Growth characterization and biostimulant potential of *Coelastrella* sp. D14 a green microalga isolated from a solar panel.

Sara Baldanta[1], Alice Ferreira[2], Luisa Gouveia[2,3], Juana Maria Navarro Llorens[1], Govinda Guevara[1]*

[1]Department of Biochemistry and Molecular Biology, c/Jose Antonio Novais 12, Universidad Complutense de Madrid, 28040 Madrid, Spain. sabaldan@ucm.es; jmnavarr@ucm.es; fguevara@ucm.es.

[2]LNEG, National Laboratory of Energy and Geology I.P., Bioenergy Unit, Estrada do Paço do Lumiar 22, 1649-038 Lisbon, Portugal. alice.ferreira@lneg.pt; luisa.gouveia@lneg.pt

[3]GreenCoLab - Green Ocean Technologies and Products Collaborative Laboratory, CCMAR, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

*Corresponding author: fguevara@ucm.es

Abstract

Extremophile environments are an important source for finding microorganisms with a potential for biotechnological applications. Among these microorganisms, microalgae contribute to several sustainable processes such as wastewater treatments or nutrition. In this work it was characterized a microalga isolated from a solar panel. The morphological and phylogenetic analysis revealed that the isolate collected was a *Coelastrella* strain. Cultivation and stress experiments has shown that *Coelastrella sp.* D14 can resist a long period of desiccation and it can grow on cheap sources such as piggery wastewaters (PWW). This work reports that a *Coelastrella* strain displays biostimulant properties with a germination index of 123% on *Lepidium sativum* when D14 biomass grown at 10% piggery effluent was used. Altogether, these results suggest that this novel strain could be a good chassis for further biotechnological applications.

Highlights

• *Coelastrella* sp. D14, a xero-tolerant strain, has been isolated from a solar panel

• This strain can grow on piggery wastewater

• *Coelastrella* sp. D14 can promote germination of *Lepidium sativum*

Keywords: *Coelastrella* sp. D14; microalga; extremophile; wastewater; biostimulant

1. Introduction

Water scarcity and pollution, recognized as significant environmental concerns, have garnered widespread attention, prompting efforts towards finding solutions (Hussain et al., 2021). Microalgae are one of the most attractive biological agents to address the

water pollution problems and greenhouse effect. Microalgae are a highly diversified group of photosynthetic microorganisms adapted to a wide range of ecological habitats that utilize solar energy to generate biomass. Among the advantages of its use, it stands out their low nutritional needs, not depending on arable land nor potable water, they can grow under several stresses and can be harvesting daily (Rizwan et al., 2018; Tang et al., 2020). Additionally, various microalgal species have demonstrated the ability to thrive in municipal and/or industrial wastewaters, effectively eliminating organic carbon, nitrogen, and phosphorus, while fixing CO_2. Moreover, many industries used microalgal feedstocks to get high-value co-products from the biomass such as antioxidants, lipids, vitamins, pigments, or carbohydrates besides biofuel to improve the economics (Sudhakar et al., 2019; Nayana et al., 2022).

Microalgal growth depends on both chemical and physical factors such as the type and concentration of carbon sources and minerals present in the medium, light intensity and regime (dark/light), pH, agitation, or temperature (Singh et al., 2015). For instance, a shortage in nitrogen or phosphorus alters the biochemical composition of the microalgae but also causes a drop in the growth rate (Procházková et al., 2014). Similarly, physical parameters affect the biomass production depending on the microbial species (Daneshvar et al., 2021; Elisabeth et al., 2021; Khanra et al., 2021). The ability of microalgae to acclimate to demanding wastewater conditions and endure oxidative stress particular to these environments differ among species. However, minimizing the cost of biomass production must be considered, and therefore, an equilibrium between growth and the use of cheap media must be reached. This strategy allows both i) wastewater remediation by recovering nutrients and removing pollutants from the environment and ii) the use of the biomass produced for different applications such as biofertilizers, bioplastics, cosmetics or ingredients in functional foods and feeds (Ferreira et al., 2017; Posadas et al., 2017; Ferreira et al., 2018; García et al., 2018; Ferreira et al., 2019; Viegas et al., 2021).

The utilization of biofertilizers and biostimulants as a natural product is particularly crucial to avoid the use of chemicals that may lead to environmental contamination, namely in soil, water and affect the quality of the food produced. Numerous efforts are being made to expand the application of these natural biostimulants (Navarro-López et al., 2020; González-Pérez et al., 2021; Sánchez-Quintero et al., 2023) under strict legislations and regulations that depends on the continent (Su et al., 2023). The use of microalgae as biostimulants has acquired importance for their role in the sustainability and circular bieconomy agenda (Ajeng et al., 2022; Sánchez-Quintero et al., 2023). This is because they are capable of sequestering CO_2, they can survive in challenging environments such as waste effluents and and their easier cultivation compared to macroalgae (Sánchez-Quintero et al., 2023).

The genus *Coelastrella* (Chlorophyta phylum, Sphaeropleales order, Scenedesmaceae family) are green microalga with reported applications for bioremediation and value-added products such as UV-protective compounds among others (Zaytseva et al., 2021). *Coelastrella* is also a better renewable energy resource feedstock with a total of 18% of their biomass made up of lipids beneficial for biodiesel conversion (Nayana et al., 2022). This genus is mainly unicellular, ellipsoidal cells with

80   a peculiar apical wart-like wall thickenings (John, 2002; Wang et al., 2019; Goecke et al.,
81   2020; Maltsev et al., 2021). It can be often found in subaerial and terrestrial habitats, and
82   it is universally distributed from the arctic boreal zone to tropical zones (Nayana et al.,
83   2022). The strains isolated from extremophilic environments display unique properties
84   for biotechnological applications as bioprospection of extremophiles have discovered
85   strains with high resistance to various stresses such as withstanding extreme dehydration,
86   salt stress, and high light exposure. Some examples are: *Coelastrella thermophila* var.
87   globulina isolated from an algerian hot spring produces n-6 and n-3 polyunsaturated fatty
88   acid of commercial interest (Boutarfa et al., 2022); *Coelastrella terrestris* collected from
89   red mucilage in a glacier foreland in Iceland is proposed for biotechnological
90   adonixanthin production (Doppler et al., 2022); a *Coelastrella* sp. isolated from an
91   ammonia-rich environment could process piggery wastewater while using its biomass for
92   other purposes such as biodiesel (Lee et al., 2021).

93   One of these extreme environments of interest are solar panels, an extreme habitat
94   subjected to different stresses, such as high irradiation, temperature fluctuations, and
95   desiccation (Dorado-Morales et al., 2016; Porcar et al., 2018; Tanner et al., 2018). The
96   present work describes the identification and characterization of a *Coelastrella* sp. D14
97   strain from an extreme environment, a solar panel. The biotechnological potential of this
98   novel strain was evaluated: D14 resists long periods of desiccation, it can grow on cheap
99   sources such as piggery wastewaters (PWW), and for the first time this work reports that
100   a *Coelastrella* xerotolerant strain can be used as a biostimulant.

101

2. Material and Methods

2.1. Strain isolation and culture conditions

104   The microalga used in this study, *Coelastrella* sp. D14, was isolated from a solar panel in
105   Valencia (Spain) (Baldanta et al., 2023). *Coelastrella* sp. D14 was grown in BG11
106   medium on 1.5% agar plates or liquid medium at 30°C $\pm$ 2 °C under 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ of
107   continuous white light, under orbital shaking (150 rpm). The BG11 medium contained
108   1.5 g/L $NaNO_3$; 0.02 g/L Na_2CO_3; 0.03 g/L K_2HPO_4; 0.075 $MgSO_4$ * 7 H_2O; 0.036 g/L
109   $CaCl_2$ * 2 H_2O; 1 g/L Na_2EDTA * 2 H_2O; 1.81 g/L $MnCl_2$* 4 H_2O; 0.05 g/L $CoCl_2$* 6
110   H_2O; 0.039 g/L Na_2MoO_4*H_2O; 0.08 g/L $CuSO_4$* 5 H_2O; 0.22 g/L $ZnSO_4$ * 7 H_2O; 2.86
111   g/L H_3BO_3; 6 g/L citric acid and 6 g/L ferric ammonium citrate (PhytotechLabs) (Rippka
112   et al., 1979). BG11 medium was buffered to pH 7.5 with 10 mM HEPES. The cultured
113   algal cells were observed and photographed under a microscope (Leica, model DM750).
114   Axenic strains were stored at -80°C in BG11 medium supplemented with 5% (v/v)
115   DMSO.

116   Cultures of *Coelastrella* sp. D14 were harvested at different growth phases and
117   preparations of these cultures were photographed and then processed by the LAS V4.2
118   software. ImageJ software was used to measure the cell size.

2.2. Molecular identification and Phylogenetic analysis

120   *Coelastrella* sp. D14 was previously identified using primers for 18rRNA
121   amplification (18S-Fw 5'-GTCAGAGGTGAAATTCTTGGATTTA-3', 18S-Rv 5'-
122   AGGGCAGGGACGTAATCAACG-3') (Baldanta et al., 2023). The 18S rRNA gene
123   sequence of *Coelastrella* sp. D14 (PP158241) was searched against homology sequences
124   in Genbank using BLAST (http://blast.ncbi.nlm.nih.gov). 18S rRNA gene sequence from
125   the identified *Coelastrella* sp. D14 and the top BLAST sequences were used for
126   phylogenetic analysis. Multiple sequence alignments were performed using MUSCLE
127   algorithm in MEGA-X. A neighbor-joining phylogenetic tree was built with the aligned
128   sequences based on the K2+G+I model with a bootstrap analysis involving 1000
129   resampling trees using MEGA-X package. *Dunaliella salina* 18S rRNA was used as
130   outgroup.

131

2.3. Autotrophic growth

133   Axenic *Coelastrella* sp. D14 was inoculated into 20 mL of BG11 medium in 100 mL
134   Erlenmeyer flasks to an initial optical density at 750 nm (OD_{750nm}) of 0.05 and grown at
135   different salinity conditions, pH, or nitrogen sources. To assess growth at different salt
136   concentrations, BG11 medium was prepared containing 0.1, 0.25, 0.5, or 1 M of NaCl.
137   The influence of pH on cyanobacterial growth was explored in BG11 buffered to pH 4,
138   6.5, 9, and 11 with 10 mM Tris adjusted to each pH. To examine the strains for growth
139   on different nitrogen sources, BG11 was modified by replacing the 16 mM of $NaNO_3$
140   with 16 mM of NH_4Cl or urea. Tolerance to urea was determined by adding this
141   compound to final concentrations of 8 and 16 mM to BG11. For temperature experiment
142   tests, 100 mL Erlenmeyer flasks with an initial OD_{750nm} of 0.20 were used. As a control,
143   strains grown under routine conditions (BG11 pH 7.5, 30°C, 150 rpm, and continuous
144   light 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) were used. The temperature effect on growth was evaluated at 4, 40,
145   and 50°C, using 30°C as control keeping the other conditions constant. Cell growth was
146   monitored by measuring the OD_{750nm} for a 10-day period. In all the growth experiments,
147   three biological replicates were performed. To define the relationship between cell
148   density per unit OD at 750 nm wavelength, a hemocytometer was used to count the cells.
149   Growth rate was determined by plotting the log OD versus time and calculating the slope
150   in the linear portion, related to the exponential growth. The beginning of the growth phase
151   was considered when the growth of the cyanobacteria was appreciable. Doubling time
152   corresponds to the log2/r. In addition, the biomass dry weight and the ash free dry weight
153   (AFDW) were determined through gravimetry by drying the samples at 105°C overnight
154   and incinerating at 550°C for 1 h, respectively.

