{dr}/V_{vv} (\% \text{ m}^2/\text{mL}) \) with \( R^2 = 0.92 \):

\[
\text{Average pit depth} = 1.17 \times (S_{dr}/V_{vv}) + 6.23
\]  

(6.2)

As shown in Table 6.2(c), the p-values of all of the terms in Equation (6.2) and the overall regression are 0.00 (< 0.05), confirming that the best-fit equation is statistically significant. By this correlation, average pit depth can be easily estimated according to the linear equation (Equation [6.2]).

However, it should be cautioned that for mildly pitted surfaces, the IFM-generated parameters were disrupted more by the surface roughness and were less sensitive to the initial appearance of few slightly deeper pits (Figure 6.1 [b]). On the other hand, as shown in Figure 6.1 (a), \( S_{vk} \) or \( V_{vv} \) on a severely pitted surface was mainly affected by the deep pits. For example, parameters for four scanned areas that all had only one pit but different pit depths are reported in Table 6.3 (a); the parameters for the scanned areas that had no pits are reported in Table 6.3 (b). The latter are used as
references for comparison. The $S_{dr}/V_{vv}$ values for Areas 1 and 2 with the pit depths of 7 µm and 9 µm were 1.94 and 1.98 (% m²/mL), respectively, which were similar to the $S_{dr}/V_{vv}$ value for the corresponding nonpitted area (2.01% m²/mL). For these areas with shallower pits, more “valleys” from surface roughness were grouped with the actual pit by the IFM equipment as the “valleys,” causing the $S_{dr}/V_{vv}$ values to be similar to the value calculated for nonpitted area. Accordingly, the $S_{dr}/V_{vv}$ ratio, between the value for a pitted surface and that for the corresponding nonpitted area, was identified as a good index for assessing the extent of surface roughness interference. A $S_{dr}/V_{vv}$ ratio of no more than 1 (p-value > 0.05, Table 6.3[a]) indicates that the existence of micropits has been masked by the surface roughness interference. Furthermore, as shown in Table 6.3, with small increases in the pit depth from 9.0 µm to 9.8 µm and then to 10.0 µm, the $S_{dr}/V_{vv}$ ratio jumped from 0.99 to 1.15 and then 1.40 (when compared to the very small increase of the $S_{dr}/V_{vv}$ ratio from 0.97 to 0.99 accompanying the larger pit depth increase from 7.0 µm to 9.0 µm). This observation suggests that once the $S_{dr}/V_{vv}$ ratio is larger than 1 (p-value <0.05, Table 6.3[a]), the surface roughness interference diminishes and can be ignored. Accordingly, the $S_{dr}/V_{vv}$ ratio of 1 was accepted as a good criterion for determining the critical pit depth associated with the surface roughness interference. In this case, the critical pit depth was approximately 9 µm, i.e., the $S_{dr}/V_{vv}$ value was insensitive to the existence of micropits with pit depths lower than approximately 9 µm.

This limitation was taken into consideration when manually identifying individual pits for determination of the average pit depth and pit density. If a surface contained at least one pit with a depth of > 9 µm, the micropits with depths smaller than 9 µm were not considered; otherwise, the pit density would increase significantly and the average pit
depth would be much smaller (with the minuscule depths of numerous micropits diluting the depths of a few deep pits). If a surface contained only micropits with depths of < 9 µm, the pits with depths between 4 µm and 9 µm were considered in calculating the average pit depth. Without these adjustments, the correlation between average pit depth and $S_{dv}/V_{vv}$, shown in Figure 6.2 (b), would fail at $S_{dv}/V_{vv} < 2.75\% m^2/mL$. Even with these adjustments, the surface roughness interference cannot be fully eliminated. Nonetheless, Equation (6.2) works reasonably well at average pit depth of ≥ 9 µm (corresponding to $S_{dv}/V_{vv} \geq 2.75\% m^2/mL$).

Good linear correlation was also found between the percentage of pitted area and the group $[S_{vk} \cdot V_{vv}/S_{dv}]$ (µm·mL/%m²), as shown in Figure 6.2 (c). Initially, the data were fit to an equation including a constant term, leading to the following: pitted area (%) = – 0.013 + 6.63 × ($S_{vk} \cdot V_{vv}/S_{dv}$). As shown in Table 6.2(d), the regression analysis showed that the constant term (–0.013) was not statistically significant (p = 0.88), while the linear term was significant (p = 0.00). The constant term was therefore dropped (Table 6.2[e]). The statistically significant (p = 0.00) correlation equation is ($R^2 = 0.96$):

$$\text{Pitted area} \% = 6.62 \times (S_{vk} \cdot V_{vv}/S_{dv}) \quad (6.3)$$

Similar to the previous discussion, surface roughness was included in the IFM-generated $S_{vk}$ and $V_{vv}$ when there were only few micropits ($S_{dv}/V_{vv} < 2.75\% m^2/mL$, average pit depth < 9 µm, Figure 6.1 (b) and Table 6.3). These $S_{vk}$ and $V_{vv}$ values were higher than the actual values caused by pitting. To exclude the effects of surface roughness, when $S_{dv}/V_{vv} < 2.75\% m^2/mL$, the $S_{vk}$ and $V_{vv}$ values, shown in Figure 6.2 (c) and used in Equation (6.3), are those that have been subtracted by the values of the nonpitted control
(given in Table 6.3 [b]). Without these subtractions, the correlation (Equation [6.3]) would fail at $S_{dr}/V_{vv} < 2.75\%$ m$^2$/mL.

The complications discussed affect the accuracy of these correlations only for very mildly pitted areas. For pitting of practical importance, the maximum pit depth, average pit depth, and pitted area percentage can all be reasonably estimated with Equations (6.1) through (6.3). As shown in Figure 6.2, Equations (6.1) through (6.3) work well for a wide variety of pitted surfaces, including both the polished and unpolished surfaces of different roughness levels and the surfaces after being incubated with the SRB for different durations (3, 21, and 43 d).

![Graphs showing correlations between various parameters and pitting characteristics.](image)
Figure 6.2. Correlations between (a) maximum pit depth and $S_v + S_k$, (b) average pit depth and $S_{dr}/V_{vv}$, and (c) pitted area percentage and $S_{vk}V_{vv}/S_{dr}$. Filled symbols are for data from polished surfaces and unfilled symbols from unpolished surfaces.

Table 6.2. Regression analysis of the data fitting to the correlation equations shown in Figure 6.2

(a) Regression: Maximum Pit Depth = $-0.44 + 0.98 \times (S_v+S_k)$

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-0.44</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>$S_v + S_k$</td>
<td>0.98</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>0.00</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

(b) Regression: Maximum Pit Depth = $0.96 \times (S_v+S_k)$ [Equation (6.1)]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_v + S_k$</td>
<td>0.96</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>0.00</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

(c) Regression: Average Pit Depth = $6.23 + 1.17 \times (S_{dr}/V_{vv})$ [Equation (6.2)]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>6.23</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>$S_{dr}/V_{vv}$</td>
<td>1.17</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>0.00</td>
<td>0.92</td>
<td></td>
</tr>
</tbody>
</table>

(d) Regression: Pitted % = $-0.013 + 6.63 \times (S_{vk}V_{vv}/S_{dr})$

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-0.013</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>$S_{vk}V_{vv}/S_{dr}$</td>
<td>6.63</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>0.00</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

(e) Regression: Pitted % = $6.62 \times (S_{vk}V_{vv}/S_{dr})$ [Equation (6.3)]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>p-value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{vk}V_{vv}/S_{dr}$</td>
<td>6.62</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>0.00</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

(A) Equations (6.1), (6.2), and (6.3) correspond to Figures 6.2(a), (b), and (c), respectively.
Table 6.3. 3D Surface Parameters Generated by IFM for a CS Coupon Pitted by *D. vulgaris*

(a) Scanned Areas with One Deeper Pit

<table>
<thead>
<tr>
<th>Area</th>
<th>Depth (µm)</th>
<th>width (µm)</th>
<th>(S_{dr}) (%)</th>
<th>(V_{vv}) (mL/m²)</th>
<th>(S_{vk}) (µm)</th>
<th>(S_v) (µm)</th>
<th>(S_{dr}/V_{vv}) ratio*</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>54.0</td>
<td>0.10</td>
<td>0.05</td>
<td>0.56</td>
<td>5.7</td>
<td>1.94</td>
<td>0.97</td>
</tr>
<tr>
<td>2</td>
<td>9.0</td>
<td>36.0</td>
<td>0.08</td>
<td>0.04</td>
<td>0.43</td>
<td>8.2</td>
<td>1.98</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>9.8</td>
<td>27.0</td>
<td>0.08</td>
<td>0.03</td>
<td>0.38</td>
<td>9.8</td>
<td>2.29</td>
<td>1.15</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>76.0</td>
<td>0.20</td>
<td>0.07</td>
<td>0.74</td>
<td>10.1</td>
<td>2.82</td>
<td>1.40</td>
</tr>
<tr>
<td>5</td>
<td>14.0</td>
<td>70.0</td>
<td>0.20</td>
<td>0.06</td>
<td>0.86</td>
<td>13.5</td>
<td>3.17</td>
<td>1.58</td>
</tr>
</tbody>
</table>

*(A) Five scanned areas (0.85 mm²) on polished surfaces; each area containing only one deeper pit (depth: 7 µm to 14 µm)*

(b) Non-pitted areas

<table>
<thead>
<tr>
<th></th>
<th>(S_{dr}) (%)</th>
<th>(V_{vv}) (mL/m²)</th>
<th>(S_{vk}) (µm)</th>
<th>(S_{dr}/V_{vv}) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polished</td>
<td>Average</td>
<td>0.06</td>
<td>0.03</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Std. Dev.</td>
<td>0.01</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>Unpolished</td>
<td>Average</td>
<td>0.09</td>
<td>0.04</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Std. Dev.</td>
<td>0.02</td>
<td>0.01</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*(B) Non-pitted areas (control) found on polished and unpolished surfaces according to 10 scanned areas on each surface*

* Ratio = [Parameter of pitted area in (a)]/[Parameter of non-pitted area in (b), i.e., 2.01]

* The p-value for comparison of the \((S_{dr}/V_{vv})\) values of the pitted area versus the non-pitted areas.

6.4.3. Four-phase diagram for categorization of pitting pattern and severity

For risk prediction, it is desirable to determine the pattern and severity of pitting corrosion. To achieve this, a four-phase diagram was generated with average pit depth as the y-axis and pitted area percentage as the x-axis, as shown in Figure 6.3(a). The average pit depth was quantified by Equation (6.2) and the pitted area percentage was estimated by Equation (6.3). With increasing severity of pitting corrosion, the four phases were defined as follows: Phase I – few or no micropits (pitted area percentage < 1.7%, average pit depth < 10 µm); Phase II – many micropits or large shallow pits (pitted area...
percentage > 2.2%, average pit depth < 10 µm); Phase III – few narrow deep pits (pitted area percentage < 1.7%, average pit depth > 14 µm); and Phase IV – deep pit cluster with or without micropits (pitted area percentage > 2.2%, average pit depth > 14 µm). Phase IV is the most dangerous phase.

The transition regions (gray areas in Figure 6.3[a]) divide the four phases. They were chosen based on the pitting data from 726 scans (0.85 mm² per scan) of the CS coupons that had been incubated with *D. vulgaris* for different times (3, 21, and 43 d). More specifically, the deep pit cluster pattern was first defined as the scanned area that contained at least 3 pits with depths higher than 15 µm (with or without micropits). The data points from areas with deep pit clusters were then used to establish the transition boundaries in Figure 6.3 (a), by the following rationale: (1) the upper limits of the transition regions were set at average pit depth = 14 µm and pitted area percentage = 2.2% because all of the scanned areas with larger values had deep pit clusters; and (2) the lower limits of the transition regions were set at average pit depth = 10 µm and pitted area percentage = 1.7% because no scanned areas with smaller values contained deep pit clusters. Following this rationale, only one pitting pattern was found in each phase (e.g., all scanned areas in Phase I only have micropits and all areas in Phase IV have deep pit clusters), while more than one pitting pattern can be found in the transition regions.

This four-phase diagram can be set up with the IFM-generated parameters and can be a very useful tool to help describe and categorize the pitting pattern and relative severity. Figures 6.3 (b) through (e) are examples that demonstrate the application of this four-phase diagram. These four (2 x 2) example diagrams are for two types (polished and unpolished) of CS coupons exposed to *D. vulgaris* culture for 2 different durations (3 d
and 21 d), respectively. The effect of longer incubation time is very clear: on both polished and unpolished surfaces, many more areas have developed deep pits (Phases III and IV) after 21 d. During the first 2 d, *D. vulgaris* grew and completely consumed the organic carbon source, pyruvate. From 2 d to 21 d, *D. vulgaris* was starved of consumable organics [70]. The finding of more deep-pit areas after 21 d indicates that *D. vulgaris* starved of organics was very damaging, forming deep pits and clusters.

The four-phase diagrams also showed some pitting pattern and progression information. Out of 124 scanned areas on each surface, most were nonpitted. These nonpitted areas were not represented in Figure 6.3. The data points shown in Phase I of Figure 6.3 are for the scanned areas containing at least one micropit. After being incubated with the SRB for 3 d, the coupons already showed different pitting patterns on the polished versus unpolished sides. For the unpolished surfaces, 10 data points (in a closely overlapping group) were found in Phase I, all of which had very small average pit depths and pitted area percentages (Figure 6.3 [c]). Micropits had initiated in these scanned areas but the pit depths and pitted areas were small. Only four scanned areas were found outside of this group. All four of these areas were located far away from the group (Figure 6.3 [c]): two had progressed into Phase IV and one into the transition region between Phases III and IV. On the other hand, there were fewer (nine in total) pitted areas on the polished surface, none of which were found in Phase IV (Figure 6.3 [b]). Three of the pitted areas were in Phase I. Compared to the data points in Phase I for the unpolished surface, these data points for the polished surface were less aggregated. One of these pitted areas had only few micropits; the other two areas contained one or two deep pits (depth > 15 µm) and few micropits. It is also apparent in the four-phase
diagram that the pitting on the polished surfaces tends to migrate toward Phase III (i.e.,
with large average pit depths but small pitted area percentages) in more scanned areas.
This is consistent with finding fewer micropits on the polished surfaces (IFM images not
shown); in the absence of micropits, the development of only one or two deeper pits can
give clearly increased average pit depths. It should also be noted that for the scanned
areas in Phase II, the pitting morphology on the polished surface is different from the
morphology on the unpolished surface: on the polished surface, the areas have one or two
broad and shallow pits, whereas the area on the unpolished surface has many micropits.
This different pitting morphology is not directly apparent in the four-phase diagram but
can be seen from the IFM images (not shown).
Figure 6.3. Four-phase diagrams constructed to show pitting characteristics according to the average pit depth and pitted area percentage obtained from IFM-generated parameters.
on the polished (filled symbols) and unpolished sides (unfilled symbols) of CS coupons incubated with *D. vulgaris* for 3, 21, and 43 d. (a) Compilation of all of the results obtained for different types of pits: deep pit clusters with micropits (●, ○), one or two deep pits with or without micropits (▲, △), and only micropits (●, ○). (b) through (e) Pits with a depth > 15 µm are defined as deep pits. Nonpitted areas are not reported in Phase I. Data points shown in (d) and (e) are random samples of all pitted areas, because there are too many to show (most surface areas on day 21 coupons are pitted).

For the coupons incubated with the SRB for 21 d (Figures 6.3 [d] and [e]), on the polished side, there are a few scanned areas in Phase IV and two areas in Phase III; on the unpolished side, there are significantly more areas in Phase IV. Pitting on rough surfaces is shown to be more dangerous.

As suggested by the four-phase diagram, pitting progressed differently on the polished and unpolished surfaces, and Phase III type pitting occurred only on the polished side. It has been reported that more sites can be activated into metastable pit growth on rougher surfaces [164]. On the unpolished surfaces, numerous micropits formed and grew deeper, mostly together, into deep pit clusters (Phase IV); on the polished surfaces, fewer pits formed and grew into deep pits more separately and presented as Phase III pitting areas. Related to this different pitting progression is the finding that on the polished side, the scanned area containing the deepest pit (46 µm) was in Phase III (with a few deep pits), whereas the area with the deepest pit (41 µm) on the unpolished side was in Phase IV (with clusters of deep pits).

The new approach developed in this work makes quantitative characterization of pitting corrosion significantly easier. Individual images from the entire surface can be stitched by IFM automatically and then the 3D surface parameters can be obtained from several scanned areas selected to exclude the sample edges (and, in this study, the hole
for coupon hanging) for avoiding misidentification of the deepest pit. The four-phase diagram will prove useful in describing and categorizing the pitting pattern and severity for risk prediction. However, this approach does not apply to corrosion features underneath the surface, such as any voids with below-surface sizes larger than their mouth sizes shown on the surface or other non-line-of-sight features that cannot be accurately measured by IFM. In addition, the new approach gives the percentage of pitted area, not the more commonly used pit density. The two would correlate only if all pits had similar mouth sizes on the surface. Nonetheless, from the viewpoint of risk resulting from corrosion, the pitted area percentage is more important to know than the pit density; the latter only gives information about pit number not size, while the former provides a measure of both pit quantity and size. As shown in the enlargements of Figures 6.4 (a) and (b), in many cases [182, 183] several smaller pits coalesce into a large pit as corrosion worsens; this phenomenon is associated with a decrease in the pit number but is more correctly reflected in the increased pitted area.
6.4.4. IFM scan-area size effect

The correlations and, accordingly, four-phase diagrams shown and described in the previous sections were developed with the 3D surface parameters generated by IFM on the scan area of 0.85 mm$^2$, which was the default at 20x objective magnification on the machine used in this study. A preliminary study was performed to evaluate how applicable the diagrams might be for different scan-area sizes. Two extreme cases were tested. Each tested area had the dimension of 0.82 mm x 2.05 mm, i.e., two side-by-side default scan areas (0.85 mm$^2$). The worst-case scenario was the test Area 1 (Figure 6.5 [a]), which was severely pitted on the left and mildly pitted on the right; the best-case scenario was the test Area 2, which was almost evenly pitted on both the left and right halves. For both Areas 1 and 2, three sets of surface parameters were generated by IFM. The first two sets were based on scanning with the default area of 0.85 mm$^2$, one on the left half of the area, the other on the right half. The third set was based on the scanning of
entire area, i.e., with a doubled scan-area size. The first two sets of surface parameters (from the small scan areas of left and right halves) were then averaged mathematically and compared with the third set of surface parameters (from the larger scan of entire area). The hypothesis tested was that if the scan area effect is insignificant such that the correlations established with the small default scan area are applicable to parameters from larger scan areas, the average values of the first two sets would be equal to the values from the third set. Figures 6.5 (c) and (d) show the comparison of these values. As expected, the standard deviation is very large for Area 1, where the left and right halves of the area have very different pitting extents, while the standard deviation is negligible for Area 2, where the left and right halves of the area are similar in pitting extents. In both the worst- and best-case scenarios, the average values of those generated from the two separate scans with the default scan area are essentially the same as the values acquired from the entire area with the doubled scan area. The correlations established in this study for quantifying the average pit depth and pitted area percentage are therefore tentatively concluded to be applicable to parameters generated from different IFM scan areas.

(a) Area 1 -- severely pitted on the left and mildly pitted on the right
Figure 6.5. IFM images of (a) Area 1 with a severely pitted left half and mildly pitted right half and (b) Area 2 with mostly even pitting on the entire area; both areas were chosen from the polished surface of a CS coupon incubated with *D. vulgaris* for 21 d. The pitted area percentage (c) and average pit depth (d) for Areas 1 and 2 obtained from two methods are compared to evaluate if the scan-area size has significant effects on the determination of these pitting properties using Equations (6.2) and (6.3) developed in this study. Using one method, the 3D surface parameter groups \( S_{vk}, V_{vv}/S_{dr} \) and \( S_{dr}/V_{vv} \) obtained by IFM scans (with a small scan area of 0.85 mm\(^2\)) on the left and right halves, separately, are used to determine the pitting properties on each half. These values are averaged to represent the properties of the entire area, shown by the solid columns in (c) and (d). Using the other method, the 3D surface parameter groups \( S_{vk}, V_{vv}/S_{dr} \) and \( S_{dr}/V_{vv} \) obtained by IFM scans of the entire area (with the doubled scan-area size) are used to directly determine the pitting properties of the entire area, shown by the striped columns in (c) and (d).
6.5. Conclusions

(1) A new method for faster characterization of pitting corrosion, including quantification of maximum and average pit depth and pitted area percentage, was developed by using correlations established with IFM-generated standard 3D surface parameters. The method can significantly reduce the workload compared with acquiring this information from individually identified and measured pits.

