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1 **Visualisation of the obligate hydrocarbonoclastic bacteria *Polycyclovorans***
2 ***algicola* and *Algiphilus aromaticivorans* in co-cultures with micro-algae by**
3 **CARD-FISH**

4
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20 Running title: Development of CARD-FISH probes

21

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23 probe, hydrocarbon degradation, phytoplankton, micro-algae, fluorescence *in situ* hybridisation

24

25 **Abstract**

26 Some studies have described the isolation and 16S rRNA gene sequence-based identification of
27 hydrocarbon-degrading bacteria living associated with marine eukaryotic phytoplankton, and thus
28 far the direct visual observation of these bacteria on micro-algal cell surfaces ('phycosphere') has
29 not yet been reported. Here, we developed two new 16S rRNA-targeted oligonucleotide probes,
30 PCY223 and ALGAR209, to respectively detect and enumerate the obligate hydrocarbonoclastic
31 bacteria *Polycyclovorans algicola* and *Algiphilus aromaticivorans* by Catalyzed Reporter
32 Deposition Fluorescence *in situ* Hybridisation (CARD-FISH). To enhance the hybridization
33 specificity with the ALGAR209 probe, a competitor probe was developed. These probes were
34 tested and optimized using pure cultures, and then used in enrichment experiments with
35 laboratory cultures of micro-algae exposed to phenanthrene, and with coastal water enriched with
36 crude oil. Microscopic analysis revealed these bacteria are found in culture with the micro-algal
37 cells, some of which were found attached to algal cells, and whose abundance increased after
38 phenanthrene or crude oil enrichment. These new probes are a valuable tool for identifying and
39 studying the ecology of *P. algicola* and *A. aromaticivorans* in laboratory and field samples of
40 micro-algae, as well as opening new fields of research that could harness their ability to enhance
41 the bioremediation of contaminated sites.

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48 **Introduction**

49 Obligate hydrocarbonoclastic bacteria (OHCB) are specialists with respect to their ability to
50 utilise hydrocarbons almost exclusively as a carbon and energy source. The occurrence of these
51 fastidious hydrocarbon degraders appears to be solely confined to the marine environment since,
52 to the best of our knowledge, they are found nowhere else on earth. They play an important role
53 in the removal and mineralization of hydrocarbon pollutants in the oceans and seas, as is
54 evidenced in the wealth of reports documenting their enrichment from near undetectable
55 abundance levels (<0.1%) to constituting up to 90% of the total bacterial community at oil-
56 impacted sites (Head *et al.*, 2006; Yakimov *et al.*, 2007). With the exception of *Planomicrobium*
57 *alkanoclasticum* (a Gram-positive Firmicute), most of the recognised OHCB are classified within
58 the order *Oceanospirillales* of the class *Gammaproteobacteria*, and comprise members of the
59 genera *Alcanivorax*, *Oleiphilus*, *Oleispira*, *Oleibacter* and *Thalassolitus* that specialize in the
60 degradation of linear or branched saturated hydrocarbons, whereas two genera, *Cycloclasticus*
61 and *Neptunomonas*, specialize in the degradation of polycyclic aromatic hydrocarbons (PAHs).
62 The detection, identification and monitoring of these types of organisms in a highly-resolved
63 manner and within samples collected *in situ* is highly advantageous to expand our knowledge on
64 their geography and ecology.

65 The phycosphere of marine eukaryotic phytoplankton is an underexplored biotope in the
66 ocean where OHCB and ‘generalist’ oil-degrading bacteria have been identified. Hydrocarbon-
67 degrading bacteria, including some comprising novel taxa, have been isolated from all three
68 major phytoplankton lineages of dinoflagellates, diatoms and coccolithophores (Green *et al.*,
69 2004; Green *et al.*, 2006; Green *et al.*, 2015; Gutierrez *et al.*, 2012a,b; 2013; 2014). This algal-
70 bacterial association raises important questions regarding the interplay and ecology between these
71 organisms, what contribution this has to the biodegradation of hydrocarbons in the ocean, and to
72 their evolutionary genesis. Enhanced degradation of hydrocarbons has been demonstrated when
73 bacteria and phytoplankton coexist (e.g., Abed and Köster, 2005; Warshawsky *et al.*, 2007), and

74 more recent work has shown that the bacterial community associated with phytoplankton may be
75 better tuned to respond to and degrade hydrocarbons when challenged with crude oil
76 (Mishamandani *et al.*, 2016; Thompson *et al.*, 2017). Whether through biogenic synthesis
77 (Andelman and Suess, 1970; Gunnison and Alexander, 1975; Zelibor *et al.*, 1988; Marlowe *et al.*,
78 1984; Shaw *et al.*, 2010; Exton *et al.*, 2012) or adsorption of hydrocarbon molecules from the
79 surrounding seawater (Mallet and Sarfou, 1964; Andelman and Suess 1970), phytoplankton cells
80 can be regarded as a “hot spot” to which hydrocarbon-degrading bacteria are attracted to and,
81 through evolution, have settled into a state of co-existence.

