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Title: Bulk phase resource ratio alters carbon steel corrosion rates and endogenously-produced extracellular electron transfer mediators in a sulfate-reducing biofilm

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Key words: biocorrosion; microbiologically-influenced corrosion; MIC; *Desulfovibrio*

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ABSTRACT

Desulfovibrio alaskensis G20 biofilms were cultivated on 316 steel, 1018 steel, or borosilicate glass under steady-state conditions in electron-acceptor limiting (EAL) and electron-donor limiting (EDL) conditions with lactate and sulfate in a defined medium. Increased corrosion was observed on 1018 steel under EDL conditions compared to 316 steel, and biofilms on 1018 carbon steel under the EDL condition had at least 2-fold higher corrosion rates compared to the EAL condition. Protecting the 1018 metal coupon from biofilm colonization significantly reduced corrosion, suggesting that the corrosion mechanism was enhanced through attachment between the material and the biofilm. Metabolomic mass spectrometry analyses demonstrated an increase in a flavin-like molecule under the 1018 EDL condition and sulfonates under the 1018 EAL condition. These data indicate the importance of S-cycling under the EAL condition and the EDL is associated to increased biocorrosion via indirect extracellular electron transfer mediated by endogenously produced flavin-like molecules.

INTRODUCTION

Microbiologically-influenced corrosion (MIC) is a widespread problem incurring significant financial cost to the petroleum industry, the country, and communities. Carbon steel oil pipelines can slowly corrode abiotically under a variety of conditions, and when sulfate-reducing communities are present, corrosion rates can be greatly accelerated (Whitney 1903, Enning et al. 2012, Enning and Garrelf 2014, and references therein). The cost of metal corrosion in the US alone is estimated to be 2% to 3% of GDP (Gross Domestic Product) (Enning and Garrelf 2014) and is particularly important to carbon steel energy pipeline infrastructure. Corrosion induced failures in infrastructure create hazards to health, safety, environment, and product

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3 58 deliverability. Adding to scale of the problem, MIC occurs under a wide variety of environmental
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5 59 conditions, including marine, freshwater and terrestrial locations.
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8 60 MIC can involve a variety of different microorganisms that include sulfate-reducing
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10 61 bacteria (SRB) and iron-reducing bacteria (IRB) (Little et al. 2007, Enning and Garrelfs 2014,
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12 62 Bonifay et al. 2017). Together with SRBs and IRBs, microbial consortia are typically involved in
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14 63 two mechanisms of MIC: EET-MIC (extracellular electron transfer, Type I), cross membrane
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16 64 electron transfer (indirect and/or direct) (Kato 2016 and references therein), and/or metabolite-
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18 65 MIC (Type II), biocorrosion caused by secreted metabolites (*e.g.*, H⁺, organic acids, sulfides) as
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20 66 opposed to chemical corrosion which can refer to direct metal-oxidant interactions (Li et al. 2018,
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22 67 Kannan et al 2018). Both EET-MIC and M-MIC are electrochemical corrosion processes (Li et
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24 68 al. 2018, Dinh et al. 2004), and different EET mechanisms can promote the transfer of electrons
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26 69 to or from extracellular solid compounds (Gralnick and Newman 2007, Kato 2016). The process
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28 70 of EET can be mediated directly with metal surfaces via cellular connections and/or conductive
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30 71 extracellular structures (*e.g.*, Gorby et al. 2006) or indirectly via diffusible redox molecules
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32 72 (Watanabe et al. 2009). The goal of this study was to elucidate nutrient ratio impacts on potential
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34 73 M-MIC and/or EET-MIC mechanisms during initial biofilm formation under continuous growth
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36 74 conditions in a defined growth medium.
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42 75 Extracellular electron transfer, which is shown to enhance MIC, is now recognized as a
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44 76 more widespread microbial phenotype and suggests that EET-MIC could be a major mechanism
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46 77 for biocorrosion world-wide (Nealson and Saffarini, 1994, Kato 2016, Huang et al. 2018).
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48 78 Previous work postulated that some SRBs contribute to MIC under various growth conditions
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50 79 through Fe⁰ oxidation via interactions with carbon steel (Gu, 2012; Venzlaff et al. 2013; Enning
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52 80 and Garrelfs 2014, Li et al. 2015). In addition to heme redox centers of cytochromes, cell-secreted
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molecules have been shown to serve as electron carriers in EET processes in both *Shewanella oneidensis* and *Geobacter sulfurreducens* (Marsili et al. 2008; Okamoto et al. 2014), and more recently, flavin mononucleotide (FMN) and riboflavin were proposed to function in a diffusion-based EET (2 e⁻) or bifurcated direct EET (1 e⁻) in *Shewanella* (Okamoto et al. 2014). Therefore, future work should focus to elucidate the physiological conditions under which EET mechanisms contribute to overall biocorrosion.

Carbon starvation was previously shown to be associated with more aggressive corrosion by *Desulfovibrio vulgaris* biofilms (Xu and Gu 2014, Chen et al. 2015) and demonstrated the prolonged survival of *D. vulgaris* biofilms in the absence of organic electron donors. Studies with *D. vulgaris* 7757 showed that the addition of flavin adenine dinucleotide (FAD) or riboflavin could accelerate corrosion of 304 stainless steel and 1018 carbon steel (Li et al. 2015; Zhang et al. 2015), and electron transfer was hypothesized to be a limiting step for biocorrosion by *D. vulgaris*. While a relationship between nutrient deprivation and continued/accelerated biocorrosion has been shown for *Desulfovibrio*, the mechanism (Type I or II) under the nutrient deprived state is not known. We recently demonstrated that nutrient ratios impacted metal interactions (*i.e.*, Cr(VI) sensitivity) in *Desulfovibrio* (Franco et al. 2018), and therefore, hypothesized that nutrient imbalance would impact reduction-oxidation reactions in *Desulfovibrio* biofilms grown on a metal surface.

