



## Influence of the algal microbiome on biofouling during industrial cultivation of *Nannochloropsis* sp. in closed photobioreactors



Javier B. Giraldo<sup>a,b,\*</sup>, Willem Stock<sup>b</sup>, Lachlan Dow<sup>c</sup>, Luc Roef<sup>a</sup>, Anne Willems<sup>d</sup>, Sven Mangelinckx<sup>e</sup>, Peter G. Kroth<sup>c</sup>, Wim Vyverman<sup>b</sup>, Mark Michiels<sup>a</sup>

<sup>a</sup> Proviron Holding NV, Georges Gilliotstraat 60, B- 2620 Hemiksem, Belgium

<sup>b</sup> Laboratory of Protistology and Aquatic Ecology, Department of Biology, Ghent University, Krijgslaan 281-S8, B-9000 Ghent, Belgium

<sup>c</sup> Department of Biology, University of Konstanz, D-78457 Konstanz, Germany

<sup>d</sup> Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

<sup>e</sup> SynBioC, Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

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### ABSTRACT

Industrial cultivation of microalgae is becoming increasingly important, yet the process is still hampered by many factors, including contamination and biofouling of the algal reactors. We characterized a subset of microorganisms occurring in the broth and different biofilm stages of industrial scale photobioreactors applied for the cultivation of *Nannochloropsis* sp. A total of 69 bacterial strains were isolated, belonging to at least 24 different species. In addition, a green microalga was isolated and identified as *Chlamydomonas hedleyi*. The effect of *C. hedleyi* and 24 of the bacterial isolates on the productivity of *Nannochloropsis* was evaluated through growth and biofilm assays. *C. hedleyi* was shown to reduce growth and induce biofilm formation in *Nannochloropsis*. These effects were however indirect as they could be attributed to the bacteria associated to *C. hedleyi* and not *C. hedleyi* itself. Although most bacterial strains reported no effect, several were able to induce biofilm formation.

### 1. Introduction

The production of a large variety of products from low-cost raw materials has raised the enthusiasm for a biobased economy. Microalgae have been proven to be a suitable option for such a bio-refinery concept: as photosynthetic single-celled microorganisms they mostly require inexpensive substrates and sunlight for growing. By growing them in seawater [1] or wastewater [2], the consumption of valuable clean freshwater can be reduced. It is expected that a large variety of products from microalgae could be obtained simultaneously, including proteins, fractionated lipids, pigments, and residual nutrient feedstocks [3]. Industrial cultivation of microalgae has become increasingly important during recent years [4].

Despite the great potential, important limitations during industrial production exist. For example, many microalgae have high growth rates, but it is still very challenging to achieve high algal densities. This is mainly due to the uneven light distribution in growing ponds or photobioreactors (PBR) [5]. Single cells absorb light and thus much less light penetration is possible at higher algal densities. Light limitation may produce changes in the algae life cycle, affecting morphology and biochemical stoichiometry [6]. This problem cannot be solved by

increasing light irradiation, which in many cases, would lead to photoinhibition [7], leading to decreased efficiency of photosynthesis and thus decreased growth rates.

The ProviAPT system, designed by Proviron Holding NV, is a promising closed PBR system for large scale microalgae production [8]. The ProviAPT system includes an array of 35 vertical flat-panel type reactors attached to a common feed and aeration layer all enclosed in a translucent plastic bag filled with water (Fig. 1). The design increases productivity compared to other systems [9] and has thus far successfully been applied for large scale cultivation of different microalgae including *Nannochloropsis* and *Isochrysis* spp. The relatively small diameter of the flat-panels reduces problems with limited light penetration at higher densities, but biofilm formation, typical for closed reactor systems, can still strongly reduce light infiltration. Known causes of biofouling and biofilm formation include mechanical problems that are typically related with a lack of aeration, and bacteria [10].

Algae-bacteria interactions are known to lead to biofilm formation [11]. The biofilm environment presents several benefits for the organisms e.g. they enhance the nutrient diffusion to the cells [12] and protect against antibiotics [13]. Bacteria typically initiate biofilm formation by the release of exopolymeric substances (EPS) in the so-called

\* Corresponding author at: Proviron Holding NV, Georges Gilliotstraat 60, B- 2620 Hemiksem, Belgium.

E-mail address: [javier.giraldo@proviron.com](mailto:javier.giraldo@proviron.com) (J.B. Giraldo).

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Fig. 1. View of ProviAPT in a production plant during 2016.

surface conditioning phase [14]. This EPS matrix is largely constituted of exopolysaccharides and proteins [15]. After the conditioning of the surface, cell attachment takes place [16], ultimately leading to the development of a mature biofilm [17]. These processes are mediated by a wide range of biochemical cascades [18]. It has been reasoned that the interference of these cascades by chemical agents can be used to inhibit biofilm formation [19].

