1 **Visualisation of the obligate hydrocarbonoclastic bacteria *Polycyclovorans***

2 ***algicola* and *Algiphilus aromaticivorans* in co-cultures with micro-algae by**

3 **CARD-FISH**

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24

Abstract

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

Introduction

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

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

4

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

5

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 (1.355×10^6 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 GGC TCC TCC AA	Genus *Polycyclovorans* (22%)	0	*Polycyclovorans algicola* (FJ176554)	55-60%
ALGAR209	S-S-ALGAR209-a-A-18	CCT CCA GCG TGA GGT CCG	Genus *Algiphilus* (33%)	0	*Algiphilus aromaticivorans* (DQ486493)	35-40%
c1ALGAR209	S-S-c1ALGAR209-a-A-18	CCT CCA GCG CGA GGT CCG			Competitor for ALGAR209	

150

151   [1]According 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] H_2O_2, 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

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

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

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

204

CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus aromaticivorans* enrichments

with micro-algae

Sub-samples fixed onto polycarbonate filters from the time-series incubations enriched with or

without phenanthrene were dipped into 0.2% (w/v) agarose, then mounted onto glass slides and

air dried following standard methods (Pernthaler *et al*., 2002). CARD-FISH was performed

directly on the agarose-embedded samples using the optimized protocol developed for the

PCY223 or ALGAR209 probes at a FA concentration of, respectively, 55% or 40%, with the

exception that 50–100 μl of hybridization buffer were used. All hybridisations were

counterstained with DAPI following standard methods (Porter and Feig, 1980) prior to

visualization under the epifluorescence microscope and analysed using Zeiss Zen-Blue image

processing software, as described above.

216

Field sample collection and CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus*

***aromaticivorans* in natural communities of phytoplankton**

During a sampling operation aboard *RV Serpula* on 24 May 2013 to a sampling station in the

lower basin of Loch Creran (56º30.820N, 5º22.817W) located on the west coast of Scotland, a

phytoplankton net (50-60 μm mesh size) was trawled near the sea surface (1–2 m depth) for

several minutes to collect the phytoplankton community. The phytoplankton net sample was

passaged through a 125-μm metal mesh filter to remove grazers, washed by gentle centrifugation

with sterile synthetic seawater, and then stored at 4°C and used within 24 hours for the

preparation of a crude oil enrichment experiment, as previously described (Thompson *et al*.,

2017). Briefly, 500 ml of the washed phytoplankton sample, with its associated bacterial

community, was added to 10 litres of filtered (0.2 μm) seawater. This was used to set up a crude

oil enrichment by adding 350 ml of the phytoplankton suspension into 1-litre Erlenmeyer flasks