82 Two species of known OHCB representing a novel genus (*Polycyclovorans*) and family
83 (*Algiphilus*) were originally isolated from eukaryotic phytoplankton, but are poorly represented in
84 16S rRNA gene sequence databases (Gutierrez *et al.*, 2012a; Gutierrez *et al.*, 2013). The type
85 species of these two genera, *Algiphilus aromaticivorans* and *Polycyclovorans algicola*, were
86 respectively isolated from the dinoflagellate *Lingulodinium polyedrum* (Gutierrez *et al.*, 2012a)
87 and the cosmopolitan marine diatom *Skeletonema costatum* (Gutierrez *et al.*, 2013). Both
88 organisms belong within the order *Xanthomonadales* and found to exhibit a narrow nutritional
89 spectrum, preferring to utilise mono- and polycyclic aromatic hydrocarbons (PAHs) and some *n*-
90 alkanes and organic acids. Using quantitative PCR primers designed to target the 16S rRNA gene
91 of *P. algicola* strain showed that this organism is also associated with other species of marine
92 diatoms and also dinoflagellates (Gutierrez *et al.*, 2013) and coccolithophores (unpublished data).
93 These organisms were identified in these studies because they were targeted based on their ability
94 to utilise hydrocarbons as a sole source of carbon and energy, and because they were searched for
95 in the right place – i.e. in cultures of marine eukaryotic phytoplankton. When isolating for these
96 types of organisms in seawater samples, in the absence of methods that could allow researchers to
97 discern if they are free living or associated with particulate matter (e.g. transparent exopolymer,
98 marine snow, phytoplankton, etc.), the common mistake is to assume these organisms exist in a
99 free-living state. In order to improve our understanding on the ecology of hydrocarbon-degrading

100 bacteria found associated with phytoplankton and their role in the event of oil contamination in
101 the marine environment, it is imperative to have a method that can identify and monitor the
102 abundance of these organisms in environmental samples.

103 CARD-FISH (Catalyzed Reporter Deposition Fluorescence *in situ* Hybridisation) is an
104 effective technique allowing phylogenetic identification, enumeration, and direct spatial
105 visualization of microorganisms in their natural environment by targeting the ribosomal RNA
106 (rRNA) of bacterial cells of interest (Pernthaler *et al.*, 2002; Schönhuber *et al.*, 1999). CARD-
107 FISH is an *in situ* amplification method utilizing horseradish peroxidase, which enhances
108 bacterial cell detection over standard FISH protocols that can be several orders of magnitude less
109 sensitive. The value of this method is in its ability to allow the observer to visualize the cells
110 being targeted by the probe(s) in natural (*in situ*) environmental samples. Genus-level
111 oligonucleotide probes were previously developed to identify members of the OHCB *Alcanivorax*
112 (Syutsubo *et al.*, 2001), *Cycloclasticus* (Maruyama *et al.*, 2003) and *Marinobacter* (Mckay *et al.*,
113 2016) by conventional FISH. CARD-FISH has been used to detect epiphytic bacteria on algae
114 (Mayali *et al.*, 2011; Simon *et al.*, 2002; Tujula *et al.*, 2005), although this method has not,
115 hitherto, been used to explore the occurrence of hydrocarbon-degrading bacteria associated with
116 eukaryotic phytoplankton. In this study, we developed and optimized new probe sets to detect *A.*
117 *aromaticivorans* and *P. algicola* by CARD-FISH. We then used these probes to examine the
118 presence and spatial association of these organisms with laboratory cultures of phytoplankton.

119

120 **Materials and methods**

121 **Oligonucleotide probe design**

122 Oligonucleotide probes targeting *Polycyclovorans algicola* or *Algiphilus aromaticivorans* were
123 designed against current 16S rRNA gene sequence databases. Using the Probe Design tool of Arb
124 v104 (Pruesse *et al.*, 2007), probe candidates were selected based on specific targeting of the 16S
125 rRNA sequences for these two species. Probe candidates were analysed using the ProbeCheck

126 server (Loy *et al.*, 2008), the Ribosomal Database Project's Probe Match tool (Cole *et al.*, 2009),
127 and the ARB-Silva TestProbe tool (Ludwig *et al.*, 2004) to evaluate their *in silico* specificity and
128 coverage. From this, one probe sequence, PCY223 (5'-TCA GAC ATA GGC TCC TCC AA-3';
129 20-mer) was selected for *Polycyclovorans algicola*, and the probe sequence ALGAR209 (5'-CCT
130 CCA GCG TGA GGT CCG-3'; 18-mer) was selected for *Algiphilus aromaticivorans*. A
131 competitor probe, c1ALGAR209 (5'-CCT CCA GCG CGA GGT CCG-3'; 18-mer), was
132 designed to improve signal intensities in hybridisations targeting *Algiphilus aromaticivorans*.
133 Table 1 summarizes the probes that were developed in this study. *In silico* analysis of both probes
134 using the SILVA 16S rRNA gene database revealed 13 uncultivated clone sequences with 100%
135 sequence match to the PCY223 probe, and 219 sequences with a 1-basepair non-weighted
136 mismatch. For the ALGAR209 probe, however, only 18 sequences with a 1-basepair non-
137 weighted mismatch were found to uncultivated clones.

138

139 **Oligonucleotide probe optimization**

140 In order to test and optimize the CARD-FISH probes, pure cultures of *Polycyclovorans algicola*
141 and *Algiphilus aromaticivorans* were grown in a defined synthetic seawater medium, ONR7a
142 (Dyksterhouse *et al.*, 1995), that was supplemented after steam-sterilization with filter-sterile (0.2
143 µm) trace elements and vitamins to final concentrations as previously described (Blackburn *et al.*,
144 1989). Cells were permeabilized by incubation in lysozyme buffer (1.355 × 10⁶ U ml⁻¹ lysozyme,
145 50 mM EDTA [pH 8.0], 300 mM Tris-HCl [pH 8.0]) at 37°C for 2 h. The slides were washed in
146 water for 1 min, incubated in 0.01M HCl for 10 min to bleach endogenous peroxidases, and then
147 washed again in water for 1 min and air dried.

148 **Table 1.** 16S rRNA-targeted oligonucleotide FISH probes developed in this study.

