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

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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 ma crobiofoulers, 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

Introduction

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

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

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

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

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

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

Materials and methods

Collection, isolation and cultivation of tropical microalgae

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

Monospecific non-axenic isolates were grown in batch conditions (from 250 mL to 5 L) in aerated appropriate media at 26°C with an irradiance of 20–40 μmol/m^2/s and a 12:12h light/dark cycle. Media 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/CH$_2$Cl$_2$) 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/CH$_2$Cl$_2$ (1:1, v/v) mixture and addition of 1g of C18 bonded-silica (Sepra 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 CH$_2$Cl$_2$. 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, $\lambda_{exc} = 628$ nm, $\lambda_{em} = 645$ nm, ThermoFischer Scientific). Fluorescence measurements for all

152 bioassays were made using a TECAN Infinit 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 μg/mL). Only fractions showing

161 EC_{50} ≤60 μg/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_{600nm}), every hour in transparent microtiter plates (sterile PS; Nunc, Fisher

168 Scientific). When reaching the stationary phase, resazurin (50 μM, 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 ± 1 °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 μg/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 × 3 mm; Phenomenex) using a 65 min linear gradient elution of H_2O/ACN/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.

NMR-based metabolomics

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 ($CDCl_3$) 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.

NMR measurements

234   The ^{1}H-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. $CDCl_3$ 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.

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 ^{1}H-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.

Results

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/H_2O (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

8

253   after SPE; aqueous fractions being the most concentrated) and their solubility in seawater (which

254   excluded the CH_2Cl_2 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 EC_{50} 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 (EC_{50} = 1–10 μg/mL), highly (EC_{50} = 11–30 μg/mL), moderately

261   (EC_{50} = 31–50 μg/mL) and weakly active (EC_{50} = 50–100 μg/mL) to not active (EC_{50} >100 μg/mL)

262   (Fig. 1). The activity of the tributyltin oxide (TBTO) was mentioned as a reference for our bioassay

263   and EC_{50} 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 EC_{50} 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 IC_{50} 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 LC_{50} 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 LC_{50} values ≤30 μg/mL (*i.e. Pavlova* sp. P-69 and *Symbiodinium* sp. P-78).

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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 LC_{50} and EC_{50}, 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 $\geq$ 10 for at least one of the

298   bacteria. Concerning the other phyla, SI were always $\leq$ 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 (IC_{50}, LC_{50} 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.

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 EC_{50} values $\leq$ 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-LC_{50} > 100 μg/mL) while dinoflagellates showed some

312   toxicity on cyprids.

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

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

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

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

Discussion

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

This study focused on benthic microalgae that were collected from different islands of the 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 tricornutum* (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.

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).

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/CH$_2$Cl$_2$ 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* ^{1}H 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   Grünewald 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

16

growth conditions, availability and concentration of nutrients in the culture medium, light intensity, temperature and/or pH (Noaman et al. 2004; Ingebrigtsen et al. 2016; Bagwell et al. 2016; Volk and Furkert 2006). Thus, the AF potential of tropical microalgae could be further increased after the optimization of culture conditions for the production of AF agents.

Biofouling is a very complex process that cannot be fully replicated by laboratory single-species bioassays. Hence confirmation of the anti-adhesion activity of selected tropical microalgae would be required using at least mixed-species biofilms *in vitro* or by field studies (Burgess et al. 2003; Briand 2009; Lee et al. 2014). Specifically, the methanolic fractions of *Amphidinium* P-43 and P-60 strains and *Navicula mollis* P-91 have shown interesting antimicro- and antimacrofouling properties thus they should be incorporated in several type of coatings for *in situ* tests.

Conclusion

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

Acknowledgements

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Figure and Table captions:

Figure 1: PCA score plot obtained from LC-MS profiles of 46 MeOH fractions of tropical microalgae.

Symbols were sized by bioactivity (*i.e.* $1/EC_{50}$). Ellipses were drawn manually according to taxa

clustering (the eukaryotic strains that clustered with Cyanobacteria were not included).

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.

Table 2: Anti-adhesion activity (EC_{50}, 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 grey scale has been

applied to ease the visualization of the results. *$n = 2$. **Published in Othmani et al. (Othmani et al.

2016b), mentioned here as a reference.

