1 **Development of a group-specific 16S rRNA-targeted probe set for the identification**

2 **of *Marinobacter* by fluorescence *in situ* hybridization**

3

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22 **Abstract**

23 Members of the *Marinobacter* genus play an important role in hydrocarbon degradation in
24 the ocean – a topic of special significance in light of the recent *Deepwater Horizon* oil spill of
25 2010. The *Marinobacter* group has thus far lacked a genus level phylogenetic probe that
26 would allow *in situ* identification of representative members. Here, we developed two new
27 16S rRNA-targeted oligonucleotide probes (Mrb-0625-a and Mrb-0625-b) to enumerate
28 *Marinobacter* species by fluorescence *in situ* hybridization (FISH). *In silico* analysis of this
29 probe set demonstrated 80% coverage of the *Marinobacter* genus. A competitor probe was
30 developed to block hybridization by Mrb-0625-a to six *Halomonas* species with which it
31 shared a one base pair mismatch. The probe set was optimized using pure cultures, and
32 then used in an enrichment experiment with a deep sea oil plume water sample collected
33 from the *Deepwater Horizon* oil spill. *Marinobacter* cells rapidly increased as a significant
34 fraction of total microbial abundance in all incubations of original contaminated seawater
35 as well as those amended with *n*-hexadecane, suggesting this group may be among the first
36 microbial responders to oil pollution in the marine environment. The new probe set will
37 provide a reliable tool for quantifying *Marinobacter* in the marine environment,
38 particularly at contaminated sites where these organisms can play an important role in the
39 biodegradation of oil pollutants.

40

41

42 ## 1. Introduction

43 The ability of marine ecosystems to recuperate from oil pollution is largely dependent on
44 the activities of indigenous communities of hydrocarbon-degrading bacteria, which often
45 varies depending on the ecosystem in question. For example, contaminants from the
46 *Deepwater Horizon* oil spill have affected several marine ecosystems, including the deep
47 water column (Diercks et al., 2010), coastal waters and beaches (Graham et al., 2010;
48 Hayworth et al., 2011), salt marshes (Silliman et al., 2012), and deep sea sediments (Liu et
49 al., 2012). Initial microbial analyses following the Macondo wellhead blowout have shown
50 diverse microbial phylotypes associated with distinct sites of oil contamination. Members
51 of the *Oceanospirillales* and the genus *Halomonas* were dominant in the deep sea
52 hydrocarbon plume (Hazen et al., 2011), Firmicutes and *Alphaproteobacteria* in open ocean
53 surface slicks (Redmond et al., 2011), and *Alcanivorax*, *Marinobacter,* and
54 *Rhodobacteracaeae* spp. were dominant in coastal beaches (Kostka et al., 2011). Detecting
55 and identifying microbial communities across different habitats is integral to the
56 assessment of the microbial degradation of oil contaminants in the marine environment.

57

58 Methods to identify and monitor the abundance of hydrocarbon-degrading microorganisms
59 in environmental samples enhance our understanding of their natural ecology, their
60 response to oil spills, and their role in degrading the oil (Head et al. 2006). Fluorescence *in*
61 *situ* hybridization (FISH) is an effective technique allowing phylogenetic identification,
62 enumeration, and direct spatial visualization of microorganisms in their natural
63 environment. It utilizes 16S rRNA-targeted oligonucleotide probes labeled with, for

64 example, a fluorophore, that bind to the complementary site on the 16S rRNA gene of a
65 target microorganism(s) (Amann et al., 1995). Genus-level oligonucleotide probes were
66 previously developed for *Alcanivorax* (Syutsubo et al., 2001) and *Cycloclasticus* (Maruyama
67 et al., 2003) – organisms which, respectively, have been shown to play an important role in
68 the degradation of aliphatic and aromatic hydrocarbons in oil-polluted seawater (Head et
69 al., 2006). Another important group of oil-degrading bacteria in the ocean is *Marinobacter*,
70 members of which have also been shown to become heavily enriched during oil spills
71 (summarized by Duran, 2010). In addition to hydrocarbon association, several clades of
72 this genus were ubiquitously found in mutual association with dinoflagellates and
73 coccolithophores originating from seas and oceans all over the world (Amin et al. 2009).
74 Despite their ubiquity in marine environments and the important role that these organisms
75 contribute to the degradation of hydrocarbons and other processes, molecular tools to
76 quantify them have had limited coverage. Previously, FISH probes or primer sets were
77 developed targeting 29% (Xiao et al., 2010), 4.2% (Brinkmeyer et al., 2003), and 9.2%
78 (Gray et al., 2011) of the *Marinobacter* genus for specific applications. So far, however, no
79 FISH probe set exists that provides a high level of coverage for this monophyletic group.
80 Here, we developed and optimized two new probes for FISH that together could be used to
81 detect up to 80% of the currently established genus, or used in concert with previously
82 published probes to examine environmental compositions and influences of distinct
83 *Marinobacter* subgroups. We then used these probes to assess the role *Marinobacter* plays
84 in degrading hydrocarbons in contaminated deep seawater from the *Deepwater Horizon* oil
85 spill.
86

87 **2. Materials and Methods**

88

89 **2.1. Oligonucleotide probe design**

90 Group-specific oligonucleotide probes for *Marinobacter* were designed against current 16S
91 rRNA gene sequence databases. Using the Probe Design tool of Arb v104 (Pruesse et al.,
92 2007), probe candidates were selected based on their provision of the greatest possible
93 coverage of 659 sequences representing the genus *Marinobacter* in August of 2011. Probe
94 candidates were analyzed using the probeCheck server (Loy et al., 2008) and the
95 Ribosomal Database Project's Probe Match tool (Cole et al., 2008) to evaluate their *in silico*
96 specificity and coverage. From this, two probe sequences, Mrb-0625-a (5'-CAG TTC GAA
97 ATG CCG TTC CCA-3'; 21-mer) and Mrb-0625-b (5'-CAG TTC GGA ATG CCG TTC CCA-3'; 21-
98 mer), were selected. Both probes converged over the same position (0625 – 0645) based
99 on the 16S rRNA *E. coli* gene (Lane, 1991) with one base pair mismatch between them.
100 Probe coverage for Mrb-0625-a was 75% of all sequences comprising the *Marinobacter*
101 genus, whereas that for Mrb-0625-b was 5% of the remaining 25% of sequences not
102 covered by Mrb-0625-a. Together, both probes covered 80% of the *Marinobacter* group.
103 Our analysis also identified six *Halomonas* clone sequences sharing one basepair mismatch
104 to probe Mrb-0625-a. A competitor probe, Hal-0625-a (5'-CAG TTC CAA ATG CCG TTC CCA-
105 3'; 21-mer), was designed to reduce hybridization of Mrb-0625-a to these non-target
106 halomonads. Table 1 summarizes the probes that were developed in this study.