149

Probe name	Probe full name ¹	Sequence 5'-3'	Coverage ²	Specificity ²	Target organism	Optimal [FA]
PCY223	S-S-PCY223-a-A-20	TCA GAC ATA	Genus	0	<i>Polycyclovorans</i>	55-60%
		GGC TCC TCC AA	<i>Polycyclovorans</i> (22%)		<i>algicola</i> (FJ176554)	
ALGAR209	S-S-ALGAR209-a-A-18	CCT CCA GCG	Genus	0	<i>Algiphilus</i>	35-40%
		TGA GGT CCG	<i>Algiphilus</i> (33%)		<i>aromaticivorans</i> (DQ486493)	
c1ALGAR209	S-S-c1ALGAR209-a-A-18	CCT CCA GCG			Competitor for	
		CGA GGT CCG			ALGAR209	

150

151 ¹According to Alm et al. (1996)

152 ²*In silico* coverage (% of target taxon) and specificity (number of matched sequences outside the target taxon for cultivated strains) were evaluated

153 using the TestProbe tool of ARB Silva with default settings (0 mismatches) and database SSU132RefNR.

154

155

156 Optimal conditions for hybridization with these probes were determined by performing
157 multiple hybridisations using increasing formamide (FA) concentrations from 0% to 70%. The
158 competitor probe c1ALGAR209 was always used in equimolar concentration with its respective
159 probe ALGAR209. Hybridization buffers contained 900 mM NaCl, 20mM Tris-HCl (pH 8), 10%
160 (w/v) dextran sulfate (Sigma-Alrich), 0.01% (w/v) sodium dodecyl sulphate and 10% blocking
161 solution. The blocking solution consisted of 10% blocking reagent (Perkin Elmer) and maleic
162 acid buffer (1.16% [w/v] maleic acid, 150 mM NaCl, pH 7.5). The washing buffer consisted of
163 NaCl (variable concentration to maintain hybridization stringency), 20 mM Tris-HCl (pH 8), 5
164 mM EDTA (pH 8) and 0.01% (w/v) SDS. Excess washing buffer was removed with blotting
165 paper. The samples were incubated for 20 min with PBS (~10 ml) and then at 46°C for 30 min
166 with 1 part fluorescently-labelled tyramide and 499 parts amplification buffer (10% [v/v]
167 blocking solution, 2 M NaCl, 10% [w/v] dextran sulfate, 0.0015% [v/v] H₂O₂, 100% [v/v] PBS
168 [pH 7.3]). Samples were then washed in PBS (pH 7.3) for 20 min, followed by three washes with
169 water for 1 min and then 96% ethanol for 3 sec before air-drying. The samples were stored at -
170 20°C for subsequent microscopic analysis.

171 Samples were covered in mountant (80% [v/v] Citifluor, 14% [v/v] Vectashield, 1 µg ml⁻¹
172 DAPI in 100% PBS [pH 9]) for visualisation using a Zeiss (Axio Scope.A1) epifluorescence
173 microscope equipped with a Zeiss digital fluorescence imaging camera (AxioCam MRm).
174 Amplified signal intensities were quantified using Zeiss Zen-Blue (2012) imaging analysis
175 software. Six to eight fields of view (~300 – 500 bacterial cells) were counted for fluorescence
176 intensity maximum quantification.

177

178 ***Polycyclovorans algicola* and *Algiphilus aromaticivorans* enrichment setup using laboratory**
179 **micro-algal cultures**

180 To directly visualize the association of *Polycyclovorans algicola* strain TG408 and *Algiphilus*
181 *aromaticivorans* strain DG125 with micro-algal cells, and to assess their response when exposed
182 to phenanthrene, a time-series enrichment incubation was setup. The marine algal strains used
183 were non-axenic laboratory cultures of the cosmopolitan diatom *S. costatum* strain CCAP
184 1077/1C and the dinoflagellate *Lingulodinium polyedrum* strain CCAP 1121/2. These micro-
185 algae were used because they were the source in the original isolation of *Polycyclovorans*
186 *algicola* strain TG408 and *Algiphilus aromaticivorans* strain DG125, respectively (Gutierrez *et*
187 *al.*, 2012a; Gutierrez *et al.*, 2013). Both strains were purchased from the Culture Collection of
188 Algae and Protozoa (CCAP; Oban, Scotland) and maintained in f/2 + Si algal medium (Guillard,
189 1975; Guillard and Ryther, 1962) in a temperature-controlled 12°C illuminated incubator.

190 Enrichments were prepared using twelve steam-sterilised 100-ml glass Erlenmeyer flasks
191 capped with cotton bungs, each containing 49 ml of f/2 + Si medium. Six of the flasks were
192 supplemented with phenanthrene (200 µg per flask) using acetone as carrier, and the acetone
193 allowed to volatilize overnight prior to adding the f/2 + Si medium. The phenanthrene was
194 allowed to equilibrate with the f/2 + Si medium for 3 days at 16°C prior to inoculation. Three of
195 these flasks were inoculated with 1 ml of an *S. costatum* culture grown to the exponential phase,
196 whereas the other three flasks were inoculated in the same way with *L. polyedrum*. The other six
197 flasks containing f/2 + Si medium, but no phenanthrene, were inoculated in the same way – three
198 with *S. costatum* and three with *L. polyedrum*. All twelve flasks were incubated in a temperature-
199 controlled illuminated incubator with gentle shaking, at 16°C, with a 12:12 light/dark cycle and at
200 a photon flux density of 150 µmol s⁻¹ m⁻²). At days 0, 3, 5 and 8, sub-samples (0.5 ml) were taken
201 from each flask and fixation performed by mixing with 3% (v/v) paraformaldehyde for 3 hours at
202 4°C on Isopore polycarbonate filters (0.22 µm). Filters were washed three times with sterile ice-
203 cold 1× PBS, air-dried and stored at 4°C for subsequent analysis within 48 hours.

204

205 **CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus aromaticivorans* enrichments**
206 **with micro-algae**

207 Sub-samples fixed onto polycarbonate filters from the time-series incubations enriched with or
208 without phenanthrene were dipped into 0.2% (w/v) agarose, then mounted onto glass slides and
209 air dried following standard methods (Pernthaler *et al.*, 2002). CARD-FISH was performed
210 directly on the agarose-embedded samples using the optimized protocol developed for the
211 PCY223 or ALGAR209 probes at a FA concentration of, respectively, 55% or 40%, with the
212 exception that 50–100 µl of hybridization buffer were used. All hybridisations were
213 counterstained with DAPI following standard methods (Porter and Feig, 1980) prior to
214 visualization under the epifluorescence microscope and analysed using Zeiss Zen-Blue image
215 processing software, as described above.

