Algal Research

Multidisciplinary integrated characterization of a native Chlorella-like microalgal strain isolated from a municipal landfill leachate

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Section/Category:	Biochemical Characterization
Keywords:	Bacterial endosymbionts; Chlorella-like organism; Light microscopy; Phylogeny; Physiological characterization; Ultrastructure
Corresponding Author:	Adriana Ciurli Dept Agriculture Food and Environment University of Pisa iversity of Pisa PISA, Italy
First Author:	Adriana Ciurli
Order of Authors:	Adriana Ciurli
	Letizia Modeo
	Alberto Pardossi
	Carolina Chiellini
Abstract:	More than a hundred taxa of green unicellular coccoid organisms have been up to now wrongly included under the genus Chlorella . This is mainly due to a kind of characterization not relying on a study approach adopting and integrating different techniques for describing cell appearance and metabolic and genomic features. In this work, we describe a new native Chlorella -like microalgal strain named “SEC_LI_ChL_1”, which was isolated from a pond where municipal landfill leachate flowed before wastewater treatment, through a multimethod study. The molecular characterization and the phylogenetic reconstructions based on two different DNA marker regions (i.e. the concatenated SSU and ITS rRNA gene and the complete 18S rRNA gene) were combined with observations with light and transmission electron microscope, and the analysis of metabolic traits under four different growing conditions (i.e., autotrophy, heterotrophy, mixotrophy and reduced autotrophy). The two separate phylogenetic analysis highlighted that our strain falls within the Chlorella - Micractinium clade, although with two different placements. According to the morphological-ultrastructural and metabolic analyses, “SEC_LI_ChL_1” shares some features with different Chlorella -like microalgae. Interestingly, two peculiar traits never or rarely previously described in Chlorella -like microalgal strains were observed: the association of the cells with endocellular bacteria, and the presence of doublets (i.e. two-celled microalgae aggregates not due to cell division).
Suggested Reviewers:	Elena Sebaneyeva e.sabaneeva@spbu.ru
	Michaela Strüder-Kypke mstruede@uoguelph.ca
	Alessandro Concas aconcasmail@gmail.com
	Cristiana Sbrana cristiana.sbrana@ibba.cnr.it
Opposed Reviewers:	

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Pisa, 30st October 2020

Algal Research – Cover letter

Dear Editor in Chief,
Please, find enclosed an original research article authored by Adriana Ciurli, Letizia Modeo, Alberto Pardossi and Carolina Chiellini entitled "Multidisciplinary integrated characterization of a native *Chlorella*-like microalgal strain isolated from a municipal landfill leachate".
We are very pleased to submit our manuscript to "Algal Research" to be considered for publication, as a result of a collaboration between the Dept. of Agriculture Food and Environment and the Dept. of Biology of the University of Pisa.
The research was aimed at describing a *Chlorella*-like microalgal strain (SEC_LI_ChL_1) isolated from a municipal landfill leachate, by using an integrated multimethod study approach in line with the most recent publications in the field. We performed its molecular phylogenetic characterization, the analysis of metabolic traits after exposure to four different trophic conditions, and its morphological-ultrastructural characterization under light and transmission electron microscope. Thus, taking this opportunity to combine molecular, physiological, and morphological data, we have indeed taken advantage of the major techniques of this century to provide a thorough description of this interesting microalgal strain. Remarkably, we found that it manifests at least two peculiar traits never or rarely previously described in *Chlorella*-like microorganisms, such as the association with endobacteria, and the formation of "doublets", i.e., aggregates formed by two cells. These findings, combined with the obtained molecular phylogenetic reconstructions and the observed metabolic features, led to the hypothesis that the strain might possibly represent a new species of the *Chlorella-Micractinium* clade.

We sincerely hope that our work could be considered suitable for publication on Algal Research.

Please, do not hesitate to contact us for any requests or clarifications.
Best regards

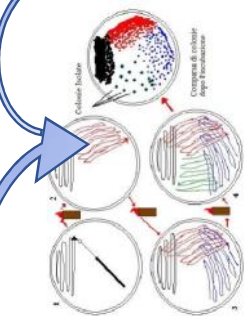
Corresponding authors: Adriana Ciurli, Carolina Chiellini
Affiliation: Department of Agriculture, Food and Environment, University of Pisa
Via del Borghetto 80, 54126 Pisa (PI) Italy
Tel.: +39 0502216537
E-mail address: adriana.ciurli@unipi.it
E-mail sddress: carolina.chiellini@phd.unipi.it

Research highlights

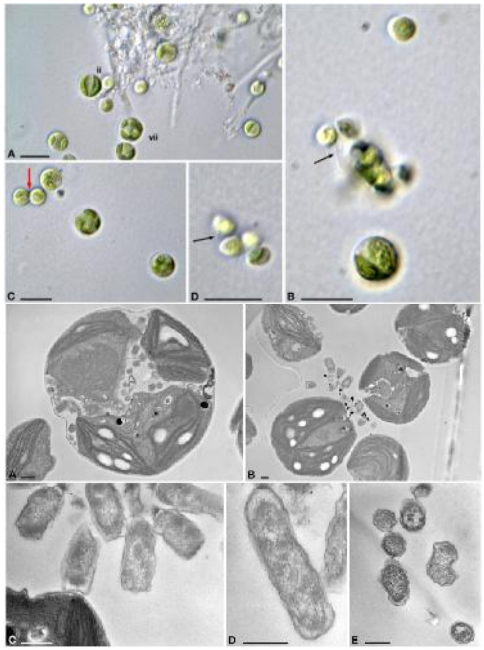
- We describe the new native microalgal strain SEC_LI_ChL_1 with a multidisciplinary approach
- It was isolated from a pond where municipal landfill leachate flowed before treatment
- We combined the study of phylogeny, morphology/ultrastructure and metabolic traits
- The association with endobacteria and the formation of "doublets" are peculiar traits
- The strain might be assigned to a novel species within the Chlorella-like clade

Graphical Abstract

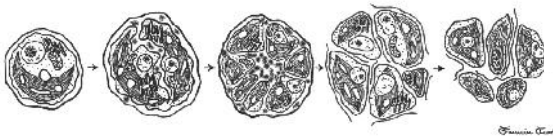
Integrated study approach

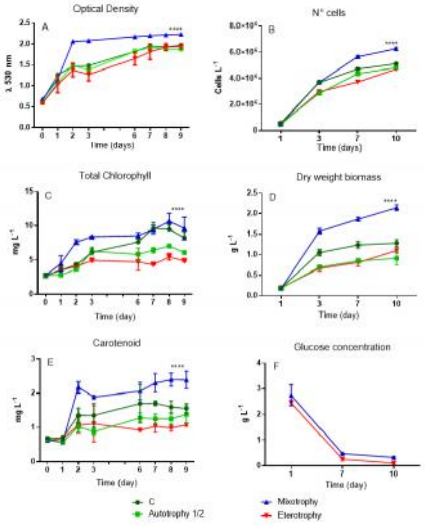


Light microscope



TEM observation





Isolation → Morphological characterization → Molecular and phylogenetic characterization → Metabolic characterization

Manuscript File

Click here to view linked References

Multidisciplinary integrated characterization of a native *Chlorella*-like microalgal strain isolated from a municipal landfill leachate

Adriana Ciurli[a]*, Letizia Modeo[bc], Alberto Pardossi[a], Carolina Chiellini[ad] *

[a] Department of Agriculture, Food and Environment (DAFE), University of Pisa, Via del Borghetto 80, 56124 Pisa, PI, Italy

[b] Department of Biology, University of Pisa, Via Volta 6, 56126 Pisa, PI, Italy.

[c] CISUP, Centro per l'Integrazione della Strumentazione, University of Pisa, Lungarno Pacinotti 43, 56126, Pisa, PI, Italy.

[d] Institute of Agricultural Biology and Biotechnology, Italian National Research Council, Via Moruzzi, 1, 56124 Pisa (Italy).

*Corresponding authors: Adriana Ciurli and Carolina Chiellini

Postal address: Department of Agriculture, Food and Environment (DAFE), University of Pisa, Via del Borghetto 80, 56124 Pisa, PI, Italy

Telephone: +39 050 2216537

E-mail address: adriana.ciurli@unipi.it, chiellini.carolina@gmail.com

Abstract

More than a hundred taxa of green unicellular coccoid organisms have been up to now wrongly included under the genus *Chlorella*. This is mainly due to a kind of characterization not relying on a study approach adopting and integrating different techniques for describing cell appearance and metabolic and genomic features. In this work, we describe a new native *Chlorella*-like microalgal strain named "SEC_LI_ChL_1", which was isolated from a pond where municipal landfill leachate flowed before wastewater treatment, through a multimethod study. The molecular characterization and the phylogenetic reconstructions based on two different DNA marker regions (i.e. the concatenated SSU and ITS rRNA gene and the complete 18S rRNA gene) were combined with observations with light and transmission electron microscope, and the analysis of metabolic traits under four different growing conditions (i.e., autotrophy, heterotrophy, mixotrophy and reduced autotrophy). The two separate phylogenetic analysis highlighted that our strain falls within the *Chlorella-Micractinium* clade, although with two different placements. According to the morphological-ultrastructural and metabolic analyses, "SEC_LI_ChL_1" shares some features with different *Chlorella*-like microalgae. Interestingly, two peculiar traits never or rarely previously described in *Chlorella*-like microalgal strains were observed: the association of the cells with endocellular bacteria, and the presence of doublets (i.e. two-celled microalgae aggregates not due to cell division).

Keywords

Bacterial endosymbionts, *Chlorella*-like organism, Light microscopy, Phylogeny, Physiological characterization, Ultrastructure

1. Introduction

Microorganisms belonging to *Chlorella* genus are morphologically defined as coccoid unicellular green algae with globular or ovoid cells, whose reproduction occurs exclusively by autosporogenesis (asexual reproduction) [1]. Autospores develop simultaneously within maternal sporangium without any flagellated or sexual stages [1], although the disclosure of meiotic genes suggest the possible occurrence of cryptic sexuality at least in *C. variabilis* and other Trebouxiophyceae [2]. Since the first description of *Chlorella vulgaris* provided in 1890 [3], more than a hundred taxa of green unicellular coccoid organisms has been wrongly included under the genus [4]. The wrong identification of microalgal strains is mainly due to the diffuse lack of a multidisciplinary approach that simultaneously adopts and integrates different techniques for describing cell morphology, metabolic characteristics, and genomic features. Indeed, traditionally, microalgal classifications were performed solely according to gross morphology as often the main interest was in describing algal physiology and growth cycle (e.g., [5]). Unfortunately, the simple morphology of *Chlorella* (and *Chlorella* like-species), always led to taxonomical difficulties [6-7]. Later, data on the cell surface features and ultrastructural identity under the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) respectively, were added to microalgal descriptions (e.g., [8-9]), also in combination with cell wall's chemical composition analysis (e.g. [7]). Finally, the development of molecular techniques and the phylogenetic approach based on 18S rRNA gene sequencing appeared (e.g., [6, 10]). Several papers pointed out that the application of single approach study in microalgal investigation may result in misidentification, since species sharing a similar morphology can be found in different phylogenetic lineages, and morphologically different species can be closely related forming monophyletic lineages [11]. Thus, as well as for other protists such as ciliates (as example see [12-14]), the use of such an integrated, multimethod study approach has gained ground (e.g. [4, 15-19]).

Chlorella-like microalgal strains are almost ubiquitous [20], being found not only in freshwater [21] and seawater environments [22] but also in terrestrial [23] and aerial niches [24]. Some of them are in symbiotic association (i.e., as endosymbionts) with other organisms [25, 26, 27]; sometimes they can also be found in extremophilic environments [7]. Many authors reported *Chlorella* sp. as the most common microalgae strains used in wastewater treatments [28]. Interestingly, *Chlorella* sp. is a good candidate for Integrated Multi-Trophic Aquaculture (IMTA) systems, where the fish consume algae and provide the inorganic carbon for their autotrophic growth while algae purify the water and generate the oxygen required by fishes [29, 30].