Industrial microalgae production is typically conducted in non-axenic conditions. Therefore, alterations in the algal microbiome may induce biofouling and biofilm formation initiated by bacterial adhesion to solid surfaces [20]. Due to lower light penetration and the subsequent lower yields, biofouling is highly undesirable in algal production reactors.

This study aims at identifying the effect of associated microorganisms from industrial bioreactors on the productivity of the system.

To this extent, microorganisms from an industrial bioreactor setup were isolated and co-cultured with a *Nannochloropsis* species. The experimental setup is summarized in Fig. 2. The goal was to identify microorganisms which affect the algal growth rate and biofilm formation. While other studies have analyzed and compared the algal microbiome in closed PBRs [21–24], here we used samples from a microalgae production plant to actually isolate microorganisms and test their individual contribution to biofouling and biofilm formation.

## 2. Materials and methods

### 2.1. Biofilm sampling protocol and selected samples

The sample set consisted of algae broth and biofilm samples from ProviAPT reactors. Each reactor has an operational volume of 160 L on an effective surface of 7 m<sup>2</sup> [25]. Semi-continuous growth of *Nannochloropsis* sp. cultures was held in outdoor conditions. Algae cultures were grown in saline media adapted from f/2 [26] by enriching with inorganic nutrients [25].

The algae broth was collected from operative reactors during

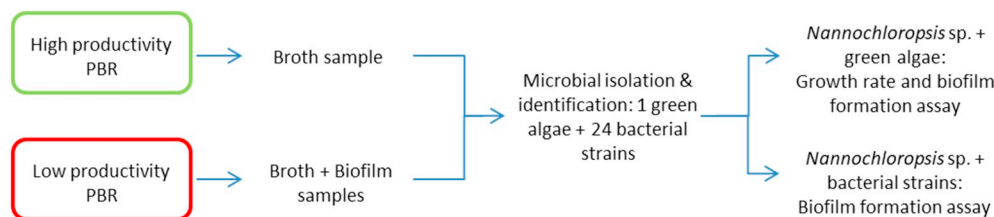


Fig. 2. Summary of the experimental approach: Microorganisms were isolated and identified from various industrial samples and their contribution to biofouling and biofilm formation was estimated.

cultivation of *Nannochloropsis* sp. strain CCAP211/78, obtained from the Culture Collection of Algae and Protozoa (CCAP, United Kingdom). Biofilm sampling was a reactor destructive process and could therefore only be performed at the end of the production season. Observations on the production facilities showed, however, that biofilms do not necessarily form uniformly in a reactor. One single reactor contains biofilm patches from initial to more mature biofilms. The biofilm sampling reported here was performed during October 2015 from reactors which were discarded for algae production due to inhibitive biofouling.

A total of five samples from two reactors with different productivities were used for microbial isolation and identification. Productivity was estimated as high or low based on overall CO<sub>2</sub> consumption, dry biomass weight and observations on reactor behavior. Values for low productivity reactors oscillated around 5 g·m<sup>-2</sup>·d<sup>-1</sup> while high productivities got up to 18 g·m<sup>-2</sup>·d<sup>-1</sup>. From an indoor inoculum reactor with high productivity, one algae broth sample was taken. From an outdoor reactor already discarded for production due to low productivity, four samples were taken: one from algae broth and three from biofilms. For the latter, sampling sites in the reactor wall were selected corresponding to areas that were visually defined as early, developed and mature biofilm stages. First, three panels from the same reactor were cut. From each panel, a relevant area with a visually homogeneous biofilm surface was chosen and marked. Each delimited area had a surface of 35 cm<sup>2</sup>. The external surface of the panels was thoroughly cleaned with 70% ethanol to prevent contamination of the sample. The biofilm surface was rinsed with sterile PBS-buffer [27] in order to remove the non-adhered cells. Afterwards, the biofilm was scraped off with a sterile spatula and re-dissolved in PBS. The content was homogenized by vigorous agitation and the homogenized solution was used for cell-culturing.

### 2.2. Microbial isolation

Artificial seawater with added inorganic nutrients and vitamins [25] was used to prepare agar medium for bacterial isolation. It was enriched with 5 g/L peptone (DUCHEFA), 1 g/L yeast extract (DUCHEFA) and 25% v/v of filter-sterilized supernatant from the particular reactor samples were taken from. The agar (Phyto-agar, DUCHEFA) concentration was 10 g/L. In this way, the enrichment of organic matter was similar to the marine agar based on the medium of Zobell (1941) [28] and nutrient proportions were similar to reactor conditions.

For each sample, a dilution series was made and spread plated on medium that was supplemented with supernatant from the corresponding reactor as described above. All plates were incubated at room temperature (23 °C) until colonies became apparent.