In the presented study, the corrosion rates and biofilm growth parameters under specific limiting nutritional ratios (*i.e.*, electron donor:acceptor imbalance) were measured with chemostat biofilms under defined culture conditions (*i.e.*, without yeast extract). *Desulfovibrio alaskensis* G20, isolated from a producing oil well in Ventura, CA (Hauser et al. 2011), was grown as a biofilm on glass, 316 stainless steel, and 1018 carbon steel with lactate as electron donor and

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3 104 sulfate as electron acceptor in a defined medium. Electron-acceptor (EAL) and electron-donor
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5 105 (EDL) limited growth was achieved by adjusting relative concentrations (EAL-50:15; EDL-15:15)
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7 106 in the chemostat medium. *Desulfovibrio* G20 demonstrated increased corrosion on 1018 carbon
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9 107 steel under the EDL condition, and biofilm attachment with the steel was required for maximal
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11 108 corrosion rates. Global metabolomic results show increased sulfolactate levels under the EAL
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13 109 condition, which may indicate recycling sulfonate compounds for use as electron acceptors when
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15 110 limited for sulfate. Additionally, increased lumichrome levels were observed under the EDL
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17 111 condition on 1018 carbon steel, and these results suggested *Desulfovibrio* G20 produced flavin
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19 112 molecules that could be used to mediate extracellular electron transfers from steel Fe⁰.
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26 114 **MATERIALS AND METHODS**

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28 115 **Microorganism.** *Desulfovibrio alaskensis* G20 was grown in a defined (without yeast
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30 116 extract) lactate/sulfate (LS4D) medium prepared anoxically as previously described under a
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32 117 variety of conditions (see below). Modifications to the original recipe included adjusting the
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34 118 lactate and sulfate concentrations to 50 mM lactate:15 mM sulfate for EAL conditions and 15 mM
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36 119 lactate: 15 mM sulfate for EDL conditions. As previously described, the medium was not prepared
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38 120 with reducing agent (Clark et al. 2006), and the use of resazurin in the culture medium served as a
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40 121 general indicator of oxidative-reduction potential (ORP). Sodium hydrosulfite (18 µg l⁻¹) was
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42 122 added to the 10% resazurin solution to shift the solution from purple to pink.
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47 123 **Planktonic Growth.** *D. alaskensis* G20 was cultivated in media with respective nutrient
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49 124 ratios at 37°C, 30°C and 20°C for initial planktonic cultures. The defined growth medium for both
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51 125 planktonic and biofilm growth was anoxically prepared and used as previously described (Clark
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53 126 et al. 2006, Clark et al. 2007, Klonowoska et al. 2008, Clark et al. 2012, DeLeon et al. 2017). All
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N₂ gas (99.995% purity) was run through an oxygen-scrubber before being used to sparge any liquids or head-space as previously described at a rate of approximately 1 ml/min (Brileya et al. 2014, Franco et al. 2018). Each batch condition was grown in triplicate in a Balch tube with anoxic N₂ headspace and sealed with butyl stoppers and a crimp seal. Optical Density (600 nm) was measured with a UNICO 1100RS spectrophotometer (New Jersey) and compared with uninoculated medium.

Biofilm Growth. Biofilm samples were grown in CDC Biofilm Reactors (Biosurface Technologies Corp., Bozeman, MT) with the headspace sparged with anoxic N₂ gas (run through oxygen-scrubber). Separate reactors were used to cultivate G20 biofilms on each surface type: glass, 316 stainless steel, and 1018 carbon steel under each limiting nutrient condition. The 1018 and 316 carbon steel coupons were not polished and used as received from Biosurface Technologies Corp. (Bozeman, MT). Reactors were inoculated with 40 ml of an exponential-phase culture and allowed to grow in batch mode for approximately 24 h. The medium pump flow rate was set to 0.3 ml min⁻¹ (~D= 0.05 h⁻¹) 24 h post-inoculation and maintained continuous flow until final samples were harvested. The dilution rate was chosen because the influent growth medium did not contain additional reducing agent as previously reported (Clark et al. 2006, Clark et al. 2012) and *in situ* growth rates are typically slower. The starting pH of medium was approximately 7.2 and during growth was 7.6 to 7.8. Glass coupon dimensions were 7.1 x 1.25 x 0.1 cm. Metal coupon dimensions were 7.6 x 1.5 x 0.1 cm. Modified coupon holders were used as previously described to provide increased material surface area (Clark et al. 2012).

Biofilm and Coupon Harvesting. Biofilm coupons were removed from the reactor and biofilm was immediately removed from the coupon using a sterile scraper. Biomass was scraped into 3 ml of dH₂O and homogenized by adding sand and vortexing prior to growth parameter

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3 150 measurements. The scraped coupon was dried and then treated according to standard practice for
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5 151 cleaning and evaluating corrosion samples (ASTM G1-03) with Clark Solution (6M HCl, 3.5 g l⁻¹
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7 152 hexamethylenetetramine) for 0.5 min before washing in nanopore H₂O and dried with dry nitrogen
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9 153 gas as previously described (Avci et al., 2013). The coupons incubated in sterile, anoxic medium
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11 154 (EAL or EDL levels of lactate and sulfate) for the same time period displayed approximately
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13 155 0.01±0.01 mm/y mass loss. The post-exposure coupon mass was subtracted from the pre-exposure
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15 156 mass to obtain the mass loss, and the value of mm y⁻¹ or mg cm⁻² was calculated as described in
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17 157 ASTM G1-03. Relative electrochemical measurements were made to the reference electrode in
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19 158 the presence of cells and compared between the EDL and EAL conditions.
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24 159 **Electrochemical measurements.** Electrochemical measurements were conducted with a
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26 160 conventional three-electrode system. A 1018 carbon steel coupon was made into a working
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28 161 electrode by spot welding the stripped end of Teflon coated wire. The exposed wire was coated
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30 162 with epoxy for insulation. The counter electrode was a platinum wire. The working electrode (the
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32 163 corroding carbon steel coupon) was not disturbed and used multiple times very briefly (<1 min
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34 164 duration) for time dependent resistance measurements. An electrochemical polarization curve was
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36 165 run with the PAR273 between 20 mV and -20 mV with respect to the approximate open circuit
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38 166 potential of 610 mV, with intervals of 0.1 mV. Readings were taken every 200 ms.
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42 167 A reference electrode was constructed because commercial probes were not the correct
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44 168 length for the reactor. A silver wire (0.25 mm or 0.5 mm) was cleaned with 600 grit sand paper to
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46 169 remove oxide coating, the bottom 2 cm of this wire (the end that forms the electrode tip) was
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48 170 submerged in 1M HNO₃ for a few seconds, then washed with nanopure water and dried with N₂
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50 171 gas. The wire was setup in a potentiostatic experiment with a EG&G Princeton Applied Research
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52 172 273 potentiostat/galvanostat (Advanced Measurement Technology, Oak Ridge, TN) was used as
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173 suggested by the manufacturer without solution resistance compensation. It should also be noted
 174 that previous work using this method confirmed predicted corrosion via atomic force microscopy
 175 measurements (Martin, 2014). The silver wire was the working electrode, a graphite rod was the
 176 counter electrode, and a purchased standard Ag/AgCl electrode was used as the reference
 177 electrode. The lower 2 cm of the silver wire was submerged in 0.1M HCl, and 1 V was applied to
 178 create 0.05-0.1 mA of current for 3 h. A 3M KCl and 3% agar solution was prepared and heated.
 179 A glass capillary tube was inserted into the agar until the agar had risen 2 cm inside the tube. The
 180 coated silver wire was pushed down the capillary until 1 cm from the bottom and in the middle of
 181 the agar. The wire-capillary assembly was removed from the agar and left to cool. Excess agar
 182 was wiped away and cut off the end of the capillary with a razor blade. A 3M KCl solution was
 183 gently added above the agar with a fine pipette. This was done carefully so no air bubbles remained
 184 in the capillary tube. The top of the newly constructed reference electrode was sealed with epoxy.