In this work, we describe the strain SEC_LI_ChL_1, a green native microalgae isolated from a pond in which the municipal landfill leachate flowed before wastewater treatment. We used an integrated study approach, in which the phylogenetic analysis of the strain based on two different molecular markers was combined with the morphological-ultrastructural characterization by means of light microscope and TEM observation, and the analysis of the metabolic traits after exposition to different environmental conditions. Four different metabolic conditions were tested through the alteration of the fundamental growth and trophic parameters (i.e., light and nutrients availability): autotrophy, heterotrophy, mixotrophy, and reduced autotrophy. Besides some features in common with different *Chlorella*-like microalgae, the morphological-ultrastructural analysis revealed the presence of interesting, peculiar morphological traits of strain SEC_LI_ChL_1.

2. Materials and methods

2.1. Strain: isolation, growth condition, characteristics

The monoclonal strain SEC_LI_ChL_1 was isolated from a water sample collected in an artificial lake within the "R.E.A. S.p.A. Company" (currently renamed "Scapigliato Energia s.r.l. Company") in Rosignano Marittimo (LI), Italy (43° 27' 45.34'' N, 10° 28' 24.42'' E), in April 2014. Since then, the strain is part of the laboratory collection at the Department of Agriculture, Food and Environment of the University of Pisa. In the artificial like, the company collected the excess of the municipal leachate. Water sample was collected in 1-liter sterile plastic bottle at 10 cm depth, and immediately brought to the laboratory for processing. The following parameters were measured: pH: 9.5; conductivity: 8.5 dS m^{-1}; B.O.D.: 20 mg O_2 L^{-1}; C.O.D.: 147 mg O_2 L^{-1}; nitrates content: 10 ppm [31]; total nitrogen content: 110 ppm [30]; phosphates: 4.5 ppm [32]; total phosphorus: 3.5 ppm [33]. Afterwards, an enrichment of the original sample by diluting it (1/1 v/v) with Tris-Ammonium Phosphate (TAP) medium [34] was performed. The enriched sample was maintained for two weeks in the growth chamber under controlled temperature (24/22 °C), and under a 16/08 h day-night cycle with PPFD of 120 µmol photons m^{-1} s^{-1} from cool-white light lamps (Gavita Lep 330 Plasma fixtures, Gavita Holland Light Emitting Plasma, Netherlands). After two weeks, some replicates (100 µl each) of the enriched water sample were streaked on a TAP agar plates, which were then kept in the growth chamber as described above. This process was further repeated until the colonies appeared well isolated and purified (i.e. until a single morphology indicating the presence of a single strain was visible). A colony was randomly chosen from one of the streaked plates and pre-inoculated in liquid TAP medium (100 ml). A massive dense pre-culture (500 ml) was obtained from the pre-inoculum by adding fresh sterile TAP medium every three days.

The culture has been maintained under laboratory conditions as described in Chiellini et al., [35]. Briefly, the strain was maintained at 1,100 ppm salinity as mono-strain culture under continuous agitation and grown in TAP medium. The exhausted medium was periodically replaced with fresh one. The microalgal culture was kept in the growth chamber under controlled temperature (23 ± 1°C), and under the following light conditions: 16/08 h day-night cycle and a PPFD of 120 µmol photons m^{-1} s^{-1} from cool-white light lamps (Gavita Lep 330 Plasma fixtures, Gavita Holland Light Emitting Plasma, Netherlands). Some liquid culture aliquots (about 200 ml each) were also periodically stored at 4°C, in dark and static conditions, to be preserved overtime. Interestingly, even after three years-long storage in such conditions, the culture was able to recover and start reproducing within two days once replaced in fresh medium and put in its optimal growth conditions (A. Ciurli, pers. obs., 2019). A periodical check under the light microscope (Nikon TMS-F 301434, Japan) was also performed to monitor the general health status of the culture and to ensure the absence of any eukaryotic contamination.

2.2. Molecular characterization of strain SEC_LI_ChL_1

2.2.1 Total DNA extraction and sequence obtainment

Total genomic DNA was extracted according to the protocol described by Saba et al., [36], starting from a frozen cellular pellet. The pellet was obtained by centrifugation (5' at 14,000 g) of 2-ml mono-strain microalgal culture, derived from a single colony grown on solid TAP medium. We first amplified a portion of ~ 1,800 bp from the nuclear DNA region comprising the final portion of 18S, the complete ITS1-5.8S-ITS2, and the initial portion of 28S. A 25-µl PCR was performed using 0.25 mM deoxynucleoside triphosphates (2.5 mM each), 0.6 pmol μl^{-1} of primer forward (18S F919: ATT GAC GGA AGG GCA CCA, [37]), 0.6 pmol μl^{-1} of primer reverse (RG D2: GGT CCG TGT TTC AAG ACG GG, [38]), 2.5 µl of template DNA, and 0.03 u μl^{-1} of taq polymerase (Ex Taq, TAKARA, Japan). Thermocycling was performed using a C1000™ Thermal Cycler (Bio-Rad) at 94°C for 10 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min with a final extension step at 72°C for 10 min [37]. The complete 18S rRNA gene was also amplified according to Chiellini et al., [39], using primers 18S F9 5'-CTG GTT GAT CCT GCC AG-3' [40] and reverse 18S R1513 Hypo 5'-TGA TCC TTC YGC AGG TTC-3' [41], following the same amplification protocol described above. A 25-µl PCR was performed using 0.25 mM deoxynucleoside triphosphates (2.5 mM each), 0.6 pmol μl^{-1} of each primer, 1 µl of the same template DNA used for the previously described amplification, and 0.03 u µl-1 of taq polymerase (Promega, U.S.A.). Thermocycling was performed using a Bio-Rad Gene Cycler Thermal Cycler. Amplicons were purified by ethanol/EDTA/Na-acetate precipitation as follows: 2.25 µl EDTA (125

mM), 2.25 µl Na-acetate (3M, pH 5.6) and 50 µl cold EtOH (95%) were added to 20 µl amplicon; after 15 min at room temperature, the mixture was centrifuged 15 min at maximum speed; the supernatant was then removed without moving the pellet; after that, the pellet was washed with 100 µl cold EtOH (70%) and left at room temperature 10 min; it was subsequently centrifuged 10 min at maximum speed and the supernatant was removed; finally, the pellet was air-dried and resuspended in 20 µl sterile distilled water.

The purified amplicons were sequenced using the same primers as for the amplification (i.e., 18S F919 and RGD2 in the first case, and F9 and R1513 Hypo for 18S rRNA gene) from the company Eurofins Genomics (Germany) in the first case, and BMR Genomics (Padova, Italy) in the case of 18S rRNA gene. The obtained sequences were deposited in GeneBank under accession numbers MT734796 and MT734757 respectively.

2.2.2 Phylogenetic analysis

The obtained DNA sequences were separately submitted to NCBI Blast analysis [42] to determine their preliminary affiliation through comparison with all the sequences present in the international databases. After an accurate review of the BLAST results, a selection of 49 high quality sequences was performed for the first amplified region (i.e., final portion of 18S, complete ITS1-5.8S-ITS2, and initial portion of 28S), according to similarity criterion. Analogously, a selection of 54 high quality sequences was performed for the complete 18S rRNA gene sequence. Then, the phylogenetic analysis was conducted as described in Chiellini et al., [43]. Briefly, a total of 50 sequences in the first case and 55 sequences in the second case were aligned with the BioEdit Software [44]. MEGA5 Software [45] was used for phylogenetic tree construction in both cases, by means of the maximum likelihood method; the robustness of the inferred trees was evaluated by 500 bootstrap resampling and the parameters chosen for the analysis were the following: Model/Method=General Time Reversible model; Rates among sites=Gamma distributed with invariant sites (G+I); Gaps=Use all sites; ML heuristic method=Nearest neighbor Interchange (NNI); Branch swap filter=Strong.

2.3 Morphological characterization of strain SEC_LI_ChL_1: light microscope and TEM observation

To perform light microscope and TEM observations, a liquid culture of the strain was prepared by resuspending a single isolated colony grown in solid TAP medium in 20 ml of sterile liquid TAP medium. The liquid culture was kept in agitation in the growth chamber, in the same conditions as

for maintenance culture, for 5 days and then employed. Live cells were observed for morphological description using Differential Interference Contrast (DIC) microscope with a Leitz Orthoplan microscope (Weitzlar, Germany). TEM analysis was carried out to investigate the subcellular structure of the strain. Cells were fixed in 1.5-ml Eppendorf tubes and all the solutions (fixatives, ethanol, acetone, and Epon araldite embedding mixture-from now on referred to as "resin"; [13]) were directly added. At each step, samples were collected by centrifugation for 3 min at 2,000 × g, except for the steps in resin diluted in acetone and the first step in pure resin, when the centrifuge speed was always set at 3,000 × g; then, after discharging the supernatant, the next solution was added, and the pellet was re-suspended. Cells were prefixed for 90 min at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4); then, after a wash in the same buffer, they were post-fixed for 1h at room temperature in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4). After a wash in distilled water, cells were ethanol-dehydrated (a single step in 30-50-70-90-95% ethanol, each step 15 min; two steps in 100% ethanol, 5 min each) and transferred to 100% acetone (30 min). Pre-embedding was accomplished transferring the cells to a graded series of resin-acetone mixture (three steps, respectively: 1:3, 20 min; 1:1, overnight; 3:1, 20 min); then, for embedding, cells were put in pure resin (two steps: first step 20 min, second-final - step up to hardening). Samples in pure resin were left under the flow hood for 4 h at room temperature to assure complete cell penetration by the resin and then put in oven at 60°C for resin polymerization (72 h). Cell ultrathin sections obtained from TEM preparations were placed on copper grids and stained with uranyl acetate and lead citrate prior to observation with a JEOL 100S TEM.

2.4. Metabolic characterization of strain SEC_LI_ChL_1

2.4.1. Trophic conditions for metabolic characteristic evaluation: autotrophy, mixotrophy heterotrophy, and reduced autotrophy.

The metabolic traits of the isolated strain were evaluated in different trophic growth conditions: autotrophy, heterotrophy, and mixotrophy. Under autotrophic growth two different light-levels were tested and indicated as "autotrophy" and "reduced autotrophy". To obtain the algal solution to be used in the different treatments, purified algal colonies from Petri dishes were selected and transferred into Pyrex glass flasks containing 100 mL of sterile liquid TAP medium. Then, fresh TAP medium was added to the algal solution to reach a 6-L volume (pre-culture) to be used for the next bioassays. When the pre-culture reached an optical density at 530 nm (OD_{530}) of 2.5, and the chlorophyll pigments were 38.7±0.1 mg L^{-1}, the microalgae were harvested by centrifugation (1000 × g, for 10 min), rinsed twice in sterile saline solution (0.9% NaCl), and re-suspended in fresh TAP medium until OD_{530} 0.7±0.2. The obtained algal solution was distributed among the treatments by

inoculation in Pyrex flasks for a volume of 500 ml each. The flasks were capped with air-permeable stoppers and constantly shaken (agitation rate of about 120 rpm) to keep the algae in suspension and to facilitate the CO_2 transfer. The cultures were continuously bubbled with an aeration rate of 0.5 L min^{-1} (ambient CO_2 concentration). All the tests were carried out in three replicates for each treatment, for a total of 12 flasks. The flasks with growing algal solution were put in a dedicated growth-chamber at the following conditions: 24/22°C, 16/08 h day-night cycle and a PPFD of 100-120 μmol photons m^{-1} s^{-1} from cool-white light lamps (Gavita Lep 330 Plasma fixtures, Gavita Holland Light Emitting Plasma, Netherlands). The microalgal growth was monitored at the beginning of the experimental set up (T_0), and after 3, 4, 7, 8, 9 days. All tests were performed in TAP medium and under constant and sterile growth conditions but modifying the light and the carbon sources according to the different test. The growth on autotrophy was assessed by cultivating the strain in glass flasks i) under a PPFD of 100-120 μmol photons m^{-1} s^{-1}, which represented the optimal growth condition ("autotrophy" and "control test" according to [35]), and ii) under a PPFD of 50-60 μmol photons m^{-1} s^{-1} ("reduced autotrophy", obtained by lining the flasks with non-woven fabric). The growth conditions of "mixotrophy" iii) were induced in the cultures by adding 3 g L^{-1} of glucose monohydrate to the growth medium, as previously described [46, 47] under a PPFD of 100-120 μmol photons m^{-1} s^{-1}. Finally, iv) the "heterotrophy" condition was induced by keeping the flasks in the dark 24h/day (0 μmol photons m^{-1} s^{-1}, obtained by lining the flasks with aluminum foil) and by adding 3 g L^{-1} of glucose monohydrate to the growth medium. The whole experiment lasted 10 days. A periodical sampling for physiological parameters measurements was performed at day 0, 3, 6, and 10.