(2) Four-phase diagrams can be generated accordingly, using average pit depth as the y-axis and pitted area percentage as the x-axis. The four-phase diagram approach may be useful for categorizing pitting pattern and predicting the risk level.

(3) Using the four-phase diagram method, the following conclusions are drawn for the pitting of CS coupons by the SRB *D. vulgaris*: (a) starving *D. vulgaris* significantly increased pitting, resulting in more scanned areas in Phases III and IV with deep pits and clusters; (b) maximum pit depth did not differ markedly on polished and unpolished surfaces (46 µm versus 41 µm) but more deep-pit clusters (Phase IV) were found on the unpolished side; (c) Phase III-type pitting, i.e., the presence of only a few deep pits, occurred only on the polished side, whereas the deep pits were formed as clusters (Phase IV pitting) on the unpolished side.
CHAPTER VII

QUANTITATIVE COMPARISON OF ANAEROBIC PITTING PATTERNS AND DAMAGE RISKS BY CHLORIDE VERSUS DESULFOVIBRIO VULGARIS USING A FAST PITTING-CHARACTERIZATION METHOD

7.1. Introduction

Anaerobic condition exists even in bulk aerobic water, especially in stagnant water where convective oxygen supply is limited [184]. Carbon steel is widely used in water environments, where anaerobic condition often occurs [185]. Sulfate reducing bacteria (SRB) are notorious in inducing pitting corrosion under anaerobic conditions [43, 49, 50, 83, 186]. Pitting corrosion may cause disastrous failures such as rupture and leakage [57, 154]. Chloride is another well-known cause of pitting [154, 187]. Steel pitting has been well studied in aerobic chloride solutions or sterilized seawater [188-190]. On the other hand, studies on chloride induced pitting under anaerobic conditions have been more limited. Reported corrosion rates of carbon steel in anaerobic chloride solutions were low [191-193]. One report included the pitting of carbon steel in sterilized anaerobic seawater but showed only 1 microscopic image without quantitative characterization [194]. Surface roughness also affects the pitting outcome. It was observed that rougher surfaces caused more but shallower micro pits, while smoother
surfaces induced fewer but deeper pits [70, 195, 196]. Pitting corrosion resulting from different causes may have different pitting patterns and pit geometries and, more importantly, may elicit different levels of failure risk. Detailed, quantitative comparisons of pitting corrosion from different causes are important to the fundamental understanding and the corrosion failure evaluation and prediction.

Literature reports on quantitative characterization of pitting corrosion are rare. Many reported only the results of weight loss measurement or microscopic observation of the pitted surfaces [148, 159, 194]. However, the few microscale images shown were not suitable for representing the pitting extents of large surfaces because pit distribution could be highly localized, and weight loss results often did not represent the pitting damages [70, 194]. ASTM G46−94 [56], a standard guide for examination and evaluation of pitting corrosion, specifies several pitting parameters that need to be determined. Maximum pit depth is the most critical parameter, since it is related with the risk of material failure [197, 198]. Pit density, average pit width and depth are also important, according to the standard rating chart for pits [56]. However, gathering these data by microscopic methods can be labor-intensive and time-consuming, especially for large surfaces with numerous pits. For example, Infinite Focus Microscopy (IFM) is widely used by corrosion researchers [67-70, 145, 199], but quantitative characterization of a pitted surface still requires manual identification and individual measurement of all the pits on the surface. In addition, the average pit depths or widths obtained typically have large standard deviations because the surfaces may contain pits with widely varying depths and widths. On surfaces with highly localized corrosion, the standard deviations
can be even larger than the average values. Drawing statistically meaningful conclusions from these data can be challenging.

Recently, Chen and Ju [200] developed correlations that allowed fast determination of maximum and average pit depths and the percentage of pitted area from the standard 3D surface parameters [134, 181] obtainable with IFM. In addition, a four-phase diagram with the average pit depth as y-axis and the pitted area percentage as x-axis was shown to be able to categorize different pitting patterns and to indicate pitting extents. This previous work was based on the results of carbon steel pitting caused by *Desulfovibrio vulgaris*.

In this study the reported correlations by the fast pitting-characterization method were evaluated for accuracy in determining the pitting parameters caused by both *D. vulgaris* and chloride on carbon steel coupons with different surface roughness. The 4-phase diagram was adopted for comparing the pitting patterns caused by *D. vulgaris* and chloride. A new diagram, with maximum pit depth as the y-axis and pit volume loss ($V_{vv}$) as the x-axis, was proposed in this study to assess the pitting-associated failure risk. To complete the comparison of pitting by chloride and *D. vulgaris*, the pit width/depth aspect ratio (AR) vs. area-box (AB) diagrams proposed by Codaro et al. [182] were also generated for pit geometry comparison. The area-box was defined as the cross-sectional area of the pit over the area of the smallest rectangular box that enclosed the pit. Preparation of these AR vs. AB diagrams still required extensive labor to measure the pit depth and cross-sectional area of each individual pit.
7.2. Materials and Methods

The methods deployed in this study were to characterize pitting corrosion of carbon steel induced by *D. vulgaris* and 3.5 % NaCl solution.

7.2.1. Carbon steel coupon preparation

Rectangular (25 × 11 × 0.3 mm) carbon steel (UNS G10100) coupons were used. Maximum contents (in mass %) of non-iron elements in the carbon steel were 0.60% manganese, 0.13% carbon, 0.030% phosphorus and 0.035% sulfur. One side of the coupons was not polished. The other side was polished with increasingly higher grit SiC sand papers, i.e., 240/P280, 600/P1200, 800/P1500, and 1200/P2500 (8” diameter, Carbimet 2, Buehler Company), using a polishing machine (Model DAP-7, Struers, Inc.) at a speed of 200 rpm. Deionized water was added to rinse and cool the coupons. Coupons were then cleaned by sequential sonication in acetone and ethanol. To avoid collision and rubbing between the coupons during sonication, only 1 layer of non-overlapping coupons were placed in a large beaker (13 cm in diameter). After sonication cleaning in acetone and then ethanol, we examined and characterized the coupons prior to corrosion test using IFM (Alicona imaging GmbH, Bartlett, IL) to ensure the coupons after cleaning were still free of pits or defects. For sterilization, coupons were soaked in pure ethanol for 2 days at room temperature (with sonication during 0 - 2 h and 46 – 48 h to remove gas bubbles on coupon surfaces). Coupons were then aseptically dried inside a laminar flow hood with filter-sterilized air (Environmental air control Inc.). More detailed description about the initial 3D surface parameters on the polished and unpolished surfaces is available elsewhere [70].

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7.2.2. Carbon steel coupon characterization

Prior to the examination by IFM, coupons were cleaned for 40 min with Clarke’s solution following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (C.3.1 in Table A1.1 of ASTM G1-03) [129]. Surface pit morphology, maximum pit depth, pit density, percentage of pitted area, average pit depth, and statistical roughness information were obtained from the 3-dimensional (3D) IFM images. The lateral resolution of the 3D IFM image was 0.4 μm and the vertical resolution was 0.2 μm. Each scan provided the following images and information required for obtaining pitting parameters in this study: (1) a 2D top view image and a side profile view for pit depth measurement; (2) a 3D image (compiled from multiple focused 2D images) with top, side and angle views for pit density and pitted area percentages measurements; and (3) the 3D surface parameters. To obtain valid 3D surface parameters, proper pre-processing of the 3D images was very important, including reference plane adjusting, form removal and roughness filtering. The reference plane was adjusted using the built-in Robust method [134]. To eliminate disturbance of sample shape (form), 3D surface filtering was carried out to extract the “roughness” profile from the “primary” profile. The “waviness” profile, which reveals the more gradual surface change, was filtered out. The cutout wavelength Le was selected according to color preview of surface filtering. The proper Le is the highest wavelength, at which the entire surface appears in the same color except for the areas with pits. Edge and areas with defects (other than pitting) were excluded from scanning. More details are available elsewhere [200].

To locate the deepest pit, the entire coupon surface was skimmed by IFM 2D view to identify the possible candidates. All deep pits were measured and compared to
determine the maximum pit depth. Average pit depth was calculated from all manually measured depths for at least 50% of the pits. Pitted area percentage was obtained by analyzing the IFM 3D top-view images using Image J (National Institutes of Health, Bethesda, MD). These experimentally measured maximum and average pit depths and pitted area percentage were later compared with the values calculated from the IFM 3D surface parameters using the correlations described in Section 7.3.1. Cross-sectional areas of pits, required for generating the AR vs. AB diagram as described in more detail in Section 7.4.6, were determined using AutoCAD 2013 (Autodesk, San Rafael, CA). Weight loss rate was calculated based on the weight loss measurements according to the ASTM standard method [131]. All coupons were weighed before the corrosion study and weighed again after being removed from the test systems and cleaned [129].

7.2.3. Corrosion experiments

Carbon steel corrosion experiments were carried out with *D. vulgaris* and 3.5% NaCl solution.

7.2.3.1. Carbon steel incubated with anaerobic *D. vulgaris* culture and abiotic medium

*Desulfovibrio vulgaris* ATCC 7757 was used in this study. The *D. vulgaris* seed culture was grown with Postgate medium C in serum bottles (Supelco, Milwaukee, WI). All cultivation procedures were conducted under anaerobic and sterile conditions [180]. The medium was sealed in a serum bottle with butyl rubber stopper, which was crimped with aluminum seal. N₂ gas was bubbled through the medium while the bottle was heated to 100 °C for 20 min. The serum bottle was then autoclaved at 121 °C for 20 min. A
cysteine stock solution (oxygen scavenger), similarly treated with N₂ and sterilized, was added to the medium to a final cysteine concentration of 2 mg l⁻¹. Stock *D. vulgaris* culture with 20% glycerol was preserved in a freezer at -70 °C. The stock culture was thawed and inoculated at 5% (by volume) to the medium. After incubating at 25 °C for 1 day, the culture would reach the early stationary phase with a cell concentration of approximately 1.4 × 10⁹ cells ml⁻¹. These cells were used as the inoculum (at 5% v/v) for the corrosion reactors with submerged carbon steel coupons. Optical density at 600 nm (OD<sub>600</sub>) was measured using a UV–Vis spectrophotometer (Model 1601, Shimadzu Corp., Canby, OR).

The corrosion experiments were done in six reactors, each containing 4 carbon steel coupons immersed in either the Postgate medium C inoculated with the *D. vulgaris* culture or a sterile Postgate medium C (without pyruvate or yeast extract to mimic the composition in the SRB system where these compounds would be depleted in the first 2 days of growth). Systems were routinely checked for contamination by confocal laser scanning microscopy (Carl Zeiss AG, Jena, Germany) and optical microscopy (Olympus America Inc., Center Valley, PA). 600 mL glass Griffin beakers with the top section containing the pour spout cut out and closed with rubber stoppers (size 14, Cole-Parmer, Vernon Hills, IL) were used as incubation vessels. Stainless steel hooks were inserted into the rubber stopper and plastic tie wraps were used to tie the coupons to the hooks through a small hole at one end of the coupon. The hooks were maintained above the liquid throughout the experiment. All 4 coupons from one reactor were taken for IFM characterization at one sampling time, on Day 3 or 21 or 43. The concentrations of pyruvate and other metabolic products were measured with a high-performance liquid
chromatography (HPLC) system (LC-10A, Shimadzu Corp., Columbia, MD) equipped with a Supelcogel H column (Sigma-Aldrich Corp., St. Louis, MO) and a UV-Vis detector (SPD-10A) [83]. The medium used in this set of SRB-inoculated experiments had 19.2 mM chloride, much lower than that in sea water (approximately 600 mM). More details about D. vulgaris cell metabolism are available elsewhere [83].

7.2.3.2. Carbon steel incubated with 3.5% NaCl solution

This second set of corrosion experiments were performed with the carbon steel exposed to 3.5% NaCl solution in deionized (DI) water, to mimic seawater, in stoppered serum vials under sterile and anaerobic condition. Two serum vials, each holding 2 carbon steel coupons, were used. The carbon steel coupons were hung on Teflon hook that was anchored on the rubber stopper and were completely submerged in the 3.5% NaCl solution. The serum vials and contents were purged with nitrogen gas for 30 min before the corrosion experiments. The serum vials and the NaCl solution were sterilized by autoclaving at 121 °C for 30 min. The pitting corrosion on these coupons was examined on both polished and unpolished sides at two exposure times, i.e., 3 and 21 days.

7.3. Pitting Characterization Methods

In this study the pitting corrosion was characterized with large numbers of IFM scans. For the D. vulgaris systems, 124 sets of images, each with the equipment (IFM) default scanning area of 0.85 mm², were recorded for each surface. These images, including both pitted and non-pitted areas, covered nearly 40% of the entire surface area,
which was 275 mm$^2$ for each side of coupon. So, 3 coupons (6 sides) were scanned at each exposure time, totaling 744 sets of images. On the other hand, in the 3.5% NaCl systems, since the coupons were found to be pitted in limited areas, only the areas having at least one micro pit with depth larger than 2 µm were located and scanned. Accordingly, at Day 3, 52 sets of images were scanned on the coupon, 26 each on the polished and unpolished sides, while at Day 21, 136 sets of images were scanned, 67 sets on the polished side and 69 sets on the unpolished side.

7.3.1. IFM obtainable surface parameters and correlations to maximum and average pit depths and percentage of pitted area

Based on the earlier results of carbon steel pitting corrosion by the SRB *D. vulgaris*, correlations were previously established between three IFM-obtainable 3D surface parameter groups and the maximum pit depth, average pit depth and pitted area percentage determined from individually identified and measured pits [200]. The IFM parameters used were [132-134]

- $S_{pk}$: mean height of the peaks above the core material (µm),
- $S_k$: height of the core material or kernel (µm),
- $S_v$: maximum valley depth (µm),
- $S_{vk}$: mean depth of the valleys below the core material (µm),
- $V_{vv}$: valley void volume of the surface (ml/m$^2$), and
- $S_d$: developed interfacial area ratio (%),

$$S_d = \frac{(\text{actual surface area} - \text{projected surface area})}{\text{(projected surface area)}} \times 100.$$
Surface was divided into three parts—peak, core and valley. $S_{pk}$ was the mean height of peak material, while $S_k$ represents surface roughness. Since valleys correspond to pits, the valley parameters ($S_v, S_{vk}$ and $V_{vv}$) correlate with pitting extent.

The correlations established were given below:

1. Maximum pit depth ($\mu$m) = $0.96 \times (S_v + S_k)$ (7.1)
2. Average pit depth ($\mu$m) = $1.17 \times \frac{S_{dr}}{V_{vv}} + 6.23$ (7.2)
3. Pitted area (%) = $6.62 \times \frac{S_{vk} \cdot V_{vv}}{S_{dr}}$ (7.3)

7.3.2. Pitting pattern diagram (four-phase diagram)

The previously developed four-phase diagram [200] was developed using around 700 sets of IFM 3D images of pits. The y axis of this diagram was the average pit depth, estimated from the $S_{dr}/V_{vv}$ value using Eq. 2, and the x axis was the percentage of pitted area, estimated from the $S_{vk} \cdot V_{vv}/S_{dr}$ value using Eq. 3. Pits in each 3D image were observed and the depths were measured manually. The manually observed and measured pitting pattern was compared with the pitting pattern indicated by the 4-phase diagram for verifying the adequacy of this diagram. According to this diagram, the pitting corrosion data could be divided into 4 phases that represent 4 different pitting patterns [200] Phase I – few or no micro pits (pitted area percentage < 1.7 %, average pit depth < 10 \( \mu \)m), Phase II – many micro pits or large shallow pits (pitted area percentage > 2.2 %, average pit depth < 10 \( \mu \)m), Phase III – few narrow deep pits (pitted area percentage < 1.7 %, average pit depth > 14 \( \mu \)m), and Phase IV – deep pit cluster with or without micro pits (pitted area percentage > 2.2 %, average pit depth > 14 \( \mu \)m). In this diagram, deep pits
are defined as those with depths of at least 15 µm and deep pit clusters are defined as to have at least 3 deep pits in one scanned area (0.85 cm²).

7.3.3. Failure risk diagram

In addition to the 4-phase pitting pattern diagram, a new diagram was developed in the current work to better differentiate the pitting-associated failure risk. The pit with maximum depth was considered the most vulnerable site for failure [56, 197, 198]. Therefore, the failure risk diagram uses the maximum pit depth \( S_v + S_k, \text{µm} \) as y axis. The pit volume loss \( V_{vv}, \text{ml/m}^2 \) was chosen as x axis because it represents the combined effects of average pit depth and pitted area percentage. For surfaces with similar maximum pit depths, the surfaces with larger \( V_{vv} \) were assumed to have higher failure risk. Together, the 4-phase pitting pattern diagram and the new failure risk diagram were used to compare and rate the pitting corrosion occurring under different conditions evaluated in this study.

7.4. Results and Discussion

In this section, pitting corrosion of carbon steel induced by \( D. vulgaris \) starved of organic nutrients and 3.5% NaCl was compared using different characterization methods. Pitting pattern and failure risk diagrams were developed based on our improved fast pitting characterization method (Chapter 6) to categorize pitting pattern and evaluate failure risk.
7.4.1. Comparison of weight loss rate by chloride and *D. vulgaris* under anaerobic condition

In an earlier study, weight loss of carbon steel was found to be mild under the anaerobic condition, no matter if the metal was incubated with the *D. vulgaris* cells or in the abiotic medium with a low chloride concentration [70]. The same was observed in this study as shown by the low weight loss rates. For both the SRB and NaCl (3.5%) systems, the rates were higher during the first 3 days (91.2 ± 16.3 µm/y for *D. vulgaris* and 29.5 ± 13.5 µm/y for NaCl) than those measured at Day 21 (10.0 ± 0.8 µm/y for the SRB and 21.2 ± 6.2 µm/y for NaCl). The corrosion induced by *D. vulgaris* and chloride was presumably caused by different mechanisms. For the *D. vulgaris* system, the biofilm thickness and coverage were significantly higher at Day 21 than at Day 3; the thicker biofilm probably protected the metal surface and lowered the weight loss rate [83]. In the NaCl system, the corrosion rates at Day 3 and Day 21 did not differ significantly (*p* = 0.53). Nevertheless, these observed weight loss rates were low; all were within the range of 0 – 100 µm/y, generally considered as excellent in relative resistance to uniform corrosion [57]. The corrosion rate of 21.2 ± 6.2 µm/y found in this study for the chloride system under the anaerobic condition tested was far less aggressive than the rate, e.g., 508 µm/y, reported for the similar chloride systems under aerobic conditions [201]. It is concluded that under the anaerobic condition, neither the *D. vulgaris* cells nor the chloride ions caused significant weight loss on carbon steel.
7.4.2. Characterization of coupons before *D. vulgaris* exposure

As shown in Table 7.1, polished surface has much lower average $S_{pk}$ and standard deviation than the unpolished surface, while $S_k$ and $S_{vk}$ were similar. This indicates that the peak material was polished out during polishing process. $S_{vk}$ values, the average valley depth, were very small on both polished (0.47 ± 0.06 μm) and unpolished sides (0.42 ± 0.10 μm), which suggested that no pit was present on coupons before *D. vulgaris* exposure. After 21d *D. vulgaris* exposure, when severe pitting was induced, both average values and standard deviations of $S_{vk}$ increased significantly (1.88 ± 1.65 μm on the unpolished side) than coupons before *D. vulgaris* exposure. 3D surface parameters measure the average roughness including pitted and unpitted areas. Due to this average nature, pit depth information was not intuitively clear.

Table 7.1. 3D surface parameters for representation of surface roughness of carbon steel coupons (a) before and (b) after 21 days *D. vulgaris* exposure.

<table>
<thead>
<tr>
<th>Systems</th>
<th>Surface treatment</th>
<th>$S_{pk}$ (μm)</th>
<th>$S_{vk}$ (μm)</th>
<th>$S_k$ (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) before <em>D. vulgaris</em> exposure</td>
<td>Polished</td>
<td>0.29±0.08</td>
<td>0.47±0.06</td>
<td>0.89±0.19</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>0.55±0.33</td>
<td>0.42±0.10</td>
<td>0.89±0.12</td>
</tr>
<tr>
<td>(b) 21 days <em>D. vulgaris</em> exposure</td>
<td>Polished</td>
<td>0.55±0.60</td>
<td>0.91±1.43</td>
<td>0.59±0.08</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>0.79±0.65</td>
<td>1.88±1.65</td>
<td>0.87±0.27</td>
</tr>
</tbody>
</table>

Note: Coupons for (a) had been cleaned by acetone and ethanol sonication and those for (b) by Clarke’s solution. One side of coupons was not polished (“unpolished” surface). Reported roughness values were averaged from 35 scanned images for (a) and 124 scanned images for (b). Standard deviations indicated the roughness variations among different scanned areas. Large standard deviations indicated the scanned surfaces included rougher or pitted area(s). $S_{pk}$: mean height of the peaks above the core material, $S_k$: height of the core material (kernel), and $S_{vk}$: mean depth of the valleys below the core material.
7.4.3. Characterization of starvation condition

Cell concentration (measured as optical density at 600 nm) and pyruvate concentration profiles were followed in a serum vial in the absence of carbon steel coupons. As shown in Figure 7.1 (a), during the first day, the carbon source pyruvate was consumed, while *D. vulgaris* cells actively grew with OD<sub>600</sub> increase from 0.03 to 0.66. After pyruvate depletion, cells experienced death phase with OD<sub>600</sub> dropping from 0.66 to 0.28. This indicated that cells were under organic electron donor starvation; otherwise cells would have continued growing or maintained a relatively stable concentration. Yeast extract, another organic nutrient in the fresh medium, was included as a source of organic nitrogen and growth factors to promote cell growth. It did not prevent cells from dying after pyruvate exhaustion.