107

108 **2.2. Oligonucleotide probe optimization**

109 Pure cultures of *Marinobacter algicola* (DSM 16394), *Marinobacter flavimarus* (DSM 16070)
110 and *Marinobacter zhanjiangensis* (KCTC 22280) were used to test and optimize the FISH
111 probes. The strains were grown on a marine broth (ZM/10) composed of ¾-strength
112 naturally aged seawater, peptone (0.05%), yeast extract (0.01%), and supplemented after
113 steam-sterilization with filter-sterile (0.2 µm) trace elements and vitamins to final
114 concentrations as previously described (Blackburn *et al.* 1989). For fixation three volumes
115 of 4% (v/v) paraformaldehyde solution in 1x phosphate buffer saline (PBS; 130 mM NaCl, 2
116 mM NaH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) were mixed with one volume of exponentially-grown
117 cells and incubated for 3 h at 4°C. After three washings with 1x PBS, the samples were
118 stored in a 1:1 (v/v) solution of 1x PBS and ethanol at -20°C.
119
120 Initial hybridizations with pure bacterial cultures and the newly-designed probes were
121 performed using formamide (FA) concentrations of 0% and 10% in order to confirm
122 permeability of the cells to the probes and adequate signal intensity. Optimal conditions for
123 hybridization with these probes were determined by multiple hybridizations using
124 increasing FA concentrations from 0% to 70%. Because the two probe sequences differ by
125 only one nucleotide and target the same binding site, they were used simultaneously in
126 competitive hybridization experiments. *M. algicola* was used as the reference strain for
127 Mrb-0625-a and as the single mismatch non-target strain for Mrb-0625-b, whereas *M.*
128 *zhanjiangensis* was used as the reference strain for Mrb-0625-b and as the single mismatch
129 non-target strain for Mrb-0625-a. Hybridization assays were performed using standard
130 methods (Daims et al., 2005). Samples hybridized with fluorescently-labeled probes were
131 visualized using an Olympus BX51 epifluorescence microscope (Tokyo, Japan) equipped

132 with a Hamamatsu C8484 digital camera (Hamamatsu City, Japan). Probe-conferred signal
133 intensities were quantified with MetaMorph image analysis software version 7.6.0.0
134 (Sunnyvale, CA, USA).
135
136 **2.3. Sample collection**
137 During a research cruise on the R/V Walton Smith (May 26 to June 8, 2010), seawater
138 samples from 1000-1250 m depth were collected by CTD rosette sampler within 1-7 miles
139 from the Macondo wellhead in the Gulf of Mexico. Some of these CTD deployments
140 recovered samples from a deepwater hydrocarbon plume that had formed early during the
141 spill and was marked by localized oxygen depletion and an increase in colored dissolved
142 organic matter which was indicative of the presence of petrochemical hydrocarbons and
143 elevated microbial activities from hydrocarbon oxidation (Diercks et al., 2010; Joye et al.,
144 2011; Yang et al., 2012—ASLO talk). Upon collection, live samples were stored at 4°C for 15
145 months until they were used as the inoculum for an enrichment experiment with *n*-
146 hexadecane to evaluate the FISH protocol described below.
147
148 **2.4. *Marinobacter* enrichment setup**
149 Enrichment cultures were prepared using steam-sterilized screw-capped 100 ml glass vials.
150 Two vials were prepared containing 6 ml of filter-sterilized (0.2 μm) ONR7a marine
151 medium (Dyksterhouse et al., 1995) supplemented with *n*-hexadecane (4% v/v). Two
152 additional vials were prepared in the same way but without *n*-hexadecane. All four vials
153 were inoculated with 1.2 ml of the plume water sample. An additional vial containing 6 ml
154 of sterile ONR7a and *n*-hexadecane was inoculated with pre-filtered (0.2 μm) and

155 autoclaved plume water to act as the killed control. All vials were incubated in the dark

156 with gentle shaking (60 rpm) at 27°C, which falls within the optimum growth temperature

157 range (25 – 30°C) for most *Marinobacter* (Duran, 2010). Fixation of subsamples (100 μl)

158 was performed by mixing with 300 μl of 4% (v/v) paraformaldehyde and incubating at 4°C

159 for 3 hrs. Cells were collected by centrifugation (15,000 x g; 5 min), mixed with ice-cold 1X

160 PBS and ethanol (1:1), and then stored at -20°C.

161

162 **2.5. FISH analysis of *Marinobacter* enrichment**

163 Subsamples from the time-series incubation were diluted (500- to 1000-fold) in 1X PBS

164 and then filtered onto 0.22 μm polycarbonate filters (25 mm, Millipore GTTP). FISH was

165 performed directly on filter sections according to previous protocols (Glöckner *et al.,* 1996;

166 Loy *et al*., 2005; Pernthaler *et al.,* 2001). Probe GAM42a was included in some

167 hybridizations to quantify the abundance of *Marinobacter* against all *Gammaproteobacteria*.

168 Since hybridization with GAM42a employs more stringent conditions (i.e. a higher FA

169 concentration) (Manz et al., 1992) than that with Mrb-0625-a, a double hybridization assay

170 was performed with GAM42a first. All hybridizations were counterstained with 4',6-

171 diamidino-2-phenylindole (DAPI) following standard methods (Porter and Feig, 1980)

172 prior to visualization under the epifluorescence microscope. Eight to twelve random fields

173 of view were counted for all time points except time point 1, for which 5 fields of view were

174 counted.

175

176 Fluorescently-labeled probes (Mrb-0625-a, Mrb-0625-b, GAM42a), labeled at the 5'-end

177 with 6-carboxyfluorescein (6-FAM) or with the sulfoindocyanine dye CY3, and unlabeled

178 probes Hal-0625-a and BET42a, were obtained from EurofinsMWG Operon (Huntsville, AL,
179 USA). *n*-Hexadecane was obtained from Acros Organics (New Jersey, USA). To distinguish
180 Mrb-0625-a fluorescence from Mrb-0625-b fluorescence, the probes were labeled with
181 6FAM and CY3, respectively. All other chemicals were of molecular biology or HPLC grade.
182

183 **3. Results and Discussion**

184 **3.1. *Marinobacter* phylogeny and probe coverage**

185 A phylogenetic tree was constructed that included all currently published type strains of
186 *Marinobacter* and other related organisms to illustrate coverage of the newly-developed
187 probes (Mrb-0625-a and Mrb-0625-b) (Figure 1). The total of *Marinobacter* sequences in
188 the Silva 104 reference database at the time (August 2011) these probes were designed
189 was 659, of which 63% are cultured isolates and the remaining 37% are uncultivated clone
190 sequences. An additional 13% of the *Marinobacter* genus—represented in the tree by *M.*
191 *maritimus* and *M. psychrophilus* (Figure 1) —is targeted by a complementary, but not yet
192 empirically tested, probe designated Mrb-0625-c (Table 1). The remaining 7% of the genus
193 does not branch together and could not be comprehensively targeted by a single probe. In
194 the tree, *Halomonas sp.* A-3 (AY914056) represents six *Halomonas* species to which it
195 shares a one base pair internal mismatch with Mrb-0625-a. For this reason, we designed
196 Hal-0625-a to bind competitively to this group of halomonads and prevent non-target
197 binding by Mrb-0625-a.
198

199 **3.2. Probe optimization and formamide series**

200 The melting curves for probes Mrb-0625-a and Mrb-0625-b when used in hybridizations
201 with target and non-target reference strains and in the absence and presence of each other
202 are shown in figure 2. In all experiments fluorescence intensity was greater for target
203 strains compared to non-target strains (with a 1-bp mismatch), demonstrating strong
204 probe specificity. Empirically optimized FA concentrations ensured specificity during
205 hybridization with these probes (Table 1). In the case of probe Mrb-0625-a, fluorescence
206 signal intensities decreased significantly at FA concentrations above 20%, indicating that
207 this concentration would be suitable for hybridizations with this probe to specifically
208 detect up to 75% of members that comprise the *Marinobacter* group. With Mrb-0625-b,
209 fluorescence signal intensities did not decrease as sharply at FA concentrations above 20%,
210 though were highest at 20-25% FA concentration and remained distinguishable from non-
211 target signals up to 40% FA concentration. Therefore, when using the probes individually
212 we recommend 20-25% FA as the ideal stringency for Mrb-0625-a and 35-40% FA for Mrb-
213 0625-b.
214
215 Two additional FA series experiments were conducted to optimize using both probes
216 together in the same hybridization. In one of these experiments, 6FAM-labeled Mrb-0625-a
217 and CY3-labeled Mrb-0625-b were applied together with *M. algicola*, while in the other
218 experiment both probes were applied together with *M. zhanjiangensis*. The melting curves
219 for 6FAM-labeled Mrb-0625-a hybridized with target strain *M. algicola* and with non-target
220 strain *M. zhanjiangensis* show that the signal intensity of the *M. algicola* cells at 25% FA
221 concentration is clearly distinguishable from the non-target fluorescence of *M.*
222 *zhanjiangensis* (Fig. 2C). Conversely, fluorescence during hybridization of CY3-labeled