216

217 **Field sample collection and CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus***
218 ***aromaticivorans* in natural communities of phytoplankton**

219 During a sampling operation aboard *RV Serpula* on 24 May 2013 to a sampling station in the
220 lower basin of Loch Creran (56°30.820N, 5°22.817W) located on the west coast of Scotland, a
221 phytoplankton net (50-60 µm mesh size) was trawled near the sea surface (1–2 m depth) for
222 several minutes to collect the phytoplankton community. The phytoplankton net sample was
223 passaged through a 125-µm metal mesh filter to remove grazers, washed by gentle centrifugation
224 with sterile synthetic seawater, and then stored at 4°C and used within 24 hours for the
225 preparation of a crude oil enrichment experiment, as previously described (Thompson *et al.*,
226 2017). Briefly, 500 ml of the washed phytoplankton sample, with its associated bacterial
227 community, was added to 10 litres of filtered (0.2 µm) seawater. This was used to set up a crude
228 oil enrichment by adding 350 ml of the phytoplankton suspension into 1-litre Erlenmeyer flasks
229 (performed in triplicate) and then amended with filter-sterilised (0.2 µm) Heidrun crude oil (ca.

230 1% v/v final concentration). A control treatment was set up in the exact same way, with the
231 exception that the oil was not added. The flasks were incubated in a temperature-controlled 15°C
232 illuminated incubator with a 12:12 light/dark cycle (photon flux density ~100 $\mu\text{mol s}^{-1} \text{m}^{-2}$).
233 Samples for CARD-FISH analysis with probes PCY223 and ALGAR209 were taken at days 2
234 and 5. The dynamics of the phytoplankton and bacterial community in these treatments, and
235 results for hydrocarbon analysis, are reported elsewhere (Thompson *et al.*, 2017).

236

237 **Probes, chemicals and reagents**

238 Probes ALGAR209 and PCY223 for CARD-FISH labelled with HRP at the 5' end were
239 purchased from Biomers and Thermo Fisher Biopolymers, respectively. The competitor probe
240 (c1ALGAR209) without HRP labelling, was purchased from Integrated DNA Technologies.
241 Cy3-labelled tyramides were purchased from Perkin Elmer. The phenanthrene (>99.5% purity)
242 was purchased from Sigma-Aldrich. All other chemicals were of molecular biology or HPLC
243 grade.

244

245 **Results and discussion**

246 ***P. algicola* and *A. aromaticivorans* phylogeny and probe design**

247 At the time of analysis, the PCY223 probe sequence was found to be 100% complementary to 43
248 *Gammaproteobacteria* and to one unclassified *Proteobacteria*. These 43 *Gammaproteobacteria*
249 are affiliated to the family *Sinobacteriaceae* and comprise uncultivated clones, with the exception
250 of 1 unclassified isolate designated strain DG1192 (EU052753) and the target organism *P.*
251 *algicola* TG408. A previously reported (Gutierrez *et al.*, 2013), 16S rRNA gene sequence
252 analysis showed strain TG408 is affiliated with the family *Sinobacteraceae*, and further
253 supported by the strain's DNA G+C content of 64.3 mol%, which is similar to that of most
254 members of the *Sinobacteraceae* family (60–65 mol%).

255 Analysis of the ALGAR209 probe sequence showed it was 100% complementary to
256 solely the target organism, *A. aromaticivorans* DG1253; it showed no match to any other
257 cultivated strains or uncultivated clones or sequences. The phylogenetic position of this organism
258 lies closest to the family *Sinobacteraceae*, but is poorly supported (low bootstrap value of <50%)
259 and indicates the strain is only moderately affiliated with this family (confidence threshold of
260 <89%) (Gutierrez *et al.*, 2012a). Hence, the phylogenetic position of the strain in the order
261 *Xanthomonadales* is unique, distinct, and probe ALGAR209 was successfully designed to target
262 solely this organism.