After one week, different colony types were visible. Three representatives of each type were picked and streaked onto a fresh agar plate. This procedure was repeated until it was ensured that all isolates were monoclonal. After 3 additional weeks the initially inoculated plates were again screened for slower growing colonies. The same procedure was repeated with these newly detected colonies. Once the identification was performed, the bacterial strains were grown in the same agar recipe without peptone, yeast extract and sterile supernatant but enriched with 20 mM of glucose at room temperature.

### 2.3. Bacterial identification by MALDI-TOF MS and 16S rRNA gene sequencing

All bacterial isolates were subjected to MALDI-TOF mass spectrometry in order to identify duplicate strains and group isolates based on their protein profile [29]. Samples were prepared and bacterial fingerprints were produced as describe previously [30] using a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems) in linear positive ion mode. Raw spectra were imported into the Data Explorer 4.0 software (Applied Biosystems) and converted to text files which were imported in BioNumerics 7.5 (Applied Maths). Cluster analysis using Pearson's product moment correlation coefficients and UPGMA clustering was performed in Bionumerics. Based on the MALDI clustering representative strains were selected for 16S rRNA gene sequencing, on the assumption that identical MALDI protein profiles corresponded to a single species [31].

From each selected isolate, DNA was extracted following an adapted phenol:chloroform extraction for marine samples [32]. The V1-V3 hypervariable region of the 16S rDNA from these extracts was amplified by PCR using primers pA (AGAGTTTGCCTGGCTCAG, positions 8–27) [33] and BKL1 (GTATTACCGCGGCTGCTGGCA, positions 536–516) [34]. Fragments were purified and sequenced, yielding fragments that were approx. 450 bp long. Identification was conducted using the EZTaxon online server [35].

### 2.4. Isolation, treatment and identification of an invasive green algae species

A green motile algae species was isolated from the outdoor reactor, following the same procedure as the bacterial isolation described above. Based on the unique colony morphology with green color and observations under the microscope, this isolate was identified as an alga. After isolation, this green alga was grown in liquid saline medium enriched with inorganic nutrients [25]. After an initial transfer from plate to flask, the culture was divided in two sub-cultures. The first sub-culture was not treated with antibiotics and the second one was treated with antibiotics over 48 h. The antibiotics (Ab) cocktail consisted of 170 µg/µL of penicillin G (Sigma-Aldrich), 85 µg/mL of streptomycin (Sigma-Aldrich), 17 µg/mL of chloramphenicol (Sigma-Aldrich), 10 µg/mL of tetracycline (Sigma-Aldrich) and 10 µg/mL of kanamycin (Sigma-Aldrich). After the Ab treatment, cells were centrifuged (10,000 rpm, 5 min, Beckman Coulter) and the pellet was suspended in antibiotic-free medium. This was repeated twice in order to ensure removal of the antibiotics from the Ab-treated culture. This culture was checked for bacterial presence with two approaches: (1) SYBR green staining followed by microscopy and (2) inoculation of algae samples in agar plates followed by incubation for one week at room temperature. In both approaches, no evidence of bacteria was detected. Despite the fact that the combination of these two methods cannot ensure absolute axenicity, the microbial population associated to the algae was considered to be dramatically reduced and therefore sufficient to prove the influence of the algal microbiome. To identify the alga, DNA was isolated using a phenol:chloroform based extraction [32]. The 'internal transcribed spacer' (ITS) region was amplified with ITS universal primers – 1800F [36], R-ITS4 [37], DITS3 and DITS2 [38] – reporting fragments of approximately 640 bp which were sent for sequencing. The obtained sequence was deposited on GenBank (accession number MH727704). A BLAST search in the NCBI-nr/nt database was performed with the resulting sequence. It produced a match with *Chlamydomonas hedleyi* (GenBank [AJ297797.1](https://www.ncbi.nlm.nih.gov/nuclot/AJ297797.1)) with a Max. score and total score of 1188 for both, a query cover of 100%, an E-value of 0.0 and an identity value of 100% (retrieved on May 14th, 2019).

### 2.5. Algae cultivation in shake flasks

The microalga *Nannochloropsis* sp., strain CCAP211/78 was

obtained from the Culture Collection of Algae and Protozoa (CCAP, United Kingdom). *Nannochloropsis* sp. and the isolated *C. hedleyi* were cultivated in 100 mL Erlenmeyer flasks (Schott-Duran) on a shaker at 70 rpm with a culture volume of 50 mL of algae growing in saline medium at 20 °C [25]. The light irradiation was 70 µmol·m<sup>-2</sup>·s<sup>-1</sup> and applied from a cool fluorescent light source in day-night cycles of 16:8.