Preparation of Samples for Imaging. Biofilm samples were fixed by treatment in
 186 Karmovsky's Fixative (3.2% w/v paraformaldehyde, 2.5% w/w glutaraldehyde, 0.05M sodium
 187 cocodylate) for 16 h. Samples were soaked 4x in dH₂O for 5 min to remove fixative. Ethanol
 188 (EtOH) dehydration involved 5 min in 25% EtOH, 5 min in 50% EtOH, 5 min in 75% EtOH, 15
 189 min in 95% EtOH, and 2 x 45 min in 100% EtOH before storage in 100% EtOH. Dehydrated
 190 samples were dried in a Tousimis Samdri-795 Critical Point Dryer (Tousimis Research
 191 Corporation, Rockland MD) using liquid CO₂ and a 10 min purge time. Dried samples were coated
 192 with Iridium for 30 s with an Emitech K575X Sputter Coater. Electron microscopy samples were
 193 imaged with a Zeiss Supra 55VP Field Emission Scanning Electron Microscopy (FE-SEM) (Carl
 194 Zeiss, Oberkochen, Germany) equipped with energy dispersive x-ray analysis (EDX). Images
 195 were false-colored using Pixelmator (Vilnius, Lithuania) and Adobe Photoshop (San Jose, CA).

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Microscopy of Biofilm Cross Sections. Biofilm coupons were incubated in acridine orange solution (4 g l⁻¹) for 1.5 h before being coated with Optimal Cutting Temperature Compound (Fisher Health Care) and frozen on a block of dry ice. The frozen biofilm was removed from the metal coupon by bending the steel coupon and stored at -80°C. The frozen biofilm samples were cut in half and sectioned with a Leica CM1850 cryostat (-20°C) at 5 µm slices and applied to a microscope slide for imaging. Images were taken with a Nikon Eclipse E800 microscope (Nikon Corporation, Minato, Tokyo, Japan) with a Photometrics Coolsnap MYO camera (Photometrics, Tuscon, AR). Images were taken at a FITC (fluorescein isothiocyanate) emission wavelength and differential interference contrast and overlaid using Metamorph software package (Molecular Devices, Sunnyvale, CA).

Growth Parameter Measurements. Protein concentrations were measured with a Qubit Protein Assay Kit (Life Technologies, Carlsbad, CA). Carbohydrate concentration was measured as previously described (Clark et al., 2006). Lactate and acetate were quantified using an Ultimate 3000 High Performance Liquid Chromatography instrument with a 300 mm x 7.8 mm HPLC Organic Acid Analysis Aminex HPX-87H Ion Exclusion Column (Thermo Scientific, Dionex Germering, Germany). Sulfate and hydrogen sulfide concentrations were measured using a Hach Colorimeter (Hach, Co., Loveland, CO) with the associated sulfate assay (Method 10248) and sulfide assay (Method 8131).

Protected 1018 Steel Coupons. Round 1018 steel coupons (127 mm) were placed in Spectra/Por Standard RC dialysis membranes (Spectrum Labs, Rancho Dominguez, CA) with a 6-8 kD molecular weight cut-off during the reactor run. The clamped membranes with steel coupons inside were suspended in the growth medium of a CDC biofilm reactor, and growth parameters and inoculation was the same as for biofilm cultivation described earlier.

219 **Metabolomics and Data Processing.** At 192 h, biofilm coupons (EAL and EDL on 1018

220 carbon steel) were dipped in degassed dH₂O and the biofilm biomass scraped into a sterile
 221 microcentrifuge tube with degassed dH₂O (4°C). The tubes were centrifuged at 10,000 rpm for 5
 222 min at 4°C. The supernatant was removed and the cell pellet frozen with liquid N₂ and stored at -
 223 80°C. Samples were processed and data analyzed as previously described (Ivanisevic et al. 2013;
 224 Benton et al. 2015; Montenegro-Burke et al. 2016; Huan et al. 2017).

226 **RESULTS and DISCUSSION**

227 **Planktonic growth under different resource ratios.** Planktonic growth was impacted
 228 by temperature, and *D. desulfuricans* G20 grew the fastest at 37°C and the slowest at 20°C in
 229 defined medium with lactate and sulfate (60:50 ratio). The specific growth rates were 0.05 h⁻¹,
 230 0.12 h⁻¹, and 0.19 h⁻¹, respectively for 20°, 30°, and 37°C, and the final yields were similar.
 231 *Desulfovibrio* G20 formed the thickest biofilm at 37°C based on protein content in biofilm samples
 232 (data not shown), which is closest to elevated temperatures of some oil pipelines. In addition,
 233 37°C is the most commonly used temperature in the literature for *D. alaskensis* G20; therefore, all
 234 subsequent experiments were done at 37°C.