155

2.4. Heterotrophic and Mixotrophic Growth

157   First, to assess the heterotrophic growth of *Coelastrella* sp. D14, BG11 agar plates
158   were prepared at final concentrations of 10 mM with different carbon sources: glucose,
159   sucrose, lactose, arabinose, maltose, fructose, galactose, mannose, and glycerol. The tests

160 were performed with spots of 10 µL at OD_{750nm}=1 onto BG11 plates to reduce the
161 possibility of contamination. Plates were incubated at 30°C in darkness for 30 days.
162 Furthermore, the photosynthesis inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-
163 dimethylurea) was added for a final concentration of 10 µM to make sure that the
164 observed growth was heterotrophic. The cell growth was evaluated by checking the
165 appearance of colonies after the incubation period.

166   Once the sugars were determined for *Coelastrella* sp. D14 cultivation, the
167 heterotrophic and mixotrophic growth were evaluated in liquid medium. Axenic
168 *Coelastrella* sp. D14 was inoculated into 20 mL of BG11 medium in 100 mL Erlenmeyer
169 flasks to an initial optical density at 750 nm (OD_{750nm}) of 0.3 and grown in light conditions
170 with no sugar, and glucose or mannose at 10 mM (mixotrophic growth). In parallel, the
171 same conditions were used adding the photosynthesis inhibitor DCMU at 10 µM
172 (heterotrophic growth). Cell growth was monitored by measuring the OD_{750nm} for a 7-day
173 period. In all the growth experiments, three biological replicates were performed.
174 Cultures were checked to ensure that they were free of contaminant bacteria before the
175 experiments.

2.5. Desiccation-Tolerance Test

177 Microalgae strain was grown on 9–10 mL of BG11 agar plates (6 cm diameter) under
178 continuous light (60-80 µE·m^{-2}·s^{-1}) at 30°C for 2 weeks at 30–35% relative humidity.
179 Then, plates were left to be air-dried under routine growth conditions by removing the
180 parafilm from the Petri dishes. After about 15 days, dried cultures were stored in the
181 laboratory bench at room temperature for 3 months, 7 months, and 1 year. For the 1-year
182 dried samples, some samples were maintained in parallel under routine growth
183 conditions. For rehydration, the dried samples were soaked with 1 mL of sterile water for
184 15 min at room light, streaked on BG11 plates, and incubated under the same initial
185 conditions (60–80 µE·m^{-2}·s^{-1} and 30°C). Results were observed after 2–3 weeks. As a
186 negative control for desiccation tolerance, *Synechocystis* sp. PCC 6803 was used.

187

2.6. Wastewater treatment and biomass production

2.6.1. Wastewater characterization

190 The piggery wastewater (PWW) was collected from a stabilization pond in a local pig
191 farm from Valorgado in Herdade do Pessegueiro (39°00009.000 N, 8°38045.500 W)
192 (Glória do Ribatejo, Portugal). This effluent corresponds to the liquid fraction of pig
193 slurry after separation (sieve 1-10mm) from solid manure. The nutrient composition of
194 PWW was determined by standard methods. The Kjeldahl nitrogen (TKN) was
195 determined by a modified Kjeldahl method adapted from the standard method 4500-Norg
196 B (Clesceri et al., 1988). Ammonium nitrogen was quantified by titration after a
197 distillation step based on standard methods 4500-NH 3 B and C (Clesceri et al., 1988). A
198 commercial kit was used for the measurement of phosphorus (Phosver 3-Powder Pillows,
199 Cat. 2125-99, HACH) using a HACH DR/2010 spectrophotometer, at 890 nm. COD

200 determination was carried out according to the open reflux method—Method 5220-B.
201 The effluent composition is shown in Table 1.

202 **Table 1.** Composition of piggery wastewater: pH, total Kjeldahl nitrogen (TKN),
203 ammonia (NH_4^+), phosphate (PO_4^{3-}), and chemical oxygen demand (COD).

pH	TKN (mg N/L)	NH_4^+ (mg/L)	PO_4^{3-} (mg/L)	COD (mg O_2/L)
7.72	1333±6	1281±1.4	218±5	4396±94

204

2.6.2. Microalga screening

206 A *screening* was carried out to determine if D14 was able to grow in PWW. 50 mL flasks
207 were inoculated using 20 mL with different dilutions (1:20, 1:10, 1:5, and 1:2.5) of PWW
208 with tap water as the cultivation medium and were kept at room temperature, under at
209 light intensity of 41 $\mu E \cdot m^{-2} \cdot s^{-1}$, and orbital agitation at 150 rpm in an incubator shaker
210 (New Brunswick Scientific Co, USA).

211

2.6.3. Biomass production

213 To obtain biomass, the microalga cultures were cultivated in 1 L bubble columns
214 photobioreactors (PBRs) using 1:20 (PWW) or BG11 as medium. The cultures were
215 maintained at room temperature (23-25°C) under continuous illumination at an average
216 light intensity of 60 $\mu E \cdot m^{-2} \cdot s^{-1}$. The aeration was supplied at 0.15 vvm (air volume (L)
217 per volume of culture medium (L) per minute (m) from aquarium pumps.

218 The microalga cultures were cultivated in 5 L bubble columns photobioreactors (PBRs)
219 using the same 1:20 and 1:10 PWW as medium, at a working volume of 1 L. The cultures
220 were at room temperature (23-25°C) under continuous illumination (3 fluorescent lamps
221 of 36 W and 6 of 18 W, Philips TL-D) at an average light intensity of 53 $\mu E \cdot m^{-2} \cdot s^{-1}$. The
222 aeration was supplied at 0.15 vvm (air volume (L) per volume of culture medium (L) per
223 minute (m) using aquarium pumps. After 9 days of cultivation the biomass was collected
224 by centrifugation (10.000×g, 10 min).

225

2.7. Biomass characterization

227 The biochemical composition of the microalgal biomass was determined in terms of
228 proteins, carbohydrates, lipids, moisture, and ash. All analyses were performed in
229 triplicate. The protein content was determined following the method described by
230 (González López et al., 2010), which is a modification of the Lowry method, with BSA
231 (Bovine serum albumin) as the standard. The sugar content was determined by the phenol-
232 sulfuric method (Dubois et al., 1956) after quantitative acid hydrolysis (Hoebler et al.,
233 1989) of the biomass. A calibration curve was prepared using standard glucose solutions.
234 Lipid content was determined gravimetrically after Soxhlet extraction with n-hexane
235 during 6h, using biomass previously submitted to bead milling (Retsch MM400, 25 Hz
236 for 3 min and 50 seconds).

2.8. Germination Index

The biostimulant activity of the microalga *Coestrella* sp. D14 was determined by measuring the germination index of seeds of *Lepidium sativum*, according to the method described by (Zucconi et al., 1981).

Microalga culture (whole biomass) and extracts obtained from the growth at different conditions (BG11, 5%, 10%, and 20% PWW) were tested at different concentrations (0.1, 0.5, 1, and 2 g/L). Microalga extracts were prepared by submitting the harvested biomass to high-pressure homogenization (1200 bar for 1 cycle) to disrupt the cells (Ferreira et al., 2022). Treatment solutions with microalga culture and extracts were then prepared with distilled water to the desired concentrations. A total of 32 treatments were tested (Fig. 1).

The germination experiments were carried out in sterilized rectangular Petri dishes (10 mm x 17 mm) with Whatman No 5 filter papers wetted with 7 mL of each treatment solution, with 10 seeds per dish in duplicates. Distilled water was used as the negative control. All samples were incubated at room temperature (25 ºC) in the dark for 3 days and the Petri dishes in a vertical position. At the end of 3 days, the seedlings were photographed and measured with the program ImageJ (Rasband, 1997). Results were registered for comparison between the microalga treatments and the control with distilled water.
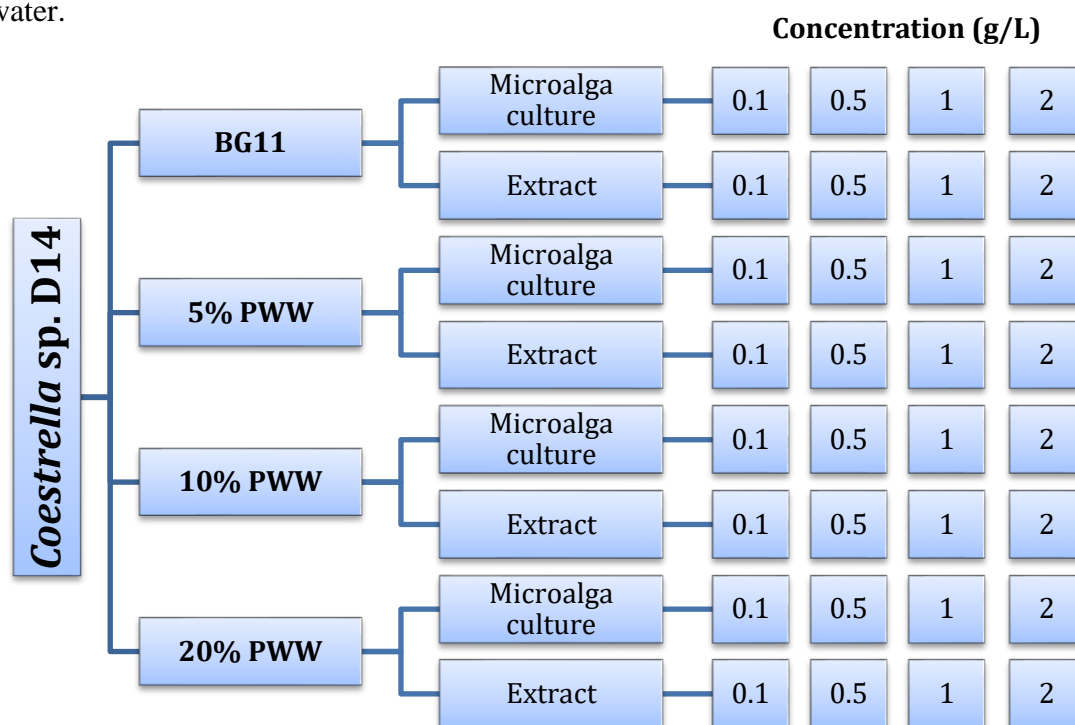
Fig. 1. Schematic diagram of the treatments of *Coestrella* sp. D14 tested in the germination trials. Different growth media (BG11, 5, 10, and 20% PWW), different biomass processing (microalga culture and extract from disrupted biomass), and different treatment concentrations (0.1, 0.5, 1, and 2 g/L) were tested.

Finally, the germination index was determined by the Equation (1), where G and L are the number of germinated seeds and their length in the case of the microalgal cultures and Gw and Lw are the same parameters but in the control (distilled water). The data shown

7

263 in the germination index experiments is, therefore, the result of the measurement of 100
264 seeds for each treatment.

265
$$GI\ (\%) = \frac{G\ x\ L}{G_w\ x\ L_w} x\ 100\ (1)$$

266

2.9. Statistical Analyses

268 One-way Anova with post-hoc Tukey HSD, with Scheffé, Bonferroni and Holm multiple
269 comparison results were calculated in the different conditions studied in this work
270 on Astatsa.com (https://astatsa.com/; Vasavada, 2016). Correlation was considered
271 statistically significant when $p < 0.05$.

