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Imaging bacterial cells and biofilms adhering to hydrophobic organic compounds-water interfaces

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1 Title

2 **Imaging bacterial cells and biofilms adhering to hydrophobic organic compounds-water**
3 **interfaces**

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30

31 **Abstract**

32 Assimilation of Hydrophobic Organic Compound (HOC) entails frequently the formation of biofilm at
33 the HOC-water interface. Knowledge on the behavior of cells at the oil-water interface and within the
34 structured biofilm is therefore important to understand the degradation of the HOC in ecosystems. The
35 adhesion and biofilm formation on oil-water interface is best documented by microscopic
36 observations. In this chapter we thus describe two methods for observation of bacterial cells and
37 biofilms growing at the HOC-water interface. The first method uses CLSM (confocal laser scanning
38 microscopy) to obtain *in situ* images of biofilm developing on thin paraffin strip which offers a flat
39 transparent surface allowing imaging directly through the bottom of the culture dish without sampling.
40 Alternatively, the biofilm can be grown on a paraffin strip deposited on a glass microscope slide and
41 then imaged from the top when high resolution is needed. The second method addresses the
42 problematic of the ultrastructure of biofilm developing on HOC. It enables to obtain by TEM
43 (transmission electron microscopy) images of cross sections of biofilms with identification of the side
44 in contact with the HOC.

45

46 **Key Words**

47 Hydrocarbons, lipids, adhesion, biofilm, oleolytic bacteria, CLSM, TEM

48

49 **1. Introduction**

50 Hydrophobic Organic Compounds (HOC), including the chemical classes of lipids and hydrocarbons
51 are ubiquitous components of the organic matter in ecosystems. The so called oleolytic bacteria have
52 the ability to use members of either one or both classes as a substrate(1,2). However, they have to face
53 the very low water solubility of these substrates. Adhesion to the HOC-water interface and the
54 subsequent formation of biofilms (3D-architectures of bacterial cells embedded within matrixes of
55 biopolymers) are strategies shared by many oleolytic bacteria to overcome the low solubility of their
56 substrates(3, 4, 5). Different mechanisms have been proposed for the stimulation of the rate of mass
57 transfer from the oily phase to cell surface. The localization of cell in the vicinity of the interface
58 results in reduction of the length of the diffusion pathway of the hydrocarbon from the interface to the
59 cell surface which in turn increases the mass transfer rate (6). Extracellular matrixes of biofilms provide
60 confined environments where biosurfactants can be accumulated and contribute to hydrocarbons
61 uptake by substrate emulsification or pseudo-solubilization. A direct contact of bacteria with
62 hydrocarbons is also possible via the modification of the cell surface (7) offering the possibility of
63 direct uptake from the hydrocarbon-water interface, although this remained to be demonstrated. In the
64 case of lipids which must undergo hydrolysis into fatty acids by extracellular lipases before uptake by
65 the cell, adhesion and biofilm formation at the water-lipid interface provide the advantage to retain
66 together the exo-enzymes, the lipids, the hydrolysis products and the cells thus preventing them from
67 rapid transportation to bulk water. The behavior of cells at the oil-water interface and within the
68 structured biofilm is therefore one important aspect of the degradation of the HOCs in ecosystems.

69 The adhesion and biofilm formation at the oil-water interface is best studied by microscopic
70 observations. Although a huge number of HOCs degrading strains have been isolated over the past
71 decades, adhesion of these strains to their substrate is rarely documented. Only few authors provided
72 images of bacteria cells adhered to HOC-water interface using light microscopy, scanning electron
73 microscopy and confocal laser scanning microscopy (CLSM) (8, 9, 10, 11, 12, 13). Studies of the
74 behavior of bacteria at the HOC-water interface and within biofilms often involve examination and
75 comparison of different strains (wild type versus mutants or different species). This can be only

76 achieved if the HOC-water interfaces generated are compatible with the quantitative measurement of
77 the adhesion or biofilm formation that is hardly the case with spherical or hemispherical droplets of
78 HOC and crystals of irregular shape. To fulfill these requirements, we propose a methodology using
79 thin films of paraffin (15 μm) to generate a substratum to grow biofilms. Ultrathin paraffin films are
80 obtained by slicing solid paraffin with a microtome. Paraffin films pasted to the bottom of a culture
81 dish offer a flat surface and are thin enough to allow in situ observation through the bottom of the
82 culture dish avoiding thus disruption of the biofilm structure. A better definition of imaging can be
83 obtained using a microscope glass slide as solid substratum to paste the paraffin; the biofilm is then
84 observed upside-down through a coverslip.

