Development of an immunoassay for the detection and diagnosis of microbially-influenced corrosion caused by methanogenic Archaea

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Abstract

Microbially-influenced corrosion (MIC) is a costly problem across several industries. The steadily rising use of advanced molecular biological methods to investigate MIC allowed ever deeper insights in the underlying microbial community structure and function. However, currently available technologies do not allow accurate detection and diagnosis of MIC in the field. Recent discovery of a special hydrogenase in certain corrosive methanogenic Archaea allowed for the development of a first ever MIC biomarker termed here *micH*. The *micH* gene encodes the large subunit of a special [NiFe] hydrogenase involved in MIC. Here we describe the development of a recombinant antibody that enable the specific and sensitive detection of the MicH protein in western blot immunoassays. Using a recombinant MicH protein we determined the lower limit of detection per assay to be around 0.3 fg MicH. The immunoassay was able to detect a strong signal for the MicH protein in *micH*-positive pure cultures of *Methanobacterium*-like. strain IM1 that was cultivated on iron granules, and the signal was over 500 times lower in a *micH*-negative *Methanococcus maripaludis* S2 culture. To further evaluate the ability to differentiate corrosive from non-corrosive microbial communities, we tested ten oil field enrichment cultures that showed a wide range of corrosion rates (0.02 – 0.48 mm/yr). We detected the MicH protein in planktonic (36.5 – 1473.5 pg/mL) and carbon steel biofilm samples (41.0 – 7971.3 pg/cm^2) from corrosive methanogenic enrichments (0.17 – 0.48 mm/yr) but did not detect MicH in any of the non-

corrosive tests (<0.08 mm/yr) despite methanogenic activity. The results indicate that corrosion was likely caused by methanogenic Archaea expressing a corrosive [NiFe] hydrogenase and that the newly developed MicH-specific immunoassay can detect and monitor their activity. The development of a specific and sensitive immunoassays to detect a MIC biomarker allow corrosion scientists and field practitioners to detect and monitor the activity of corrosive methanogenic Archaea.

Introduction

Microbially-influenced corrosion (MIC) negatively affects steel infrastructures and causes significant costs across multiple industries ever year. In anaerobic systems, such as pipelines used for the production and transportation of mixtures of oil, water and gas, MIC is often linked to the presences and activity of sulfate-reducing bacteria (SRB) and methanogenic Archaea[1–5].

The monitoring of corrosive biofilms in industrial settings often involves the quantification of cell numbers by culture-based or molecular biological methods such as the quantification of adenosine triphosphate (ATP) or universal and functional genes by quantitative PCR (qPCR)[6–9]. Despite the introduction of the latter into industrial operations, reliable detection of MIC during operation is still challenged. Firstly, not all microorganisms are causing industrially relevant corrosion and secondly, even members of the same metabolic groups (e.g. SRB or methanogenic Archaea) have been shown to have vastly different corrosion rates[1,10]. This suggest likely genetic differences that enable some microorganisms to cause higher rates of corrosion than others.

A recent study identified a specific gene islands in genomes of corrosive methanogenic Archaea that are absent in non-corrosive close relative strains. The gene islands encode among other genes a unique and phylogenetically highly conserved bidirectional [NiFe] hydrogenase (termed 'MIC hydrogenase'), a Tat system for protein translocation as well as enzymes with potential

46 involvement in protein glycosylation[5,11–13]. The 'MIC hydrogenase' was only detected in corrosive
47 pure cultures and corrosive oil field enrichment cultures but not in the ones showing low corrosion,
48 explaining the corrosive character of some methanogenic Archaea.

49 We recently proposed to use the large subunit of the 'MIC hydrogenase' as biomarker (termed
50 *micH*) to detect and monitor certain corrosive methanogenic Archaea in the field[5]. By utilizing
51 specific qPCR assays, the MIC biomarker showed excellent correlation with MIC in both
52 laboratory tests and field applications[5,14]. Here, we discuss the development of a MicH-specific
53 recombinant antibody to detect and monitor corrosive methanogenic Archaea in laboratory tests
54 and in the field. In addition, protein detection may reduce potential false positive that could be
55 associated with qPCR-based detection. A recombinant antibody able to detect MIC protein
56 biomarker would also allow the development of a field-friendly rapid test assays (e.g. lateral flow
57 tests) to support MIC monitoring and detection in the field.

