

Screening and selection of growth-promoting bacteria for it *Dunaliella* cultures

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1 Algal Research

2 Screening and selection of growth-promoting bacteria for *Dunaliella* cultures

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23 **Abstract**

24 Previous studies have demonstrated that bacteria influence microalgal metabolism, suggesting that the
25 selection and characterization of growth-promoting bacteria should offer a new strategy for improving
26 industrial algal cultivation. In the present study, 48 cultivable bacteria were isolated from marine
27 microalgae species and identified using 16S rRNA phylogenetic analysis. The recovered bacteria were
28 found to be members of the α - and γ -Proteobacteria, *Cytophaga-Flavobacterium-Bacteroides* (CFB)
29 and gram-positive monophyletic clusters. To address the effect of these bacteria on the growth of
30 *Dunaliella sp.* individually, an experimental high-throughput tool was developed to simultaneously
31 compare replicated associations. A two-step approach was used to monitor growth rate and biomass
32 accumulation of *Dunaliella sp.* in mixed culture with bacteria, which proved the high-throughput
33 device to be an efficient tool for the selection of growth-promoting bacteria. Depending on the
34 bacterial strain involved, inhibitory effects were recorded for maximal microalgal growth rate,
35 whereas inhibitory and stimulating effects were registered on microalgal biomass accumulation and
36 nitrogen incorporation. Organic nitrogen remineralization by *Alteromonas sp.* SY007 and *Muricauda*
37 *sp.* SY244 are discussed to explain the higher biomass and ammonium incorporation of *Dunaliella sp.*
38 obtained under nitrogen-limited conditions. These bacteria could be considered as helpers for N
39 accumulation in *Dunaliella sp.* cells.

40 **Keywords:** Microalgae, *Dunaliella*, bacteria, interaction, bacterial diversity

41 **Abbreviations:** μ_{\max} , maximal growth rate; ΔX_{\max} , maximal biomass increase at stationary phase; C:N,
42 carbon:nitrogen ratio; Chl *a*, chlorophyll *a* ; RAPD, random amplification of polymorphic DNA.

43

43 1. Introduction

44 There is a diverse array of current and potential applications for microalgae, which include food,
45 animal feed, healthcare, energy and phycoremediation [1–3]. The boom of microalgal value-adding
46 over recent decades has drawn attention to the study of bacteria-microalgae interactions in applied
47 algal cultivation [4,5]. Bacteria can compete with microalgae for the limited resources [6,7] or even
48 produce toxic substances against microalgae [8], all of which can decrease culture yields. Axenic
49 microalgae cultures appear to be too unrealistic and labour-intensive for large-scale cultivation, but the
50 addition of selected probiotic bacteria may be beneficial to cultures of microalgae as a preventive
51 action against an inhibiting bacterial population [9,10]. Such added bacteria may also increase
52 microalgae growth rates, and thus enhance culture yields, through the synthesis of growth-promoting
53 compounds [11–13] such as vitamins, or by improving nutrient supply through remineralization of
54 organic nitrogen excreted by microalgae [14]. The strong influence that bacteria can have on maximal
55 growth rate and cell density of different microalgae species was demonstrated by Liu *et al.* (2008) by
56 the addition of a *Bacillus* strain to microalgae cultures [15]. Aside from growth, other aspects of
57 microalgal metabolism may be affected by bacteria such as cell size, pigment and lipid content, and
58 variety of fatty acids, observed in the association of *Chlorella vulgaris* cells with *Azospirillum*
59 *brasiliense* [16], for example. In addition, toxin production [17], extracellular secretions [18] and cell
60 aggregation [19] are all parameters that may potentially be affected in microalgae grown in association
61 with some heterotrophic prokaryotes.

62 Recently, the coupling of anaerobic digestion to microalgae production has been suggested as an
63 efficient means to reduce the huge amounts of nitrogen required for microalgae-based biofuel
64 production [20]. The proposed process results in the recycling of nitrogen and flux of ammonium back
65 to the microalgae culture. Additionally, the use of *Dunaliella sp.* has been proposed for carbon dioxide
66 and ammonium remediation [21], biofuel production [22] and methane production [23]. Indeed,
67 *Dunaliella sp.* exhibit ecological valence for major environmental factors such as irradiance, pH,
68 salinity and temperature: this makes them good candidates for large-scale cultivation [24] and means
69 that they could be coupled to anaerobic digestion and nutrient recycling. However, to the best of our

70 knowledge, the selection of growth-promoting bacteria has not been used, to date, as a method to
71 increase the industrial production of *Dunaliella sp.*.

72 In the present study, we focused on selecting bacteria that promote growth for *Dunaliella sp.* SAG
73 19.3 in a specific context : the coupling of anaerobic digestion to microalgae production. In particular,
74 we tested the ability of bacteria to increase growth and nitrogen incorporation for this microalgae.
75 Accordingly, ammonium-limiting conditions were used to evaluate the effects of bacteria. This study
76 was also conducted without vitamin enrichment in order to test for bacteria ability to supply vitamins
77 to microalgae. The first part of this research consisted of isolating and characterizing cultivable
78 bacteria from various microalgal cultures for subsequent testing in association with *Dunaliella sp.*
79 SAG 19.3. In a high-throughput experiment we first screened a large number of microalgae - bacteria
80 associations for their effect on microalgae growth. Three selected bacteria strains with potential
81 growth altering effects on *Dunaliella sp.* were further tested in a flask experiment. Results highlighted
82 the growth altering effects on *Dunaliella sp.* SAG 19.3 and influence of these bacteria on nitrogen
83 incorporation in microalgae.

