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**Screening and selection of growth-promoting bacteria for *Dunaliella* cultures**M. Le Chevanton<sup>a</sup>, M. Garnier<sup>a,\*</sup>, G. Bougaran<sup>a</sup>, N. Schreiber<sup>a</sup>, E. Lukomska<sup>a</sup>, J.-B. Bérard<sup>a</sup>,  
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email address : [matthieu.garnier@ifremer.fr](mailto:matthieu.garnier@ifremer.fr)**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  $\alpha$ - and  $\gamma$ -*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 is 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**Highlights** ► We developed a high-throughput tool to evaluate microalgae-bacteria interactions ► We selected growth-promoting bacteria for *Dunaliella* sp. ► The growth promoting bacteria enhanced nitrogen incorporation in *Dunaliella* cultures.**Abbreviations** :  $\mu_{\max}$ , maximal growth rate ;  
 $\Delta X_{\max}$ , maximal biomass increase at stationary phase ;  
C:N, carbon:nitrogen ratio ;  
Chl a, chlorophyll a ;  
RAPD, random amplification of polymorphic DNA

## 1. Introduction

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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.

New strategies for the production of biogas through anaerobic digestion lead to a high amount of ammonium. A coupling with the production of microalgae has been suggested as an effective way to use these huge quantities 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

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### 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  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). Cultures were performed in sterile Erlenmeyer flasks filled with artificial seawater (ASW, salinity 35) [25] filtered at 0.22  $\mu\text{m}$  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 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  $\mu\text{g}$  ampicillin, 250  $\mu\text{g}$  gentamycin, 500  $\mu\text{g}$  kanamycin, and 2500  $\mu\text{g}$  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 cultures maintained in the laboratory. Microalgae were cultivated in sterilized seawater enriched with Walne's medium at 20 °C under continuous light (50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) 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  $\mu\text{L}$ ) was composed of 100 ng DNA, 50 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM of  $\text{MgCl}_2$ , 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 being based on RDP naïve Bayesian rRNA Classifier and 95% confidence threshold was selected [30]. BLAST analysis was performed on public nr database (Expect threshold 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<sub>680</sub> 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. Accordingly, we mixed microalgal to bacterial suspensions in order to obtain different microalgal : bacterial concentration ratios meeting 2<sup>2\*</sup> central composite design requirement and we further measured *in vivo* Chl *a* fluorescence and OD<sub>680</sub>. Five levels were used for the respective factors (*i.e.* microalgal and bacterial concentrations) by adding so-called star-points to the simple (square) 2-level factorial design points in order to assess quadratic component (figure 1). Three center point replicates were added to evaluate experimental variance. The distance between center points and star-points was calculated using the axial distance  $\alpha=1.414$ . Finally, 11 experiments were needed to incorporate this 2<sup>2\*</sup> central composite design. . The general quadratic model fitted to the data is given in equation 1.

$$R = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon \quad \text{equation 1}$$

where  $\beta_i$ ,  $\beta_{ij}$ , and  $\beta_{ii}$  are model coefficients,  $X_i$  the main effect for the factor  $i$ ,  $X_{ij}$  the interaction between the factors  $i$  and  $j$ ,  $X_i^2$  the quadratic effect of the factor  $i$ ,  $\varepsilon$  the residual error and  $R$  the response (either *in vivo* Chl *a* fluorescence or OD<sub>680</sub>). Here,  $X_1$  was chosen for microalgal concentration and  $X_2$  for bacterial concentration.

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 96-wells microplate, as shown in Figure 1. *In vivo* Chl *a* fluorescence (wavelength: excitation = 450 nm, emission = 685 nm) and OD<sub>680</sub> 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. The device consists of an illuminating plate with 10 fluorescent tubes (OSRAM L13W/954). A PMMA diffusion plate is placed on the illuminating plate. The three clear-bottom microplates are then placed above. We tested a diversity of microplate and, ultimately, special 96 well 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. 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 measuring parameters inside the wells filled with ASW. This set-up

provided a mean irradiance of  $50 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  and irradiance field homogeneity with a 5.3 % coefficient of variation ( $n = 117$ ). Temperature was set at  $19.4 \text{ }^\circ\text{C}$ , while temperature variation between wells, estimated by the coefficient of variation, was 2.3 % ( $n = 38$ ). Cultures were performed statically. Prior to readings with Tecan, cultures were homogenized by automatic shaking.

To start the experiment, bacterial strains were precultured in Marine Broth for 48 hours at  $20 \text{ }^\circ\text{C}$ . Bacterial cells were then harvested by centrifugation at  $3000 \text{ g}$  for 5 min at  $20 \text{ }^\circ\text{C}$ . 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  $2 \times 10^5 \text{ cell.mL}^{-1}$  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  $547.8 \mu\text{M}$ , 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  $10 \text{ mM}$  of  $\text{NaHCO}_3$ .