58 **Materials and Methods**

59 *Growth of pure cultures and enrichments*

60 Actively growing cultures of *Methanococcus maripaludis* strain S2 (= DSM 14266), *Desulfovibrio*
61 *ferrophilus* strain IS5 (= DSM 15579) and *Oleidesulfovibrio alaskensis* strain G20 (= DSM 17464)
62 were acquired from the German strain collection DSMZ. Culture aliquots of *Methanobacterium*-
63 like strain IM1 were donated by Dr Florin Musat (Helmholtz Centre for Environmental Research,
64 Leipzig, Germany). Pure cultures and enrichments in this study were routinely grown in the
65 presence of 1 – 2 mm iron granules (40 µg/ml medium; Thermo Fisher Scientific, Waltham,
66 Massachusetts, US), 0.5 mM sodium acetate, 0.5 mM sodium sulfide and 10 mM sodium sulfate
67 in anaerobic synthetic brine that was modelled after an oil field produced water as previously
68 described[5].

69 For establishing field enrichment cultures 100 ml Duran® glass bottles were filled with 2 g iron
70 granules and dry heat sterilized at 180° C for 1 h. After cooling down the bottle headspace was
71 exchanged with N_2/CO_2 (79:21) and sealed with sterile butyl rubber stoppers and screw cups. The
72 sterile anoxic glass bottles were inoculated with 50 ml of produced water on site. Production fluids
73 (oil, gas and water) were collected in sterile polypropylene bottles and oil and water allowed to
74 separate by gravity. Once separated, the water was collected using sterile syringes with attached
75 sterilized polypropylene tubes. After collection the tube was carefully replace by a sterile needle
76 and the anoxic glass bottles inoculated. After arrival in the destination lab, the cultures were
77 incubated at 32° C for at least two weeks before used as inoculum for corrosion bottle tests.

78 Enrichment cultures were established this way from eight pipelines of two separate West African
79 oil field operations. In addition, two additional enrichment were established using pipeline solids
80 ('pigging debris') collected as described previously[5] from a pipeline from an U.S. West Coast
81 operation and a U.S. onshore oil field operation during maintenance cleaning. Upon arrival in the
82 lab approximately 3 g solids were transfer into sterile bottle containing 50 ml of the previously
83 mentioned synthetic produced water[5] and closed with sterile butyl rubber stoppers under N_2/CO_2
84 (79:21) atmosphere.

85 *Corrosion bottle tests*

86 Corrosion tests were performed in one-liter Duran® glass bottles that were filled with 600 ml
87 anoxic synthetic brine to which three pre-cleaned and sterilized API 5L X52 steel coupons were
88 added in polyether ether ketone coupon holder as described earlier[5,15]. The bottles were sealed with
89 butyl rubber stoppers using sterile Hungate techniques and a N_2/CO_2 (79:21) headspace. Test were
90 inoculated with 1% of respective cultures and incubated on a rotary shaker at 75 rpm and 32°C for
91 eight weeks. Planktonic samples were collected for protein and DNA analysis using sterile syringes

92 and 0.2 µm polyethersulfone filter membranes or sterile swabs for biofilm collection as mentioned
93 previously[5]. Samples were immediately frozen at −80°C until further analysis. Removal of
94 corrosion products and weigh loss corrosion analysis of corrosion coupons was performed at the
95 end of the test as outlined earlier[5].