272

273 ## 3. Results

274 ### 3.1. Isolation and identification of *Coelastrella* sp. D14

275   Solar panel samples were collected during the summertime of 2013 and 2014 for
276 screening cyanobacteria and microalgae. The first isolation was made in Castenholz-D
277 medium (Baldanta et al., 2023) and afterwards, microalgae and cyanobacteria were
278 maintained growing on BG11. At this time, a consortium among microorganisms,
279 bacteria and cyanobacteria/microalgae, was evident on BG11 plates. After several
280 streaks, different strains were isolated (Baldanta et al., 2023). One of these strains, a
281 unicellular green microalga, named D14, was identified by PCR 18S rRNA amplification
282 reaching a homology of 99% with other *Coelastrella* strains.

283   A phylogenetic analysis based on the 18S rRNA gene sequence and a comparison to
284 similar strains in the GenBank database indicated that the D14 had a high similarity with
285 other strain sequences of *Coelastrella* (Fig. 2).
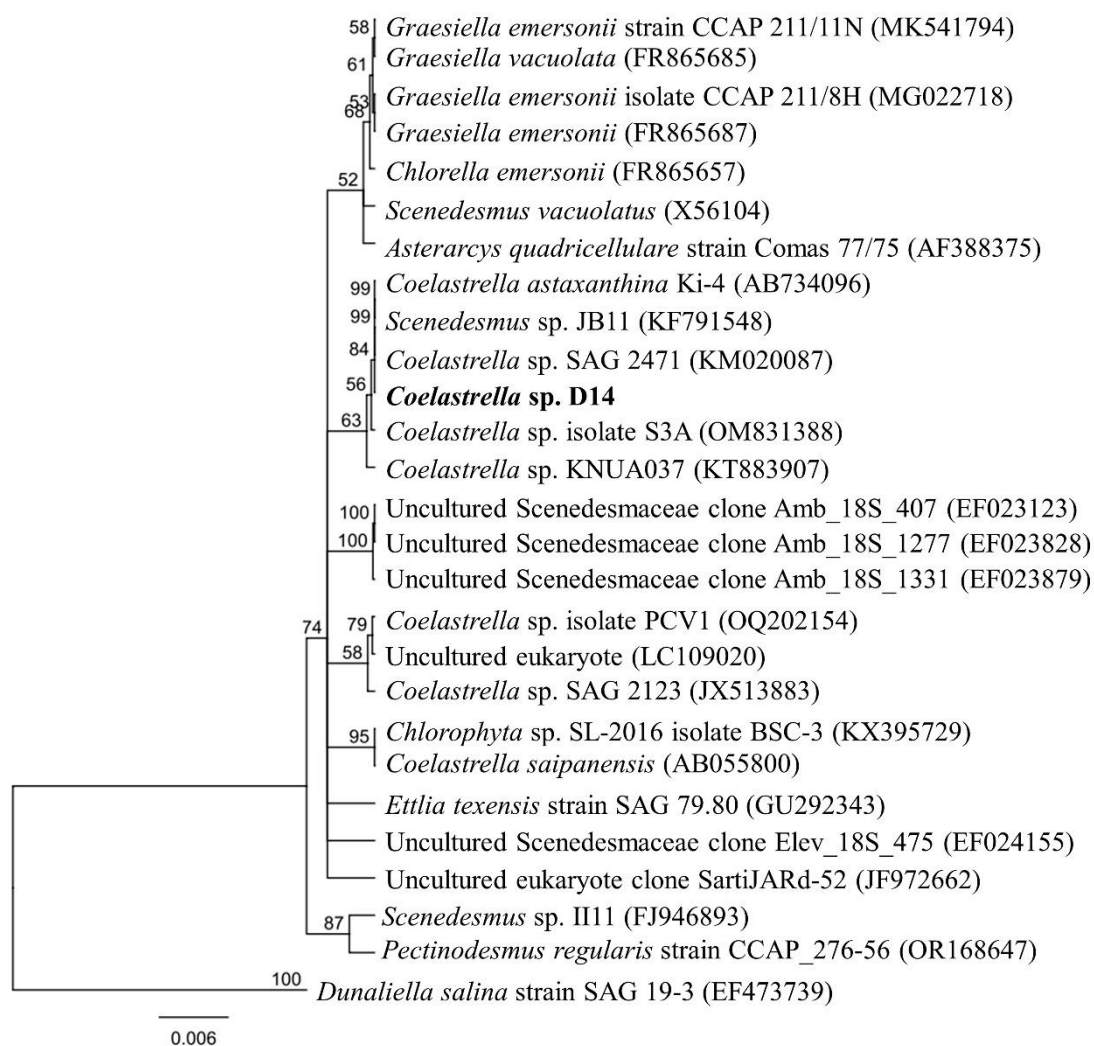
286 **Fig. 2. Phylogenetic tree inferred from 18S rRNA gene sequence from *Coelastrella***
287 **sp. D14 isolated from the solar panel.** The tree was constructed using the neighbor-

288 joining method in MEGA-X. The length of the scale bar indicates 0.006 substitutions per
289 site. The percentages of bootstrap support of branches (>50%) are indicated at each node.
290 The numbers in the parenthesis are the accession numbers of respective 18S rRNA gene
291 sequences obtained from Genbank. *Coelastrella* sp. D14 is shown in bold.

292

293 Streaked microalgal colonies on agar plate, and microscopic observation is shown in
294 Fig. 3 (A-H). Light microscopical observations showed that *Coelastrella* sp. D14 was
295 unicellular green coccoid microalgae. The cells showed usually as single oval cells, but a
296 large degree of variation in cell sizes was observed ranging between 4.2 to 14.8 µm, with
297 a mean diameter of 8.68 ± 1.96 µm. Single cells are smaller, have a lemon-shaped after
298 division, have a thin wall, and the pyrenoid is clearly noted. Also, in some of them, wart-
299 like wall thickenings are observed (Fig. 3E). As the culture grew, the cells appeared round
300 shaped and formed small groups of 2-6 cells. Fig. 3G shows 2-3 daughter cells after cell
301 division with the cell wall of the mother cell surrounding the new cells. In mature cells,
302 the chloroplast is dissected into blades.

303
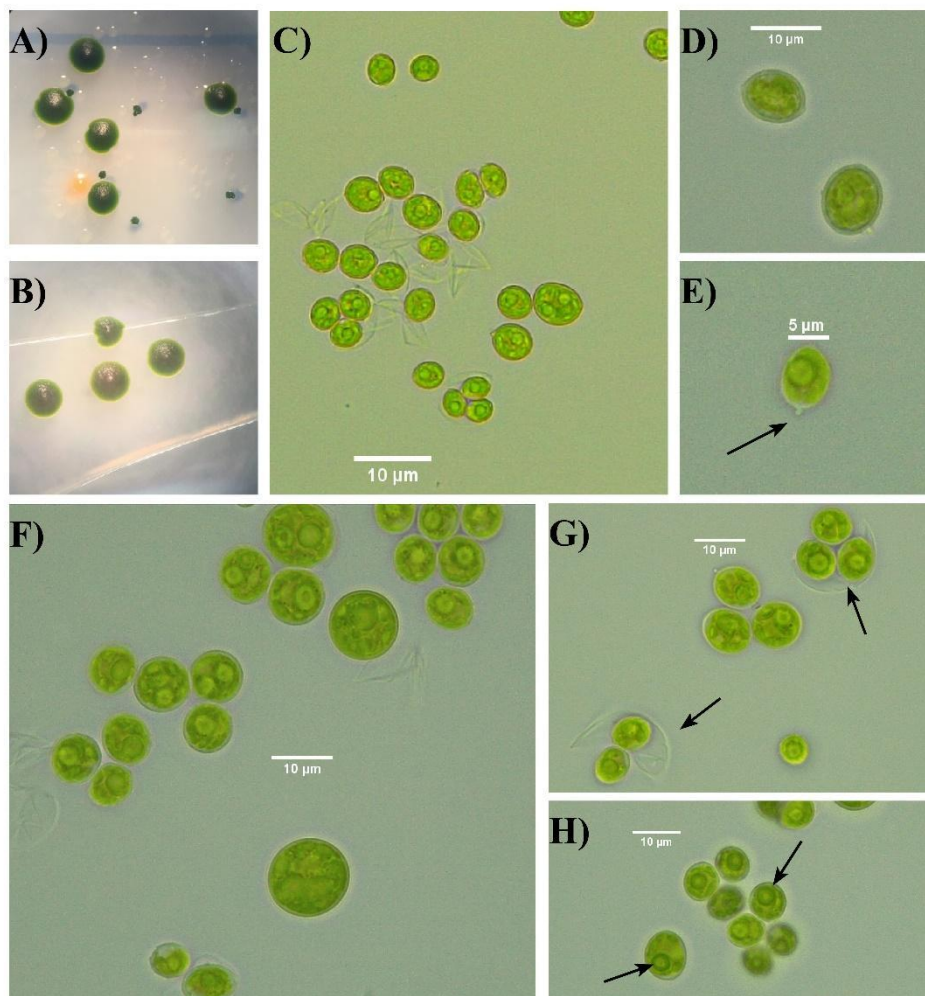
304 **Fig. 3. Isolation of *Coelastrella* sp. D14. A)** Streak colonies from the original
305 consortium microalgae-bacteria. **B)** Streak colonies from the axenic microalgae. **D-H)**

10

306   Bright-field photomicrographs of *Coelastrella* sp. D14 on different days of culture. Scale

307   bar is shown in all the pictures. D and E, lemon-shaped cells, arrow indicates a wart-like

308   wall thickening. F, mature and round-shaped cells. G and H, cells after division. In G

309   arrows indicate the cell wall of the mother cell and in H, the pyrenoids are pointed.

310

3.2. Autotrophic growth conditions for *Coelastrella s*p. D14

312   The growth evolution of *Coelastrella* sp. D14 in BG11 medium (pH 7.5) within 10

313   days at 30ºC under 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ were studied (Fig. 4, Table S1). A mild lag phase of

314   two days was observed and then, the microalgae reached 2.60 ± 0.01 days of doubling

315   time. The calculated correlation between the OD_{750nm} and cells per mL was: Nºcells/mL

316    $= 3.191 * 10^6$ OD_{750} ($R^2 = 0.99$). In addition, the correlation of the biomass dry weight

317   with OD_{750nm} was determined through gravimetry (Dry weight=0.5314* OD_{750}-0.0127;

318    $R^2 = 0.99$). After 10 days of growth in BG11, a total of 2.53 g/L of *Coelastrella* sp. D14

319   biomass was obtained.

320
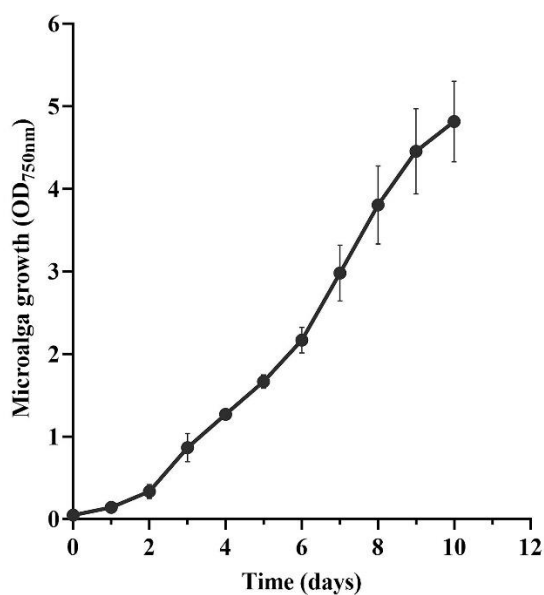
321

322   **Fig. 4. Growth curve of *Coelastrella* sp. D14 in BG11.** Average OD_{750nm} of three

323   biological replicates together with the standard deviation.