Growth of microalgae was monitored by *in vivo* Chl *a* fluorescence (wavelength: excitation =  $450 \text{ nm}$ , emission =  $685 \text{ nm}$ , 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  $20 \text{ }^\circ\text{C}$  and  $300 \text{ rpm}$ . After centrifugation ( $10000 \text{ g}$ , 5 min,  $20 \text{ }^\circ\text{C}$ ), 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  $25 \text{ mm}$  GF/C filters (Whatman,  $1.2 \mu\text{m}$ ). Filters were then dried for 24 hours at  $70^\circ\text{C}$  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  $20 \text{ }^\circ\text{C}$  before experiment 2, with successive batch cultures on enriched ASW under a continuous irradiance of  $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ . Cells were harvested to eliminate residual nutrients, and then transferred into flasks. Triplicate cultures were conducted in sterile  $1\text{L}$ -glass flasks with a supply of  $0.22 \mu\text{m}$

filtered air (Midisart, Sartorius). We specifically paid attention to reproduce culture conditions as close as possible to that in experiment 1 in order to readily compare results. The medium used was similar to that for experiment 1 (N source, N:P ratio, NaHCO<sub>3</sub> enrichment, no vitamins added). Temperature was set at 20 °C and irradiance was set to a higher level (250 μmol photons m<sup>-2</sup>s<sup>-1</sup>) than in experiment 1 to compensate for the higher optical path length in flasks.

Cultures were sampled daily for microalgae cell density and cell size, as measured with a HIAC cell counter (Hach Ultra, USA). Cell biovolume was computed from mean cell diameter under the assumption of a spherical shape for *Dunaliella sp.* Total particulate C and N were also estimated as previously described for bacteria (see 2.4.1). Microalgal N and C recovered on GF/C filters were then calculated as the difference between total particulate and bacterial N and C. Finally, N incorporation was computed as the percentage of initial N-NH<sub>4</sub> enrichment (547.8 μM) incorporated in microalgae cells at stationary phase.

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

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

Absence of bacterial contamination at the end of the experiment was assessed by RAPD analysis on randomly selected strains after isolation on Marine Agar plates. Extraction and PCR reactions were performed using the same mixture as described above. Analyses were applied twice with Amersham@ RAPD Analysis Primer 1 (5'-GGTGCGGGAA-3') and 4 (5'-AAGAGCCCGT-3'). The cycling 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 at 72 °C. PCR products were separated on 2 % TAE agarose gels and the profiles obtained were compared to the original bacterial reference strain from the collection. RAPD analyses were produced with the Bionumerics software (V6.01) by using a band-based similarity index.

## 2.5. Estimation of growth and statistics

Maximal growth rate,  $\mu_{\max}$  (d<sup>-1</sup>) of *Dunaliella sp.* was computed according to equation 1 from the linear part of the ln-transformed growth curve:

$$\mu_{\max} = \frac{\Delta \ln X}{\Delta t} \quad \text{Equation 2}$$

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  $\mu_{\max}$  could be computed from total particulate carbon data in experiment 2.

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

$$\Delta X_{\max} = X_f - X_i \quad \text{Equation 3}$$

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

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

**Equation 4**

Where  $\bar{\Delta X}_{\max}$  is mean maximal biomass increase and  $\sigma$  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 ( $\alpha=5\%$ ). The comparison of growth parameters computed from the two experiments was carried out using Spearman's rank correlation coefficient ( $\alpha=5\%$ ).

### 3. Results

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#### 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 1).

#### 3.3. High-throughput selection (experiment 1)

##### 3.3.1. optical measurement for microalgae population

The factorial approach used to compare OD<sub>680</sub> to fluorescence as proxies for microalgae population resulted in both models explaining more than 99% of the variability observed on data. Both OD<sub>680</sub> and fluorescence were significantly ( $\alpha=0.01$ ) and positively related to microalgal concentration (Table 2). For  $\alpha=0.01$ , neither quadratic effects nor interaction between microalgae and bacterial concentration were found significant for both measurements. However, as shown in Table 2, OD<sub>680</sub> 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 ( $\mu_{\max}$ ) of *Dunaliella* sp. ranged from 0.23 d<sup>-1</sup> to 0.36 d<sup>-1</sup> depending on bacterial association, with 0.36 d<sup>-1</sup> (0.01) for the axenic control (Figure 2). Addition of bacteria to *Dunaliella* sp. cultures mostly resulted in slight negative effects on  $\mu_{\max}$ , although some other bacterial strains did not alter microalgae  $\mu_{\max}$ . No bacterial enhancement of growth rate was observed in this experiment. The growth-inhibiting bacteria were broadly distributed across taxonomic groups (Figure 2 ; Table 1). 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  $\mu_{\max}$  (-18 %). The addition of certain strains resulted in lesser reductions in  $\mu_{\max}$ , such as with *Halomonas* sp. SY003 (-12 %) and *Alteromonas* sp. SY007 (-7 %).

Fourteen strains exhibited  $\mu_{\max}$  close to the control, such as the strains affiliated to *Arthrobacter* sp. SY004 and to *Bacillus foraminis* SY097, for example (Figure 2).

### 3.3.3. Effect of bacteria on maximal biomass increase

Maximal biomass increase ( $\Delta X_{\max}$ ) measured at stationary phase was more strongly altered by bacterial addition than  $\mu_{\max}$  (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  $\Delta X_{\max}$  with strong effects (-42 % and -44 %).

Twenty-one bacterial strains resulted in  $\Delta X_{\max}$  close to that of the axenic control (Figure 3). However,  $\Delta X_{\max}$  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 ( $\Delta X_{\max}$ ) at the stationary phase (Figure 3). *Halomonas* sp. SY003 was also selected as an example of a  $\Delta X_{\max}$ -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 showed 100 % similarity with the corresponding reference bacterial strain (Figure 4), regardless of the primer used (RAPD1 or RAPD4). Similarity was lower (90 %) only for *Halomonas* SY003 C1 strain isolated from flask F1, when RAPD1 was used, but was 100% with RAPD4. Considering the high sensitivity of the technique, we concluded that bacterial strains at the end of experiment 2 were similar to that inoculated.