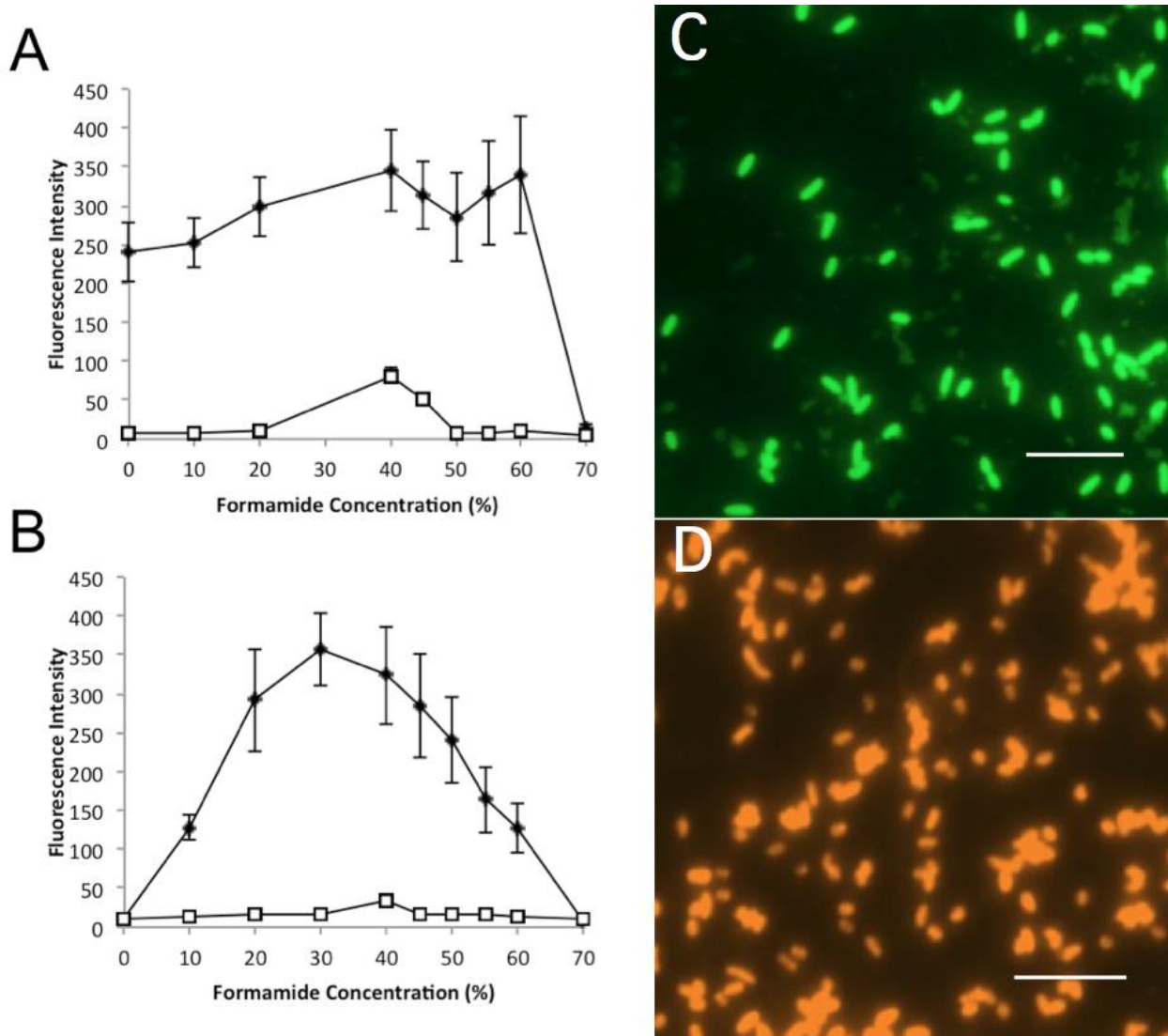
263

264 **PCY223 and ALGAR209 probe optimization**

265 The hybridization conditions for probes PCY223 and ALGAR209 were optimised by
266 hybridisations with the target strain (Figure 1A and 1B). As expected, the fluorescence intensity
267 for the two target strains was greater than that employing the nonsense probe over a wide range
268 of formamide concentrations. In the case of probe PCY223, fluorescence signal intensities
269 dramatically decreased at formamide concentrations higher than 60%. The formamide series
270 experiments indicated that concentrations of 40-60% would be suitable for use with this probe.
271 Whilst a 60% formamide concentration appears as the ideal stringency for probe PCY223, this
272 concentration, however, is at the maximal point where specificity of the probe dramatically
273 decreases with higher formamide concentrations. Therefore, 55% was selected as the empirically
274 optimized formamide concentration for this probe.

275 For the formamide series experiments with probe ALGAR209, highest fluorescence
276 signal intensities occurred with formamide concentrations between 20-40%. Decay of the
277 fluorescence signal occurred rapidly with formamide concentrations <20% and >40%. An
278 optimised formamide concentration of 40% was, hence, selected that would be at the high end of
279 stringency and still produce relatively strong fluorescence signals. Use of these optimised
280 formamide concentrations for probe PCY223 (55% FA) and for ALGAR209 (40% FA) were

281 tested in subsequent hybridization experiments using pure cultures for each of their target strains
282 – respectively, *P. algicola* TG408 (Fig. 1C) and *A. aromaticivorans* DG1253 (Fig. 1D) – and as
283 expected showed strong cell signal intensities for both probes.
284



285
286 **Figure 1.** Dissociation profiles of 16S rRNA-targeted probes PCY223 and ALGAR209 for
287 CARD-FISH evaluated against the perfectly-matching (target) strains *P. algicola* and *A.*
288 *aromaticivorans*, respectively, over a range of formamide concentrations (%). Black diamonds
289 represent hybridization intensities for *P. algicola* (A) or *A. aromaticivorans* (B) with their
290 respective probes. White squares correspond to hybridization intensities for these same
291 organisms, but using the NON338 probe. These probes were labeled with CY3 in all experiments.

292 Each data point represents the average fluorescence intensity value \pm standard deviation up to
293 eight randomly-selected fields of view. In some cases the standard deviation is smaller than the
294 symbol. Images of the target cells were captured from hybridisations performed with probe
295 PCY223 (C) or ALGAR209 (D) using the formamide concentration determined optimal for each
296 probe – 55% for PCY223 and 40% for ALGAR209. The competitor probe c1ALGAR20 was
297 included together in hybridisations with ALGAR209. Bar, 4 μ m.

298

299 **Detection and response of *P. algalicola* and *A. aromaticivorans* in micro-algal cultures** 300 **enriched with phenanthrene**