324

325   Regarding salt stress, *Coelastrella* sp. D14 grew up to 0.5 M of NaCl (Fig. 5A). The

326   growth was slightly affected at 0.1 M and 0.25 M of NaCl, as the statistical analysis

327   revealed (Table S1). At 0.5 M of NaCl, the lag phase was 3 days longer and the maximal

328   OD was lower compared to control. However, the doubling time was comparable to the

329   control. No growth was observed at 1 M NaCl.

330

11
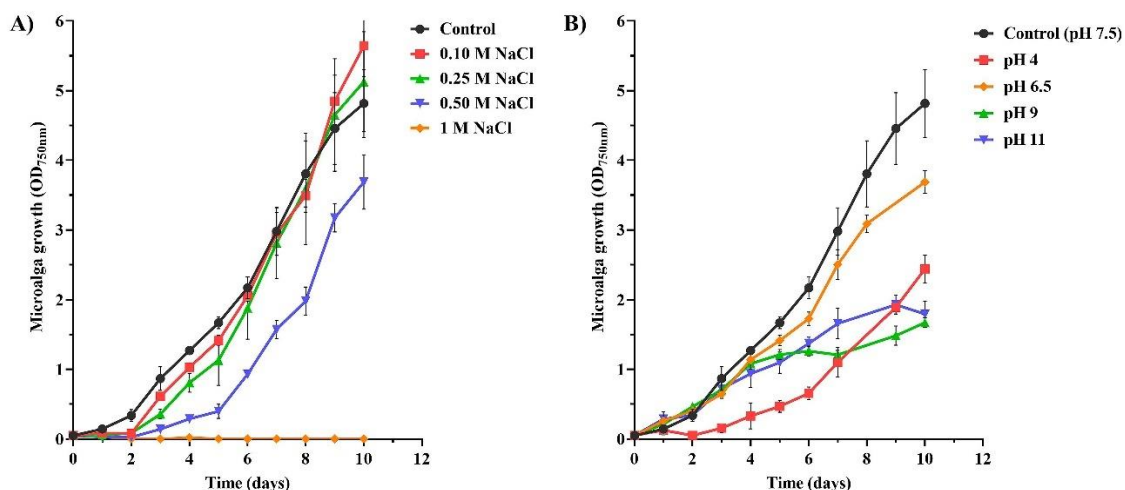
Fig. 5. Growth characterization of *Coelastrella* sp. D14 in different conditions. **A)** Effect of NaCl concentration on microalgae growth. As a control, growth in BG11 medium was used. **B)** Effect of pH on microalga growth. BG11 buffered with 10mM Tris pH 4, pH 6.5, pH 9, and pH 11 was used instead of 10 mM HEPES at pH 7.5 (control). In all cases, the graphs show the average OD_{750nm} of three biological replicates together with the standard deviation (n=3).

The best pH to grow *Coelastrella* sp. D14 was 7.5 (control) as it is shown in Fig. 5B. non-statistical differences were observed in the doubling time for pH 6.5 when compared to the control; however, a lower growth was reached after 10 days. Interestingly, *Coelastrella* sp. D14 grown at pH 4 despite having an extended lag phase, the doubling time was similar to that calculated for pH 7.5, after 5 days of grown. On the other hand, the microalga was able to tolerate a pH 9 and 11, with a statistically significant higher doubling time and reaching a lower OD than the control (5.91 and 3.39 days, respectively vs. 2.57 days at pH 7.5, Table S1). The results demonstrate that *Coelastrella* sp. D14 is pH broadly resistant (from 4 to 11). More specifically, in those cultures, the final pH was ~6.3 and ~9.5, respectively and the cultures remained green after the experiment (Fig. S1).

Temperature greatly affected the growth of *Coelastrella* sp. D14 (Fig. S2). No growth was observed at 50ºC, and the cultures were bleached on the second day of the experiment. At 4 and 40ºC, we had a small increase in the microalga growth, and the cultures remained pale green, indicating they were highly stressed.

The effect of the absence or the use of other nitrogen sources at 16 mM (the same concentration as $NaNO_3$ is used in BG11) for the cell growth was assessed in liquid cultures. Fig. 6A shows the growth of the microalga for 10 days using alternative nitrogen sources, such as urea or ammonium. *Coelastrella* sp. D14 was not able to grow without any nitrogen source, which may be because they lacked the ability to fix atmospheric nitrogen in the conditions tested. Growth in the presence of ammonium was also closer to the result observed in $BG11_0$, despite an initial increase of the OD after 6 days. However, even though the microalgae displayed the best growth with nitrate, it seems

12

362 that urea could also be used as a nitrogen source, with no statistical differences and with
363 a similar doubling time (2.52 days) compared to the control but with a longer lag phase
364 (4 days instead of 2 days).

365
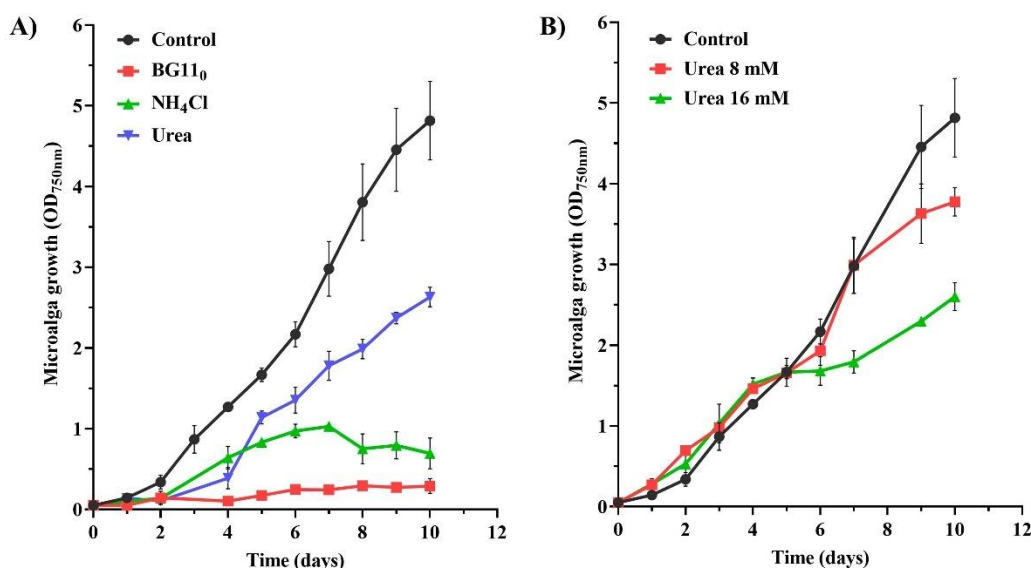
Fig. 6. Effect of nitrogen source on 10 days growth of *Coelastrella* sp. D14. A)
NaNO$_3$ in BG11 was substituted for urea or NH$_4$Cl (16 mM). Alternatively, no nitrogen
was added (BG11$_0$). **B)** *Coelastrella* sp. D14 tolerance to urea. Urea at 8 and 16 mM was
added to BG11 complete medium. As a control, strains grown under routine conditions
(BG11 pH 7.5, 30°C, 150 rpm, and continuous light 100 μE·m^{-2}·s^{-1}) were used. The
graphs show the average OD$_{750nm}$ of three biological replicates together with the standard
deviation (n=3).

373

Considering that urea was the best nitrogen source for growing the microalga (Fig. 6A)
and can be abundant in different types of wastewaters, various concentrations of urea
were tested to grow *Coelastrella* sp. D14 (Fig. 6B). The microalga was sensitive to 16
mM urea, while 8 mM allowed a better growth, although lower than the control after 10
days of growth. These results show that even though urea cannot replace NaNO$_3$ as a
nitrogen source, some strains can tolerate it.

380

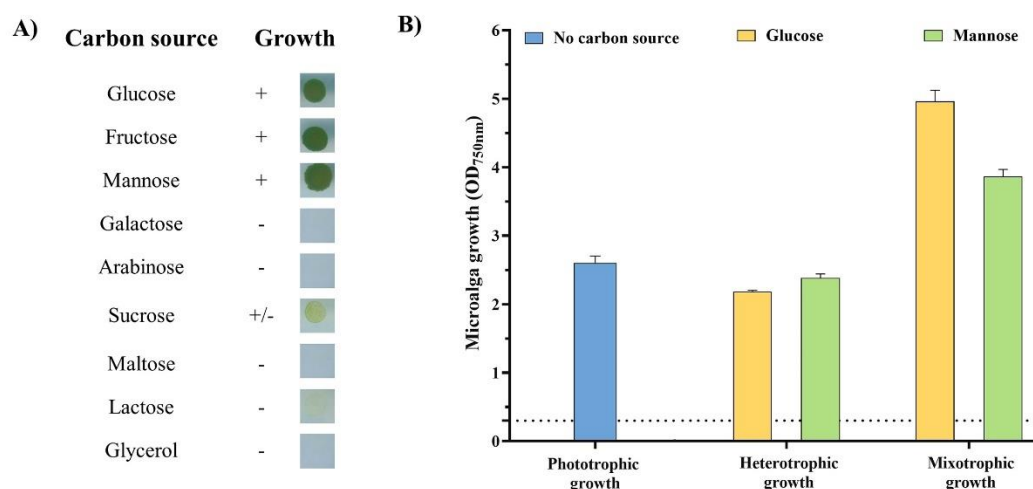### 3.3. Heterotrophic and Mixotrophic Growth

The growth of *Coelastrella* sp. D14 was tested heterotrophically, as this strategy is
widely used to increase microalgae biomass. First, it was valuated growth using glycerol
and eight different sugars at 10 mM in BG11 agarized medium, complete darkness and
with the photosynthesis inhibitor DCMU at 10 μM final concentration, for 30 days. The
microalga was able to use glucose, fructose, and mannose as carbon source. *Coelastrella*
sp. D14 showed a weak growth using sucrose but could not grow on maltose or lactose
(Fig. 7A). Erlenmeyer flask experiments were done to find accelerated mixotrophic

389　biomass growth compared to an autotrophic condition. The investigated C-sources were
390　glucose and mannose as they contain the same amount of carbon (both are hexoses). In
391　parallel, as control, heterotrophic growth was performed by adding DCMU to cultures
392　with carbon source and light, to ensure the growth was due to the consumption of the
393　sugar. Cultures without DCMU and carbon source represent the phototrophic growth.

394　　As it is shown in Fig. 7B, the culture supplemented with glucose reached the highest
395　OD_{750nm} compared to mannose (mixotrophic) and phototrophic growth. No differences
396　were observed between the autotrophic control and the heterotrophic growth (DCMU
397　condition), for both carbon sources.

398

399

Fig. 7. Growth of *Coelastrella* sp. D14 using different carbon sources. A)
Heterotrophic growth of *Coelastrella* sp. D14 in agar BG11. Microalga was grown to
$OD_{750nm} = 1$, 10 µL were plated and let the microalga grow for a month. **B)** Evaluation of
phototrophic, heterotrophic and mixotrophic growth in *Coelastrella* sp. D14. For
heterotrophic growth, the photosynthesis inhibitor DCMU was used at 10 µM
concentration. Sugars were added at 10 mM. The circle crossed indicates that no sugar
was added. OD_{750nm} was measured after 5 days of growth from an initial OD_{750nm} of 0.3
(dashed line). In all cases, the graphs show the average OD_{750nm} of three biological
replicates together with the standard deviation (n=3).