223 probe Mrb-0625-b to target strain *M. zhanjiangensis* and non-target strain *M. algicola*
224 resulted in distinguishable signals between target and non-target fluorescence within a
225 range from 15% to at least 40% FA, while non-target fluorescence was greatly reduced in
226 the competitive hybridization (Figure 2D). Competitive interference by Mrb-0625-a is not
227 likely beyond 35% FA, since Mrb-0625-a targeted fluorescence is greatly diminished at
228 greater FA concentrations (Figure 2A, C). As a result, Mrb-0625-b shows a gradual decay of
229 fluorescence signal (Figure 2D) not unlike the previous experiment without use of
230 competitive Mrb-0625-a (Figure 2B). When using the two probes together in the same
231 hybridization reaction, we recommend a common stringency of 25% FA for the
232 simultaneous detection of their target *Marinobacter* species.
233
234 The competitive use of alternately labeled Mrb-0625-a and Mrb-0625-b at their
235 empirically-determined optimal FA concentration of 25% was then tested with a mixed
236 population of *M. algicola* and *M. zhanjiangensis* (Figure 3). Consistent with the FA series
237 results from Figure 2, the two *Marinobacter* reference strains could be clearly distinguished
238 from one another. Since an overlay of figures 3A (showing Mrb-0625-a targeted signals)
239 and 3B (showing Mrb-0625-b targeted signals) did not yield any double-labeled cells that
240 were orange or yellow, but rather that were either distinctly green or distinctly red (Figure
241 3C), we conclude that non-target binding is negligible and that the probes are highly
242 specific for their respective target organisms. Further confirmation comes from
243 morphological observations: from previous pure (non-mixed) culture experiments, *M.*
244 *zhanjiangensis* cells were larger than *M. algicola* cells, and this can be observed in the
245 difference between red and green cell size in Figure 3.

246

247 ### 3.3. *Marinobacter* spp. response to oil-contaminated seawater

248 A microbial enrichment with plume water samples obtained from 1000-1250 m depth in
249 the Gulf of Mexico during the Deepwater Horizon oil spill was characterized with the
250 *Marinobacter* probe set. Since previous work with the plume water samples from the Gulf
251 of Mexico showed that it contained *Marinobacter* species, as revealed in 16S rRNA clone
252 libraries and isolation experiments (Yang et al, in review), it was considered a suitable field
253 sample for application of the *Marinobacter* FISH protocol employing these new probes. *n*-
254 Hexadecane has been used in several studies to enrich for and isolate *Marinobacter* species
255 (Gauthier et al., 1992; Nguyen et al., 1999; Green et al., 2006; McGowan et al., 2004; Abed et
256 al., 2007), and was therefore selected as a model hydrocarbon to enrich for these
257 organisms. At 27°C, the incubation temperature was set within the optimum range for
258 growth of most *Marinobacter* cultures (25 – 30°C, Duran, 2010). After enrichment with *n*-
259 hexadecane, subsamples taken during this experiment were analyzed using probes Mrb-
260 0625-a and Mrb-0625-b to provide information on the detection and abundance of
261 *Marinobacter*. Hybridizations with 6FAM-labeled Mrb-0625-a revealed that a substantial
262 fraction (e.g., ca. 30% in vial 1, day 3) of the entire DAPI-stained microbial population in the
263 plume water inoculum was composed of *Marinobacter* species (Figure 4A; Table 2).
264 Samples taken from these experiments and hybridized with the Mrb-0625-b probe did not
265 yield any signals (results not shown). Therefore, the *Marinobacter* population in this
266 enrichment was quantified using only the Mrb-0625-a probe (discussed below). Hazen and
267 colleagues (2010) showed that *Halomonas spp*. were significantly enriched in plume waters
268 during the Deepwater Horizon oil spill – by as much as 140% compared to their abundance

269 in non-plume water samples. In order to block false positive Mrb-0625-a hybridization to
270 halomonads, FISH experiments with this probe included the unlabeled Hal-0625-a
271 competitor probe (Table 1) which shares 100% sequence homology to halomonads with a
272 1 base pair mismatch at the 16S rRNA region targeted by Mrb-0625-a.
273
274 We recognize that the identification of organisms using FISH that employs a probe(s)
275 targeting only a single binding site is not always sufficiently robust. Since no other realistic
276 combination of comprehensive genus-level *Marinobacter* probes could be developed, a
277 double hybridization with the class-level cy3-labeled GAM42a probe and unlabeled BET42a
278 as competitor (Manz et al., 1992) was performed on selected samples from the plume
279 water enrichment experiment. FISH analysis of a sample taken from vial 2 at day 4
280 hybridized with 6FAM-labeled Mrb-0625-a and CY3-labeled GAM42a showed that
281 *Marinobacter* cells targeted by Mrb-0625-a represented 30% of the gammaproteobacterial
282 population (Figure 4B). Superimposition of duplicate fields of view under red and green
283 light filters demonstrated *Marinobacter* cells in yellow due to Mrb-0265-a fluorescence in
284 green and GAM42a fluorescence in red.
285
286 The abundance of *Marinobacter* cells quantified with probe Mrb-0625-a had markedly
287 increased as a result of enrichment with *n*-hexadecane (Figure 5A) compared to the
288 untreated (i.e. no added *n*-hexadecane) controls (Figure 5B). The most dramatic increase
289 (ca. 1 order of magnitude) in abundance occurred between days 2 and 3 and coincided with
290 a significant increase (ca. 1.5 order of magnitude) in the total bacterial population detected
291 by DAPI counts. This was consistent with our observation of an increase in the turbidity of

292 the culture liquid of vials 1 and 2 (amended with *n*-hexadecane) occurring during this time
293 (Supplementary Figure S1). Bacterial abundance remained elevated in the *n*-hexadecane-
294 amended incubations, reaching approximately 3.0 x 10^9 cells ml^{-1} by the termination of
295 these experiments at day 21. From day 3 to day 21 the *Marinobacter* fraction of the total
296 microbial population had steadily decreased from 30%±5% to 2.7%±0.8% in vial 1, and
297 from 29%±6% to 20%±5% in vial 2. Due to the relatively rapid response of *Marinobacter*
298 spp. in both hexadecane-amended vials 1 and 2 and environmentally contaminated vials 3
299 and 4 at the start of the incubation and subsequent lesser involvement (ie, lesser fraction of
300 the total microbial community) towards the end of the incubation, we conclude that
301 *Marinobacter* is one of the initial responding groups to hydrocarbon presence in the marine
302 environment.
303
304 **3.4. Trophic cascading by marine hydrocarbon degraders**
305 Although our enrichment experiment had the primary objective of testing the newly
306 designed *Marinobacter* probe set, our data offers some insight into the dynamics of marine
307 hydrocarbon degradation. The overall increase in the bacterial population and relative
308 decrease in *Marinobacter* (Fig. 5A) suggests a trophic cascade of distinct microorganisms
309 that participated in the degradation of the *n*-hexadecane, and possibly also of other
310 hydrocarbons that were inherently present in the plume water inoculum. A community-
311 level collaboration and succession of different microbial groups is not atypical following an
312 oil spill in marine waters (Head et al., 2006; Yakimov et al. 2007). Initial degradation of the
313 *n*-hexadecane by *Marinobacter* in these enrichments may have yielded intermediates that
314 fueled the growth of secondary degraders. Such a trophic cascade may in part explain the

