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Exploring the chemodiversity of tropical microalgae for the discovery of natural antifouling compounds

Réveillon Damien^{1,2}, Tunin-Ley Alina³, Grondin Isabelle⁴, Othmani Ahlem¹, Zubia Mayalen⁵,
Bunet Robert⁶, Turquet Jean³, Culioli Gérald^{1,*}, Briand Jean-François^{1,*}

¹ MAPIEM EA 4323, Université de Toulon, Toulon, France

² Laboratoire Phycotoxines, Ifremer, Nantes, France

³ Hydrô Réunion, CBEM c/o CYROI, Sainte Clotilde, La Réunion, France

⁴ Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments (LCSNSA), Université de La Réunion, Saint-Denis, France

⁵ Université de Polynésie Française, UMR-EIO, LabEx-CORAIL, BP 6570, 98702 Faa'a, Tahiti, French Polynesia

⁶ Institut Océanographique Paul Ricard, Ile des Embiez, Six-Fours-les-Plages, France

* Corresponding author : Gérald Culioli, email address : culioli@univ-tln.fr ; Jean-François Briand, email address : briand@univ-tln.fr

Abstract :

Marine microalgae and cyanobacteria have largely been studied for their biotechnological potential and proved their ability to produce a wide array of bioactive molecules. We investigated the antifouling potential of unexplored benthic tropical microalgae using anti-adhesion and toxicity bioassays against two major micro- and macrobiofoulers, namely bacteria and barnacles. Fifty strains belonging to six phyla [Cyanobacteria, Miozoa (Dinoflagellata), Bacillariophyta, Cryptophyta, Rhodophyta and Haptophyta] were isolated from southwestern Islands of the Indian Ocean. They were chosen in order to represent as much as possible the huge biodiversity of such a rich tropical ecosystem. The associated chemodiversity was highlighted by both NMR- and LC-MS-based metabolomics. The screening of 84 algal fractions revealed that the anti-adhesion activity was concentrated in methanolic ones (i.e. 93% of all active fractions). Our results confirmed that microalgae constitute a promising source of natural antimicrofoulants as 17 out of the 30 active fractions showed high or very high capacity to inhibit the adhesion of three biofilm-forming marine bacteria. Dinoflagellate-derived fractions were the most active, both in terms of number and intensity. However, dinoflagellates were also more toxic and may not be suitable as a source of environmentally friendly antifouling compounds, in contrast to diatoms, e.g. *Navicula mollis*. The latter and two dinoflagellates of the genus *Amphidinium* also had interesting anti-settlement activities while being moderately toxic to barnacle larvae. Our approach, combining the bioprospecting of a large number of tropical microalgae for their anti-settlement potential and metabolomics analyses, constituted a first step towards the discovery of alternative ecofriendly antifoulants.

Keywords : Microalgae, Antifouling, Bioassay, Chemodiversity, Bioprospecting, Metabolomics

47 **Introduction**

48 Biofouling is a natural multistep process leading to the rapid colonization of any underwater surface
49 by a multitude of micro- (e.g. bacteria, diatoms, fungi) and macrofoulers (e.g. macroalgae, barnacles)
50 (Abarzua and Jakubowski 1995). The formation of biofilms on man-made structures is a global major
51 issue causing huge economic impacts for marine industries (Yebra et al. 2004; Schultz et al. 2011).
52 Efficient organotin-based antifouling (AF) paints have been banned since 2008 due to their high
53 toxicity and adverse environmental effects (Dafforn et al. 2011). They were mainly replaced by
54 coatings including copper and booster biocides. Unfortunately, the environmental impact of such
55 coatings remains of concern (Bellas 2006; Fernández-Alba et al. 2002) as the decreasing number of
56 European authorized AF biocides testifies (Biocidal Products Regulation, PT 21). Therefore, new
57 biomimetic alternatives have arisen driven by an increased awareness of environmental protection, like
58 the development of fouling release coating technology or the research of natural or bioinspired
59 antifoulants (e.g. (Lejars et al. 2012; Qian et al. 2010).

60 Particularly, marine natural products have been the subject of considerable bioprospecting studies as
61 they usually have unique chemical structures and a wide range of biological activities (Hu et al. 2015).
62 To date, hundreds of molecules have shown varying degree of activity against a broad range of fouling
63 organisms (Fusetani 2011; Qian et al. 2015). In fact, any natural product capable of inhibiting one or
64 several stages of fouling (e.g. antibacterial, antifungal, antialgal, antilarval) may have a potential as
65 antifoulant. Compounds inhibiting the bacterial adhesion could be viewed as a way to interfere with
66 the first conditioning phase of the biofouling process but also with the subsequent cross-kingdom
67 signaling between biofilms and macrofoulers propagules (Briand 2009; Houdai et al. 2004; Viles et al.
68 2000; Salta et al. 2013).

69 In opposition to macroalgae (Pérez et al. 2016) and benthic invertebrates (Fusetani 2004), the AF
70 potential of microalgae has been overlooked. Compared to human and foodborne pathogens, only few
71 microalgal compounds with antibacterial activity against marine fouling bacteria have been
72 characterized to date (Falaise et al. 2016; Mazur-Marzec et al. 2015; Montalvao et al. 2016). While the
73 development of natural product-based paints has been hampered by the difficulty to obtain antifoulants
74 from a sustainable source, microalgae-derived compounds can be obtained via short time large-scale
75 controlled cultures (Qian et al. 2010; Dafforn et al. 2011). This main advantage of microalgae over
76 macroorganisms could often circumvent expensive and challenging laboratory syntheses or the
77 restriction of collection and the inherent difficulty to obtain an adequate supply of invertebrate-sourced
78 molecules (Qian et al. 2010; Pulz and Gross 2004). Such culture-based approach is shared with
79 cultivable marine bacteria that have already been more investigated for AF purpose (Dobretsov et al.
80 2006).

81 In addition, photosynthetic microorganisms are represented by a huge diversity of species living in a
82 wide range of ecological habitats (Barra et al. 2014). To survive in such highly diversified and

83 competitive environments, tropical microalgae have developed adaptive and defensive strategies. In
84 particular, benthic biofilm-forming microalgae, mainly identified as pennate diatoms (e.g. (Salta et al.
85 2013), have been reported to produce deterrent chemicals that reduce or prevent biofilm formation
86 (Gademann 2007). A large number of marine microalgae have not been bioprospected yet, especially
87 tropical benthic species, with the exception of species of the genus *Lyngbya* or *Moorea* identified as
88 outstanding producers of bioactive metabolites (Taylor et al. 2014). This unexplored chemodiversity
89 from tropical environments constitute a natural heritage that should be preserved and promoted (Hay
90 and Fenical 1996).

91 Therefore, this study focused on tropical benthic microalgae from the southwest Indian Ocean that
92 may represent an unexplored source of natural and diversified antifoulants. A collection of fifty strains
93 covering six different phyla (Table 1) was screened to study their AF properties. To this end, we used
94 a bioassay targeting the anti-adhesion activity against three marine biofilm-forming bacteria that was
95 designed to discover effective antimicrofouling compounds (Camps et al. 2011; Othmani et al. 2014).
96 Three of the most promising microalgal strains were then selected to test their capacity to inhibit the
97 settlement of barnacle larvae (Othmani et al. 2016b). The toxicity of active fractions was also tested to
98 discuss their potential as sources of environmentally friendly antifoulants. Besides, the chemodiversity
99 of our collection of fifty strains was investigated using metabolomics analyses to explore the
100 differences in metabolic profiles between and within taxa. We expected that this polyphasic approach
101 would allow us to select the most promising strains in order to purify novel ecofriendly antifoulants in
102 further studies.

103

104 **Materials and methods**

105 ***Collection, isolation and cultivation of tropical microalgae***

106 Fifty strains of tropical benthic microalgae (*i.e.* including Cyanobacteria), collected from several
107 islands of the southwest Indian Ocean and conserved at the Phytobank collection (Hydrô Reunion,
108 Reunion Island, France) were used in this study. Half of them were isolated directly from
109 environmental biofilm samples (Table 1). They corresponded to 21 strains of Cyanobacteria (*i.e.*
110 prokaryotes) and 29 strains of microalgae belonging to 5 different phyla [*i.e.* 19 strains of
111 dinoflagellates (Miozoa), 4 of diatoms (Bacillariophyta), 3 of Cryptophyta, 2 of Rhodophyta and 1 of
112 Haptophyta]. Their isolation and identification were performed as explained in Zea Obando *et al.* (Zea
113 Obando et al. 2016).