2.4.2. Physiological parameters measured for metabolic characterization

The evaluation of the microalgal response in the tested metabolic conditions has been assessed through: i) the measurement of the variation of OD_{530} during the experiment and in presence of the different treatments; ii) the measurement of biomass variation; iii) the quantification of photosynthetic pigments; iv) the growth rate and v) the cell count in all the tests. Glucose quantification in the culture medium was performed in "mixotrophy" and "heterotrophy" tests. The biomass production was determined by measuring the dry weight (DW) of the culture during the whole experiment. For each sampling point, three 3-ml replicate aliquots of each culture-test were collected under sterile conditions and centrifuged at 1,500 × g for 10 min. The pellet was dried in oven at 60°C until the complete evaporation of the liquid phase. The dried biomass was then weighted in an analytical balance (Ohaus® Pioneer™ Plus Model PA114C). Total chlorophyll and carotenoids were extracted with methanol (Sigma Aldrich, Michigan, U.S.A.) and quantified [48],

as described in Chiellini et al., [35]. Briefly, 1-ml samples were centrifuged at 1500 × g for 5 min at 4 °C (Speedmaster 14R, Euro Clone, Milano, Italy). The pellet was resuspended in 1-ml methanol, sonicated for 10 min (Branson 1210, Bransonic Ultrasonic Cleaner, U.S.A.), and kept in the dark at 4 °C. After an overnight time period, they were centrifuged at 10,000 × g for 5 min (Speedmaster 14R, Euro Clone, Milano, Italy) and the absorbance of the supernatant was spectrophotometrically analyzed (UV-1800 Spectrophotometer, Shimadzu, Japan) with respect to blank at 665.2, 652.4, and 470.0 nm. Cell count was performed for all tests at each sampling point through optical microscope observation (Nikon TMS-F 301434, Japan), using a Burker counting chamber. Finally, glucose quantification was carried out in "mixotrophy" and "heterotrophy" treatments, according to the colorimetric method described in Miller [49], followed by a spectrophotometric measurement with respect to the blank. Briefly, a 5-ml aliquot for each sample was collected and centrifuged (1,500 × g, 5 min); 3 ml of the supernatant were collected and added to 3 ml of 1% 3,5-dinitrosalicylic acid solution. Samples were left in a warm bath (about 40°C) for 15 min, and then 1 ml of 40% potassium sodium tartrate solution (Rochelle salt) was added. Samples were rapidly cooled and the spectrophotometric measurement at OD 575 nm was performed and compared to the previously obtained calibration curve (i.e., constructed with known glucose concentrations).

The collected data were subject to two-way Anova analysis of variance, with the time and the treatments (trophic growth conditions) as the sources of variation. Statistical analysis was performed using Prism version 6.05 (GraphPad Software, Inc). Data are shown as mean values ± SD of three replicates.

3. Results and discussion

3.1 Molecular characterization and phylogenetic analysis

The molecular characterization of strain SEC_LI_ChL_1 was performed in parallel on two different markers: the concatenated SSU and ITS rRNA gene sequences and the complete 18S rRNA gene sequence. The use of multiple gene sequencing for phylogenetic reconstruction has been demonstrated to be more resolutive for microalgal taxonomic identification [50]. This is especially true for *Chlorella* like-strains, whose taxonomy has been widely revised during the past decades [51, 52]. A 1,860 bp long sequence including the final portion of 18Sr RNA gene, the complete ITS1-5.8S-ITS2, and the initial portion of 28S rRNA gene were obtained and submitted to NCBI Blast analysis. According to NCBI, our strain is phylogenetically related to the species *Micractinium inermum*, *Pseudochlorella pringsheimii*, *Chlorella vulgaris*, and *Micractinium reisseri* (Supplementary Table S1). After the analysis of Blast results, a total of 50 sequences (including the sequence obtained in this work) were used for ML phylogenetic reconstruction To

better visualize the position of our strain in the phylogenetic tree, a "subtree" was extrapolated with the specific tool in MEGA 5 software (Fig. 1). The phylogenetic tree revealed a 100% similarity of strain SEC_LI_ChL_1 with *Chlorella vulgaris* strain CCAP 211/79 (Acc. FR865683.1). The latter is the only *C. vulgaris* included in a clade of *Micractinium* (Supplementary Fig. S1 and Fig. 1); moreover, it is phylogenetically not related with other *C. vulgaris* strains that are included in the analysis (i.e., AB699112.1, FR865682.1, MH703753.1, FR865681.1 and AY948419.1). Thus, in our opinion, it cannot be excluded that strain CCAP 211/79 (FR865683.1) deposited in GenBank as "*Chlorella vulgaris*" was incorrectly assigned. However, Germond and colleagues [53] somehow previously made the same observation because they considered CCAP 211/79 a *Micractinium* strain without bristles, instead of a *Chlorella* strain. Unfortunately, the lack of morphological and ultrastructural data about CCAP 211/79 precludes any comparison of this strain with our isolate. Based on the phylogenetic analysis of the concatenated SSU and ITS rRNA gene sequences, the *Chlorella* clade can be divided into six distinct lineages representing six genera: *Chlorella*, *Micractinium*, *Didymogenes*, *Actinastrum*, *Meyerella*, and *Hegewaldia* [54]. According to available literature and both to phylogenetic and morphological data on these six lineages, and the whole investigation herein presented (as for morphological data see below), our strain SEC_LI_ChL_1 should not be included in any of them. The second phylogenetic analysis based on the complete 18S rRNA gene sequence allowed us to obtain a 1,702 bp long sequence. According to NCBI Blast analysis, the first 10 queries to which the obtained 18S rRNA gene sequence is similar to, are phylogenetically related to *Chlorella sorokiniana*, *Micractinium sp.* and *Chlorella sp.* (Supplementary Table S2). A total of 55 sequences (including the 18S rRNA gene sequence obtained from our strain) were accurately selected on the base of their length and quality and used for ML phylogenetic reconstruction (Fig. 2). Analogously to the previous reconstruction (Fig.1), based on 18S rRNA gene our strain clusters with both *Micractinium* spp. and *Chlorella* spp. (Fig. 2). Interestingly, the 18S rRNA gene sequence of *C. vulgaris* strain CCAP 211/79 (FR865683.1) does not show a 100% similarity with the one of our strain, in disagreement with results obtained with concatenated SSU and ITS rRNA gene sequences (Fig. 1). Accordingly, strain SEC_LI_ChL_1 is phylogenetically more closely related with strains *Chlorella* sp. MDL4-1 (AY197624.1), *Chlorella* sp. NMX37N (JF767012.1), *Micractinium* sp. KNUA034 (KM243325.1), *C. sorokiniana* NKH6 (LC505542.1) and *Micractinium* sp. KSF0094 (MN414469.1). As shown in Fig. 2, our hypothesis that strain CCAP 211/79 (FR865683.1) might have been wrongly attributed to *C. vulgaris* is supported by 18S rRNA gene analysis as well, as it is the only *C. vulgaris* strain clustering with *Micractinium* sp., *Chlorella* sp., and *C. sorokiniana*. Interestingly, *C. sorokiniana* strain LC505542.1, which is the most similar to our strain, is the only representative of the species

within the whole clade. Indeed, *C. sorokiniana* strains are mainly grouped in the upper side of the tree (although with weak support) (Fig.2). Therefore, the sequence LC505542.1 might represent another wrongly attributed species. The molecular phylogenetic investigation carried out on two different gene markers disclosed two different placements of our strain SEC_LI_ChL_1. Although its phylogenetic placement was not univocally determined, the strain was always included in the *Chlorella-Micractinium* clade (Fig. 1, 2; Supplementary Tables S1 and S2). According to the available literature, some authors preferentially selected one molecular marker respect to another for phylogenetic analysis. For this reason, not all the genes of a single strain are sequenced and deposited in public database. This brings to a possible lack of information since for all the described strains there are not all the genetic data available. This might be an explanation to the fact that the use of two different molecular markers in phylogenetic reconstruction brings to different taxonomic attributions. Moreover, when the amplified regions are merged in a single 3,006 bp-long sequence comprising the complete 18S gene, ITS1, 5.8S, ITS2 and the first portion of 28S, Blast analysis provides a third different result (i.e. the first most similar sequence is *Micractinium inermum* KF597304.1, with 98.42% similarity on 99% query coverage, followed by Chlorella-like strain KU641127.1 with 98.26% similarity on 99% query coverage). Unfortunately, just three out of the 100 best hits are of the same length as this merged sequence (C. Chiellini, pers. obs., 2020), preventing any kind of possible sequence comparison. Thus, in this context, we preferred to provide two separated phylogenetic reconstructions instead of a single one. However, in this work, we would like to stress that each single study approach (i.e. molecular *vs* morphological *vs* physiological) is not resolutive for the description of a microalgal strain, and multiprospectivity is far more appropriate.

3.2 Light microscope and TEM observation

Under the light microscope, cells of strain SEC_LI_ChL_1 usually show a solitary life form; vegetative cells are spherical in shape, and ~ 3-7 µm in diameter (Fig. 3A-C). Cell diameter varies based on cell age: younger cells appear smaller than older ones. All these characteristics agree with previous descriptions of *C. vulgaris* [7, 55]. In Fig. 3 most of the cells appear in division and contain different autospores, each apparently surrounded by its membrane. The diameter of autospore-forming cells ranges between 7 and 9 µm. Inside the mother cells, the number of autospores observed under the light microscope ranges from 2 to 7, which, according to binary fission, indicates that the "lacking" 8^{th} autospore was out of visible plane (respectively indicated with "ii" and "vii" in Fig. 3A). This feature disagrees with previous descriptions of *C. vulgaris*, that produces a maximum of 4 autospores (mainly 2) before their release through mother cell breakage