### 2.6. Co-cultivation of *Nannochloropsis* sp. with the green algae species

The growth rates of *Nannochloropsis* sp. and the two subcultures (Xenic and Ab treated) of *C. hedleyi* were estimated by daily measurements of OD<sub>750</sub> during 9 days. The initial OD<sub>750</sub> for *Nannochloropsis* sp. was 0.550 ± 0.005 and for the two *C. hedleyi* cultures 0.140 ± 0.005. The volume in every flask was 50 mL. For the co-cultivation flasks, 1 mL from the same *C. hedleyi* was inoculated in a *Nannochloropsis* sp. culture. All treatments were setup in triplicate. At the end of the 9 days, growth rates were calculated as the logarithmic ratio of cell densities divided by the time and expressed as d<sup>-1</sup>.

### 2.7. Biofilm formation assay

Biofilm formation assays were performed in flat bottomed 96-well plates (Sarstedt) that were inoculated with *Nannochloropsis* sp. and co-cultures of *C. hedleyi* or the bacterial isolates depending on the case [39]. Each well was inoculated with 150 µL of algae suspension with an optical density of OD<sub>630</sub> 1.50 ± 0.05 and OD<sub>750</sub> 1.40 ± 0.05. Each bacterial culture was transferred from a colony growing on agar to liquid medium and used after three days. Bacterial isolates were cultivated in saline media [25] enriched with 20 mM glucose as an organic carbon source is necessary to maintain bacterial cultures. Since addition of glucose might alter the capacity of different microorganisms to induce biofilm formation, prior to co-cultivation bacterial cultures were washed twice with fresh medium via centrifugation (10000 rpm, 5 min, Beckman Coulter) in order to remove traces of glucose. A volume of 30 µL of the glucose-free bacterial suspension was used to inoculate each well which later was brought to a final volume of 250 µL with fresh sugar-free culture media. Well plates were manually prepared and the well position of each treatment was randomized. Plates were incubated for 10 days under the same conditions as those used for shake flasks, however without shaking.

Well plates were prepared for biofilm quantification using a Freedom EVO pipetting robot (Tecan) along with a Tecan Infinite F500 plate reader. Growth media and non-adherent cells were removed via both robotic and manual pipetting, and biofilm density measured using absorbance at wavelengths 630 nm and 750 nm, as proxies for chlorophyll *c* concentration [40] and biomass [41] respectively. In order to quantify extracellular polysaccharide secretion, well plates were then stained with 40 µL of 0.02% (w/v) aqueous crystal violet (CV) solution (Sigma-Aldrich), and incubated for one minute. Excess CV solution was removed, well plates rinsed with water and excess liquid removed via manual pipetting. CV staining intensity was quantified via absorbance at 580 nm. The absorbance of each well was measured in twelve locations. The average value of these twelve measurements was used as a single replicate.

### 2.8. Statistical analysis

The growth rates and each wavelength used in biofilm absorbance measurements were separately compared by means of a one-way ANOVA followed by a Tukey HSD post-hoc test if significant (p-value < 0.05). The ODs from the biofilm formation assays using the different bacterial strains were not compared by means of an ANOVA since residuals were not normally distributed and group sizes were not always equal. A non-parametric Kruskal-Wallis test was performed for each wavelength followed by a Dunn's test (R-package RVAideMemoire 0.9–66) if significant (p-value < 0.05). Only p-values comparing each

**Table 1**

Overview of the 69 isolates that were identified by MALDI-TOF MS and/or 16S rRNA gene sequence analysis. Strains that were used in the co-cultivation experiments are shown in bold. Numbers in brackets at the identification column refer to different strains on the same genus. Asterisks in the accession number column refer to strains identified by MALDI-TOF MS.