(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 μmol s^{-1} 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
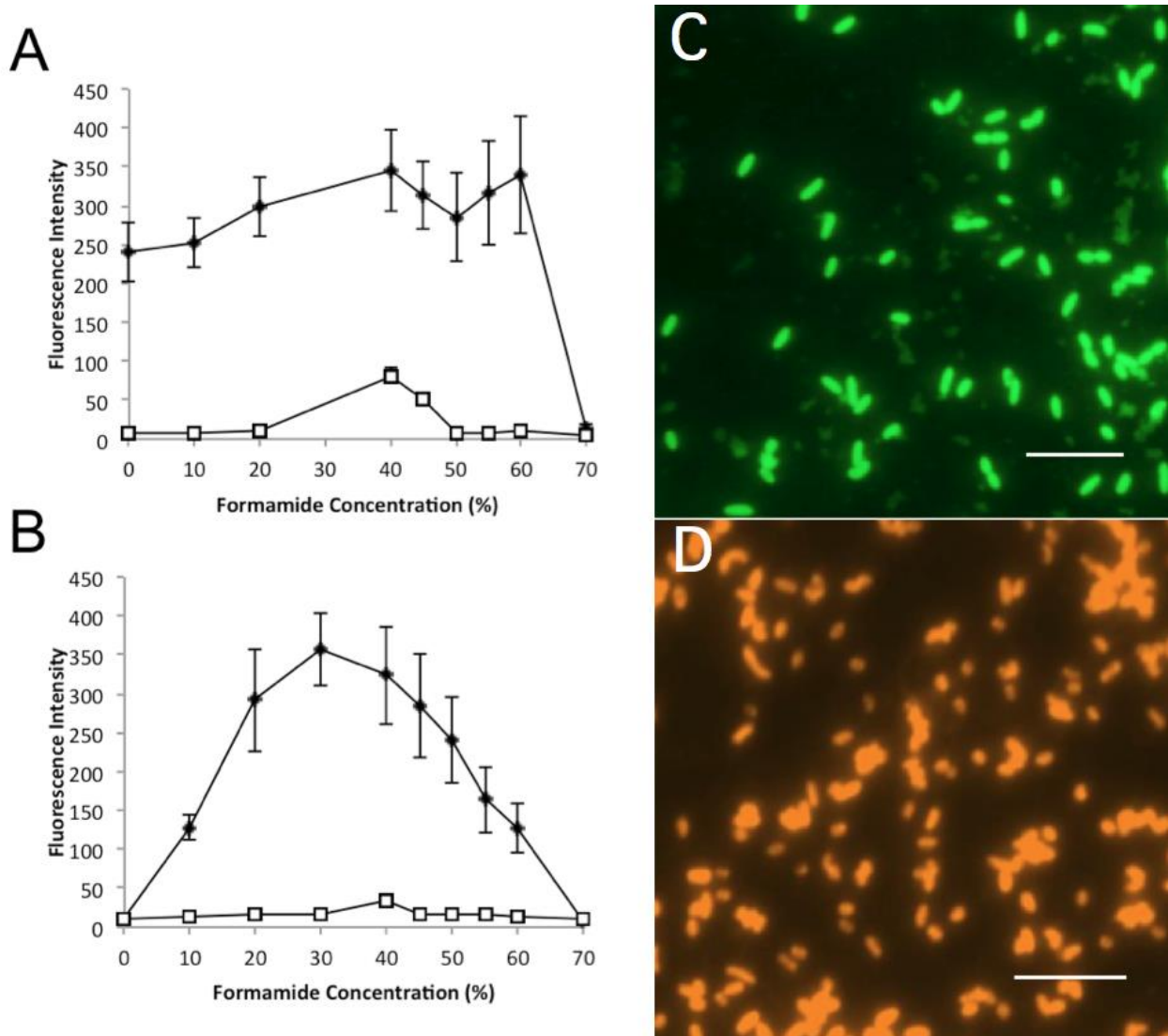
285

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 ± 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 μm.

298

299 **Detection and response of *P. algicola* and *A. aromaticivorans* in micro-algal cultures**