84 **2. Materials and methods**

85 ***2.1. Algal strain, maintenance and purification***

86 *Dunaliella sp.* SAG 19.3 was obtained from the culture collection of algae at the University of
87 Goettingen (SAG) Germany, and maintained at 20 °C under continuous light with daylight fluorescent
88 tubes (50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). Cultures were performed in sterile Erlenmeyer flasks filled with
89 artificial seawater (ASW, salinity 35) [25] filtered at 0.22 μm and enriched with modified Walne's
90 medium [26]. Ammonium was used as a nitrogen source (1.17 mM) and vitamins were omitted. The
91 initial *Dunaliella sp.* culture obtained from the SAG collection will hereinafter be referred to as ? the
92 xenic culture.

93 To eliminate bacteria initially associated with *Dunaliella sp.* SAG 19.3 and to obtain axenic cultures,
94 cells were harvested by centrifugation (500 g for 3 min at 20 °C) just before the stationary phase, then
95 transferred to a fresh Erlenmeyer flask containing enriched ASW (as described above) and a specific
96 mix of antibiotics based on Cho *et al* [27]: 1250 μg ampicillin, 250 μg gentamycin, 500 μg

97 kanamycin, and 2500 µg neomycin were added per mL of culture. A first 7-day antibiotic treatment
98 was conducted, followed by a 20-day batch culture without treatment. Cells were then washed with
99 sterile seawater to eliminate remaining free bacteria and a second 7-day treatment was conducted.
100 Absence of bacteria was verified by epifluorescence microscopy using SYBRGreen® I Stain (Lonza,
101 USA) and by plating on Marine Agar (BD Difco™ 212185, Becton Dickinson and Company, USA).
102 Plates were incubated for 10 days at 20 °C before observation.

103 **2.2. Bacterial collection from microalgae culture: isolation and 16S rRNA analysis**

104 Bacteria were isolated from 19 marine monospecific microalgae cultures maintained in the laboratory.
105 Microalgae were cultivated in sterilized seawater enriched with Walne's medium at 20 °C under
106 continuous light (50 µmol photons m⁻²s⁻¹) and isolation was performed at the early stationary phase to
107 select bacteria that grow well together with microalgae. Free living bacteria were isolated by plating
108 xenic microalgae culture on Marine Agar (BD Difco™ 212185, Becton Dickinson and Company,
109 USA) at 20 °C. Isolates were cultured in liquid Marine Broth (BD Difco™ 279110, Becton Dickinson
110 and Company, USA) and stored at -80 °C after addition of 5 % Dimethyl sulfoxide (D 8779, Sigma-
111 Aldrich, USA).

112 For each strain, nucleic acids were extracted by phenol/chloroform extraction followed by isopropanol
113 precipitation [28]. Amplification of the bacterial 16S rRNA gene was performed using universal
114 primers SAdir (5'-AGAGTTTGATCATGGCTCAGA-3') and S17 Rev (5'-
115 GTTACCTTGTTACGACTT-3') [29]. The PCR mixture (25 µL) was composed of 100 ng DNA, 50
116 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM of MgCl₂, 1x of GoTaq™ Buffer (GoTaq™ kit,
117 Promega, USA) and 1.25 units of Taq Polymerase (GoTaq™ kit, Promega, USA). Amplification was
118 carried out on a thermocycler (MyCycler, BIO-RAD) according to the following procedure: 5 min at
119 94 °C, then 35 cycles including 35 s at 94 °C, 1 min at 54 °C and 1 min 30 s at 72 °C, and a final step
120 of 7 min at 72 °C. PCR products were checked on a 0.8% agarose electrophoresis gel.

121 The amplified lengths of DNA were then sequenced at 'Plateforme Biogenouest' (Roscoff, France,
122 <http://www.sb-roscoff.fr/plateformes-techniques/genomique-sbr.html>) on an ABI Prism™ 3100 GA,
123 using BigDye® Terminator v3.1 chemistry (Applied Biosystems) and the SAdir primer. Taxonomic

124 classification was performed online with Ribosomal Database Project Classifier Version 2.5 software,
125 hierarchical taxa assignment being based on RDP naïve Bayesian rRNA Classifier and 95%
126 confidence threshold was selected [30]. BLAST analysis was performed on public nr database (Expect
127 treshold 10 ; word size 28; Match/Mismatch Scores 1,-2) and culturable species that gave the closest
128 sequence was used for specie identification.

129 **2.3. High-throughput experiment (experiment 1)**

130 **2.3.1. Optical measurement for microalgae population**

131 Bacterial effects on *Dunaliella sp.* SAG 19.3 growth were assessed by a screening experiment
132 (experiment 1) using microplates cultures. Because direct measurement for carbon biomass was not
133 available in microplate wells, we first tested *in vivo* Chl *a* fluorescence (450 nm - 685 nm) and OD₆₈₀
134 in order to assess microalgal biomass. The experiment aimed at defining whether either optical
135 measurement gave reliable estimation for microalgae population in mixed culture, regardless of
136 bacterial population : we designed a 2^{2*} central composite design approach where microalgal and
137 bacterial concentrations were the two factors. Five levels were used for the respective factors by
138 adding so-called star-points to the simple (square) 2-level factorial design points in order to assess
139 quadratic component. Three center point replicates were added to evaluate experimental variance. The
140 distance between center points and star points was calculated using the axial distance $\alpha=1.414$.
141 Finally, 11 experiments were needed to incorporate this 2^{2*} central composite design.

142 An axenic culture of *Dunaliella sp.* was grown on enriched ASW. Bacteria (strain SY183) were grown
143 on Marine Broth for 48 h at 20 °C, then centrifuged (10000 g, 5 min, 20 °C) to remove growth
144 medium and resuspended in ASW. By mixing the axenic microalgal culture to the bacterial
145 suspension, we were able to achieve different microalgae and bacterial concentration in samples, as
146 shown in Table 1. *In vivo* Chl *a* fluorescence (wavelength: excitation = 450 nm, emission = 685 nm)
147 and OD₆₈₀ were measured in mixed cultures with a TECAN (Mannedorf Switzerland)
148 spectrofluorimeter.