[7, 55]. However, our findings are in agreement with previous findings ([56]. In fact, Yamamoto and colleagues [56] reported that, during autospore-forming, the protoplast of *C. vulgaris* Beijerinck divides into two, four, eight and so on within the mother cell wall. In our strain, several newly divided daughter cells can be observed (Fig. 3B, D). In some cases, just after the rupture of the mother cell wall and autospore release in the environment, some transparent, filamentous structures are present and seem to keep somehow together the daughter cells (Fig. 3B, D, black arrow). These are likely the residuals of the mother cell-wall remaining stuck with daughter cells as also observed in TEM processed cells (see below). The putative cell-wall residuals were no more visible once the cells undergo the next phase of their life cycle (Fig. 3). This observation suggests that neither true connecting strands nor bristle-like structures are present among the cells as typical of *Chlorella* at difference from *Chlorella*-like genera (such as *Micractinium*; [16]),Observation with TEM revealed cells in different vegetative/reproductive stages. Diameter of vegetative cells is about 3.5-6 µm (Supplementary Fig. S2 A). Some cell measurement reduction with respect to in vivo data was expected since TEM procedure generally brings to cell shrinkage (as example in protists see [11, 13]). In our case, cell shrinkage was about 1.5% with respect to the maximum diameter measured in vivo. Nuclear size is variable; in cells soon before the first division nuclear size is ~ 1.5-2.5 µm in diameter, while in triangular autospores soon after their release from mother cell the diameter is ~ 1.3-1.8 µm. Cells do not show mucilaginous envelopes or other cell wall ornamentation which are distinctive characters for other taxa according to [54] (Supplementary Fig. S2, S7, Fig. 4, 5A-C). Cell wall is well visible and monolayered, while in the description of *C. vulgaris* Beijerinck 1890 strain R-06/2 it has a multilayered structure [7]. We did not investigated the possible presence of layered microfibrils in cross-sections of adult cell walls, which was reported by Němcová and Kalina [9] in *C. vulgaris* and other *Chlorella* spp. (i.e., *C. sorokiniana* and *C. luteoviridis*). As for the cytoplasm, starch granules of variable shape (mainly oval), sizes and abundance are present inside the unique chloroplast which shows a double membrane, in agreement with previous description of *C. vulgaris* [7, 55]. Lipid globules, ~ 0.2 µm in diameter, can often be visible in cell cytoplasm, while they were never observed inside the chloroplasts (Supplementary Fig. S2 A) as previously described in *C. vulgaris* [55], although Moriyama and colleagues [57] demonstrated that lipid droplets only exist in close association with the chloroplast (or are largely engulfed by the chloroplasts), not inside the chloroplast. A single chloroplast with a pyrenoid (~1.5-2 µm in length) is observed. Several elongated mitochondria are located close to the chloroplast. The latter is usually parietally positioned and spindle shaped; only seldom, in cells at early stage of development it appears cup-shaped (Supplementary Fig. S2A, 5B) as previously reported in *C. vulgaris* [7]. The chloroplast divides with the typical binary fission pattern, in which a central constriction ring is

formed. The single pyrenoid is covered by a starch envelope, usually consisting in 2/3 large, distinct, irregular pieces. It is traversed by 1-3 similarly oriented double-layered thylakoid membranes. The latter features are in disagreement with the study by Gärtner and colleagues [7], who reported that in typical *C. vulgaris* two large concave convex cup-shaped starch plates cover the pyrenoid which is divided into two almost similar halve by only a single thylakoid. As for other *Chlorella* and *Chlorella*-like spp., in *C. luteoviridis* the pyrenoid is bisected by four or two thylakoids, and in *Parachlorella keissleri* (previously known as *C. keissleri*), it is bisected by only two thylakoids [9]. TEM observations confirmed light microscope findings concerning the presence of a variable number of autospores derived from a single cell (i.e., 2-8 autospores at maximum, included autospores out of visible plan) (Fig. 4). In sporangia with a higher number of autospores, these are usually packed in a triangular shape (Fig.4 C-E), which is unknown in *Chlorella,* but described in *Heveochlorella hainangensis* [58]. Less frequently autospores appear spherical; smaller autospores are often observed (i.e., unequal division of the parent cell often occurred, Fig 4 F). The latter feature, observed in *H. hainangensis* as well, has been attributed to an unequal division of the chloroplast [58]. Although we did not perform any analysis of the cell's wall chemical composition, along with the study of cell wall's ultrastructure, we roughly investigated its development (Fig. 4). According to several studies [7, 9, 56, 59], in *C. vulgaris,* when the mother cell wall breaks, each daughter cell continues the synthesis of its own cell wall started within mother cell, finalizing it shortly after the release from the autosporangium. The observation that young autospores within the sporangium are covered by an electron dense layer of the developing cell wall, clearly visible around the young daughter protoplasts, is a feature shared by *C. vulgaris* with other *Chlorella* spp. such as *C. sorokiniana* and *C. luteoviridis P. kessleri* [9], independently from the chemical composition of cell wall. According to our TEM images, this should not be the case of our strain SEC_LI_ChL_1. Indeed, often no electron-dense layer is visible on the young daughter protoplasts inside the mother cell (Fig. 4 D, F). Although it might be possible that they start synthetizing their own cell wall before being released in the environment, inside the mother cell, autospores with uncomplete cell walls are usually visible. According to Němcová and Kalina [9], at difference with *Chlorella* spp., *P. keissleri* shows the lack of this electron-dense layer as well; however, the latter species differs from our strain in several other ultrastructural traits, such as the visibility of the adult cell wall (hardly visible *vs* well visible respectively) (Fig. 4 A-B; 5A-B; S2; S7). After hatching and before autospore release, mother cells show a thicker wall with respect to previous stages (~ 0.10-0.13 µm vs ~ 0.02-0.03 µm) (Supplementary Fig. S3), as previously reported [59]. Interestingly, after their development, autospores seem to be released in the environment by cell wall rupture not due to apical rupturing of sporangia, as previously described in

C. vulgaris and other Chlorophyta (i.e., [59-61]). However, according to Bock and colleagues [51], mother cell rupture might occur in different ways in different *Chlorella* spp.. In *C. pituita* and *C. pulchelloides* autospores are horizontally or obliquely released; in *C. singularis* the release of autospores occurs after ruptures of mother cell wall into four flaps; in *C. chlorelloides* the rupture of mother cell wall occurs by 180° slanting. As for strain SEC_LI_ChL_1 the pathway seems to be somehow different from the above-mentioned ones: indeed, the process presumably start simultaneously in different sites of the cell wall, next to the border areas between daughter cells (Fig. 4 B, C, F). However, as a result, residual limbs of mother cell wall still remain stuck with daughter cell wall as remnants (Fig. 4 E), likely until daughter cells undergo the next phase of their life cycle (see also Fig. 3 B, C). The presence of remnants of the broken maternal cell walls persisting in culture medium has apparently been reported only in species of *Chlorella* (i.e., *C. viridis*, *C. sorokiniana*, and *C. luteoviridis*) [9]. In Fig. 6, a graphical artwork representation of the strain SEC_LI_ChL_1 is provided; the image is based on the observation of cells from liquid culture. Different stages during the lifetime of a typical representative are shown according to both in vivo and TEM observation.

3.3 Physiological characterization

Microalgae are eukaryotic autotrophic unicellular organisms able to convert sunlight energy and inorganic compounds into organic matter (energy supply) by means of the photosynthetic process, analogously to plant species [62]. Some microalgal strains are also able to perform heterotrophy in the dark and to obtain energy from organic compounds produced by other organisms or supplied artificially. Among them, *C. vulgaris* [63, 64], *C. pyrenoidosa* [65], *Scenedesmus acutus* [66], and *Haematococcus pluvialis* [63] were observed to grow on glucose and/or acetate, although their specific growth rate was lower than under autotrophic or mixotrophic conditions [67]. Our strain SEC_LI_ChL_1 showed the ability to grow under autotrophic, mixotrophic, and heterotrophic conditions (Fig. 7), and to consume almost completely the exogenous glucose in “mixotrophy” and “heterotrophy” tests after 10 days of experimentation (Fig. 7f). Accordingly, our native SEC_LI_Chl_1 strain demonstrated a high adaptation tendence; this was also demonstrate by its ability to grow under excess of trace elements in the growth medium [68]. All our data demonstrated that both the time and the treatments (i.e. trophic conditions) influence the growth of the microalgal strain (Two way-Anova, $p<0.05$ in all the tests, Supplementary Table S3). On the other side, in the glucose consumption, the interaction between treatment and time is not statistically significant (p=0.9117, Supplementary Table S3). The OD values of “autotrophy” and “reduced autotrophy” treatments shared similar trends while the lowest OD values were measured

in the "heterotrophy" test (Fig. 7a). It has been reported that the maximum specific growth rate of algae heterotrophically cultured on simple sugars and organic acids is lower than in autotrophic cultures [62]. *C. vulgaris* is one of the exceptions as autotrophic and heterotrophic maximum specific growth rates are comparable [63, 64, 67]. Our strain deviates from what expected for a *C. vulgaris* strain, suggesting that it might be a different *Chlorella*-like strain not yet described. On the other side, the highest OD_{530} values of our experiment have been registered in "mixotrophy" test (Fig. 7a). Mixotrophy in microalgae is a growth strategy in which CO_2 and organic carbon are simultaneously assimilated and both respiration and photosynthesis operate at the same time [67]. The cell count monitored over the 10-days experiment (Fig. 7b) reflects the same trend as the total chlorophyll content quantification (Fig. 7c): the highest number of cells was counted in "mixotrophy" test while the lowest in "heterotrophy" test. Similarly to that observed in our experiment, in *C. sorokiniana* mixotrophy led to a rapid increase in the cell number compared with photoautotrophy [69]. The same observation can be formulated for the biomass quantification (Fig. 7d). These results are in agreement with the observation that the growth rate of a mixotrophic microalgal culture is about the sum of the specific growth rates of the culture grown under photoautotrophic and heterotrophic conditions, suggesting that under mixotrophic metabolism the microalgal population grows more than in autotrophy and/or heterotrophy [67]. One of the most distinctive characteristics of *Chlorella* sp. is its color due to the chlorophyll pigments, which can reach 1-2% of the dry weight [55]. *Chlorella* contains chlorophyll a and b, along with a range of carotenoids such as β-carotene, lutein, zeaxanthin, violaxanthin, neoxanthin and antheraxanthin [70]. Analogously to the OD and biomass quantification results, the highest chlorophyll content was measured in "mixotrophy" test during almost the whole experimental period, while the lowest one was measured in "heterotrophy" test (Fig 7c). As expected, since lowest light values bring to a decrease in photosynthesis, the "autotrophy" treatment showed higher total chlorophyll content values with respect to "reduced autotrophy" (Fig. 7c). In parallel, the total content of carotenoids (Fig. 7e) reached its highest values in "autotrophy" and "mixotrophy" treatments. Interestingly, the latter two tests were performed at PPDF level of 100-120 μmol m^{-2} s^{-1}: this suggests that an enhanced synthesis of photosynthetic pigments by microalgae may improve the light use efficiency with simultaneous protection against the negative effects of excessive excitation energy in chloroplasts and the over-production of radicals [71].

Our research disclosed that strain SEC_LI_ChL_1 has better growth performances under a mixotrophic metabolism than under heterotrophy or autotrophy. Mixotrophy is a nutritional strategy widely adopted by microorganisms (both eukaryotes and prokaryotes). It represents an evolutionary successful modality to overcome stress conditions associated with the shortage of light and nutrients

[72, 73] and represents a competitive advantage in terrestrial habitats [74]. Some examples of freshwater microalgae that could be cultured mixotrophically are *Chlorella* spp. (i.e., *C. vulgaris*, *C. sorokiniana*, *C. minutissima*, *C. regularis*), *Euglena gracilis*, *Chlorococcum* sp. [67]. The green alga *Apatococcus lobatus*, typically inhabiting terrestrial environments such as concrete walls, showed the ability to grow mixotrophically [74].