96 *Recombinant antibody development*

97 The recombinant antibody development workflow was conducted by Genscript Biotech
98 (Piscataway, NJ) and will be briefly outline below. For the initial development of polyclonal
99 antibodies, two peptide sequences were chosen from the protein sequence of the target gene *micH*
100 of the corrosive methanogen *M. maripaludis* strain OS7 (see supplementary material for details)
101 and conjugated to the metalloprotein keyhole limpet hemocyanin (KLH). Per peptide four rabbits
102 were immunized with three additional consecutive boosters. After four months, blood was
103 harvested from the rabbits and polyclonal antibodies partially purified for evaluation. Tests were
104 performed by indirect enzyme-linked immunosorbent assay (ELISA) using either of the two
105 peptides as target and via Western blotting as outlined below using protein extracts from
106 *Methanobacterium*-like strain IM1 as positive control[16] and protein extracts from *M. maripaludis*
107 strain S2 was used as MicH-negative control (Fig. S1)[11]. Animals with best performing polyclonal
108 antibodies were selected for cell fusion of spleen B cells with myeloma cell line. Screening and
109 selection of clones were performed via immunoassays as described above. The best performing
110 monoclonal antibody 'A11-1' was chosen for DNA sequencing (see supplementary material) and
111 recombinant antibody production by Genscript Biotech.

112 *Protein extraction and detection via Western blot*

113 Proteins were extracted from filters or swabs by placing them in sterile 2 ml screw cup
114 microcentrifuge tubes. 900 µl lysis buffer (10 mM HEPES, 1% Triton X-100, 5 mM EDTA, 100

115 mM DTT and 100mM Na_2CO_3, 1% SDS, 12% sucrose) were added to the filters or swabs and
116 placed in a heating block (105°C) for exactly 2 min. The tubes were removed and cooled to room
117 temperature and then vigorously mixed and centrifuged for 5 min at 21,100 g. 200 µl of the
118 supernatant was further processed to remove detergents using Pierce™ HiPPR Detergent Removal
119 Spin Column Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocol.
120 The detergent-free protein samples were further processed using Pierce™ Protein Concentrators
121 with a 10 kDa molecular weight cut-off polyether sulfone membrane (Thermo Fisher Scientific,
122 Waltham, MA). Protein samples were concentrated to an approximate volume of 25 µl and either
123 directly used for Wester blotting or stored at −80°C.
124 Western blotting was performed on a Protein Simple JESS Western blot platform (Protein Simple,
125 San Jose, CA) using the 12 – 230 kDa Separation Module and the Jess Anti-Rabbit Detection
126 Module. Protein analysis was performed according to the manufacturer protocol using a 1:20
127 antibody dilution (approx. 25 µg/ml) of A11-1 and 3 µl of protein sample. Protein Simple's Total
128 Protein module was used for normalization. For quantification a recombinant MicH protein was
129 purchased from Genscript Biotech (Piscataway, NJ) developed following their large-scale *E. coli*
130 expression protocol and His-tag purification.

131 *DNA extraction and qPCR analysis*

132 For DNA extraction cells were collected via filtration and filters transferred to 5 ml cryo vials
133 containing 1 ml DNA/RNA shield (Zymo Research, Irvine, CA) as preservative. Filters were
134 stored at −80°C until further analysis. DNA extraction and *micH* qPCR analysis were performed
135 by Microbial Insights Inc. (Knoxville, TN) as described previously[5].

136 *Adenosine triphosphate analysis*

137 ATP was quantified using the 2nd Generation ATP® test kit and PhotoMaster™ luminometer
138 (LuminUltra Technologies Ltd., Fredericton, Canada). For analysis of planktonic cells 20 ml of
139 medium was collected via filtration using the provided filters and syringes following the
140 manufacturers protocol. Biofilms were collected using a minimum of two sterile swabs per coupon
141 and the swab tips used for ATP quantification as outlined in the kit's manual.

142 *Gas chromatography*

143 Gas chromatographic analysis of methane was performed on an Agilent 490 Micro gas
144 chromatograph using a thermal conductivity detector. Separation was achieved on a heated Agilent
145 PoraPlot U column (50°C, 22.1 lb/in^2, and 10 m) in argon gas.