149 2.3.2. *Experimental culture*

150 A specific high-throughput experimental set-up was devised to allow the use of three microplates to
151 perform mixed cultures with replicates under homogenous conditions of irradiance and temperature.
152 We tested a diversity of microplate and, ultimately, special black microplates (Costar[®] 3615,
153 Corning[®], USA) were chosen with clear bottoms made of 60% thinner polystyrene than standard,
154 resulting in lower background fluorescence readings. To prevent contamination, wells were sealed
155 with adhesive film (MicroAmp[®] Optical Adhesive Film, Applied Biosystems[®], USA) selected for its
156 additional protection against evaporation, which can reach as much as 85 % in 11 days when using
157 some other tissue culture films. Light was provided by 10 fluorescent tubes (OSRAM L13W/954)
158 through white PMMA diffusion plates. Irradiance and temperature were measured using a Li-Cor
159 LI193 quantum scalar meter and a LM 35DZ sensor, respectively. The small size of the two sensors
160 allowed to measure parameters inside wells filled with ASW. This set-up provided a mean irradiance
161 of 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and irradiance field homogeneity with a 5.3 % coefficient of variation (n =
162 117). Temperature was set at 19.4 °C, while temperature variation between wells, estimated by the
163 coefficient of variation, was 2.3 % (n = 38). Cultures were performed statically with automatic
164 shaking prior to readings with Tecan to homogenize cultures and to prevent biofilm formation in
165 wells.

166 To start the experiment, bacterial strains were precultured in Marine Broth for 48 hours at 20 °C.
167 Bacterial cells were then harvested by centrifugation at 3000 g for 5 min at 20 °C. Associations with
168 the axenic culture of *Dunaliella sp.* were made-up at the initial ratio of around 10 bacterial cells per
169 microalgal cell, with a concentration of *Dunaliella sp.* cells of $2 \cdot 10^5 \text{ cell.mL}^{-1}$ in both the axenic and
170 mixed cultures. The culture medium consisted of ASW enriched with Walne's medium without
171 vitamins and modified for nitrogen. To test bacteria for their ability to remineralize nitrogen, cultures
172 were grown under nitrogen limitation, with the addition of ammonium to obtain a nitrogen
173 concentration of 547.8 μM , resulting in a molar nitrogen:phosphorus ratio of 3.3:1. Since the adhesive
174 film was not permeable to gas, carbon limitation was prevented by adding 10 mM of NaHCO_3 .

175 Growth of microalgae was monitored by *in vivo* Chl *a* fluorescence (wavelength: excitation = 450 nm,
176 emission = 685 nm, TECAN Mannedorf Switzerland). Three measurements per day were performed
177 during the first 17 days and one or two measurements per day until day 20. Testing for bacterial
178 contamination was done on Marine Agar plates at the end of the experiment.

179 **2.4. Flask cultures (experiment 2)**

180 **2.4.1. Bacterial quotas**

181 Preliminary microscopic observations revealed that bacterial size and shape were different for the
182 three bacterial strains SY003, SY007 and SY244. We recorded that more than 95% of bacteria cells in
183 mixed cultures were retained on precombusted GF/C filters. In order to evaluate the contribution of
184 bacteria to total particulate C and N recovered on GF/C filters, we assessed carbon (Q_C) and nitrogen
185 (Q_N) quotas for the three bacterial strains tested: bacterial cultures were incubated in Marine Broth
186 medium for 48 h at 20 °C and 300 rpm. After centrifugation (10000 g, 5 min, 20 °C), cells were
187 resuspended in fresh ASW and cell concentration was assessed by cytometer (BD Accuri Cytometer).
188 Bacterial particulate N and C were estimated : a given volume of cell suspension was filtered in
189 triplicate on precombusted 25 mm GF/C filters (Whatman, 1.2 μ m). Filters were then dried for 24
190 hours at 70°C and further analysed using a CN Elemental Analyzer (Flash 2000, ThermoScientific).
191 Since all bacterial strains in the xenic culture could not be cultivable in Marine Broth medium, we
192 estimated mean quotas from cell volume (as assessed from microscopic observations). Indeed, these
193 bacteria demonstrated size and shape very similar to that observed for SY003. We therefore
194 considered same quotas for SY003 and bacteria in the xenic culture.

195 **2.4.2. experimental culture**

196 Following the screening in experiment 1, three microalgae-bacteria associations and the initial xenic
197 and axenic cultures were selected for further comparative investigation (experiment 2). The axenic
198 culture was considered as the control. Algae-bacteria associations were maintained for several months
199 at 20 °C before experiment 2, with successive batch cultures on enriched ASW under a continuous
200 irradiance of 100 μ mol photons $m^{-2}s^{-1}$. Cells were harvested to eliminate residual nutrients, and then
201 transferred into flasks. Triplicate cultures were conducted in sterile 1L-flasks with a supply of bubbled

202 air. The medium used was similar to that for experiment 1 (N source, N:P ratio, NaHCO₃ enrichment,
203 no vitamins added). Temperature was set at 20 °C and irradiance was set to a higher level (250 μmol
204 photons m⁻²s⁻¹) than in experiment 1 to compensate for the higher optical path length in flasks.