315 changes in the microbial community composition of the deep oil plume from the *Deepwater*
316 *Horizon* spill which have been observed by different researchers (Redmond and Valentine
317 2012; Kessler et al. 2011; Yang et al., in review).
318
319 Vials 3 and 4 (not amended with *n*-hexadecane) also exhibit overall increases in DAPI-
320 stained microbial cell counts up until day 10 for vial 3 and day 5 for vial 4, but these
321 numbers are approximately 2 orders of magnitude lower compared to those from the *n*-
322 hexadecane-amended vials (Table 2; Supplementary Figure S2). Interestingly,
323 *Marinobacter* appeared to represent the dominant fraction of the lower-density total
324 microbial community in non-amended incubations – ca. 40% by day 1, then peaking at
325 83%±9% in vial 3 and 94%±6% in vial 4 by days 2 and 4 before dropping back down to ca.
326 40-50% by day 21. This is not implausible when considering that C16 hydrocarbons (like
327 hexadecane) were found to constitute a significant fraction (2nd and 4th highest) of the total
328 C10 to C35 presence in two deep sea plume water samples (Wade et al., 2011). Hence, low-
329 density pre-enrichment of *Marinobacter* in our deep water plume inoculum was likely
330 attributed to the endogenous presence of these types of hydrocarbons and to *Marinobacter*
331 seed populations that responded well to sample containment. Prolonged bottle storage for
332 15 months might also have had an effect; bottle incubation of marine water samples
333 resulted in elevated transcription within the Alteromonadales order, which includes the
334 *Marinobacter* genus (Stewart et al., 2012).
335

336 **4. Conclusion**

16S rRNA oligonucleotide probes targeting an important group of hydrocarbon-degrading and micro-algal associated bacteria, the *Marinobacter*, provide a useful tool with which to study the occurrence and ecological response of these organisms during major perturbations in the marine environment. The newly developed FISH probe set (Mrb-0625-a + Mrb-0625-b + competitor Hal-0625-a) was developed to target up to 80% of species comprising the *Marinobacter* genus, and tested empirically for hybridization stringency. Our observations of the *Marinobacter* and total microbial community response to oil contamination indicate that *Marinobacter* spp. may be among the first responders to the presence of hydrocarbons in marine systems. We posit that hybridizations combining our newly developed probe set with previously published *Marinobacter* probes MB-IC022 (Brinkmeyer et al., 2003) and MB115 (Xiao et al., 2010) could provide more detailed analysis on the dynamics of *Marinobacter* subgroups in the environment. Furthermore, the new probe set provides a useful expansion to the current collection of oligonucleotide probes by allowing in-situ identification of microbial groups contributing to important metabolic processes, such as the breakdown of hydrocarbons.

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366

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494

495

496 **Figure and Table Legends**

497 **Figure 1.** Small subunit ribosomal RNA (16S rRNA) phylogeny of members of the

498 gammaproteobacterial marine hydrocarbon-degrading genus *Marinobacter* and six other

499 hydrocarbon-degrading *Gammaproteobacteria*. Of the 659 *Marinobacter* species in the

500 current 16S Silva database, 27 cultured representatives were chosen to demonstrate the

501 overall diversity of the genus as well as approximate percent coverage by our

502 oligonucleotide probes. Type strains are indicated by ^{T}. Strains with known hydrocarbon-

503 degrading capabilities are shown in bold (summarized by Duran, 2010) while species

504 marked with $^{\Sigma}$ were not able to growth on *n*-tetradecane or crude oil (Guo et al., 2007).

505 *Roseobacter litoralis* (X78312) was used as the outgroup. The species used to test and

506 optimize our probes, *Marinobacter algicola* and *Marinobacter zhanjiangensis*, are indicated

507 by asterisks.

508 **Figure 2.** Dissociation profiles of 16S rRNA-targeted FISH probes Mrb-0625-a and Mrb-
509 0625-b evaluated against a perfectly-matching (target) and one basepair-mismatching
510 (non-target) strain. Black diamonds represent hybridization intensities for *M. algicola*;
511 white squares correspond to hybridization intensities for *M. zhangjiangensis*. In two
512 separate experiments Mrb-0625-a was hybridized with its target strain, *M. algicola*, and
513 with its single base pair mismatch strain, *M. zhanjiangensis* (2A). In two separate
514 experiments Mrb-0625-b was hybridized with its target strain, *M. zhanjiangensis*, and with
515 its single base pair mismatch strain, *M. algicola* (2B). In additional experiments both
516 probes were used together as competitors for the same 16S rRNA motif on both target
517 strains. Dissociation curves are shown for Mrb-0625-a hybridized to its target and non-
518 target strain in the presence of Mrb-0625-b (2C), and for Mrb-0625-b hybridized to its
519 target and non-target strain in the presence of Mrb-0625-a (2D). Each data point
520 represents the average fluorescence intensity value ± standard deviation from ten
521 randomly-selected fields of view. Mrb-0625-a is labeled with 6FAM, and Mrb-0625-b is
522 labeled with CY3 in all experiments. See supplementary text for energetic considerations of
523 probe competition. Linear interpolation was excluded for *M. zhanjiangensis* in 2C and *M.*
524 *algicola* in 2D to denote lower resolution in formamide concentration changes.
525

526 **Figure 3.** Competitive hybridization experiment showing Mrb-0625-a and Mrb-0625-b
527 fluorescence in a mixed culture of their respective target strains, *M. algicola* and *M.*
528 *zhanjiangensis*. (A) FITC filtered image of Mrb-0625-a (labeled with 6FAM) targeting *M.*
529 *algicola*. (B) TRITC filtered image of Mrb-0625-b probe (labeled with CY3) targeting *M.*
530 *zhanjiangensis*. (C) Overlay of images from 3A and 3B, representing the same field of view.
531

532 **Figure 4.** Hybridization of samples from the *n*-hexadecane enrichment experiment with

533 (A) Mrb-0625-a (green) amongst the entire DAPI-stained microbial population (blue) in a

534 sample taken from vial 1 after 3 days, and (B) Mrb-0625-a (yellow-green) amongst the

535 entire GAM42a-targeted gammaproteobacterial population (red) in a sample taken from

536 vial 2 after 4 days incubation.

537

538 **Figure 5.** Absolute DAPI-stained (white and grey) and Mrb-0625-a-targeted (grey only)

539 cell numbers throughout the time course of the enrichment experiment. All experiments

540 were performed in parallel in two sets of duplicate vials (Vials 1 and 2; Vials 3 and 4); DAPI

541 and FISH counts for these duplicated assays are plotted separately in two neighboring

542 columns for all time points, to show the consistency of the microbial growth and

543 enrichment response. (A) Average cell numbers for hexadecane-amended vial 1 (lefthand

544 column) and vial 2 (right-hand column) from days 0, 1, 2, 3, 4, 5, 10, and 21 of the

545 enrichment. (B) Average cell numbers for non-hexadecane-amended vial 3 (left-hand

546 column) and vial 4 (right-hand column) from days 0, 1, 2, 3, 4, 5, 10, and 21 of the

547 enrichment. Error bars represent standard deviation from mean cell counts. The x-axis is

548 abbreviated between days 5 and 10 and between days 10 and 21.