85 Determination of the ultrastructure of biofilms adhered to HOC-water interface are also of great
86 interest. In general, biofilm cells exhibit heterogeneity in their physiology which can be reflected by
87 the presence of different morphotypes. In the case of biofilm developing on hydrocarbons, intracellular
88 inclusions of storage lipids have been often observed (14). For instance, biofilm cells of *Marinobacter*
89 *hydrocarbonoclasticus* SP17 growing on hexadecane accumulate wax ester in their cytoplasm. These
90 storage lipids persist after detachment from the biofilm and provide cells with energy for colonization
91 of unoccupied interfaces (15). Lipid inclusions can be investigated using CLSM using specific
92 fluorescent dye (figure 1). However, the ultrastructure of growing cells on HOCs is best studied using
93 transmission electron microscopy (TEM) (1, 16). The distribution and arrangement of cells containing
94 inclusions within the biofilm can be investigated by TEM provided that the biofilm structure is
95 preserved and the orientation of the biofilm in respect to the HOC surface is traceable. Here, we
96 describe a method where the biofilm is grown in a petri dish whose the bottom has been coated with
97 paraffin. TEM requires sectioning the biofilm included in epoxy resin. As paraffin is incompatible
98 with epoxy resin, the biofilm is removed from the paraffin surface by embedding it in agarose. Then
99 agarose pieces are processed for TEM by paying attention to keep track of the side bearing the biofilm.

100 This procedure enables to obtain images of cross sections of biofilms with identification of the side in
101 contact with the paraffin. The imaging methods were both applied on a marine gram negative
102 bacterium, *Marinobacter hydrocarbonoclasticus* SP17 and on a gram positive bacterium *Rhodococcus*
103 *equi* NapRu1.

104 **2. Materials**

105 **2.1 Observation of biofilms grown on paraffin by CLSM**

106 2.1.1 Strains, culture media and substratum for biofilm growth

107 1. The protocol described here has been developed with the bacterial strains *M. hydrocarbonoclasticus*
108 SP17(17) and *R. equi* NapRu1 (3) (Note 1)

109 2. Synthetic Sea Water (SSW): NaCl 0,2 mol.L⁻¹, KCl 10 mmol.L⁻¹, Tris-HCl 50 mmol.L⁻¹ pH 7,8,
110 NH₄Cl 56 mmol.L⁻¹, K₂HPO₄ 427 μmol.L⁻¹, FeSO₄ 8 μmol.L⁻¹, MgSO₄ 65 mmol.L⁻¹ et CaCl₂
111 13 mmol.L⁻¹) (Note 2)

112 3. Sodium lactate 2 mol.L⁻¹ adjusted to pH 7

113 4. Paraffin Normal Q Path® (VWR International, France)

114 5. Petri dishes (55 and 90 mm diameter) (Note 3)

115 6. Washed glass microscopic slides (25x75 mm) (Note 4)

116 7. Ultramicrotome (UC6, Leica microsystems, Germany)

117

118 2.1.2 CLSM observation of the biofilm

119 1. Fluorescent dye: Syto®9 (L-10316, Life Technologies, France), Syto®61 (S-11343, Life
120 Technologies), BODIPY® (D-3922, Life technologies) (Note 5)

121 2. Glass coverslips N° 1.5 (Knittel Gläser, Germany)

122 3. Silicon spacer for coverslip, Press-to-Seal™, 1 mm thick (Invitrogen™, Life Technologies)

123 4. Inverted Confocal Laser Scanning Microscope, Leica TCS SP8 AOBS (Leica Microsystems,
124 Germany) (Note 6)

125 5. Image analysis software IMARIS 7.7.2 software (Bitplane, Switzerland) and Fiji (Fiji.sc/Fiji)(Note
126 7)

127 **2.2 Observation of biofilms grown on paraffin by TEM**

128 2.2.1 Strains, culture media and substratum for biofilm growth

129 See subheading 2.1.1

130 2.2.2 TEM observation of the biofilm

131 1. Sodium cacodylate buffer 0.15 or 0.1 mol.L⁻¹, pH 7.4 (Sigma-Aldrich, France) (Note 8). The pH is
132 adjusted with HCl

133 2. Fixative solution (prepared extemporaneously): 2.5% glutaraldehyde (from glutaraldehyde solution
134 Grade I, 25% in H₂O, Sigma-Aldrich) in 0.15 M sodium cacodylate buffer at pH 7.4 or 0.10 M sodium
135 cacodylate buffer at pH 7.4 (Note 8)