235 Growth rates were measured for planktonic cultures under the varied nutritional ratios that
 236 were tested in the described experiments. Maximum growth rates were similar at 0.21 h⁻¹ for EAL,
 237 0.19 h⁻¹ for EDL, and 0.20 h⁻¹ for the 60:50 condition. As expected, final yields (OD values) were
 238 higher in media that had increased lactate concentrations. The 60:50 condition had the highest
 239 final biomass yield due to increased lactate and sulfate concentrations. The 60:50 condition is the
 240 standard SRB media recipe used in numerous *Desulfovibrio* studies (Zhou et al. 2011) and was
 241 used as a basis for comparison in the described study. When planktonic biomass yields (protein)

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3 242 were compared in terms of sulfate or acetate (Y^{Sulfate} or Y^{Acetate}) consumed, the EAL and EDL
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5 243 conditions were similar (Table 1). When the conditions were compared based upon Y^{Lactate} , the
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7 244 EDL condition had a higher yield that was twice the Y^{Lactate} for the EAL condition. However, in
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9 245 terms of Y^{Acetate} , the EAL condition was 1.5-fold higher compared to EDL and similar to the 60:50
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11 246 condition. The planktonic culture results suggested that biomass generation from a given amount
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13 247 of electrogenic flow to sulfate was more efficient under the altered ratios for batch planktonic cells.
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15 248 The observations coincide with the theoretically low value of 0.06 mole of biomass ($\text{C}_5\text{H}_7\text{O}_2\text{N}$)
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17 249 produced per mole of lactate consumed (Eq 1) based upon assumed cellular stoichiometries for
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19 250 bacterial growth (McCarty 1971). The results suggest that planktonic cultures grown under the
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21 251 altered resource ratios (EAL and EDL) processed resource differently compared to the 60:50
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23 252 condition in terms of lactate oxidized to acetate produced.

33 255 **Biofilm on metal and glass.** *Desulfovibrio* G20 formed and maintained visible biofilm on
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35 256 glass, 316 steel, and 1018 carbon steel within 48 h under the tested conditions of EDL and EAL
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37 257 growth conditions; and as expected, the 1018 carbon steel surface appeared to have visible biofilm
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39 258 form earlier. The FE-SEM images show the presence of biofilm cells and other material that is
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41 259 most likely a combination of cellular material and mineral precipitates (Figure 1). In addition, the
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43 260 biofilms on glass and 316 steel had lower cell density compared to the biofilms on 1018 steel
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45 261 (Figure 1), and this result corresponded to measured protein levels (Figure 2). As expected, the
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47 262 biofilm is visually less pronounced on the 316 steel surface, and biofilm/metal precipitates were
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49 263 easier to remove from the 316 steel compared to the 1018 steel under both EAL and EDL. The
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51 264 observed biofilms on 1018 steel were visually more complex and heterogeneous under both EDL
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and EAL conditions. False-colored FE-SEM images showed a mixture of aggregated cells embedded within mineral precipitates for the EAL and EDL conditions (Figure 3a and b). Energy dispersive x-ray spectroscopy was used to differentiate materials based on elemental composition (data not shown), and the corrosion products (colored in orange) are abundant and likely composed of iron-sulfide minerals that have been previously shown to be conductive (Thauer 2007). The EDL biofilm displayed a combination of organic material that was most likely carbohydrate-based EPS and extracellular filaments in addition to cells. Filaments appeared similar to structures previously observed in *Desulfovibrio vulgaris* biofilms cultivated on glass under a nutrient ratio of 60:50 (Clark et al. 2006); however, the exact role of these structures is not known. However, *flaG* (gene DVU1442 annotated as a flagellin in *D. vulgaris* genome) was up-expressed in *D. vulgaris* biofilms (Clark et al. 2012); and a Δ *flaG* mutant was deficient in biofilm formation but not motility (unpublished data). Further work is needed to determine the exact role(s) of extracellular structures in SRB biofilms grown on different surfaces and conditions.

Biofilm biomass. Carbohydrate and protein were quantified every 48 h for glass, 316 steel, and 1018 steel surfaces for both EAL and EDL conditions. The two metal surfaces under both EAL and EDL conditions displayed increasing biofilm (both protein and carbohydrate) over the tested time course (192 h) with a trend of more biofilm under EAL conditions and the most biofilm overall on 1018 steel (Figure 2). Under EAL conditions, glass and 316 steel biofilms had higher carbohydrate to protein (C:P) ratios compared to 1018 steel, and the biofilms on all three surfaces increased carbohydrate levels from 144 to 192 h as the biofilm matured (Figure 2). Under EDL conditions, carbohydrate and protein levels remained low on the glass and 316 steel biofilms. For EDL 1018 steel biofilms at 192 h, the C:P ratio was higher (4.36) compared to the 1018 EAL 192 h biofilms (2.86). The C:P ratios were higher for 192 h glass and 316 steel EAL biofilms (4.0

288 and 6.0, respectively) than the 192 h glass and 316 steel EDL biofilms (1.5 and 1.2, respectively)
289 (Figure 2). These results suggest that carbon allocation is altered in biofilms on different surfaces
290 and nutrient ratios, namely carbohydrate allocation under EAL conditions and protein in EDL
291 conditions. The location (intracellular v. extracellular) is unknown for the protein and
292 carbohydrate allocation. The biofilm composition data suggests that the biofilms responded
293 differently to different types of energy restriction and coincides with previous work that has shown
294 *Desulfovibrio* biofilm has altered physiology (*i.e.*, electron-flow is altered) even when compared
295 to sulfate-reducing planktonic cells (Clark et al. 2012).