205 Cultures were sampled daily for microalgae cell density and cell size, as measured with a HIAC cell
206 counter (Hach Ultra, USA). Cell biovolume was computed from mean cell diameter under the
207 assumption of a spherical shape for *Dunaliella sp.* Total particulate C and N were also estimated as
208 previously described for bacteria (see 2.4.1). Microalgal N and C recovered on GF/C filters were then
209 calculated as the difference between total particulate and bacterial N and C. Finally, N incorporation
210 was computed as the percentage of initial N-NH₄ enrichment (547.8 μM) incorporated in microalgae
211 cells at stationary phase.

212 In order to validate N starvation at the end of the experiments, cultures were re-enriched with
213 547.8 μmol of ammonium, and the biomass increase was verified over the following days.

214 The density of bacterial cells was measured by cytometric analysis at the beginning of the experiment,
215 during the growth phase and at the stationary phase. The bacterial population was identified by
216 cytometer (BD Accuri Cytometer) after coloration with SYBRgreen. Bacteria to microalgae ratio
217 (B:A, cell:cell) was calculated from cytometric data for bacteria and from HIAC data for algae.

218 Absence of bacterial contamination at the end of the experiment was assessed by RAPD analysis on
219 randomly selected strains after isolation on Marine Agar plates. Extraction and PCR reactions were
220 performed using the same mixture as described above. Analyses were applied twice with Amersham@
221 RAPD Analysis Primer 1 (5'- GGTGCGGGAA-3') and 4 (5'- AAGAGCCCGT-3'). The cycling
222 program was as follows: 5 min at 94 °C, 45 cycles including 1 min at 94 °C, 1 min at 36 °C and 2 min
223 at 72 °C. PCR products were separated on 2 % TAE agarose gels and the profiles obtained were
224 compared to the original bacterial reference strain from the collection.

225 **2.5. Estimation of growth and statistics**

226 Maximal growth rate, μ_{\max} (d⁻¹) of *Dunaliella sp.* was computed according to equation 1 from the
227 linear part of the ln-transformed growth curve:

Equation 1

$$\mu_{\max} = \frac{\Delta \ln X}{\Delta t}$$

228 where X is either Chl a fluorescence (experiment 1) or particulate carbon (experiment 2) during the
229 exponential growth phase and t is time in days. Since bacterial carbon contributed to a low level to
230 total particulate carbon recovered on GF/C filters, microalgae μ_{\max} could be computed from total
231 particulate carbon data in experiment 2.

232 Maximal biomass increase ΔX_{\max} of *Dunaliella sp.* was computed at stationary phase according to
233 equation 2:

$$\Delta X_{\max} = X_f - X_i$$

Equation 2

234 where X_f and X_i are either Chl a fluorescence (experiment 1) or particulate carbon (experiment 2),
235 respectively at the stationary phase and at the beginning of the culture.

236 Since ΔX_{\max} was computed from fluorescence readings in experiment 1 and from particulate carbon in
237 experiment 2, comparison for ΔX_{\max} between the two experiments was performed after normalization
238 according to equation 3:

$$\Delta X_{\max}^{\text{norm}} = \frac{\Delta X_{\max} - \Delta \bar{X}_{\max}}{\sigma}$$

Equation 3

239 Where $\Delta \bar{X}_{\max}$ is mean maximal biomass increase and σ is standard deviation in experiment.

240 Statistical analyses were performed with Statgraphics® software for the factorial design approach and
241 R software (GNU project) elsewhere. Since experiments 1 and 2 involved only triplicate cultures,
242 results are expressed hereafter as median and interquartile range (IQR) rather than mean and standard
243 error. In experiment 2, effects of bacteria on microalgal parameters were tested using the Kruskal
244 Wallis test ($\alpha=5\%$). The comparison of growth parameters computed from the two experiments was
245 carried out using Spearman's rank correlation coefficient ($\alpha=5\%$).

246 **3. Results**

247 **3.1. Purification of *Dunaliella sp.* SAG 19.3 culture**

248 An axenic culture of *Dunaliella sp.* strain SAG 19.3 was obtained successively to the repeated
249 antibiotic treatment. No cultivable bacteria were observed on the Marine Agar plates inoculated with
250 samples of this algal culture. In addition, before the use of this *Dunaliella sp.* culture for experiments,
251 absence of uncultivable strains was systematically verified by epifluorescence microscopy after
252 SYBRgreen staining.

253 **3.2. Bacterial collection**

254 Forty-eight strains of bacteria were isolated from 19 microalgae species, of which 71% were acquired
255 from diatoms. In the collection, analysis for partial 16S rRNA revealed that 37 strains were gram-
256 negative, of which 8 belong to *Cytophaga-Flavobacterium-Bacteroides* (CFB) including 2
257 sphingobacteria and 6 flavobacteria, 17 to *Alphaproteobacteria* and 12 to *Gammaproteobacteria*.
258 Eleven strains were gram-positive including 10 *Actinobacteria* and one *Bacilli* (Table 2).

259 **3.3. High-throughput selection (experiment 1)**

260 **3.3.1. optical measurement for microalgae population**

261 The factorial approach used to compare OD₆₈₀ to fluorescence as proxies for microalgae population
262 resulted in both models explaining more than 99% of the variability observed on data. Both OD₆₈₀ and
263 fluorescence were significantly ($\alpha=0.01$) and positively related to microalgal concentration (Table 3).
264 For $\alpha=0.01$, neither quadratic effects nor interaction between microalgae and bacterial concentration
265 were found significant for both measurements. However, as shown in Table 3, OD₆₈₀ readings
266 increased with bacterial concentration, while fluorescence was not significantly affected.