Table 3: Growth inhibition (IC_{50}), mortality (LC_{50}) and selectivity index ($SI = LC_{50}/EC_{50}$) of tropical

microalgae MeOH fractions on the three biofilm-forming bacteria (TC8: *Pseudoalteromonas lipolytica*,

TC5: *Polaribacter* sp. and TC11: *Shewanella* sp.). Two distinct grey scales (one for IC_{50} and LC_{50} and

one for SI) have been applied to ease the visualization of the results. *Published in Othmani et al.

(Othmani et al. 2016b), mentioned here as a reference.

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 ± SD).

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	*Pavlova*	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	*Amphidinium*	*carterae*	Reunion	F/2	**P-38**
		Amphidinium	*massartii*	Mauritius	F/2	**P-41**
		Amphidinium	*massartii*	Europa	F/2	**P-42**
		Amphidinium	*gibbosum*	Europa	F/2	**P-43**
		Amphidinium	*massartii*	Glorioso	F/2	**P-44**
		Amphidinium	*carterae*	Glorioso	F/2	**P-45**
		Amphidinium	*carterae*	Glorioso	F/2	**P-46**
		Amphidinium	*carterae*	Madagascar	F/2	**P-59**
		Amphidinium	*carterae*	Madagascar	F/2	**P-60**
		Amphidinium	*carterae*	Madagascar	F/2	**P-63**
		Amphidinium	*massartii*	Reunion	F/2	**P-80**
	Prorocentraceae	*Prorocentrum*	*lima*	Reunion	F/2	**P-04**
		Prorocentrum	*lima*	Reunion	F/2	**P-08**
		Prorocentrum	*lima*	Reunion	F/2	**P-37**
	Suessiaceae	*Symbiodinium*	sp. (Clade F)	Reunion	F/2	**P-72**
		Symbiodinium	sp. (Clade B)	Reunion	F/2	**P-73**
		Symbiodinium	sp.	Reunion	F/2	**P-76**
		Symbiodinium	sp. (Clade D)	Reunion	F/2	**P-78**
		Symbiodinium	sp. (Y6-1)	Reunion	F/2	**P-79**
Bacillariophyta (Diatoms)	Naviculaceae	*Navicula*	*mollis*	Reunion	F/2+Si	**P-91***
		Navicula	sp.	Reunion	F/2+Si	**P-92***
	Bacillariaceae	*Nitzschia*	sp.	Reunion	F/2+Si	**P-89***
		Psammodictyon	sp. aff. *constrictum*	Reunion	F/2+Si	**P-90***
Cyanobacteria	Merismopediaceae	*Synechocystis*	sp.	Madagascar	F/2	**C-02**
	Chroococcaceae	*Gloeocapsopsis*	sp.	Glorioso	BG11	**C-61***
	Cyanothecaceae	*Cyanothece*	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***
	Coleofasciculaceae	*Roseofilum*	sp.	Reunion	BG11	**C-07***
		Roseofilum	sp.	Reunion	BG11	**C-32***
	Leptolyngbyaceae	*Leptolyngbya*	sp.	Mayotte	BG11	**C-10***
		Leptolyngbya	sp.	Mayotte	Z8	**C-13***
		Leptolyngbya	sp. (RS01)	Mayotte	BG11	**C-16***
		Leptolyngbya	sp.	Reunion	BG11	**C-18***
		Leptolyngbya	sp.	Reunion	BG11	**C-23***
	Spirulinaceae	*Spirulina*	*subsalsa*	Reunion	BG11	**C-17***
		Spirulina	sp.	Reunion	BG11	**C-27***
	Pseudanabaenaceae	*Pseudoanabaena*	sp.	Reunion	BG11	**C-24***
		Pseudoanabaena	sp.	Reunion	BG11	**C-30***
		Limnothrix	sp.	Reunion	BG11	**C-33***
	Synechococcaceae	*Synechococcus*	*elongatus*	Madagascar	F/2	**C-01**
		Synechococcus	*elongatus*	Glorioso	BG11	**C-60***
Rhodophyta	Porphyridiaceae	*Porphyridium*	sp.	Glorioso	BG11	**C-64***
	Stylonemataceae	*Choodactylon*	sp. aff. *ornatum*	Mayotte	BG11	**C-03***