136 3. Post-fixative solution (prepared extemporaneously): 1% osmium tetroxide (Electron Microscopy
137 Sciences, France) in 0.15 M sodium cacodylate buffer at pH 7.4 or 0.10 M sodium cacodylate buffer
138 pH 7.4 (accordingly to the fixative solution)

139 4. 2.5% agarose solution in distilled water (Seakem® LE agarose, Lonza, France). Agarose solution
140 must be maintained at 50 °C in an incubator for example (Note 9)

141 5. Ethanol 99.8% (AnalaR NORMAPUR, VWR International)

142 6. Fresh or thawed epoxy resin (R1165, Low Viscosity Resin - Medium grade, Agar Scientific, France)
143 (Note 10)

144 7. A filtered solution of 0.05% oolong tea extract (OTE) in distilled water (OTE, Eloïse, France)
145 (Note 11)

146 9. Whatman® grade n°1 cellulose filter paper (Sigma-Aldrich)

147 10. 2 mL Eppendorf® microcentrifuge tubes (Eppendorf, France) (Note 12)

- 148 11. Rotator for Eppendorf microcentrifuge tubes
- 149 12. Vacuum desiccator
- 150 13. Diamond knife for ultrathin sectioning (35 ° angle, DU3520 Diatome 2 mm) and ultramicrotome
151 (UC6, Leica microsystems, Germany)
- 152 14. 200 and 300 mesh copper grids (G200-Cu and G300-Cu, Electron Microscopy Sciences)
- 153 15. Flat embedding molds for TEM (70900 silicone clear Electron Microcopy Sciences or 70907
154 Dykstra blue, Electron Microscopy Sciences) (Note 13)
- 155 16. HT7700 transmission electron microscope (Hitachi, Japan) equipped with an 8 million pixels
156 format CCD camera driven by the image capture engine software AMT version 6.02.
- 157

158 **3. Methods**

159 **3.1 Observation of biofilms grown on paraffin by CLSM**

160 3.1.1. Pre-culture of bacteria

161 1. Inoculate 100 μL of a glycerol stock of bacteria (strain SP17 or NapRu1) stored at $-80\text{ }^{\circ}\text{C}$ into
162 10 mL of SSW supplemented with $20\text{ mmol}\cdot\text{L}^{-1}$ sodium lactate as a carbon source in a 50 mL tube and
163 incubate overnight at 180 rpm, $30\text{ }^{\circ}\text{C}$.

164 2. Transfer 100 μL of the culture in 10 mL of SSW plus $20\text{ mmol}\cdot\text{L}^{-1}$ sodium lactate and incubate
165 overnight at 180 rpm, $30\text{ }^{\circ}\text{C}$.

166 3. Centrifuge the overnight culture at $20\text{ }^{\circ}\text{C}$, $10\ 000\text{ g}$ for 15 min, wash the cell pellet twice in SSW and
167 resuspend the cells in SSW at an $\text{OD}_{600\text{nm}}=0.01$.

168

169 3.1.2. Biofilm culture

170 1. Prepare the substratum

171 Melt the paraffin at $65\text{ }^{\circ}\text{C}$ and pour it into cassettes for microtome. Let the paraffin cooled-down on a
172 cool top board to allow solidification. Cut the solid paraffin with a microtome in $15\text{ }\mu\text{m}$ thick strips.
173 Strips must be transported on ice (for example in a Petri dish) then stored at $4\text{ }^{\circ}\text{C}$ until use. A thin strip
174 of paraffin is placed on a microscope glass slide within a Petri dish (90 mm) or directly on the bottom
175 of a Petri dish (55 mm). Put the Petri dish in an incubator at $45\text{ }^{\circ}\text{C}$ for 10 min to soften the paraffin and
176 make it adhere to the solid support. Before use, decontaminate by exposing the Petri dish to UV light for
177 5 min.

178 2. Inoculate the solid support

179 Pour 30 mL of the bacterial suspension ($\text{OD}_{600\text{nm}}=0.01$) in the Petri dish (90 mm) containing the thin
180 paraffin strip stuck on a microscopic slide or 5 mL in a Petri dish (55 mm) containing the thin paraffin

181 strip stuck on the bottom. Make sure that the bacterial suspension covers the whole surface of the solid
182 substratum. Incubate at 30° C without shaking for 5 days (Note 14).