Strain	Identification	Broth/ biofilm (BF)	Reactor productivity	Accession number
R-67403	<i>Idiomarina loihiensis</i>	Broth	High	MH974272
R-67404	<i>Halomonas</i> sp. (1)	Broth	High	*
R-67405	<i>Roseivirga ehrenbergii</i>	Broth	High	*
R-67406	<i>Alcanivorax</i> sp.	Broth	High	*
R-67407	<i>Alcanivorax</i> sp.	Broth	High	MH974273
R-67408	<i>Halomonas</i> sp. (1)	Broth	High	MH974238
R-67409	<i>Alcanivorax</i> sp.	Broth	High	MH974274
R-67410	<i>Halomonas</i> sp. (2)	Broth	High	MH974239
R-67411	<i>Halomonas venusta</i>	Broth	High	*
R-67413	<i>Alcanivorax</i> sp.	Broth	High	MH974262
R-67415	<i>Halomonas venusta</i>	Broth	High	*
R-67416	<i>Halomonas</i> sp. (1)	Broth	High	MH974240
R-67417	<i>Celeribacter baekdonensis</i>	Broth	High	MH974241
R-67418	<i>Devosia subequoris</i>	Broth	High	MH974242
R-67419	<i>Alcanivorax</i> sp.	Broth	High	MH974243
R-67420	<i>Paracoccus</i> sp.	Broth	High	MH974263
R-67421	<i>Roseovarius pacificus</i>	Broth	High	*
R-67423	<i>Stappia indica</i>	Broth	High	MH974244
R-67424	<i>Halomonas venusta</i>	Broth	High	*
R-67425	<i>Halomonas venusta</i>	Broth	High	MH974245
R-67426	<i>Halomonas venusta</i>	Broth	High	MH974246
R-67427	<i>Marinobacter adhaerens</i>	Broth	High	MH974264
R-67428	<i>Marinobacter adhaerens</i>	Broth	High	MH974247
R-67429	<i>Alcanivorax</i> sp.	Broth	High	MH974265
R-67430	<i>Roseivirga ehrenbergii</i>	Broth	High	MH974266
R-67431	<i>Roseivirga ehrenbergii</i>	Broth	High	MH974267
R-67432	<i>Halomonas venusta</i>	Broth	High	MH974248
R-67434	<i>Muricauda</i> sp.	Broth	High	MH974249
R-67436	<i>Alcanivorax</i> sp.	Broth	Low	MH974250
R-67437	<i>Pseudoalteromonas</i> sp.	Broth	Low	MH974251
R-67438	<i>Pseudoalteromonas</i> sp.	Broth	Low	MH974252
R-67439	<i>Halomonas</i> sp. (1)	BF	Low	*
R-67440	<i>Halomonas alkaliphila</i>	BF	Low	*
R-67441	<i>Marinobacter adhaerens</i>	BF	Low	*
R-67442	<i>Halomonas</i> sp. (3)	BF	Low	MH974253
R-67443	<i>Halomonas</i> sp. (3)	BF	Low	*
R-67444	<i>Marinobacter adhaerens</i>	BF	Low	*
R-67445	<i>Halomonas alkaliphila</i>	BF	Low	*
R-67446	<i>Idiomarina loihiensis</i>	BF	Low	MH974254
R-67447	<i>Idiomarina loihiensis</i>	BF	Low	MH974255
R-67448	<i>Halomonas alkaliphila</i>	BF	Low	*
R-67449	<i>Idiomarina loihiensis</i>	BF	Low	MH974268
R-67450	<i>Idiomarina loihiensis</i>	BF	Low	*
R-67451	<i>Halomonas alkaliphila</i>	BF	Low	*
R-67452	<i>Roseovarius pacificus</i>	BF	Low	*
R-67453	<i>Idiomarina loihiensis</i>	BF	Low	*
R-67454	<i>Halomonas alkaliphila</i>	BF	Low	MH974256
R-67471	<i>Nitratireductor aquimarinus</i>	Broth	Low	*
R-67472	<i>Labrenzia aggregata</i>	Broth	Low	MH974257
R-67473	<i>Halomonas</i> sp. (3)	BF	Low	*
R-67474	<i>Nitratireductor aquimarinus</i>	BF	Low	*
R-67475	<i>Halomonas</i> sp. (3)	BF	Low	*
R-67476	<i>Idiomarina</i> sp.	BF	Low	MH974258
R-67477	<i>Roseovarius pacificus</i>	BF	Low	MH974269
R-67478	<i>Alcanivorax</i> sp.	BF	Low	*
R-67479	<i>Halomonas</i> sp. (1)	BF	Low	MH974259
R-67480	<i>Donghicola</i> sp.	BF	Low	MH974270
R-67481	<i>Roseovarius pacificus</i>	BF	Low	*
R-67482	<i>Halomonas alkaliphila</i>	BF	Low	*
R-67483	<i>Idiomarina loihiensis</i>	BF	Low	MH974260
R-67484	<i>Roseovarius mucosus</i>	BF	Low	MH974261
R-67486	<i>Celeribacter halophilus</i>	BF	Low	MH974271
R-67487	<i>Nitratireductor kimnyeongensis</i>	BF	Low	MH974275
R-67488	<i>Poseidonocella</i> sp.	BF	Low	MH974276

bacterial strain with the control were retained and corrected by means of false discovery rate multiple test correction. All statistical tests were run in R (3.4.1). Figures were also designed in this software with ggplot 2 (2.2.1).

### 3. Results and discussion

#### 3.1. Microbial isolation

Sixty-nine bacterial isolates were obtained from the sampling of the reactor setups under study. Using MALDI-TOF MS profile clustering in combination with partial 16S rRNA gene sequencing, 24 different species-level taxa were identified among 64 strains. For 5 isolates, a good quality MALDI profile, nor good sequence, could be obtained. Most isolates belonged to the Gamma- and Alphaproteobacteria and the rest were Bacteroidetes (Table 1).

Furthermore, a green motile alga strain was isolated from the low productivity reactor and identified as *Chlamydomonas hedleyi*. The presence of green algae at the production site is often correlated with an increase in biofouling as well as a dramatic drop in productivity. Observations in the production plant suggested that *C. hedleyi* is the principal species invading the reactors.