In the corrosion study, *D. vulgaris* was incubated in the presence of carbon steel coupons. As shown in Figure 7.1 (b), pyruvate was completely consumed during the first 2 days. Similar to the observations in the coupon-free system, cells in liquid phase of the corrosion-study reactors experienced starvation of organic electron donor after complete depletion of pyruvate.
Figure 7.1. Changes of *D. vulgaris* cell concentration, shown with optical density (OD), and pyruvate concentration in inoculated Postgate medium C in the absence of carbon steel (a) and pyruvate concentration change in the presence of carbon steel (b). Infinite focus microscope (IFM) images of the polished surfaces of carbon steel coupons that had been incubated under anaerobic condition for 21 days with 3.5% NaCl solution (c), and with the *D. vulgaris* culture in Postgate medium C (d), and for 43 days with abiotic Postgate medium C without carbon source (e). The area highlighted in (c) with the small white rectangle was enlarged as the arrow-indicated inset.
7.4.4. Comparison of pitting characteristics induced by chloride and \textit{D. vulgaris} under anaerobic condition

Although the weight loss rates were low in both the \textit{D. vulgaris} and chloride systems, pitting was clearly observed in these systems. For carbon steel that had been incubated with 3.5\% NaCl for 21 days, pitting was seen along some scratch lines presumably generated during polishing (Figure 7.1 [c]). On the other hand, random clusters of deep pits were found on the carbon steel incubated with \textit{D. vulgaris} (Figure 7.1 [d]). Chloride and \textit{D. vulgaris} induced different pitting characteristics presumably because they involved different corrosion mechanisms. Chloride ion is considered as an aggressive chemical that can breakdown the passive corrosion product film [154, 202, 203]. Defects such as polishing lines or scratches influence the protective property of passive film [196] and the areas near these defects may be at higher risk for chloride ion penetration. This may be responsible for the observed pitting along the polishing lines in the NaCl systems. For \textit{D. vulgaris} systems, pyruvate, the organic electron donor in the initial medium, was completely consumed in the first 2 days (Figure 7.1 [b]). The HPLC analysis results showed no consumption of organic compounds in the liquid broth afterwards, indicating that the cells were starved of exogenous organic substrates after the initial growth. The proposed mechanism of SRB-induced corrosion was that the organic-starving cells attached to the steel surface could derive energy for their survival by coupling iron oxidation with sulfate reduction, which caused the pitting [51, 83, 145]. Because pitting corrosion induced by \textit{D. vulgaris} was associated with biofilm attachment [83], the resultant pits were more randomly dispersed on the carbon steel surface (Figure 7.1 [d]). According to Figure 7.1 (c) and (d), both average pit depth and pit density
seemed to be higher in the *D. vulgaris* system than in the NaCl solution. However, a few SEM images from some randomly picked small areas are not enough to conclude on the different extents of pitting. Information gathered from an entire surface would be more meaningful, as described in the following sections.

Coupons removed after 43 days from the abiotic control were covered with corrosion products. Entire surfaces of these coupons, after cleaning, were examined with IFM. There was no clear pitting attack. The representative surface morphology is shown in Figure 7.1 (e) for a coupon sample taken after 43 days.

### 7.4.5. ASTM standard method specified pitting parameters in chloride versus *D. vulgaris* systems on carbon steel of different surface roughness

ASTM standard method [56] specifies several characteristic parameters to measure for quantifying pitting corrosion. These parameters were measured and compared in Table 7.2 for the anaerobic carbon steel corrosion by the *D. vulgaris* cells versus the 3.5% NaCl. When focused only on pit density and maximum pit depth, the following conclusions can be clearly drawn from the results summarized in Table 7.2: (1) Compared to the 3.5% NaCl, *D. vulgaris* induced deeper pitting on carbon steel, giving consistently higher pit density and maximum pit depth for the same exposure time and surface roughness. For example, the *D. vulgaris* system caused a pit density of $4.78 \times 10^5$ pits/m$^2$ and a maximum pit depth of 46 µm at 21 days on the polished surface, whereas the NaCl system caused only a pit density of $3.44 \times 10^4$ pits/m$^2$ and a maximum pit depth of 32 µm. (2) In both systems the coupons incubated for 21 days showed more severe pitting than the coupons incubated for only 3 days. For example, on the unpolished
carbon steel surface exposed to the NaCl solution, the pit density \((2.72 \times 10^5 \text{ pits/m}^2)\) and maximum pit depth \(24 \mu\text{m}\) at 21 days were higher than those \((1.98 \times 10^5 \text{ pits/m}^2, 14 \mu\text{m})\) at 3 days. (3) Unpolished surfaces have consistently higher pit density than their polished counterparts. For example, after 21 days in the \(D. vulgaris\) system, the pit density on the unpolished surface \((3.00 \times 10^7 \text{ pits/m}^2)\) was higher than that on the polished side \((4.78 \times 10^5 \text{ pits/m}^2)\).

On the other hand, neither average pit depth nor average pit width showed any clear trends or differences because of the large standard deviations associated (Table 7.2). For example, the polished carbon steel surface in the \(D. vulgaris\) system had an average pit depth of \(9 \pm 7 \mu\text{m}\) and an average pit width of \(46 \pm 32 \mu\text{m}\) at Day 3, which were not statistically different from the average pit depth of \(12 \pm 9 \mu\text{m}\) and average pit width of \(55 \pm 17 \mu\text{m}\) at Day 21. These uncertainties cause incomplete characterization of pitted surfaces, if relying on just maximum pit depth and pit density. For example, a surface with many pits of depths similar to the maximum pit depth is clearly different from a surface with only one deep pit at the maximum depth and many micro pits. Besides the issue of large standard deviations for average pit depth and width, determining these parameters from measurements of individually identified pits required excessive time and effort. A faster and improved method was needed for quantitative characterization of pitted surfaces.
Table 7.2. Important pitting parameters determined by measurements of individually identified pits for the anaerobic *D. vulgaris* and chloride systems investigated in this study

<table>
<thead>
<tr>
<th>System</th>
<th>Surface treatment</th>
<th>Time (day)</th>
<th>Pit density (pits m$^{-2}$)</th>
<th>Maximum pit depth (µm)</th>
<th>Average pit depth (µm)</th>
<th>Average pit width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. vulgaris</em></td>
<td>Polished</td>
<td>3</td>
<td>$1.76 \times 10^5$</td>
<td>27</td>
<td>$9 \pm 7$</td>
<td>$46 \pm 32$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>$4.78 \times 10^5$</td>
<td>46</td>
<td>$12 \pm 9$</td>
<td>$55 \pm 17$</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>3</td>
<td>$1.10 \times 10^6$</td>
<td>37</td>
<td>$7 \pm 6$</td>
<td>$52 \pm 19$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>$3.00 \times 10^7$</td>
<td>41</td>
<td>$11 \pm 9$</td>
<td>$60 \pm 19$</td>
</tr>
<tr>
<td>3.5% NaCl</td>
<td>Polished</td>
<td>3</td>
<td>$2.71 \times 10^4$</td>
<td>15</td>
<td>$5 \pm 5$</td>
<td>$29 \pm 12$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>$3.44 \times 10^4$</td>
<td>33</td>
<td>$6 \pm 6$</td>
<td>$36 \pm 17$</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>3</td>
<td>$1.98 \times 10^5$</td>
<td>14</td>
<td>$4 \pm 4$</td>
<td>$42 \pm 20$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>$2.72 \times 10^5$</td>
<td>24</td>
<td>$8 \pm 4$</td>
<td>$46 \pm 19$</td>
</tr>
</tbody>
</table>

7.4.6. AR vs. AB diagram for pit geometry classification

Pitting corrosion of the systems with *D. vulgaris* or 3.5% NaCl was evaluated using the AR vs. AB diagram proposed by Codaro et al. [182] as shown in Figure 7.2 and Table 7.3. For the AR vs. AB diagram, the y-axis AR is the width/depth aspect ratio and the x-axis is the area-box (AB) parameter defined as the ratio between pit area and the area of the smallest rectangle (the box) that encloses the pit. Codaro et al. [182] classified pit geometry, i.e., conical, cylindrical, and spherical or hemispherical, according to their 2D side-view shapes, i.e., triangle, rectangle, and circle or semicircle. Each shape family contains around 20 test pictures with fixed width but different AR (0-15). AR vs. AB diagram was calibrated with these test pictures for the three shape families. It was shown that all the triangles (conical pits) have AB values of about 0.5, all the circles or semicircles (spherical or hemispherical pits) have AB values of about $\pi/4$, and all the rectangles (cylindrical pits) have AB values of about 1. Other than these well-defined pit geometries, some pits had irregular shapes (with AB < 0.5) or were during shape
transition \((0.54 < AB < 0.72 \text{ or } 0.86 < AB < 1)\). Accordingly, the diagram was divided into 7 regions (I to VII, as labeled in Figure 7.2), each corresponding to a specific pit shape: (I) irregular pits, \(AB < 0.5\); (II) conical pits, \(0.5 < AB < 0.54\); (III) shape transition A, \(0.54 < AB < 0.72\); (IV) hemispherical pits, \(0.72 < AB < 0.86\); (V) shape transition B, \(0.86 < AB < 1\); (VI) cylindrical pits, \(AB = 1\); and (VII) spherical pits, \(0.72 < AB < 0.86 \) and \(1 < AR < 2\). Figure 7.2 (c) showed the representative geometries for the 6 regions observed in the \(D. vulgaris\) systems. The shape transition phases A and B refer to the pits that fell into the range between the two nearby phases. For example, as shown in Figure 7.2 (c), shape transition B (phase V) showed a pit with a morphology combining hemispherical (phase IV) and cylindrical pit shapes (VI). As shown in Figure 7.2, most scanned areas in this study fell in Regions I, II and III, particularly concentrated in or near Region II. Thus, the NaCl and \(D. vulgaris\) systems induced mostly conical or nearly conical pits.

The percentages of pits in different regions (shapes) for the pits shown in Figure 7.2 are summarized in Table 7.3. Between the \(D. vulgaris\) and 3.5% NaCl systems, the main difference of pit geometry was that consistently higher percentages of cylindrical pits were observed in the \(D. vulgaris\) systems than for the surfaces exposed to the NaCl systems under the same experimental conditions. For example, on the unpolished surfaces observed at 21 days, the percentage of cylindrical pits in the \(D. vulgaris\) system was 20.8%, while it was only 0.8% in the NaCl system. Surface roughness also had a significant effect: cylindrical pits appeared at higher percentages on the polished side than on the unpolished side in both NaCl and \(D. vulgaris\) systems. For example, in the \(D. vulgaris\)
vulgaris system the percentage of cylindrical pits on the polished side (13.3%) was much higher than that on the unpolished side (3.7%).
Figure 7.2. Diagrams of width/depth aspect ratio (AR) versus area-box parameter (AB) constructed for pit geometry evaluation for the carbon steel coupons corroded by exposure to the SRB *D. vulgaris* culture or 3.5% NaCl for 3 days and 21 days: (a) for polished surfaces; (b) for unpolished surfaces. (c) 2D (left) and 3D (right) views of IFM images for showing representative pit geometry observed in the *D. vulgaris* systems. The seven regions (I – VII) [182] indicated in the diagrams corresponded to different pit geometry: (I) irregular pits, AB < 0.5; (II) conical pits, 0.5 < AB < 0.54; (III) shape transition A, 0.54 < AB < 0.72; (IV) hemispherical pits, 0.72 < AB < 0.86; (V) shape transition B, 0.86 < AB < 1; (VI) cylindrical pits, AB = 1; (VII) spherical pits, 0.72 < AB < 0.86 and 1 < AR < 2.
Table 7.3. Percentage of different types of pit geometry formed in the *D. vulgaris* and chloride systems

<table>
<thead>
<tr>
<th>Systems</th>
<th>Surface treatment</th>
<th>Time (day)</th>
<th>Irregular</th>
<th>Conical</th>
<th>Transition A</th>
<th>Hemispherical</th>
<th>Transition B</th>
<th>Cylindrical</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. vulgaris</em></td>
<td>Polished</td>
<td>3</td>
<td>5</td>
<td>40</td>
<td>35</td>
<td>1.7</td>
<td>5</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>5.0</td>
<td>35.6</td>
<td>24.8</td>
<td>0.0</td>
<td>5.9</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>3</td>
<td>8.6</td>
<td>56.8</td>
<td>25.9</td>
<td>3.7</td>
<td>1.2</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>4.0</td>
<td>37.6</td>
<td>22.8</td>
<td>6.9</td>
<td>7.9</td>
<td>20.8</td>
</tr>
<tr>
<td>3.5% NaCl</td>
<td>Polished</td>
<td>3</td>
<td>14.9</td>
<td>28.4</td>
<td>55.2</td>
<td>1.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>6.3</td>
<td>62.5</td>
<td>18.8</td>
<td>0.0</td>
<td>4.5</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Unpolished</td>
<td>3</td>
<td>28.9</td>
<td>39.0</td>
<td>31.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>14.2</td>
<td>54.2</td>
<td>24.2</td>
<td>0.0</td>
<td>6.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>
7.4.7. Pitting pattern and failure risk diagrams

In this section, correlations between individually measured pit parameters and 3D surface parameters were established and validated according to IFM images of pits induced by *D. vulgaris* and chloride. Comparison of pitting corrosion induced by *D. vulgaris* and chloride was demonstrated using pitting pattern and failure risk diagrams.

7.4.7.1. Correlations for maximum pit depth, average pit depth, and pitted area percentage

As described earlier, linear correlations for maximum pit depth (with $S_v+S_k$, Eq. 1), average pit depth (with $S_{dr}/V_{vv}$, Eq. 2), and pitted area percentage (with $S_{vk}V_{vv}/S_{dr}$, Eq. 3) were successfully developed for the pitting corrosion on carbon steel induced by the *D. vulgaris* [200]. When analyzing the correlation between average pit depth and $S_{dr}/V_{vv}$, the pits with pit depth < 4 µm do not increase $S_{dr}/V_{vv}$ significantly, so a minimum of 4 µm pit depth was selected during average pit depth calculation [200]. This criterion was applied for both the chloride and *D. vulgaris* systems in the correlation development. As shown in Figure 7.3, the results of the current study confirmed that these correlations work very well for the pitting induced by chloride ions and for the pitting on carbon steel coupons with different levels of surface roughness. This finding significantly improves the potential applicability of these correlations to a wider range of pitting corrosion systems and conditions.
Figure 7.3. Correlations between (a) maximum pit depth and $S_v + S_k$, (b) average pit depth and $S_{dr}/V_{vv}$, and (c) pitted area percentage and $S_{vk}V_{vv}/S_{dr}$. Filled symbols are for data from polished surfaces and unfilled symbols from unpolished surfaces. Carbon steel coupons were incubated with 3.5% NaCl (●, ○) or the *D. vulgaris* culture in Postgate medium C (◆, ◇). Average pit depth can be estimated by $S_{dr}/V_{vv}$ and pitted area percentage can be estimated by $S_{vk}$ and $S_{dr}/V_{vv}$. Average pit depth calculations excluded micro pits (pit depth < 9 µm) when there was at least 1 significant pit (pit depth > 9 µm). If only micro pits were found, average pit depth was calculated based on all the pits with pit depth between 4 to 9 µm. To exclude effects of surface roughness on the area with only micro pits, $S_{vk}$ and $V_{vv}$ values used in (c) had been subtracted by the corresponding values found on the non-pitted area on the same *D. vulgaris* or 3.5% NaCl incubated coupon. More details on average pit depth calculations were discussed elsewhere [200]. $S_v$: height of the core material or kernel (µm), $S_v$: maximum valley depth (µm), $S_{vk}$: Mean depth of the valleys below the core material (µm), $V_{vv}$: valley void volume of the surface (ml m$^{-2}$), and $S_{dr}$: developed interfacial area ratio (%), = (actual surface area – projected surface area)/(projected surface area)×100.
7.4.7.2. Pitting pattern four-phase diagram

Shown in Figure 7.4 are the pitting pattern four-phase diagrams with average pit depth as the y-axis and pitted area percentage as the x-axis. The diagrams were generated using the IFM scanning results for coupons incubated with *D. vulgaris* or 3.5% NaCl for 3 or 21 days. These diagrams are useful to reveal the pitting patterns resulting from different pitting causes (*D. vulgaris* versus chloride) and different surface roughness levels. For the effects of *D. vulgaris* versus chloride, the SRB systems had significantly more scanning areas in Phases II and IV than the chloride systems on both polished (Figure 7.4 [a]) and unpolished (Figure 7.4 [b]) surfaces. Phases II and IV correspond to high pitted area percentages. The finding indicated that *D. vulgaris* was significantly more potent than chloride in initiating carbon steel pitting and causing higher pitted area percentages.

Regarding the surface roughness effects, on the polished surfaces, the few pitted areas on coupons from the chloride systems were all in Phases I and III (Figure 7.4 [a]) whereas the large number of pitted areas from the *D. vulgaris* systems were in all four phases, with many in Phases II and IV. On the unpolished surfaces, the majority of pitted areas from the NaCl systems were in Phase I, except one in Phase II and another in the transition between Phases I and III (Figure 7.4 [b]). There were clearly fewer Phase III areas compared to the pattern seen on the polished surfaces. A similar trend was found for the SRB-induced pitting: while there were many Phase III pitting areas on the polished surfaces, there were none on the unpolished surfaces (instead, pit clusters developed). This observation is consistent with the reports that metastable pits are easier to form on rough surfaces because of the lower pitting potential (thus, more areas in
Phases I and II) while the metastable pit formed on smooth surfaces has higher chance to be activated into a stable pit (thus, more areas in Phases III) [163, 164]. Accordingly, with increasing exposure time, the dominant phase transition path on the polished surfaces appeared to be Phase I→III for the chloride systems and Phase I→III→IV for the SRB systems, whereas on the unpolished surfaces the common transition path might be Phase I→II for the chloride systems and Phase I→II→IV for the SRB systems.

As described above, the four-phase diagram can be used to distinguish pitting patterns caused by different pitting mechanisms, exposure times and surface roughness levels. However, it may sometimes be misleading if used for failure risk interpretation. For example, in Figure 7.4 (a) the area with the largest average pit depth (33 µm) on polished carbon steel was found in the NaCl systems. On the contrary, the maximum pit depth was clearly higher in the *D. vulgaris* systems (46 µm) than in the NaCl systems (33 µm, Table 7.2). With close examination of the IFM scanned area having the highest average pit depth in the NaCl systems, the area had only a single narrow and deep pit. So, for that particular area the average pit depth was also the maximum pit depth. On the other hand, for the *D. vulgaris* systems, pit clusters were formed and the average pit depth could be substantially lower than the maximum pit depth due to the presence of many shallower pits. Therefore, the pitting pattern diagram is not the best tool for failure risk indication.
Figure 7.4. Pitting pattern four-phase diagrams for (a) polished (filled symbols) and (b) unpolished (unfilled symbols) carbon steel surfaces; and pitting risk diagrams for (c) polished (filled symbols) and (d) unpolished (unfilled symbols) carbon steel surfaces. Carbon steel coupons were incubated with 3.5% NaCl solution (●, ○) or *D. vulgaris* (▲, △) for 3, 21 or 43 days. In (c) and (d), *V* _vv_ (pit volume loss) was presented in a logarithmic scale. Each data point corresponded to an IFM scan with default scan area of 0.85 mm² at 20× objective magnification. Arrows in (a) and (b) indicated the assumed phase transition pathways; arrows in (c) and (d) indicated the areas with the highest risk. Non-pitted areas were not shown in the figures.