146 **Results**

147 *Recombinant Anti-MicH antibody showed excellent affinity for recombinant MicH protein.*

148 In order to optimize the signal to noise ratio (S/N) we performed first a dilution series with the
149 Anti-MicH antibody and a fixed concentration of 1 ng/ml of $MicH_{rec}$. A signal was detected
150 between approximately 50 – 60 kDa molecular weight that is in good agreement with the expected
151 molecular weight of the MicH protein containing the His-tag used for purification (54.5 kDa; Fig.
152 1A). Test performed with different antibody dilutions of Anti-MicH showed best results for S/N
153 ratio and low baseline with dilutions 1:50, 1:20 and 1:10 (Fig. 1B). For subsequent tests an
154 antibody dilution of 1:20 was chosen to minimize potential background noise while achieving an
155 overall strong signal.

156 In addition, a calibration curve of recombinant MicH protein showed excellent linear response
157 with concentrations between 20 pg/ml and 10 ng/ml (Fig. 2 and Fig. S2). A clearly detectable signal
158 with acceptable S/N ration (>30) was still detected at 20 pg/ml $MicH_{rec}$ therefore the assay was

159 able to reliably detect as little as 0.3 fg of recombinant MicH corresponding to approximately $3 \cdot 10^3$
160 protein molecules.

161 *MicH-specific immunoassay showed excellent selectivity for corrosive methanogenic cultures*

162 Unlike qPCR assays, immunoassays are less selective and may show potential background signal
163 due to cross-reactivity with non-target proteins or peptides in absence or low concentration of their
164 specific target[17]. To test for assay specificity and the ability to distinguish between corrosive and
165 non-corrosive methanogenic Archaea as well as non-methanogenic cultures, we tested the MicH-
166 specific immunoassay on protein extracts from methanogenic and sulfate-reducing pure cultures
167 (Fig. 3).

168 Protein extract of *Methanobacterium*-like strain IM1 obtained from cultures, that inflicted
169 significant corrosion of 0.3 mm/yr, showed a strong signal in the Western blot (Fig. 3 and Fig.
170 S3A). Furthermore, the *micH* gene was also detected by qPCR in DNA extracted from cultures of
171 strain IM1, confirming both the presence and expression of the 'MIC hydrogenase' during
172 corrosion. In contrast, the MicH-specific assay showed a more than 500x lower signals for protein
173 extracts from *M. maripaludis* strain S2 known to lack the 'MIC hydrogenase'[11]. In agreement,
174 strain S2 caused only negligible corrosion of 0.04 mm/yr (Fig. 3). Similar low signals were
175 observed in protein extracts from *Desulfovibrio ferrophilus* IS5 and *Desulfovibrio alaskensis* G20.
176 DNA analysis via qPCR confirmed absence of the *micH* gene in those cultures (Fig. 3). The results
177 confirm that the MicH-specific immunoassay selectively detects the MicH protein while showing
178 low cross-reactivity with proteins extracted from non-corrosive methanogenic strain S2 and SRB
179 pure cultures (Fig. 3 and Fig. S3A).

180 *MicH-specific immunoassay allowed detection of MicH in corrosive methanogenic field cultures*

181 Next, we wanted to better understand the performance of the MicH-specific immunoassay and
182 selectivity of the Anti-MicH antibody in more complex context of oil field produced water
183 enrichment cultures. Therefore, ten field enrichment cultures that showed varying degree of MIC
184 activity were compared in terms of corrosion as well as presence of the *micH* gene and expression
185 of the MicH protein (Fig. 4). Five of the ten cultures were classified as '+MIC' showing averaged
186 corrosion rates between 0.48 – 0.17 mm/yr, which according to the Association for Materials
187 Protection and Performance (AMPP) is considered high to severe (AMPP-SP0775-2013). The
188 other five cultures were classified as '-MIC' showing rates between 0.02 – 0.08 mm/yr, considered
189 as low to moderate corrosion according to AMPP (Fig. 4A).

