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Algal Research

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

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Abstract

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

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Algal strain, maintenance and purification

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

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

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

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

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

For each strain, nucleic acids were extracted by phenol/chloroform extraction followed by isopropanol precipitation [28]. Amplification of the bacterial 16S rRNA gene was performed using universal primers SAdir (5'-AGAGTTTGATCATGGCTCAGA-3') and S17 Rev (5′-GTTACCTTGTTACGACTT-3') [29]. The PCR mixture (25 µL) was composed of 100 ng DNA, 50 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM of $MgCI_2$, 1x of GoTaq™ Buffer (GoTaq™ kit, Promega, USA) and 1.25 units of Taq Polymerase (GoTaq™ kit, Promega, USA). Amplification was carried out on a thermocycler (MyCycler, BIO-RAD) according to the following procedure: 5 min at 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 of 7 min at 72 °C. PCR products were checked on a 0.8% agarose electrophoresis gel.

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

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

2.3. High-throughput experiment (experiment 1)

2.3.1. Optical measurement for microalgae population

Bacterial effects on *Dunaliella sp*. SAG 19.3 growth were assessed by a screening experiment (experiment 1) using microplates cultures. Because direct measurement for carbon biomass was not available in microplate wells, we first tested *in vivo* Chl *a* fluorescence (450 nm - 685 nm) and OD_{680} in order to assess microalgal biomass. The experiment aimed at defining whether either optical measurement gave reliable estimation for microalgae population in mixed culture, regardless of bacterial population : we designed a 2^2* central composite design approach where microalgal and bacterial concentrations were the two factors. Five levels were used for the respective factors by adding so-called star-points to the simple (square) 2-level factorial design points in order to assess quadratic component. Three center point replicates were added to evaluate experimental variance. The distance between center points and star points was calculated using the axial distance α=1.414. Finally, 11 experiments were needed to incorporate this 2^2* central composite design.

An axenic culture of *Dunaliella sp*. was grown on enriched ASW. Bacteria (strain SY183) were grown on Marine Broth for 48 h at 20 °C, then centrifuged (10000 g, 5 min, 20 °C) to remove growth medium and resuspended in ASW. By mixing the axenic microalgal culture to the bacterial suspension, we were able to achieve different microalgae and bacterial concentration in samples, as shown in Table 1. *In vivo* Chl *a* fluorescence (wavelength: excitation = 450 nm, emission = 685 nm) and OD_{680} were measured in mixed cultures with a TECAN (Mannedorf Switzerland) spectrofluorimeter.

2.3.2. Experimental culture

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

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

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

2.4. Flask cultures (experiment 2)

2.4.1. Bacterial quotas

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

2.4.2. experimental culture

Following the screening in experiment 1, three microalgae-bacteria associations and the initial xenic and axenic cultures were selected for further comparative investigation (experiment 2). The axenic culture was considered as the control. Algae-bacteria associations were maintained for several months at 20 °C before experiment 2, with successive batch cultures on enriched ASW under a continuous irradiance of 100 µmol photons $m^{-1}s^{-1}$. Cells were harvested to eliminate residual nutrients, and then 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, $NaHCO_3$ enrichment,
203 no vitamins added). Temperature was set at 20 °C and irradiance was set to a higher level (250 µmol
204 photons $m^{-2}s^{-1}$) 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 sperical 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-NH_4 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

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

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

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

Equation 2

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

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

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

Equation 3

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

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

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

3. Results

3.1. Purification of Dunaliella sp. SAG 19.3 culture

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

3.2. Bacterial collection

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

3.3. High-throughput selection (experiment 1)

3.3.1. optical measurement for microalgae population

The factorial approach used to compare OD_{680} to fluorescence as proxies for microalgae population resulted in both models explaining more than 99% of the variability observed on data. Both OD_{680} and fluorescence were significantly (α=0.01) and positively related to microalgal concentration (Table 3). For α=0.01, neither quadratic effects nor interaction between microalgae and bacterial concentration were found significant for both measurements. However, as shown in Table 3, OD_{680} readings increased with bacterial concentration, while fluorescence was not significantly affected.

3.3.2. Effect of bacteria on maximal growth rate

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

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

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

3.3.3. Effect of bacteria on maximal biomass increase

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

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

3.4. Flask cultures (experiment 2)

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

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

3.4.1. Bacterial populations

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

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

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

3.4.2. Microalgae growth

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

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

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

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

3.4.3. Nitrogen incorporation

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

353 population we could substract 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 developped 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].

4.2. Methodological aspects

4.2.1. High-throughput selection of growth-promoting bacteria

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

Unlike OD_{680} measurement, fluorescence (450nm - 685 nm) was insensitive to bacteria concentration (Table 3) and could be seen as a reliable proxy for microalgae population assessment in microplate. Additionally, comparison between microplate and flask experiments revealed similar trends for both growth parameters, as illustrated by the Spearman's rank correlation coefficients found here. These results confirmed that indirect measurements for microalgae growth using *in-vivo* fluorescence gave consistent results with direct measurements for microalgal particulate carbon.

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

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

4.2.2. Assessment of compartimentation between microalgae and bacteria

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

4.3. Effect of bacteria on growth of Dunaliella sp.

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

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

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

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

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

5. Conclusion

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

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

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

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

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

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

Table 2 : Bacterial collection isolated from microalgae cultures.

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

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

Table 5 : Growth parameters and nitrogen incorporation for *Dunaliella sp*. in flask cultures (experiment 2) for 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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