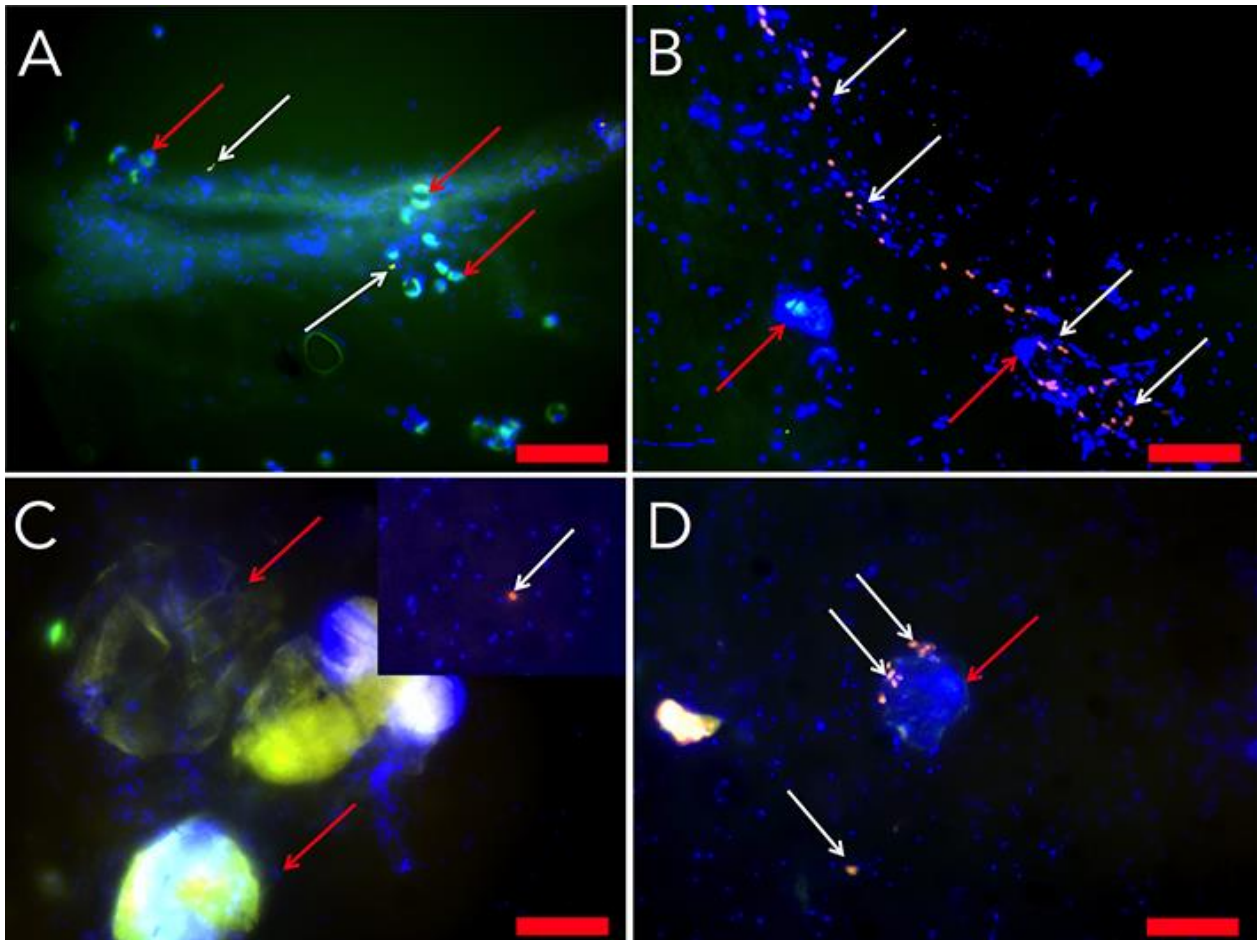
301 In the present study the aim was to visualize, for the first time, the presence of these OHCB on
302 the surface (phycosphere) of micro-algae. The localization of the hydrocarbonoclastic bacterial in
303 relation to their micro-algal hosts and during petroleum hydrocarbon enrichment may be
304 important factors governing the relationship of these organisms. The CARD-FISH probes
305 designed and optimized for the detection of *P. algalicola* and *A. aromaticivorans* were used to
306 directly visualize these bacteria in association with marine eukaryotic phytoplankton (micro-
307 algae), which is likely their natural biotope in the ocean. For this, micro-algal cultures *S.*
308 *costatum* and *L. polyedrum* were used as they represent the original source from which *P.*
309 *algalicola* and *A. aromaticivorans*, respectively, were isolated (Gutierrez *et al.*, 2012a; Gutierrez *et*
310 *al.*, 2013). Subsamples taken prior to and during enrichment were analysed in order to examine
311 the response of these bacteria to phenanthrene, which was shown as a preferred carbon source for
312 these obligate hydrocarbon-degrading bacteria (Gutierrez *et al.*, 2012a; Gutierrez *et al.*, 2013)
313 and considered a model hydrocarbon to enrich for these organisms.

314 In the enrichment employing *S. costatum*, subsamples analysed with Cy3-labelled
315 PCY223 revealed an extremely low abundance of *P. algalicola*, with either none or one to three
316 cells detected in any of the fields of view analysed (Figure 2A). By day 5, the abundance of *P.*
317 *algalicola* cells quantified with probe PCY223 had increased as a result of enrichment with

318 phenanthrene, with *P. algalicola* signals observed attached with, and detached from, the diatom
319 (Figure 2B). Hybridisations performed with subsamples taken at day 8 (results not shown) did not
320 noticeably differ to those examined at day 5. In the enrichment employing *L. polyedrum*,
321 subsamples analysed with Cy3-labelled ALGAR209 also revealed a very low abundance of *A.*
322 *aromaticivorans* cells, none of which were observed attached to dinoflagellate cells in any of the
323 fields of view analysed (Figure 2C). By day 3, the abundance of these bacteria, as quantified with
324 probe ALGAR209, was observed to have moderately increased as a result of enrichment with
325 phenanthrene (results not shown), and then markedly increased by day 8 (Figure 2D). Signals for
326 *A. aromaticivorans* cells were observed attached to cells of *L. polyedrum* (Figure 2D), and in
327 some fields of view some cells were found associated with clusters of other (DAPI-stained)
328 bacteria. After incubation with phenanthrene, cells of the hydrocarbonoclastic bacteria in both the
329 *L. polyedrum* and *S. costatum* cultures appeared to be arranged in clusters (or arranged in
330 streaks), where previously (on day 0) they were present as unattached single cells, sometimes
331 appearing near micro-algal cells. These micro-aggregate (<5 – 500 µm) arrangements of bacteria
332 and micro-algae have also been reported in the literature (Simon *et al.*, 2002). The distribution of
333 these OHCB appears uneven among the rest of the DAPI-stained bacterial community,
334 suggesting that these bacteria are possibly either aggregating towards a hydrocarbon source that
335 may be adsorbed onto the micro-algal cells and/or are being held together by some transparent
336 extracellular polymer (as proposed by Thornton, 2002).