183

184 3.1.3. CLSM observation of the biofilm

185 1. Biofilm staining with fluorescent markers (Note 15)

186 Remove the culture medium leaving about 1 mL to keep the biofilm wet. It is important to avoid
187 dehydration to conserve the biofilm structure. Add the selected fluorescent markers to the
188 recommended concentration (see manufacturer's instructions) and incubate at room temperature in
189 dark for 15 min. Here, either Syto®9 (diluted 1:1000) or the mixture Syto®61 (1:1000)/BODIPY®
190 (final concentration 0.05 mg.mL⁻¹) are used.

191 2. Biofilm observation

192 Biofilms grown on paraffin thin film deposited on the bottom of the petri dish are imaged directly,
193 without mounting, through the bottom of the Petri dish with an inverted microscope. This approach is
194 preferred when biofilms are loosely attached to paraffin and mounting would result in loss in the
195 biofilm integrity. However, if a maximal resolution is desired, the biofilm is grown on paraffin film
196 pasted on a microscope slide. Then the biofilm is mounted between the slide and a coverslip with a
197 silicon spacer to avoid compressing it and observed upside down on the inverted microscope. Biofilms
198 mounted or not are observed with a water immersion HCX APO L, 0.80 NA, W U-V-I, Leica 40 X
199 objective with a working distance of 3.3 mm. Adjust the laser excitation and fluorescence signal
200 recuperation according to the characteristic of the markers (absorption and fluorescence emission
201 maxima Syto®9 482/500 nm, Syto®61 628/645 nm, BODIPY® 493/503 nm).

202 3. Acquire xy image or 3D xyz-stack and process the image series using IMARIS 7.7.2 software and Fiji
203 (Figure 1).

204

205

206 **3.2 Observation of biofilms grown on paraffin by TEM**

207 The basic principles and techniques of electron microscopy for biological applications are described
208 elsewhere(18). This protocol is designed to visualize more specifically the spatial distribution in the
209 biofilm of cells containing intracytoplasmic inclusions. In order to visualize the extracellular matrix,
210 bacterial capsules or appendices, other fixative cocktails and other electron microscopy techniques
211 have to be used (19, 20).

212 3.2.1. Pre-culture of bacteria

213 See subheading 3.1.1.

214 3.2.2. Biofilm culture

215 1.Coat the bottom of petri dishes with paraffin by pouring 15 mL of liquid paraffin heated at 50 °C.

216 Let the paraffin solidify by cooling at room temperature.

217 2.Twenty milliliters of bacterial suspension prepared as described in 3.1.1 are used to inoculate the
218 paraffin coated Petri dishes. Make sure that the whole surface of the solid paraffin is covered by the
219 cell suspension.Incubate at 30 °C without shaking for 5 days (Note 14).

220

221 3.2.3. TEM observation of the biofilm

222 To prevent dehydration, the samples must be kept immersed in solution during the all procedure,
223 except for step 3. During the change of solutions, it is therefore recommended to not discard all the
224 solution from the previous step. Addition and removal of solutions must be performed with extreme
225 care to avoid biofilm alteration. Be aware of the toxicity of the compounds used (Note 16).

226 1.Fix the biofilm

227 Remove the culture medium from the petri dish and pour 20 mL of the fixative solution, incubate at
228 room temperature for 1 h. The cacodylate buffer concentration in fixative solution is 0.15 mol.L⁻¹for
229 *M. hydrocarbonoclasticus*SP17 and 0.10 mol.L⁻¹for *R. equi* NapRu1. At this stage samples can be
230 stored few months in the fixative solution, at 4°C. Wash three times during 5 min with the cacodylate
231 bufferat the corresponding concentration.

232

233 2.Post-fix the biofilm

234 Remove the fixative solution,add 20 mL of post-fixative solution and incubate at room temperature for
235 1 h. Wash twice for 10 min in distillated water. At this stage samples can be stored few days
236 maximum, at 4 °C.

237

238 3.Embed the biofilm in agarose

239 Discard the distilled water and quickly embed the fixedbiofilm in 30 mL of a 2.5% agarose solution
240 maintained above its gelling point at 50 °C (Note 9). Tilt the Petri dish and carefully swirl the melted
241 agarose to obtain a homogenous surface. Let the agarose gellify for 1 h at 4 °C.

242 Detach the agarose gel from the petri dish wall by moving all around the edges of the dish a thin blade
243 inserted between the agarose and the dish wall. Unmold carefully the agarose by detachingit from the
244 paraffin. The biofilm normally separates easily from the paraffin staying in the agarose layer.