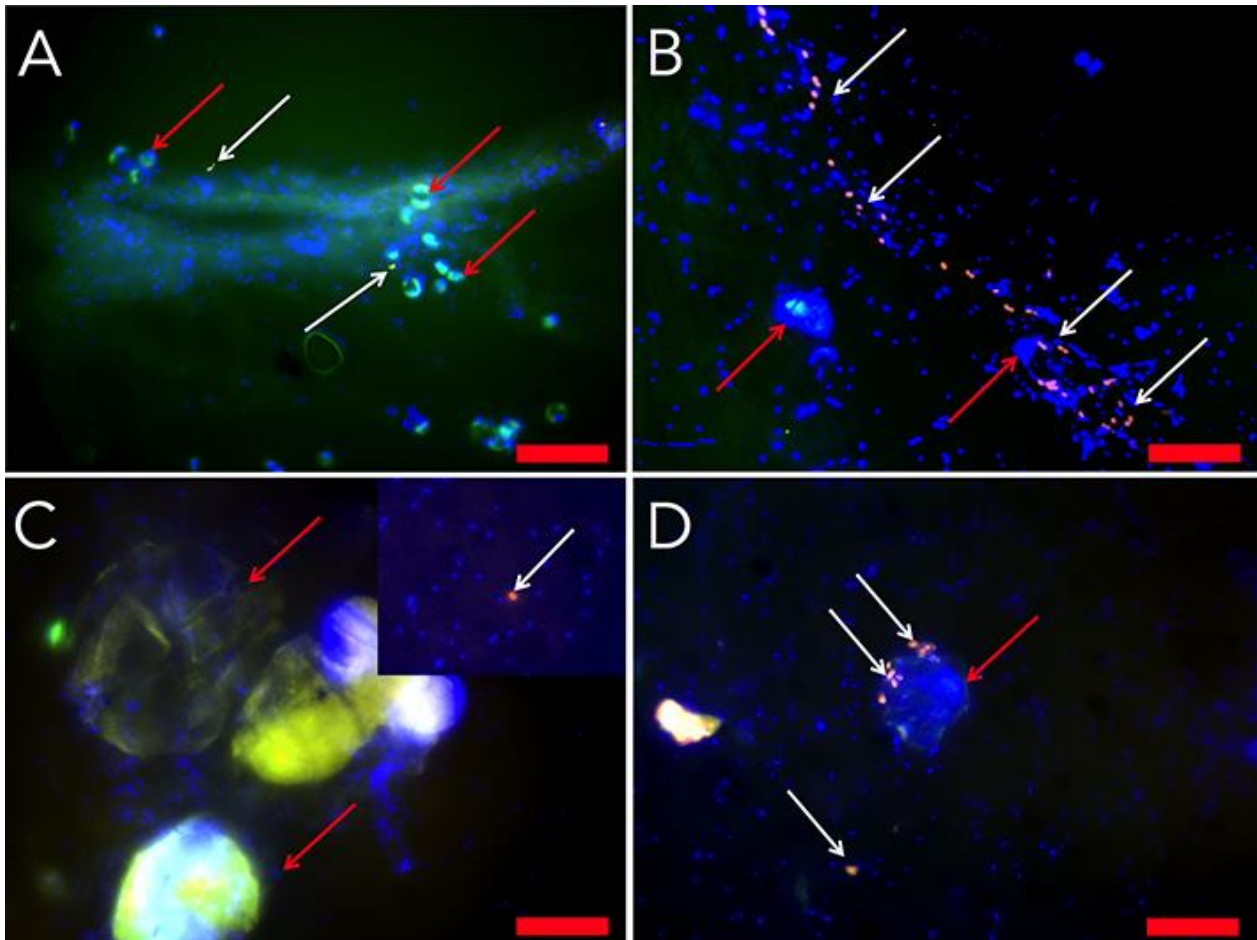300 **enriched with phenanthrene**

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. algicola* 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 *algicola* 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. algicola*, 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 *algicola* cells quantified with probe PCY223 had increased as a result of enrichment with

14

318   phenanthrene, with *P. algicola* 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).

337

338

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

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

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

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

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

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

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

arrows. Bar, 20 μm.

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

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

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

16

associated with these micro-algae. The observed attachment of some cells for both types of

bacteria to their micro-algal host cells infers that they may share more than just a physical

attachment, as reported for other types of bacteria living associated with micro-algae. The

interaction of micro-algae with bacteria in the ocean is believed to play an important role to their

ecological success (Amin *et al*., 2012; Amin *et al*., 2015; Buchan *et al*., 2014). Algal-associated

bacteria have been shown to acquire algal exudates as carbon and energy sources (Bell and

Mitchell, 1972; Myklestad, 1995), where the algae have been reported to benefit from bacterial-

produced vitamins, trace metals and other nutrients (Kazamia *et al*., 2012; McGenity *et al*.,

2012). In a study by Amin et al. (2009), the authors reported a mutual sharing of iron and fixed

carbon between several species of phytoplankton and bacteria, including members comprising the

hydrocarbon-degrading genus *Marinobacter*. Taking into account the narrow nutritional spectrum

of strains TG408 and DG1253 living associated with micro-algae (Gutierrez *et al*., 2012a;

Gutierrez *et al*., 2013), it is possible that they too may co-exist through some form of nutrient-

sharing, though further work will be needed to determine this.

Detection and response of *P. algicola* and *A. aromaticivorans* in natural seawater

populations of phytoplankton enriched with crude oil

The enrichment and spatial localization of *P. algicola* and *A. aromaticivorans* was investigated in

a natural population of phytoplankton from coastal water. Since these obligate

hydrocarbonoclastic bacteria were originally isolated and shown to live associated with

laboratory cultures of micro-algae (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013), the application

of CARD-FISH employing the new probes (PCY223 and ALGAR209) allowed us to observe

these bacteria in natural seawater samples, including associated with phytoplankton cells. This

was examined in enrichments with and without crude oil in order to detect for these bacteria

under, respectively, perturbed and unperturbed environmental conditions. Samples taken at days

0 (results not shown) and 2 of these enrichment experiments and analysed with probes PCY223