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341 **Figure 2.** Hybridisation of samples from a culture of *Skeletonema costatum* (A, B) or

342 *Lingulodinium polyedrum* (C, D) enriched with phenanthrene using, respectively, probe PCY223

343 targeting *P. algicola* or probe ALGAR209 (and competitor probe cALGAR209) targeting *A.*

344 *aromaticivorans* (orange) among cells of the diatom/dinoflagellate and other bacteria (blue).

345 Samples were taken for analysis on day 0 (A, C), day 5 (B) or day 8 (D). Cells of *P. algicola* and

346 *A. aromaticivorans* (ca. 1 µm size) are indicated with white arrows in the panels, whereas cells of

347 *S. costatum* cells (ca. 10 µm) or *Lingulodinium polyedrum* (ca. 30 µm) are indicated with red

348 arrows. Bar, 20 µm.

349

350 Overall, in both enrichments with the *S. costatum* and *L. polyedrum* cultures, most fields

351 of view rarely revealed any signals for, respectively, *P. algicola* and *A. aromaticivorans*. This

352 indicates that these bacteria are in very low abundance to the total bacterial composition

353 associated with these micro-algae. The observed attachment of some cells for both types of
354 bacteria to their micro-algal host cells infers that they may share more than just a physical
355 attachment, as reported for other types of bacteria living associated with micro-algae. The
356 interaction of micro-algae with bacteria in the ocean is believed to play an important role to their
357 ecological success (Amin *et al.*, 2012; Amin *et al.*, 2015; Buchan *et al.*, 2014). Algal-associated
358 bacteria have been shown to acquire algal exudates as carbon and energy sources (Bell and
359 Mitchell, 1972; Mykkestad, 1995), where the algae have been reported to benefit from bacterial-
360 produced vitamins, trace metals and other nutrients (Kazamia *et al.*, 2012; McGenity *et al.*,
361 2012). In a study by Amin *et al.* (2009), the authors reported a mutual sharing of iron and fixed
362 carbon between several species of phytoplankton and bacteria, including members comprising the
363 hydrocarbon-degrading genus *Marinobacter*. Taking into account the narrow nutritional spectrum
364 of strains TG408 and DG1253 living associated with micro-algae (Gutierrez *et al.*, 2012a;
365 Gutierrez *et al.*, 2013), it is possible that they too may co-exist through some form of nutrient-
366 sharing, though further work will be needed to determine this.

367

368 **Detection and response of *P. algalicola* and *A. aromaticivorans* in natural seawater** 369 **populations of phytoplankton enriched with crude oil**

370 The enrichment and spatial localization of *P. algalicola* and *A. aromaticivorans* was investigated in
371 a natural population of phytoplankton from coastal water. Since these obligate
372 hydrocarbonoclastic bacteria were originally isolated and shown to live associated with
373 laboratory cultures of micro-algae (Gutierrez *et al.*, 2012a; Gutierrez *et al.*, 2013), the application
374 of CARD-FISH employing the new probes (PCY223 and ALGAR209) allowed us to observe
375 these bacteria in natural seawater samples, including associated with phytoplankton cells. This
376 was examined in enrichments with and without crude oil in order to detect for these bacteria
377 under, respectively, perturbed and unperturbed environmental conditions. Samples taken at days
378 0 (results not shown) and 2 of these enrichment experiments and analysed with probes PCY223