549

550 **Table 1.** According to the standard nomenclature for the naming of new probes (Alm *et al.*,
551 1996), official names of probes Mrb-0625-a, Mrb-0625-b, Mrb-0625-c and Hal-0625-a are
552 S-G-Mrb_0625-a-A-21, S-G-Mrb_0625-b-A-21, S-G-Mrb_0625-c-A-21, and S-G-Hal_0625-a-
553 A-21, respectively. Also indicated are percent genus coverage, representative strains and
554 accession numbers, 5'-3' sequences for each probe, and optimal FA concentrations for
555 hybridization. Sequence mismatches between probes are indicated in white lettering. Mrb-
556 0625-c is a suggested third probe that targets an additional 13% of the *Marinobacter* group
557 not covered by Mrb-0625-a and Mrb-0625-b is also included in this table, but was not
558 empirically evaluated in this study.
559

560 **Table 2.** Microbial cell counts show changes in total microbial abundance and the

561 corresponding fraction of *Marinobacter* spp. abundance in each vial over the 21 day time

562 course. Values are in 10^6 cells ml^{-1}. The first row indicates the day of incubation and the

563 first column indicates the vial # and either DAPI, for total microbial community, or 6FAM,

564 for Mrb-0625-a conferred fluorescence. Asterisks demarcate spikes in microbial

565 abundance in vials 1 and 2 between days 2 and 3, concomitant with noticeable changes in

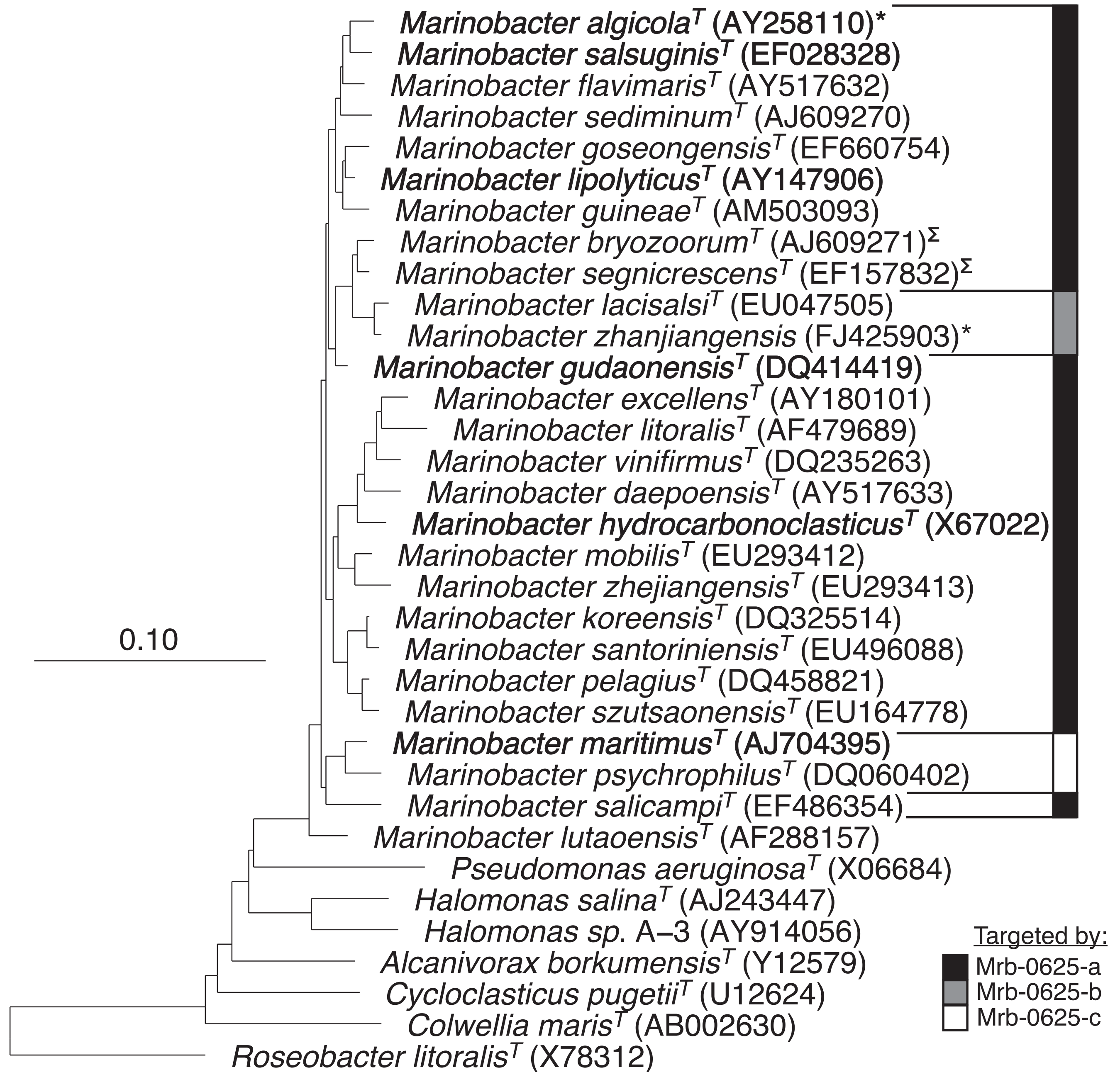
566 turbidity (Supplementary Figure S1).