114 Monospecific non-axenic isolates were grown in batch conditions (from 250 mL to 5 L) in aerated
115 appropriate media at 26°C with an irradiance of 20–40 $\mu\text{mol}/\text{m}^2/\text{s}$ and a 12:12h light/dark cycle. Media
116 were prepared in filter-sterilized natural seawater and cells were harvested at stationary phase.

117 ***Extraction and fractionation***

118 Freeze-dried material (500 mg) was extracted sequentially with mixtures of methanol/dichloromethane
119 (MeOH/CH₂Cl₂) of increasing polarity [1:2, 1:1 and 2:1 (v/v); 3 × 25 mL]. Each macerate was
120 vigorously shaken and then subjected to ultrasounds for 5 min (20°C). These macerates were then
121 filtered, pooled and concentrated to dryness under reduced pressure. The resulting crude extract was
122 further fractionated by solid phase extraction (SPE) using dry loading, *i.e.* dissolution of the extract in
123 a MeOH/CH₂Cl₂ (1:1, v/v) mixture and addition of 1g of C18 bonded-silica (Septra C18-E, 50 μm,
124 Phenomenex) before evaporation to complete dryness. SPE cartridges (Strata C18-E, 55 μm, 12g,
125 Phenomenex) were conditioned with 10 mL of MeOH and water, then dried samples were loaded and
126 eluted successively with 10 mL of water, water/MeOH (1:1, v/v), MeOH and CH₂Cl₂. The four
127 fractions were separately collected, evaporated to dryness and stored at -20°C.

128 ***Bacterial bioassays***

129 Microalgal fractions giving the best yields were tested for their anti-adhesion property and toxicity
130 against three marine biofilm-forming bacterial strains (*i.e.* depending on their activity and the amount
131 of extracts available).

132 The three fouling bacterial strains *Polaribacter* sp. (TC5), *Pseudoalteromonas lipolytica* (TC8) and
133 *Shewanella* sp. (TC11) were isolated from the Toulon Bay (Mediterranean Sea, France) between
134 February 2008 and June 2010 (Brian-Jaisson et al. 2014; Camps et al. 2011) and conserved at the
135 Toulon Collection (MAPIEM, Toulon, France). All strains were stored at -80°C in 30% glycerol
136 medium until use. They were chosen for their different taxonomic, physiological, and biochemical
137 properties (Brian-Jaisson et al. 2014; Camps et al. 2011).

138 For both anti-adhesion and toxicity assays, TC8 was first screened (*i.e.* lab reference strain) followed
139 by TC11 and TC5.

140 ***Anti-adhesion assay***

141 Anti-adhesion assay was adapted from Camps et al. 2011. The bacterial strains were grown in
142 Väätänen nine salt solution (VNSS) (Holmström et al. 1998) at 20 °C and 120 rpm and collected at
143 their stationary phase. Growth was monitored by measuring the turbidity (OD_{600 nm}, Genesis 20
144 spectrophotometer, ThermoFischer Scientific). After centrifugation 10 min at 6000 rpm, cell pellet
145 was suspended in sterile artificial seawater (ASW, 36 g/L, Sea Salts, Sigma) and introduced into 96-
146 well black microtiter plates (sterile PS, Nunc, Fisher Scientific). Fractions were tested in triplicates at
147 5–8 concentrations from 10 to 100 μg/mL concurrently with three controls: (1) non-specific staining
148 control, (2) adhesion control, and (3) solvent control [*i.e.* ASW/MeOH (95:5, v/v)]. After incubation
149 during an optimized adhesion time (ca. 15h) non-adhered bacteria were eliminated by three successive
150 washes (sterile NaCl solution, 36 g/L) and adhered cells were quantified using SYTO 61 staining
151 (1 μM, λ_{exc} = 628 nm, λ_{em} = 645 nm, ThermoFischer Scientific). Fluorescence measurements for all

152 bioassays were made using a TECAN Infini 200 microplate fluorescence reader and Tecan i-control
153 1.10 software.

154 A percent of adhesion was calculated per well, subtracting the value of appropriate non-specific
155 staining control and normalized to the adhesion control. Finally, a sigmoid dose-response curve was
156 obtained by plotting the percentage of adhesion with the log of fraction concentrations. After mean
157 ($n = 3$) and standard deviation (SD) calculation for each concentration, EC_{50} values (effective
158 concentration for 50% inhibition of bacterial adhesion) were calculated.

159 Biological replicates were performed on three independent bacterial cultures when sufficient amount
160 was available and if a bioactivity was first detected (*i.e.* $EC_{50} < 100 \mu\text{g/mL}$). Only fractions showing
161 $EC_{50} \leq 60 \mu\text{g/mL}$ were used to perform the toxicity tests.

162 *Toxicity assays*

163 Bacterial growth inhibition and viability assays were performed based on previous studies of our
164 group (Othmani et al. 2014; Camps et al. 2011). The microtiter plates were filled as for the anti-
165 adhesion assay.

166 Briefly, for growth inhibition assay, bacterial growth was monitored in VNSS medium, using the
167 turbidity as a proxy ($OD_{600\text{nm}}$), every hour in transparent microtiter plates (sterile PS; Nunc, Fisher
168 Scientific). When reaching the stationary phase, resazurin ($50 \mu\text{M}$, Sigma-Aldrich) was added and
169 fluorescence measured after 2h incubation. The growth rate μ (h^{-1}) was calculated during the
170 exponential phase and a percentage of inhibition was deduced. Finally, after mean and SD calculation
171 per triplicate for each concentration, a sigmoid dose-response curve was obtained and IC_{50} (inhibitory
172 concentration for 50% of the bacteria) was determined. For viability assay, the same procedure was
173 applied to calculate a LC_{50} (lethal concentration for 50% of the bacteria) using resazurin fluorescence.

174 ***Barnacle anti-settlement and larval toxicity assays***

175 Adult and larval barnacle cultures as well as anti-settlement and naupliar toxicity assays were
176 performed with *Amphibalanus* (= *Balanus*) *amphitrite* as explained in Othmani et al. (2016b) and
177 adapted from Rittschof et al. (1992). Solvent controls were 0.1% MeOH in filtered seawater (FSW,
178 salinity 37 ppm). Aliquots of the test solutions were pipetted into 24-well polystyrene culture plates
179 (ThermoScientific, France) and air-dried prior addition of 2 mL of FSW containing 10 organisms. For
180 anti-settlement assays, culture plates containing cyprids were incubated in the dark at $22 \pm 1 \text{ } ^\circ\text{C}$ for up
181 to 7 days before attached or metamorphosed individuals were counted under a binocular microscope.
182 Toxicity assays were carried out with both nauplii (stage V/VI) and cyprids. After 24 h, larvae were
183 examined for swimming activity or movement under a binocular microscope and categorized as alive,
184 morbid or dead (for calculation of the percentage of mortality, morbid larvae were classified as dead).
185 Only MeOH fractions of three microalgae were selected for the barnacle bioassays, based on both the
186 antimicrofouling and metabolomics analyses and due to the limitation of extracts. Concentrations

187 between 5 and 100–150 $\mu\text{g/mL}$ were tested in triplicate and repeated three times (*i.e.* twice for P-91)
188 with different nauplii/cyprids batches. Results were expressed as effective concentration inhibiting the
189 settlement of 50% cyprids (EC_{50}) and concentration inducing 50% morbidity (LC_{50}).

190 ***Statistical analysis***

191 Sigmoidal dose-response curves and determination of EC_{50} , IC_{50} and LC_{50} values for each fraction
192 were conducted with GraphPad Prism 5® (GraphPad Software).

193 ***Metabolomics analyses***

194 *LC-MS-based metabolomics*

195 MeOH eluates from the SPE fractionation of 47 microalgal extracts (*i.e.* all that remained after
196 bioassays) were used to perform a metabolomics analysis. All samples were standardized at a
197 concentration of 10 mg/mL. Blank samples were MeOH while quality control samples (QCs)
198 consisted of homogeneous pools of each sample.

199 LC-MS analyses were conducted on a LaChrom Elite HPLC system (VWR-Hitachi, France)
200 comprising an L-2130 quaternary pump, an L-2200 autosampler and an L-2300 column oven coupled
201 to an ion trap mass spectrometer (Esquire 6000; Bruker Daltonics) fitted with an electrospray
202 ionisation (ESI) interface.

203 The mass spectrometer parameters were set as follows: dry temperature, 350 °C; capillary voltage,
204 4000 V; nebulizer, 50 psi; dry gas, helium at 12 L/min. Ion trap full-scan analysis in the positive
205 detection mode was conducted from m/z 50 to 1200 with an upper fill time of 200 ms.