The bacterial population, estimated at  $t_0$ ,  $t_5$  and  $t_{10}$  by cytometry analysis, developed in all mixed cultures (Figure 5B). 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.* Epifluorescence microscopy observations after sybrgreen dyeing revealed that, for all mixed cultures, bacteria cells were free in the medium and not attached to the surface of living algal cells.

Nitrogen ( $Q_N$ ) and carbon ( $Q_C$ ) cell quotas measured for the three bacterial strains are presented in Table 3. 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 3).

### 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 5A by the different growth curves recorded during experiment 2. Microalgae  $\mu_{max}$  computed on a per-carbon basis in experiment 2 (Table 4) 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  $\mu_{max}$  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  $\mu_{max}$  when compared with the axenic cultures of *Dunaliella sp.* (Table 4). The lowest  $\mu_{max}$  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  $\Delta X_{max}$  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  $\Delta X_{max}$  between the two experiments. The lowest  $\Delta X_{max}$  were observed in experiment 2 for xenic cultures (-25 %) and when *Halomonas sp.* SY003 (-33 %) was associated with *Dunaliella sp.* (Table 4). 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 4) on the basis of Hiac data, assuming a spherical shape for *Dunaliella sp.* We found a positive correlation ( $\rho=0.98$ ; P value=0.003) 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 population we could subtract bacterial N to total particulate N and estimate N incorporation for microalgae. At the stationary phase, the resulting microalgal C:N (Table 4) was high (22.8 to 29.6) for the different cultures, compared to the C:N ratio recorded at  $\mu_{\max}$  (C:N = 6, data not shown).

It followed that bacterial addition significantly altered N incorporation for *Dunaliella sp.* (Table 4). The lowest N incorporation in microalgae was obtained in SY003 cultures (19 %) while axenic (26 %) and xenic (34 %) demonstrated intermediate N incorporation. In mixed cultures with *Muricauda sp.* SY244 and *Alteromonas sp.* SY007, N incorporation was significantly enhanced up to 56 % of the initial N enrichment.

## 4. Discussion

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### 4.1. Microalgae culture-based bacterial collection

Isolation of bacteria from a diversity of monospecific microalgal cultures provided a bacterial collection of 48 strains. Since these bacteria strains developed in microalgae cultures without organic carbon supplementation, we suspected that they were able to grow on the organic carbon released by microalgae. This suggested interactions between bacteria and *Dunaliella sp.*. A high bacterial diversity with low redundancy was recorded. Indeed, bacterial strains isolated from different microalgal cultures were mostly different, with the strains well distributed among four phylogenetic clusters:  $\alpha$ - and  $\gamma$ - *Proteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* and gram-positive mainly affiliated to *Actinobacteria*. This study aimed at providing a bacterial collection for further interaction studies and did not encompass an ecological scope since only dominant and cultivable bacteria were recovered from microalgal cultures. However, it should be noted that we isolated and identified bacterial groups that were previously observed elsewhere, following isolation of bacteria from microalgae cultures in hatcheries [31] and in bacterioplankton communities [32–34]. No members of the  $\beta$ -*Proteobacteria* were recovered from this collection, although this cluster has been recorded in several ecological studies [35,36]. Again, this absence could result from the experimental set-up, as only dominant cultivable bacteria were considered here. In addition, the marine origin of this bacterial cluster has 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 NaHCO<sub>3</sub> addition in culture medium we were able to prevent cross-contaminations between wells and carbon limitation in the absence of gas exchange. The novelty of this setup consists in the possibility to screen microalgal and

bacterial mixed cultures and to evaluate associated yields thanks to the selection of adapted materials, controlled environmental parameters (temperature, light, contamination, evaporation) and the use of fluorescence to monitor microalgae growth without bacteria population disturbance.

Unlike  $OD_{680}$  measurement, fluorescence (450nm - 685 nm) was insensitive to bacteria concentration (Table 2) 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  $\mu$ L) 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 compartmentation 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 not 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 3). We point out that since bacterial population remained low in the xenic culture (see Figure 5B), bacteria contributed to a very low level to N and C recovered on GF/C filters (Table 4), irrespective of the assumption for quotas. We then subtracted the bacterial compartment from total particulate 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 2 and 3) on *Dunaliella sp.*  $\mu_{max}$  and  $\Delta X_{max}$ . Most of the 48 bacterial strains tested in experiment 1 negatively affected microalgal