296 Images were taken of the EAL and EDL biofilm cross-sections to measure biofilm
297 thickness and show distribution of *Desulfovibrio* G20 cells. The EAL biofilm (~200µm) is
298 approximately 4-fold thicker than the approximately ~50 µm EDL biofilm (Figure 4). The
299 accumulation of corrosion products may aid in forming a textured surface to attach as well as an
300 extracellular matrix to grow within; however, the accumulation of thicker biofilms under EAL
301 conditions was not associated with increased corrosion. The availability of more carbon source
302 likely contributed to thicker biofilm at the 192 h time point on glass and 316 steel under EAL
303 conditions than compared to EDL conditions. The 1018 steel biofilms had higher hexose levels
304 than protein under both EAL and EDL conditions. Previous research by Clark et al. (2006) showed
305 protein to be the major component of *Desulfovibrio vulgaris* Hildenborough (DvH) biofilms, with
306 carbohydrate levels being relatively low. Our current observations with glass and steel under EAL
307 and EDL demonstrate that hexose levels can be altered in *Desulfovibrio* biofilms (up to 50% of
308 biomass), and in several samples accounted for a majority of the biomass. Though DvH and G20
309 are both members of the *Desulfovibrio* genus, there are significant physiological differences
310 between the two microorganisms. Under the EDL condition, there is significantly less protein and

hexose on the glass and 316 steel surfaces than on the 1018 steel. Though G20 is being fed the same levels of nutrients on all surfaces under the EDL condition, the protein levels were increased on 1018 compared to glass and 316.

Lactate/acetate levels. Lactate and acetate levels were measured from the biofilm reactors to assess growth physiology under biofilm reactor conditions (Figure S1, S2, and S3). Typically for *Desulfovibrio* species, lactate is converted to acetate in a 1:1 ratio (Eq.1). Because G20 consumes lactate and sulfate in a 2:1 ratio, the 15 mM of available sulfate allows G20 to oxidize 30 mM (of the 50 mM available in the EAL condition) lactate producing 30 mM of acetate. Measured levels of lactate and acetate were similar to predicted stoichiometries (Figure S1). Under the EAL condition (50 mM lactate: 15mM sulfate) *Desulfovibrio* G20 used slightly more than 30mM of the 50mM lactate available, corresponding to approximately 15mM lactate in the effluent for all three surface types. Theoretically, there should be 20mM lactate remaining under this condition and the missing carbon likely was used for biomass biosynthesis. The EAL acetate levels are approximately 30 mM at 50 h and remain near this level at the last time point (192 h).

Under the EDL condition, which contains 15 mM lactate: 15 mM sulfate, growth is limited by lactate, which is expected to be entirely consumed. Within 50 h, lactate is not detectable in the EDL reactors after inoculation and remains below detection during reactor operation. With all of the lactate being consumed, the EDL acetate levels are expected to be at 15mM. Acetate levels reached approximately 12 mM at 50 h and remained at this level during reactor operation, likely representing carbon incorporation into biomass (Figure S1).

Sulfate levels. Under the EAL condition (50:15), as expected, the sulfate levels approach 2 mM or lower at 50 h and remain almost non-detectable during reactor operation for all three surface types (Figure S2). According to the 2:1 ratio of lactate: sulfate consumption, the EDL

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3 334 condition (which contains 15mM lactate:15mM sulfate) would theoretically have 7.5 mM sulfate
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5 335 remaining in the medium. The EDL reactors (all surface types) approached 4 to 6 mM during
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7 336 reactor operation (Figure S2). While the measured sulfate levels are close to theoretical, there
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10 337 could have been some additional sulfate reduction dependent upon the oxidation of biomass in the
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12 338 reactor system (or Fe⁰ from the carbon steel).

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14 339 **Sulfide levels.** *Desulfovibrio* G20 reduces sulfate to sulfide, and sulfide levels were
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16 340 measured for both the planktonic and biofilm fractions normalized to protein levels for a given
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18 341 volume (planktonic) or surface area (biofilm) (Figure S3). Within each nutritionally limiting
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20 342 condition, the three surface types had similar planktonic sulfide concentrations; however, greater
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22 343 variation was observed in the last time point for EAL samples. The EAL planktonic samples
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24 344 maintained approximately 1 to 2 µg µg⁻¹ (sulfide/protein) for each surface type, and the EDL
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26 345 condition maintained approximately 0.5 µg µg⁻¹ (sulfide/protein) for each surface type (Figure S3).
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28 346 The results indicate that aqueous phase sulfide levels are higher under EAL conditions compared
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30 347 to EDL conditions for all tested surface types, and from a metal interaction perspective, higher
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32 348 sulfide levels generally translate to increased corrosion rates of carbon steel (Dinh et al. 2004).

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34 349 The concentration of sulfide differed in biofilms grown on different surfaces (Figure S3).
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36 350 Under both EAL and EDL conditions, sulfide levels were higher on 1018 than on 316 steel or
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38 351 glass. EDL biofilms on average had higher sulfide levels, especially on 1018 carbon steel, than
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40 352 EAL biofilms. Despite the fact there was a thinner biofilm on 1018 under the EDL condition
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42 353 (Figure 2), the G20 biofilm produced more sulfide per unit surface area than under the EAL
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44 354 condition. This result was unexpected because the planktonic sulfide was lower under EDL
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46 355 compared to the EAL condition and suggested altered physiological conditions under the different
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48 356 energy restriction conditions (e.g., more sulfide associated with biofilm biomass).

Corrosion rates. Corrosion for 316 stainless steel was minimal while the 1018 carbon steel showed a general corrosion under the tested conditions and time (data not shown). Corrosion rates calculated from mass loss (ASTM G1-03) were higher for the 1018 carbon steel compared to the 316 stainless steel coupons under both EAL and EDL conditions and less biofilm (protein and carbohydrate) was maintained on the 316 over the tested time period. These results suggested that the anti-corrosive properties of 316 can impede the type of corrosion observed with 1018 steel under the EDL condition. After 8 days, the estimated corrosion rate was the highest for 1018 steel under the EDL condition (Figure 5). Despite the higher planktonic sulfide levels under EAL conditions and thinner biofilm under EDL (Figure 4 and S3), the highest corrosion rate was observed under the EDL condition on 1018 steel (Figure 5). The increased EDL corrosion was further confirmed by electrochemical measurements and differences in $1/R$ values between EAL and EDL conditions on 1018 carbon steel ($\sim 3\times$). Electrochemical measurements can quantify the instantaneous corrosion rate, and the initial spike in corrosion occurred when the reactor was inoculated and a passivation layer of FeS_2 had not yet formed, likely due to abiotic processes. The EDL condition on 1018 steel had elevated electrochemical corrosion rate, and increased corrosion has also been observed by Xu and Gu (2014) and Chen et al. (2015) when *Desulfovibrio* biofilms were starved for nutrients under batch conditions.