409

3.4. Resistance of *Coelastrella* sp. D14 to desiccation

411　　Considering that the microalga has been isolated from a solar panel, it was of interest
412　to study its capacity to resist desiccation, so it was tested the desiccation-tolerance of
413　*Coelastrella* sp. D14 for 3 months, 7 months, and 1 year. As shown in Fig. 8A, in terms
414　of ability to form colonies and grow, the rewetted samples differed little from the non-
415　dried cultivated form, indicating that *Coelastrella* sp. D14 was drought-resistant.
416　*Synechocystis* sp. PCC 6803, which was used as a non-resistant strain, was unable to grow

14

417   after 3 months, the minimum time tested (data not shown). Just after the rewetting, all the

418   cells exhibited the same morphology, with a thick sheath surrounding the cells (Fig. 8D).
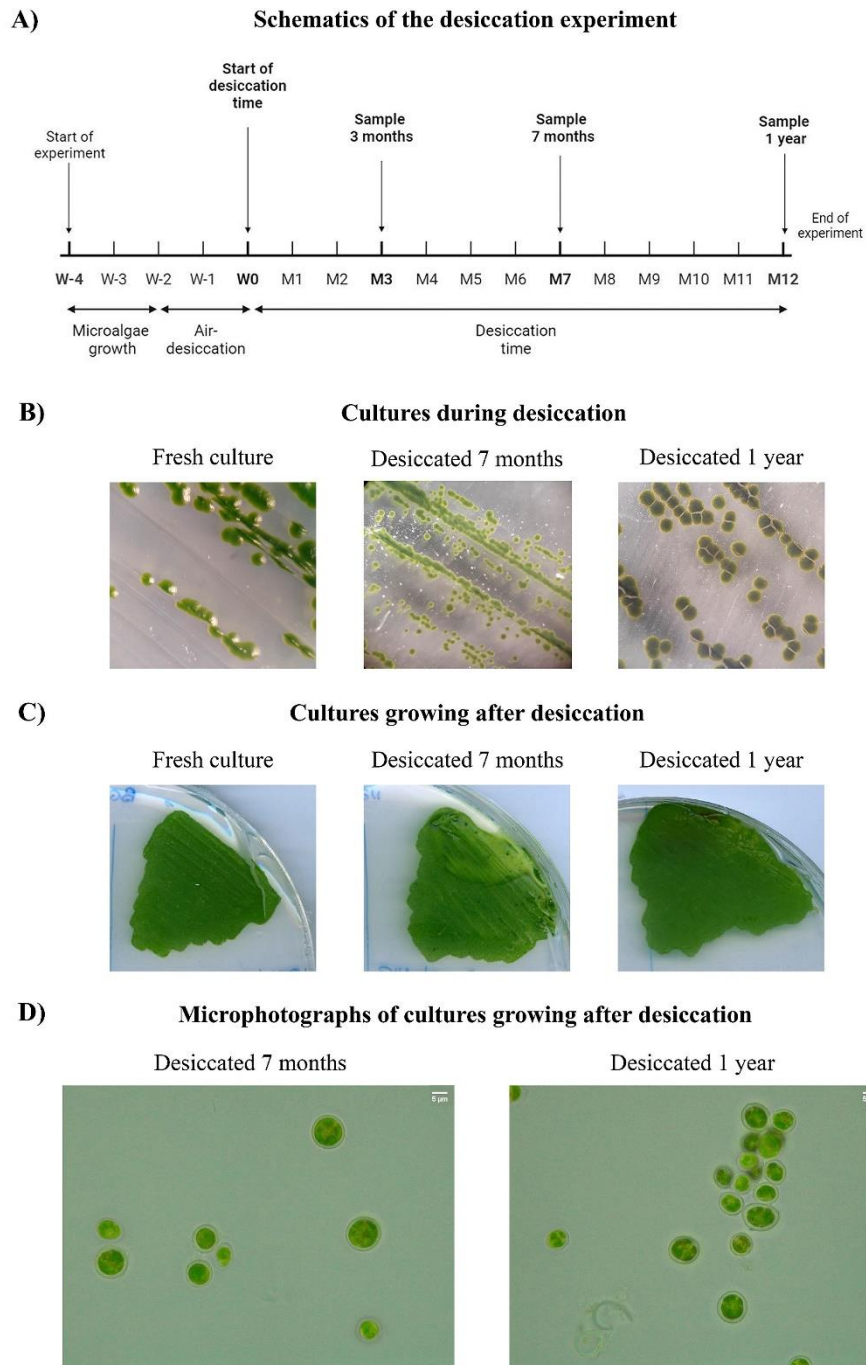
419   **Fig. 8. Long-term desiccation tolerance in *Coelastrella* sp. D14**. **A)** Diagram of the

420   desiccation process. W represents weeks, M represents months. **B)** Microphotographs of

421   *Coelastrella* sp. D14 during the experiment. **C)** Growth observed after 2 weeks on BG11

422   post rewetting for 7-month and 1-year desiccated culture; a freshly streaked strain was

423   included as a control. **D)** Light microscopical observation of *Coelastrella* sp. D14 just

424   after rewetting 7 months and 1 year-desiccated samples.

425

15

3.5. Evaluation of *Coelastrella* sp. D14 for wastewater treatment

After changing carbon and nitrogen sources in BG11 medium, the growth of *Coelastrella* sp. D14 in wastewater was evaluated, more specifically in a piggery effluent. The composition of the piggery effluent used in the trials is shown in Table 1.

Coelastrella sp. D14 was submitted to an initial screening in different PWW concentrations (Fig. 9). Experiments demonstrated that D14 could grow in this effluent at concentrations up to 30% (v/v). However, the initial lag phase becomes longer with increasing PWW concentration. At 40% PWW, no visible growth was observed during the 15 days of the experiment. Native microorganisms could be found in the medium and co-cultivated with the inoculated D14. Nevertheless, in all experiments the inoculated microalga rapidly outcompeted other organisms present in the wastewater. Thus, their impact on growth and biomass concentration was considered insignificant.
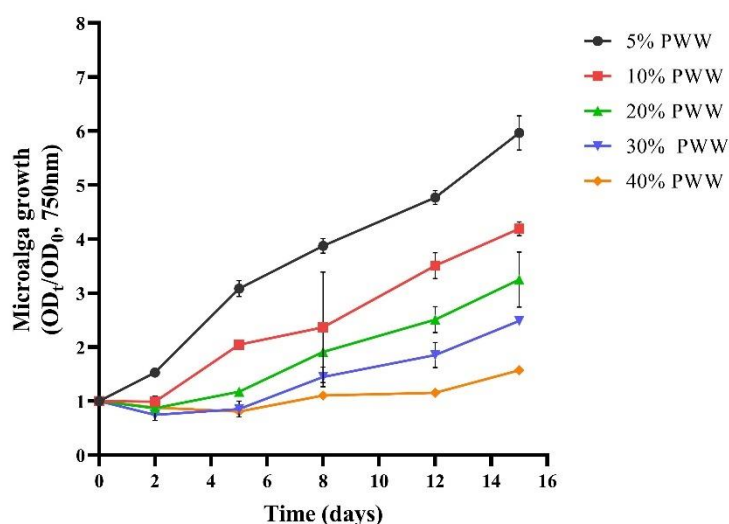

Fig. 9. Growth of *Coestrella* sp. D14 at different concentrations of piggery wastewater (5, 10, 20, 30, and 40% v/v). In all cases, the graphs show the normalized value of the average OD_{750nm} together with the standard deviation (n=2).

Coelastrella sp. D14 grown in synthetic medium (BG11) presents a high amount of carbohydrates (37.8%), followed by 23.2% of protein, 13.9% lipids, and 7.1% ash. When grown in PWW, it has a similar composition profile for biomass grown in 5 and 10% PWW (30-34% carbohydrates, ~21% protein, and 14-16% lipids).

### 3.6. Effect of *Coelastrella* sp. D14 as biostimulant for seed germination

Plant growth is affected by phytohormones, amino acids and polysaccharides, along with other nutrients, available from various sources, including microalgae. Here, the effect of *Coelastrella* sp. D14 biomass was evaluated on germination of cress seeds

16

462   (*Lepidium sativum*). In the biostimulant assay, a germination index (GI) of 100% was
463   attributed to distilled water (control). Values higher than the control were considered to
464   have a biostimulant activity (Fig. 10). The highest GI values obtained were 128%
465   corresponding to non-disrupted biomass trials at 1 or 2 g/L on BG11 medium and 132%
466   at 1g/L culture on 5% PWW. Cell disruption causes a significant drop, for instance, at 2
467   g/L of D14 grown on BG11, yield dropped a 45% of the GI value with respect to the
468   whole biomass.

469   This tendency is not seen when grown on 20% PWW. In this case, there are not so many
470   differences between both culture and extracts GI values, not reaching in any case the GI
471   100% value. In general, values under 100% may suggest that the microalga concentration
472   or their biochemical composition might be excessive or toxic for *L. sativum*, negatively
473   affecting their growth (Navarro-López et al., 2020).

474   Therefore, these results highlighted that 1 g/ L of whole algal suspension grown at 5%
475   PWW may be the best treatment for root lengths. However, whole D14 biomass grown at
476   10% PWW and used at 0.1%, also yielded a GI value of approximately 123%. The latter
477   is more interesting since it uses a higher percentage of PWW to grow, lower amount of
478   non-processed biomass, decreasing also downstream costs.

479
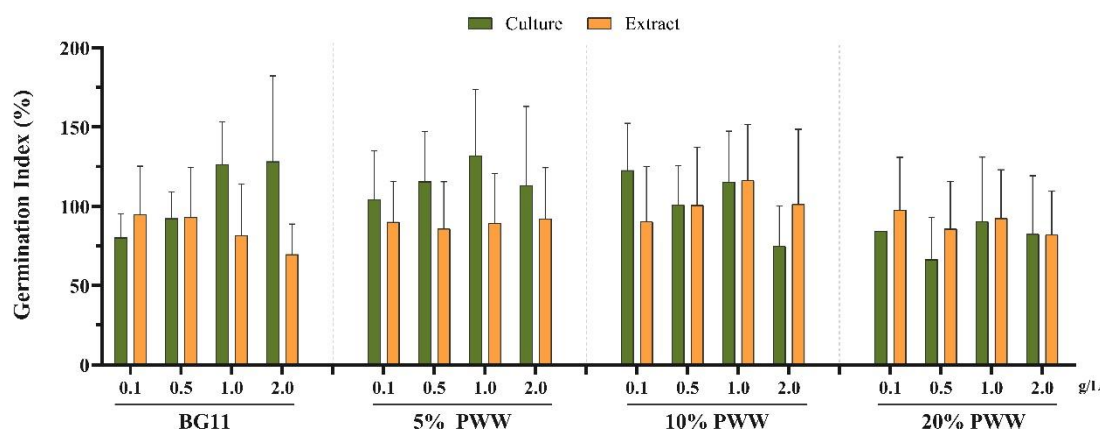
480   **Fig. 10. Germination index (%) of *Lepidium sativum* seeds using of *Coelastrella***
481   **sp. D14**. The graph shows the mean of germination index (GI) value, either using initial
482   biomass or an extract of D14 growth in four different media (BG11 or BG11 containing
483   different concentrations of piggery wastewater, PWW). GI value obtained when using
484   distilled water as medium was considered as control (GI, 100%). Error bars indicate,
485   standard deviation (n = 20).