$\Delta X_{\max}$  and  $\mu_{\max}$ . However, effect on microalgal growth rate was only slight compared to the wide range we recorded for  $\Delta X_{\max}$ . Since cultures were grown without vitamin supplementation, we expected that some associations could result in increased microalgae  $\mu_{\max}$ . Yet, we did not record any microalgal  $\mu_{\max}$  improvement, demonstrating that synthesis of growth-promoting compounds [11–13] by bacteria did not occur 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  $\mu_{\max}$ , while altering  $\Delta X_{\max}$ : *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  $\mu_{\max}$  and  $\Delta X_{\max}$ . 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  $\mu_{\max}$  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  $\mu_{\max}$  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 *Muricauda sp.* SY244 were added. Bacterial remineralization of extracellular organic matter, originating from algal cells death and/or algal organic excretion, could provide ammonium and delay nitrogen starvation for *Dunaliella sp.*. Indeed mineralization of microalgal organic N is well documented in the presence of bacteria [53–55]. Brussaard and Riegman [56] demonstrated that bacteria reduced death rates of N-starved *Ditylum brightwellii* and the authors assumed that mineralized ammonium was probably partly utilized by microalgal cells. Furthermore, they pointed out that, under N-limited conditions, both bacteria and microalgae could benefit from each other. Our hypothesis is strengthened by the higher N incorporation in *Dunaliella sp.* cells when mixed with one of the two bacterial strains, as compared with the axenic control. With the same nitrogen supply in the medium at the beginning of the experiment for all cultures, a higher amount of mineral nitrogen is available for microalgae growth thanks to algal organic matter recycling by bacteria. We consequently obtained a higher algal biomass reached at the stationary phase, compared to the axenic control. From these results, bacteria SY007 and SY244 could be considered as helpers for N assimilation for *Dunaliella sp.* cells. However, there is a need for further experiments with measurements for dissolved inorganic and organic nitrogen and microalgal particulate nitrogen to test this assumption.

Bacterial strains can also decrease  $\Delta X_{\max}$  of microalgae by competing for a limiting nutrient. Such an effect has been previously reported by Meseck *et al.* for nitrogen, and by Rhee *et al.* and Danger *et al.* for phosphorus [7,50,57]. Alternatively, release of toxic compounds by bacteria could also be involved in the inhibitory effect observed at the stationary phase

[49,58]. We assumed that the low microalgal biomass accumulation recorded for SY003 cultures could result from competition between bacteria and microalgae for the limited nitrogen. Indeed, the latter hypothesis was supported, since high bacterial concentration occurred at stationary phase in SY003 cultures, with 24 % of the supplemented N incorporated in bacterial cells, while N incorporation in microalgae (19 %) was lower than that recorded in axenic cultures (26 %).

Xenic cultures exhibited significantly lower  $\mu_{\max}$  and  $\Delta X_{\max}$  than axenic cultures in both experiments. *Rhodococcus fascians* (SY001 and SY002) and *Dietzia* sp. (SY250) isolated from the xenic culture of *Dunaliella* sp. SAG 19.3 and assayed individually also depressed microalgal growth performance. Interestingly, we identified *Rhodococcus fascians* strains in the xenic culture that had been previously described by Sim-Mateo *et al.* as a phytopathogenic bacteria involved in gall formation [59]. In addition, *Dietzia* bacteria are also known as algicidal bacteria [48]. This result highlights the usefulness of testing bacterial populations in microalgae cultures. Indeed, at the industrial scale where axenic conditions can hardly be attainable, especially in open culture systems, sustainable association of microalgae with selected bacteria could improve performance for microalgae culture.

Interactions between bacteria and microalgae are complex mechanisms and may result in different pattern depending on conditions and protagonists involved in. Indeed, our results highlighted that interactions between microalgae and bacteria are highly species specific. This was already pointed out by Brussaard and Riegman who suggested that “species-specific differences in the response of nutrient deficient phytoplankton to the presence of bacteria do exist” [56]. Culture conditions can also substantially affect the outcome of interactions in mixed cultures, since they may affect composition and rates of algal exudation. Hence, it is suspected that the outcome of mixed cultures under N-limited conditions would differ depending on batch or continuous culture mode as well as microalgal growth rate. Additionally, environmental factors such as temperature can affect microalgal organic N remineralization rates [53,54]. Finally, we also suspect that initial microalgae : bacteria ratio is another central issue in mixed culture and may turn symbiosis into competition for the mineral N resource : high ratios would favor competition for the mineral N resource, while low ratios could result in higher N availability for microalgae, as reported here for SY244 and SY007.

## 5. Conclusion

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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.

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## Tables

Table 1 : bacterial collection isolated from microalgae cultures.