3.4 Peculiar features of strain SEC_LI_ChL_1

3.4.1 Association with putative endosymbiotic bacteria

In the majority of in vivo (Supplementary Fig. S4) and TEM observed (Supplementary Fig. S2 C, D; Fig.4B; 5A) mother cells with still intact wall (i.e., before autospore release), rod shaped structures were observed in the inner portion. These structures were considered putative bacterial endosymbionts (with symbiosis simply meaning an even-temporary association of organisms from different species) according to their general appearance and morphology. In TEM processed SEC_LI_ChL_1 cells, endobacteria were ~ 1.0-1.5 µm x ~ 0.25-0.4 µm in size and showed an inner electron dense network-like structure, delimited by a surrounding clearer layer covered by a wavy, double membrane (Fig. 5 B-E). Inside microalgae, endobacteria were localized in the space among forming autospores without any association to any cellular structure except for some mucous (Supplementary Fig. S2 C, D; Fig. 4F; 5A). Several bacterial cells with the same morphology and size of endosymbionts were also observed outside the cells, among the microalgae (Supplementary Fig. S4 and Fig. 5 B-E), and sometimes, almost in direct contact with microalgal wall with their membrane (Fig. 5C). Endobacterial release outside of the mother cell into the medium was also observed; in this case, the mother cell wall appeared broken and some mucous was concurrently produced (Fig. 5B). The observation of endobacteria in both light microscope and TEM investigated cells is in line with laboratory tests. Indeed, experiments demonstrated that even when microalgae were cultivated for one week in agar plates in presence of antibiotics (ciprofloxacin and penicillin, up to 10 µg/ml, Supplementary Fig. S5) and re-inoculated in liquid medium, the presence of these bacteria persisted both in liquid culture and in agar plates. Interestingly, when we inoculated in fresh TAP liquid medium a single algal colony picked up from a TAP medium agar plate, the algal culture started to grow very soon. Initially the inoculum was clear, and the algal cells appeared green, but as soon as the algal cells grew, the medium became turbid and the algal strain concentrated on the bottom of the flask. The culture turbidity during algal growth was presumably due to the release of endobacterial cells into the medium. Once free in the medium, the bacterial cells were still alive and duplicating, thus they were able to grow when re-streaked in bacterial agar

medium as shown in schematic representation in Supplementary Fig. S6 (C. Chiellini, pers. obs., 2020).

Although we cannot *a priori* exclude a transmission of bacteria from a mother cell to daughter cells, it could be hypothesized that the endobacterial release in the environment might possibly due not only to mother cell division but also due to cell death, and the re-entering the eukaryotic host might possibly occur in a specific phase of the algal life cycle. In 2005 Watanabe and colleagues [75] observed with SEM the presence of bacteria on the surface of *C. sorokiniana* (i.e. ectobacteria) representing an associated natural microbial consortium. More recently, Haberkorn and colleagues [76] observed cells of *Tistrella* sp., a polyhydroxyalkanoate-producing alphaproteobacterium on the surface of SEM processed *C. vulgaris* cells. The bacterial cells were observed i) within aggregates of extracellular structures, ii) in proximity of some portion of the microalgal external layer exhibiting rough surfaces, and iii) closely attached to *C. vulgaris* cells losing outer membranous structures. No descriptions of endobacteria associated to *Chlorella-Micractinium* clade members, and particularly to *C. vulgaris*, are available in literature, while associations with ecto/endobacterial symbionts are very frequent in other protists such as ciliates (e.g. [26, 77-80]). Other microalgae known for the presence of bacterial endosymbionts are: i) the euglenophycean *Euglena spirogyra*, *Lepocinclis ovum*, *Strombomonas conspersa*, and *Trachelomonas oblonga* var. punctate [81] hosting rod-shaped inclusions in the nuclei and/or cytoplasm identified as living populations of bacteria by reference to size, shape and ultrastructure; ii) the freshwater diatom *Rhopalodia gibba*, hosting cyanobacteria [82]; iii) *Carteria cerasiformis* and colonial *Pleodorina japonica* [83], the Euglenophycean *Eutreptiella* sp. [84], *Volvox carteri* [85] and *Mesostigma viridae* [86], all hosting *Rickettsiales* endosymbionts.

At present we could only putatively consider the relationship between the endocellular bacteria and the microalgae as a kind of “mutualistic non-obligate” symbiosis. Indeed, this interesting aspect, especially the physiological aspects of bacteria-algae interaction, will be a matter of investigation of future research, along with the investigation aimed at characterizing the bacterial endosymbionts from a multidisciplinary viewpoint (i.e., covering endobacterial molecular, morphological-ultrastructural, physiological, and metabolic features).

3.4.2 Doublets

Under the light microscope we could occasionally observe doublets, i.e. aggregates formed by two cells; these somehow contacted each other along a small portion of their walls but maintaining their spherical shape (Fig. 3C red arrow). TEM analysis confirmed this observation, which might indicate

a higher degree of phenotypic plasticity then previously supposed, although this feature was highlighted since the first produced monograph [1] (Supplementary Fig. S7). Only a few cells of the strain were observed in such a connection, which was not the result of mother cell division as neither cells were enveloped in any mother cell wall, nor any residual limb of mother cell wall was visible (Supplementary Fig. S7). To the best of our knowledge, only two works have been published with evidences of the occurrence of dobluetes in *C. pyrenoidosa* [87, 88]. The first article includes a picture ([87], Fig. 3, p. 542) of a doublet during a study on the influence of environmental conditions of algal ultrastructure. The second research [88] reports that doublets are visible in culture among other algal aggregates formed in response to nutrient stress (glyoxylate), but unfortunately no pictures are available for a morphological comparison. According to the picture provided by Budd et al. [87], the doublet cells possess their own wall and it is not fully clear whether some thin space is still present between the facing walls, while in our work cells were observed before the first division, coupled by their adhering walls. In the doublets the upper cell's lateral side contacts the apical portion of the lower cell (Supplementary Fig. S7). In this work, we could not focus on the analysis of these aggregates. Without any specific analysis, we cannot exclude that these doublets represent stages of algal cells development/life cycle, perhaps less common, or play a role in the cell-to-cell communication by means of or in response to, for instance, specific molecule (e.g. secondary metabolites) like in coenobial microalgae [89]. Moreover, we cannot *a priori* exclude the possible capability of SEC_LI_ChL_1 strain to occasionally perform oogamy (i.e., sexual reproduction) as well. Indeed, it has been previously documented that *Micractinium* can facultatively reproduce by oogamy [90, 91]. On the other side, within the *Chlorella* subclade, *H. parvula* is the only species that can reproduce sexually by oogamy [54, 92]. However, according to morphological data, such as the presence of bristles, the colonial life style, and to the molecular phylogenetic investigation, our strain is not related to the genus *Hegewaldia*.

Conclusions

The native *Chlorella*-like strain SEC_LI_ChL_1 has been characterized through a multidisciplinary integrated approach including phylogenetic, morphological-ultrastructural, and metabolic analyses. The phylogenetic reconstructions performed on two marker genes (i.e., the concatenated SSU and ITS rRNA and the complete 18S rRNA genes) agree with each other including our strain in the *Chlorella-Micractinium* clade. Although this evidence, the molecular analysis based on the first marker gene indicated as closest relative a *Chlorella vulgaris* strain, while the second pointed out a similarity with *Chlorella* sp., *Micractinium* sp. and *Chlorella sorokiniana*. The complete 3006 bp

long sequence comprising both the over mentioned molecular markers gave a third result providing as closest relative *Micractinium inermum*. Morphological-ultrastructural investigation showed that strain SEC_LI_ChL_1 shares some characteristics not only with *C. vulgaris* but also with other C*hlorella* and *Micractinium* microalgae. Moreover, we identified two peculiar traits that were never or rarely previously described in *Chlorella*-like microalgal strains: i) the presence of associated bacteria (i.e, putative endosymbionts) that might be transmitted along the different generations; ii) the presence of doublets (i.e., two-celled aggregates), which might suggest a kind of cell-to-cell communication system or some kind of sexual reproduction (e.g. oogamy) Up to now, no described species belonging to the *Chorella*-like clade share all the described characteristics with our strain. These findings suggest that the strain SEC_LI_ChL_1 might possibly be assigned to a novel species within the clade. Work is in progress for the species assignment of the strain by means of the genome sequencing, and for the deep characterization of the bacteria-microalgae interaction.

Author Contributions

A.C., C.C. and A.P. conceived the idea and the experimental design. A.C. collected and maintained in culture the strain. L.M. performed optical microscopy observation, TEM observation and analysis, ultrastructure data interpretation. A.C. performed the physiological analysis and interpretation. C.C. performed molecular analysis, phylogenetic reconstructions and data interpretation. C.C., L.M. and A.C. interpreted the results. All authors wrote parts of the manuscript and contributed towards revision and final approval of the manuscript.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The research was funded by the project "SIMTAP - Self-sufficient Integrated Multi-Trophic AquaPonic systems for improving food production sustainability and brackish water use and recycling", which is part of the PRIMA programme supported by the European Union. Prof. Giulio Petroni from Department of Biology, University of Pisa, is gratefully acknowledged for providing the primers for PCR amplification, and for his precious help during phylogenetic analysis. Mr

Simone Gabrielli from Department of Biology, University of Pisa, is acknowledged for the assistance with photographic work. Dr. Teodoro Francia is gratefully acknowledged for the original artwork in Fig. 6. We gratefully acknowledge Eng. Stefano Ricci (Scapigliato Energia s.r.l.) for technical assistance during water sampling in Rosignano.

References

[1] Fott B, Nováková BM. A monograph of the genus *Chlorella*. The freshwater species. In Studies in Phycology, pp. 10–74. Edited by B. Fott. Praha: Academia, 1969.

[2] Fučíková K, Pažoutová M, Rindi F. Meiotic genes and sexual reproduction in the green algal class Trebouxiophyceae (Chlorophyta). J. Phycol.2015; 51: 419-430.

[3] Beijerinck MW. Kulturversuche mit Zoochlorellen, Lichenengonidien und anderen niederen Algen I-III. Bot. Z. 1890;48: 726–740.

[4] Krienitz L, Huss VA, Bock C. *Chlorella:* 125 years of the green survivalist. Trends Plant Sci. 2015;20: 67-69.

[5] Pearsall WH, Loose L. The growth of *Chlorella vulgaris* in pure culture. P. Roy. Soc. Lond B Bio. 1937;121: 451-501.

[6] Huss VA, Frank C, Hartmann EC, Hirmer M, Kloboucek A, Seidel BM, et al. Biochemical taxonomy and molecular phylogeny of the genus Chlorella sensu lato (Chlorophyta). J. Phycol. 1999;35: 587-598.

[7] Gärtner G, Uzunov B, Ingolic E, Kofler W, Gacheva G, Pilarski P, et al. Microscopic investigations (LM, TEM and SEM) and identification of Chlorella isolate R-06/2 from extreme habitat in Bulgaria with a strong biological activity and resistance to environmental stress factors. Biotechnol. Biotec. Eq. 2015;29(3):536-540.

[8] Nadakavukaren MJ, McCracken DA. An ultrastructural survey of the genus *Prototheca* with special reference to plastids. Mycopathologia, 1977;61: 117-119.

[9] Němcová Y, Kalina T. Cell wall development, microfibril and pyrenoid structure in type strains of Chlorella vulgaris, C. kessleri, C. sorokiniana compared with C. luteoviridis (Trebouxiophyceae, Chlorophyta). Archiv fur Hydrobiologie-Supplementband Only. 2000;136: 95-106.

[10] Heeg JS, Wolf M. ITS2 and 18S rDNA sequence-structure phylogeny of *Chlorella* and allies (Chlorophyta, Trebouxiophyceae, Chlorellaceae). Plant Gene 2015;4: 20-28.

[11] Darienko T, Gustavs L, Eggert A, Wolf W, Pröschold, T. Evaluating the species boundaries of green microalgae (Coccomyxa, Trebouxiophyceae, Chlorophyta) using integrative taxonomy and DNA barcoding with further implications for the species identification in environmental samples. PloS one 2015;10: 6.

[12] Modeo L, Fokin SI, Boscaro V, Andreoli I, Ferrantini F, Rosati G, et al. Morphology, ultrastructure, and molecular phylogeny of the ciliate Sonderia voraxwith insights into the systematics of order Plagiopylida. BMC Microbiol. 2013;1: 13(1):40.

[13] Nitla, V., Serra, V., Fokin, S.I., Modeo, L., Verni, F., Sandeep, B.V., et al. Critical revision of the family Plagiopylidae (Ciliophora: Plagiopylea), including the description of two novel species, Plagiopyla ramani and Plagiopyla narasimhamurtii, and redescription of Plagiopyla nasuta Stein, 1860 from India. Zool. J. Linn. Soc-Lon. 2019;186: 1-45.