### 7.4.7.3. Failure risk diagram

For indicating the failure risk associated with pitting corrosion, the diagram with maximum pit depth as the y axis and pit volume loss (*V*_vv_) as the x axis was proposed to be more useful (Figures 7.4 [c] and [d]). The figures are plotted with *V*_vv_ values in a
logarithmic scale. This rather arbitrary choice is made because the $V_{vv}$ values are all very small for the chloride system and, when plotted in the normal scale, they mostly fall in a closely packed spot or on a single vertical line and, therefore, cannot be differentiated. The semi-logarithmic plots also allow more emphasis on the difference in maximum pit depth (shown in the normal scale), which is believed to be the most important factor for risk level prediction. The scanned areas with higher maximum pit depths are the more susceptible locations on the sample. When several scanned areas have similar maximum pit depths, the $V_{vv}$ values in the x-axis, indicating the overall extents of pitting (total volumes pitted away), can further differentiate their risk levels as a secondary factor. (Intuitively, the area with higher pit volume loss is considered to have a higher risk level.) Thus, in the failure risk diagram, the bottom left corner has the lowest risk and the risk increases as the data move toward the top right corner.

As shown in Figures 7.4 (c) and (d), the systems with *D. vulgaris* gave higher maximum pit depth and pit volume loss ($V_{vv}$) than the NaCl systems, on both polished and unpolished surfaces. It is concluded that the *D. vulgaris* starving from organic electron donors is more damaging than the 3.5% NaCl solution under the anaerobic condition investigated. Furthermore, chloride mostly induced few deep pits, whereas both the maximum pit depth and pit volume loss were high in *D. vulgaris* systems due to deep pit cluster formation.

As for the effects of surface roughness, on the polished surface, there were two areas (Areas 1 and 2 in Figure 7.4 [c]) with maximum pit depth higher than 40 µm. Between these areas of similar maximum pit depths, Area 2 had higher pit volume loss (0.45 ml/m²) than Area 1 (0.14 ml/m²); therefore, Area 2 was considered to have the
highest risk. On the unpolished side, there was only one area with maximum pit depth slightly higher than 40 µm (Area 3 in Figure 7.4 [d]). The pit volume loss of Area 3 (0.65 ml/m²) was higher than those of Areas 1 and 2. As confirmed by the IFM 3D images (not shown), Area 3 contained many deep pits interspersed with many micro pits. Area 2 also had many deep pits but only few micro pits. Area 1 contained only two deep pits. The IFM images matched the order of pit volume loss in these areas very well.

In Figures 7.4 (c) and (d), for both the *D. vulgaris* and chloride systems the scanned areas on the polished and unpolished surfaces have similar ranges of pit volume loss but the maximum pit depth is generally higher on the polished side than the unpolished side. There are more pitted areas on the unpolished side (18 areas) compared with the polished side (10 areas). This trend is consistent with that of pit density shown in Table 7.2, obtained from manual identification and counting of pits. The finding again suggests that higher pit density tended to form on the unpolished surface, whereas higher maximum pit depths were reached on the polished surface.

7.4.8. Effects of exposure time shown by pitting pattern and failure risk diagrams

Effects of exposure time were compared between 3 and 21 days (Figure 7.5). For the *D. vulgaris* system, the cells became starved of organic electron donors after about 2 days. So the pitting compared between 3 and 21 days was affected by the presence or absence of organics to the *D. vulgaris*. On the other hand, the abiotic chloride systems did not have this microorganism starvation effect. The scanned areas shown for the chloride systems were all the pitted areas with at least 1 pit with depth > 4 µm. For the *D. vulgaris* systems, all the pitted areas with pit depth > 4 µm (a manageably small percentage of all
areas) at Day 3 were considered but at Day 21 most of the areas were pitted so only a small portion of representative areas were evaluated.

As shown by the pitting pattern diagrams, in the chloride systems most areas on the carbon steel surfaces were not pitted at Day 3 and only few areas appeared in Phase I with low average pit depths and pitted area percentages (Figures 7.5 [a] and [b]); after 21 days some Phase III areas (with very few deep pits) appeared on the polished surfaces and one Phase II area (with several shallow pits) appeared on the unpolished surfaces (Figures 7.5 [c] and [d]). Propagation of the chloride-induced pitting under the anaerobic condition was shown. Similarly, for the *D. vulgaris* systems (Figures 7.5 [a] through [d]), there were significantly fewer pitted areas at Day 3 than at Day 21. At Day 3, there were only 1 Phase III area on the polished surfaces and 3 Phase IV areas on the unpolished surfaces. Pits in these few localized areas had quickly propagated to Phases III and IV, with average depths > 10 μm. At Day 21, after *D. vulgaris* cells had been starved for 19 days, more areas appeared in Phases III and IV on the polished side, whereas on the unpolished side more areas moved into Phase IV but not Phase III. The organics-starving *D. vulgaris* cells were shown to be capable of causing formation of deep pits and clusters when they were surviving as attached biofilm on the metal surface [83]. These conclusions derived from the pitting pattern diagrams cannot be seen from just the pitting parameters specified in the standard method (Table 7.2).

The exposure time effect on the risk level is clearly demonstrated by the failure risk diagrams shown in Figures 7.5 (e) through (h). For the chloride systems, after 3 days, neither maximum pit depth nor pit volume loss was high on the polished or unpolished surfaces. After 21 days the maximum pit depths and pit volume losses increased
significantly in several areas, especially on the polished surface (Figures 7.5 [g] and [f]). With increasing exposure time (from 3 days to 21 days) the pitting-associated failure risk increased in the chloride system. A similar effect of exposure time in increasing the failure risk was found in the *D. vulgaris* system but the pattern of risk increase was different on the polished and unpolished surfaces: On the polished side, both maximum pit depth and pit volume loss increased significantly (Figures 7.5 [e] and [g]); on the unpolished side, maximum pit depth did not change much whereas the pit volume loss increased significantly (Figures 7.5 [f] and [h]). The organics-starving *D. vulgaris* continued to contribute significantly to the increased pitting-associated risk level.
Figure 7.5. Pitting pattern four-phase diagrams [(a) – (d)] and pitting risk diagrams [(e) – (h)] generated for illustrating the effects of exposure time (3 versus 21 days), from the
same pitting data shown in Figure 4. Carbon steel coupons with polished (filled symbols) and unpolished (unfilled symbols) surfaces were incubated with 3.5% NaCl solution (●,○) or D. vulgaris (▲,△) for 3, 21 or 43 days. For the D. vulgaris systems, the cells were starved of organic electron donor (pyruvate) after about 2 days. The scan areas for D. vulgaris systems shown in (c), (d), (g) and (h) were randomly selected pitted areas; almost entire surfaces were pitted after 21 days incubation with D. vulgaris.

7.4.9. Significance of the methods

Obtaining the pitting parameters specified in the ASTM standard method [56] by IFM generally required significant time and manual effort in the past. And the average pit depths obtained by measuring depths of individually identified pits were associated with large standard deviations (Table 7.2). These difficulties limited the reported quantitative pitting corrosion data in the literature [148, 159]. The methods used in this work allowed fast characterization of pitting by calculating percentage of pitted area and maximum and average pit depths from standard 3D surface parameters using Eqs. 1-3. An earlier study showed that the scan area did not significantly affect the outcomes of the methods [200]. Further, two types of diagrams were generated: one for categorizing pitting patterns, the other for indicating risk associated with the pitting corrosion. Failure risk diagram is not expected to be affected by the scan area size because the maximum pit depth and $V_{vv}$ (pit volume loss per area) are independent of the scan area size (as long as the scanning resolution remains the same). Thus, the failure risk diagram can be very easily constructed using the parameters generated by reconstructing the full surface by combination of the individual partial surface scans (done with built-in capability of IFM). For example, in our study the whole coupon can be imaged by combining several individual images by IFM with 20x magnification. Then, for data analysis, 4 to 6 areas can be selected to cover the surface while excluding edges and other defect areas (such as...
the holes used for hanging the coupons in this study). Furthermore, as shown by the AR vs. AB diagrams (Figure 7.2 (a), (b) and Table 7.3), pits formed in the *D. vulgaris* and chloride systems on surfaces with different roughness had different pit morphologies but the proposed methods appeared to work well for all these pitting systems. 3D surface parameters were also obtainable by other equipment (i.e. 3D profilometer), which provided potential applicability of the method to other instruments.

The methods used in this study may also assist the development of online monitoring of pitting corrosion using, for example, electrochemical techniques. Several kinetic models for pit growth were proposed, based on correlations between current density, pit depth, pit radius, and exposure time [196]. Many researchers used the change of current density versus time to qualitatively indicate pit growth based on the established models [61, 204, 205]. Electrochemical noise measurement was also used to indicate occurrence of pitting [206-208]. But it is difficult to estimate pit depth quantitatively from current density measurements especially when different environments and/or pit shapes are involved, because most of the models are developed for certain assumed pit shape (cylindrical or spherical) and are valid only for the same material and environment [196]. The methods developed in this work offer a fast way to get pit depth and pitted area percentage information on sample surfaces. The information obtained from some representative samples can provide more realistic pitting characteristics for the intended monitoring target. These characteristics may be incorporated into advanced models to allow more accurate online monitoring using electrochemical techniques. Furthermore, the methods may assist gathering experimental data for pit modeling work [176-179, 209, 210].
7.5. Conclusions

The recently developed correlations, which used IFM-measured parameters to obtain maximum and average pit depths and pitted area percentage, were found to be applicable for the analysis of pitting occurring in several systems, including both polished and unpolished carbon steel surfaces exposed respectively to a SRB *D. vulgaris* and a 3.5% NaCl solution. Chloride ions induced predominantly conical pits while *D. vulgaris* cells induced pits of varying morphology: conical, hemispherical and cylindrical pits. The correlations worked well despite the differing pit morphology. This validation is important for expanding the use of the newly developed correlations. Further, in 21 days *D. vulgaris* caused the formation of deep pit clusters (Phase IV in the pitting pattern diagram) while chloride induced noticeably fewer deep pits along the surface scratches or defects. *D. vulgaris* also generated pits with larger maximum pit depth and pit volume loss $V_{vv}$. For the effects of surface roughness, with increasing exposure time, the pits on polished surfaces tended to evolve through the transition path of Phase I→III→IV while those on unpolished surfaces transitioned through Phase I→II→IV. For both surfaces, the final transition to Phase IV, i.e., developing deep pit clusters, occurred only in the *D. vulgaris* system, not in the chloride system. The pitting pattern diagram was for the first time employed to follow and differentiate the pitting development paths in different corroding systems. Finally, in both *D. vulgaris* and chloride systems, the maximum pit depth was generally higher on the polished surfaces while the lost pit volumes were similar. Although numerous micro pits formed on the unpolished surfaces, the deeper pit clusters on the polished surfaces would likely be the sources of higher failure risk.
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CHAPTER VIII

ELUCIDATION OF INTERFACIAL LAYER EVOLUTION ON CARBON STEEL ALONG SULFATE REDUCING BACTERIUM-INDUCED PITTNG CORROSION BY CORRELATING ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY MONITORING WITH MEASUREMENTS OF BIOACTIVITIES, BIOFILM AND CORROSION DEPOSITS

8.1. Introduction

Microbiologically influenced corrosion (MIC) involves complex phenomena that require multidisciplinary understanding of biological activity, metal surface and electrochemistry. Sulfate reducing bacteria (SRB) are among the most commonly found culprits of MIC[1]. Most SRB are anaerobes[16]; therefore, sterile and anaerobic conditions are generally required when designing laboratory experiments that involve SRB. Because it is hard to take a coupon sample from the bioreactor without risking contamination with oxygen and other microbes, sacrificial systems are typically used, i.e., each bioreactor can provide only one set of samples. This restriction makes it difficult to observe, with frequent samples, the progress of SRB-induced MIC in laboratory experiments. In actual field environments, it is even more difficult to take a large number of coupon samples for examination and analysis. It is desirable to develop techniques
capable of online detection or monitoring of the presence and accumulation of biofilm and corrosion product.

Electrochemical impedance spectroscopy (EIS) is a powerful technique that has been explored for online monitoring of interfacial layer evolution in many systems such as coatings, fuel cells and batteries [135]. Alternating current (AC) EIS measures the impedance response of an electrochemical cell as a function of frequency, when a sinusoidal excitation potential is applied on the electrode [135]. EIS is usually measured by a potentiostat. Open circuit potential (OCP, by a 3-electrode system) or potential difference (PD, by a 2-electrode system) can also be monitored online with the potentiostat. PD indicates the potential difference between the working and reference electrodes.

Existing literatures on MIC are either focused on characterization of the corroded metal surface with or without biological activity information [70, 78, 79] or solely based on the measurements by electrochemical techniques [80-82]. For the latter, EIS was used in most reports. The correlations between biological activities and electrochemical signals are scarce in the current state of knowledge to allow EIS to detect and monitor MIC reliably. Halim et al. [85] measured results of EIS and open circuit potential in four systems with carbon steel samples exposed to abiotic aqueous control, an SRB isolate, a nitrate reducing bacteria (NRB) isolate, and a mixed SRB and NRB culture in production water, respectively. They compared these electrochemical results with scanning electron microscopy (SEM) images to study the layer formation. Xu et al. [86] compared EIS Bode plots with corrosion products of carbon steel under disbonded coating in the presence and absence of Desulfovibrio desulfuricans. Desulfovibrio vulgaris is a common
H$_2$ utilizing SRB species. The strain ATCC 7757 may also be capable of direct electron uptake from iron under starvation of organic electron donors [83]. During the organic starvation, the biofilm attached on the metal surface was observed to undergo a morphological change. The innermost layer of cells that were directly attached on the steel surface could survive for at least 43 days, while the cells in the outer layers would die and detach gradually. The above observations were however made rather infrequently (once every 2-3 weeks) with sacrificial samples. It is desirable to have more frequent information and to follow the biofilm and corrosion progression in the same experimental system to avoid random variations between different sacrificial systems.

The aim of this work was to evaluate the potential of EIS in meeting this continuous monitoring need and in furthering the understanding of interfacial layer formation on carbon steel coupon surfaces in SRB-relevant corroding systems. EIS signals obtained were correlated with the results from direct characterization of the biological activity and metal surface; the characterization included microscopic imaging of biofilm structure attached on metal, analysis of liquid samples for concentrations of planktonic cells and metabolites, and metal surface characterization by confocal laser scanning microscopy (CLSM), SEM and energy dispersive microscopy (EDS). EIS data were fitted with electrical analog models to obtain the number of equivalent layers and the magnitudes of the resistance and constant phase element of each layer [84, 135, 211]. These EIS derived information were then compared with the observations and results from the other experimental techniques to identify any potential correlations. Since the phosphate (KH$_2$PO$_4$) concentration in the culture medium was proved to affect the biofilm morphology on the metal surface [83], the experiments were conducted with two
media of significantly different phosphate concentrations (0.5 and 10 g/L) to investigate if and how the EIS results would change with the different biofilm morphologies developed on the coupons.

8.2. Materials and Methods

Multi-disciplinary techniques were used in this study. Microbiological methods were used for cell cultivation and incubation. Surface characterization techniques were deployed to characterize carbon steel surface and biofilm. Electrochemical techniques were used to elucidate interfacial layer evolution of biofilm-metal interface.

8.2.1. Cell cultivation and incubation

The bacterial strain used in this work was *Desulfovibrio vulgaris* ATCC 7757. The medium for bacterial cultivation and corrosion study was Postgate medium C[16]. Postgate medium C contained, per L: sodium pyruvate 6 g (56 mM), NaSO\(_4\) 4.5 g (32 mM), yeast extract 1 g, NH\(_4\)Cl 1 g (54 mM), KH\(_2\)PO\(_4\) 0.5 g (4 mM) or 10 g (80 mM), sodium citrate dihydrate 0.3 g, MgSO\(_4\)-7H\(_2\)O 0.06 g (0.24 mM), CaCl\(_2\)-6H\(_2\)O 0.04 g (0.27 mM), FeSO\(_4\)-7H\(_2\)O 0.004 g (0.01 mM) and deionized water 1 L. Cells were normally cultured in the low-phosphate Postgate medium C with 0.5 g/L KH\(_2\)PO\(_4\). For the experiments made with the high-phosphate medium (10 g/L KH\(_2\)PO\(_4\)), cells were first transferred to the high phosphate medium in a serum vial for 18 h and then inoculated into the reactor for corrosion study. This process was used to adapt the cells to the high osmotic pressure environment before the corrosion study. The pH was pre-adjusted to 7.5. All systems were maintained under anaerobic and sterile conditions [70].
8.2.2. Coupons and materials

The carbon steel (CS) coupons used for corrosion study were UNS G10100. The CS coupons employed in the low phosphate systems were from Q-Lab (Westlake, OH) with dimensions of $25 \times 11 \times 0.3$ mm. The CS coupons used in the high phosphate systems were from Metal Samples (Munford, AL) with dimensions of $20.3 \times 10.2 \times 1.6$ mm. Every coupon had a hole near one end, to allow hanging by a platinum wire. Epoxy coating was used to prevent galvanic corrosion in the contacting area between the coupon and the platinum wire. For sterilization coupons were soaked in pure ethanol for 2 days at room temperature, with 2 h sonication at the beginning and the end to exclude gas bubbles and ensure direct contact between ethanol and the coupon surface.

8.2.3. Experimental setup

There were ten systems in the corrosion study. Four had the medium with $10$ g/L KH$_2$PO$_4$ (high phosphate systems); the other six had $0.5$ g/L KH$_2$PO$_4$ (low phosphate systems). Half of the high phosphate and low phosphate systems were with *D. vulgaris* cells (SRB systems), while the others were their bacterium-free counterparts (abiotic control systems). For EIS measurements, four of the systems, one each for the high phosphate SRB, low phosphate SRB, high phosphate control and low phosphate control groups, were made in electrochemical cells equipped as the typical two-electrode systems. The carbon steel coupon served as the working electrode and a platinum wire connected with a platinum mesh functioned as the counter and pseudo-reference electrodes. The electrochemical cell was a glass reactor constructed with two halves that were secured together by metal clamps and sealed with an O-ring. The working volume of the
The other corrosion study systems were carried out in 600 ml glass Griffin beakers (Fisherbrand, Thermo Fisher Scientific Inc., Pittsburgh, PA) with the pour spouts removed and sealed with rubber stoppers. Each system contained 4 carbon steel coupons that were hung vertically by the platinum wires, onto stainless steel hooks (SS304, McMaster Carr, Cleveland, OH) anchored on the reactor ceilings. The coupons were completely submerged in the medium during the corrosion study.

The media and reactors were sterilized by autoclaving at 120°C for 30 min. The ethanol-sterilized coupons were then hung in the autoclaved reactors. The media were next bubbled with humidified and filtered nitrogen gas (at least 4 h) and then further reduced by addition of a sterile cysteine stock solution (to a final concentration of 150 mg/L). Afterwards, the SRB cell culture was inoculated at 5% (v/v) into the five SRB systems (2 with high phosphate and 3 with low phosphate media). All the tubing and fittings for gas bubbling or sampling were made of stainless steel 316 (McMaster Carr, Cleveland, OH) to avoid oxygen penetration.

8.2.4. Analytical techniques

Cell concentration was quantified by optical density measurement using a UV-vis spectrophotometer (Model 1601, Shimadzu Corp., Canby, OR) at a wavelength of 600 nm (OD\textsubscript{600}). Cells were first separated from the medium by 10 min centrifugation at 8000 rpm (5896g; Eppendorf, Hamburg, Germany). Cell pellet was resuspended in deionized water and centrifuged again. The washed cell pellet was resuspended in an adjusted volume of deionized water to have the OD\textsubscript{600} reading in the range of 0.05-0.6. Cell number concentration can be roughly estimated by a pre-established equation: cell
number concentration (cells/mL) = (3 \times 10^9) \text{OD}_{600} - (8 \times 10^7) \text{ with } R^2 = 0.99. \text{ The equation was generated using triplicate samples taken from a batch } D. \text{ vulgaris culture, where the cell number concentration was obtained by using a counting chamber (Petroff-Hausser counter, Hausser Scientific, Horsham, PA) under an optical microscope (Olympus, Center Valley, PA).}\

Sulfide concentration was quantitated using the methylene blue assay [128]. A 1-mL sample was mixed with 1 mL 10% zinc acetate solution immediately after collection from the reactors. Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY) was used for the mixing. The zinc sulfide precipitate was collected by centrifugation and then used for the sulfide assay. If not preserved and converted into zinc sulfide precipitate by the above procedure, sulfide would be oxidized by atmospheric oxygen to give inaccurate analysis results. The collected liquid samples were separately measured for pH (Model 420A, Orion, Watsonville, CA).