Strain	Origin	Partial 16S rRNA sequence ID	Class	Family	Blast results	
SY022	<i>Sketetonema costatum</i>	HE589500	Sphingobacteria	Cyclobacteriaceae	<i>Cytophaga sp.</i>	
SY049	<i>Rhodella violacea</i>	HE589505		Cyclobacteriaceae	<i>Cytophaga sp.</i>	
SY010	<i>Odontella aurita</i>	HE589497	Flavobacteria	Flavobacteriaceae	<i>Flexithrix sp.</i>	
SY121	<i>Odontella aurita</i>	HE589507		Flavobacteriaceae	<i>Flexithrix sp.</i>	
SY088	<i>Emiliana huxleyi</i>	HE589506		Flavobacteriaceae	<i>Maribacter sp.</i>	
SY221	<i>Chaetoceros sp</i>	HE589511		Flavobacteriaceae	<i>Maribacter sp.</i>	
SY186	<i>Chaetoceros pumilum</i>	HE589509		Flavobacteriaceae	<i>Muricauda sp.</i>	
SY244	<i>Thalassiosira sp</i>	HE589517		Flavobacteriaceae	<i>Muricauda sp.</i>	
SY097	<i>Chaetoceros calcitrans</i>	HE995401	Bacilli	Bacillaceae 1	<i>Bacillus foraminis</i>	
SY233	<i>Thalassiosira pseudomona</i>	HE589528	Actinobacteria	Intrasporangiaceae	<i>Janibacter limosus</i>	
SY234	<i>Thalassiosira pseudomona</i>	HE589529		Intrasporangiaceae	<i>Janibacter sp.</i>	
SY004	<i>Isochrysis galbana</i>	HE589520		Micrococcineae	<i>Arthrobacter sp</i>	
SY240	<i>Phaeodactylum</i>	HE589530		Micrococcineae	<i>Micrococcus sp</i>	
SY241	<i>Phaeodactylum</i>	HE589531		Micrococcineae	<i>Micrococcus sp</i>	
SY208	<i>Chaetoceros gracilis</i>	HE589525		Nocardiaceae	<i>Actinobacter sp</i>	
SY001	<i>Dunaliella sp</i>	HE589518		Nocardiaceae	<i>Rhodococcus fascians</i>	
SY002	<i>Dunaliella sp</i>	HE589519		Nocardiaceae	<i>Rhodococcus fascians</i>	
SY090	<i>Chaetoceros pumilum</i>	HE589522		Dietziaceae	<i>Dietza mari</i>	
SY250	<i>Dunaliella sp</i>	HE589534		Dietziaceae	<i>Dietza sp.</i>	
SY086	<i>Rhodomonas salina</i>	HE589541		Alphaproteobacteria	Rhodospirillaceae	<i>Thalassospira sp</i>
SY093	<i>Rhodomonas salina</i>	HE589542			Rhodospirillaceae	<i>Thalassospira xianheensis</i>
SY180	<i>Skeletonema marinoi</i>	HE589555			Rhodospirillaceae	<i>Nisaea sp.</i>
SY127	<i>Nannochloris sp</i>	HE995403			Erythrobacteraceae	<i>Citromicrobium sp</i>
SY183	<i>Chaetoceros pumilum</i>	HE589556			Phyllobacteriaceae	<i>Hoeflea sp</i>
SY148	<i>Chaetoceros pumilum</i>	HE589550	Phyllobacteriaceae		<i>Hoeflea sp</i>	
SY191	<i>Chaetoceros pumilum</i>	HE589558	Phyllobacteriaceae		<i>Hoeflea sp</i>	
SY228	<i>Chaetoceros reptans</i>	HE589562	Rhodobacteraceae		<i>Stappia sp.</i>	
SY052	<i>Chaetoceros pumilum</i>	HE589538	Rhodobacteraceae		<i>Haematobacter massiliensis</i>	
SY133	<i>Skeletonema marinoi</i>	HE589545	Rhodobacteraceae		<i>Roseobacter sp.</i>	
SY029	<i>Odontella aurita</i>	HE589537	Rhodobacteraceae		<i>Thalassococcus sp.</i>	
SY245	<i>Chaetoceros gracilis</i>	HE589563	Rhodobacteraceae		<i>Sulfobacter sp.</i>	
SY143	<i>Emiliana huxleyi</i>	HE589548	Rhodobacteraceae		<i>Roseobacteraceae</i>	
SY255	<i>Chaetoceros calcitrans</i>	HE589564	Rhodobacteraceae		<i>Roseobacteraceae</i>	
SY118	<i>Nannochloris sp</i>	HE995402	Hyphomonadaceae		<i>Hyphomonas sp</i>	
SY145	<i>Chaetoceros sp</i>	HE589549	Hyphomonadaceae		<i>Algimonas sp.</i>	
SY196	<i>Chaetoceros sp</i>	HE589559	Hyphomonadaceae		<i>Algimonas sp.</i>	
SY007	<i>Phaeodactylum</i>	HE589567	Gammaproteobacteria		Alteromonadaceae	<i>Alteromonas sp.</i>
SY102	<i>Odontella aurita</i>	HE589577			Pseudoalteromonadaceae	<i>Pseudoalteromonas sp.</i>
SY182	<i>Nannochloris sp</i>	HE995404			Pseudomonadaceae	<i>Pseudomonas sp</i>
SY003	<i>Odontella aurita</i>	HE589565		Halomonadaceae	<i>Halomonas sp.</i>	
SY213	<i>Emiliana huxleyi</i>	HE589587		Halomonadaceae	<i>Halomonas sp.</i>	
SY135	<i>Chaetoceros minus</i>	HE589581		Alteromonadaceae	<i>Marinobacter alkaliphilus</i>	
SY171	<i>Chaetoceros minus</i>	HE589584		Alteromonadaceae	<i>Marinobacter alkaliphilus</i>	
SY185	<i>Chaetoceros minus</i>	HE589585		Alteromonadaceae	<i>Marinobacter alkaliphilus</i>	
SY032	<i>Odontella aurita</i>	HE589572		Alteromonadaceae	<i>Marinobacter sp.</i>	
SY089	<i>Skeletonema marinoi</i>	HE589576		Alteromonadaceae	<i>Marinobacter sp.</i>	
SY106	<i>Chaetoceros pumilum</i>	HE589578		Alteromonadaceae	<i>Marinobacter sp.</i>	
SY260	<i>Thalassiosira sp</i>	HE589591		Alteromonadaceae	<i>Marinobacter sp.</i>	

Table 2 : ANOVA table resulting from the factorial design approach used for comparison of OD<sub>680</sub> to fluorescence as proxy for microalgal population in mixed culture. Significant p-values ( $\alpha=0.01$ ) 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.

	<i>A</i>	<i>B</i>	<i>AA</i>	<i>BB</i>	<i>AB</i>	R <sup>2</sup> (%)
OD <sub>680</sub>	<b>0.0003</b> (+)	<b>0.0000</b> (+)	0.4271 (-)	0.1432 (-)	0.0985 (+)	99.88
fluorescence	<b>0.0006</b> (+)	0.0277 (-)	0.0223 (-)	0.9183 (-)	0.1790 (-)	99.62

Table 3 : 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.