206 HPLC separation was achieved on an analytical reversed-phase column (Gemini C6-phenyl, 5 μm ,
207 250 \times 3 mm; Phenomenex) using a 65 min linear gradient elution of $\text{H}_2\text{O}/\text{ACN}/\text{formic acid}$. The
208 gradient started from 90:10:0.1 (v/v/v, isocratic from 0 to 5 min) to 0:100:0.1 (v/v/v) in 35 min, held
209 for 20 min before re-equilibration of the system for 10 min with the initial conditions. The flow rate
210 was 0.5 mL/min, the column temperature 30 °C and the injection volume was 10 μL . All solvents
211 were of LC-MS-grade. Ultrapure water was prepared using a Milli-Q water system (Millipore).
212 QCs were injected at both the beginning and the end and every 5 samples of the sequence injection to
213 ensure analytical repeatability. To limit time-dependent changes in LC-MS chromatographic analyses,
214 microalgal extracts and blank samples were randomly injected.

215 *Data preprocessing and filtering*

216 Data Analysis 4.3 software (Bruker Daltonics) was used to convert chromatograms to netCDF files as
217 line spectra. Data preprocessing was then performed with the XCMS software (Smith et al. 2006)
218 under the R 3.1.0 environment. Peak picking was performed with the “*matchedFilter*” method
219 (“*snthresh*” = 5), retention time correction with the “*obiwarp*” method (“*profstep*” = 0.1), peak

220 grouping with “*bw*” = 30 and “*mzwidth*” = 0.25 and gap filling with the “*FillPeaks*” method (using
221 default parameters).

222 The matrix containing the list of 797 features with their retention time, *m/z* value and intensity was
223 exported to SIMCA-P 13.0.3 software (Umetrics, Sweden). Data were log₁₀-transformed and Pareto-
224 scaled. Principal component analysis (PCA) was used to explore the chemical diversity across the
225 studied tropical microalgae.

226 ***NMR-based metabolomics***

227 *Sample preparation*

228 Extraction of microalgae for NMR-based metabolomics was different. Freeze-dried material (50 mg)
229 of the 47 strains was extracted with 1.5 mL of deuterated chloroform (CDCl₃) containing 0.03%
230 tetramethylsilane (TMS) by vortexing 5 min at 25 °C and 1700 rpm. Samples were ultrasonicated for
231 15 min (45 kHz, 35 °C) and centrifuged at 13000 rpm for 20 min. Aliquots of 0.7 mL were used for
232 NMR analysis.

233 *NMR measurements*

234 The ¹H-NMR experiments were performed at 25 °C on an Avance II 600 NMR spectrometer (Bruker,
235 Rheinstetten, Germany) equipped with a cryoprobe operating at a proton NMR frequency of
236 600.18 MHz. CDCl₃ was used for internal lock. A total of 128 scans spectra with a spectral width of
237 16,019 Hz were recorded with pulse width = 30° and relaxation delay = 1 s.

238 *Data and statistical analyses*

239 Prior to Fourier transformation, the free induction decays (FIDs) were zero-filled to 65536 points and
240 an exponential window function with a line broadening of 0.3 Hz was applied. The resulting spectra
241 were manually phased, baseline corrected and referenced to internal TMS at 0.00 ppm using
242 MestReNova 10.0 software (Mestrelab Research S.L., Spain). The ¹H-NMR spectra were then
243 automatically binned. Spectral intensities were normalized to total intensity and reduced to integrated
244 regions of equal width (0.04 ppm) corresponding to the region of δ 0.58–10.02 ppm. The region
245 δ 7.06–7.42 ppm was removed because of the residual solvent signal. PCA was performed on the
246 Pareto-scaled dataset using SIMCA 13.0.

247 **Results**

248 ***Anti-adhesion activity against bacteria***

249 Crude extracts of the 50 microalgal strains were further fractionated by SPE, leading to 200
250 fractions. In total, 84 fractions [*i.e.* 35 aqueous, 19 MeOH/H₂O (1:1, v/v), and 30 MeOH eluates] were
251 assessed for their anti-adhesion activity against one, two or the three pioneer biofilm-forming marine
252 bacteria. The choice for the fractions to be tested was guided by their amount (*i.e.* the mass obtained

253 after SPE; aqueous fractions being the most concentrated) and their solubility in seawater (which
254 excluded the CH₂Cl₂ fractions). No aqueous fractions were active while only two of the MeOH 50%
255 fractions were weakly or moderately active on *Polaribacter* sp. TC5 with EC₅₀ values of 83 and
256 47 µg/mL for the unidentified Cyanobacteria C-09 and the dinoflagellate *Symbiodinium* sp. P-73,
257 respectively (data not shown). The anti-adhesion activity was concentrated in MeOH fractions as they
258 represented 28 out of the 30 active fractions while only two MeOH fractions were not active.

259 Depending on both the microalgae and the targeted bacterium, different degrees of activity were
260 observed, spanning from very highly (EC₅₀ = 1–10 µg/mL), highly (EC₅₀ = 11–30 µg/mL), moderately
261 (EC₅₀ = 31–50 µg/mL) and weakly active (EC₅₀ = 50–100 µg/mL) to not active (EC₅₀ >100 µg/mL)
262 (Fig. 1). The activity of the tributyltin oxide (TBTO) was mentioned as a reference for our bioassay
263 and EC₅₀ values were between 1.2 and 14 × 10⁻³ µg/mL, ca. 1000 times lower than the most active
264 microalgal fraction. Interestingly, anti-adhesion activity was detected among all phyla. Indeed, 8/21
265 Cyanobacteria, 14/19 dinoflagellates, the four diatoms, 2/3 Cryptophyta, 1/2 Rhodophyta and the only
266 Haptophyta showed some antimicrofouling potential. Nevertheless, most highly active fractions were
267 obtained from dinoflagellates, especially from species of the genus *Amphidinium*. Notably, two strains
268 (*i.e.* *Amphidinium gibbosum* P-43 and *Symbiodinium* sp. P-78) were very highly active against the
269 three bacteria while only two non-dinoflagellate fractions (*i.e.* one of the cryptophyte P-68 and one of
270 the Cyanobacteria C-59) showed very high activities but only against one of the bacteria. Noteworthy,
271 the fractions derived from microalgae isolated from biofilms also had high activities, particularly two
272 out of the four diatoms and some Cyanobacteria (e.g. C-59 and C-61).

273 ***Toxicity against bacteria***

274 We evaluated the impact of the MeOH microalgal fractions on both the growth and the viability of
275 the three biofilm-forming marine bacteria. Bioassays were performed if an EC₅₀ value ≤60 µg/mL was
276 obtained for at least one bacterium and when sufficient material was still available (Fig. 2).
277 MeOH fractions with promising anti-adhesion activities also exhibited important growth inhibition as
278 42, 71 and 100% of the tested fractions were active on TC11, TC8 and TC5, respectively. Even though
279 TC5 seemed more sensitive than the two other strains, the comparison of bacterial strains sensitivity is
280 hampered by the fact that the number of tested fractions differed between the three strains (17/18 for
281 TC8, 12/18 for TC11 and 9/18 for TC5, *i.e.* due to the lack of material). Strains of the genus
282 *Amphidinium* gave the most active fractions with IC₅₀ values ≤50 µg/mL for all but two bacterial
283 fractions (*i.e.* P-44 and P-63 on TC11), followed by *Pavlova* sp. P-69 and the cryptophyte P-68.
284 However, few fractions were lethal to the bacteria. Again, *Polaribacter* sp. TC5 appeared more
285 sensitive than the other two strains, with LC₅₀ values ≤50 µg/mL for the six out of the nine active
286 fractions. Only two fractions were weakly toxic to *Shewanella* sp. TC11. *P. lipolytica* TC8 had an
287 intermediate sensitivity as 41% of the tested fractions were active, including two that were highly
288 toxic with LC₅₀ values ≤30 µg/mL (*i.e.* *Pavlova* sp. P-69 and *Symbiodinium* sp. P-78).

289 While almost all toxic microalgae also had some growth inhibition activity, four strains were only
290 toxic to the bacteria (*i.e.* the diatoms *Nitzschia* sp. P-89 and *Psammodictyon* sp. aff. *constrictum* P-90
291 and the Cyanobacteria *Pseudanabaena* sp. C-30 and *Synechococcus elongatus* C-60), suggesting a
292 specific mode of action. The opposite trend was observed for the unidentified cryptophyte P-68 which
293 was non-toxic but inhibited the growth of the three bacteria. The underpinning mechanisms of both
294 growth inhibition and toxicity were beyond the scope of this study.