[14] Serra V, Gammuto L, Nitla V, Castelli M, Lanzoni O, Sassera D, et al. Next generation taxonomy: integrating traditional species description with the holobiont concept and genomic approaches-The in-depth characterization of a novel Euplotes species as a case study. bioRxiv, 666461. doi: 10.1101/666461 (2019).

[15] Huss VA, Ciniglia C, Cennamo P, Cozzolino S, Pinto G, Pollio A. Phylogenetic relationships and taxonomic position of Chlorella-like isolates from low pH environments (pH< 3.0). BMC Evol. Biol. 2002;2: 13.

[16] Chae H, Lim S, Kim HS, Choi HG, Kim JH. Morphology and phylogenetic relationships of Micractinium (Chlorellaceae, Trebouxiophyceae) taxa, including three new species from Antarctica. Algae. 2019;34(4): 267-75.

[17] Malavasi V, Škaloud P, Rindi F, Tempesta S, Paoletti M, Pasqualetti M. DNA-based taxonomy in ecologically versatile microalgae: a re-evaluation of the species concept within the coccoid green algal genus Coccomyxa (Trebouxiophyceae, Chlorophyta). PLoS One. 2016;30: 11(3):e0151137.

[18] Darienko T, Pröschold T. Reevaluation and discovery of new species of the rare genus *Watanabea* and establishment of *Massjukichlorella* gen. nov. (Trebouxiophyceae, Chlorophyta) using an integrative approach. J. Phycol. 2019;55: 493-499.

[19] Mikhailyuk T, Holzinger A, Tsarenko P, Glaser K, Demchenko E, Karsten U. Dictyosphaerium-like morphotype in terrestrial algae: what is Xerochlorella (Trebouxiophyceae, Chlorophyta)?. J. Phycol. 2020;28: 56(3):671-86.

[20] Darienko T, Rad-Menéndez C, Campbell C, Pröschold T. Are there any true marine *Chlorella* species? Molecular phylogenetic assessment and ecology of marine *Chlorella*-like organisms, including a description of *Droopiella* gen. nov., Syst. Biodivers. 2019;17: 811-829.

[21] Lee KK, Lim PE, Poong SW, Wong CY, Phang SM, Beardall J. Growth and photosynthesis of Chlorella strains from polar, temperate and tropical freshwater environments under temperature stress. J. Oceanol. Limnol. 2018;1: 36(4):1266-79.

[22] Singh D, Puri M, Wilkens S, Mathur AS, Tuli DK, Barrow CJ. Characterization of a new zeaxanthin producing strain of *Chlorella saccharophila* isolated from New Zealand marine waters. Biores. Technol. 2013;1: 143:308-14.

[23] Rindi F, Hodkinson T, Jones M. Terrestrial green algae: systematics, biogeography and expected responses to climate change. Climate change, ecology and systematics. 2011;78: 201.

[24] Abe K, Matsumura I, Imamaki A, Hirano M. Removal of inorganic nitrogen sources from water by the algal biofilm of the aerial microalga *Trentepohlia aurea*. World J. Microb. Biot. 2003;19: 325-328.

[25] Reisser, W. The metabolic interactions between Paramecium bursaria Ehrbg. and *Chlorella* spec. in the *Paramecium bursaria*-symbiosis. Arch. Microbiol. 1980;125: 291-293.

[26] Lanzoni O, Fokin SI, Lebedeva N, Migunova A, Petroni G, Potekhin A. Rare freshwater ciliate Paramecium chlorelligerum Kahl, 1935 and its macronuclear symbiotic bacterium "Candidatus Holospora parva". PloS one. 2016;16: 11(12):e0167928.

[27] McAuley PJ. Cell-to-cell interactions during the establishment of the *Hydra-Chlorella* symbiosis. In: Scannerini S., Smith D., Bonfante-Fasolo P., Gianinazzi-Pearson, V. (eds) Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. NATO ASI Series (Series H: Cell Biology), vol 17. Springer, Berlin, Heidelberg, 1988.

[28] Emparan Q, Harun R, Danquah MK. Role of phycoremediation for nutrient removal from wastewaters: A review. Appl Ecol Environ Res 2019;17:889–915. doi:10.15666/aeer/1701_889915.

[29] Gilles S, Fargier L, Lazzaro X, Baras E, De Wilde N, Drakides C, et al. An integrated fish-plankton aquaculture system in brackish water. Animal. 2013;1: 7(2):322-9.

[30] Tossavainen M, Lahti K, Edelmann M, Eskola R, Lampi AM, Piironen V, et al. Integrated utilization of microalgae cultured in aquaculture wastewater: wastewater treatment and production of valuable fatty acids and tocopherols. J. Appl. Phycol. 2019;15: 31(3):1753-63.

[31] Cataldo DA, Haroon M, Sehrader LE, Youngs VL. Rapid colorimetric determination of nitrate in plant tissue by titration of salicylic acid. Commun. Soil Sci. Plant. Anal. 1975;6: 71-80.

[32] Valderrama JC. The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem. 1981;10: 109-122.

[33] Olsen SR, Sommers EL. Phosphorus, In: Methods of Soil Analysis, Wisconsis, USA, 403-430, 1982.

[34] Gorman DS, Levine RP. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. PNAS. 1965;54: 1665-1669.

[35] Chiellini C, Guglielminetti L, Pistelli L, Ciurli A. Screening of trace metal elements for pollution tolerance of freshwater and marine microalgal strains: Overview and perspectives. Algal Res. 2020;45: 101751.

[36] Saba F, Papizadeh M, Khansha J, Sedghi M, Rasooli M, Amoozegar MA, et al. A rapid and reproducible genomic DNA extraction protocol for sequence-based identification of archaea, bacteria, cyanobacteria, diatoms, fungi, and green algae. J. Med. Bacteriol. 2016; 22-8.

[37] Rosati G, Modeo L, Melai M, Petroni G, Verni F. A multidisciplinary approach to describe protists: a morphological, ultrastructural, and molecular study on *Peritromus kahli* Villeneuve-Brachon, 1940 (Ciliophora, Heterotrichea). J. Eukaryot. Microbiol. 2004;51: 49-59.

[38] Boscaro V, Fokin SI, Verni F, Petroni G. Survey of *Paramecium duboscqui* using three markers and assessment of the molecular variability in the genus *Paramecium*. Mol. Phylogenet. Evol. 2012;65: 1004-1013.

[39] Chiellini C, Iannelli R, Modeo L, Bianchi V, Petroni G. Biofouling of reverse osmosis membranes used in river water purification for drinking purposes: analysis of microbial populations. Biofouling 2012;28: 969-984.

[40] Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified 16S-like rRNA-coding regions. Gene 1988;71: 491–499.

[41] Petroni G, Dini F, Verni F, Rosati G. A molecular approach to the tangled intrageneric relationships underlying phylogeny in Euplotes (Ciliophora, Spirotrichea). Mol. Phylogenet. Evol. 2002;22: 118–130.

[42] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;1: 25(17):3389-402.

[43] Chiellini C, Miceli E, Bacci G, Fagorzi C, Coppini E, Fibbi D, et al. Spatial structuring of bacterial communities in epilithic biofilms in the Acquarossa river (Italy). FEMS Microbiol. Ecol. 2018;94(12): fiy181.

[44] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95 /98/NT. Nucl. Acid Res. 1999;41: 95–98.

[45] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 2011;28: 2731-2739.

[46] Perez-Garcia O, Escalante FM, de-Bashan LE, Bashan Y. Heterotrophic cultures of microalgae: metabolism and potential products. Water Res. 2011;45: 11-36.

[47] Scarsella M, Bravi M. Microalgae culturing in thin-layer photobioreactors. Chem. Eng. 2010;20.

[48] Lichtenthaler HK. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, Methods in Enzymology, Academic Press. 1987;148: 350–382.

[49] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 1959;31: 426.

[50] Zou S, Fei C, Wang C, Gao Z, Bao Y, He M, Wang C. How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in Scenedesmus (Chlorophyceae). Sci. Rep. 2016;6: 36822.

[51] Bock C, Krienitz L, Proeschold T. Taxonomic reassessment of the genus Chlorella (Trebouxiophyceae) using molecular signatures (barcodes), including description of seven new species. Fottea 2011;11: 293-312.

[52] Champenois J, Marfaing H, Pierre R. Review of the taxonomic revision of Chlorella and consequences for its food uses in Europe. J. Appl. Phycol. 2015;27: 1845-1851.

[53] Germond A, Hata H, Fujikawa Y, Nakajima T. The phylogenetic position and phenotypic changes of a *Chlorella*-like alga during 5-year microcosm culture. Eur. J. Phycol. 2013;48: 485-496.

[54] Luo W, Pröschold T, Bock C, Krienitz L. Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). Plant Biol. 2010;12: 545-553.

[55] Safi C, Zebib B, Merah O, Pontalier PY, Vaca-Garcia C. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renew. Sust. En. Rev. 2014;35: 265-278.

[56] Yamamoto M, Kurihara I, Kawano S. Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae). Planta 2005;221: 766-775.

[57] Moriyama T, Toyoshima M, Saito M, Wada H, Sato N. Revisiting the algal "chloroplast lipid droplet": the absence of an entity that is unlikely to exist. Plant Physiol. 2018;176: 1519-1530.

[58] Zhang J, Huss VA, Sun X, Chang K, Pang D. Morphology and phylogenetic position of a trebouxiophycean green alga (Chlorophyta) growing on the rubber tree, Hevea brasiliensis, with the description of a new genus and species. Eur. J. Phycol. 2008;43: 185-193.

[59] Yamamoto M, Fujishita M, Hirata A, Kawano S. Regeneration and maturation of daughter cell walls in the autospore-forming green alga Chlorella vulgaris (Chlorophyta, Trebouxiophyceae). J. Plant Res. 2004;117: 257-264.

[60] Darienko, T, Lukešová A, Proeschold T. The polyphasic approach revealed new species of *Chloroidium* (Trebouxiophyceae, Chlorophyta). Phytotaxa, 2018;372: 51-66.

[61] Dong Q, Xing XY. *Chroococcidiorella tianjinensis*, gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a green alga arises from the cyanobacterium TDX16. 2020; bioRxiv.

[62] Masojıdek J, Koblızek M, Torzillo G. Photosynthesis in microalgae. Handbook of microalgal culture: biotechnology and applied phycology, p.20, 2004.

[63] Martinez F, Orus MI. Interactions between glucose and inorganic carbon metabolism in *Chlorella vulgaris* strain UAM101. Plant Physiol. 1991;95: 1150–55.

[64] Ogawa T. Aiba S. Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. Biotechnol. Bioeng. 1981;23: 1121–32.

[65] Droop MR. Heterotrophy of Carbon. In: Algal Physiology and Biochemistry (ed. W.D.P. Stewart), pp. 530–59. Blackwell, Oxford, 1974.

[66] Kobayashi M, Kakizono T, Yamaguchi K, Nishio N, Nagai S. Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. J. Ferm. Bioeng. 1992;74: 12–20.

[67] Lee YK. Algal Nutrition–Heterotrophic Carbon Nutrition. Handbook of microalgal culture: Biotechnology and Applied Phycology, pp.116-124, 2003.

[68] Ciurli A, Di Baccio D, Scartazza A, Grifoni M, Pezzarossa B, Chiellini C, Mariotti L, Pardossi A. Influence of zinc and manganese enrichments on growth, biosorption and photosynthetic efficiency of Chlorella sp. Environ. Sci. Pollut. R. 2020; 1-17.

[69] Marchello AE, dos Santos AC, Lombardi AT, de Souza CWO, Montanhim GC. Physiological and Ecological aspects of *Chlorella sorokiniana* (Trebouxiophyceae) under photoautotrophic and mixotrophic conditions. Microb. Ecol. 2018;76: 791-800.