[a]Culture 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); [b]ID: Phytobank identification number; [c]ND: not determined; *strains isolated from biofilms or growing while isolating biofilms

Table 2: Anti-adhesion activity (EC$_{50}$, 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	*Pavlova* 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	*Amphidinium carterae*	22 ± 11	5.8	20 ± 14
P-41	*Amphidinium massartii*	>100	16	18 ± 13
P-43	*Amphidinium gibbosum*	10 ± 2.3	1.0	6.3 ± 3.2
P-44	*Amphidinium massartii*	7.1 ± 5.8	2.3	12 ± 7.6
P-45	*Amphidinium carterae*	9.8 ± 1.0	15	14 ± 5.5
P-46	*Amphidinium carterae*	15 ± 0.2	19	>100
P-59	*Amphidinium carterae*	15 ± 2.4	29	15 ± 3.0
P-60	*Amphidinium carterae*	24 ± 15	4.5	28 ± 9.1
P-63	*Amphidinium carterae*	13 ± 4.7	2.6	14 ± 8.6
P-08	*Prorocentrum lima*	13 ± 2.7	na	13
P-37	*Prorocentrum lima*	>100	na	15 ± 9.1
P-76	*Symbiodinium* sp.	>100	8	17 ± 6.9
P-78	*Symbiodinium* sp.	9.6 ± 4.7	6.9	9.3 ± 7.7
P-91	*Navicula mollis*	28 ± 3.3	22 ± 18	24
P-92	*Navicula* sp.	75 ± 14	57 ± 18	15
P-89	*Nitzschia* sp.	33 ± 22	47 ± 9.0	13
P-90	*Psammodictyon* cf. *constrictum*	13 ± 2.1	42 ± 19	21
C-58	cf. *Cyanothece* sp.	63 ± 18	83 ± 26	na
C-59	Cyanobacteria sp.	10	68	na
C-61	Cyanobacteria sp.	25	50	na
C-32	*Roseofilum* sp.	47 ± 16	59	43
C-18	cf. *Leptolyngbya* sp.	na	>100	na
C-27	*Spirulina* sp.	>100	na	na
C-30	*Pseudoanabaena* sp.	41 ± 20	42	na
C-60	*Synechococcus elongatus*	79 ± 9	57	48