245 Checkvisually that all the biofilm has been removed from the surface of the paraffin.Transfer the
246 agarose slab, biofilm facing down, in a Petri dish containing the first solution of dehydration (30%
247 ethanol). Make sure that the biofilm bearing face is always immersed in the solution. Cut the agarose
248 slab into big pieces(1 cm³) with a scalpel and choose pieces where the biofilm seems thicker and
249 homogeneous.

250

251 4. Dehydrate the agarose pieces in ethanol solutions series: 50%, 70%, 90% and 2x100% v/v, 15 min
252 for each step at room temperature. Removal and addition of ethanol solution must be carried out with
253 care to not damage the biofilm. Dehydration procedure can be stopped at the stage of 70% ethanol.
254 Samples are stable for few days maximum, at 4°C.

255 After dehydration reduce the thickness of the agarose layer above the biofilm to 1 mm and cut small
256 pieces of 3 mm long by 1 mm wide. Transfer each piece in an Eppendorf™ microcentrifuge tube
257 containing 700 µL of a 100% ethanol (third bath).

258

259 5. Impregnate successively the dehydrated samples with epoxy resin solutions in ethanol of increasing
260 concentrations: 25% and 33% for 10 min, 50% overnight, then 66% and 75% for 10 min.

261 Impregnations are performed at room temperature under rotary shaking (Notes 17 and 18). Then
262 impregnate with pure (100%) fresh resin, for 10 min up to few hours, with the caps of the
263 microcentrifuge tubes opened to let evaporate the last traces of ethanol. Impregnate once more with
264 pure fresh resin overnight on rotary shaking. Finally, impregnate with pure fresh resin with the caps of
265 the microcentrifuge tubes opened in under vacuum to maximize resin penetration (from 10 min to few
266 hours). After dehydration and impregnation the biofilm bearing face of agarose pieces can be easily
267 recognized by their black coloration.

268

269 6. Include samples in epoxy resin.

270 Pour a thin layer of pure fresh epoxy resin into TEM mold and allow to polymerize for 2 h at 60 °C.

271 Place each impregnated sample, in a mold, on the top of the polymerized epoxy layer in such a way
272 that the biofilm black layer is below and parallel to the bottom of mold. The viscous thin layer of resin
273 in the bottom of the mold will help to stabilize the sample during polymerization (Note 19).

274 Fill completely the cavity with pure fresh epoxy resin, considering volume reduction during
275 polymerization. Put the mold under vacuum for 10 min up to few hours to maximize resin penetration

276 and eliminate air bubbles. Polymerize at 60 °C, during 16 to 18 h, depending on the resin hardness
277 preferred for the cutting. Resin blocks can be stored *ad vitam æternam* in a temperate and dry place.

278

279 7. Make ultrathin sections (60 to 80 nm) with a diamond knife and an ultramicrotome. Deposit on 200
280 or 300 mesh copper grids (Note 20). Stain each grid with one drop of 0.05% OTE solution for 30 min,
281 washed with 4 successive drops of distilled water and dried on Whatman grade n°1 cellulose filter
282 paper.

283

284 8. Observe in a TEM microscope between 80 to 120 kV (Figure 2). Contrast increases when
285 accelerating voltage is decreased and objective aperture is more closed (*2I*).

286

287 **4. Notes**

288 Note 1. Although this method was originally developed for *M. hydrocarbonaclasticus* SP17 and *R.*
289 *equi* NapRu1, it can be adapted, by using the appropriated culture medium, to any strain that is able to
290 form biofilm on paraffin or other HOCs, hydrocarbon or lipidsthat are solid at the growth temperature.
291 The HOC must be soft enough to be cut in very thin slices. Crystallized compounds (such as pure
292 alkanes or aromatics) are not suitable since they are not sliceable.

293 Note 2. Any other medium that allows the studied strain to grow, adhere and form biofilm on HOCcan
294 be employed.

295 Note 3. Classical Petri dishes are employed. Other diameters can be used. If the biofilm is observed
296 directly through the bottom of the dish, specific Petri dishes with a bottom designed for high-end
297 microscopic analyses have to be used (μ -Dish50 mm, low, Ibidi) for higher resolution imaging.

298 Note 4. Microscopic slides and coverslips are cleaned by acetone/ethanol (50/50 V/V), dried and
299 stored in sterile conditions in Petri dishes until used.