Uniform (weight-loss) corrosion rate was calculated according to the ASTM standard method [131]. All coupons were marked and weighed before the study. For each reactor sacrificed at one sampling point, all of the four coupons in the reactor were weighed after being cleaned according to the ASTM G1-03 standard chemical cleaning procedure for iron and steel [129]. Each coupon was weighed twice and the average weight loss for that specific system was calculated from the weight loss values obtained for all 4 coupons.
8.2.5. Characterization of biofilm and coupons

Biofilm 3D structure was characterized using confocal laser scanning microscopy (CLSM). A Zeiss LSM 510 CLSM (Carl Zeiss AG, Jena, Germany) was used for the coupons from the low phosphate systems, while an Olympus FV1000 CLSM (Olympus America Inc., Center Valley, PA) was used for the coupons from the high phosphate systems. FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen, Life Technologies, Grand Island, NY) was used for observation of live and dead cells at excitation wavelengths of 488 and 559 nm, respectively. The live cells appeared green and the dead cells red.

Surface analysis of the carbon steel coupons with or without SRB biofilm was performed with a scanning electron microscope (TM-3000 Tabletop SEM, Hitachi High Technologies America, Dallas, TX) attached with an energy dispersive X-ray spectrometer (Hitachi EDS, Quantax 70). Coupons with biofilm were fixed by anoxic glutaraldehyde solution (2% w/w) and dehydrated using the ethanol gradient method [83]. Coupons without biofilm were directly dried with nitrogen gas. All coupons (with or without biofilm) were examined by both SEM and EDS. Afterwards, they were cleaned following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (See C.3.1 in Table A1.1 of ASTM G1-03) [129] and then examined again by SEM and EDS.

8.2.6. Electrochemical measurements

Potential difference (PD) and impedance measurements (EIS) were made using New Research Grade Potentiostat/Galvanostat/EIS (Model SP-200, Bio-Logic Science Instruments, Knoxville, TN). At each measurement time, PD and EIS were measured
sequentially, each for 30 min. EIS was measured under a sinusoidal excitation potential of 10 mV and varying frequencies in the range of 200000 to 0.01 Hz. The PD and EIS measurements were performed at least twice each day. The averaged results and standard deviations from independent measurements during the same day were reported. The impedance data were fitted using the EC-Lab software V.10.12 (Bio-Logic Science Instruments, Claix, France) for three types of equivalent circuits (Figure 8.1), depending on the number of layers formed between the carbon steel surface and the liquid medium. Bode phase angle diagrams were used to assess the number of layer formation. Each layer was simulated with one resistor and one constant phase element (i.e., an equivalent electrical circuit that represents an imperfect capacitor). The following symbols (widely used in the impedance spectroscopy field [135]) were used to represent the equivalent circuit properties: Rct, the charge transfer resistance, represents the resistance of uniform dissolution of the metal into solution. Rs, the solution resistance, is the electrical resistance of the liquid medium. Rp, the porous resistance, describes the resistance caused by a layer formed on the coupon. Owing to the complex multilayers of biofilm and corrosion product precipitates formed on the coupons in this study, up to 3 layers of porous resistance were considered: Rp_{in} for the inner layer, Rp_{mid} for the middle layer, and Rp_{out} for the outer layer. Corresponding symbols for the constant phase element, in place of the resistance, were similarly used by replacing R with Q (i.e., Qct, Qs and Qp).
Figure 8.1. Equivalent circuits of impedance spectroscopy used for modeling the layer evolution of carbon steel incubated with or without *D. vulgaris*. Rct: charge transfer resistance; Rp: porous resistance; Rs: electrolyte resistance; in: inner layer (low frequency component); mid: middle layer (middle frequency component); out: outer layer (high frequency component); Q: constant phase element.

8.3. EIS Data Analysis

Nyquist and Bode plots are commonly used for presenting EIS data [135]. In a Nyquist plot, the imaginary phase (Z″) of impedance (Z) is plotted against the real phase counterpart (Z′) according to Equation 8.1:

$$Z(\omega) = Z' + jZ''$$  \hspace{1cm} (8.1)

where \(\omega\) is the angular frequency and \(j^2 = -1\). In a Bode phase plot, the phase angle is plotted against the frequency in a log scale [212].

For a parallel resistor-capacitor (R-C) circuit, the characteristic response of a Nyquist plot is a single semicircle. However, most of the interfacial corrosion layers do not resemble pure capacitors; therefore, constant phase elements (CPE), instead of ideal
capacitors, are used in the equivalent circuits and the shape of Nyquist plots can deviate significantly from semicircles. The impedance \( Z \) of a CPE is represented by Equation 8.2 [135]:

\[
Z_{\text{CPE}} = \frac{1}{Q(j\omega)^n}
\]  

(8.2)

where \( Q \) is the magnitude of CPE, a property independent of frequency, and \( n \) is a factor describing how similar the CPE is to an ideal capacitor. If \( n = 1 \), the CPE is an ideal capacitor and \( Q \) equals to the capacitance (C); \( n < 1 \) in most cases. The average thickness \( d \) of the layer can be estimated by \( Q \), based on Equation 8.3 [211]:

\[
d = \frac{\epsilon\epsilon_0}{Q}
\]  

(8.3)

where \( \epsilon \) is the dielectric constant (relative permittivity) of the solution and \( \epsilon_0 \) is the permittivity of vacuum (8.8542 \times 10^{-14} \text{ F/cm}).

Multiple layer formation at the metal-solution interface is simulated with the multiple resistor-CPE circuits shown in Figure 8.1. Each R-CPE circuit corresponds to a layer. Take Figure 8.1 (b) as an example. Ideally, the corresponding Nyquist plot would feature three semicircles and the Bode phase plot would have three peaks in the phase angle profile. In the Nyquist plot, the radius of each semicircle corresponds to the layer’s resistance multiplied by the coupon surface area; in the Bode phase plot, the absolute value of each phase angle peak is related to the layer’s porosity: a more porous layer gives a lower absolute value of phase angle and a layer with a higher absolute value is more similar to an ideal capacitor [84]. The features (semicircle and peak) at the higher frequency correspond to the properties of the outer layer, and those at the middle and lower frequencies correspond to the properties of the middle and inner layers, respectively. For the innermost (lowest-frequency) layer, the resistance is usually
associated with charge transfer, i.e., dissolution of metal, and thus named as the charge transfer resistance (Rct). Rct has been reported to correlate inversely with the uniform corrosion rate [211]. The corresponding Qct describes the magnitude of double layer CPE at the metal-solution (electrolyte) interface. Qct has been reported to correlate inversely with Rct and proportionally with the surface area available for charge transfer [84].

In this study, most of the interfacial biofilm and corrosion layers did not resemble pure capacitors and the Nyquist plots did not show distinct semicircles. It was easier to recognize the number of interfacial layers by the number of peaks in the Bode phase angle plots. Once the layer number was determined, the EIS data were fitted by the corresponding multilayer R-CPE equivalent circuit model (Figure 8.1) to determine the model parameters in all layers, i.e., Rct, Qct, Rs, Rp’s and Qp’s. The thickness of the layer, or the semi-quantitative change of the layer thickness over time, could be estimated from Q (the magnitude of CPE) according to Equation 8.3.

8.4. Results and Discussion

As described in Section 8.3, Nyquist and Bode phase diagrams were generated from the EIS data (shown in the Supplementary Materials). The diagrams were used to determine the number of layers for fitting of the EIS data with the equivalent circuit model. The best-fit parameters obtained for the equivalent circuit model are used, together with the results of other measurement and characterization techniques, to interpret the corrosion and interfacial layer evolution, as reported in the following sections.
8.4.1. Effects of active planktonic *D. vulgaris* cells on carbon steel corrosion

The carbon source pyruvate could only support 2 d growth of *D. vulgaris* cells; afterward, cells were starved of organic electron donor [83]. The SRB grew actively during the first 12 h, increasing the optical density OD$_{600}$ from 0.07 to 0.76 (Figure 8.2 [a]). Sulfide was produced; the concentration increased from 0.008 to 0.140 mM in the liquid phase during the first 40 h (Figure 8.2 [a]). To elucidate the potential corrosion effects of the actively growing *D. vulgaris* cells, EIS measurements were made before, just after, and 12 h after the SRB inoculation. The Nyquist impedance and Bode phase angle plots measured in the high- phosphate system are shown in Figure 8.3; values of the fitted equivalent-circuit parameters are given in Table 8.1. The plots for measurements made before and just after the inoculation were found to be similar. There were two apparent peaks in each of the phase angle plots, suggesting the existence of two equivalent R-CPE circuits of different properties; an inner layer/circuit associated with the charge transfer resistance and an outer layer. After 12 h the corrosion rate increased significantly, indicated by the clear decrease of the lower-frequency semicircle radius in the Nyquist impedance plot (Figure 8.3 [a]) and the corresponding decrease of charge transfer resistance (Rct) from $4.2 \times 10^7$ to $4.8 \times 10^5$ Ohm.cm$^2$ (Table 8.1). In addition, the outer layer turned more porous as indicated by a smaller absolute value of phase angle for the higher-frequency peak in the Bode phase diagram and the decrease of outer layer porous resistance (Rp) from $7.6 \times 10^5$ to $1.5 \times 10^5$ Ohm.cm$^2$. According to Equation 8.3, this layer might have also become thinner, corresponding to the small increase of Qp from $1.1 \times 10^{-6}$ to $1.3 \times 10^{-6}$ F.cm$^{-2}$. 

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Figure 8.2. Profiles of (a) liquid-phase cell and sulfide concentrations and (b) pH in systems with carbon steel coupons submerged in Postgate medium C with 0.5 g/L KH\textsubscript{2}PO\textsubscript{4} (low phosphate) and 10 g/L of KH\textsubscript{2}PO\textsubscript{4} (high phosphate). Figure (a) showed results only in the systems inoculated with \textit{D. vulgaris} (SRB); Figure (b) showed pH profiles for both SRB and abiotic control systems.
Figure 8.3. Impedance diagrams of (a) Nyquist plot, (b) the detailed plot at high frequencies and (c) the phase diagram at different times of carbon steel coupon exposed to Postgate medium C with 10 g/L KH$_2$PO$_4$ measured before *D. vulgaris* inoculation (◆), 0.1h after inoculation (▲), 12 h after inoculation (○), and 12 h in abiotic control (△).

For comparison, Figure 8.3 and Table 8.1 also include results of the abiotic control systems. The outer layer in the control systems also turned more porous and thinner; Rp decreased from $9.3 \times 10^6$ to $4.7 \times 10^4$ Ohm.cm$^2$ and Qp increased from $1.1 \times 10^{-6}$ to $7.1 \times 10^{-5}$ F.cm$^2$. The coupon Rct in the control at 12 h was $3.7 \times 10^5$ Ohm.cm$^2$, smaller than the Rct of $4.8 \times 10^5$ Ohm.cm$^2$ found in the SRB system at 12 h, indicating a faster uniform corrosion in the abiotic control. It was therefore concluded that despite the high planktonic cell concentration at 12 h in the SRB system, the increase of uniform corrosion rate was caused by the liquid medium, not by the SRB cells. Instead, SRB cells and/or metabolism (which altered the medium composition) seemed to have a protective effect against uniform corrosion of carbon steel. Planktonic *D. vulgaris* cells were not a damaging factor in terms of uniform corrosion.

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<th>Qp (F cm$^2$)</th>
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<td>Before SRB inoculation</td>
<td>157.4 ± 5.4</td>
<td>$2.0 \times 10^3 \pm$</td>
<td>$2.7 \times 10^7 \pm$</td>
<td>$2.5 \times 10^7 \pm$</td>
<td>$2.7 \times 10^7 \pm$</td>
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<tr>
<td>After SRB inoculation</td>
<td>149.7 ± 1.1</td>
<td>$4.3 \times 10^7 \pm$</td>
<td>$5.8 \times 10^{10} \pm$</td>
<td>$4.2 \times 10^9 \pm$</td>
<td>$4.0 \times 10^{10} \pm$</td>
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<tr>
<td>12 h with SRB</td>
<td>159.5 ± 1.1</td>
<td>$2.5 \times 10^6 \pm$</td>
<td>$8.5 \times 10^{10} \pm$</td>
<td>$4.8 \times 10^7 \pm$</td>
<td>$1.3 \times 10^6 \pm$</td>
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<td>12 h control</td>
<td>88.4 ± 0.9</td>
<td>$9.6 \times 10^3 \pm$</td>
<td>$1.4 \times 10^6 \pm$</td>
<td>$3.7 \times 10^3 \pm$</td>
<td>$7.1 \times 10^2 \pm$</td>
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8.4.2. Rs for indication of corrosion product formation

Rs, the solution resistance, depends strongly on the ionic strength of medium [213], which could be affected by SRB metabolic activities and other reactions. Precipitation of corrosion product and removal of volatile compounds by the continuous purge with nitrogen gas might also decrease the ionic strength. To evaluate these effects, the model-fitted Rs values from EIS data were considered together with the measured sulfide concentrations and pH of medium samples and the coupon surface characterization by SEM and EDS. Rs values in abiotic control and SRB systems are shown in Figure 8.4.

(i) Abiotic control systems

Rs was lower in the high-phosphate abiotic control than the low-phosphate one: the former remained around 85 Ohm.cm$^2$ during the entire experiment, the latter increased almost continuously. Corresponding to the increasing Rs in the low phosphate control, a clear corrosion product layer formed on the entire coupon, seen in Figures 8.5 (a) and (b). This layer was thick (~1 mm) and visible to naked eyes. The EDS results in Figure 8.5 (c) indicated that the area with corrosion products had significantly higher oxygen content and lower iron content, i.e., 30.6 ± 5.7% O and 52.0 ± 8.8% Fe, than the bare metal without apparent corrosion products, i.e., 13.4 ± 4.0% O and 71.1 ± 5.6% Fe. The finding is consistent with the increased formation of dark iron oxides. As Rs could increase if the ionic strength was lowered, these findings suggested that some ions precipitated out and formed the dark corrosion product layer.

In the high-phosphate abiotic system, Rs did not change significantly. This could be partially because the high phosphate concentration kept the liquid ionic strength high.
Also, the SEM images (Figure 8.5 [d]) showed that corrosion products (darker area) covered a much lower percentage of the coupon surface in this system.

Figure 8.4. Dynamic responses of Rs from equivalent circuit modeling of EIS results in (a) abiotic control systems and (b) SRB systems.
(ii) SRB systems

Rs changed much more in the SRB systems than in the abiotic controls, as compared in Figure 8.4. The change was again significantly higher in the low phosphate system, increasing from 154 Ohm.cm$^2$ at the beginning to 1276 Ohm.cm$^2$ after 43 days. In comparison, Rs in the high phosphate system remained relatively constant during the first 10 days, dropped to about 60 Ohm.cm$^2$ at 21 days, and then continuously increased to about 938 Ohm.cm$^2$ after 45 days. The much larger changes of Rs reflected the changes of medium components by the SRB metabolism.

In both high and low phosphate systems, the sulfide concentration increased initially and then decreased; the change was higher in the low phosphate system: it began with 0.008 mM, increased to 0.241 mM at 21 days, and then decreased to 0.014 mM at 43 days, as shown in Figure 8.2 (a). So, one contributing factor to the increase of Rs could be that sulfate ions in the original medium were removed by *D. vulgaris*’ sulfate
reduction and part of the sulfide formed was precipitated out as FeS or gradually removed as H₂S by the N₂ gas flow. Also, as discussed above for the abiotic controls, Rs could increase when more ions were removed from the liquid medium into forming insoluble corrosion products. The increasing corrosion product formation with time in the SRB systems is clearly seen with the SEM and EDS results, as described in the following:

In the low phosphate system, as shown in Figures 8.6 (a) and (b), thick biofilm formed on the coupon incubated with the SRB for 21 days. The EDS results in Figure 8.6 (g) show that the biofilm was rich in carbon (33.1 ± 2.8%) and oxygen (30.8 ± 4.5%). Iron was also found, at 22.1 ± 9.7%. The biofilm layer contained a mixture of SRB cells and corrosion products. After 43 days, pits were observed to have SRB cells distributed around or inside the pits (Figure 8.6 [c]). Although not always clear, thick corrosion product layers could be found in areas with fewer attached SRB cells, as shown in Figure 8.6 (d). The EDS analysis results are given in Figure 8.6 (g). The data for inside and outside the pit were gathered from the areas shown in Figure 8.6 (c); the elemental compositions inside and outside the pit were not significantly different. The EDS data for the corrosion product layer was gathered from areas shown in Figure 8.6 (d). The oxygen content of corrosion product layer (32.5 ± 4.0%) was significantly higher than that inside (14.6 ± 11.3%) or outside the pit (13.3 ± 1.3%). This trend of increasing corrosion product formation from 0 to 43 days (Figures 8.6 [b] and [d]) in the low phosphate SRB system is consistent with the trend of continuous Rs increase (Figure 8.4 [b]).

In the high phosphate SRB system, the number of sessile cells was much lower than in the low phosphate system during the entire exposure time, as shown in Figures
8.6 (e) and (f). Only a few cells were seen inside or around the pits. Corrosion products were not nearly as visible as in the low phosphate system; nonetheless, some mixtures of SRB cells and corrosion products could be seen after 46 days, as shown in Figure 8.6 (f). The EDS results again did not show statistically significant difference between the areas inside and outside the pits (Figure 8.6 [h]). The much lower corrosion product formation is also consistent with the milder Rs increase in this high phosphate SRB system (Figure 8.4 [b]).

In summary, much more corrosion products were formed in the SRB systems than in the abiotic controls and, between the SRB systems, the formation was clearly more in the low phosphate system than in the high phosphate system. The extents and trends of Rs increase in all these systems matched those of the corrosion product formation. Gradual purge of the H$_2$S produced in the SRB systems might have also contributed to the Rs increase. The findings indicate that Rs can indirectly reflect corrosion product formation and other MIC activities that alter the liquid medium composition. The sensitivity may be lower if the ion concentration of the medium is high and the composition change represents only a small percentage of the total ionic strength, as observed in the high-phosphate abiotic control. For an open system in flowing liquid, because the medium at the point of EIS monitoring changes constantly, it can be hard to correlate the Rs measurement with the MIC activities. Porous resistance is a more direct indicator of the formation of biofilm and corrosion product layer. This is described later in Section 8.4.4.
(a) 21 d SRB low phosphate

(b) Enlargement of (a)

(c) 43 d SRB low phosphate

(d) 43 d SRB low phosphate cell corrosion product mixture

(e) 21 d with SRB high phosphate

(f) 46 d with SRB high phosphate
Figure 8.6. SEM images of *D. vulgaris* biofilm on carbon steel incubated with Postgate medium C with 0.5 g/L KH$_2$PO$_4$ for (a), (b) 21 days and (c), (d) 43 days and those with 10 g/L KH$_2$PO$_4$ for (e) 21 days and (f) 46 days; and (g), (h) summary of EDS results on 21 and 43 or 46 days. Each average weight percentage in EDS analysis was based on 3 images.

8.4.3. Rct for indication of uniform corrosion

Charge transfer resistance is associated with metal dissolution [135]; in principle, it may correlate with the weight loss rate. However, the weight loss measurement gives an average rate that reflects the cumulative effects over the entire exposure history of the coupon, while Rct obtained from EIS indicates the condition right at the time of measurement. Data processing is necessary before they can be meaningfully correlated.

The raw, instantaneous Rct values are shown in Figures 8.7 (a) and (c) for the abiotic and SRB systems, each with high and low phosphate concentrations respectively. These Rct values were fitted with polynomial equations, which were then used to get the time-averaged Rct values at the times of weight loss measurements; for example, the average Rct value during Day 0 to 21 was calculated by numerically integrating the polynomial equation with time over that period and then dividing the integral by the time interval of 21 days. The cumulatively averaged Rct values obtained this way are plotted together.
with the measured weight loss rates in Figures 8.7 (b) and (d). The averaged Rct and uniform corrosion rate were found to correlate well for the low phosphate systems, with increasing Rct values corresponding to lower uniform corrosion rates (Figure 8.7 [b] and [d]). The correlation was unclear for the high phosphate systems; at the two measurement times, neither the weight loss rates nor the averaged Rct values changed significantly.