190 Of the ten cultures eight showed detectable methanogenic activity having formed between 0.1 and
191 1.0 mmol of methane at the end of the test (Fig. 4A). ATP results indicated that biofilms have
192 formed on steel surfaces to a varying degree with generally higher numbers detected on corroded
193 steel surfaces (+MIC; 278 – 71299 pg/cm^2) compared to less corroded ones (-MIC; 6 – 1762
194 pg/cm^2; Fig. 4B). The *micH* gene was detected in planktonic cells as well as all five biofilms
195 collected from corroded steel coupons (>0.17 mm/yr; Fig. 4C). The MicH protein was similarely
196 detected in planktonic (36.5 – 1473.5 pg/mL) as well as biofilm samples (41.0 – 7971.3 pg /cm^2)
197 collected from '+MIC' enrichments, indicating active expression of the 'MIC hydrogenase' in
198 those cultures (Fig. 4D and Fig. S3B). Interestingly, we detected a small amount of the *micH* gene
199 in one of the enrichments showing low corrosion (0.04 mm/yr). However, the MicH protein has
200 not been detected, suggesting that the *micH* gene may not have been expressed and no corrosive
201 biofilm formed (Fig. 4).

202 **Discussion**

203 Multiple studies have highlighted the importance of understanding the underlying MIC
204 mechanisms to better detect and diagnose MIC[5,7,18,19]. Here we presented the development of a
205 MicH-specific immunoassay that showed strong affinity for the large subunit of a the 'MIC
206 hydrogenase' shown to be involved in corrosion by certain methanogenic Archaea[5,11]. The results
207 obtained with pure and field enrichment cultures suggest that the Anti-MicH antibody is highly
208 selective for the MicH protein found in certain corrosive methanogenic Archaea while showing
209 little to no cross-reactivity with proteins from SRB or non-corrosive methanogenic Archaea. This
210 also included oil field enrichment cultures showing methanogenic activity. In addition, the assay
211 confirmed that corrosive methanogenic cultures indeed actively expressed the 'MIC hydrogenase'
212 thereby accelerating the iron oxidation of carbon steel as previously shown in pure cultures and
213 genetically modified *Methanococcus maripaludis* strains[11,12].

214 Interestingly, we were able to detect the *micH* gene as well as the MicH protein in cultures of the
215 corrosive *Methanobacterium*-like strain IM1[16]. To our knowledge this is the first dataset that
216 allowed a deeper insight into the underlying molecular mechanisms of this corrosive methanogenic
217 archaeon. Previous potential step measurements have suggested a biologically catalyzed H_2
218 production pathway on graphite electrodes[20]. The identification of the *micH* gene and protein in
219 strain IM1 further supports an enzymatic catalyzed pathway for proton reduction on steel surfaces.

220 The detecting of the MicH protein not only in biofilms but also planktonic cells suggest that the
221 assay may be applicable to both MIC monitoring in biofilms as well as water samples. The latter
222 is often readily available in the field, while it is often challenging to obtain relevant biofilm
223 samples in the field to monitor pipeline corrosion. Biofilms act as planktonic cell factories
224 continuously shedding cells into fluids streams[21,22] that can then be collected at pipeline outlets
225 and can be analyzed for presents and expression of the MIC biomarker. We could previously show

226 the feasibility of using qPCR assays to detect MIC biomarkers in produced water samples as well
227 as pipeline solids from various global operations [5,14] and it would be interesting to see whether
228 similar can be accomplished using the novel immunoassay.

229 Overall, the development of a MicH-specific immunoassay offers an additional tool for corrosion
230 scientists and field practitioners to detect, monitor and understand corrosive methanogenic
231 biofilms. Furthermore, the detection of the MicH protein in multiple field enrichment cultures from
232 different locations together with previous qPCR results[5] highlight the importance of this MIC
233 mechanism in anaerobic systems.

234 **Conclusion**

235 Here, we built on previous research and findings to develop specific immunoassay for the detection
236 of an industrially important MIC mechanism. The assay offers corrosion scientists and field
237 practitioners a novel way to detect, monitor and understand corrosive methanogenic biofilms.
238 Furthermore, the detection of the MicH protein in multiple field enrichment cultures from different
239 locations together with previous qPCR results5 highlight the importance of this MIC mechanism
240 in anaerobic systems on a global scale.