295 Finally, the selectivity index (SI) also called therapeutic ratio (Rittschof et al. 1992) and defined as
296 the ratio of LC₅₀ and EC₅₀, was calculated. Eight out of the nine dinoflagellate-derived fractions and
297 only one non-dinoflagellate fraction (*i.e.* for the cryptophyte P-68) had a SI ≥ 10 for at least one of the
298 bacteria. Concerning the other phyla, SI were always ≤ 4, independently of the bacterial strains. SI
299 values were strain-dependent but highest for TC11 as this strain was more resistant.
300 Values for TBTO (IC₅₀, LC₅₀ and SI) were added as references. While being less active in the anti-
301 adhesion bioassay, MeOH fractions of microalgae were about 3500–30 000 times less acutely toxic
302 than TBTO and thus had generally better SI.

303 ***Anti-settlement activity and toxicity against barnacle larvae***

304 Two dinoflagellates of the genus *Amphidinium* (P-43 and P-60) and the diatom *Navicula mollis* P-
305 91 were selected to perform barnacle bioassays (Table 2). These strains were chosen based on the
306 availability of the remaining extracts and both their antimicrofouling potential and chemodiversity
307 (Fig. 3, Appendices A and B). The three MeOH fractions showed a significant anti-settlement activity
308 with EC₅₀ values ≤ 45 µg/mL. *Navicula mollis* P-91 fraction was ca. 5 times more active than those of
309 the dinoflagellates P-43 and P-60. Cytotoxicity towards *A. amphitrite* stage V/VI nauplii and cyprids
310 was strain-dependent. Interestingly, no acute toxicity for both nauplii and cyprids was observed for P-
311 91, even at the highest concentrations (24h-LC₅₀ > 100 µg/mL) while dinoflagellates showed some
312 toxicity on cyprids.

313 ***Metabolomics analyses***

314 Metabolomics analyses were performed to evaluate the chemodiversity of the tropical microalgae.
315 Only chromatographic profiles obtained with MeOH fractions were considered for LC-MS-based
316 metabolomics as anti-adhesion activity was predominantly detected in these fractions.
317 The PCA score plot of 46 out of the 50 strains initially studied was represented on Fig. 1. The four
318 missing fractions corresponded to three fractions that were no longer available (*i.e.* cyanobacterial
319 strains C-30, C-33 and C-58) and the exclusion of C-64 (*Porphyridium* sp.) as it clustered with the
320 blanks. The total variance due to the two main axis accounted for 45% on the PCA score plots. The
321 distinction between prokaryotes (*i.e.* Cyanobacteria) and eukaryotes is clearly visible on the first
322 component (27%), with few exceptions (*i.e.* the eukaryotes C-03, P-04, P-67 and P-92 clustered with

323 Cyanobacteria) while the second component (17%) allowed the distinction between diatoms and
324 Cryptophyta.

325 Although the sizing of symbols by bioactivity confirmed the better anti-adhesion activity of
326 dinoflagellates, other strains (e.g. the Cyanobacteria C-59 or the diatoms P-90 and P-91) had
327 interesting activities while being in separate clusters, thus displaying different chemical profiles.
328 To verify that the clustering obtained with LC-MS-based metabolomics was not biased by the specific
329 sample preparation, we also performed an NMR-based metabolomics analysis using organic crude
330 extracts (Supp Figure 1). The two main axes accounted for a better total variance of 71.3%. In these
331 conditions, C-64 was not an outlier and a few exceptions with the taxa-based clustering were also
332 noted. The presence of the C-03 and P-67 strains in the Cyanobacteria group was in agreement with
333 the LC-MS-based metabolomics. However, considering diatoms, P-89 was clearly in the
334 dinoflagellates group considering NMR profiles, whereas P-92 did not cluster with the three other
335 diatom strains considering LC-MS profiles. Both metabolomics approaches revealed that
336 Cyanobacteria and dinoflagellates were scattered groups, indicating metabolic specificity thus
337 chemodiversity within these two phyla.

338 A more specific analysis of the metabolomics dataset of these two phyla allowed, through the building
339 of OPLS-DA models, to highlight some putative biomarkers. A focus was made on the most
340 discriminating m/z variables and NMR chemical shifts (Supp Figures 2 and 3). More precisely,
341 variables with m/z 587.5 and m/z 589.5 were specifically produced in cyanobacterial samples and
342 could be related to the occurrence of phycobilins in such extracts (Fu et al. 1979; Singh and Verma
343 2012). In the case of NMR data, several of the ^1H NMR bins overexpressed in dinoflagellates ranged
344 between 4.00 and 3.50 ppm and between 5.00 and 5.50 ppm and thus could be attributed to
345 acylglycerols with polyunsaturated fatty acids (Nieva-Echevarría et al. 2014).

346 Discussion

347 The discovery of environmentally friendly molecules that effectively prevent biofouling remains a
348 challenge in the marine antifoulant research field (Dafforn et al. 2011; Qian et al. 2015). New
349 biomimetic solutions could be developed when considering the natural mechanisms implemented by
350 marine organisms to protect themselves (e.g. against epibiosis) (Aguila-Ramirez et al. 2014; Scardino
351 and de Nys 2011). A key point could be that a single molecule would never have a chance to inhibit
352 the huge diversity of biofoulers colonizing surfaces in the marine environment. For this reason, this
353 work aimed to study extracts/fractions and not pure compounds which required time-consuming and
354 costly purification and characterization steps while not necessarily being ecologically relevant. In
355 addition to the possibility to inhibit the adhesion of several organisms, considering extracts/fractions
356 allowed to benefit from likely synergism between compounds.

357 This study focused on benthic microalgae that were collected from different islands of the
358 southwest Indian Ocean (*i.e.* 23 from Reunion Island, 14 from the Scattered Islands, 7 from Mayotte, 5

359 from Madagascar and 1 from Mauritius) between 1992 and 2013. Hitherto, studies about microalgae
360 from this were mainly restricted to toxic dinoflagellates (e.g. *Ostreopsis* spp. which produce one of the
361 most potent non-protein toxin (Lenoir et al. 2004)). Thus, the biotechnological potential of such
362 organisms in the field of AF was unknown. Our collection was very diverse, although dominated by
363 two out of the six phyla (dinoflagellates and Cyanobacteria represented 38% and 42% of the 50
364 strains, respectively). The four diatoms and almost all Cyanobacteria (*i.e.* 19/21 strains) were isolated
365 directly from marine biofilms (Landoulsi et al. 2011; Salta et al. 2013). These autotrophs, especially
366 Cyanobacteria, are major components of tropical biofilms (Viles et al. 2000; Paul et al. 2005). It has
367 also been suggested that these biofilm-forming microalgae can produce a variety of chemical
368 deterrents for defense purposes. Hence, they may show some AF properties (Dobretsov et al. 2006;
369 Leao et al. 2012).

370 ***Anti-adhesion activity***

371 When searching for AF compounds intended to protect man-made devices, bacteria adhering on
372 artificial substrata should be tested (Briand 2009; Camps et al. 2011). The three bacterial strains used
373 here (TC5, TC8 and TC11) were isolated from marine biofilms formed after a few hours on immersed
374 artificial substrata in a temperate area (Brian-Jaisson et al. 2014). We have also isolated a set of
375 bacterial strains from artificial surfaces immersed at the Reunion Island (*i.e.* tropical area), which
376 showed a similar sensitivity against AF biocides compared to the three temperate ones (data not
377 shown). Therefore, we considered that TC5, TC8 and TC11 temperate strains, for which previous data
378 were available (e.g. Brian-Jaisson et al. 2014; Camps et al. 2011; Othmani et al. 2014; Othmani et al.
379 2016a; Othmani et al. 2016b; Favre et al. 2017), were suitable targets to assess AF potential of tropical
380 microalgae. Interestingly, targeting the inhibition of fouling bacteria adhesion may further prevent or
381 reduce both the micro- and macrofouling of submerged structures. Indeed, some microfoulers (e.g.
382 bacteria, especially of the genus *Pseudoalteromonas* and diatoms) have been reported to regulate the
383 settlement of macrofoulers via chemical cues (Hadfield 2011; Qian et al. 2007).

384 The screening of anti-adhesion activity among the 50 microalgal strains revealed that the AF
385 potential varied widely depending on the polarity of the crude extract, the species and, to a lesser
386 extent, the fouling bacteria tested. The higher sensitivity of TC5 in all bioassays was already reported
387 [22, 24, 68] and may be related to its lower ability to form substantial biofilms *in vitro* (Brian-Jaisson
388 et al. 2014). Bioactivity was considered fraction-specific as 93% of the 30 active samples were
389 methanolic fractions and none of the 35 aqueous fractions inhibited the adhesion of bacteria. In
390 agreement with our results, Falaise et al. reviewed that antimicrobial activity of microalgae was
391 generally found in methanolic and other organic extracts (Falaise et al. 2016).