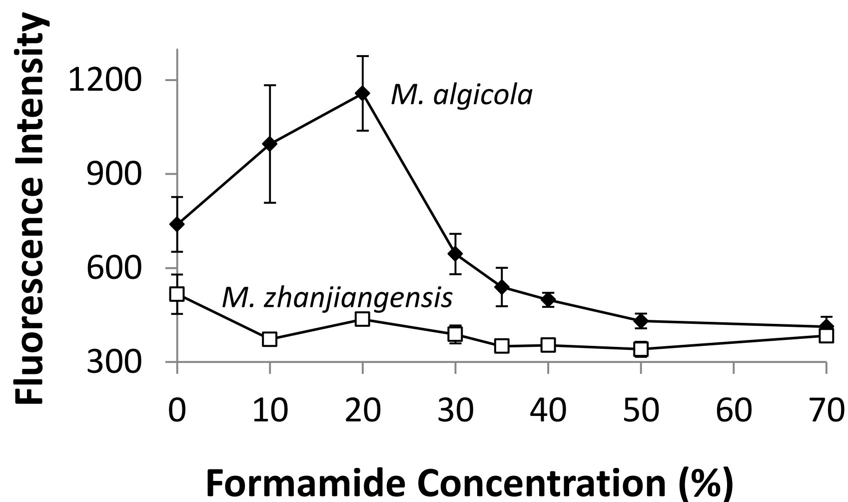
567

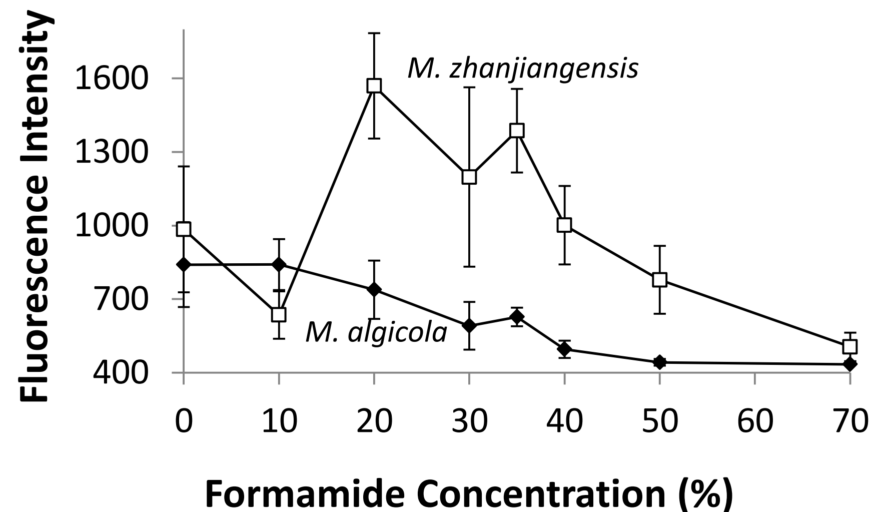
Probe	Genus coverage	Representative strain	Sequence 5' – 3'	Optimal [FA]
S-G-Mrb_0625-a-A-21	75%	*Marinobacter algicola* (AY258110)	CAGTTCGAAATGCCGTTCCCA	20-25%
S-G-Mrb_0625-b-A-21	5%	*Marinobacter zhanjiangensis* (FJ425903)	CAGTTCGGAATGCCGTTCCCA	35%
S-G-Mrb_0625-c-A-21	13%	*Marinobacter maritimus* (AJ704395)	CAGTTCGAAGTGCCGTTCCCA	ND
S-G-Hal_0625-a-A-21	0%	*Halomonas sp. A-3* (AY914056)	CAGTTCCAAATGCCGTTCCCA	ND

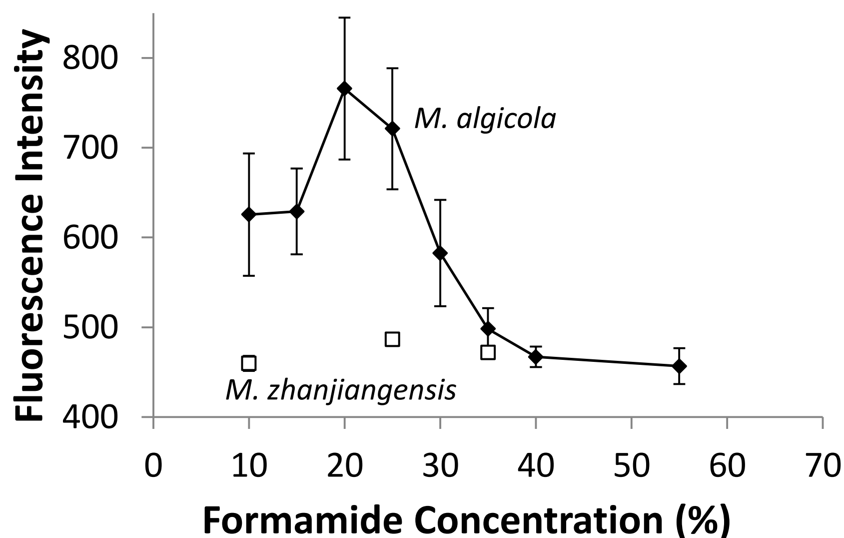
Day of Incubation

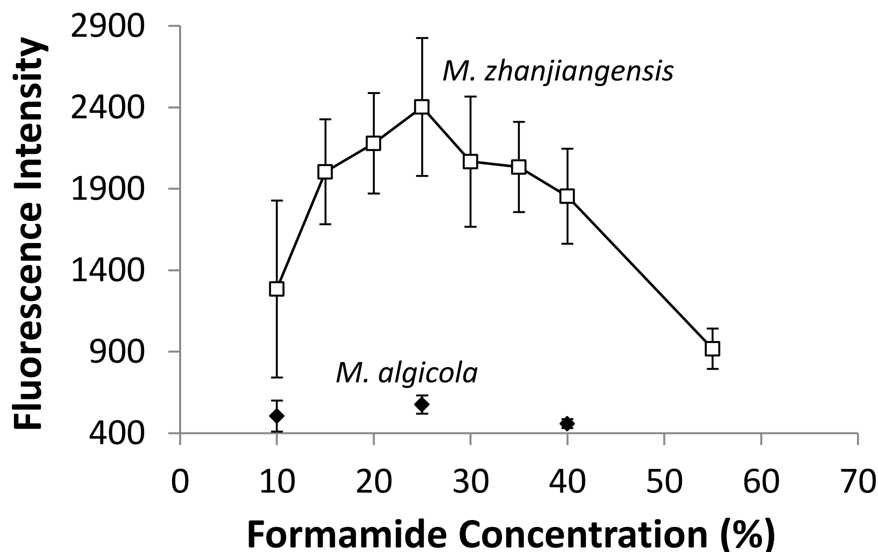
	0	1	2	3	4	5	10	21
1-6FAM **1-DAPI**	3.56 12.0	29.9 96.9	62.0 132	* 663 2200	759 2710	413 2260	254 2320	96.2 3570
2-6FAM **2-DAPI**	3.56 12.0	11.4 59.9	56.3 99.0	* 473 1650	384 1710	442 2020	457 2170	496 2480
3-6FAM **3-DAPI**	3.56 12.0	12.5 29.4	68.6 82.3	61.5 74.5	44.2 57.5	54.7 74.1	31.4 109	27.4 54.9
4-6FAM **4-DAPI**	3.56 12.0	7.13 20.7	35.1 40.8	7.83 20.0	115 122	141 153	9.26 20.7	12.1 30.3

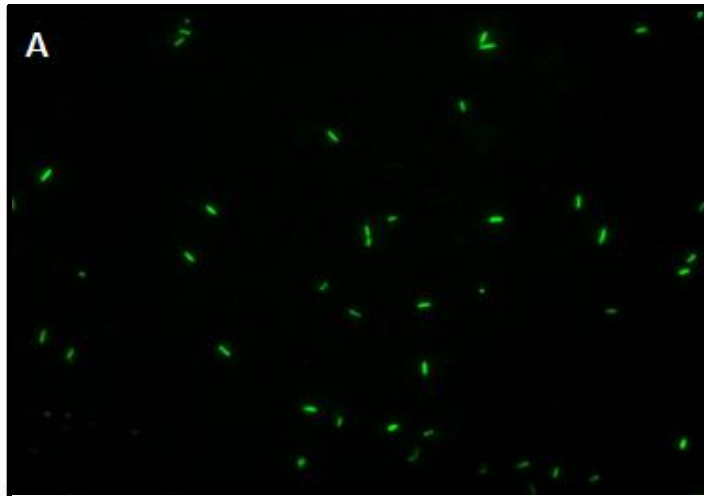

Marinobacter algicola^T (AY258110)*
Marinobacter salsuginis^T (EF028328)
Marinobacter flavimaris^T (AY517632)
Marinobacter sediminum^T (AJ609270)
Marinobacter goseongensis^T (EF660754)
Marinobacter lipolyticus^T (AY147906)
Marinobacter guineae^T (AM503093)
Marinobacter bryozoorum^T (AJ609271)$^\Sigma$
Marinobacter segnicrescens^T (EF157832)$^\Sigma$
Marinobacter lacisalsi^T (EU047505)
Marinobacter zhanjiangensis (FJ425903)*
Marinobacter gudaonensis^T (DQ414419)
Marinobacter excellens^T (AY180101)
Marinobacter litoralis^T (AF479689)
Marinobacter vinifirmus^T (DQ235263)
Marinobacter daepoensis^T (AY517633)
Marinobacter hydrocarbonoclasticus^T (X67022)
Marinobacter mobilis^T (EU293412)
Marinobacter zhejiangensis^T (EU293413)
Marinobacter koreensis^T (DQ325514)
Marinobacter santoriniensis^T (EU496088)
Marinobacter pelagius^T (DQ458821)
Marinobacter szutsaonensis^T (EU164778)
Marinobacter maritimus^T (AJ704395)
Marinobacter psychrophilus^T (DQ060402)
Marinobacter salicampi^T (EF486354)
Marinobacter lutaoensis^T (AF288157)
Pseudomonas aeruginosa^T (X06684)
Halomonas salina^T (AJ243447)
Halomonas sp. A–3 (AY914056)
Alcanivorax borkumensis^T (Y12579)
Cycloclasticus pugetii^T (U12624)
Colwellia maris^T (AB002630)
Roseobacter litoralis^T (X78312)
0.10
Targeted by:
Mrb-0625-a
Mrb-0625-b
Mrb-0625-c