[70] Sansawa H, Endo H. Production of intracellular phytochemicals in Chlorella under heterotrophic conditions. J. Biosci. Bioeng. 2004;98: 437-444.

[71] Masojídek J, Torzillo G, Koblížek M. Photosynthesis in microalgae. In: Richmond A & Hu Q (eds) Handbook of Microalgal Culture: Applied Phycology and Biotechnology, 2nd edn. John Wiley y Sons, Ltd. GBR, pp 21-36, 2013.

[72] Jones RI. Mixotrophy in planktonic protists as a spectrum of nutritional strategies. Fresh. Biol. 1994;8: 87–96.

[73] Stoecker DK. Conceptual model of mixotrophy in planktonic protists and some ecological and evolutionary implications. Eur. J. Protistol. 1998;34: 281–290.

[74] Gustavs L, Schumann R, Karsten U, Lorenz M. Mixotrophy in the terrestrial green alga *Apatococcus lobatus* (Trebouxiophyceae, Chlorophyta). J. Phycol. 2016;52: 311-314.

[75] Watanabe K, Takihana N, Aoyagi H, Hanada S, Watanabe Y, Ohmura N, et al. Symbiotic association in Chlorella culture. FEMS Microbiol. Ecol. **51,** 187-196 (2005).

[76] Haberkorn I, Böcker L, Mathys A, Walser JC, Helisch H, Belz S, et al. Characterization of Chlorella vulgaris (Trebouxiophyceae) associated microbial communities. 2020; J. Phycol.

[77] Castelli M, Sabaneyeva E, Lanzoni O, Lebedeva N, Floriano AM, Gaiarsa S, et al. Deianiraea, an extracellular bacterium associated with the ciliate Paramecium, suggests an alternative scenario for the evolution of Rickettsiales. ISME J. 2019a;**13:** 2280-2294.

[78] Castelli, M., Serra, V., Senra, M.V., Basuri, C.K., Soares, C.A., Fokin, et al. The hidden world of Rickettsiales symbionts: "Candidatus Spectririckettsia obscura," a novel bacterium found in Brazilian and Indian Paramecium caudatum. Microb. Ecol. 2019;77: 748–758.

[79] Fokin SI, Serra V, Ferrantini F, Modeo L, Petroni G. ''Candidatus Hafkinia simulans'' gen. nov. sp. nov., a novel Holospora-like bacterium from the macronucleus of the rare brackish water ciliate Frontonia salmastra (Oligohymenophorea, Ciliophora): multidisciplinary characterization of the new endosymbiont and its host. Microb. Ecol. 2019;77: 1092–1106.

[80] Modeo L, Salvetti A, Rossi L, Castelli M, Szokoli F, Krenek S, et al. "Candidatus Trichorickettsia mobilis", a Rickettsiales bacterium, can be transiently transferred from the unicellular eukaryote Paramecium to the planarian Dugesia japonica. PeerJ 2020;8: e8977.

[81] Leedale GF. Observations on endonuclear bacteria in euglenoid flagellates. *Österreichische botanische Zeitschrift*, 1969;116: 279-294.

[82] Prechtl J, Kneip, C, Lockhart P, Wenderoth K, Maier UG. Intracellular spheroid bodies of *Rhopalodia gibba* have nitrogen-fixing apparatus of cyanobacterial origin. Mol. Biol. Evol. 2004;21: 1477-1481.

[83] Kawafune K, Hongoh Y, Hamaji T, Nozaki H. Molecular identification of rickettsial endosymbionts in the non-phagotrophic volvocalean green algae. PLoS One 2012;7: 2.

[84] Kuo RC, Lin S. Ectobiotic and endobiotic bacteria associated with *Eutreptiella* sp. isolated from Long Island Sound. Protist 2013;164: 60-74.

[85] Kawafune K, Hongoh Y, Nozaki H. A rickettsial endosymbiont inhabiting the cytoplasm of *Volvox carteri* (Volvocales, Chlorophyceae). Phycologia 2014;53: 95-99.

[86] Yang A, Narechania A, Kim E. Rickettsial endosymbiont in the "early-diverging" streptophyte green alga Mesostigma viride. J. Phycol. 2016;52: 219-229.

[87] Budd TW, Tjostem JL, Duysen ME. Ultrastructure of Chlorella pyrenoidosa as affected by environmental changes. Am. J. Bot. 1969;56: 540-545.

[88] Yang Z, Liu Y, Ge J, Wang W, Chen Y, Montagnes D. Aggregate formation and polysaccharide content of Chlorella pyrenoidosa Chick (Chlorophyta) in response to simulated nutrient stress. Biores. Technol. 2010;101: 8336-8341.

[89] Lin WJ, Ho HC, Chu SC, Chou JY. Effects of auxin derivatives on phenotypic plasticity and stress tolerance in five species of the green alga Desmodesmus (Chlorophyceae, Chlorophyta). PeerJ 2020;8: e 8623.

[90] Korshikov AA. On the sexual reproduction (oogamy) in the Micractinieae. Proc. Charkov State Univ. 1937;10: 109-126.

[91] Hegewald E, Schnepf E. Ergänzungen und Korrekturen zur Struktur und Taxonomie bestachelter Chlorellales. Nova Hedwigia, 1987;44: 537-541.

[92] Pröschold T, Bock C, Luo W, Krienitz L. Polyphyletic distribution of bristle formation in Chlorellaceae: Micractinium, Diacanthos, Didymogenes and Hegewaldia gen. nov. (Trebouxiophyceae, Chlorophyta). Phycol. Res. 2010;58: 1-8.

Figure Captions

Figure 1: Subtree extrapolated from the phylogenetic tree reconstruction (fully shown in Supplementary Fig. S1) based on the concatenated SSU and ITS rRNA gene obtained with maximum likelihood method (see Materials and methods for details) on a total of 50 high quality sequences selected among those most similar to strain SEC_LI_ChL_1 sequence (Acc. N° MT734757).

Figure 2: Phylogenetic tree reconstruction based on complete 18S rRNA gene, obtained with maximum likelihood method (See Materials and Methods for details) on a total of 55 high quality sequences selected among those most similar to strain SEC_LI_ChL_1 sequence (Acc. N° MT734796).

Figure 3: *In vivo* observation under DIC microscope of strain SEC_LI_ChL_1 cells from liquid culture. A. Several cells with different age and size: younger cells are smaller than older; ii: a mother cell containing two autospores; vii: a mother cell containing 7 visible autospores (the 8^{th} autospore is out of the focal plane). B. Daughter cells after sporulation; some cells appear somehow kept together by filamentous structures (black arrow), likely remnants of mother cell wall. C. Red arrow: a doublet (two-celled aggregate) with cells adhering by their walls. D. Black arrow: filaments (shorter with respect to those in picture b) apparently derive from a single cell. Scale bars represent 10 µm.

Figure 4: TEM observation of strain SEC_LI_ChL_1 cells from liquid culture: mother cells and autospores. A. Mother cell with three visible autospores (the 4^{th} autospore is not visible in the section); in two of them the chloroplast is in division. B-D. Mother cell with respectively 7, 5, and 6 autospores; B. Mother cell is still intact with autospores without any clear electrodense layer (i.e., developing wall) covering their protoplast. (the 8^{th} autospore is not visible in the section). C. Fresh released autospores are still in contact to each other with their own wall now visible; the 6^{th} autospore is not visible in the section. D. The triangular autospores have been already separated from each other. E. Several mother cell wall remnants (electron-dense covering pieces) are visible on the released autospores. F. The autospore is larger than the other two daughter cells due to unequal division of the mother cell. Scale bars represent 1 µm.

Figure 5: TEM observation of strain SEC_LI_ChL_1 cells from liquid culture: the bacterial endosymbionts. A. Mother cell with autospores: in the space among the forming daughter cells several putative endosymbiotic bacteria (arrow) are visible. Note that autospores do not show any clear electrodense layer (i.e., developing wall) covering their protoplast. B. Bacteria in the medium

apparently released from the cell in the middle, whose wall appears somehow broken (arrowhead). Arrow indicates a remnant of the broken maternal cell walls. C-E. Putative endosymbiotic bacteria released in the medium. C. Several bacteria in longitudinal section; a bacterium is in proximity, almost in contact, with the microalgal wall. D, E. Bacteria in longitudinal (D) and cross (E) sections respectively; they show an inner electron dense network-like structure, delimited by a surrounding clearer layer covered by a wavy, double membrane. Scale bars represent 500 nm.

Figure 6: Artwork representing strain SEC_LI_ChL_1 cells from liquid culture. Different stages during the lifetime of a typical representative are shown according to both i*n vivo* and TEM observation.

Figure 7: Physiological growth parameters of strain SEC_LI_ChL_1 measured during the 10-days experiment under different trophic conditions:. OD_{530} of the growing culture cell number count, dry biomass and the concentration of total chorophills, carotenoids and glucose in the TAP medium.

Supporting Material

Supplementary Figure Captions

Supplementary Figure S1: Phylogenetic tree reconstruction obtained with maximum likelihood method (see Materials and Methods for details) on a total of 50 high quality sequences selected among those most similar to strain SEC_LI_ChL_1 sequence (Acc. N° MT734757).

Supplementary Figure S2: TEM observation of strain SEC_LI_ChL_1 cells from liquid culture. A. Cells in early stage of development, with a cup-shaped chloroplast (Ch); Lg, lipid globules; Sg, starch granules; star indicates starch envelope around pyrenoid (Py). B. A cell after chloroplast division; N, nucleus. C. cell after its first division leading to two autospores; these do not show any clear electrodense layer covering their protoplast; note the apical presence of putative endosymbiotic bacteria (arrow) and mucous material in the clear space between the autospores; arrowheads indicate thylakoid membranes traversing pyrenoid. D. enlargement of the apical part of mother cell in C; arrow indicates putative bacteria. Scale bars represent 1 μm.

Supplementary Fig. S3: TEM observation of strain SEC_LI_ChL_1 cells from liquid culture. Two mother cells during autospore development showing different wall thickness depending on their different growth phases: the cell on the right side (older according to its morphology) show a thicker cell wall with respect to the cell on the left side (younger). Note that autospores do not show any clear electrodense layer (wall) covering their protoplast. Scale barsrepresent 1 μm.

Supplementary Figure S4: In vivo observation under the DIC microscope of strain SEC_LI_ChL_1 cells from liquid culture. A group of algae where putative endosymbiotic bacteria are visible inside (red arrow) all the cells and are apparently released outside by the cell on the bottom (black arrow). Scale bar represents 10 μm.

Supplementary Figure S5: 1-week growth of strain SEC_LI_ChL_1 in presence of different concentration of antibiotics. Once the strain was grown in solid medium, it was re-inoculated in liquid medium where the presence of associated bacteria was persistent.

Supplementary Figure S6: schematic representation of release and recovery in solid medium of the putative endosymbiotic bacterial cells associated to strain SEC_LI_ChL_1. Bacteria are released in the liquid medium during microalgal growth. The morphology of the bacterial colonies is unique, suggesting the presence of a single bacterial species.

Supplementary Figure S7: TEM observation of strain SEC_LI_ChL_1 cells from liquid culture. A doublet (i.e., two-celled aggregate). The two cells, each showing a duplicated chloroplast, are coupled by their contacting wall;upper cell's lateral side contacts the apical portion of the lower cell. Scale bar represents 1 μm.

Supplementary Tables

Supplementary Table S1: NCBI blast analysis results of amplicon sequence final 18S-ITS1-5.8S-ITS2-initial 28S, showing the 10 closely related hits in public databases. Max Score: the highest alignment score calculated from the sum of the rewards for matched nucleotides and penalities for mismatches and gaps; Tot Score: the sum of alignment scores of all segments from the same subject sequence. Query Cover: the percent of the query length that is included in the aligned segments. Ident: the highest percent identity for a set of aligned segments to the same subject sequence.