Due to the likely unculturability of certain strains, the isolates identified in this study may represent only part of the microbiome [42]. Our findings are however in line with previous efforts to identify bacteria present in *Nannochloropsis* cultures. A study based on microbial communities associated to *Nannochloropsis salina* in open ponds [43,44], concluded that the microbial community is highly dynamic, depending on growth phase and external conditions. The authors showed that Alteromonadales dominated in exponential phase while Alphaproteobacteria and Flavobacteria dominated in the stationary phase. This is in line with the results presented here, since the reactors were in late exponential to stationary phase when sampled.

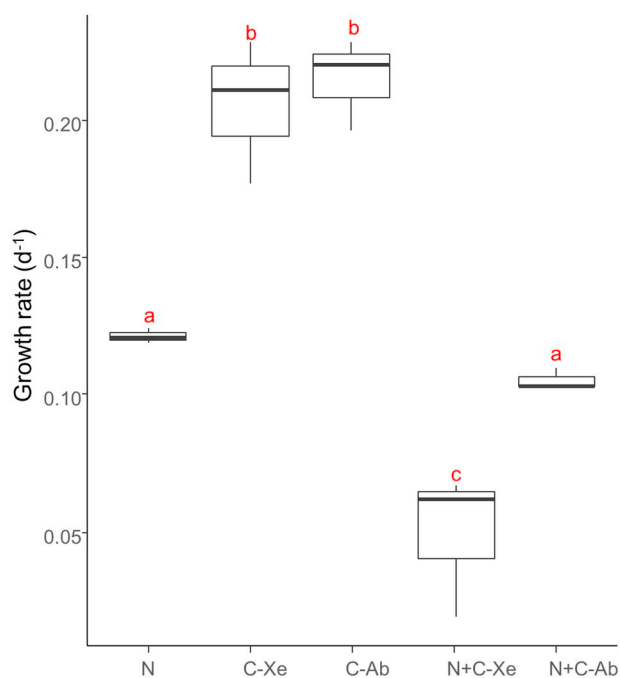
The dominance of Alphaproteobacteria in all samples may be related to the secretion of signal molecules. Members of the *Roseobacter* clade have been described to produce acylated homoserine lactones (AHL) [45] which are quorum sensing compounds, which are well-known signals that can be involved in the regulation of biofilm formation processes [46]. Further studies are desirable to better understand signaling between bacteria and algae in closed PBRs.

#### 3.2. Effect of the microbiome on the productivity of *Nannochloropsis*

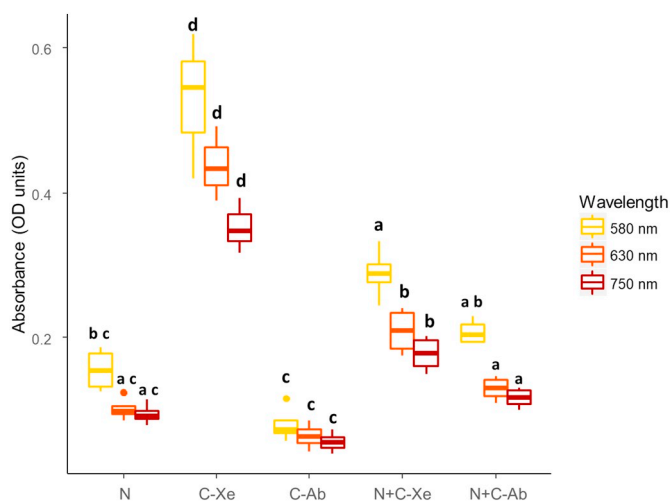
The detection of *C. hedleyi* in the PBRs was often correlated with a dramatic decrease of their productivity. Therefore, testing the influence of *C. hedleyi* on *Nannochloropsis* sp. growth was the first step to understand biofouling in the ProviAPT production reactors. It was decided to compare the growth of *Nannochloropsis* sp., an axenic and a non-axenic *C. hedleyi* culture, and both *Nannochloropsis*-*C. hedleyi* co-cultures (Fig. 3).

When the algal monocultures were compared, both *C. hedleyi* subcultures grew faster than *Nannochloropsis* sp. (p-value < 0.05). This was expected, since from previous observations it is known that *C. hedleyi* may overgrow the reactors shortly after its detection. However, the *C. hedleyi* subcultures behaved differently in co-cultivation with *Nannochloropsis* sp. The growth rate of the co-culture with Ab treated *C. hedleyi* is comparable to the *Nannochloropsis* sp. culture, while the growth rate of the *Nannochloropsis* sp. with xenic *C. hedleyi* is dramatically lower (p-value < 0.05) compared to all other treatments. These observations suggest that the microbiome associated to *C. hedleyi* might not affect its own growth but negatively affect the algal growth of *Nannochloropsis* sp. during co-cultivation.