Biofilm contact. In order to evaluate the importance of biofilm formation and presence on the metal surface for corrosion, biofilm formation on the metal was prevented by incubating 1018 carbon steel coupons in sealed 6-8 kD molecular weight cutoff dialysis tubing suspended in a CDC biofilm reactor growing *Desulfovibrio* G20. In addition, the same reactor contained coupons not in dialysis tubing (normal) to allow direct biofilm colonization and planktonic cell growth in the bulk medium. Under the normal condition where biofilm can form directly on the

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3 380 metal surface, EDL showed increased corrosion (mass loss) compared to EAL; however, under the
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5 381 protected condition corrosion was decreased for both EDL and EAL (Figure 6). Moreover, the
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7 382 reduction in corrosion was greater (~3-fold decline) for the protected metal under the EDL
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9 383 condition (Figure 6). The results demonstrated that SRB biofilm attachment to and biofilm
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11 384 formation on the 1018 carbon steel surface was needed for maximal corrosion rates.
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15 385 **Metabolomic analysis on biofilm**

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17 386 Having observed a difference in corrosion rate between EAL and EDL biofilms on 1018
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19 387 carbon steel, the mechanism involved in the increased corrosion caused by EDL-grown
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21 388 *Desulfovibrio* G20 was unknown. Metabolomic mass spectrometry analyses combined with
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23 389 XCMS data processing (Smith et al. 2012) was done on biofilm scraped from 1018 carbon steel
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25 390 grown under EAL and EDL conditions. A total of 1,157 features were detected between EDL and
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27 391 EAL ($p < 0.01$) (Figure 7). Based on significance values and MS/MS confirmation, three
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29 392 metabolites of interest displayed significant fold changes (10 to 20-fold) (Table 2).
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33 393 **Sulfur cycling.** One of the confirmed dysregulated metabolites, sulfolactate, was
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35 394 increased 15.7-fold under EAL conditions on 1018 steel (Figure 7). Sulfolactate is a sulfonate
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37 395 compound related to the cysteine/methionine processing pathways in some microorganisms. A
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39 396 complete pathway from cysteine to sulfolactate is not predicted in the *Desulfovibrio* G20 genome
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41 397 whereas the genome is annotated to contain an aminotransferase that could convert cysteate to
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43 398 sulfopyruvate (E.C. 2.6.1.1; KEGG). However, the genome annotation does not contain an
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45 399 identified enzyme to catalyze the conversion of sulfopyruvate to sulfolactate. The commonly
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47 400 identified enzymes with this activity include sulfolactate dehydrogenase (R and S) and malate
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49 401 dehydrogenase and would provide additional electron acceptor (reduction of sulfopyruvate to
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51 402 sulfolactate) via recycling of cysteine through cysteate and could account for the elevated
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sulfolactate levels that were detected under EAL conditions. The *Desulfovibrio* G20 genome is annotated to have a gene that encodes a protein with potential malate dehydrogenase activity (Dde1008, NAD⁺-linked). Sequence comparisons and biochemical studies have recently expanded the functionality of previously annotated malate and lactate dehydrogenases to have other activities such as sulfolactate dehydrogenase (Muramatsu et al. 2005); however, the potential role of Dde1008 in conversion of sulfopyruvate to sulfolactate is unknown.

The source of sulfopyruvate may be from the degradation of sulfur-containing amino acids cysteine and methionine, taking advantage of normal amino acid recycling as a means for generating sulfite (an additional electron acceptor). Other members of the *Desulfovibrio* family have demonstrated growth capabilities on sulfonate compounds such as cysteate or isethionate, as is the case with *Desulfovibrio desulfuricans* IC1 (Lie et al. 1996). Cysteate is the product from cysteine degradation and can be converted to 3-sulfopyruvate by cysteine lyase. However, cysteine lyase has not been identified in the *Desulfovibrio* G20 genome via annotation. The *Desulfovibrio* G20 genome is annotated to have the gene for aspartate aminotransferase (EC 2.6.1.1) that can convert cysteate to 3-sulfopyruvate. To explore whether G20 was able to utilize cysteate, planktonic growth with cysteate alone or with lactate was tested at similar ratios. Significant growth with cysteate was not observed with the tested growth medium (data not shown), and these results suggest that *Desulfovibrio* G20 cannot utilize cysteate as a sole source of carbon and energy.

Under the EAL condition, another significant metabolite that was identified at increased levels with high confidence was a cysteine-S-sulfate-related molecule (Table 2). Given the complexity of S-cycling, particularly in sulfate-reducing bacteria, the exact role of this compound is not known. A cysteine-S-sulfate molecule could be involved in cycling any available

intracellular sulfate, but a known annotated pathway has not been identified in *Desulfovibrio* G20. The presented results demonstrate the importance of S-cycling in *Desulfovibrio* G20 under EAL conditions, and further work is needed to fully elucidate the turnover of methionine/cysteine in SRBs and the potential effect on growth when limited for sulfate.

Role of electron shuttles. Under the EDL condition with increased 1018 steel corrosion, the metabolite lumichrome was increased 10-fold compared to the EAL condition (Table 2; Figure 7). Lumichrome is a by-product of riboflavin biosynthesis, and riboflavin is a predicted intermediate for the biosynthesis of FAD and FMN in both *D. vulgaris* and *Desulfovibrio* G20 genomes (Figure 8). *Desulfovibrio* G20 has annotated genes for the biosynthesis of riboflavin to FMN and FAD but does not have an annotated gene for the riboflavinase that can catalyze the conversion of riboflavin to lumichrome and ribitol (Figure 8). However, photolysis has been shown to drive the formation of lumichrome from riboflavin (Treadwell and Metzler 1972; Birss et al. 1997), particularly in anoxic conditions even under normal laboratory lighting, and the current anoxic reactors were exposed to ambient light during growth and the samples were processed in the light. The solubility of riboflavin, FMN, and FAD in water (0.12 g l^{-1} , 92 g l^{-1} , and 50 g l^{-1} , respectively) is much higher than lumichrome (0.0048 g/L). In addition, lumichrome has been shown to be an inhibitor of flavin oxidoreductase in *Escherichia coli* (Fieschi et al. 1995), and a protein family (dodecins) with high preference for lumichrome has been suggested to play a role in “trapping” riboflavin photolytic degradation products (*i.e.*, lumichrome) (Grininger et al. 2006). While lumichrome could play an unknown role in biofilm physiology, it is more likely that lumichrome is a photolytic by-product from riboflavin that has been shown previously to impact metal corrosion in SRBs (Kato 2016, Chen et al. 2015). Based upon these data and given that