Rct profiles in abiotic control and SRB systems showed different patterns. In the low phosphate systems, a clear increase of Rct appeared between 7 days ($9.5 \times 10^4$ Ohm cm$^2$) and 43 days ($2.6 \times 10^6$ Ohm.cm$^2$) in the abiotic control (Figure 8.7 [a]), which indicates the decrease of corrosion rate caused by the formation of protective corrosion product layer (Figures 8.5 [a] and [b]). Rct of the low-phosphate SRB system fluctuated between $1.8 \times 10^5$ and $2.3 \times 10^7$ Ohm.cm$^2$ caused by the biofilm and corrosion product layer evolution. On average, Rct was higher in the SRB system ($8.0 \times 10^6$ Ohm.cm$^2$) than in the abiotic control ($9.8 \times 10^5$ Ohm.cm$^2$), consistent with the lower overall weight loss rate in the SRB system (20.5 μm.y$^{-1}$) than in the control (43.2 μm.y$^{-1}$). In the high phosphate systems, Rct in the control was relatively stable in the range of $4.2 \times 10^5$ to $6.1 \times 10^5$ Ohm.cm$^2$ (Figure 8.7 [a]) while Rct in the SRB system fluctuated much more, from $1.8 \times 10^5$ to $5.2 \times 10^7$ Ohm.cm$^2$. Similar to the trend observed in the low phosphate systems, the abiotic control had significantly lower average Rct ($6.2 \times 10^5$ Ohm.cm$^2$) and higher overall weight loss rate (68.6 μm.y$^{-1}$) than the SRB system (average Rct = $1.8 \times 10^7$ Ohm.cm$^2$ and corrosion rate = 17.8 μm.y$^{-1}$). In both low and high phosphate systems, the formation of interfacial SRB biofilm appeared to have a protective effect against the weight loss corrosion of carbon steel. In addition, the SRB biofilm and associated
formation of corrosion products were evolving, causing the Rct to fluctuate significantly in the SRB systems.

In Figures 8.7 (a), (c), (e) and (f), the magnitudes of constant phase element (Qct) were observed to correlate inversely with the Rct values. Qct in the abiotic controls (Figure 8.7 [e]) also fluctuated less than the Qct in SRB systems (Figure 8.7 [f]). More discussion about the correlation between Rct, Qct and the interfacial layer formation is included in the following section.
Figure 8.7. Dynamic responses for equivalent circuit elements of (a) instantaneous Rct, (b) cumulatively time-averaged Rct compared with cumulative corrosion rates (CR) based on weight loss measurements in the abiotic control systems and (c) instantaneous Rct, (d) cumulative Rct compared with cumulative corrosion rates (CR) based on weight loss measurements in the SRB systems. The corresponding Qct in (e) control and (f) with SRB systems are also shown.
8.4.4. Layer evolution and Rp for representation of biofilm and corrosion product layers

In this section, layer evolutions of abiotic control and *D. vulgaris* systems represented by Rp were validated with scanned images. Qualitative correlations of different fitting parameters and measured results were also established.

8.4.4.1 Layer evolution in abiotic control systems

Two layers, modeled with Rct, Rp, Qct and Qp values (Figure 8.1 [a]), were observed in the low- and high-phosphate abiotic systems (Figures 8.8 [c] and [f]). pH remained around 7.8 in the high-phosphate control but increased to 9.2 at 43 days in the low-phosphate control (Figure 8.2 [b]). Corresponding to the different pH conditions, much more insoluble corrosion products formed in the low-phosphate system (Figures 8.5 [a] through [c]) than in the high-phosphate system (Figures 8.5 [d]). With more solid corrosion products, the low-phosphate system also showed a higher absolute value of phase angle, ~80° after 13 days (Figure 8.8 [c]), than the high-phosphate system, ~70-73° (Figure 8.8 [f]). A higher absolute phase angle is expected from a layer that is more similar to an ideal capacitor, i.e., with more complete coverage and/or lower porosity [135]. The phase angle data were consistent with the extents of corrosion product formation observed in the SEM images.
(a) Nyquist control low phosphate

(b) Nyquist control low phosphate

(c) Phase control low phosphate

(d) Nyquist control high phosphate

(e) Nyquist control high phosphate
Figure 8.8. Impedance diagrams of (a) Nyquist plot, (b) the detailed plot at high frequencies and (c) the phase diagram at different times of carbon steel coupon exposed to abiotic Postgate medium C with 0.5 g/L KH$_2$PO$_4$, (d) Nyquist plot, (e) the detailed plot at high frequencies and (f) the phase diagram of those with 10 g/L KH$_2$PO$_4$.

As described earlier, corrosion product formation was protective against further uniform corrosion [57]. Accompanying the formation of a thick corrosion product layer in the low-phosphate system, further corrosion slowed down, from 81.3 μm.y$^{-1}$ at 7 days to 43.2 μm.y$^{-1}$ at 43 days; Rct increased from 5.4 × 10$^5$ Ohm.cm$^2$ to 2.6 × 10$^6$ Ohm.cm$^2$ (Figure 8.7 [a]); and Rp increased from 2.0 × 10$^5$ to 2.2 × 10$^6$ Ohm.cm$^2$ during 21-43 days (Figure 8.9 [a]). On the other hand, in the high-phosphate system where corrosion product formation was minimal, Rct, Rp and the uniform corrosion rate remained relatively constant, the latter being 63.5 and 68.6μm.y$^{-1}$ at 21 and 46 days, respectively.

As given in Equation 8.3, Q correlates inversely with the layer thickness [211]. The thick corrosion product layer formed in the low-phosphate system indeed corresponded to decreases of Qct and Qp, the former from 1.3 × 10$^{-4}$ to 2.9 × 10$^{-6}$ F.cm$^{-2}$ and the latter from 8.8 × 10$^{-5}$ to 7.9 × 10$^{-6}$ F.cm$^{-2}$; without thick corrosion product layer formation in the high-phosphate system, Qct and Qp remained stable around 5.9 ×
10^{-5} and 8.3 \times 10^{-5} \text{ F.cm}^{-2}, respectively (Figures 8.7 [e] and 8.9 [b]). The corrosion product formation also affected the potential difference (PD). As shown in Figure 8.10, PD increased in the low-phosphate control from 7 days (-0.36 V) to 43 days (0.24 V) but PD was mostly stable (around -0.5 V) in the high-phosphate control.

In summary, the impedance phase angle, Rp, Rct, weight loss rate, and PD were all found to correlate well with the different extents of corrosion product formation in the abiotic control systems.
Figure 8.9. Dynamic responses of (a), (c), (d) Rp (porous resistance) and (b), (e), and (f) Q (corresponding constant phase elements) of carbon steel coupons that were incubated with (a) and (b) abiotic Postgate medium C and *D. vulgaris* in Postgate medium C with (c), (e) 0.5 g/L and (d), (f) 10 g/L KH$_2$PO$_4$. 
8.4.4.2 Layer evolution in *D. vulgaris* systems

Layer evolution in the *D. vulgaris* systems was more complex because corrosion products and biofilms were both involved. Medium composition (phosphate concentration in this study) changed biofilm attachment and cell viability, and the layer evolution differed clearly in the high- and low-phosphate SRB systems.

In the low-phosphate system, as shown in Figure 8.10 (a), thick biofilms formed at 21 days (starved of organics for 19 days). The cells that were directly attached on carbon steel were mostly alive, whereas those in the outer layer were all dead. In the high-phosphate system after 21 days, as shown in Figure 8.10 (b), only one layer of scattered cells were attached and most of the cells were alive. The cell survival under organics starvation was sustained by direct electron uptake from the steel surface in contact, coupled with sulfate reduction[83]. After 43 or 46 days, as shown in Figures 8.10 (c) and (d), sessile cell numbers decreased significantly from those at 21 days in both high- and low-phosphate systems, and the dead cells in outer layers in the low-phosphate system had also mostly detached. The above description indicates that up to two different layers of cells, i.e., an outer layer of dead cells and an inner layer of live cells, could be found in the low-phosphate system, while only one layer of live cells was found in the high-phosphate system. But, as described earlier in Section 8.4.2, additional layers of corrosion products as well as mixed SRB cells and corrosion products could be present. These were particularly clear in the low-phosphate system at 21 and 43 days (Figures 8.6 [a]-[d] and [g]) and in the high-phosphate system at 43 days (Figures 8.6 [e], [f] and [h]). The numbers of layers at different times and in different systems were determined from
the Bode phase plots and the EIS data fitted with the corresponding multilayer equivalent circuit models, as described in the following.

Figure 8.10. Potential difference measurements of carbon steel coupons incubated with or without *D. vulgaris* in Postgate medium C with (a) 0.5 g/L KH$_2$PO$_4$ or (b) 10 g/L KH$_2$PO$_4$ at different exposure time.

The best-fit Rp results for the multiple porous resistance layers in the low- and high-phosphate SRB systems are shown in Figures 8.9 (c) and (d), respectively. (Rct results for the charge-transfer layer have been described in Section 8.4.3.) The number of Rp layers in the low-phosphate system was 2 prior to 13 days, 3 during 13 to 23 days, and 2 during 23 to 43 days, whereas that in the high-phosphate system was 1 during 0 to 40 days and 2 during 40 to 46 days. The potential makeup of these layers was tentatively assigned with the help of CLSM and SEM images obtained.

For the low-phosphate systems, the two Rp layers prior to 13 days were plausibly made of corrosion products and the relatively thick biofilm (for comparison, only one corrosion product layer was present in the abiotic control, as shown in Figure 8.9 [a]). For the 3 Rp layers during 13-23 days, it was hypothesized that the inner layer consisted of a mixture of live cells and corrosion products, the middle layer a mixture of dead cells and
corrosion products, and the outer layer mainly dead cells. As described earlier, after initial attachment and development into a thick biofilm in the low-phosphate system, the biofilm would evolve into a thin inner layer of live cells and a thick outer layer of dead cells, responding to the starvation of organic electron donors (Figure 8.10 [a]). The presence of corrosion products in the midst of the SRB biofilm has also been shown with the SEM and EDS results obtained at 21 days (Figures 8.6 [a], [b] and [g]). During this period, the biofilm was thicker than that was necessary for accommodating the small amounts of corrosion products formed and dead cells could be clearly observed in the outermost layer (without being covered by corrosion products). The outer Rp layer was therefore considered to be this layer of additional dead cells. During 23-43 days, many of the outer dead cells had detached (Figures 8.6 [c] and 8.10 [c]) while more corrosion products precipitated (Figures 8.6 [d] and [g]). The 2 Rp layers during this period were therefore considered to be an inner layer of mixed live cells and corrosion products and an outer layer of mixed dead cells and corrosion products, similar to the first two layers in the previous period (13-23 days) but with fewer cells and more corrosion products in the mixtures.

For the high-phosphate SRB system, the EIS results suggested only 1 porous resistance layer during the first 40 days; a second (outer) layer appeared afterward (Figure 8.9 [d]). Properties of the single layer during the first 40 days changed significantly, as indicated by the fluctuating Rp in a wide range ($2.1 \times 10^3$ to $4.6 \times 10^6$ Ohm.cm$^2$). There were much fewer sessile SRB cells in this high-phosphate (i.e., high-phosphate) system than in the low-phosphate system (Figures 8.11); so this layer could not be attributed simply to the biofilm. Corrosion products, such as iron phosphate, iron
sulfide and iron hydroxide [104, 214], should also be present in this layer. SEM images confirmed the presence of a layer of mixed SRB cells and corrosion products (Figures 8.6 [e] and [f]). Up to 29 days, RP was high in the range of $1.0 \times 10^5 - 4.6 \times 10^6$ Ohm.cm$^2$; for comparison, RP reported for a coupon with iron phosphate coating was $2.7 \times 10^7$ Ohm.cm$^2$ and, as the coating degraded, RP decreased to $9.8 \times 10^4$ Ohm.cm$^2$ after 14 days [215]. The significant decrease of RP to $1.0 \times 10^5 - 1.6 \times 10^5$ Ohm.cm$^2$ during 14-21 days might be related to corrosion pit formation; pits were observed on the coupons taken at 21 days (Figure 8.6 [e]). RP values observed at 36-40 days were even lower, $2.1 \times 10^3 - 4.5 \times 10^3$ Ohm.cm$^2$. The sharp drop of RP likely indicated the passivity breakdown of the corrosion products. A two-layer model described the EIS data during 42-46 days better, with $R_{P_{\text{out}}}$ of $7.6 \times 10^3$ to $9.1 \times 10^3$ Ohm.cm$^2$ and $R_{P_{\text{in}}}$ of $1.2 \times 10^5$ to $9.2 \times 10^5$ Ohm.cm$^2$. The outer layer coverage was likely incomplete as compared to the inner layer (Figure 8.9 [c]). Digital images (not shown) taken immediately after the CS coupons were removed from the reactor also showed that loose black precipitates (FeS) covered only a small portion (around 4%) of the surface, while other areas were not. In the Postgate medium C, FeS precipitate would form only after the citrate chelating agent was exhausted. One possibility was that after the citrate capacity was exhausted, the FeS precipitates accumulated and formed an incomplete outer layer.

Bode phase angle diagrams offer some more information about the compactness of these layers. Phase angles with higher absolute values correspond to more compact, less porous layers. In the low phosphate SRB system, most phase angles were between $-30^\circ$ and $-50^\circ$ for the inner and middle layers (each being a mixture of live or dead cells with corrosion products) and between $-10^\circ$ and $-25^\circ$ for the outer layer (loose layer of
dead cells, Figure 8.12 [c]). Furthermore, the absolute phase angle was lower at earlier stages (e.g., 21 days) with thicker biofilm and smaller amounts of corrosion products. Results suggested that it was the corrosion products that made the layers more compact. In the high phosphate SRB system, the phase angles of the interfacial layers were consistently larger in absolute values (between -50° and -80°), suggesting that the layers contained more corrosion products. This was in agreement with the finding that the high phosphate system had much fewer sessile SRB cells than the low phosphate system (Figures 8.11 [b] and [d]).

![Figure 8.11. CLSM images of SRB biofilm on carbon steel coupons incubated with Postgate medium C for 21 days [(a), (b)] and 43 days [(c), (d)] with 0.5 g/L [(a), (c)] and 10 g/L [(b), (d)] KH₂PO₄. SRB biofilm in the low phosphate systems was significantly thicker than that in the high phosphate systems.](image-url)
(a) Nyquist SRB low phosphate

(b) Nyquist SRB low phosphate

(c) Phase angle SRB low phosphate

(d) Nyquist SRB high phosphate

(e) Nyquist SRB high phosphate
Figure 8.12. Impedance diagrams of (a) Nyquist plot, (b) the detailed plot at high frequencies and (c) the phase diagram at different times of carbon steel coupon exposed to \textit{D. vulgaris} in Postgate medium C with 0.5 g/L KH$_2$PO$_4$, (d) Nyquist plot, (e) the detailed plot at high frequencies and (f) the phase diagram of those with 10 g/L KH$_2$PO$_4$.

Some more information about the layer thickness could be found from the model-fitted Q results. Q results for the low-phosphate SRB system are shown in Figure 8.9 (e). The inner layer capacitance $Q_{in}$ maintained relatively stable around $2.5 \times 10^{-5}$ F.cm$^{-2}$ during 7 to 43 days, which indicated that the inner layer was relatively stable under the organic nutrient starvation. During this time period, only the innermost layer of cells that were directly attached on carbon steel survived. $Q_{mid}$ of the middle layer, a mixture of dead cells and corrosion products, decreased from $1.8 \times 10^{-5}$ F.cm$^{-2}$ at 13 days to $1.5 \times 10^{-6}$ F.cm$^{-2}$ at 43 days, suggesting an increase of middle layer thickness. This thickness increase was mostly because more and more corrosion products were formed during the later period (Figures 8.6 [c], [d] and [g]).

Q results for the high-phosphate SRB system are shown in Figure 8.9 (f). A comparison of Figures 8.9 (d) and (f) showed that for both inner and outer layers, Q and Rp roughly correlated inversely, reflecting that thicker layers (smaller Q) had higher
resistance. The correlation did not hold only during 36 to 40 days, when both \(R_{p_{in}}\) and \(Q_{in}\) were low. The absolute value of phase angle was lower at day 36 (33.5°) comparing with day 25 (77.3°, Figure 8.12 [f]), which indicated that the layer was more porous at day 36. The results suggest that during this period the interfacial layer turned thicker but more porous.

The PD of the low-phosphate SRB system is shown in Figure 8.10 (a). During the first 2 days, PD increased sharply from -0.2 to 0.12 V, when the organic nutrients supported active cell growth and biofilm formation on the coupon. From 2 to 23 days, PD fluctuated slightly between 0.12 and 0.16 V, when the layer evolution was presumably mainly related with biofilm (Figures 8.6 [a], [b] and 8.10 [a]). PD increased again after 23 days, from 0.13 V to 0.39 V at 43 days, when significant precipitation of corrosion products took place presumably because the chelating capacity of the citrate present in the medium was exhausted (Figure 8.6 [c], [d]). Each fast increase of PD appeared to correspond to a type of layer formation (biofilm or corrosion products). The PD profile for the high-phosphate SRB system is shown in Figure 8.10 (b). In this system, the initial PD increase was similar to that in the low-phosphate system. However PD started to decrease after about 8 days, likely corresponding to the faster cell lysis and biofilm sloughing under the osmotic stress caused by the high phosphate. Overall, except in the first 8 days, PD was much lower in the high-phosphate system than in the low-phosphate system. This is consistent with the thinner interfacial layer formation in the high-phosphate system, as described in previous sections.

In the high-phosphate SRB system, where only scattered thin biofilm was attached on the coupon surfaces, the similarity between trends of PD (Figure 8.10 [b])
and $R_{p_{in}}$ (Figure 8.9 [d]) was apparent. Both showed two periods of clear drops, roughly during 13-20 days and 31-38 days, respectively. The possibility of these sharp drops being related with pitting is considered, because the SRB-induced pitting occurred here and pitting is associated with breakdown of passive layer on the surface. If so, PD and Rp monitoring has the potential of indicating pitting occurrence. In the low-phosphate SRB system, the PD trend was also similar to the trend of total Rp (i.e., sum of all serial Rp’s modeled, not shown). The SRB-induced pitting was even more severe in this system but neither PD nor Rp trend showed clear drops like those seen in the high-phosphate SRB system. The thick biofilm (and perhaps corrosion product layer) developed on the coupon surface might be responsible for preventing EIS signals from reflecting the pitting occurrence.

In summary, interfacial layer evolution observed by SEM and CLSM correlated reasonably well with Rp, Qp, Rct, Qct, phase angle diagram, weight loss rate, and PD. The layer evolution in the low-phosphate system was 2 layers (presumably, biofilm and biofilm-corrosion product mixture) during 0-13 days, 3 layers (dead cells in the outer layer, dead cells with corrosion products in the middle layer, and live cells with corrosion products in the inner layer) during 13 – 23 days, and 2 layers (dead cells with corrosion products in the outer layer and live cells with corrosion products in the inner layer) during 23 - 43 days. In the high-phosphate system, there was only 1 layer (corrosion products with few SRB cells) during the first 40 days and 2 layers from 42 to 46 days. The two layers were possibly developed by the accumulation of corrosion product precipitates after the chelating capacity of citrate in the medium was exhausted.
8.4.4.3. Role of *D. vulgaris* on interfacial layer evolution

As demonstrated earlier, *D. vulgaris* had strong effects on the interfacial layer evolution observed. For the low-phosphate abiotic system, a single compact Rp layer was formed with Rp value increasing from $3.7 \times 10^4$ to $2.2 \times 10^6$ Ohm.cm$^2$ in 43 days (Figure 8.9 [a]), which was in agreement with the SEM images shown in Figures 8.5 (a) and (b). The low-phosphate SRB system, on the other hand, formed multiple layers (Figure 8.9 [c]) according to the biofilm and corrosion product formation on the coupon surfaces (Figures 8.6 [a]-[d] and 8.10 [a] and [c]). The final (43-day) Rp value in the abiotic control ($2.2 \times 10^6$ Ohm.cm$^2$) was higher than the $R_{p_{in}}$ value in the *D. vulgaris* system ($3.9 \times 10^5$ Ohm.cm$^2$) while the final Qp value was lower in the abiotic control ($7.9 \times 10^6$ F.cm$^{-2}$) than $Q_{p_{in}}$ value in the *D. vulgaris* system ($1.6 \times 10^5$ F.cm$^{-2}$). These indicated that the final corrosion product layer in abiotic control had higher resistance and larger thickness than the final interfacial layer remaining on the coupon in the *D. vulgaris* system, which can be explained by the porous nature and gradual thinning of biofilm over long-term organics starvation. Pits formed in the low-phosphate *D. vulgaris* system were mostly filled with corrosion products and biofilm (Figures 8.6 [b] and [c]), which might have interfered the detection of pitting occurrence by EIS results (Rp and PD profiles). For the high-phosphate abiotic systems, Rp and Qp were stable around $8.0 \times 10^4$ Ohm.cm$^2$ and $9.5 \times 10^5$ F.cm$^{-2}$, respectively, throughout the 46-day experiment, which suggested the existence of a relatively stable iron hydroxide layer without significant further accumulation of solid corrosion products. In the high-phosphate *D. vulgaris* system, maintenance of SRB cell survival and biofilm attachment was inhibited by the higher osmolality (Figures 8.11 [b] and [d]) and only a single Rp layer was suggested by
the EIS results during the first 40 days. The layer properties were likely affected by both attached cells and solid corrosion products (Figure 8.6 [e]), with the contributions from the two groups varying with time. Pits formed in the high-phosphate SRB system were not filled with corrosion products or covered by thick biofilm (Figure 8.6 [e]). This property might have allowed clearer detection of Rp and Qp fluctuations associated with pitting occurrence. From 42 to 46 days, a second porous layer was formed (Figure 8.9 [d]), which might be associated with more corrosion product precipitation after the citrate chelating capacity was exhausted.