267 **3.3.2. Effect of bacteria on maximal growth rate**

268 High-throughput experiment was carried out for 20 days, until all cultures have reached stationary
269 phase. Bacterial isolation on Marine Agar plates confirmed that no contamination occurred at the
270 beginning or the end of the experiment in mixed or axenic cultures. Maximal growth rate (μ_{\max}) of
271 *Dunaliella sp.* ranged from 0.23 d⁻¹ to 0.36 d⁻¹ depending on bacterial association, with 0.36 d⁻¹ (0.01)
272 for the axenic control (Figure 1). Addition of bacteria to *Dunaliella sp.* cultures mostly resulted in

273 slight negative effects on μ_{\max} , although some other bacterial strains did not alter microalgae μ_{\max} . No
274 bacterial enhancement of growth rate was observed in this experiment. The growth-inhibiting bacteria
275 were broadly distributed across taxonomic groups (Figure 1, Table 2). The strongest negative effect (-
276 36 %) was obtained for the xenic *Dunaliella sp.* culture. Interestingly, 3 bacterial strains isolated from
277 this xenic culture (*Rhodococcus fascians* SY001, SY002 and *Dietzia sp.* SY250) resulted in negative
278 effects (-18 %, -11 % and -22 %, respectively) when tested individually. *Muricauda sp.* strain SY244
279 isolated from *Thalassiosira sp.*, resulted in a 22 % decrease in *Dunaliella sp.* growth rate. Another
280 *Muricauda* strain (SY186) also had an inhibitory effect on μ_{\max} (-18 %). The addition of certain strains
281 resulted in lesser reductions in μ_{\max} , such as with *Halomonas sp.* SY003 (-12 %) and *Alteromonas sp.*
282 SY007 (-7 %).

283 Fourteen strains exhibited μ_{\max} close to the control, such as the strains affiliated to *Arthrobacter sp.*
284 SY004 and to *Bacillus foraminis* SY097, for example (Figure 1).

285 3.3.3. Effect of bacteria on maximal biomass increase

286 Maximal biomass increase (ΔX_{\max}) measured at stationary phase was more strongly altered by bacterial
287 addition than μ_{\max} (Figure 2). Effects ranged from -57 % to +26 % and were mainly negative. The
288 strongest negative effect (-57 %) was observed for the xenic *Dunaliella sp.* culture. Strains SY001 and
289 SY002 isolated from the xenic culture and affiliated with *Rhodococcus fascians* also decreased ΔX_{\max}
290 with strong effects (-42 % and -44 %).

291 Twenty-one bacterial strains resulted in ΔX_{\max} close to that of the axenic control (Figure 2). However,
292 ΔX_{\max} was enhanced by 22% and 26% when *Dunaliella sp.* was associated with bacteria SY007 and
293 SY244 affiliated to *Alteromonas sp.* and *Muricauda sp.*, respectively. These bacteria were isolated
294 from diatom cultures: *Thalassiosira sp.* for the *Alteromonas sp.* SY007, and *Phaeodactylum*
295 *tricornutum* for the *Muricauda sp.* SY244.

296 3.4. Flask cultures (experiment 2)

297 Following experiment 1, three bacterial strains were selected for the different alteration pattern they
298 brought about in *Dunaliella sp.*. *Alteromonas sp.* SY007 and *Muricauda sp.* SY244 were selected for
299 their enhancing effect on *Dunaliella sp.* maximal biomass increase (ΔX_{\max}) at the stationary phase

300 (Figure 2). *Halomonas sp.* SY003 was also selected as an example of a ΔX_{\max} -inhibiting bacteria.
301 These three mixed cultures were compared to the control axenic strain and to the original xenic strain
302 SAG19.3.

303 3.4.1. Bacterial populations

304 First of all, no bacterial contamination was observed on Marine Agar plates along the course of
305 experiment. Cytometry analysis confirmed these results since we observed no events corresponding to
306 bacteria in the axenic cultures, and only one uniform bacterial population on cytograms for mixed
307 cultures. RAPD profile analyses of bacteria isolated at the end of the experiment 2 were similar to the
308 reference bacterial strains (Figure 3).

309 The bacterial population, estimated at t_0 , t_5 and t_{10} by cytometry analysis, developed in all mixed
310 cultures (Figure 4). The highest bacterial cell density was recorded in the mixed culture SY003. At the
311 stationary phase, the bacteria to microalgae ratio (B:A, cell:cell) in this culture was also particularly
312 high: 777 bacteria cells per algae. Differences were observed at the stationary phase in B:A for strains
313 SY007 and SY244, being 39 for SY007 and only 8 for SY244. Finally, the lowest bacteria increase
314 and B:A level, 2 bacteria per algae, was recorded in the xenic cultures of *Dunaliella sp.*

315 Nitrogen (Q_N) and carbon (Q_C) cell quotas measured for the three bacterial strains are presented in
316 Table 4. From quotas and bacterial cell population data we could compute bacterial contribution to
317 total particulate N and C recovered on GF/C filters. It followed that bacterial carbon represented less
318 than 4 % of total particulate carbon in all mixed cultures, except for SY003 where it was 10 % of total
319 particulate carbon. Bacteria contributed to higher level of total particulate N, bacterial N being as high
320 as 34 % for SY003 and 15 % for SY007 (Table 4).

321 3.4.2. Microalgae growth

322 As already mentioned above, contribution of bacteria to total particulate carbon was low in mixed
323 cultures. Therefore we assumed that total particulate carbon recovered on GF/C filters was a suitable
324 proxy for microalgal carbon and in the following, we further compare microalgae growth computed
325 from total particulate carbon data.