Desulfovibrio G20 does not have an annotated riboflavinase, the detected lumichrome is likely related to riboflavin or flavin family molecules (*i.e.*, FMN and/or FAD)

Moreover, two metabolic features with the likely identity of FAD were increased 2.8 and 3.6-fold under the EDL condition. The p-values for these were 0.2, and while not meeting the statistical cutoff of 0.01, the FAD-molecules likely have biological (and corrosion) significance. Recent work in *Shewanella oneidensis* has demonstrated a role for FMN and riboflavin in which one-electron and two-electron mechanisms can play extracellular electron transfer roles under different conditions (Brutinel and Gralnick 2012; Okamoto et al. 2013). Interestingly, recent research demonstrated that adding FAD or riboflavin to *Desulfovibrio vulgaris* cultures increased metal corrosion rates in batch (Kato 2016, Chen et al. 2015). Our data further extends the role for flavin co-factors in metal corrosion and demonstrates a physiological condition (*i.e.*, EDL) in which biofilm would produce elevated levels of these compounds under metal-corroding conditions.

When considering the EDL condition and increased corrosion, elemental Fe could serve as a source of electrons for the biofilm under EET-MIC. Our data further supports the hypothesis that endogenous flavin compounds (*i.e.*, riboflavin, FMN, and FAD) could serve the role of extracellular electron transfer mediators for biofilm grown on 1018 carbon steel. When considering riboflavin, a one-electron transfer (semiquinone flavin) from Fe^0 ($E_m = -0.167$ V; Ksenzhek and Petrova 1983) would provide a favorable $\Delta G^{0'}$ value (-52 kJ) and if the semiquinone was further reduced the $\Delta G^{0'}$ value of the subsequent reaction would be -34 kJ. For the FMN/FMNH₂ couple ($E_0' = -0.21$ V; Mayhew 1999), a two-electron transfer from Fe^0 would provide a favorable $\Delta G^{0'}$ value (-44 kJ). For the FAD/FADH₂ couple ($E_0' = -0.3$ V; Curley et al. 1991), a two-electron transfer from Fe^0 would provide a favorable $\Delta G^{0'}$ value (-27 kJ). Based

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3 471 solely upon thermodynamic estimations under standard conditions, riboflavin would be the
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5 472 preferred molecule, and this corresponds to more corrosion previously observed with riboflavin
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7 473 compared to FAD with *D. vulgaris* on 1018 carbon steel (Li et al. 2015). The results indicate that
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9 474 *Desulfovibrio* G20 biofilms likely produced endogenous flavin-like molecules when grown under
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12 475 EDL conditions on a metal surface.
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17 477 **CONCLUSION**
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19 478 The presented results demonstrate that energy restriction (EAL v. EDL) greatly impacted
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21 479 biofilm growth and physiology of *Desulfovibrio* G20 biofilms grown on carbon steel. Less
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23 480 corrosion was observed on 316 stainless steel compared to 1018 carbon steel for both EAL and
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25 481 EDL condition; however, the EDL condition promoted more corrosion of 1018 steel despite a
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27 482 thinner biofilm with decreased aqueous sulfide levels compared to the EAL condition. However,
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29 483 despite being limited for electrons under the EDL condition, more biofilm-associated sulfide was
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31 484 observed and corrosion was increased. In addition, the increased corrosion was dependent upon
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33 485 biofilm interaction with the metal surface. These results suggested that the metal surface was
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35 486 serving as an additional electron source (*i.e.*, Fe⁰). Untargeted metabolomics indicated that the
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37 487 EAL-grown biofilm on 1018 steel altered cysteine/methionine cycling while the EDL-grown
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39 488 biofilm had elevated levels of flavin-like compounds. The flavin molecules likely serve a role in
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41 489 extracellular electron transfer when interacting with the metal surface (Fe⁰) in EET-MIC (Figure
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43 490 9), and this was further supported by the need for biofilm to interface directly with the metal
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45 491 surface for elevated corrosion. The endogenously-produced flavin molecules likely play a role to
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47 492 harvest electrons from Fe⁰ when limited for electron donor, allowing continued biofilm growth
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49 493 under nutrient-restricted conditions. In essence, when lactate was limiting, sulfate reduction was
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coupled to Fe^0 oxidation at the steel surface, thus promoting increased carbon steel corrosion. The results also suggest that the EDL condition could be relevant to the common practice of seawater injection into reservoir formations for enhanced oil extraction. Seawater has an average sulfate level of 28 mM, nearly twice the concentration used in this study. In hydrocarbon environments carbon/electron sources can be limiting due to slower degradation rates and/or competition for resources. Thus, the introduction of increased sulfate levels could shift the nutrient ratio in the EDL direction, thereby promoting increased corrosion rates via EET-MIC. Further work is needed to better understand the impact of energy imbalance on sulfate-reducing communities in different environments and the outcome on carbon steel materials.

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Figure Legends

Figure 1. Field Emission Scanning Electron Microscopy images were taken at 15,000X magnification for each condition (EAL and EDL) on glass, 316 stainless steel, and 1018 carbon steel.

Figure 2. (a) Hexose and protein levels on glass, 316 stainless steel, and 1018 carbon steel coupons under EAL conditions. (b) Hexose and protein on glass, 316 stainless steel, and 1018 carbon steel coupons under EDL conditions.

Figure 3. False colored Field Emission-Scanning Electron Microscopy image for (a) EAL and (b) EDL conditions. The colorized structures correspond to: Purple: G20 cells, Gold: iron sulfides, Blue: carbon-containing EPS, Green: extracellular filaments. The cells in the EAL condition are encrusted with iron-sulfide minerals.