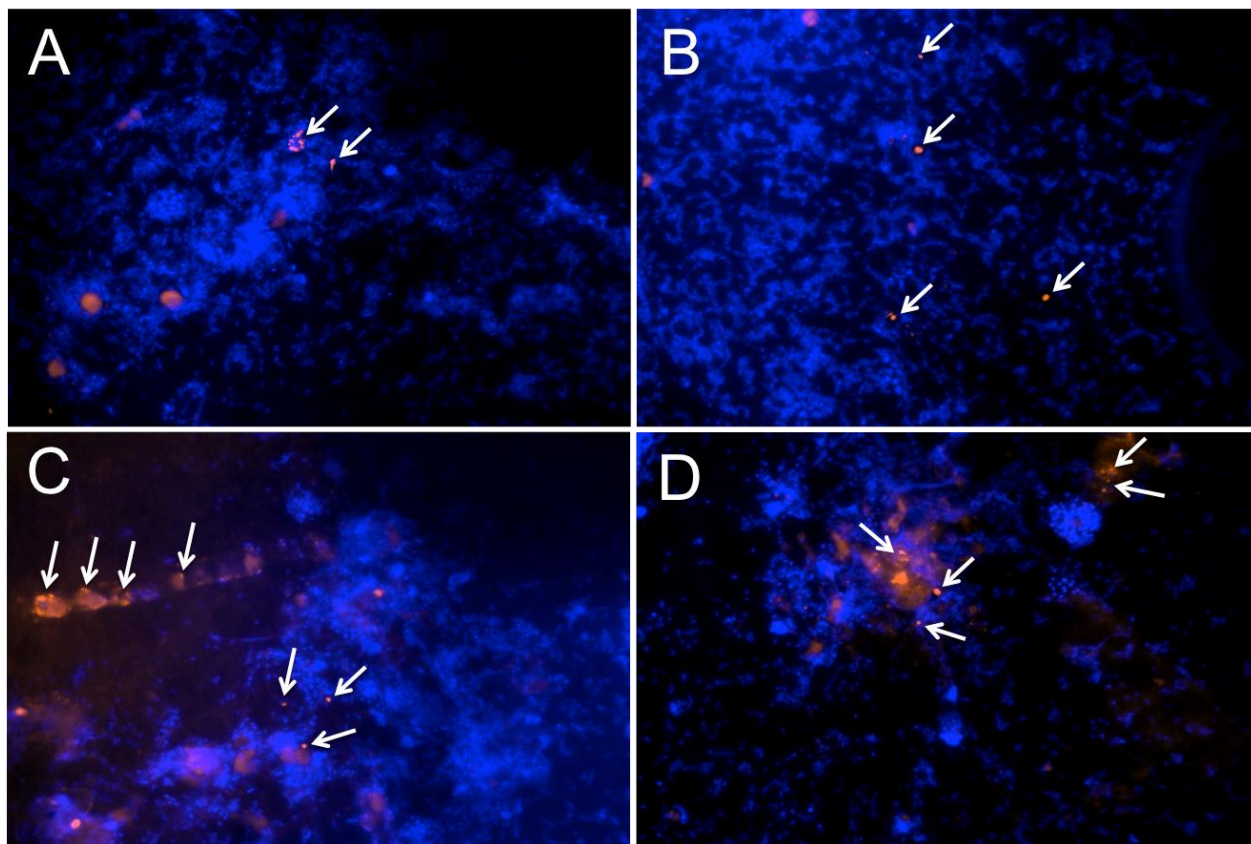
379 or ALGAR209 rarely revealed signals for *P. algicola* (Figure 3A) or *A. aromaticivorans* (Figure
380 3C), respectively, but were nonetheless detected in the field sample. In samples taken at day 5,
381 however, cell signals for these bacteria were slightly more abundant, though still rare to find in
382 the many fields of view observed under the microscope (Figure 3B and D). At this time point,
383 cells of *P. algicola* and *A. aromaticivorans* were observed among the free-living bacterial
384 population that was counterstained with DAPI, as well as attached to cells of micro-algae,
385 including cells of *Skeletonema* spp., likely *S. costatum*. It was apparent that the crude oil had
386 enriched for these bacteria, but to heightened levels of cell abundance that were still very low
387 compared to their abundance in the original unperturbed phytoplankton field sample. These
388 results corroborate those from our analysis of these bacteria in laboratory cultures of micro-algae
389 where very few cell signals had been detected, and where the abundance of these bacteria
390 appeared to have only slightly increased after enrichment with phenanthrene.

391 Although the primary objective of these enrichment experiments was to test the newly
392 designed PCY223 and ALGAR209 probes, and to directly visualize the target organisms (*P.*
393 *algicola* and *A. aromaticivorans*) with micro-algae, our data offers some insight into the ecology
394 and dynamics of these obligate hydrocarbon-degrading bacteria during exposure to a
395 hydrocarbon. In a previous study, qPCR probes were used to quantify the abundance of *P.*
396 *algicola* 16S rRNA gene sequences during enrichment of *S. costatum* CCAP 1077/1C – the same
397 strain used in the present study – with crude oil, and showed these genes to have increased by one
398 order of magnitude within eight days of exposure to the oil (Mishamandani *et al.*, 2016). This
399 corroborates our CARD-FISH analysis with the PCY223 probe that showed a distinct increase in
400 the abundance of *P. algicola* signals within 8 days exposure to hydrocarbons. The study by
401 Mishamandani *et al.*, (2016) further showed that the abundance of *P. algicola* 16S rRNA genes
402 continued to increase, by a further three orders of magnitude, by day 42 of the crude oil
403 enrichment experiment with *S. costatum*.

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408 **Figure 3.** Hybridization of samples from the crude oil enrichment with probe PCY223 at days 2
409 (A) and 5 (B), and with probe ALGAR209 at days 2 (C) and 5 (D) amongst the entire DAPI-
410 stained microbial population (blue). White arrows indicate cell signals targeted by the respective
411 probe. Small spherical micro-algal cells (orange autofluorescence) are shown in (A) with no
412 apparent associated *P. algalicola* cell signals; a *Skeletonema* chain (orange autofluorescence) is
413 observed in (C) with several *A. aromaticivorans* cell signals associated with it.

414

415 Based on initial abundance of these bacteria and their response to PAH/crude oil
416 enrichment, it is possible that these types of obligate hydrocarbonoclastic bacteria may contribute
417 to background levels of hydrocarbon biodegradation in the ocean rather than as major responders
418 to oil spills. This is supported by the fact that they are poorly represented in 16S rRNA gene

419 sequence databases, including datasets from sequencing surveys performed on samples collected
420 at natural oil seeps and oil spill sites (e.g. Deepwater Horizon).

421 **Conclusions**

422 The low abundance of *P. algalicola* and *A. aromaticivorans* observed in our phenanthrene
423 enrichment experiments, and the fact that 16S rRNA gene sequences of these bacteria are poorly
424 represented in sequencing surveys in the literature, suggests that they are likely to contribute to
425 the biodegradation of hydrocarbons in the water column at background levels. These organisms
426 have been shown to be associated with a range of other species of diatoms, dinoflagellates and
427 coccolithophores (Gutierrez *et al.*, 2013; and unpublished data). It may be inferred that they have
428 eluded detection in sequencing surveys because they occupy a specific biotope in the ocean (i.e.,
429 the phycosphere of phytoplankton), which has not been sufficiently explored in this respect, and
430 because these bacteria may comprise the rare biosphere in the ocean. The design and
431 development of 16S rRNA oligonucleotide probes for CARD-FISH provides a useful tool to
432 study the occurrence and ecology of hydrocarbon-degrading bacteria in laboratory cultures of
433 micro-algae and in fresh field samples. These new probes (PCY223 and ALGAL209) also
434 expand the current inventory of FISH probes for targeting hydrocarbon-degrading organisms that
435 will contribute to improving our understanding on the role of these bacteria in the ocean
436 hydrocarbon cycle.

437

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448

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