	Xenic	SY003	SY007	SY244
Shape	cocci	cocci	bacilli	bacilli
Q <sub>C</sub> (fmol C.cell <sup>-1</sup> )	6.4* (0.3)	6.4 (0.3)	27.7 (4.7)	18.3 (0.3)
Q <sub>N</sub> (fmol N.cell <sup>-1</sup> )	1.3* (0.1)	1.3 (0.1)	5.1 (0.9)	3.6 (0.1)
% of particulate C	0.05 (0.05)	9.6 (0.2)	3.6 (1.2)	0.41 (0.2)
% of particulate N	0.2 (0.2)	33.8 (0.9)	14.6 (5.1)	2.1 (0.81)

\* on the basis of microscopic observation, same quotas were considered for bacteria in the xenic cultures and for SY003

Table 4 : physiological results for *Dunaliella sp.* in experiment 2, including growth parameters ( $\mu_{\max}$  and  $\Delta X_{\max}$ ) computed on a per carbon basis, cell biovolume, carbon quota, computed C:N ratio, and nitrogen incorporation. *Dunaliella sp.* was cultivated in different conditions : xenic and axenic strains and associated with the selected bacteria SY003, SY007 and SY244. For each column, the values presented are median and interquartile range (IQR) in brackets. Values with the same superscript letters are not statistically different (Kruskal Wallis test;  $\alpha = 5\%$ ).

	Axenic	SY003	SY007	SY244	Xenic	P value
$\mu_{\max}$ (d <sup>-1</sup> )	0.36 <sup>a</sup> (0.05)	0.26 <sup>b</sup> (0.05)	0.33 <sup>a</sup> (0.01)	0.27 <sup>b</sup> (0.01)	0.22 <sup>b</sup> (0.01)	0.018 (n=3)
$\Delta X_{\max}$ (mM)	8.0 <sup>a</sup> (0.5)	5.4 <sup>b</sup> (0.5)	10.5 <sup>c</sup> (0.3)	10.8 <sup>c</sup> (0.5)	6.0 <sup>d</sup> (0.3)	2.2 10 <sup>-5</sup> (n=6)
Cell biovolume ( $\mu\text{m}^3$ )	384 <sup>a</sup> (15)	350 <sup>b</sup> (15)	337 <sup>c</sup> (8)	411 <sup>d</sup> (21)	285 <sup>e</sup> (16)	3.8 10 <sup>-7</sup> (n=9)
Q <sub>C</sub> (pmol C.cell <sup>-1</sup> )	20.6 <sup>a</sup> (0.6)	19.6 <sup>b</sup> (0.6)	17.2 <sup>c</sup> (0.9)	22.4 <sup>d</sup> (1.0)	14.7 <sup>e</sup> (0.9)	3.6 10 <sup>-8</sup> (n=9)
C:N	29.6 <sup>a</sup> (1.4)	24.4 <sup>b</sup> (1.3)	24.1 <sup>bc</sup> (2.4)	25.6 <sup>d</sup> (0.5)	22.8 <sup>c</sup> (1.7)	8.9 10 <sup>-7</sup> (n=9)
N incorporation (%)	26 <sup>a</sup> (9)	19 <sup>b</sup> (2)	56 <sup>c</sup> (3)	56 <sup>c</sup> (4)	34 <sup>d</sup> (5)	6.91 10 <sup>-7</sup> (n=9)

**Figures**

Figure 1 : microalgal and bacterial concentrations tested in mixed suspensions during the central composite experiments. Light grey dots represent experiments for the 2-level factorial design. Dark grey dots represent experiments at the center of the experimental domain, used to compute experimental variance. Distance between star-points (white dots) and the center of the experimental domain was calculated using the axial distance  $\alpha = 1.414$ . Measurements for  $DO_{680}$  and *in-vivo* Chl *a* fluorescence were made for the 11 experiments.