(A) Probe Mrb-0625-a fluorescence
Fluorescence Intensity
1200
900
600
300
M. algicola
M. zhanjiangensis
0
10
20
30
40
50
60
70
Formamide Concentration (%)


(B) Probe Mrb-0625-b fluorescence
Fluorescence Intensity
1600
1300
1000
700
400
M. zhanjiangensis
M. algicola
0
10
20
30
40
50
60
70
Formamide Concentration (%)


(C) Probe Mrb-0625-a fluorescence
in presence of Mrb-0625-b
Fluorescence Intensity
800
700
600
500
400
M. algicola
M. zhanjiangensis
0
10
20
30
40
50
60
70
Formamide Concentration (%)


(D) Probe Mrb-0625-b fluorescence
in presence of Mrb-0625-a
Fluorescence Intensity
2900
2400
1900
1400
900
400
M. zhanjiangensis
M. algicola
0
10
20
30
40
50
60
70
Formamide Concentration (%)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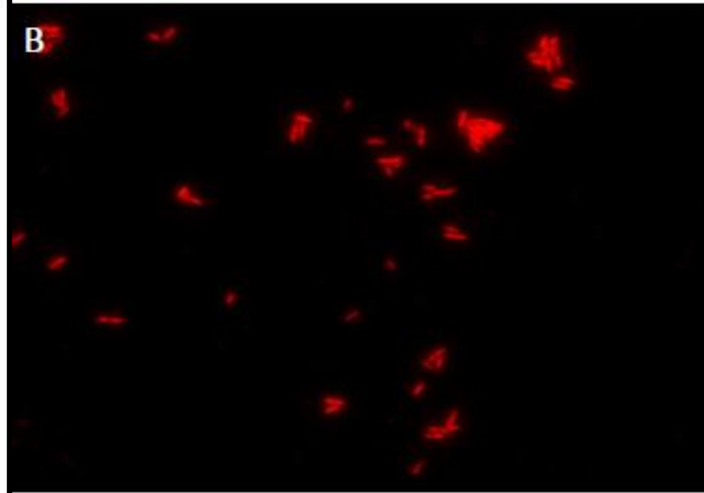
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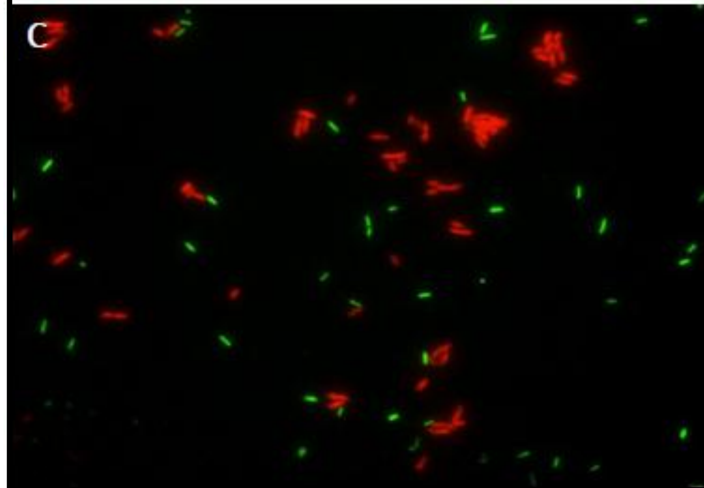
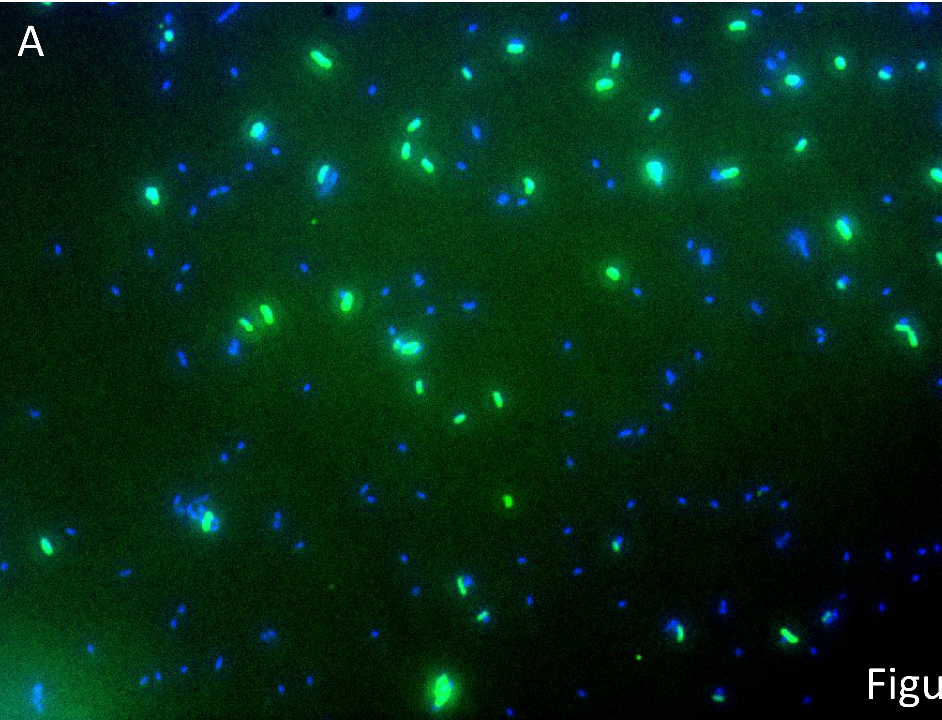