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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 eights 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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406
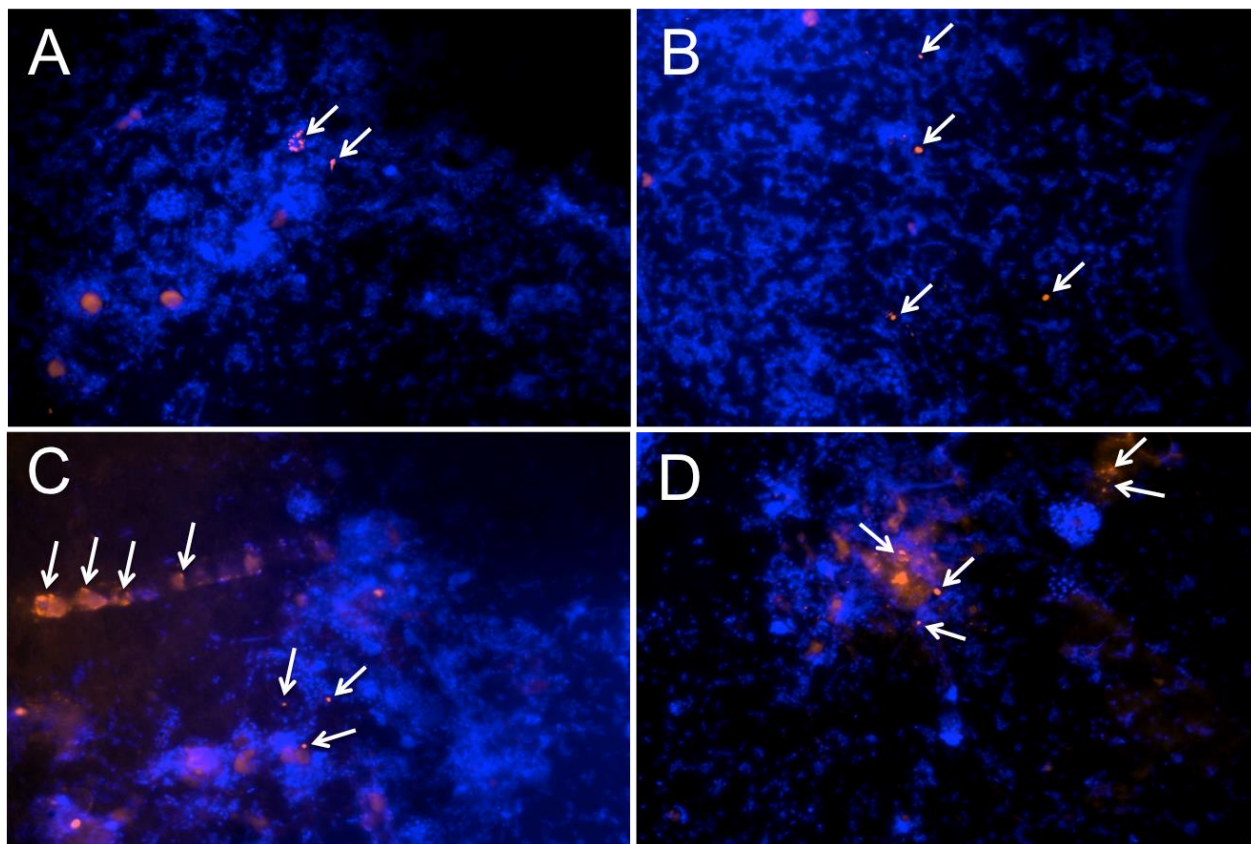
Figure 3. Hybridization of samples from the crude oil enrichment with probe PCY223 at days 2 (A) and 5 (B), and with probe ALGAR209 at days 2 (C) and 5 (D) amongst the entire DAPI-stained microbial population (blue). White arrows indicate cell signals targeted by the respective probe. Small spherical micro-algal cells (orange autofluorescence) are shown in (A) with no apparent associated *P. algicola* cell signals; a *Skeletonema* chain (orange autofluorescence) is observed in (C) with several *A. aromaticivorans* cell signals associated with it.

Based on initial abundance of these bacteria and their response to PAH/crude oil enrichment, it is possible that these types of obligate hydrocarbonoclastic bacteria may contribute to background levels of hydrocarbon biodegradation in the ocean rather than as major responders 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. algicola* 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.

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445 accommodating all of our research needs. We would also like to acknowledge Alexander Loy for

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447 thorough revision of the manuscript. The authors declare no conflict of interest.

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