241 Establishment and optimization of a novel Western blot-based immunoassay to detect an
242 important MIC biomarker ('MicH') found in certain highly corrosive methanogenic Archaea.

243 Analysis of recombinant MicH protein and pure cultures of corrosive and non-corrosive
244 methanogens and SRB showed high sensitivity and selectivity of the immunoassay

245 Test with corrosive and non-corrosive oil field enrichment cultures showed applicability
246 for Mic detection in the field.

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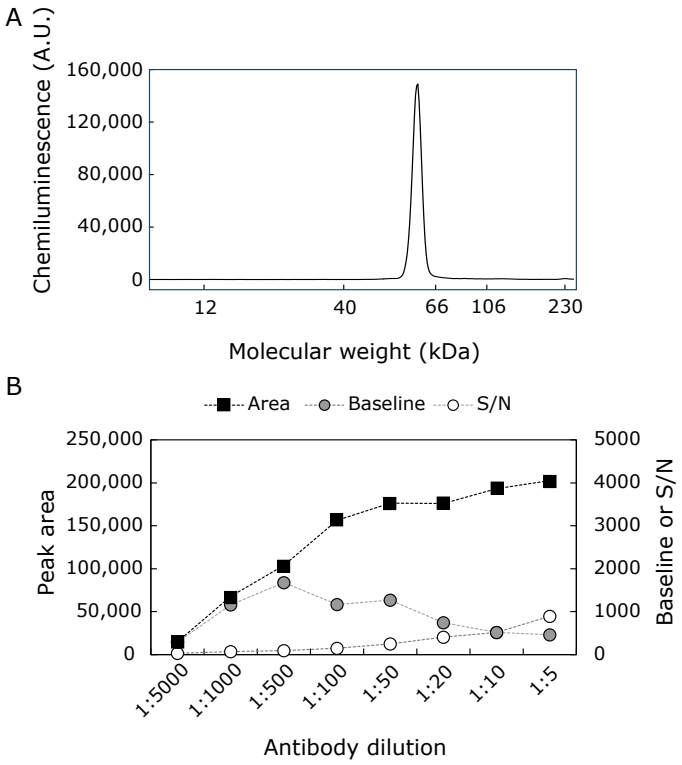

Figure 1 Performance of recombinant antibody Anti-MicH using a recombinant MicH protein as target. A) Chemiluminescence measured as arbitrary units (A.U.) showing signal at the expected molecular weight of $MicH_{rec}$. Test was performed with a 1:20 dilution of the antibody. B) Results of tests performed with varying dilutions of Anti-MicH and 1 ng/ml $MicH_{rec}$.

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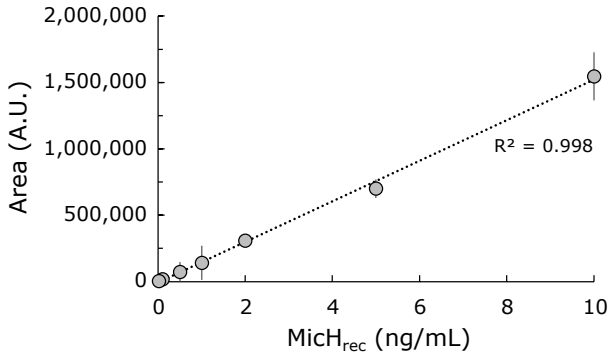

305 Figure 2 Western Blots performed with dilution series of recombinant MicH protein and Anti-
306 MicH antibody dilutions of 1:20. The error bar represent the standard deviation of technical
307 triplicates.