C



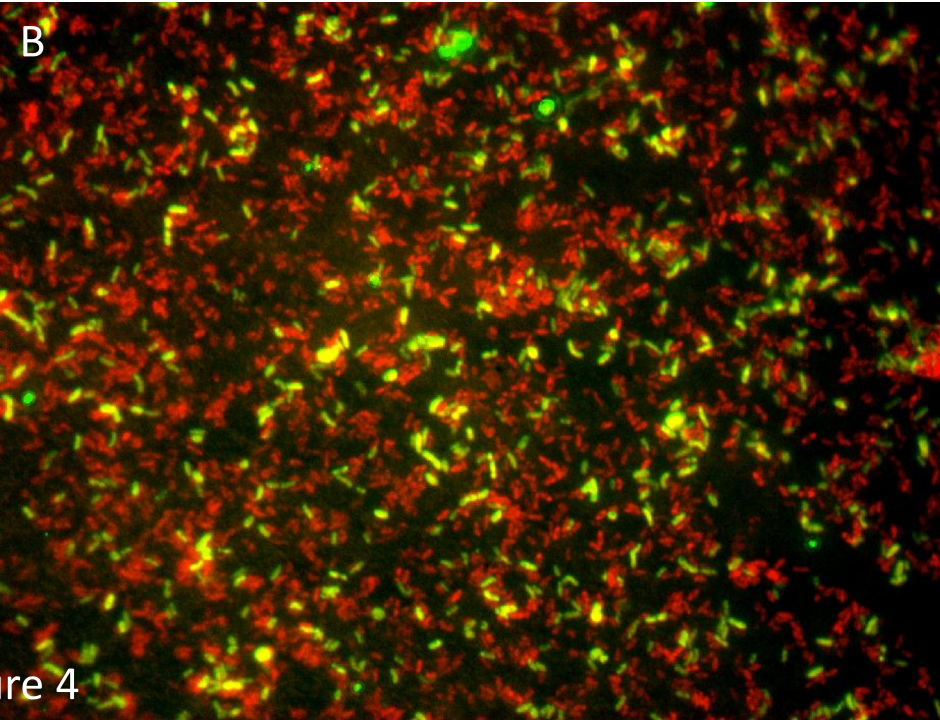
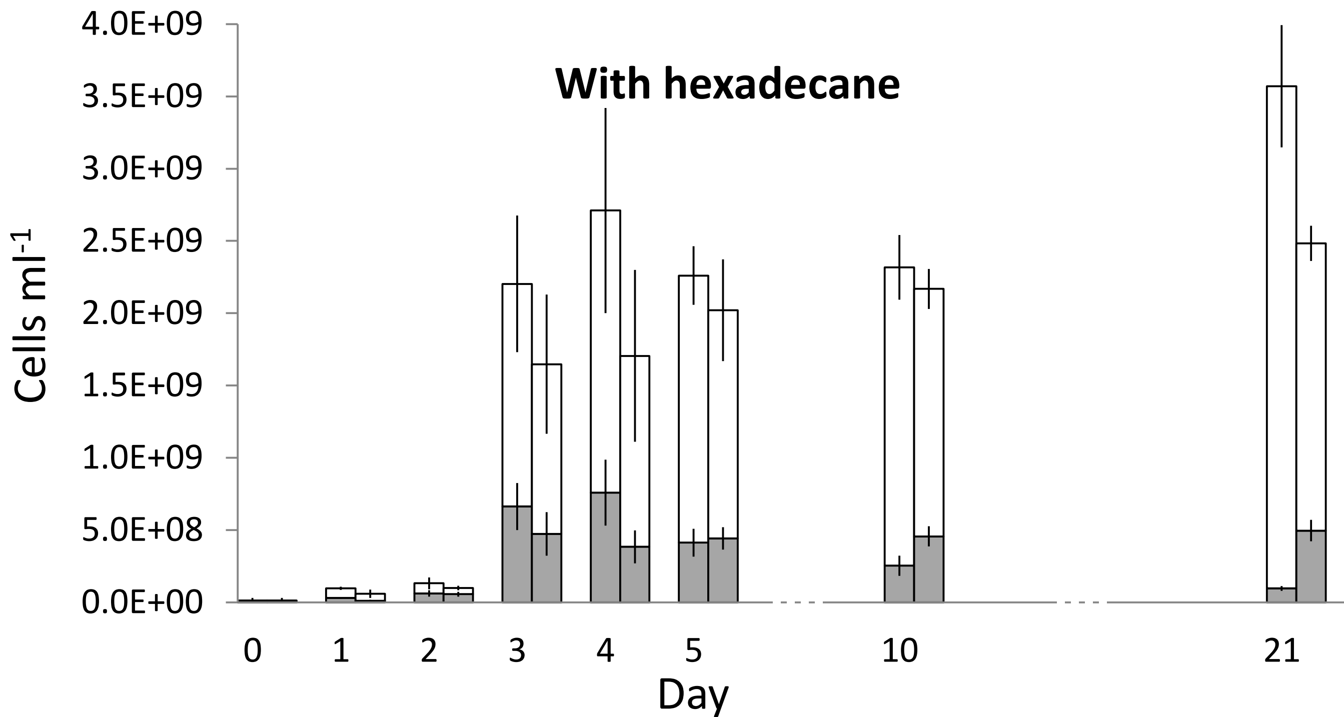
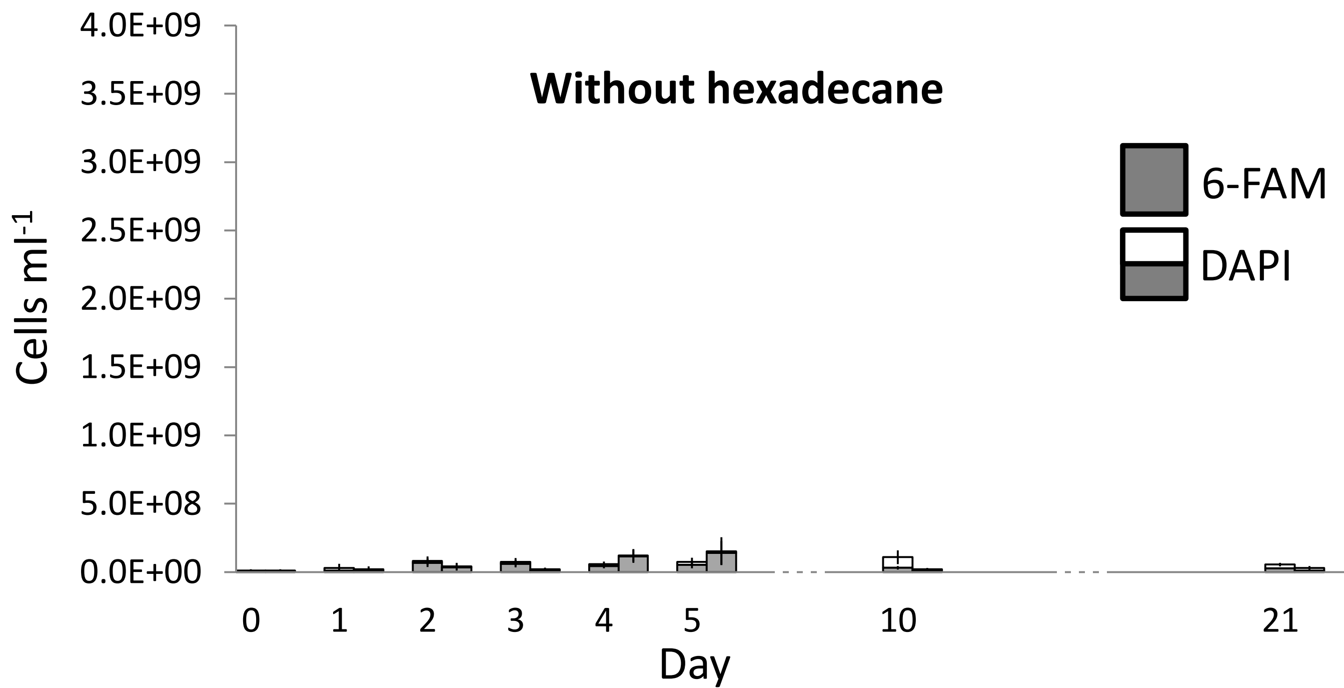


Figure 4


With hexadecane
Cells ml-1
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0
1
2
3
4
5
10
21
Day


Without hexadecane
Cells ml-1
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
0
1
2
3
4
5
10
21
Day
6-FAM
DAPI