392 It is interesting to note that microalgae from all taxa, independently of their origin, showed some
393 activity. Mudimu and collaborators hypothesized that the capacity to produce antibacterial and
394 antifungal compounds has evolved independently of phylogenetic relationship in both microalgae and

395 Cyanobacteria (Mudimu et al. 2014). However, dinoflagellate species assayed here, especially
396 *Amphidinium* and *Symbiodinium* strains, were the most active. While marine dinoflagellates have
397 proved to be an important source of bioactive natural products (e.g. Van Wagoner et al. 2014),
398 antimicrobial activity derived from dinoflagellate extracts or compounds has rarely been reported so
399 far. Only a few studies observed antimicrobial properties for compounds isolated from *Amphidinium*
400 spp., e.g. amphidinins C–F and luteophanol A (Doi et al. 1997; Kubota et al. 2014). Most of the
401 dinoflagellates screened in this study are sand-dweller or benthic species with predominantly motile
402 cells. From an ecological point of view, their capacity to inhibit the growth of bacterial competitors
403 could be less critical than for sessile benthic taxa which settle on surfaces in more diversified
404 microbial communities.

405 Concerning the microalgae isolated directly from biofilms, Cyanobacteria had surprisingly a
406 general moderate activity while diatoms, and in particular *Navicula mollis* P-91, were highly active.
407 Cyanobacteria are a very prolific source of bioactive metabolites (Leao et al. 2012) and they are
408 known to display a rich chemodiversity (Burja et al. 2001). Even if some reports seemed to prone their
409 potential application as antimicrofoulants (e.g. Bhadury and Wright 2004; Gademann 2007), others
410 reached similar conclusions to our own. For example, during a bioprospecting of Cyanobacteria from
411 the Baltic Sea, only three out of 27 strains were reported to weakly inhibit the growth of fouling γ -
412 Proteobacteria (Mazur-Marzec et al. 2015). However, it should be noted that only nine of the
413 Cyanobacterial strains gave sufficient material to perform bioassays in our study. Thus, the
414 antimicrofouling potential of the twelve other strains cannot be ruled out.

415 Diatoms may hide a potential for AF compound production according to Dobretsov et al. (2006). In
416 previous studies, extracts from *Attheya longicornis* (Ingebrigtsen et al. 2016), *Amphiprora paludosa*
417 (Sanchez-Saavedra et al. 2010), *Nitzschia communis* and *Amphora* cf. *capitellata* (Montalvao et al.
418 2016), and polyunsaturated fatty acids (e.g. hexadecatetraenoic, eicosapentaenoic and
419 hexadecatrienoic acids) from *Chaetoceros muelleri* (Falaise et al. 2016), *Navicula delognei* (Findlay
420 and Patil 1984) and *Phaeodactylum tricorutum* (Desbois et al. 2008; Desbois et al. 2009) have shown
421 interesting antibacterial activities. Moreover, blue pigments from *Haslea ostrearia* and *H.*
422 *karadagensis* were found to be active against three marine fungi implicated in biofouling (Gastineau et
423 al. 2012a, b).

424 Among strains belonging to the three other taxa, moderate anti-adhesion activity was detected for
425 the rhodophyte C-64 *Porphyridium* sp. whereas the haptophyte *Pavlova* sp. P-69 was highly active
426 against the three bacteria. The two strains of Cryptophyta (P-68 and P-70) showed in-between
427 activities. To date, some *Porphyridium* spp. have shown antibacterial activities, e.g. organic extracts of
428 *P. purpureum*, *P. aerugineum* or *P. cruentum* (Falaise et al. 2016; Sanchez-Saavedra et al. 2010;
429 Guedes et al. 2011) while phycobiliproteins of *P. aerugineum* and *P. cruentum* were both antibacterial
430 and antifungal (Najdenski et al. 2013). AF potential of haptophytes is poorly documented and only
431 two antibacterial chlorophyll a degradation products from *Isochrysis galbana* have been reported

432 (Falaise et al. 2016). Finally, we could not find any report of bioactivity concerning Cryptophyta.
433 However, one of our unidentified strains (P-68) showed significant anti-adhesion activity against the
434 three bacteria tested that may involve some growth inhibition mechanism considering toxicity results.

435 It is well recognized that AF activity should include the capacity to disturb the adhesion of a wide
436 range of foulers. Among them, barnacle represents a key species, especially because of its wide
437 distribution and, as encrusting taxa, the huge damage associated with its settlement (Aldred and Clare
438 2008). Thus, the potential to prevent adhesion of the barnacle model *A. amphitrite* was also assessed
439 with three microalgal fractions selected for their interesting antibacterial adhesion activity. The two
440 dinoflagellate (*i.e.* from *Amphidinium gibbosum* P-43 and *A. carterae* P-60) showed moderate activity
441 while the diatom *Navicula mollis* P-91 exhibited a remarkable ability to inhibit the adhesion of *A.*
442 *amphitrite*. This is the first time to our knowledge that a benthic diatom isolate coming from a marine
443 biofilm was shown to be potentially involved in the prevention of invertebrate adhesion.

444 It is of great interest for our screening to identify natural extracts inhibiting several target species.
445 Some other interesting activities from microalgae have been reported against biofouling, in addition to
446 antibacterial compounds. For example, antifungal or algicidal molecules have been purified from
447 *Amphidinium* species (Houdai et al. 2004; Washida et al. 2006; Kong et al. 2016). Similar activities
448 have also been observed in Cyanobacteria and Rhodophyta (Najdenski et al. 2013; Berry et al. 2008).
449 Besides, Almeida et al. noted that aqueous extracts of *Synechocystis* strains isolated from Atlantic
450 coasts showed interesting molluscicidal activities (Almeida et al. 2015). That result should also be
451 considered regarding the interactions between biofilms and invertebrate larval recruitment. If the
452 bacterial role has already been investigated as previously mentioned (Hadfield 2011; Qian et al. 2007;
453 Salta et al. 2013), the role of major autotrophs in biofilms, *i.e.* the diatoms, in the control of larval
454 recruitment has been overlooked.

455 ***Toxicity of the tropical microalgae***

456 Inhibition of bacterial adhesion (*i.e.* biofilm formation) or cyprid settlement may result either from
457 specific inhibition of the adhesion process itself or as a consequence of toxicity. In order to discuss the
458 actual potential of tropical microalgae as sources of environmentally friendly antifoulants, we studied
459 the toxicity of active fractions on the three fouling bacteria and on two stages of barnacle life cycle.
460 Generally, when SI, *i.e.* the ratio of bioactivity and toxicity, is higher than 10 to 15 (Rittschof et al.
461 1992; Qian et al. 2010), extracts or compounds are assumed to be non-toxic.

462 Regarding the toxicity towards bacteria, this criterion was met only for dinoflagellates while other
463 strains had selective indexes lower than four. These lower SI reflected more the less potent anti-
464 adhesion activity of non-dinoflagellate microalgae rather than their toxicity, except for the haptophyte
465 *Pavlova* sp. P-69 which was highly toxic against TC8 and TC5.

466 Therefore, considering only SI, almost all dinoflagellate strains studied here may be sources of
467 environmentally friendly antimicrofoulants. However, they also had important growth inhibition

468 activity on the three marine bacteria, in contrast to diatoms and Cyanobacteria. Moreover, many toxic
469 compounds have been isolated from dinoflagellates of the genus *Amphidinium*, *Symbiodinium* and
470 *Prorocentrum* (Kobayashi and Kubota 2007; Gordon and Leggat 2010; Ten-Hage et al. 2002; Neves et
471 al. 2017) that might explain their relative higher toxicity in this study, including on barnacles.
472 Indeed, the anti-settlement activity of dinoflagellate fractions appeared to be associated with toxic
473 compounds. Thus, resulting therapeutic ratio (*i.e.* > 10 for P-91 and < 3 for P43 and P-60) suggested
474 that anti-settlement activity of *Navicula mollis* P-91 was likely due to molecules that may specifically
475 hinder adhesion mechanisms of barnacle, in opposition to intrinsic toxicity of *Amphidinium* P-43 and
476 P-60 fractions. Noteworthy, toxicity of the two dinoflagellates was noted only for cyprid larvae (*i.e.*
477 the stage at which barnacle larva are able to attach to surfaces).