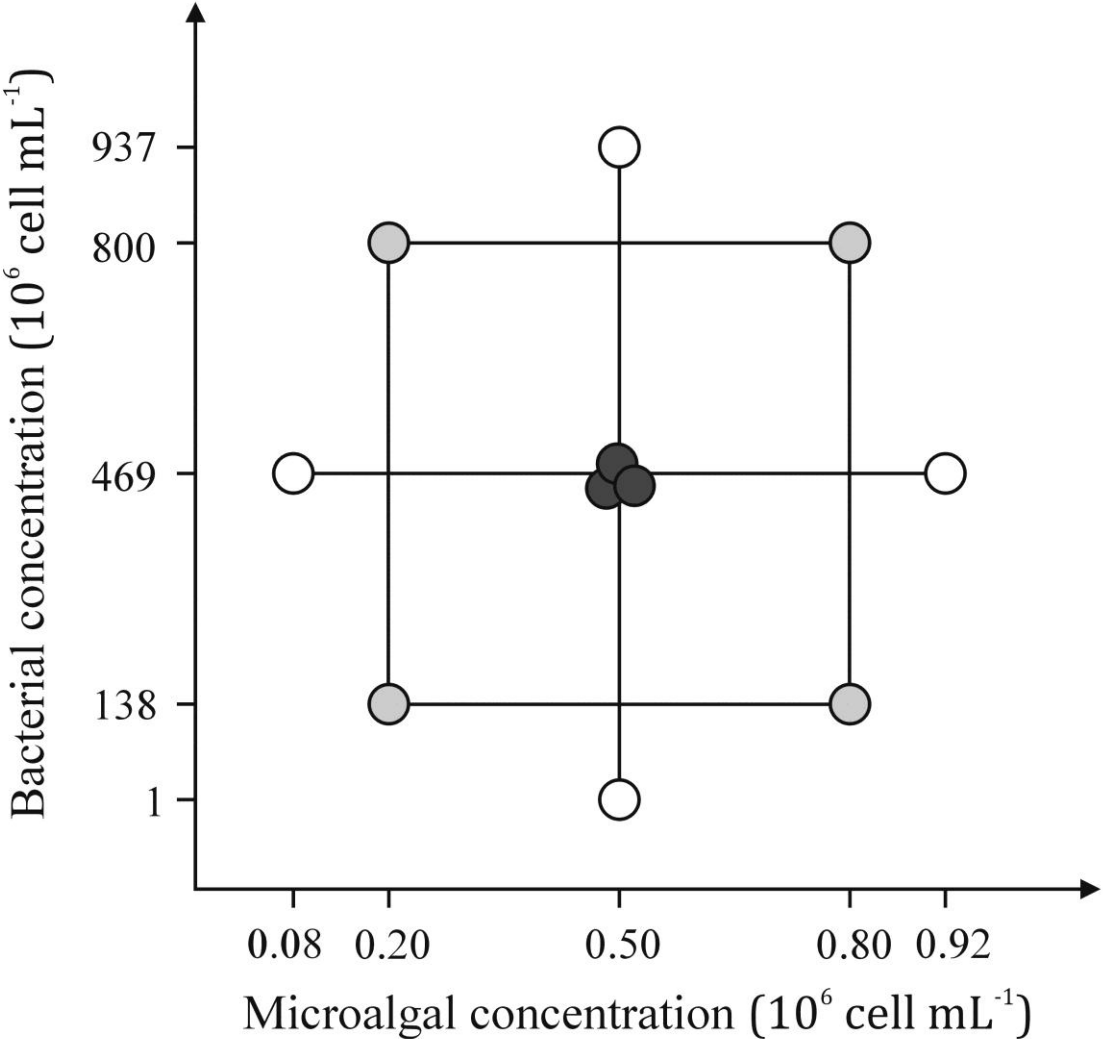


Figure 2 : maximal growth rate ( $\mu_{max}$ ) for *Dunaliella sp.* SAG 19.3, calculated in the high-throughput experiment (experiment 1) for axenic, xenic or mixed cultures with different bacterial strains assayed individually. For each culture, raw data for the three replicates are connected by a vertical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

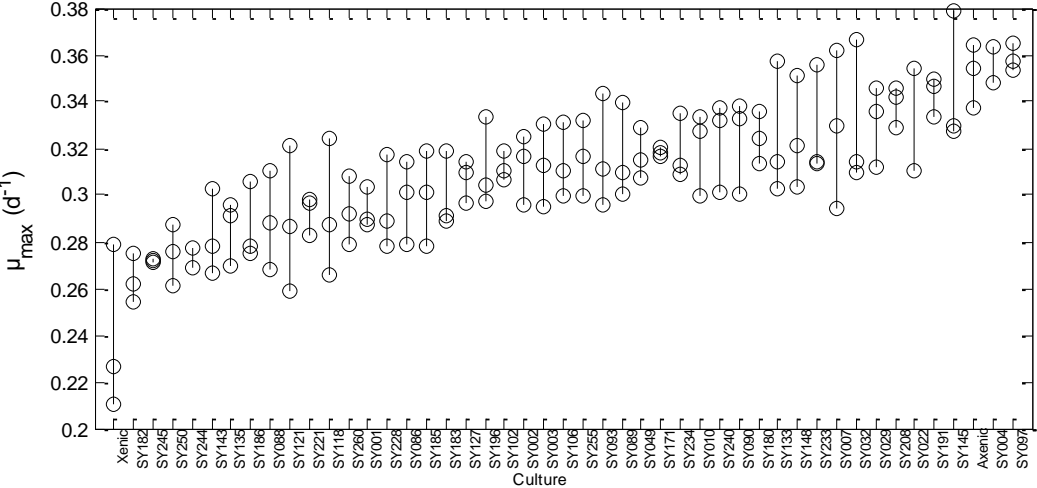


Figure 3: maximal biomass increase ( $\Delta X_{max}$ ) for *Dunaliella sp.* SAG 19.3 estimated in the high-throughput experiment (experiment 1) for axenic, xenic or mixed cultures with different bacterial strains assayed individually. For each culture, raw data for the three replicates are connected by a vertical line to facilitate reading. Reference numbers in the collection are given on the X-axis.