486

487

4. DISCUSSION

The isolate obtained from the solar panel was identified as a *Coelastrella* sp. based on morphological features and phylogenetic analysis. The morphological features of the studied strain (single oval cells with a mean diameter of 8.68 ± 1.96 μm) match those of the genus *Coelastrella* which is characterized as unicellular and occasionally forming aggregates with vegetative cells spherical to subspherical, from 4.2–14.8 μm in diameter (Shetty et al., 2021; Doppler et al., 2022). The phylogenetic tree was obtained using conventional loci such the nuclear 18S rRNA. This novel strain belongs to the Chlorophyceae class, Sphaeropleales order, Scenedesmaceae family, *Coelastrella* genus. The closest relatives of D14 are *Coelastrella* sp. isolate 3A (*Coelastrella thermophila* var. globulina) (Boutarfa et al., 2022) and the unclassified *Coelastrella* sp. SAG 2471(GenBank: KM020087). The clade to whom D14 belongs contains in general *Coelastrella* strains isolated from harsh environments (Ki4- from a Japanese asphalt surface in midsummer, (Kawasaki et al., 2020); *Scenedesmus* sp. JB11 from an extreme saline-alkali soil, data from NICB; *Coelastrella* sp. isolate 3A isolated from an algerian hot spring, (Boutarfa et al., 2022)) and some of them with remarkable properties such as for producing n-6 and n-3 PUFA fatty acids (Boutarfa et al., 2022) or astaxanthin production (Kawasaki et al., 2020).

In the conditions tested, D14 displayed a doubling time of 2.6 days (Table S1). A novel freshwater *Coelastrella* strain isolated in Belgium, presented a rapid growth in phototrophy, with a doubling-time of 6.8 ± 0.30 h hours at a light intensity of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 5% CO_2 (Corato et al., 2022). The specific growth rate seems to depend on dosing times of the carbonic solution added to the culture unless indoor. This causes an increase on the lipids and proteins content with major carbon source dosing times, while the carbohydrate content decreased, suggesting that the carbon source is a critical parameter for algal growth (Razooki et al., 2019). The doubling time of D14 is given without any extra C source, which could explain the low value obtained and leaves room for improvement by increasing the % of CO_2 or adjusting the amount of light during growth.

D14 growth was assayed under several conditions as microalgal growth and biomass production depends on nutrient availability and amounts, light intensities, pH or temperature value among others. Most microalgae can grow over the range of pH values from 6.8 to 8.0, the suitable pH value depending on the microalgal species (Daneshvar et al., 2021). Concretely, a range from 5.0 to 9.0 is reported for *Coelastrella* KKU-P1 strain (Thepsuthammarat et al., 2023). D14 grown in flasks is viable from a wide range of pH, from 4.0 to 11.0, although the highest production was obtained under a pH of 7.5 (Fig. 5). *Coelastrella* sp. strain D3–1 has been reported to resist from pH 2 to pH 11, but the experiments were performed in different conditions to this work, mainly, after the stress treatment on a diluted medium 0.2XBG11, cells were spotted on BG11 plates and grown for 7 days (Saito et al., 2023) while in this work data was taken in liquid medium with the pH corrected. Regarding the temperature, it greatly affected the growth of *Coelastrella* sp. D14 (Fig. S2) as no growth was observed over 50 ºC. On the contrary, several

530 *Coelastrella* have been reported to be able to grow on 50ºC or over, for instance,
531 *Coelastrella* sp. M60 (Nayana et al., 2022) or *Coelastrella* sp. strain D3–1 (Saito et al.,
532 2023) resisted temperatures of up to 50 °C. However, D14 was able to resist desiccation
533 up to one year (the maximum time tried). There are no similar experiments done with
534 other *Coelastrella*. However, a heat-dry stress done in *Coelastrella* sp. D3–1, in which
535 cell pellet was exposed to 42 ∘C for 3 h in a dryer, showed that the microalga was able to
536 grow when later was spotted on BG11 plates (Saito et al., 2023). The xerotolerance that
537 D14 showed is consistent with its original habitat, a solar panel, in which the water
538 activity is logically low. It is quite possible that other *Coelastrella* isolated from solid
539 environments such that characterized from an asphalt surface (Kawasaki et al., 2020)
540 could display a similar behavior.

541 To enhance the biomass production, the growth of *Coelastrella* sp. D14 was investigated
542 under both heterotrophic and mixotrophic conditions. The microalga was able to grow
543 well heterotrophically using glucose, fructose, and mannose as carbon sources, hardly
544 with sucrose and could not grow on maltose or lactose (Fig. 4A). In mixotrophic
545 conditions, the growth of D14 reached the highest OD_{750nm} when the medium was
546 supplemented with glucose, in comparison to the use of mannose or phototrophic growth.
547 In general, green microalgae can efficiently use glucose and fructose for growing but they
548 usually lack sucrose transporter systems (Pang et al., 2019). However, there are some
549 reports as *Coelastrella* sp. KKU-P1 in which it is capable of sucrose consumption and
550 could be used for growing on unhydrolyzed molasses as a low-cost carbon source that is
551 rich in sugars, mainly sucrose, glucose, and fructose (Thepsuthammarat et al., 2023). D14
552 could be subjected to adaptive laboratory evolution experiments in a future to improve its
553 mild growth on sucrose and in this way, being able to grow on more low-cost substrates.

554 In autotrophic conditions, the absence of nitrogen in the medium, D14 was not able to
555 survive (Fig. 7). This is expected as nitrogen is an essential component for microalgae
556 growth, needed for macromolecules synthesis among other things and *Coelastrella* is not
557 able to fix it, at least in the conditions tested. When $NaNO_3$ from BG11 was replaced by
558 urea or NH_4Cl (16 mM), growth was restored, although it was not as good as using the
559 original $NaNO_3$ of BG11. Ammonium is the preferred nitrogen source for algae since it
560 consumes less energy, as it does not require a redox reaction (Nayana et al., 2022).

561 Furthermore, the use of other cheap sources of nitrogen as urea, is convenient for reducing
562 economical costs of the microalgae growth. Due to the ability of microalgae to grow in
563 very diverse environments, and considering the idea of circular economy, wastewaters
564 that are rich in nutrients can be used as a culture medium. In fact, growing microalgae in
565 wastewater is a suitable alternative to reduce freshwater expenses and valorize residual
566 nutrients (Ahmed et al., 2022; Sánchez-Quintero et al., 2023). (Sharma et al., 2022)
567 enumerates several economic and growth considerations when cultivating microalgae in
568 wastewater pointing to that appropriate strains' selection is crucial for the whole process.
569 Therefore, it is important to count on different strains or consortiums able to bioremediate
570 the effluents for choosing the best one for a certain valorization, for instance, using the
571 produced biomass as plant biostimulant.

One kind of wastewater that causes big concern is the one from piggery industry (Ferreira et al., 2021), which is a complex effluent rich in nutrients, such as ammonia and organic matter. It causes eutrophication and toxicity of freshwater ecosystems while the deep dark color hampering photosynthesis of this medium when discharged into rivers without complete treatment (Li et al., 2019; Ferreira et al., 2021; Lee et al., 2021). On the other hand, when using piggery effluents directly in composting for agriculture, greenhouse gas emissions are generated (such as CO_2 and N_2O) (Mohedano et al., 2019; Hu et al., 2020). For this reason, several attempts have been reported for the biological treatment of raw PWW with microalgae with different approaches. The studies at a pilot scale treating undiluted raw PWW with microalgae, also resulted in an efficient removal of nutrients and an enhancement in the clarity of wastewater (Lee et al., 2022). Another *Coelastrella* sp. isolated from an ammonia-rich environment was used for PWW treatment in a 4-day two-step process: heterotrophic plus mixotrophic steps in a narrow transparent photobioreactor (Lee et al., 2021). In these conditions, *Coelastrella* sp. could remove 99% of ammonia, 92% of chemical oxygen demand (COD), and 100% of phosphorus. In this case, the microalgal biomass was oriented towards the production of biodiesel of high quality (Su et al., 2023). This study showed that D14 can grow on piggery wastewater up to 20%. More studies must be done to evaluate how the microalga biochemical composition could be affected.

In this work, besides assessing the potential of *Coelastrella* sp. D14 to grow in piggery wastewater, it was explored, the use of the resulting biomass to stimulate plant growth. A bioassay was performed based on the germination index of *Lepidium sativum* seeds. The results have shown that D14 has potential as a biostimulant product acting as a gibberellin-like when growing on BG11 at 1-2 g/L or 5% PWW at 1 g/L, yielding GI values up to 132%. Other bioassays in agricultural models, such as examining root formation in mung beans and cucumbers, will be necessary to further demonstrate the auxin-like effect. On the other hand, data obtained with the whole biomass yielded higher GI values with respect to the broken cells. For other strains such as the microalga *Scenedesmus obliquus* a similar behavior has been reported: the highest GIs were obtained with the initial biomass in the absence of any pre-treatment (Navarro-López et al., 2020). For this microalga, grown on brewery effluents, a GI of 139% was obtained.

There are recent strategies of culturing microalgae using wastewater and CO_2 to produce large quantities of biomass at moderate costs while integrating local and circular economy approaches (Sánchez-Quintero et al., 2023). The fact that the microalgal D14 biomass could be used directly as a biostimulant implies a reduction in economic costs and a sustainable application, avoiding synthetic stimulants. Regarding the use of raw piggery wastewater, despite that microalgal treatment of reduces the risk of pathogens by 63% (Lee et al., 2022), recent reports highlight their persistence. To align with EU Regulation 2019/1009 on plant biostimulants, it is recommended to use extracts to mitigate potential pathogen presence (Lee et al., 2022; Sánchez-Quintero et al., 2023). This needs for optimization studies on the preparation of alga extracts, as stated procedures may impact the final bioavailability of microalga compounds. These factors are influenced by the microalga species, cultivation medium, and culture state (Ferreira et al., 2018; Ferreira et

615 al., 2019). More specifically, bioactive compounds reported as biostimulants, such as
616 phytohormones, heteropolysaccharides, amino acids, or vitamins (as reviewed by
617 (Sánchez-Quintero et al., 2023)), are produced in various phases of growth (Tan et al.,
618 2021). This variability could explain why extracts with the same concentration but grown
619 in different media (resulting in distinct growth curves) do not exhibit the same
620 biostimulant behaviour. For this reason, it could be convenient in the next future to have
621 more studies on *Coelastrella* sp. D14 broken cells at different conditions to optimize its
622 use as biostimulant.

623

5. Conclusion

625 Microalgae are a promising feedstock to produce valuable products, but it is important to
626 isolate strains with the ability to grow in stressful conditions, to avoid contaminations and
627 having a particular biochemical profile, and to widen their biotechnological applicability.
628 This research highlights the potential of the strain *Coelastrella* sp. D14 for growing on
629 low-cost resources such as PWW while removing nutrients from this effluent. If grown
630 on 5% PWW, D14 biomass could also be used as a biostimulant allowing a more
631 sustainable process.

632

Author contributions

634 All authors have contributed to the manuscript. SB and GG contributed equally to the
635 conception and design of the study. SB, GG, and AF conducted the characterization and
636 studies on the strain isolates and performed the studies. All authors analyzed and
637 discussed the data. SB, GG and JMN wrote the draft of the manuscript, and AF and LG
638 made the revisions. All authors read and approved the submitted version.

Funding

CRediT author contribution statement

Sara Baldanta: Conceptualization, Investigation, Methodology, Software, Writing-Original draft preparation. **Alice Ferreira**: Investigation, Software, Visualization, Writing- Original draft preparation. **Luisa Gouveia**: Conceptualization, Visualization, Supervision, Resources, Writing- Reviewing and Editing. **Juana Maria Navarro Llorens**: Writing- Reviewing and Editing, Resources, Supervision. **Govinda Guevara**: Conceptualization, Investigation, Data curation, Writing- Reviewing and Editing.