308

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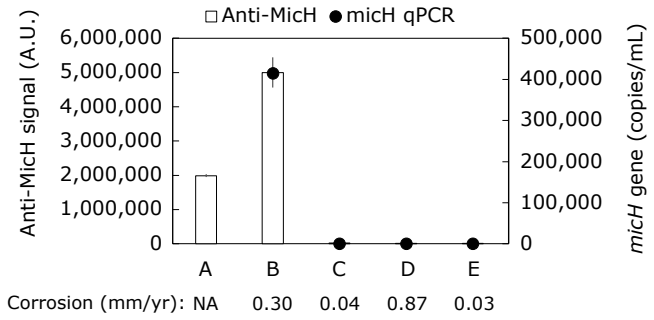

310 Figure 3 Analysis of protein extracts with MicH-specific Western blot immunoassay and DNA
311 extracts with micH qPCR assay. A) Recombinant MicH Protein (10 ng/mL), B) *Methanobacterium*
312 sp. strain IM1, C) *Methanococcus maripaludis* strain S2, D) *Desulfovibrio ferrophilus* IS5, E)
313 *Desulfovibrio alaskensis* G20. Average corrosion rates are shown below the bar plot. The error
314 bars where seen indicate the standard deviations of triplicate cultures or technical triplicates in
315 case of recombinant MicH.

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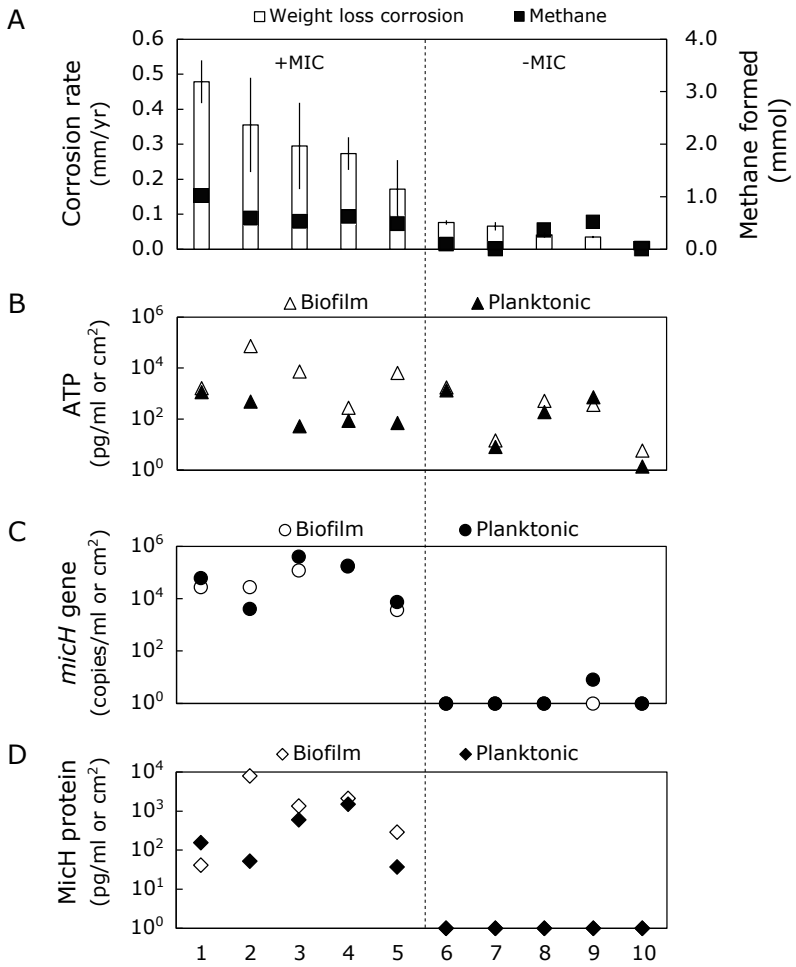

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319 Figure 4 A) Weight loss corrosion rates and methane formation in oil field produced water
320 enrichment cultures classified as corrosive [‘+MIC’] and non-corrosive [‘-MIC’] enrichments. B)
321 MicH protein numbers detected in corrosion coupon biofilms (open diamonds) and planktonic
322 cells (closed diamonds). Enrichments 1, 2, 4, 6, 7, 9, 10 – First West African oil field. Enrichment
323 3 – Second West African oil field. Enrichment 5 – U.S. offshore oil field. Enrichment 8 – U.S.
324 onshore oil field. Error bars in A) indicate standard deviation of weight loss analysis from three
325 corrosion coupons.