In addition to monitoring growth effects, a biofilm formation assay was used to estimate the ability of *C. hedleyi* to induce biofilm formation of *Nannochloropsis* sp. (Fig. 4). Although growth rates of both *C. hedleyi* subcultures were similar (Fig. 3), the two cultures differ in terms of



**Fig. 3.** Estimated growth rates ( $d^{-1}$ ) of five different cultures ( $n=3$ ). Error bars correspond to standard deviation. Boxes indicate median and first and third quartile: (N) *Nannochloropsis* sp.; (C-Xe) *C. hedleyi* (Xenic); (C-Ab) *C. hedleyi* (Ab treated); (N + C-Xe) *Nannochloropsis* sp. + *C. hedleyi* (Xenic); (N + C-Ab) *Nannochloropsis* sp. + *C. hedleyi* (Ab treated). Lower case letters indicate groups that are not significantly different (Tukey HSD post-hoc test,  $p$ -values  $>0.05$ ).



**Fig. 4.** Estimated biofilm densities of five different cultures ( $n=6$ ). Error bars correspond to standard deviation. Boxes indicate median and first and third quartile: (N) *Nannochloropsis* sp.; (C-Xe) *C. hedleyi* (Xenic); (C-Ab) *C. hedleyi* (Ab treated); (N + C-Xe) *Nannochloropsis* sp. + *C. hedleyi* (Xenic); (N + C-Ab) *Nannochloropsis* sp. + *C. hedleyi* (Ab treated). Lower case letters indicate groups that are not significantly different (Tukey HSD post-hoc test,  $p$ -values  $>0.05$ ).

biofilm formation (Fig. 4). Biofilm production was most pronounced in the xenic *C. hedleyi* cultures, while the lowest values were recorded for the Ab treated *C. hedleyi*. The presence of bacteria stimulated biofilm formation of *C. hedleyi*. It is concluded that the presence of bacteria contributed to the attachment of algae on the substrate, which is in line with the classical mechanisms described for algae biofilm formation, where it is suggested that bacteria play an important role in initiating and developing biofilms [47]. A similar pattern was observed when *C. hedleyi* was co-cultivated with *Nannochloropsis* sp. The xenic culture reported higher optical densities when compared to the Ab treated ( $p$ -

value  $<0.05$ ). Only the xenic *C. hedleyi* and the co-cultivation of the xenic *C. hedleyi* and *Nannochloropsis* sp. reported higher values compared to axenic *Nannochloropsis* sp. at all three wavelengths ( $p$ -value  $<0.05$ ). These results together with those from the growth rate experiments highlight the effect of bacteria associated with *C. hedleyi*, suggesting they negatively affect the growth rate of *Nannochloropsis* sp. and initiate biofilm formation. Despite several *Chlamydomonas* sp. strains are used for industrial applications [48,49], the potential of *C. hedleyi* remains unexplored. This might be a promising candidate for harvesting microalgae by means of bioflocculation [50,51] although this suggestion requires further investigation.

### 3.3. *Poseidonocella* sp. is the strongest biofilm inducer among 24 isolated bacterial strains

Twenty-four bacterial strains representative of the isolated diversity (Table 1) were selected for the biofilm formation assay with the *Nannochloropsis* sp. (Fig. 5).

Most bacterial strains did not affect biofilm formation compared to the axenic *Nannochloropsis* sp. The strain R-67488 – closely related to *Poseidonocella pacifica* (Table 1), an alphaproteobacterium first described from shallow sandy sediments of the Sea of Japan [52] – is the exception here. It very strongly induced biofilm formation in co-culture with *Nannochloropsis* sp. Although the different values obtained for the three wavelengths are not significant due to the high variation associated with this method at higher ODs, the high values obtained at OD 630 and 750 indicate the ability of this bacterium to induce pronounced biofilms within a short amount of time. Although to a lesser extent, R-67480 (*Donghicola* sp.) and R-67418 (*Devosia subaequoris*) also significantly induced the development of biofilms.