Declaration of competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. No conflicts, informed consent, human or animal rights are applicable. All authors confirmed the manuscript's authorship and agreed to submit it for peer review.

Data availability

The 18S rRNA data has been submitted on Genbank under accession number PP158241.

Data will be made available on request.

Acknowledgments

Supplementary material

Table S1. Growth parameters of *Coelastrella* sp. D14 growing in different conditions

Condition		Doubling time[1]	Lag Phase[2]	OD_{750nm} Max[3]
Control		2.57 ± 0.11	2	4.94 ± 0.70
NaCl	0.1 M	1.99 ± 0.09	2	5.64 ± 0.36
	0.25 M	1.54 ± 0.08	2	5.13 ± 0.59
	0.5 M	1.60 ± 0.08	5	3.69 ± 0.31
	1 M	nd	nd	nd
pH	4	2.09 ± 0.12	5	2.22 ± 0.33
	6.5	2.34 ± 0.07	3	3.69 ± 0.15
	9	5.91 ± 0.26	3	1.67 ± 0.06
	11	3.39 ± 0.30	3	1.93 ± 0.11
Nitrogen sources	$BG11_0$	5.83 ± 0.75	4	0.30 ± 0.04
	NH_4Cl	3.31 ± 0.34	2	1.02 ± 0.04
	Urea	2.53 ± 0.23	4	2.63 ± 0.10
Urea	8 mM	3.61 ± 0.23	2	4.01 ± 0.34
	16 mM	8.65 ± 2.27	4	2.60 ± 0.14

[1]Mean of doubling time in days $\pm$ the standard deviation (n=3). [2]Lag phase expressed in days. [3]OD_{750nm} maximal after 10 days of growth. nd: not determined (no growth). Conditions correspond to Figure 4, Figures 5AB Figure 6AB, respectively.
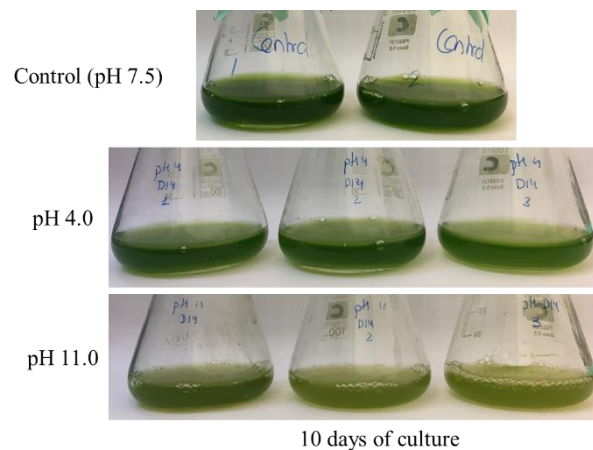

10 days of culture

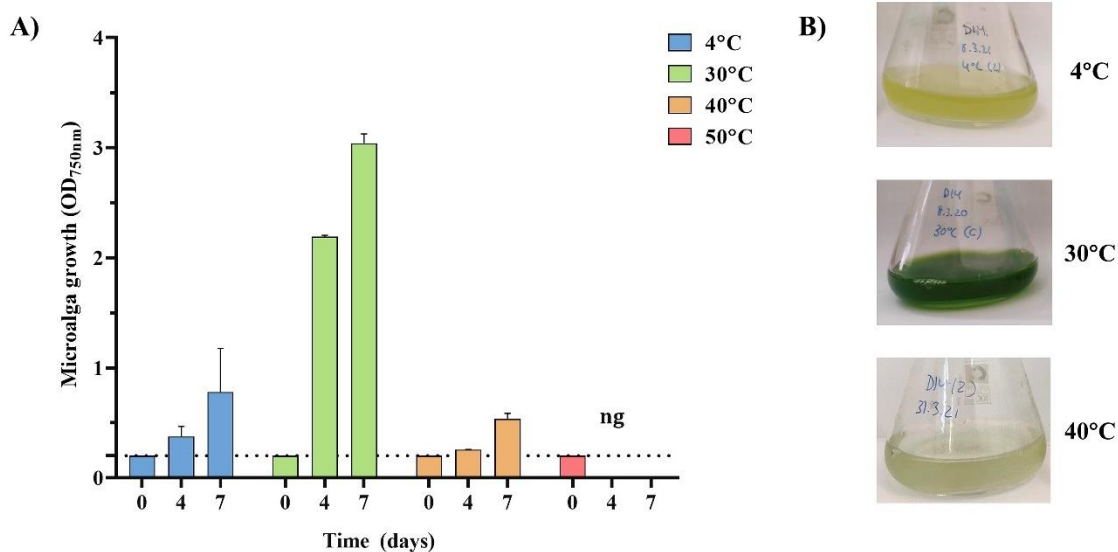**Fig. S1. Growth of *Coelastrella* sp. D14 at different pH.**

23

Fig. S2. Growth of *Coelastrella* sp. D14 at different temperatures. A. OD_{750nm} reached after 10 days of growth. Initial OD_{750nm} was 0.2 (dashed line). B. Pictures taken from different points of the experiment. ng: no growth.

References

Ahmed, S.F., Mofijur, M., Parisa, T.A., Islam, N., Kusumo, F., Inayat, A., Le, V.G., Badruddin, I.A., Khan, T.M.Y., Ong, H.C., 2022. Progress and challenges of contaminate removal from wastewater using microalgae biomass. Chemosphere;286,131656. https://doi.org/10.1016/j.chemosphere.2021.131656.

Ajeng, A.A., Rosli, N.S.M., Abdullah, R., Yaacob, J.S., Qi, N.C., Loke, S.P., 2022. Resource recovery from hydroponic wastewaters using microalgae-based biorefineries: A circular bioeconomy perspective. J. Biotechnol.;360,11-22. https://doi.org/10.1016/j.jbiotec.2022.10.011.

Baldanta, S., Arnal, R., Blanco-Rivero, A., Guevara, G., Navarro Llorens, J.M., 2023. First characterization of cultivable extremophile *Chroococcidiopsis* isolates from a solar panel. Front. Microbiol.;14,982422. https://doi.org/10.3389/fmicb.2023.982422.

Boutarfa, S., Senoussi, M.M., Gonzalez-Silvera, D., López-Jiménez, J., Aboal, M., 2022. The green microalga *Coelastrella thermophila* var. globulina (Scenedesmaceae, Chlorophyta) isolated from an algerian hot spring as a potential source of fatty acids. Life (Basel);12(4)https://doi.org/10.3390/life12040560.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1988. Standard methods for the examination of water and wastewater. Washington DC., American Public Health Association.

Corato, A., Le, T.T., Baurain, D., Jacques, P., Remacle, C., Franck, F., 2022. A fast-growing pleaginous strain of *Coelastrella* capable of astaxanthin and canthaxanthin accumulation in phototrophy and heterotrophy. Life (Basel);12(3)https://doi.org/10.3390/life12030334.

Daneshvar, E., Sik Ok, Y., Tavakoli, S., Sarkar, B., Shaheen, S.M., Hong, H., Luo, Y., Rinklebe, J., Song, H., Bhatnagar, A., 2021. Insights into upstream processing of microalgae: a review. Bioresour. Technol.;329,124870. https://doi.org/10.1016/j.biortech.2021.124870.

Doppler, P., Kriechbaum, R., Käfer, M., Kopp, J., Remias, D., Spadiut, O., 2022. *Coelastrella terrestris* for adonixanthin production: physiological characterization and evaluation of

730 secondary carotenoid productivity. Mar Drugs;20(3).
731 https://doi.org/10.3390/md20030175.

732 Dorado-Morales, P., Vilanova, C., Pereto, J., Codoner, F.M., Ramon, D., Porcar, M., 2016. A
733 highly diverse, desert-like microbial biocenosis on solar panels in a Mediterranean city. Sci.
734 Rep.;6,29235. https://doi.org/10.1038/srep29235.

735 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for
736 determination of sugars and related substances. Anal. Chem.;28(3),350-356.

737 Elisabeth, B., Rayen, F., Behnam, T., 2021. Microalgae culture quality indicators: a review.
738 Crit. Rev. Biotechnol.;41(4),457-473. https://doi.org/10.1080/07388551.2020.1854672.

739 Ferreira, A., Melkonyan, L., Carapinha, S., Ribeiro, B., Figueiredo, D., Avetisova, G., Gouveia,
740 L., 2021. Biostimulant and biopesticide potential of microalgae growing in piggery
741 wastewater. Environ. Adv.;4,100062. https://doi.org/10.1016/j.envadv.2021.100062.

742 Ferreira, A., Figueiredo, D., Ferreira, F., Ribeiro, B., Reis, A., da Silva, T.L., Gouveia, L., 2022.
743 Impact of high-pressure homogenization on the cell integrity of *Tetradesmus obliquus* and
744 seed germination. Molecules;27(7). https://doi.org/10.3390/molecules27072275.

745 Ferreira, A., Ribeiro, B., Marques, P.A.S.S., Ferreira, A.F., Dias, A.P., Pinheiro, H.M., Reis, A.,
746 Gouveia, L., 2017. *Scenedesmus obliquus* mediated brewery wastewater remediation and
747 CO2 biofixation for green energy purposes. J. Clean Prod.;165,1316-1327.

748 Ferreira, A., Marques, P., Ribeiro, B., Assemany, P., de Mendonca, H.V., Barata, A., Oliveira,
749 A.C., Reis, A., Pinheiro, H.M., Gouveia, L., 2018. Combining biotechnology with circular
750 bioeconomy: From poultry, swine, cattle, brewery, dairy and urban wastewaters to
751 biohydrogen. Environ Res;164,32-38. https://doi.org/10.1016/j.envres.2018.02.007.

752 Ferreira, A., Ribeiro, B., Ferreira, A.F., Tavares, M.L.A., Vladić, J., Vidovic, S.S., Cvetković, D.D.,
753 Melkonyan, L.H., Avetisova, G.E., Goginyan, V., Gouveia, L., 2019. *Scenedesmus obliquus*
754 microalga-based biorefinery – from brewery effluent to bioactive compounds, biofuels and
755 biofertilizers – aiming at a circular bioeconomy. Biofuels;13.
756 https://doi.org/10.1002/bbb.2032.

757 García, D., Posadas, E., Blanco, S., Acién, G., García-Encina, P., Bolado, S., Muñoz, R., 2018.
758 Evaluation of the dynamics of microalgae population structure and process performance
759 during piggery wastewater treatment in algal-bacterial photobioreactors. Bioresour.
760 Technol.;248(Pt B),120-126. https://doi.org/10.1016/j.biortech.2017.06.079.

761 Goecke, F., Noda, J., Paliocha, M., Gislerød, H.R., 2020. Revision of *Coelastrella*
762 (Scenedesmaceae, Chlorophyta) and first register of this green coccoid microalga for
763 continental Norway. World J. Microbiol. Biotechnol.;36(10),149.
764 https://doi.org/10.1007/s11274-020-02897-0.

765 González-Pérez, B.K., Rivas-Castillo, A.M., Valdez-Calderón, A., Gayosso-Morales, M.A., 2021.
766 Microalgae as biostimulants: a new approach in agriculture. World J. Microbiol.
767 Biotechnol.;38(1),4. https://doi.org/10.1007/s11274-021-03192-2.

768 González López, C.V., García Mdel, C., Fernández, F.G., Bustos, C.S., Chisti, Y., Sevilla, J.M.,
769 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour.
770 Technol.;101(19),7587-7591. https://doi.org/10.1016/j.biortech.2010.04.077.