Figure 4. Confocal images of thin-sectioned biofilms grown on 1018 carbon steel under (a) EAL and (b) EDL conditions. Biofilm ranges from 116-220 μm in thickness under EAL condition (a) to 50 μm under EDL condition (b). G20 cells are stained green. Substratum (carbon steel surface) is on the right.

Figure 5a. Corrosion rate (mm y^{-1}) for 316 stainless steel and 1018 carbon steel coupons during exposure to biofilm growth under EDL and EAL conditions.

Figure 5b. Corrosion rate (mg cm^{-2}) for 316 stainless steel and 1018 carbon steel coupons during exposure to biofilm growth under EDL and EAL conditions.

Figure 6. Corrosion rate of 1018 carbon steel coupons under normal exposure to *Desulfovibrio* G20 culture compared to coupons protected inside of dialysis tubing submerged in culture.

Figure 7. (a) Cloud plot of dys-regulated features comparing EAL versus EDL condition on 1018 carbon steel. All features shown have a P-value <0.01 . **(b)** Intensities of sulfolactate peaks shown in EAL versus EDL. Lumichrome is increased 10.1-fold under EDL condition where increased corrosion rate is observed. **(c)** Sulfolactate is increased 15.7 fold under EAL condition.

Figure 8. Predicted riboflavin biosynthesis pathway in *Desulfovibrio* G20 and *D. vulgaris* based on KEGG predictions.

Figure 9. Classic model of corrosion with the addition of cycling endogenously produced flavin-based extracellular electron transfer molecules (Fm) with elemental Fe^0 within the biofilm as a form of EET (extracellular electron transfer)-MIC. The dashed arrow to H_2 could occur when electron acceptors (*e.g.*, sulfates) are at low levels.

Figure S1. (a) Lactate levels are shown under EAL condition on glass, 316 stainless steel, and 1018 carbon steel surfaces; as well as **(b)** Lactate levels under EDL condition on all three surface types. **(c)** Acetate levels under EAL condition on all 3 surfaces are shown with **(d)** Acetate levels under EDL condition on all three surface types.

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741 **Figure S2.** (a) Planktonic sulfate concentrations under EAL conditions. (b) Planktonic sulfate
742 concentrations under EDL conditions.

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744 **Figure S3.** Sulfide was measured and normalized to protein levels. Biofilm sulfide levels were
745 also normalized to surface area. (a) Planktonic sulfide concentrations under EAL conditions. (b)
746 Planktonic sulfide levels under EDL conditions. (c) Biofilm sulfide levels under EAL conditions.
747 (d) Biofilm sulfide levels under EDL conditions.

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Figure 2. (a) Hexose and protein levels on glass, 316 stainless steel, and 1018 carbon steel coupons under EAL conditions. (b) Hexose and protein on glass, 316 stainless steel, and 1018 carbon steel coupons under EDL conditions.

238x211mm (72 x 72 DPI)

Figure 3. False colored Field Emission-Scanning Electron Microscopy image for (a) EAL and (b) EDL conditions. The colorized structures correspond to: Purple: G20 cells, Gold: iron sulfides, Blue: carbon-containing EPS, Green: extracellular filaments. The cells in the EAL condition are encrusted with iron-sulfide minerals.

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Figure 5a. Corrosion rate (mm y-1) for 316 stainless steel and 1018 carbon steel coupons during exposure to biofilm growth under EDL and EAL conditions.

190x190mm (300 x 300 DPI)

Figure 5b. Corrosion rate (mg cm⁻²) for 316 stainless steel and 1018 carbon steel coupons during exposure to biofilm growth under EDL and EAL conditions.

190x190mm (300 x 300 DPI)

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190x190mm (300 x 300 DPI)

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103x68mm (300 x 300 DPI)

Figure 8. Predicted riboflavin biosynthesis pathway in *Desulfovibrio* G20 and *D. vulgaris* based on KEGG predictions.

279x215mm (300 x 300 DPI)

Figure 9. Classic model of corrosion with the addition of cycling endogenously produced flavin-based extracellular electron transfer molecules (Fm) with elemental Fe⁰ within the biofilm as a form of EET (extracellular electron transfer)-MIC. The dashed arrow to H₂ could occur when electron acceptors (e.g., sulfates) are at low levels.

143x175mm (300 x 300 DPI)

Table 1. Planktonic growth yields (mg protein/M substrate or product) are shown from EAL (electron-acceptor limited), EDL (electron-donor limited), and 60:50 media calculated from sulfate consumption, lactate consumption, and acetate production.

	<u>Sulfate Yield</u>	<u>Lactate Yield</u>	<u>Acetate Yield</u>
EAL	0.042 ± 0.006	0.014 ± 0.002	0.033 ± 0.004
EDL	0.042 ± 0.008	0.032 ± 0.006	0.021 ± 0.004
60:50	0.025 ± 0.003	0.018 ± 0.002	0.027 ± 0.003

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Table 2. Metabolites that differed significantly between EAL vs. EDL and have been confirmed with MS/MS.

<u>Metabolite</u>	<u>Condition</u>	<u>Fold Change</u>	<u>p-value</u>	<u>q-value</u>
Sulfolactate	Up in EAL	15.7	0.00003	0.0023
Cysteine-S-sulfate related	Up in EAL	20.1	0.0025	0.0122
Lumichrome	Up in EDL	10.1	0.0019	0.0019

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Figure S1. (a) Lactate levels are shown under EAL condition on glass, 316 stainless steel, and 1018 carbon steel surfaces; as well as (b) Lactate levels under EDL condition on all three surface types. (c) Acetate levels under EAL condition on all 3 surfaces are shown with (d) Acetate levels under EDL condition on all three surface types.

Figure S2. (a) Planktonic sulfate concentrations under EAL conditions. (b) Planktonic sulfate concentrations under EDL conditions.

Figure S3. Sulfide was measured and normalized to protein levels. Biofilm sulfide levels were also normalized to surface area. (a) Planktonic sulfide concentrations under EAL conditions. (b) Planktonic sulfide levels under EDL conditions. (c) Biofilm sulfide levels under EAL conditions. (d) Biofilm sulfide levels under EDL conditions.