478 Despite their weak bacterial growth inhibition described here, toxicity of Cyanobacteria and
479 diatoms has also been reported against several types of organisms. For examples, extracts of
480 *Synechocystis* and *Leptolyngbya* spp. were found to induce acute toxicity to *Artemia salina nauplii*, to
481 sea urchin larvae while they completely inhibited the embryogenesis of the Mediterranean mussel
482 (Martins et al. 2007; Lopes et al. 2010). Concerning diatoms, even though none of the *Navicula* and
483 *Nitzschia* strains screened by Wichard et al. produced oxylipins (Wichard et al. 2005), many diatoms
484 can produce these very reactive aldehydes that were highly cytotoxic to six different phyla including
485 copepods, oysters and sea urchins (Adolph et al. 2004). Fortunately, the active diatom strains tested in
486 this work showed no toxicity towards bacteria and barnacle larvae but ecotoxicological tests should be
487 performed on a wider diversity of marine organisms, as well as screening for domoic acid production.
488 Indeed, this neurotoxin responsible for Amnesic Shellfish Poisoning (ASP) in humans and
489 accumulating in organisms of higher trophic levels (Saeed et al. 2017), has recently been discovered in
490 *Nitzschia bizertensis* sp. (Smida et al. 2014) and *Nitzschia navis-varingica* (Suriyanti and Usup 2015).
491 Toxicity information should be considered before the selection of tropical microalgae for future
492 possible costly works (e.g. cultivation at large scale, purification and assessment of *in situ* activity of
493 AF compounds).

494 ***Chemodiversity of tropical microalgae***

495 Metabolomics is the global measurement of metabolites in biological systems that reflect the
496 phenotype. It is ultimately the result of underlying genomic, transcriptomic, and proteomic networks
497 (Fiehn 2002). Combining PCA with analytical tools such as liquid chromatography tandem mass
498 spectrometry is an attractive method to provide a visual representation of differences between LC-MS
499 profiles (Hou et al. 2012; Othmani et al. 2016a; Favre et al. 2017). Indeed, microalgae producing
500 similar metabolites would group together and vice versa. Considering our sample preparation
501 procedure (*i.e.* fractionation of MeOH/CH₂Cl₂ crude extracts into four fractions of decreasing polarity
502 for bioassay purpose), the clustering observed on the PCA score plots was very interesting. Indeed, the
503 groups observed were confirmed by a NMR-based metabolomics approach with the whole organic

504 extracts and corresponded to the four main taxa (*i.e.* Cyanobacteria, dinoflagellates, diatoms and
505 Cryptophyta while Rhodophyta and Haptophyta cannot be considered as only one strain was available
506 for both phyla). These results suggested that both methods were valuable to evaluate the
507 chemodiversity of tropical microalgae.

508 Previously, the clear separation between our Cyanobacteria and dinoflagellate strains had already
509 been reported using *in vivo* ¹H HR-MAS NMR spectroscopy (Zea Obando et al. 2016). However, we
510 unexpectedly observed the presence of four eukaryotic strains in the Cyanobacteria cluster with LC-
511 MS-based metabolomics; some (e.g. the rhodophyte C-03) were confirmed by the NMR-based
512 approach. It may result from the presence of common compounds like phycobiliproteins, or, more
513 probably, from the degradation of these fractions. The biodiversity of microalgae was thus reflected by
514 both the inter- and intraspecific metabolic profiles which is interesting for a bioprospecting purpose.
515 Using metabolomics, even with low resolution mass spectrometry on fractionated extracts, we were
516 able to highlight the chemodiversity of tropical microalgae without identifying the composition of
517 each extract or having the precise identification of all strains. Indeed, the heatmap of metabolites
518 showed only partial overlap between selected strains (Supp Figure 4). As evidenced by Hou et al.
519 (2012) and supported by this preliminary study, the LC/MS-PCA combination should be useful for the
520 selection of strains displaying maximal chemical diversity for the discovery of novel antifoulants.

521 ***Selection of the most promising tropical microalgae***

522 Based on all our results, *i.e.* anti-adhesion activity, toxicity and chemodiversity, up to five strains
523 could be selected for in-deep study of their AF potential, namely the unidentified cryptophyte P-68,
524 the Cyanobacteria *Gloeocapsopsis* sp. C-61, the dinoflagellates *Amphidinium gibbosum* P-43 and *A.*
525 *carterae* P-60 and finally the diatom *Navicula mollis* P-91.

526 Benthic marine microalgae have been neglected for bioprospecting purpose, possibly due to culture
527 issues (Barra et al. 2014). However, the fifty strains studied here were successfully cultivated in up to
528 5 L flasks. Besides, *A. carterae* has shown a good potential regarding culture scale-up (Fuentes-
529 Grünwald et al. 2016) as well as Cyanobacteria like *Spirulina* (Xu et al. 2009) or benthic diatoms
530 (Raniello et al. 2007). Further studies on the purification and identification of active compounds from
531 the selected strains will be conducted to characterize natural eco-friendly antifoulants. So far,
532 antimicrobial compounds isolated from microalgae were mainly fatty acid-related compounds or, to a
533 lesser extent, pigments (Falaise et al. 2016). Other chemical families structures, e.g. lactones are
534 promising like for honaucins isolated from the marine Cyanobacteria *Leptolyngbya crossbyana* (Choi
535 et al. 2012) which inhibited quorum sensing, a form of cell-cell communication essential in microbial
536 surface colonization (Dang and Lovell 2016).

537 Another advantage of microalgae is that culture conditions can be manipulated and optimized to the
538 overexpression or accumulation of metabolites of interest, which is known as metabolic induction
539 concept (Abida et al. 2013). The production of bioactive compounds by microalgae varies according to

540 growth conditions, availability and concentration of nutrients in the culture medium, light intensity,
541 temperature and/or pH (Noaman et al. 2004; Ingebrigtsen et al. 2016; Bagwell et al. 2016; Volk and
542 Furkert 2006). Thus, the AF potential of tropical microalgae could be further increased after the
543 optimization of culture conditions for the production of AF agents.
544 Biofouling is a very complex process that cannot be fully replicated by laboratory single-species
545 bioassays. Hence confirmation of the anti-adhesion activity of selected tropical microalgae would be
546 required using at least mixed-species biofilms *in vitro* or by field studies (Burgess et al. 2003; Briand
547 2009; Lee et al. 2014). Specifically, the methanolic fractions of *Amphidinium* P-43 and P-60 strains
548 and *Navicula mollis* P-91 have shown interesting antimicro- and antimacrofouling properties thus they
549 should be incorporated in several type of coatings for *in situ* tests.

550 **Conclusion**

551 In this study, we presented the results of the large-scale screening of the AF properties of fifty
552 tropical microalgae including Cyanobacteria. The metabolomics analyses revealed the untapped
553 chemodiversity of our collection of tropical strains. Methanolic fractions from strains of all six phyla
554 inhibited to some extent the adhesion of the three fouling bacteria tested. Dinoflagellates were highly
555 active but also the more toxic. One diatom strain especially exhibited a promising potential for the
556 inhibition of the adhesion of both biofilm bacteria and barnacle larvae. Together, these results allowed
557 us to select five strains with very promising potential as sources of environmentally friendly
558 antifoulants. However, in addition to the complete chemical characterization of the active extracts,
559 further field studies with microalgae extract-based coatings are required to better characterize the
560 actual AF potential of these tropical microalgae.

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568

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853

854 **Figure and Table captions:**

855 **Figure 1:** PCA score plot obtained from LC-MS profiles of 46 MeOH fractions of tropical microalgae.
856 Symbols were sized by bioactivity (*i.e.* $1/EC_{50}$). Ellipses were drawn manually according to taxa
857 clustering (the eukaryotic strains that clustered with Cyanobacteria were not included).

858

859 **Table 1:** Origin, identification (*i.e.* names accepted according to Guiry and Guiry 2017), culture media
860 and identification number of the 50 tropical microalgae used in this study.

861 **Table 2:** Anti-adhesion activity (EC_{50} , mean \pm SD) of microalgal fractions on the three fouling bacteria
862 (TC8: *Pseudoalteromonas lipolytica*, TC5: *Polaribacter* sp. and TC11: *Shewanella* sp.). Only data for
863 MeOH fractions showing activity against at least one bacterium were given. A grey scale has been
864 applied to ease the visualization of the results. * $n = 2$. **Published in Othmani et al. (Othmani et al.
865 2016b), mentioned here as a reference.