300 Note 5. Syto®9 (green fluorescent dye) and Syto®61 (red fluorescent dye) are nucleic acid stains and
301 nonpolar BODIPY® (green fluorescent dye) labels neutral and non-polar lipid components. Other
302 fluorescent dyes can be used according to the structures to be observed as previously described (22).

303 Note 6. The use of an inverted CLSM is required to observe directly biofilms *in situ* without
304 perturbing their architecture during staining and mounting.

305 Note 7. Various softwares can be used as previously described (22).

306 Note 8. Adapt and control the osmolarity of the buffer for each type of sample.

307 Note 9. The agarose solution is maintained at the liquid state at a temperature above its gel point but
308 not too high to avoid the paraffin from melting or any damage to the biofilm.

309 Note 10. This resin is prepared accordingly to the manufacturer's instructions, aliquotedand stored at -
310 20°C.

311 Note 11. OTE is a non-hazardous reagent used instead of the hazardous uranyl acetate(23).

312 Note 12. Smaller microcentrifuge tubes are not suitable for dehydration and next steps.

313 Note 13. Pay attention to the compatibility between the type of resin and the mold materials.

314 Note 14. Time and temperature depend on the strain cultivated and on the conditions studied. If
315 incubation is very long, prevent the evaporation by humidifying the atmosphere.

316 Note 15. Wear a lab coat and gloves to manipulate fluorescent markers. Use specific disposal bins.
317 Read the material safety data sheet (MSDS) of the products.

318 Note 16. Work under a fume hood wearing lab coat, gloves and glasses. Use specific disposal bins.
319 Read the MSDS of the products before use.

320 Note 17. While facilitating the early stages of impregnation, propylene oxide traditionally employed is
321 not used in this protocol. It is a hazardous product (boiling point at 34°C) that reacts with many
322 plastics. Instead, ethanol is used for mixed baths with epoxy resin although they mix less easily.

323 Note 18. Number and length of the impregnation steps are adjusted to our models and our epoxy resin.
324 They can be adapted in case of troubles during the sample cutting (block too soft to an ultrathin cut,
325 dissolution of the ultrathin section in the water tank of the knife) or during TEM observations (holes
326 visible in the ultrathin section). Other standardized protocols describing impregnation steps with other
327 resins can be found (24).

328 Note 19. When placing the samples into the cavities of the molds, it is possible to reference them by
329 adding a small paper with a printed code (pen ink diffuses in the resin) on the opposite side of the
330 sample.

331 Note 20. Use the lowest possible mesh copper grids to maximize the observation area (i.e. 200 mesh
332 copper grids). Use 300 mesh copper grids for unstable sections under electron beam.

333

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337

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408

409 **Figure legends**

410 Figure 1. Observation of paraffin-grown biofilms by CLSM

411 Biofilm of *M. hydrocarbonoclasticus* SP17 (A) and of *R. equi* NapRu1 (C) stained by Syto®9
412 observed directly through the bottom of a plastic Petri dish and paraffin (optical lens x 40, numerical
413 zoom 1, bar = 50 µm). Biofilm of *M. hydrocarbonoclasticus* SP17 (B) and of *R. equi* NapRu1 (D)
414 stained by the mixture BODIPY®/Syto®61 observed upside down through a microscopic cover slip.
415 (optical lens x 40, numerical zoom 4, bar = 10 µm). Intracytoplasmic lipids are abundant during
416 growth of *M. hydrocarbonoclasticus* SP17 on paraffin (green staining in B) but not for *R. equi* NapRu1
417 (D).

418 Images in A and C are three-dimensional projections of biofilm structures reconstructed using the Easy
419 3D function (in blend mode) of the IMARIS 7.7.2 software. B and D are xy-images for which the
420 overlay of the two channels and the scale bar were performed using Fiji software.

421

422 Figure 2. TEM observation of *M. hydrocarbonoclasticus* SP17 (A) and *R. equi* NapRu1 (B).

423 Only the bottom of the *R. equi* NapRu1 biofilm (i.e. the side in contact with paraffin during growth) is
424 shown as the biofilm is very thick with no stratification within the ultrastructure of cells. The *R. equi*
425 NapRu1 biofilm (Fig. 2B) contain few cells, as compared with the *M. hydrocarbonoclasticus* SP17
426 biofilm (Fig. 2A), and probably a lot of extracellular matrix. Numbers of cells of *M.*
427 *hydrocarbonoclasticus* SP17 contained big inclusions in contrast to *R. equi* NapRu1 cells. These
428 observations are in agreement with CLSM imaging (Fig. 1B, D).