771 Hoebler, C., Barry, J.L., David, A., Delort-Laval, J., 1989. Rapid acid hydrolysis of plant cell
772 wall polysaccharides and simplified quantitative determination of their neutral
773 monosaccharides by gas-liquid chromatography. J. Agric. Food Chem.;37(2),360-367.
774 https://doi.org/10.1021/jf00086a020.

775 Hu, H., Li, X., Wu, S., Yang, C., 2020. Sustainable livestock wastewater treatment via
776 phytoremediation: Current status and future perspectives. Bioresour.
777 Technol.;315,123809. https://doi.org/10.1016/j.biortech.2020.123809.

778 Hussain, F., Shah, S.Z., Ahmad, H., Abubshait, S.A., Abubshait, H.A., Laref, A., Manikandan, A.,
779 Kusuma, H.S., Iqbal, M., 2021. Microalgae an ecofriendly and sustainable wastewater
780 treatment option: Biomass application in biofuel and bio-fertilizer production. A review.
781 Renew. Sust. Energ. Rev.;137,110603. https://doi.org/10.1016/j.rser.2020.110603.

782 John, D.M., 2002. Filamentous and plantlike green algae. In: Wehr J,Sheath R, Kociolek JP
783 editors. Freshwater Algae of North America, Ecology and Classification. Cambridge, MA,
784 USA: Academic Press. p. 311–352.

785 Kawasaki, S., Yoshida, R., Ohkoshi, K., Toyoshima, H., 2020. *Coelastrella astaxanthina* sp. nov.
786 (Sphaeropleales, Chlorophyceae), a novel microalga isolated from an asphalt surface in
787 midsummer in Japan. Phycological Res.;68(2),107-114.
788 https://doi.org/10.1111/pre.12412.

789 Khanra, A., Vasistha, S., Kumar, S., Rai, M.P., 2021. Cultivation of microalgae on unhydrolysed
790 waste molasses syrup using mass cultivation strategy for improved biodiesel.
791 Biotech.;11(6),287. https://doi.org/10.1007/s13205-021-02823-7.

792 Lee, S.-A., Kim, M., Kim, H.-S., Ahn, C.-Y., 2022. Extra benefit of microalgae in raw piggery
793 wastewater treatment: pathogen reduction. Microbiome;10(1),142.
794 https://doi.org/10.1186/s40168-022-01339-3.

795 Lee, S.A., Ko, S.R., Lee, N., Lee, J.W., Le, V.V., Oh, H.M., Ahn, C.Y., 2021. Two-step microalgal
796 (*Coelastrella* sp.) treatment of raw piggery wastewater resulting in higher lipid and
797 triacylglycerol levels for possible production of higher-quality biodiesel. Bioresour.
798 Technol.;332,125081. https://doi.org/10.1016/j.biortech.2021.125081.

799 Li, X., Li, W., Zhai, J., Wei, H., Wang, Q., 2019. Effect of ammonium nitrogen on microalgal
800 growth, biochemical composition and photosynthetic performance in mixotrophic
801 cultivation. Bioresour. Technol.;273,368-376.
802 https://doi.org/10.1016/j.biortech.2018.11.042.

803 Maltsev, Y., Krivova, Z., Maltseva, S., Maltseva, K., Gorshkova, E., Kulikovskiy, M., 2021. Lipid
804 accumulation by *Coelastrella multistriata* (Scenedesmaceae, Sphaeropleales) during
805 nitrogen and phosphorus starvation. Sci. Rep.;11(1),19818.
806 https://doi.org/10.1038/s41598-021-99376-9.

807 Mohedano, R.A., Tonon, G., Costa, R.H.R., Pelissari, C., Belli Filho, P., 2019. Does duckweed
808 ponds used for wastewater treatment emit or sequester greenhouse gases? Sci. Total.
809 Environ.;691,1043-1050. https://doi.org/10.1016/j.scitotenv.2019.07.169.

810 Navarro-López, E., Ruiz-Nieto, A., Ferreira, A., Acién, F.G., Gouveia, L., 2020. Biostimulant
811 potential of *Scenedesmus obliquus* grown in brewery wastewater. Molecules;25(3).
812 https://doi.org/10.3390/molecules25030664.

813 Nayana, K., Sudhakar, M.P., Arunkumar, K., 2022. Biorefinery potential of *Coelastrella*
814 biomass for fuel and bioproducts—a review. Biomass Convers. Biorefin.
815 https://doi.org/10.1007/s13399-022-02519-9.

816 Pang, N., Gu, X., Chen, S., Kirchhoff, H., Lei, H., Roje, S., 2019. Exploiting mixotrophy for
817 improving productivities of biomass and co-products of microalgae. Renew. Sust. Energ.
818 Rev.;112,450-460. https://doi.org/10.1016/j.rser.2019.06.001.

819 Porcar, M., Louie, K.B., Kosina, S.M., Van Goethem, M.W., Bowen, B.P., Tanner, K., Northen,
820 T.R., 2018. Microbial ecology on solar panels in Berkeley, CA, United States. Front.
821 Microbiol.;9,3043. https://doi.org/10.3389/fmicb.2018.03043.

822 Posadas, E., Alcántara, C., García-Encina, P.A., Gouveia, L., Guieysse, B., Norvill, Z., Acién, F.G.,
823 Markou, G., Congestri, R., Koreiviene, J., Muñoz, R., 2017. Microalgae cultivation in
824 wastewater. In: González-Fernández C, Muñoz R editors. Microalgae-Based Biofuels and
825 Bioproducts: Woodhead Publishing. p. 67-91.

826 Procházková, G., Brányiková, I., Zachleder, V., Brányik, T., 2014. Effect of nutrient supply
827 status on biomass composition of eukaryotic green microalgae. J. Appl. Phycol.;26(3),1359-
828 1377. https://doi.org/10.1007/s10811-013-0154-9.

829 Rasband, W.S., 1997. ImageJ. Bethesda, Maryland, USA: U. S. National Institutes of Health.
830 vol. 1997-2018.

831 Razooki, Z.H., Abed, I.J., Al-Mashhadani, M.K.H., 2019. Efect of the aqueous carbon source on
832 growth rate of the microalgae *Coelastrella* sp. MH923012. Plant Arch.;19,1420-1421.

833 Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y., 1979. Generic
834 assignments, strain histories and properties of pure cultures of cyanobacteria.
835 Microbiol.;111(1),1-61. https://doi.org/10.1099/00221287-111-1-1.

836 Rizwan, M., Mujtaba, G., Memon, S.A., Lee, K., Rashid, N., 2018. Exploring the potential of
837 microalgae for new biotechnology applications and beyond: A review. Renew. Sust. Energ.
838 Rev.;92(C),394-404. https://doi.org/10.1016/j.rser.2018.04.03.

839 Saito, M., Watanabe, H., Sasaki, M., Ookubo, M., Yarita, T., Shiraiwa, M., Asayama, M., 2023.
840 Coproduction of lipids and carotenoids by the novel green alga *Coelastrella* sp. depending
841 on cultivation conditions. Biotechnol. Rep.;37,e00769.
842 https://doi.org/10.1016/j.btre.2022.e00769.

843 Sánchez-Quintero, A., Fernandes, S.C.M., Beigbeder, J.B., 2023. Overview of microalgae and
844 cyanobacteria-based biostimulants produced from wastewater and CO(2) streams towards
845 sustainable agriculture: A review. Microbiol. Res.;277,127505.
846 https://doi.org/10.1016/j.micres.2023.127505.

847 Sharma, R., Mishra, A., Pant, D., Malaviya, P., 2022. Recent advances in microalgae-based
848 remediation of industrial and non-industrial wastewaters with simultaneous recovery of
849 value-added products. Bioresour. Technol.;344,126129.
850 https://doi.org/10.1016/j.biortech.2021.126129.

851 Shetty, P., Farkas, A., Pap, B., Hupp, B., Ördög, V., Bíró, T., Varga, T., Maróti, G., 2021.
852 Comparative and phylogenomic analysis of nuclear and organelle genes in cryptic
853 *Coelastrella vacuolata* MACC-549 green algae. Algal Res.;58,102380.
854 https://doi.org/10.1016/j.algal.2021.102380.

855 Singh, P., Gupta, S.K., Guldhe, A., Rawat, I., Bux, F., 2015. Microalgae isolation and basic
856 culturing techniques. Amsterdam, The Netherlands, Elsevier.

857 Su, M., Bastiaens, L., Verspreet, J., Hayes, M., 2023. Applications of microalgae in foods,
858 pharma and feeds and their use as fertilizers and biostimulants: legislation and regulatory
859 aspects for consideration. Foods;12(20). https://doi.org/10.3390/foods12203878.

860 Sudhakar, M.P., Kumar, B.R., Mathimani, T., Arunkumar, K., 2019. A review on bioenergy and
861 bioactive compounds from microalgae and macroalgae-sustainable energy perspective. J.
862 Clean Prod.;228,1320-1333. https://doi.org/10.1016/j.jclepro.2019.04.287.

863 Tan, C.-Y., Dodd, I.C., Chen, J.E., Phang, S.-M., Chin, C.F., Yow, Y.-Y., Ratnayeke, S., 2021.
864 Regulation of algal and cyanobacterial auxin production, physiology, and application in
865 agriculture: an overview. J. Appl. Phycol.;33,2995-3023. https://doi. org/10.1007/s10811-
866 021-02475-3.

867 Tang, D.Y.Y., Khoo, K.S., Chew, K.W., Tao, Y., Ho, S.H., Show, P.L., 2020. Potential utilization of
868 bioproducts from microalgae for the quality enhancement of natural products. Bioresour.
869 Technol.;304,122997. https://doi.org/10.1016/j.biortech.2020.122997.

870 Tanner, K., Martí, J.M., Belliure, J., Fernández-Méndez, M., Molina-Menor, E., Peretó, J., Porcar,
871 M., 2018. Polar solar panels: Arctic and Antarctic microbiomes display similar taxonomic
872 profiles. Environ. Microbiol. Rep.;10(1),75-79.

873 Thepsuthammarat, K., Reungsang, A., Plangklang, P., 2023. Microalga *Coelastrella* sp.
874 cultivation on unhydrolyzed molasses-based medium towards the optimization of
875 conditions for growth and biomass production under mixotrophic cultivation. Molecules.
876 vol. 28.

877 Viegas, C., Gouveia, L., Gonçalves, M., 2021. Aquaculture wastewater treatment through
878 microalgal. Biomass potential applications on animal feed, agriculture, and energy. J.
879 Environ. Manage.;286,112187. https://doi.org/10.1016/j.jenvman.2021.112187.

880 Wang, Q., Song, H., Liu, X., Liu, B., Hu, Z., Liu, G., 2019. Morphology and molecular phylogeny
881 of coccoid green algae *Coelastrella sensu lato* (Scenedesmaceae, Sphaeropeales), including
882 the description of three new species and two new varieties. J. Phycol.;55(6),1290-1305.
883 https://doi.org/10.1111/jpy.12915.

884 Zaytseva, A., Chekanov, K., Zaytsev, P., Bakhareva, D., Gorelova, O., Kochkin, D., Lobakova, E.,
885 2021. Sunscreen effect exerted by secondary carotenoids and mycosporine-like amino acids
886 in the aeroterrestrial chlorophyte *Coelastrella rubescens* under high light and UV-A
887 irradiation. Plants (Basel);10(12). https://doi.org/10.3390/plants10122601.

888 Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Biological evaluation of compost
889 maturity. BioCycle;22,27-29.

890

28