Results of the study helped develop the application of EIS for online monitoring of the biofilm and metal surface interactions and interfacial evolution, but also indicated the significant signal complexity. Concerted future work is required to keep improving the understanding and correlation between the EIS results and the MIC activities.

8.5. Conclusions

1. Planktonic D. vulgaris cells did not cause significant uniform corrosion of carbon steel.
2. Rs change might be used as an indirect indicator for corrosion product formation, especially when the ion concentration of water/medium was low.
3. Time-averaged Rct inversely correlated with the uniform corrosion rate.
4. In all systems investigated, the interfacial layer evolution observed by SEM and CLSM could correlate with the EIS results, including the phase angle diagram, PD and the model-fitted Rp, Qp, Rct, and Qct.
(5) The results suggested the following layer evolution processes in the high- and low-phosphate SRB systems. In the low-phosphate system, the coupon surface had two interfacial layers prior to 13 days – an inner layer of live cells mixed with corrosion products and an outer layer of predominantly porous biofilm; developed into 3 layers during 13 to 23 days – an inner layer of live SRB cells mixed with corrosion products, a middle layer of dead cells and corrosion products, and an outer layer of dead SRB cells; and then reduced back to 2 layers during 23 to 43 days, after the outer layer of dead cells detached and more corrosion products precipitated. In the high-phosphate system, there were much fewer and more scattered cells attached. The interfacial layer remained as a single layer for 40 days, composed of the corrosion products and SRB cells; and then developed into 2 layers during 42–46 days as more corrosion product deposits accumulated.
CHAPTER IX

INHIBITION OF SRB-INDUCED CORROSION ON CARBON STEEL BY PHOSPHATE

9.1. Introduction

Sulfate reducing bacteria (SRB) are well-known to cause microbiologically influenced corrosion (MIC), especially on steel [11]. Two mechanisms are more commonly accepted to explain the SRB-associated MIC [43]. One involves the chemical effects of H$_2$S [43], the other relies on SRB’s ability to couple sulfate reduction with direct electron uptake from steel [49, 83, 216]. Current MIC mitigation approaches face effectiveness concerns. The most widely used approach is biocide treatment [10]. It is more effective in killing planktonic bacteria but not sessile bacteria [217]. According to our previous finding with *Desulfovibrio vulgaris*, pitting corrosion of carbon steel would worsen during long-term survival of sessile cells under conditions of no external organic electron donors, and cell attachment on steel surface was essential for this long-term survival [83]. It is therefore more critical to kill the sessile bacteria than planktonic ones, which is unfortunately an MIC mitigation effect harder to achieve by biocide treatment. Another approach is to use mechanical devices such as “pigs” to remove biofilm [10]. However, “pigs” may not fit into narrow pipes and they are expensive. Nitrate treatment
has also been proposed as a strategy to control SRB-induced corrosion [92]. Nitrate may enable nitrate reducing bacteria to outcompete SRB for available organic electron donors [93-95]. However, this approach produced inconsistent results in field settings [10, 96]. Many SRB can also use nitrate as electron acceptor; this weakens the basic premise of nitrate treatment [12, 13]. SRB’s ability to survive without organic electron donors would further limit the effectiveness of this approach [83]. There is a critical need to develop more effective MIC mitigation approaches that target at inhibiting/minimizing sessile SRB.

Phosphate is a known corrosion inhibitor, which stabilizes the rust layer by forming binuclear phosphate-metal complexes on metal surfaces [97-99]. It is more effective when oxygen is present. Phosphate solution passivated pits with oxygen to form a mixed layer of iron oxides and iron phosphates [100, 101]. In the absence of oxygen, observations with mild steel in Na₂HPO₄ solutions suggested that phosphate inhibited pitting by forming a physical barrier (green rust), which was reported to contain vivianite [Fe₃(PO₄)₂·8H₂O] as a component [104, 105]. Although vivianite was less protective than iron oxides, it was still more corrosion resistant than the soluble complex formed by steel submerged in a 0.1 N sodium tartrate solution [104]. Despite the broad application of phosphate in corrosion inhibitors, the effects of phosphate on MIC were scarcely known. Volkland et al. [106] showed the formation of a vivianite layer due to bioactivities of *Rhodococcus* sp. C125 and *Pseudomonas putida* mt2 and the associated inhibition of mild steel corrosion. They evaluated corrosion only by weight loss. There was no literature report on the effects of phosphate on SRB-induced pitting.
In this work, biofilm activities of *D. vulgaris*, electrochemical potential, pitting corrosion and intergranular corrosion (IGC) of carbon steel were studied at two phosphate concentrations, 0.5 and 10 g/L KH$_2$PO$_4$. Results were compared to elucidate the effects of phosphate. Correlations among the observed biofilm activities, potential, pitting corrosion and IGC were investigated and discussed.

9.2. Materials and Methods

This study was characterized by multi-disciplinary techniques. Bacterial cultivation and phosphate effects on growth of *D. vulgaris* were monitored by microbiological methods. Biofilm, coupon surface were characterized by material science techniques. Pitting corrosion was characterized using improved fast characterization method as described in Chapters 6 and 7. Electrochemical techniques were used to measure potential difference.

9.2.1. Bacterium, medium and setup

*Desulfovibrio vulgaris* (ATCC# 7757) was routinely cultivated under sterile and anaerobic conditions in Postgate medium C [16], containing sodium pyruvate (6 g/L), Na$_2$SO$_4$ (4.5 g/L), yeast extract (1 g/L), NH$_4$Cl (1 g/L), KH$_2$PO$_4$ (0.5 g/L), Na$_3$C$_3$H$_5$O(COO)$_3$·2H$_2$O (0.3 g/L), MgSO$_4$·7H$_2$O (0.06 g/L), CaCl$_2$·6H$_2$O (0.04 g/L), and FeSO$_4$·7H$_2$O (0.004 g/L). The KH$_2$PO$_4$ concentration was altered for study purpose when specified otherwise. Medium pH was adjusted to 7.0 using 5N NaOH. Resazurin (1 mg/L) was added as a redox (E$_h$) indicator. Serum bottles sealed with butyl rubber stoppers were typically used. To maintain anaerobic condition, N$_2$ was bubbled through the medium in
serum bottles, while they were heated to 100 °C. This treatment ensured removal of dissolved oxygen in the liquid and gaseous oxygen in the bottle headspace. The treated systems were then sterilized by autoclaving at 120 °C for at least 30 min. Cysteine (150 mg/L) was finally added as an oxygen scavenger, right before inoculation of the seed culture.

*D. vulgaris* stock culture was stored at a -80 °C freezer. With 2% inoculation in sterilized anaerobic Postgate medium C, the culture was grown at 25 °C for 26 h to reach the late exponential growth phase or early stationary phase with a cell concentration of 1.4 (± 0.1) × 10⁹ cells/mL [70]. The fresh culture was used as the inoculum (5%) for experiments in which *D. vulgaris* was cultivated with or without carbon steel coupons.

9.2.2. Study of phosphate concentration effect on *D. vulgaris* growth

A batch culture study was carried out at 37 °C to examine the effects of phosphate on initial cell growth of *D. vulgaris*. Four media, modified from Postgate medium C, with different phosphate concentrations (0.5, 5, 8 and 10 g/L KH₂PO₄) were tested. Crimp top serum bottles with 80 mL media and no steel coupons were used in this study. Samples were taken periodically using sterile syringes flushed with filtered nitrogen. pH was measured with a pH meter (Model 420A, Orion, Watsonville, CA) immediately after sampling. Samples were then centrifuged to collect cells and supernatant separately. Cells were washed once, resuspended in deionized (DI) water, and then measured for optical density at 1 cm path length and 600 nm (OD₆₀₀) using a UV-Vis spectrophotometer (Model 1601, Shimadzu Corp., Canby, OR). The suspension might need dilution to adjust OD₆₀₀ to the linear range (lower than 0.6) of measurement. The measured OD₆₀₀ values,
after multiplication by dilution factors, were used to indicate the planktonic cell concentrations. The sample supernatant collected by centrifugation was measured for pyruvate concentration. Pyruvate analysis was done with a high-performance liquid chromatography (HPLC) system (Model LC-10A, Shimadzu Corp., Columbia, MD) equipped with a Supelcogel H column (Sigma-Aldrich Corp., St. Louis, MO) and a UV-Vis detector (SPD-10A). The mobile phase used was 0.1% H$_3$PO$_4$ at a flow rate of 0.14 ml/min.

9.2.3. Corrosion study with *D. vulgaris*

Ten systems with carbon steel coupons were included in the study. Six of them had the low phosphate concentration (0.5 g/L KH$_2$PO$_4$), the other four had the high phosphate concentration (10 g/L). For both low and high phosphate systems, half of the systems had *D. vulgaris* cells; the other half were abiotic controls. The reactors used were 600 mL glass Griffin beakers closed with rubber stoppers. (The top part of beaker with the pour sprout was removed.) The study was made at room temperature (22 ± 1 °C). Humidified and filter-sterilized (0.2 µm) N$_2$ was constantly bubbled through the liquid broth to maintain anaerobic conditions. All the tubing and fittings for gas addition and sampling were made of stainless steel 304 (McMaster Car, Aurora, OH). Teflon tape was used at all connections and contacts to prevent leakage. A 1 mg/L resazurin solution was used to pre-test the reactors for maintaining anaerobic under experimental operation. After purging with nitrogen gas for 30 min, the resazurin solution turned from blue to pink. Once the cysteine solution (0.002 g/L) was added, the color turned into colorless. Reactors used in the study were thus confirmed to provide the anaerobic condition.
Each reactor contained 4 carbon steel coupons (C1010, Q-Panel, Cleveland, OH; rectangular shape 25 × 11 × 0.3 mm). Coupons employed in the low phosphate systems were prepared in the following procedure. One side of coupon was polished using SiC sand papers as described elsewhere [180], and the unpolished side was numbered using stainless steel tweezers. Coupons used in the high phosphate systems were purchased from Metal Samples Company (Munford, AL; rectangular shape 20.3 × 10.2 × 1.6 mm). They were custom made with lap finish on one side and 120-grit finish on the other side. Coupons were cleaned by 20 min sonication in acetone and then in ethanol. For sterilization, the coupons were soaked in pure ethanol for 2 days at room temperature. Sonication was done during 0 to 2 h and then during 46 to 48 h of the soaking period to avoid gas bubbles adhering to the coupon surface. Each coupon had a centered hole of 5.1 mm diameter near one end (5.7 mm away from the end). Through the hole a plastic tie-wrap was used to hang the coupon vertically on a stainless steel hook (type 304) that was anchored on the rubber stopper. Coupons were completely submerged in the liquid medium during the experiments.

All 4 coupons from an SRB-containing reactor and those from a cell-free control were removed at every sampling time. Each coupon was used for one respective evaluation as described in section 2.4. Liquid samples were taken periodically from the reactors. They were analyzed to determine cell growth, pH change, nutrient consumption and metabolite production. Samples for sulfide concentration measurement were preserved with equal volume of a zinc acetate solution (100 g/L) immediately after the samples were collected from the reactors. Samples were vortexed for 30 s to ensure mixing and reaction. ZnS precipitates formed were separated from the liquid by
centrifugation and then resuspended in DI water for sulfide analysis using the methylene blue assay [128]. Duplicate samples were taken for analyses from each system at every sampling time. All reported results for liquid samples were averaged from 4 to 6 measurements.

Four out of the 10 reactors, one from each type of systems studied, i.e., high phosphate-SRB, low phosphate-SRB, high phosphate-control and low phosphate-control, were equipped for electrochemical measurements using two-electrode systems [84]. Carbon steel coupons served as the working electrode. A platinum wire connected with a platinum mesh served as the counter and pseudo-reference electrodes. Potential difference (PD) was measured at least twice a day by a New Research Grade Potentiostat/Galvanostat (Model SP-200, Bio-Logic Science Instruments, Knoxville, TN). At each measurement, PD was monitored for 30 min. Data reported for each day was averaged from all measurements made in the same system during that day.

9.2.4. Biofilm and CS coupon characterization

Coupons removed from reactors for analyses were handled in an anaerobic glove bag (Coy Laboratory Products, Grass Lake, MI) with 97.5% N$_2$ and 2.5% H$_2$. Biofilm formed on the coupon was characterized by a confocal laser scanning microscope (CLSM, Model FV1000, Olympus America Inc., Center Valley, PA). Immediately after removal from the reactor, biofilm was stained with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Life Technologies Corp.; Grand Island, NY). Stain and stained biofilm were always protected from light. Stained biofilm was scanned using the CLSM with 488 nm and 559 nm excitation wavelengths. Live cells appeared green while dead cells
appeared red. For enumeration of sessile SRB, five to ten sets of CLSM images were scanned for each coupon. Four sets of the scanned images with good quality were selected for counting the number of sessile SRB using Image J (National Institutes of Health, USA), and the averaged results were reported. For biofilms with multiple cell layers, cell numbers from all layers were summed and the total sessile SRB number on each coupon was obtained.

Coupons were also examined by a scanning electron microscope (TM-3000 Tabletop SEM, Hitachi High Technologies America, Dallas, TX). For preparation, biofilm was fixed by an anoxic glutaraldehyde solution (2% w/w), dehydrated in ethanol (over a gradient of increasing ethanol concentration), and then stored in a desiccator for 1 day at room temperature. The anoxic glutaraldehyde solution was prepared by the following procedure. Sterilized DI water was stored in a loosely capped glass bottle, which was placed in an anaerobic glove box for 3 days. A 50% glutaraldehyde solution was treated with N₂ gas for 2 min in a serum vial and then added into anoxic DI water in the glove box. Aerobic glutaraldehyde solution was avoided because it might corrode the coupons. More detailed description for biofilm fixation was available elsewhere [83]. For examination of grain structures, coupons were cleaned following the ASTM G1-03 standard chemical cleaning procedure for iron and steel (C.3.1 in Table A1.1 of the ASTM G1-03) [129].

Pitting corrosion of coupons was quantified and compared based on measurements made with an infinite focus microscope (IFM, Alicona imaging GmbH, Bartlett, IL), using a fast characterization method established previously in our group [200, 218]. In this method, the surface parameters obtained by IFM were used to
calculate the maximum pit depth, average pit depth and pitted area percentage according
to the following equations:

\[
\text{Maximum pit depth (\(\mu m\))} = 0.96 \times (S_v + S_k) \tag{9.1}
\]

\[
\text{Average pit depth (\(\mu m\))} = 1.17 \times (S_{dr}/V_{vv}) + 6.23 \tag{9.2}
\]

\[
\text{Pitted area (\%)} = 6.62 \times (S_{vk} \cdot V_{vv}/S_{dr}) \tag{9.3}
\]

Physical meaning of the surface parameters used in Equations 1-3 are briefly
described here [132-134]. Surface features can be divided into three parts – peak, core
and valley. The core (or kernel), with a height/thickness of \(S_k\) (\(\mu m\)), represents the
random surface roughness. “Peaks” and “valleys” are the features above and below the
core, respectively. \(S_{vk}\) (\(\mu m\)) denotes the mean depth of all valleys (below the core) on a
surface (and \(S_{pk}\) the mean depth of all peaks above the core). \(S_v\) is the maximum depth
among all valleys found on a surface (\(\mu m\)). \(V_{vv}\) is the valley void volume of the surface
(\(ml/m^2\)). \(S_{dr}\) is the developed interfacial area ratio (\%), \(=\) (actual surface area – projected
surface area)/(projected surface area) \(\times\) 100. For pitting corrosion, valleys are the results
of pitting; therefore, parameters associated with the valleys are used in the above
equations for calculation of the maximum and average pit depths and the percentage of
pitted areas.

Failure risk and pitting pattern diagrams [218] were generated based on the
average results from entire coupon surface excluding edges and holes. Pitting could be a
highly localized phenomenon. Standard deviations reported in this work reflected mainly
the varying pitting extents detected from different areas scanned. Failure risk diagram
compared the different risk levels of different coupons; the failure risk was higher for the
coupon with more data point found in the right top corner of diagram [218]. Pitting
pattern diagram divided pitting into four phases [200]: Phase I – few or no micro pits (pitted area percentage < 1.7 %, average pit depth < 10 µm), Phase II – many micro pits or large shallow pits (pitted area percentage > 2.2 %, average pit depth < 10 µm), Phase III – few narrow but deep pits (pitted area percentage < 1.7 %, average pit depth > 14 µm), and Phase IV – deep pit clusters with or without micro pits (pitted area percentage > 2.2 %, average pit depth > 14 µm). More details about the pit characterization method and diagrams are available elsewhere [200, 218].

The differences of sessile SRB numbers on carbon steel in the low versus high phosphate systems and the differences of pit initiation percentages at different zones were analyzed for statistical significance [130]. \( p \) values were determined by using Minitab (Minitab Inc., USA).

9.3. Results and Discussion

Results of this study indicated that phosphate inhibited pitting and intergranular corrosion (IGC) via its effects on the planktonic and sessile cell numbers of the SRB and on the potential of the metal. Individual effects are described in detail in the following sections. A summary diagram is given in Figure 9.1 to help organize and envision the complex structures of these inter-related effects.
Figure 9.1. Mechanisms of phosphate inhibition on *D. vulgaris* induced corrosion of carbon steel. SRB: sulfate reducing bacteria (*D. vulgaris*); IGC: intergranular corrosion; EMIC: electrical microbiologically influenced corrosion; CMIC: chemical microbiologically influenced corrosion; GB: grain boundary.

9.3.1. Effects of phosphate on *D. vulgaris* in the absence of carbon steel

Four KH$_2$PO$_4$ concentrations, i.e., 0.5, 5, 8 and 10 g/L, in Postgate medium C were tested. As shown in Figure 9.2, cells grew well and the carbon source pyruvate was completely consumed in all systems by 25 h. pH dropped faster and to lower values in systems with lower phosphate concentrations (Figure 9.2 [c]), which had weaker phosphate phosphate capacities. The rather similar culture behaviors enabled the selection of 0.5 and 10 g/L KH$_2$PO$_4$ as two significantly different concentrations for comparison in the subsequent corrosion study.
9.3.2. Effects of phosphate on *D. vulgaris* in the presence of carbon steel

During the first 2 days, pyruvate was completely consumed and cells grew to exhibit a maximum optical density of approximately 0.9 in both systems of low and high phosphate concentrations (Figure 9.3). In the following 2 to 43 days, optical density decreased moderately to 0.7 in the low phosphate systems but, much more significantly, to 0.09 in the high phosphate systems. Presumably, the osmotic pressure stress of high...
phosphate medium caused the organics-starving SRB cells to lyse more readily. Concentration profiles of sulfide formed by sulfate reduction are shown in Figure 9.3 (b), which however may underrepresent the actual formation because of the sulfide stripping by N₂ continuously bubbling through the cell culture. Higher sulfide production, up to 0.24 mM, was found in the low phosphate systems, compared to the 0.17 mM in high phosphate systems. This is consistent with the previous cell concentration observations, i.e., more cells were present in the low phosphate systems to carry out sulfide production. Further, low pH favors formation of more strippable H₂S according to the sulfide equilibrium (HS⁻ + H⁺ ↔ H₂S). pH in the low phosphate systems rose to significantly higher values (8.8-9.4) while pH in the high phosphate systems remained around 7.2-7.4 [Figure 9.3 (c)]. Less sulfide removal was expected from the low phosphate systems. For both low and high phosphate systems, during 2 to 21 days, sulfide concentrations increased, although pyruvate was already completely depleted. The sulfide increase might be from sulfate reduction driven by two potential electron donors: (1) H₂, if accumulated from previous pyruvate metabolism and not completely purged out by N₂ bubbling [27]; and (2) direct electron uptake from steel by cells attached on the metal surface [83]. After 21 days, sulfide concentrations decreased in both systems, presumably because the sulfide production rate was slower than the removal rate by N₂ bubbling.