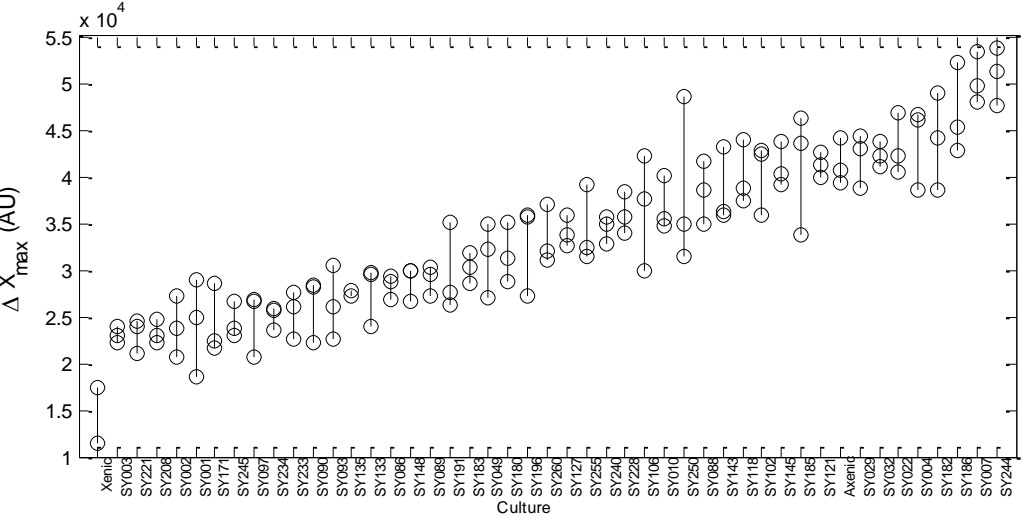


Figure 4: RAPD-PCR profiles for isolates from flask mixed cultures SY003, SY007 and SY244 (experiment 2) compared with the relevant SY003, SY007 and SY244 controls from the collection. Results were obtained with the two primers RAPD 1 and RAPD 4. Two bacteria colonies (C1 and C2) were analysed for the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

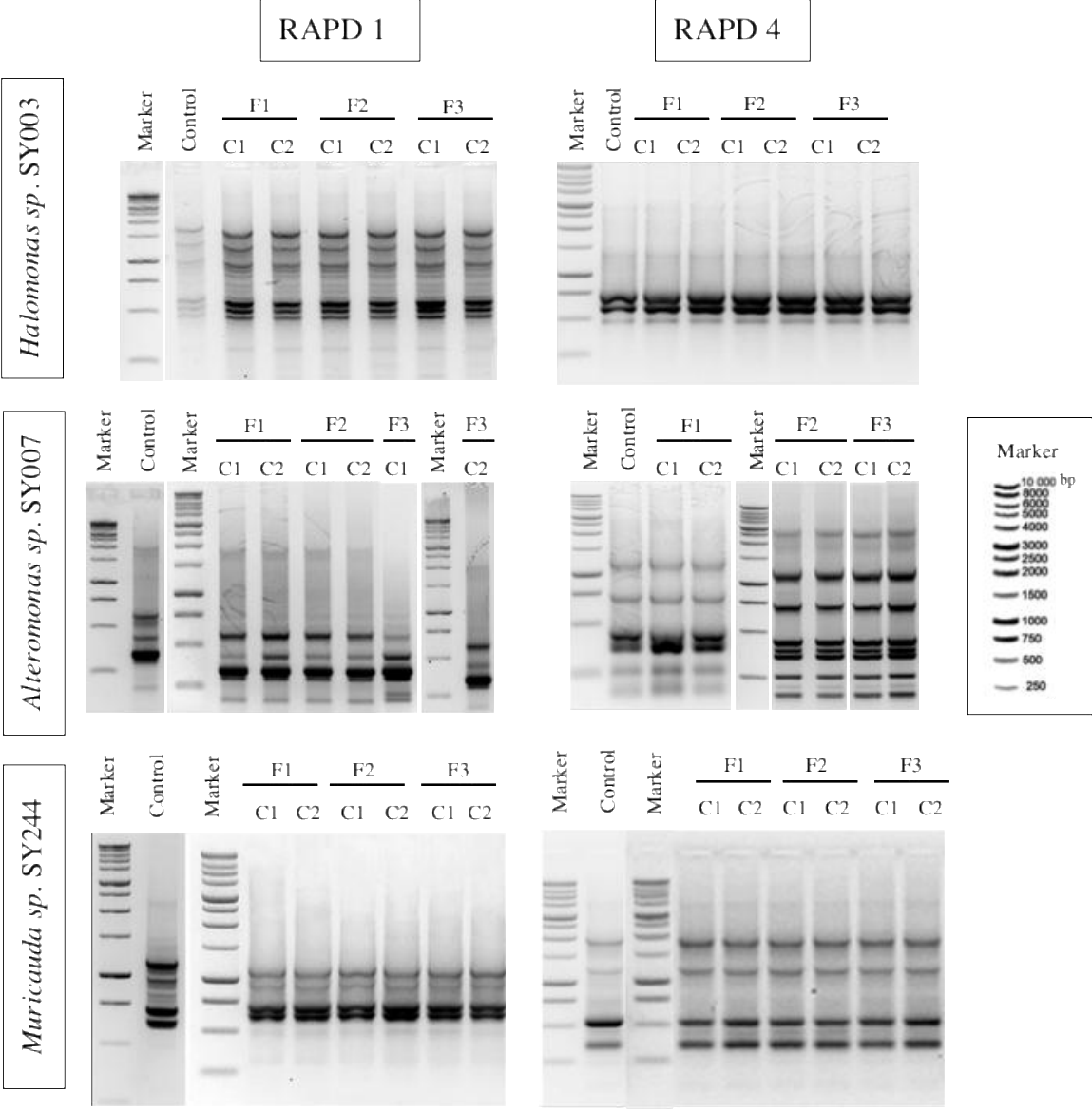


Figure 5 : A) Particulate carbon growth curves in flask cultures (experiment 2) for axenic, xenic strains and mixed cultures (SY003, SY007 and SY244). B) Bacterial concentration in mixed cultures at the beginning of the experiment and after 5 and 10 days of culture.