326 Growth of *Dunaliella sp.* in flasks was very sensitive to the bacterial strain added in the culture, as
327 illustrated in Figure 4 by the different growth curves recorded during experiment 2. Microalgae μ_{\max}
328 computed on a per-carbon basis in experiment 2 (Table 5) were very similar to those computed from
329 *in vivo* Chl *a* fluorescence in experiment 1. Indeed, a positive correlation ($\rho = 0.91$; P value = 0.042 ;
330 slope = 1.0) was found for μ_{\max} recorded in the two experiments. As previously observed in the high-
331 throughput experiment, the addition of bacteria to the cultures did not result in an enhancing effect for
332 μ_{\max} when compared with the axenic cultures of *Dunaliella sp.* (Table 5). The lowest μ_{\max} were
333 observed in xenic cultures and when *Halomonas sp.* SY003 and *Muricauda sp.* SY244 were added to
334 cultures. Interestingly, no significant difference with the axenic control was obtained when *Dunaliella*
335 *sp.* was associated to *Alteromonas sp.* SY007.

336 At the stationary phase in experiment 2, bacterial addition resulted in altered ΔX_{\max} for *Dunaliella sp.*
337 Again, results recorded in experiment 2 were similar to those of experiment 1 and a positive
338 correlation ($\rho = 0.95$; P value = 0.042 ; slope =1.0) was obtained for normalized ΔX_{\max} between the
339 two experiments. The lowest ΔX_{\max} were observed in experiment 2 for xenic cultures (-25 %) and
340 when *Halomonas sp.* SY003 (- 33 %) was associated with *Dunaliella sp.* (Table 5). In addition,
341 similar enhancing effects were observed in mixed cultures SY007 (+31 %) and SY244 (+35 %). These
342 two bacteria significantly increased carbon accumulation in microalgae cultures compared with the
343 axenic control and, more strongly, when compared to the original SAG 19.3 xenic strain.

344 Microalgae cell size was also significantly affected by bacterial addition. We were able to compute
345 biovolume of microalgae cells (Table 5) on the basis of Hiac data, assuming a spherical shape for
346 *Dunaliella sp.* We found a positive correlation ($\rho=0.98$; P value=0.88) between biovolume and carbon
347 quota in microalgae. We recorded high microalgal biovolume for SY244 and the axenic cultures,
348 while cells in the xenic cultures were significantly smaller.

349 3.4.3. Nitrogen incorporation

350 In order to estimate nitrogen incorporation in microalgae, we corrected total particulate N for bacterial
351 N. Indeed, we found that bacterial N could contribute to high level to total particulate N recovered on
352 GF/C filters (up to 34 % at stationary phase for SY003). From data of bacterial N quota and bacterial

353 population we could subtract bacterial N to total particulate N and estimate N incorporation for
354 microalgae. At the stationary phase, the resulting microalgal C:N (Table 5) was high (22.8 to 29.6) for
355 the different cultures, compared to the C:N ratio recorded at μ_{\max} (C:N = 6, data not shown).

356 It followed that bacterial addition significantly altered N incorporation for *Dunaliella sp.* (Table 5).
357 The lowest N incorporation in microalgae was obtained in SY003 cultures (19 %) while axenic (26 %)
358 and xenic (34 %) demonstrated intermediate N incorporation. In mixed cultures with *Muricauda sp.*
359 SY244 and *Alteromonas sp.* SY007, N incorporation was significantly enhanced up to 56 % of the
360 initial N enrichment.

361 4. Discussion

362 4.1. Microalgae culture-based bacterial collection

363 Isolation of bacteria from a diversity of monospecific microalgal cultures provided a bacterial
364 collection of 48 strains. Since these bacteria strains developed in microalgae cultures without organic
365 carbon supplementation, we suspected that they were able to grow on the organic carbon released by
366 microalgae. This suggested interactions between bacteria and *Dunaliella sp.*. A high bacterial diversity
367 with low redundancy was recorded. Indeed, bacterial strains isolated from different microalgal cultures
368 were mostly different, with the strains well distributed among four phylogenetic clusters: α - and γ -
369 *Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* and gram-positive mainly affiliated to
370 *Actinobacteria*. This study aimed at providing a bacterial collection for further interaction studies and
371 did not encompass an ecological scope since only dominant and cultivable bacteria were recovered
372 from microalgal cultures. However, it should be noted that we isolated and identified bacterial groups
373 that were previously observed elsewhere, following isolation of bacteria from microalgae cultures in
374 hatcheries [31] and in bacterioplankton communities [32–34]. No members of the β -*Proteobacteria*
375 were recovered from this collection, although this cluster has been recorded in several ecological
376 studies [35,36]. Again, this absence could result from the experimental set-up, as only dominant
377 cultivable bacteria were considered here. In addition, the marine origin of this bacterial cluster has
378 been debated in previous studies [37,38].

379 **4.2. Methodological aspects**

380 **4.2.1. High-throughput selection of growth-promoting bacteria**

381 Most of the previous studies conducted on interactions between microalgae and bacteria have been
382 carried out in Erlenmeyer or larger flasks [12,39]. However, these culture volumes are not suitable for
383 the screening of a large number of species at once. Therefore, we developed a specific experimental
384 device based on microplates. Similar tools have been previously used to assess growth for microalgae
385 [40]. However, in this study, we had to face specific constraints, including the presence of bacteria that
386 can affect optical measurements for microalgae concentration and bacterial cross-contamination
387 between wells. With the use of the impermeable film together with NaHCO₃ addition in culture
388 medium we were able to prevent cross-contaminations between wells and carbon limitation in the
389 absence of gas exchange.

390 Unlike OD₆₈₀ measurement, fluorescence (450nm - 685 nm) was insensitive to bacteria concentration
391 (Table 3) and could be seen as a reliable proxy for microalgae population assessment in microplate.
392 Additionally, comparison between microplate and flask experiments revealed similar trends for both
393 growth parameters, as illustrated by the Spearman's rank correlation coefficients found here. These
394 results confirmed that indirect measurements for microalgae growth using *in-vivo* fluorescence gave
395 consistent results with direct measurements for microalgal particulate carbon.