Number of sessile SRB cells was significantly affected by the phosphate concentration difference, especially during the first 21 days. D. vulgaris biofilm was clearly denser in the low phosphate systems (Figures 9.4 [a] and [b]) than in the high phosphate systems (Figures 9.4 [c] through [f]). The counted sessile cell numbers in the low phosphate systems were also significantly higher (Table 9.1); p values for
comparison between low and high phosphate systems were lower than 0.05 (0.004 at 3 or 7 days and 0.000 at 21 days). The planktonic cell concentration (shown as optical density) in the low phosphate systems at 3 days was only around 1.5 times higher than that in the high phosphate systems at 7 days; however, the sessile cell number was 7.7 times higher. The preferential cell attachment in the low phosphate systems was less pronounced after 21 days; with a 2.3 times higher planktonic cell concentration, the low phosphate systems had a 3.9 times higher sessile cell number. It is clear that the lower cell attachment number in the high phosphate systems was not only caused by its lower planktonic cell concentration. Cells grown in the high phosphate systems seemed to have lower tendency to attach to steel surface. Phosphate was suggested to form a passive film under anaerobic conditions [102, 103]. The possible composition of the passive film was reported as Fe₃(PO₄)₂·8H₂O (vivianite) [104, 105]. It is unknown if vivianite formation would interfere with cell attachment.

In summary, although the higher phosphate concentration did not clearly affect cell growth and pyruvate consumption, it caused much faster lysis of organics-starving cells and lower sulfide production and cell attachment. These findings are included in Figure 9.1 [1], i.e., at the high phosphate concentration, planktonic and sessile SRB numbers were lower.
Figure 9.3. Profiles of (a) planktonic cell concentration (optical density at 600 nm) and pyruvate consumption, (b) sulfide concentration, (c) pH and (d) potential difference observed for systems with *D. vulgaris* in Postgate medium C containing 10 g/L (high phosphate) and 0.5 g/L KH$_2$PO$_4$ (low phosphate) in the presence of CS coupons. For comparison, pH and potential difference profiles for corresponding abiotic control systems (without *D. vulgaris*) are also shown in (c) and (d).
(a) 21 d SRB low phosphate

(b) 43 d SRB low phosphate

(c) 21 d SRB high phosphate

(d) 46 d SRB high phosphate

(e) 21d SRB high phosphate

(f) 46d SRB high phosphate
Figure 9.4. SEM (a, b, e, f) and CLSM (c, d) images of carbon steel coupons incubated with *D. vulgaris* in Postgate medium C with low (a, b) or high (c, d, e, f) phosphate (0.5 or 10 g/L KH$_2$PO$_4$) for 21 (a, c, e, f), 43 (b) or 46 (d) days; revealing clear effects of phosphate on biofilm and pit morphologies.

Table 9.1. Number of sessile *D. vulgaris* cells on carbon steel coupons incubated in Postgate medium C with low (0.5 g/L) and high (10 g/L) KH$_2$PO$_4$ buffer, and *p* values calculated by Minitab for statistical significance (< 0.05) of the phosphate effect on cell attachment number, compared at different days.

<table>
<thead>
<tr>
<th>KH$_2$PO$_4$ (g/L)</th>
<th>Time (day)</th>
<th>Sessile cell numbers per cm$^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low (0.5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>(2.3 ± 0.7) × 10$^7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>(3.5 ± 0.2) × 10$^7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>(9.3 ± 6.2) × 10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High (10)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(3.0 ± 0.4) × 10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>(9.0 ± 3.1) × 10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>(6.7 ± 1.2) × 10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>0.5 vs. 10</strong></td>
<td>3 or 7</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>43 or 46</td>
<td>0.439</td>
<td></td>
</tr>
</tbody>
</table>

9.3.3. Effects of phosphate on solution pH and potential difference

pH in high phosphate systems maintained relatively stable around 7.8 in abiotic control and 7.3 in SRB systems (Figure 9.3 [c]). pH in low phosphate systems increased from 7.4 to 9.2 in control systems and from 6.5 to 8.3 in SRB systems. During the first 3 days, potential difference (PD, Figure 9.3 [d]) profiles of the SRB systems were similar in low and high phosphate systems; both showed similar increases from -0.15 to 0.16 V. This was the characteristic potential ennoblement caused by thick biofilm formation [219]. Afterwards, PD was always higher in the low phosphate (final PD around 0.38 V)
than the high phosphate systems (final PD around -0.33 V), which could be attributed to the thicker layer of biofilm and/or corrosion products formed in the low phosphate systems. For abiotic control systems, PD was always higher in the low phosphate systems. The high phosphate concentration caused the medium to have a lower solution resistance and, correspondingly, a lower initial PD. Over 7 days, a thick corrosion product layer developed on coupons in the low phosphate control systems (image not shown), which caused PD to increase from -0.4 to 0.2 V. On the other hand, PD in the high phosphate systems fluctuated around -0.5 V, where the corrosion product layer was not very thick. In summary, both pH and PD were relatively stable in high phosphate systems, while those in low phosphate systems showed clear increases. These findings are summarized in Figure 9.1, i.e., the higher phosphate concentration resulted in lower PD due to lower solution resistance (Figure 9.1 [2]) and lower sessile SRB numbers (Figure 9.1 [3]).

9.3.4. Inhibition of pitting by phosphate ions

Effects of phosphate on pitting of carbon steel caused by *D. vulgaris* with high and low phosphate concentrations were investigated using pitting pattern and failure risk diagrams. Mechanism of phosphate inhibition of *D. vulgaris* induced pitting was also discussed in this section.

9.3.4.1. Pitting characterization by pitting pattern and failure risk diagrams

The pitting pattern diagram shown in Figure 9.5 (a) compared the pitting patterns induced by *D. vulgaris* in low versus high phosphate systems. Data points in high phosphate systems fell in Phase I, which corresponded to the pattern of few micro pits
(pitted area percentage < 1.7%, average pit depth < 10 µm). Data points for low phosphate systems were in Phase IV, which corresponded to the pattern of deep pit clusters (pitted area percentage > 2.2%, average pit depth > 14 µm) [200, 218]. Figure 9.5 (b) evaluated failure risks caused by *D. vulgaris* in the two phosphate systems. Maximum pit depth and pit volume loss were clearly lower in the high phosphate systems (Figure 9.5 [b]), which suggested that high phosphate systems have lower failure risk than the low phosphate systems. The above pitting patterns were consistent with IFM images: coupons in low phosphate systems had mostly deep pit clusters (Figure 9.5 [c]), whereas only one micro pit was formed on the examined coupon collected from the high phosphate systems (Figure 9.5 [d]). In summary, high phosphate clearly inhibited pitting induced by *D. vulgaris*, supported by the less severe pitting indicated by both pitting pattern and failure risk diagrams.
Figure 9.5. (a) Pitting pattern diagram — SRB

(b) Failure risk diagram — SRB

(d) 43d SRB — low phosphate

(b) 46 d SRB — high phosphate

(e) Average viable sessile SRB number per m²

y = 2 × 10⁻¹¹x - 1.14
R² = 0.95

Average pit volume loss rate (µL/(m²·day))

Average pitted area percentage (%)

Maximum pit depth (µm)

V_p (pit volume loss, ml/m²)

Low PO₄³⁻ 21d
High PO₄³⁻ 21d
Low PO₄³⁻ 43d
High PO₄³⁻ 46d

Low PO₄³⁻ 21d
High PO₄³⁻ 21d
Low PO₄³⁻ 43d
High PO₄³⁻ 46d

Figure 9.5. (a) Pitting pattern diagram, (b) failure risk diagram, (c) and (d) IFM images of carbon steel coupons incubated with *D. vulgaris* in Postgate medium C with (c) 0.5 g/L
and (d) 10 g/L KH$_2$PO$_4$ for 21, 43 or 46 days; and (e) correlation between average pit volume loss rate and average viable sessile SRB number per m$^2$. Average pit volume loss rate was calculated from the $V_{vv}$ change that had occurred between two sampling days. For viable sessile SRB number per m$^2$, 4 representative CLSM images were considered at each sampling day. Standard deviations represented the variations with different scanned areas. The average numbers reported were averages of all results obtained for samples taken within the specific periods indicated.

9.3.4.2. Effects of $D. vulgaris$ on pitting

As described in Section 9.3.2, the higher concentration of phosphate ions significantly decreased the bacterial attachment number. Average sessile SRB number per m$^2$ was also found to correlate linearly with the average pit volume loss rate (Figure 9.5 [e]). This correlation can be explained by two mechanisms: (1) electrical microbiologically influenced corrosion (EMIC), where organics-starving $D. vulgaris$ cells could attach to carbon steel and survive by extracting electrons from metal and, thus, induced pitting [83]; (2) chemical microbiologically influenced corrosion (CMIC), where H$_2$S produced by viable cells caused pitting. These two mechanisms are indicated in Figure 9.1 [4]. Both mechanisms depend on the viable sessile SRB cells to enhance pitting. Thus, by decreasing viable sessile SRB numbers, high phosphate would inhibit pitting (Figure 9.1 [1] and [4]).

9.3.4.3. Effects of potential difference on pitting

When the phosphate ion concentration was lower, PD was higher as described in Section 9.3.3. When PD was higher than the pitting potential, more severe pitting might occur [220]. This was seen in the $D. vulgaris$ systems; PD was higher and pitting was more severe in the low phosphate systems than in the high phosphate systems. However,
no pits were found on the examined coupons taken from the abiotic control systems despite the high PD (around 0.26 V) in the low phosphate control. This observation suggested that higher PD only enhanced pitting when other pitting agent/mechanism(s) (i.e. *D. vulgaris* and H$_2$S) existed (Figure 9.1 [5]). If aggressive bacterium or chemical was not present to cause pitting, high PD alone might not be sufficient to enhance pitting. Thus, higher phosphate concentration could inhibit pitting by lowering PD (Figure 9.1 [5]).

9.3.5. Inhibition of intergranular corrosion (IGC) by phosphate ions

IGC was observed in both control and *D. vulgaris* systems. As shown in Figure 9.6, the systems with higher PD suffered more severe IGC attack. At 43 or 46 days, values of PD in the low phosphate abiotic control (0.24 V) and *D. vulgaris* systems (0.39 V) were higher than the values in high phosphate systems (-0.66 V for control and -0.33 V for *D. vulgaris* systems). Correspondingly, IGC was more severe in low phosphate control and *D. vulgaris* systems (Figures 9.6 [a] and [c]) than in the high phosphate systems (Figures 9.6 [b] and [d]). For the low phosphate *D. vulgaris* systems, when PD reached the maximum value, boundaries of several grains were severely corroded and several micro pits formed, as noted by the circles in Figure 9.6 (c). Thus, high phosphate could inhibit IGC by lowering PD (Figures 9.1 [2], [3] and [6]) [220]. IGC tended to initiate at higher potentials, whereas pitting usually occurred at lower potentials [77].
9.3.6. Effects of intergranular corrosion on pitting

In systems with *D. vulgaris* cells, at 21 days IGC was only observed inside the pits (Figure 9.7 [a]) but IGC became widespread at 43 days (Figure 9.7 [b]). This might also be related with PD. PD was lower at 21 days (0.13 V) than 43 days (0.38 V). It was reported that potential at the pit bottom was the highest [221]. The fact that IGC was seen only inside pits at 21 days (Figure 9.7 [a]) might be because the potential was sufficiently
high only at pit bottoms, not on top coupon surfaces. On the other hand, at 43 days PD was high enough to allow IGC to take place on the entire coupon surface.

Many micro pits were observed to initiate at grain boundaries when IGC was severe (Figure 9.7 [b]). Micro pits tended to initiate at three locations, as summarized in Figures 9.7 (c), (d) and (e). Figure 9.7 (c) shows that one of the pits initiated at the triple junction of grain boundaries. Figure 9.7 (d) shows 3 micro pits. Two of them initiated at triple junctions, while the other one (noted with arrow) formed at a two phase boundary. Figure 9.7 (e) shows a pit inside the grain body. To clarify the effects of IGC on pit initiation, 200 pits were observed and categorized. Only micro pits that were smaller than the grain size were considered. Standard deviations were calculated for results obtained from 10 scanned areas (0.01 mm²/area). 87.6% (± 8.8%) of pits initiated at the grain boundaries, whereas only 12.4% (± 8.8%) of pits initiated inside the grain bodies. For the pits at grain boundaries, 64.8% (± 7.3%) of them initiated at triple junctions and 35.2% (± 7.3%) at two phase boundaries. The above values were averaged from multiple scanned areas. The \( p \) value for comparison of pit initiation inside grain bodies versus at grain boundaries for these different scanned areas was 0.00. The \( p \) value for comparison between triple junctions and two phase boundaries was also 0.00. The extremely low \( p \) values proved that the different tendencies for pit to initiate at different locations were statistically significant. Thus, more severe IGC provided more sites for micro pit initiation.

For some deeper pits, IGC was more severe at parts of the pitted surface (right inset of Figure 9.7 [b]). The grain structure was revealed as the grain boundaries were corroded out. As shown in Figure 9.7 (f), several empty grain structures were observed
on a coupon exposed to the high phosphate SRB systems. All boundaries of a grain structure were completely corroded out, as shown by the inset of Figure 9.7 (f). The sizes of the grain and the empty grain structures were very similar (Figures 9.7 [b] and [f]), which suggested that grain dislodgement might have happened inside the pits. The grain structures before (Figure 9.7 [b]) and after dislodgement (Figure 9.7 [g]) were also observed in the low phosphate SRB systems. Therefore, grain dislodgement caused by IGC deepened the depth of large pits.

In summary, IGC enhanced pitting by the following two mechanisms: (1) Triple junctions and two phase boundaries exposed by IGC provided more sites for micro pit initiation. (2) For pits with larger sizes and depths, severe IGC could lead to grain dislodgement and, thus, further increase the pit depth. Accordingly, by inhibiting IGC, the higher phosphate concentration also reduces pitting severity (Figure 9.1 [7]).
(c) Pits initiated at triple junction

(d) Pits initiated at two phase grain boundary

(e) Pits initiated inside the grain

(f) 21 d SRB, polished, high phosphate

(c) 21 d SRB, unpolished, low phosphate
Figure 9.7. SEM images for (a) polished and (b) unpolished sides of carbon steel coupons exposed to *D. vulgaris* in low phosphate medium for (a) 21 and (b) 43 days; micro pits initiated at three locations: (c) triple junction, (d) two-phase grain boundary and (e) inside the grain; and pit structures on (f) polished and (g) unpolished sides of coupons exposed to *D. vulgaris* in (f) high and (g) low phosphate media for 21 days.

9.4. Conclusions

(1) Higher phosphate concentrations tested up to 10 g/L KH$_2$PO$_4$ did not affect initial growth of *D. vulgaris*, whereas at 10 g/L KH$_2$PO$_4$ planktonic cell concentration decreased over long-term starvation of organic electron donors (from 6 to 46 days) and cell attachment on carbon steel coupons was inhibited.

(2) At 10 g/L KH$_2$PO$_4$, phosphate ions inhibited pitting of carbon steel, characterized with maximum pit depth, average pit depth and pitted area percentage, by the following ways: (a) Since number of viable sessile SRB cells correlated linearly with pit volume loss rate, high phosphate inhibited pitting by decreasing the viable sessile SRB number. (b) When potential was higher than pitting potential, pitting might happen. The high phosphate concentration resulted in a lower potential and, thus, might inhibit pitting.

(3) Similar to the above pitting inhibition mechanism, phosphate ions inhibited IGC by decreasing potential.

(4) IGC enhanced pitting by the following two mechanisms: (a) IGC provided more sites for pit initiation and increased pit density. Pits tended to initiate at grain boundaries (87.6%) instead of grain bodies; among the pits initiated at grain boundaries, significantly more pits initiated at triple junctions (64.8%). (b) When IGC was severe,
grain dislodgement might happen inside deep and large pits, which further deepened the pit depth.
CHAPTER X

CONCLUSIONS AND RECOMMENDATIONS

10.1. Summary of conclusions

Four main achievements were reached in this work. The first discovery was that *D. vulgaris*, a common organotrophic H\textsubscript{2} utilizing SRB was capable of uptaking electrons directly from steel to reduce sulfate for its respiration and help its survival for up to 55 days of starvation. The associated pitting corrosion was evaluated under organic starving and nutrients supplemented condition. Effects of surface roughness, KH\textsubscript{2}PO\textsubscript{4} and FeS crust were also investigated. Mechanism of SRB-induced corrosion under organic starving condition was proposed, which explained the wide-spread presence of *D. vulgaris* and the sustained steel pitting in natural environment.

The second achievement is that EIS was investigated as an online monitoring tool of MIC (biofilm, corrosion product, pitting). Correlations between biological, corrosion product layer formation and electrochemical responses were established. Interfacial layer evolution observed by SEM and CLSM could correlate with the EIS results, including the phase angle diagram, PD and the model-fitted $R_p$, $Q_p$, $R_{ct}$, and $Q_{ct}$. Time-averaged $R_{ct}$ inversely correlated with the uniform corrosion rate. Also, interfacial layer evolution processes in the high- and low-phosphate SRB systems were elucidated.
The third discovery was that an improved method for pit characterization on large surface area was proposed and validated by corrosion pits induced by *D. vulgaris* and 3.5 % NaCl. This method allowed fast and reliable quantification of pitted area percentage, maximum and average pit depths. Failure risk diagram and pitting pattern diagrams were proposed for risk level prediction and pitting pattern categorization.

The fourth achievement is development of MIC mitigation approach according to MIC mechanism proposed in Chapter 4. KH$_2$PO$_4$ was demonstrated to significantly reduce SRB attachment number, decrease potential and thus inhibit pitting and intergranular corrosion (IGC).

10.2. Recommendations for future work

As demonstrated in this work, direct attachment of SRB on carbon steel is essential for long term cell survival and associated pitting corrosion under organic-starvation. It is critical to prevent SRB attachment for MIC mitigation approach development. Although chemical treatment (i.e. addition of phosphate) is effective to inhibit SRB attachment and pitting, it increases cost of wastewater treatment. A more environmental friendly approach is needed. Rhamnolipids were reported to have antimicrobial properties on many strains of bacteria [117, 222]. Although rhamnolipids were not effective in inhibiting SRB growth, it is still promising in prevention of bacterial attachment [223]. Comparative study of cell attachment between with and without rhamnolipid addition would clarify the effects of rhamnolipid on SRB attachment. The
associated corrosion can be evaluated and compared by the newly developed method for fast pit characterization.

Furthermore, if rhamnolipids were proved to inhibit SRB cell attachment, whether they can minimize biofilm build up became more interesting. This study can be done by growing SRB with CS coupons and rhamnolipids with constant supplement of nutrients. The carbon steel coupons without rhamnolipids will be control system. After certain period of incubation, biofilm structure can be characterized by both CLSM and SEM to determine whether the system with rhamnolipids has thinner and looser biofilm.

Another important factor to be studied is whether rhamnolipid solution can destroy the established SRB biofilm. In this study, carbon steel coupons will be incubated with SRB culture for several days to let biofilm grow and develop. Afterwards, rhamnolipid solution will be added to incubate with biofilm for certain period of time. This study can provide evidence on whether rhamnolipid can inhibit corrosion by disruption of established biofilm.

If the studies above prove that rhamnolipid has clear inhibition effect on MIC, a co-culture study with both SRB and nitrate reducing bacteria can be implemented to simulate the condition in oil pipeline. As reported earlier, the minimum inhibiting concentration of rhamnolipids ranged from 32 to 256 mg/L for gram-negative bacteria [222] and around 0.9 g/l for *Bacillus pumilus* (gram-positive bacteria). 0.9 g/l of rhamnolipids were able to disrupt 60% of *Bacillus pumilus* biofilm compared with no rhamnolipid system [120]. Our previous result showed that *P. aeruginosa* can produce $2.59 \pm 0.08$ g/l rhamnolipids under P-limitation (results not shown). Trigger of in-situ
rhamnolipid production by *P. aeruginosa* is promising for disruption of SRB biofilm and thus inhibit MIC. Experiments were planned to grow both *P. aeruginosa* (a rhamnolipid over-producing strain) or *P. chlororaphis* (a non-pathogenic rhamnolipid producing strain) and *D. vulgaris* ATCC 7757 with carbon steel coupons in P or Mg-limited media. Organic carbon and nitrate solution will be added accordingly to provide enough carbon and nitrate for cells to survive. Biofilm structure can be characterized by CLSM with live and dead cell information. Limiting nutrients for rhamnolipid production can be identified. Comparison between co-culture system and pure SRB system can be made to see the difference of biofilm development. The associated corrosion will be characterized and compared.

Combination of addition of rhamnolipid solution and inoculating nitrate reducing bacteria can be a promising MIC mitigation approach, which deserves in-depth study. Rhamnolipids can also be applied in microbial enhanced oil recovery (MEOR), which has been reviewed comprehensively by Banat et al. [224]. Although this study will not focus on MEOR, but it can be a side benefit, which give more value on application of rhamnolipids in oil and gas industry.
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