866 **Table 3:** Growth inhibition (IC_{50}), mortality (LC_{50}) and selectivity index ($SI = LC_{50}/EC_{50}$) of tropical
867 microalgae MeOH fractions on the three biofilm-forming bacteria (TC8: *Pseudoalteromonas lipolytica*,
868 TC5: *Polaribacter* sp. and TC11: *Shewanella* sp.). Two distinct grey scales (one for IC_{50} and LC_{50} and
869 one for SI) have been applied to ease the visualization of the results. *Published in Othmani et al.
870 (Othmani et al. 2016b), mentioned here as a reference.

871 **Table 4:** Anti-settlement activity (EC_{50}) and mortality (LC_{50}) of the MeOH fraction of three tropical
872 microalgae against *A. amphitrite* nauplii and cyprids (expressed as mean \pm SD).

873

874

Table 1: Origin, identification (*i.e.* names accepted according to Guiry and Guiry 2017), culture media and identification number of the 50 tropical microalgae used in this study.

Phyla	Family	Genus	Species	Collection Site	Culture Media ^a	ID ^b
Haptophyta	Pavlovaceae	<i>Pavlova</i>	sp.	Glorioso	F/2	P-69
Cryptophyta	ND ^c	Cryptophyta	sp.	Glorioso	F/2	P-67
		Cryptophyta	sp.	Glorioso	F/2	P-68
		Cryptophyta	sp.	Glorioso	F/2	P-70
Miozoa (Dinoflagellates)	Gymnodiniaceae	<i>Amphidinium</i>	<i>carterae</i>	Reunion	F/2	P-38
		<i>Amphidinium</i>	<i>massartii</i>	Mauritius	F/2	P-41
		<i>Amphidinium</i>	<i>massartii</i>	Europa	F/2	P-42
		<i>Amphidinium</i>	<i>gibbosum</i>	Europa	F/2	P-43
		<i>Amphidinium</i>	<i>massartii</i>	Glorioso	F/2	P-44
		<i>Amphidinium</i>	<i>carterae</i>	Glorioso	F/2	P-45
		<i>Amphidinium</i>	<i>carterae</i>	Glorioso	F/2	P-46
		<i>Amphidinium</i>	<i>carterae</i>	Madagascar	F/2	P-59
		<i>Amphidinium</i>	<i>carterae</i>	Madagascar	F/2	P-60
		<i>Amphidinium</i>	<i>carterae</i>	Madagascar	F/2	P-63
	<i>Amphidinium</i>	<i>massartii</i>	Reunion	F/2	P-80	
	Prorocentraceae	<i>Prorocentrum</i>	<i>lima</i>	Reunion	F/2	P-04
		<i>Prorocentrum</i>	<i>lima</i>	Reunion	F/2	P-08
		<i>Prorocentrum</i>	<i>lima</i>	Reunion	F/2	P-37
	Suessiaceae	<i>Symbiodinium</i>	sp. (Clade F)	Reunion	F/2	P-72
		<i>Symbiodinium</i>	sp. (Clade B)	Reunion	F/2	P-73
		<i>Symbiodinium</i>	sp.	Reunion	F/2	P-76
<i>Symbiodinium</i>		sp. (Clade D)	Reunion	F/2	P-78	
<i>Symbiodinium</i>		sp. (Y6-1)	Reunion	F/2	P-79	
Bacillariophyta (Diatoms)	Naviculaceae	<i>Navicula</i>	<i>mollis</i>	Reunion	F/2+Si	P-91*
		<i>Navicula</i>	sp.	Reunion	F/2+Si	P-92*
	Bacillariaceae	<i>Nitzschia</i>	sp.	Reunion	F/2+Si	P-89*
		<i>Psammodictyon</i>	sp. aff. <i>constrictum</i>	Reunion	F/2+Si	P-90*
	Merismopediaceae	<i>Synechocystis</i>	sp.	Madagascar	F/2	C-02
	Chroococcaceae	<i>Gloeocapsopsis</i>	sp.	Glorioso	BG11	C-61*
	Cyanothecaceae	<i>Cyanothece</i>	sp.	Glorioso	BG11	C-58*
	ND	ND	sp.	Glorioso	BG11	C-59*
		LPP-group	sp. (LPP1)	Mayotte	BG11	C-09*
		LPP-group	sp. (LPP1)	Mayotte	BG11	C-12*
		LPP-group	sp. (LPP1)	Mayotte	BG11	C-14*
Cyanobacteria	Coleofasciculaceae	<i>Roseofilum</i>	sp.	Reunion	BG11	C-07*
		<i>Roseofilum</i>	sp.	Reunion	BG11	C-32*
	Leptolyngbyaceae	<i>Leptolyngbya</i>	sp.	Mayotte	BG11	C-10*
		<i>Leptolyngbya</i>	sp.	Mayotte	Z8	C-13*
		<i>Leptolyngbya</i>	sp. (RS01)	Mayotte	BG11	C-16*
		<i>Leptolyngbya</i>	sp.	Reunion	BG11	C-18*
		<i>Leptolyngbya</i>	sp.	Reunion	BG11	C-23*
	Spirulinaceae	<i>Spirulina</i>	<i>subsalsa</i>	Reunion	BG11	C-17*
		<i>Spirulina</i>	sp.	Reunion	BG11	C-27*
	Pseudoanabaenaceae	<i>Pseudoanabaena</i>	sp.	Reunion	BG11	C-24*
		<i>Pseudoanabaena</i>	sp.	Reunion	BG11	C-30*
		<i>Limnothrix</i>	sp.	Reunion	BG11	C-33*
Synechococcaceae	<i>Synechococcus</i>	<i>elongatus</i>	Madagascar	F/2	C-01	
	<i>Synechococcus</i>	<i>elongatus</i>	Glorioso	BG11	C-60*	
Rhodophyta	Porphyridiaceae	<i>Porphyridium</i>	sp.	Glorioso	BG11	C-64*
	Stylonemataceae	<i>Choodactylon</i>	sp. aff. <i>ornatum</i>	Mayotte	BG11	C-03*

^aCulture media: F/2 and F/2+Si: Guillard's medium without or with silicate (Guillard 1975); BG11: Blue Green Medium

(Stanier et al. 1971); Z8 medium (Kotai 1972); ^bID: Phytobank identification number; ^cND: not determined; *strains isolated from biofilms or growing while isolating biofilms

Table 2: Anti-adhesion activity (EC₅₀, mean ± SD) of microalgal fractions on the three fouling bacteria (TC8: *Pseudoalteromonas lipolytica*, TC5: *Polaribacter* sp. and TC11: *Shewanella* sp.). Only data for MeOH fractions showing activity against at least one bacterium were given. A colour scale has been applied to ease the visualization of the results (red: <10; orange: 11-30; yellow: 31-50; green: 51-99; blue: >100). **n* = 2. **Published in Othmani et al. [29], mentioned here as a reference.

Phytobank ID	Microalgae	EC ₅₀ (µg/mL)		
		TC8	TC11	TC5
P-69	<i>Pavlova</i> sp.	15 ± 1.1	33	27 ± 5.9
P-68	Cryptophyta sp.	60 ± 17	7.0	24 ± 12
P-70	Cryptophyta sp.	>100	na	13 ± 2.4
P-38	<i>Amphidinium carterae</i>	22 ± 11	5.8	20 ± 14
P-41	<i>Amphidinium massartii</i>	>100	16	18 ± 13
P-43	<i>Amphidinium gibbosum</i>	10 ± 2.3	1.0	6.3 ± 3.2
P-44	<i>Amphidinium massartii</i>	7.1 ± 5.8	2.3	12 ± 7.6
P-45	<i>Amphidinium carterae</i>	9.8 ± 1.0	15	14 ± 5.5
P-46	<i>Amphidinium carterae</i>	15 ± 0.2	19	>100
P-59	<i>Amphidinium carterae</i>	15 ± 2.4	29	15 ± 3.0
P-60	<i>Amphidinium carterae</i>	24 ± 15	4.5	28 ± 9.1
P-63	<i>Amphidinium carterae</i>	13 ± 4.7	2.6	14 ± 8.6
P-08	<i>Prorocentrum lima</i>	13 ± 2.7	na	13
P-37	<i>Prorocentrum lima</i>	>100	na	15 ± 9.1
P-76	<i>Symbiodinium</i> sp.	>100	8	17 ± 6.9
P-78	<i>Symbiodinium</i> sp.	9.6 ± 4.7	6.9	9.3 ± 7.7
P-91	<i>Navicula mollis</i>	28 ± 3.3	22 ± 18	24
P-92	<i>Navicula</i> sp.	75 ± 14	57 ± 18	15
P-89	<i>Nitzschia</i> sp.	33 ± 22	47 ± 9.0	13
P-90	<i>Psammodictyon</i> cf. <i>constrictum</i>	13 ± 2.1	42 ± 19	21
C-58	cf. <i>Cyanothece</i> sp.	63 ± 18	83 ± 26	na
C-59	Cyanobacteria sp.	10	68	na
C-61	Cyanobacteria sp.	25	50	na
C-32	<i>Roseofilum</i> sp.	47 ± 16	59	43
C-18	cf. <i>Leptolyngbya</i> sp.	na	>100	na
C-27	<i>Spirulina</i> sp.	>100	na	na
C-30	<i>Pseudoanabaena</i> sp.	41 ± 20	42	na
C-60	<i>Synechococcus elongatus</i>	79 ± 9	57	48
C-64	cf <i>Porphyridium</i> sp.	31 ± 20	49	na
	TBTO** (x10 ⁻³)	4.2 ± 3.0	1.2 ± 0.60	14 ± 7.2

na: not analyzed (*i.e.* insufficient material); >100: no anti-adhesion activity was observed