396 The high correlation coefficient mentioned above for both growth parameters in the two experiments
397 also suggested that the low culture volume (300 µL) in microplate, combined with the use of
398 impermeable adhesive film, is a reliable culture system for *Dunaliella sp.* By paying particular
399 attention to light and temperature variability between plates and wells, we managed to reduce the
400 coefficient of variation (CV) to 5.3% and 2.3%, respectively. As a consequence, we recorded only low
401 variability between culture replicates, particularly for growth rate. Finally, the high throughput
402 technique confirmed to be a time-saving approach since set-up of experiment can be achieved within
403 hours easily and fluorescence reading is fast enough to allow several readings per day. Together, these
404 benefits may afford the use of a higher number of replicates to even increase system reliability. As

405 such, the proposed high-throughput device proved to be an efficient tool to qualitatively assay the
406 effect of a high number of bacterial strains on microalgae growth.

407 *4.2.2. Assessment of compartmentation between microalgae and bacteria*

408 In experiment 2, the use of GF/C filters did not allow to separate microalgae from bacteria. Hence, in
409 order to estimate N and C incorporation in microalgae, we first measured bacterial N and C quotas
410 with pure bacterial cultures, as already reported elsewhere [41]. We did not have evidence for growth
411 capacity on Marine Broth of all bacteria strains found in the xenic culture. Hence, we could no reliably
412 measure quotas for these bacteria. However, bacteria strains in the xenic culture and SY003 exhibited
413 similar shape and size and we considered C and N quotas similar to that for SY003 (Table 4). We
414 point out that since bacterial population remained low in the xenic culture (see Figure 4), bacteria
415 contributed to a very low level to N and C recovered on GF/C filters (Table 5), irrespective of the
416 assumption for quotas. We then substracted the bacterial compartment from total particular matter
417 recovered on filters to compute microalgae N and C. This approach resulted in high C:N for
418 microalgae at stationary phase in mixed and axenic cultures, a result in accordance with the Droop
419 quota theory [42] that N-limited microalgae cells stop growth at a given maximum C:N. By the way,
420 this result and the good correlation found between microalgae biovolume and Q_C , also supported our
421 approach for microalgae N and C computation. Finally, the high C:N recorded here were in
422 accordance with N limitation for microalgae at stationary phase as assessed by N re-supplementation
423 at the end of the experiment.

424 *4.3. Effect of bacteria on growth of Dunaliella sp.*

425 The high-throughput experiment (experiment 1) was designed so as to rapidly focus on microalgae-
426 bacteria associations altering growth performance for *Dunaliella sp.*, that could be further
427 characterized in the successive flask experiment. The experiment resulted in a number of inhibition
428 and/or promotion effects (Figure 1 and 2) on *Dunaliella sp.* μ_{\max} and ΔX_{\max} . Most of the 48 bacterial
429 strains tested in experiment 1 negatively affected microalgal ΔX_{\max} and μ_{\max} . However, effect on
430 microalgal growth rate was only slight compared to the wide range we recorded for ΔX_{\max} . Since
431 cultures were grown without vitamin supplementation, we expected that some associations could

432 result in increased microalgae μ_{\max} . Yet, we did not record any microalgal μ_{\max} improvement,
433 demonstrating that synthesis of growth-promoting compounds [11–13] by bacteria did not occur or
434 was not efficient. Finally, we were unable to find connection between bacterial taxonomic position and
435 effect on *Dunaliella sp.* growth. However, as pointed out by Mayali and Azam [43] for algicidal
436 bacteria, the question of metabolic properties common to broad bacterial taxa remains largely
437 unanswered.

438 In experiment 2, we focused on three bacterial strains that produced various effects on microalgae
439 μ_{\max} , while altering ΔX_{\max} : *Alteromonas sp.* SY007, *Muricauda sp.* SY244 and *Halomonas sp.* SY003;
440 Effects that were recorded with the bacterial strains in experiment 1 were confirmed in the flask
441 experiment for both μ_{\max} and ΔX_{\max} . Assumptions for the underlying mechanisms are discussed in the
442 following.

443 It is well known that bacteria can modify microalgal growth by affecting either growth rate or biomass
444 accumulation. Maximal growth rate of microalgae is likely to be affected by bacterial population,
445 possibly with an enhancing effect, as previously observed in the literature [39,44], but not in this
446 study. Several authors have demonstrated that the negative effects of bacteria on μ_{\max} are the result of
447 the excretion of toxic bacterial compounds; this issue has been frequently addressed in studies dealing
448 with the impact of algicidal bacteria on algal blooms [43,45,46]. Several bacterial genera (*Cytophaga*,
449 *Dietzia*, *Janibacter*, *Micrococcus*, *Pseudoalteromonas*) referenced in our collection that led to
450 decreased μ_{\max} for *Dunaliella sp.* in culture, have been precisely described as algicidal bacteria in the
451 literature [47,48].

452 Bacterial effects on biomass accumulation at the stationary phase have also been previously reported
453 in the literature [49,50]. Mouget *et al.* observed a strong increase (+50 %) in maximal cell density for
454 *Scenedesmus bicellularis* associated with a *Brevundimonas diminuta* strain [51]. Tai *et al.* suggested
455 the occurrence of *Vibrio* species in ammonium production, supporting *Synechococcus sp.* growth [14].
456 Besides, it is well known that nitrogen excretion occurs during microalgae batch culture [52]. Since
457 nitrogen-limited conditions were used in this study, it was assumed that nitrogen remineralization of
458 organic nitrogen released by microalgae occurred in cultures where *Alteromonas sp.* SY007 and

459 *Muricauda sp.* SY244 were added. Bacterial remineralization of extracellular organic matter could
460 provide nitrogen and delay starvation for *Dunaliella sp.* This hypothesis is strengthened by the higher
461 N incorporation in *Dunaliella sp.* cells when mixed with one of the two bacterial strains, as compared
462 with the axenic control. From these results, bacteria SY007 and SY244 could be considered as helpers
463 for N assimilation for *Dunaliella sp.* cells. However, there is a need for further experiments with
464 measurements for dissolved inorganic and organic nitrogen and microalgal particulate nitrogen to test
465 this assumption.