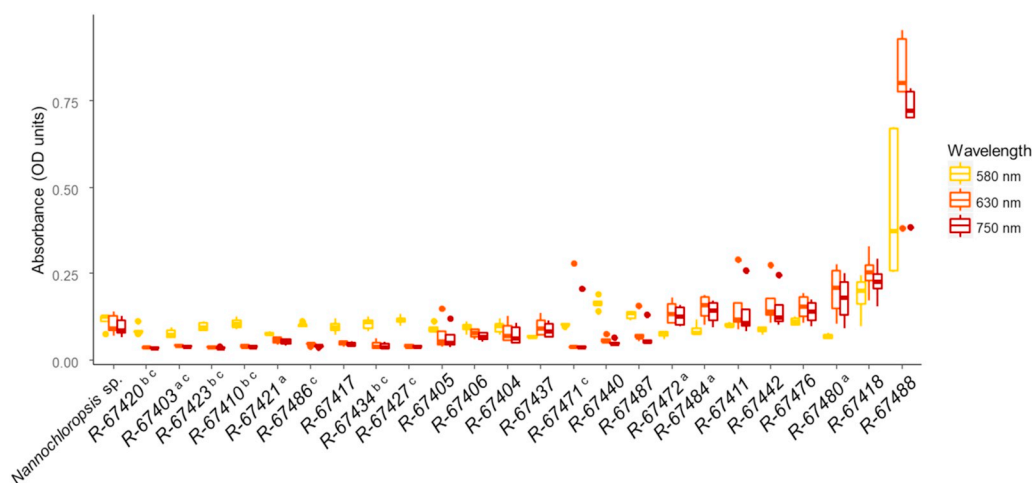
In this study, *Poseidonocella* sp. was only found in biofilms from low productivity reactors. The observed effect on *Nannochloropsis* sp. during laboratory co-cultivation is similar to that in low productivity reactors during industrial microalgae production. This bacterium was able to induce attachment of a considerable amount of algal biomass, which can be concluded from the high 630/750 ratio, if this ratio is considered as an indication of chlorophyll content per algal biomass.

Co-cultivations resulting in lower biofilm formation than axenic *Nannochloropsis* sp. cultures could be interpreted as an indication that the bacterial strains involved may have prevented biofilm formation by *Nannochloropsis* sp. or could be indicative of an algicidal effect of the bacteria. This could be the case when values taken at 580 nm are much higher than the ones at 630 nm and 750 nm since this is an indication of a higher relative abundance of bacterial cells and EPS compared to algae due to CV staining. These hypotheses would need to be confirmed by experiments on larger scale including co-cultivation.

Moreover, a study on microbial communities associated to *Nannochloropsis oceanica* proved the importance of environmental parameters such as growth temperature [10]. These authors found that certain *Halomonas* sp., *Muricauda* sp., *Rhodobacteriales* and an unknown gammaproteobacteria strain caused aggregation of *N. oceanica*. These conclusions emphasize the role of bacteria in inducing biofouling on *Nannochloropsis* sp. This is in line with this study, where certain Proteobacteria strains were found able to induce biofouling even at lower temperatures.

Biofouling and biofilm formation can lead to important losses during algae cultivation [53]. This can be prevented by improving technical aspects and biological control. The reactor wall composition influences the algal adhesion on the solid surface [54]. Nevertheless, this study emphasizes the relevance of the microbiome in non-axenic production and opens the exploration of ways to control it in order to prevent biofilm formation during cultivation in closed PBRs.

This study suggests that bacteria can both positively and negatively impact biofilm formation in a PBR. This has important implications for industrial algal cultivation, where working in axenic conditions is hardly feasible. The inoculation of bacteria which inhibit biofouling



**Fig. 5.** Biofilm formation assay of the bacterial-algae co-cultivations ( $n=6$ ). Error bars correspond to standard deviation. Boxes indicate median and first and third quartile. The R-codes from bacterial names (x-axis) indicate co-cultivation of that strain together with *Nannochloropsis* sp. *Nannochloropsis* sp. was used as a control. Small letters next to bacterial names indicate p-values  $< 0.05$  in comparison with the control (a for 580 nm, b for 630 nm and c for 750 nm). Strains are ordered by increasing biofilm formation capacity.

might suppress biofouling and extend the lifetime of the reactors. At the same time, culturing conditions can be tweaked to suppress biofilm inducers or their activity. Identification of the bacteria involved and their biochemical pathways will be crucial to develop such strategies.

#### 4. Conclusions

*Chlamydomonas hedleyi* was identified as a typical contaminant at a microalgal production site and proved to have higher growth rate than *Nannochloropsis* sp., which allows it to rapidly overgrow the reactors. The contaminant had a microbiome itself, which stimulated biofilm development, indicating a dual threat of *C. hedleyi* to the yield of the PBRs. The bacterial communities of the *Nannochloropsis* sp. reactors were dominated by Proteobacteria, of which some were able to induce biofilm formation, a *Poseidonocella* sp. in particular. Further studies on the isolates obtained in this study will help to identify the pathways involved in biofilm inhibition and formation.

#### Authors' contributions

JG cultured *Nannochloropsis* sp. and isolated bacterial strains, performed experiments and drafted this manuscript. AW contributed to the identification of bacterial strains. JG and LD performed biofilm formation assays. WS performed statistical analysis. LR, SM, AW, PK, WV and MM participated in the design of the study and helped to revise the manuscript. All of the authors read and approved the final manuscript.

#### Declaration of Competing Interest

None.

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