C-64	*cf Porphyridium* 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 (IC$_{50}$), mortality (LC$_{50}$) and selectivity index (SI = LC$_{50}$/EC$_{50}$) 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 IC$_{50}$ and LC$_{50}$: 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.
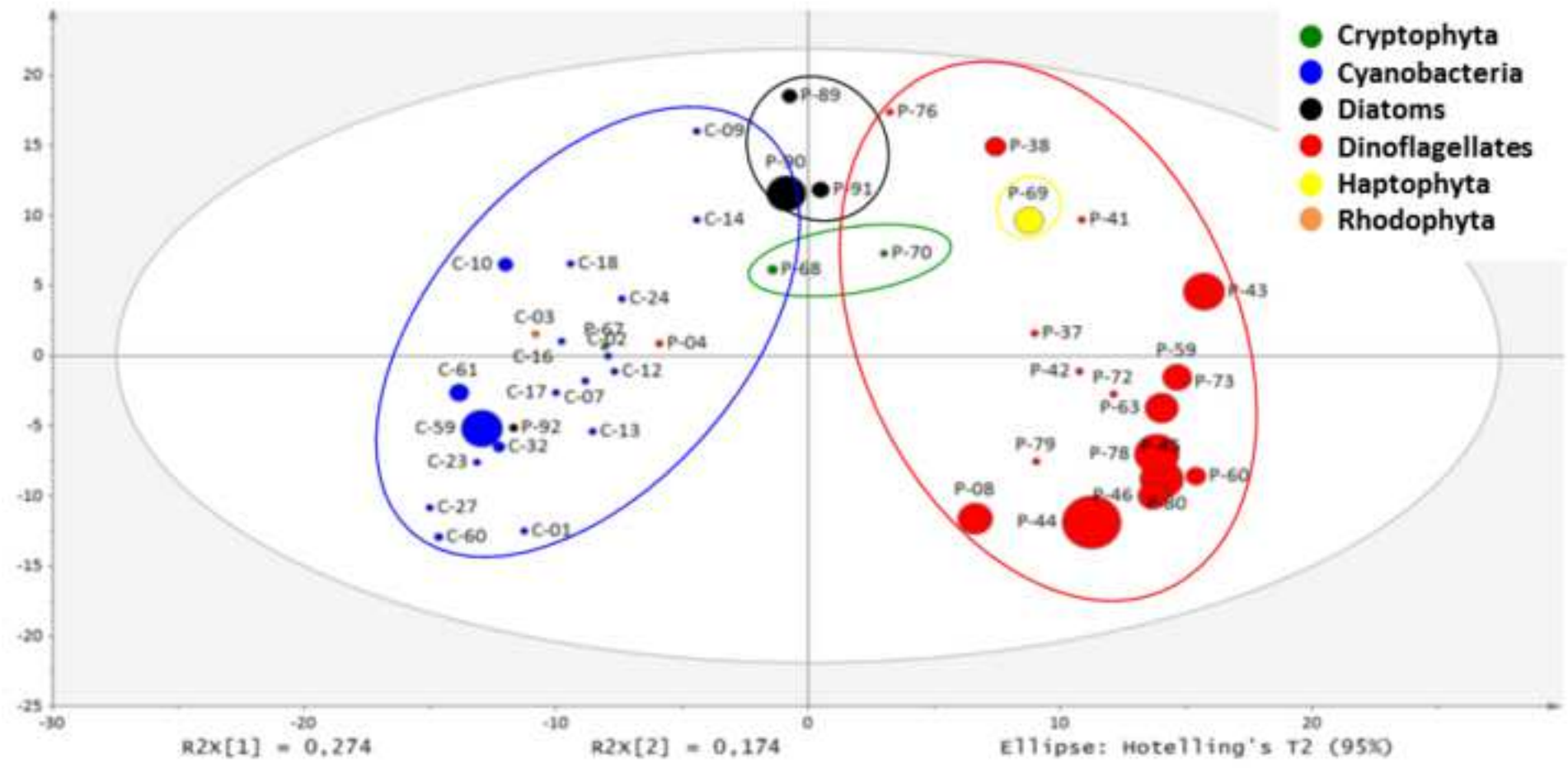
ID	Microalgae	IC50 (µg/mL)			LC50 (µg/mL)			Selectivity Index		
		TC8	TC11	TC5	TC8	TC11	TC5	TC8	TC11	TC5
P-69	*Pavlova* sp.	55	>100	30	29	>100	37	1.9	3.0	1.4
P-68	Cryptophyta sp.	42	47	46	>100	>100	>100	1.7	14	4.2
P-38	*Amphidinium carterae*	48	42	39	>100	>100	30	4.5	17	1.5
P-43	*Amphidinium gibbosum*	27	44	42	>100	>100	>100	9.6	98	16
P-44	*Amphidinium massartii*	41	>100	24	49	>100	23	6.9	44	1.9
P-45	*Amphidinium carterae*	47	41	46	>100	>100	>100	10	6.7	7.3
P-60	*Amphidinium carterae*	26	44	18	>100	>100	32	4.2	22	1.2
P-63	*Amphidinium carterae*	40	>100	35	>100	>100	33	7.6	39	2.4
P-37	*Prorocentrum lima*	>100	na	na	>100	na	na	1.0	-	-
P-78	*Symbiodinium* sp.	28	>100	58	22	>100	44	2.3	15	4.8
P-91	*Navicula* sp.	85	na	na	>100	na	na	3.6	-	-
P-92	*Navicula* sp.	54	>100	na	>100	>100	na	1.3	1.8	-
P-89	*Nitzschia* sp.	>100	>100	na	58	84	na	1.7	1.8	-
P-90	*Psammodictyon* cf. *constrictum*	>100	na	na	48	na	na	3.8	-	-
C-32	*Roseofilum* sp.	55	na	na	67	na	na	1.4	-	-
C-30	*Pseudoanabaena* sp.	na	>100	na	na	80	na	-	1.9	-
C-60	*Synechococcus elongatus*	>100	na	na	60	na	na	0.8	-	-
C-64	*cf Porphyridium* 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 ± SD).

ID	EC_{50} (μg/mL)	24h-LC_{50} (μg/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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