466 Bacterial strains can also decrease ΔX_{\max} of microalgae by competing for a limiting nutrient. Such an
467 effect has been previously reported by Meseck *et al.* for nitrogen, and by Rhee *et al.* and Danger *et al.*
468 for phosphorus [7,50,53]. Alternatively, release of toxic compounds by bacteria could also be involved
469 in the inhibitory effect observed at the stationary phase [49,54]. We assumed that the low microalgal
470 biomass accumulation recorded for SY003 cultures could result from competition between bacteria
471 and microalgae for the limited nitrogen. Indeed, the latter hypothesis was supported, since high
472 bacterial concentration occurred at stationary phase in SY003 cultures, with 24 % of the supplemented
473 N incorporated in bacterial cells, while N incorporation in microalgae (19 %) was lower than that
474 recorded in axenic cultures (26 %).

475 Xenic cultures exhibited significantly lower μ_{\max} and ΔX_{\max} than axenic cultures in both experiments.
476 *Rhodococcus fascians* (SY001 and SY002) and *Dietzia sp.* (SY250) isolated from the xenic culture of
477 *Dunaliella sp.* SAG 19.3 and assayed individually also depressed microalgal growth performance.
478 Interestingly, we identified *Rhodococcus fascians* strains in the xenic culture that had been previously
479 described by Sim-Mateo *et al.* as a phytopathogenic bacteria involved in gall formation [55]. In
480 addition, *Dietzia* bacteria are also known as algicidal bacteria [48]. This result highlighted the
481 usefulness of testing bacterial populations in microalgae cultures. Indeed, at the industrial scale where
482 axenic conditions can hardly be attainable, especially in open culture systems, sustainable association
483 of microalgae with selected bacteria could improve performance for microalgae culture.

484 **5. Conclusion**

485 A specific microplate-based experimental design was developed to screen bacteria-*Dunaliella sp.*
486 associations and to select microalgae growth-promoting bacteria. From the comparison of results in a
487 flask experiment, it was concluded that the experimental device was a powerful tool for high-
488 throughput examination of the bacterial effect on microalgal growth. Two bacteria strains affiliated to
489 *Alteromonas sp.* and *Muricauda sp.* particularly enhanced biomass accumulation for *Dunaliella sp.* A
490 strong increase was also recorded in N incorporation, which suggested that N availability for
491 microalgae was affected by these bacteria. Further research is needed for a precise assessment of the
492 underlying mechanisms of these interactions. Nevertheless, the results of the present study suggest that
493 culture performance can be substantially modified by bacteria, resulting in increased culture
494 productivity, which is of particular interest for industrial production.

495

495 Figure 1 : Maximal growth rate (μ_{max}) for *Dunaliella sp.* SAG 19.3, calculated in the high-throughput experiment
496 (experiment 1) for axenic, xenic or mixed cultures with different bacterial strains assayed individually. For each
497 culture, raw data for the three replicates are connected by a vertical line to facilitate reading. Reference numbers
498 in the collection are given on the X-axis.

499 Figure 2: Maximal biomass increase (ΔX_{max}) for *Dunaliella sp.* SAG 19.3 estimated in the high-throughput
500 experiment (experiment 1) for axenic, xenic or mixed cultures with different bacterial strains assayed
501 individually. For each culture, raw data for the three replicates are connected by a vertical line to facilitate
502 reading. Reference numbers in the collection are given on the X-axis.

503 Figure 3: RAPD-PCR profiles for isolates from flask mixed cultures SY003, SY007 and SY244 (experiment 2)
504 compared with the relevant SY003, SY007 and SY244 controls from the collection. Results were obtained with
505 the two primers RAPD 1 and RAPD 4. Two bacteria colonies (C1 and C2) were analysed for the three replicated
506 flasks (F1, F2 and F3).

507 Figure 4 : A) Particulate carbon growth curves in flask cultures (experiment 2) for axenic, xenic strains and
508 mixed cultures (SY003, SY007 and SY244). B) Bacterial concentration in mixed cultures at the beginning of the
509 experiment and after 5 and 10 days of culture.

510 Table 1 : Factor levels in the central composite design, where $\alpha = 1.414$ is the axial distance between star points
511 and the center of the experimental domain.

512 Table 2 : Bacterial collection isolated from microalgae cultures.

513 Table 3 : ANOVA table resulting from the factorial design approach used for comparison of OD_{680} to
514 fluorescence as proxy for microalgal population in mixed culture. Significant p-values ($\alpha=0.01$) are given in
515 bold. (+) and (-) symbols depict positive or negative effects for the corresponding factor. *A* stands for
516 microalgae concentration, *B* for bacterial concentration, *AA* and *BB* for the corresponding quadratic effects and
517 *AB* for interaction.

518 Table 4 : shape, quotas and contribution for bacteria to particulate C and N recovered on GF/C filters. For each
519 column, the values presented are median and interquartile range (IQR) in brackets.

520 Table 5 : Growth parameters and nitrogen incorporation for *Dunaliella sp.* in flask cultures (experiment 2) for
521 xenic and axenic strains, and for mixed cultures (SY003, SY007 and SY244). For each column, the values

522 presented are median and interquartile range (IQR) in brackets. Values with the same superscript letters are not
523 statistically different (Kruskal Wallis test; $\alpha = 5\%$).

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