Table 3: Growth inhibition (IC₅₀), mortality (LC₅₀) and selectivity index (SI = LC₅₀/EC₅₀) of tropical microalgae MeOH fractions on the three biofilm-forming bacteria (TC8: *Pseudoalteromonas lipolytica*, TC5: *Polaribacter* sp. and TC11: *Shewanella* sp.). Two distinct colour scales (one for IC₅₀ and LC₅₀: red: <29; orange: 30-49; yellow: 50-99; green: >100 and one for SI: green: <5; yellow: 6-10; orange: 11-15; red: >15) have been applied to ease the visualization of the results. *Published in Othmani et al. [29], mentioned here as a reference.

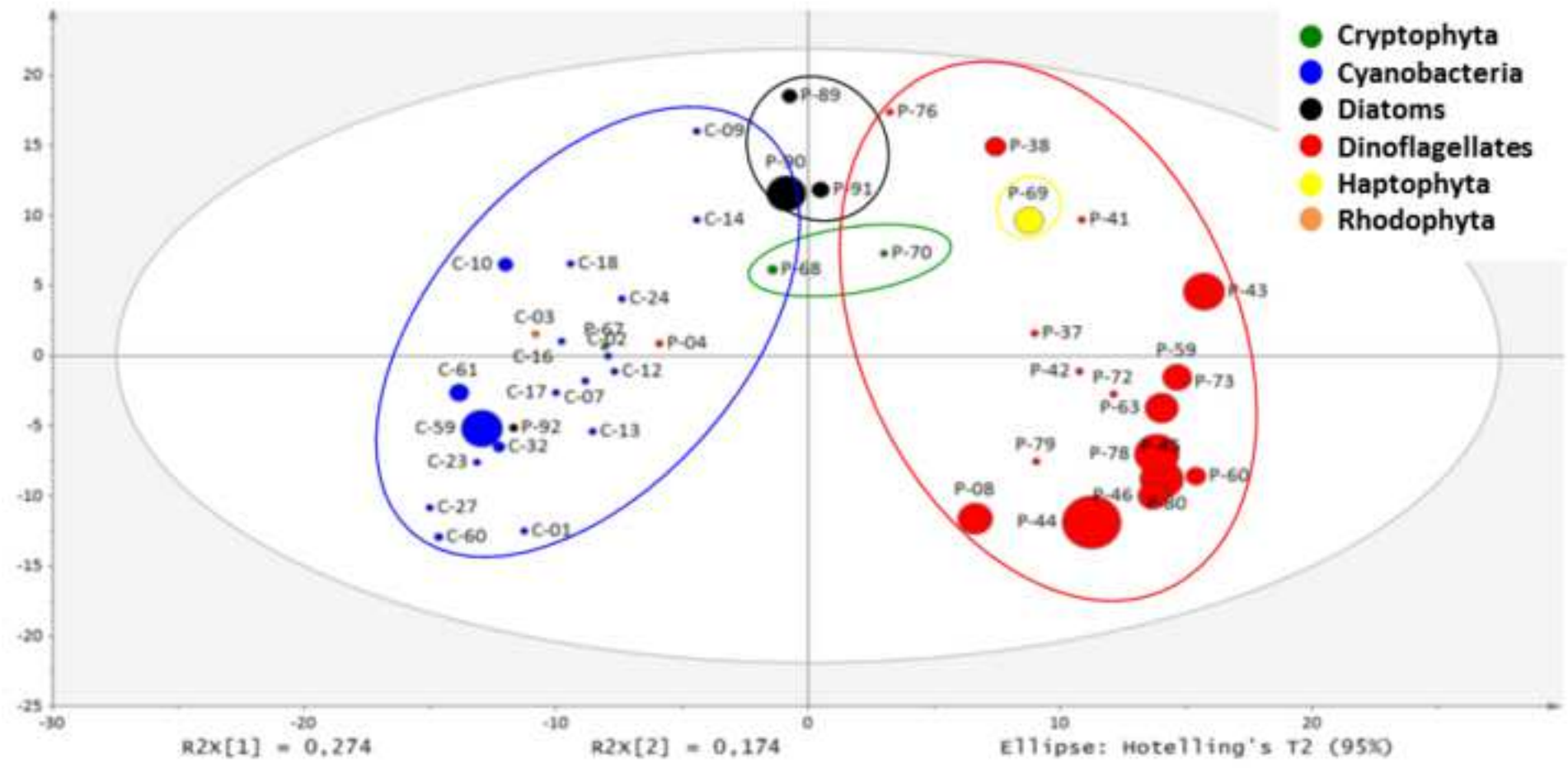
ID	Microalgae	IC ₅₀ (µg/mL)			LC ₅₀ (µg/mL)			Selectivity Index		
		TC8	TC11	TC5	TC8	TC11	TC5	TC8	TC11	TC5
P-69	<i>Pavlova</i> sp.	55	>100	30	29	>100	37	1.9	3.0	1.4
P-68	Cryptophyta sp.	42	47	46	>100	>100	>100	1.7	14	4.2
P-38	<i>Amphidinium carterae</i>	48	42	39	>100	>100	30	4.5	17	1.5
P-43	<i>Amphidinium gibbosum</i>	27	44	42	>100	>100	>100	9.6	98	16
P-44	<i>Amphidinium massartii</i>	41	>100	24	49	>100	23	6.9	44	1.9
P-45	<i>Amphidinium carterae</i>	47	41	46	>100	>100	>100	10	6.7	7.3
P-60	<i>Amphidinium carterae</i>	26	44	18	>100	>100	32	4.2	22	1.2
P-63	<i>Amphidinium carterae</i>	40	>100	35	>100	>100	33	7.6	39	2.4
P-37	<i>Prorocentrum lima</i>	>100	na	na	>100	na	na	1.0	-	-
P-78	<i>Symbiodinium</i> sp.	28	>100	58	22	>100	44	2.3	15	4.8
P-91	<i>Navicula</i> sp.	85	na	na	>100	na	na	3.6	-	-
P-92	<i>Navicula</i> sp.	54	>100	na	>100	>100	na	1.3	1.8	-
P-89	<i>Nitzschia</i> sp.	>100	>100	na	58	84	na	1.7	1.8	-
P-90	<i>Psammodyctyon</i> cf. <i>constrictum</i>	>100	na	na	48	na	na	3.8	-	-
C-32	<i>Roseofilum</i> sp.	55	na	na	67	na	na	1.4	-	-
C-30	<i>Pseudoanabaena</i> sp.	na	>100	na	na	80	na	-	1.9	-
C-60	<i>Synechococcus elongatus</i>	>100	na	na	60	na	na	0.8	-	-
C-64	cf <i>Porphyridium</i> sp.	>100	na	na	>100	na	na	3.2	-	-
	TBTO*	0.0027	0.0015	0.0050	0.0029	0.0022	0.0038	0.7	1.8	0.3

ID: PhytoBank identification number; na: not analyzed; >100: not active

Table 4: Anti-settlement activity (EC_{50}) and mortality (LC_{50}) of the MeOH fraction of three tropical microalgae against *A. amphitrite* nauplii and cyprids (expressed as mean \pm SD).

ID	EC_{50} ($\mu\text{g/mL}$)	24h- LC_{50} ($\mu\text{g/mL}$)	
		Stage V/VI nauplii	Cyprids
P-91	7.5	> 100	> 100
P-43	31 ± 3	> 150	89 ± 5
P-60	45 ± 4	> 150	46 ± 6
TBTO*	$(4.5 \pm 0.3) \times 10^{-5}$	$(11 \pm 0.2) \times 10^{-5}$	$(14 \pm 1) \times 10^{-5}$

* published in Othmani et al. 2016b, mentioned here as a reference





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