Description	Accession	Max score	Total score	Query Cover	Per. Ident
Chlorella-like algae voucher HS26	KU641127.1	3165	3165	99.00%	98.00
Micractinium inermum strain NLP-F014	KF597304.1	3160	3160	99.00%	98.05
Pseudochlorella pringsheimii	KY364701.1	2686	2686	99.00%	92.06
Chlorella vulgaris	FR865683.1	2648	2648	79.00%	99.86
Micractinium reisseri	AB506071.1	2612	2612	99.00%	91.15
Micractinium reisseri	AB506070,1	2612	2612	99.00%	91.15
Micractinium reisseri	AB437244.1	2609	2609	99.00%	91.10
Micractinium inermum	KM114868.1	2476	2476	78.00%	97.87

Chlorellaceae sp. MCWWS28	KP204570.1	2351	2351	74.00%	97.82
Micractinium sp. NIES-2171	JX889641.1	2335	2335	74.00%	97.61

Supplementary Table S2: NCBI blast analysis results of complete 18S rRNA gene sequence, showing the 10 closely related hits in public databases. Max Score: the highest alignment score calculated from the sum of the rewards for matched nucleotides and penalities for mismatches and gaps; Tot Score: the sum of alignment scores of all segments from the same subject sequence. Query Cover: the percent of the query length that is included in the aligned segments. Ident: the highest percent identity for a set of aligned segments to the same subject sequence.

Description	**Accession**	**Max score**	**Total score**	**Query Cover**	**Per. Ident**
Chlorella sorokiniana NKH6	LC505542.1	3048	3048	100%	99.76
Chlorophyta sp. SL-2016 isolate BSC-24	KX395732.1	3048	3048	100%	99.76
Micractinium sp. KNUA034	KM243325.1	3048	3048	100%	99.76
Chlorella sp. NDem 9/21 T-13d	AY197628.1	3048	3048	100%	99.76
Chlorella sp. MDL4-1	AY197624.1	3048	3048	100%	99.76
Chlorella sp. NMX37N	JF767012.1	3047	3047	99%	99.76
Micractinium sp. KNUA029	KM243319.1	3044	3044	100%	99.71
Chlorella sp. EO5-4C	FJ946889.1	3044	3044	100%	99.71
Chlorella sp. WO10-1	FJ946886.1	3044	3044	100%	99.71
Chlorella sp. VI11	FJ946884.1	3044	3044	100%	99.71

Supplementary Table S3: Results of Two-Way ANOVA analysis; α=0.05. Data were analyzed considering the sampling time and the trophic conditions (treatments) as factor affecting the microalgal growth. Data are shown as mean values ± SD of three replicates. P-values highlighted in bold indicate statistically significant values ($p<0.05$).

		p value	SS	DF	F
Optical density	Interaction	**0.021**	1.007	21	F (21. 64) = 1.958
	Treatment	< **0.0001**	2.306	3	F (3. 64) = 31.39
	Time	< **0.0001**	20.34	7	F (7. 64) = 118.7
	Residuals		1.567	64	
Number of cells	Interaction	< **0.0001**	4.46E+12	9	F (9. 32) = 85.50
	Treatment	< **0.0001**	7.96E+12	3	F (3. 32) = 457.8
	Time	< **0.0001**	1.58E+14	3	F (3. 32) = 9079
	Residuals		1.85E+11	32	
Total chlorophyll	Interaction	**0.0009**	69.67	21	F (21. 62) = 2.788
	Treatment	< **0.0001**	152.5	3	F (3. 62) = 42.71
	Time	< **0.0001**	310.4	7	F (7. 62) = 37.25
	Residuals		73.79	62	
Dry biomass	Interaction	< **0.0001**	1.747	9	F (9. 32) = 12.98
	Treatment	< **0.0001**	4.647	3	F (3. 32) = 103.6
	Time	< **0.0001**	9.667	3	F (3. 32) = 215.5
	Residuals		0.4785	32	
Carotenoids	Interaction	< **0.0001**	4.725	21	F (21. 64) = 5.241
	Treatment	< **0.0001**	10.79	3	F (3. 64) = 83.80
	Time	< **0.0001**	13.74	7	F (7. 64) = 45.72
	Residuals		2.748	64	
Glucose concentration	Interaction	0.9117	0.005911	2	F (2. 12) = 0.09317
	Treatment	**0.0127**	0.2713	1	F (1. 12) = 8.554
	Time	< **0.0001**	21.41	2	F (2. 12) = 337.5
	Residuals		0.3807	12	

Figure 1

Click here to access/download;Figure;Fig. 1.tiff

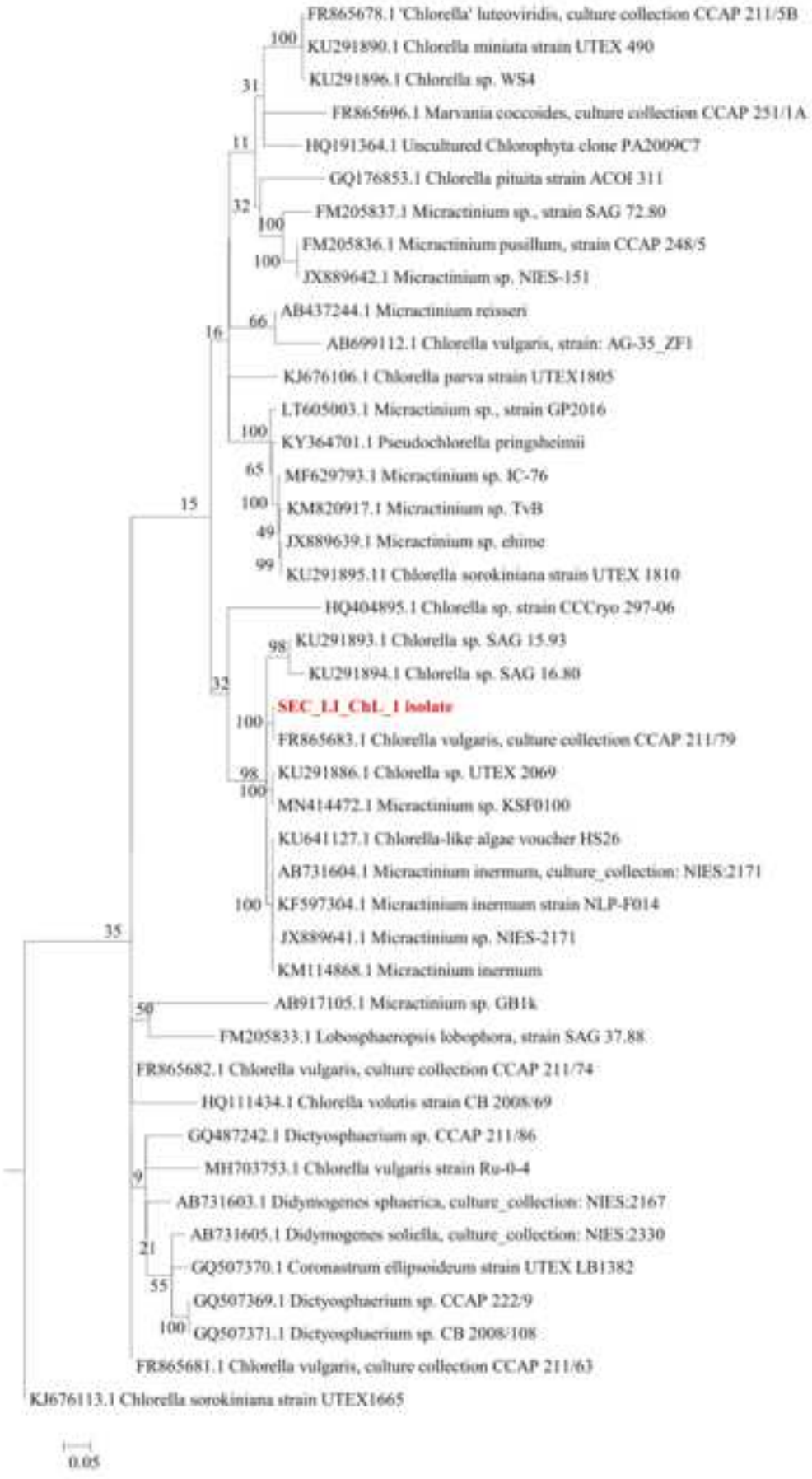


Figure 2

Click here to access/download;Figure;Fig. 2.tiff

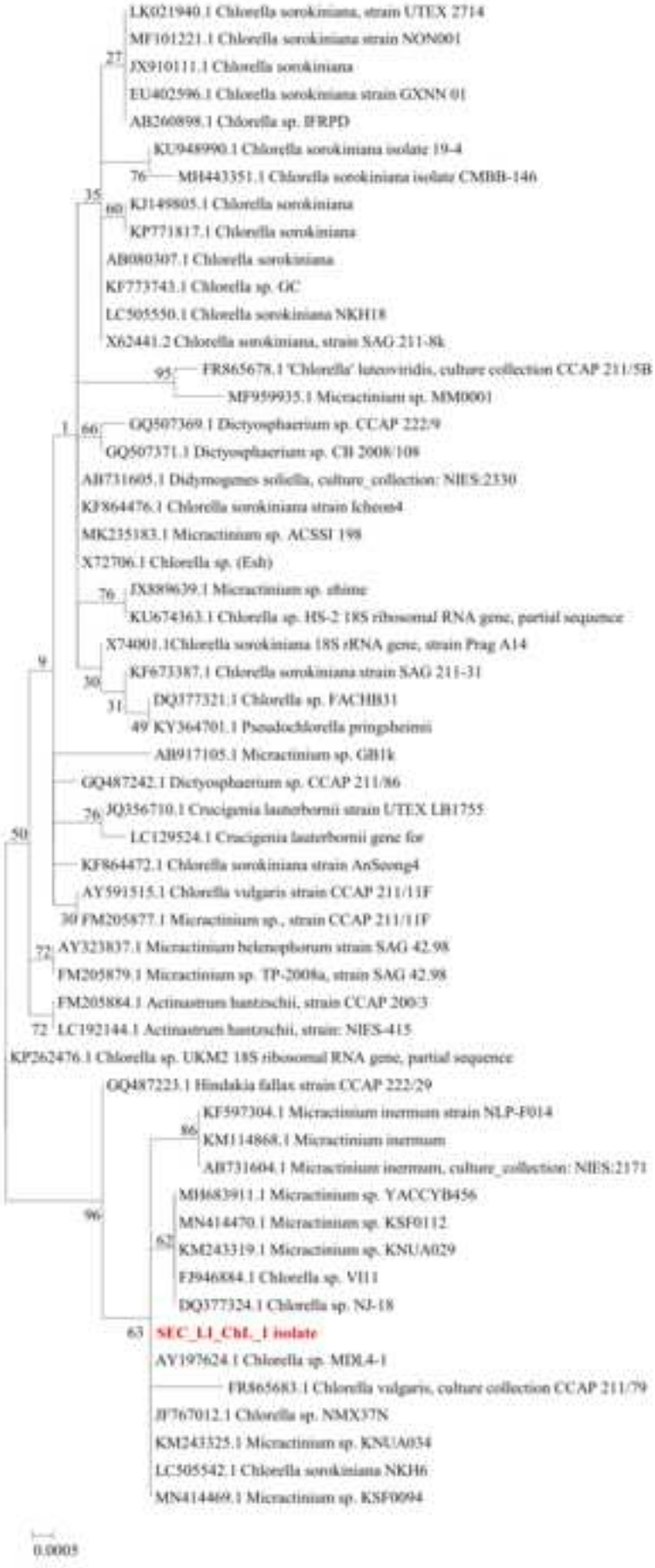


Figure 3

Click here to access/download;Figure;Fig. 3.tif

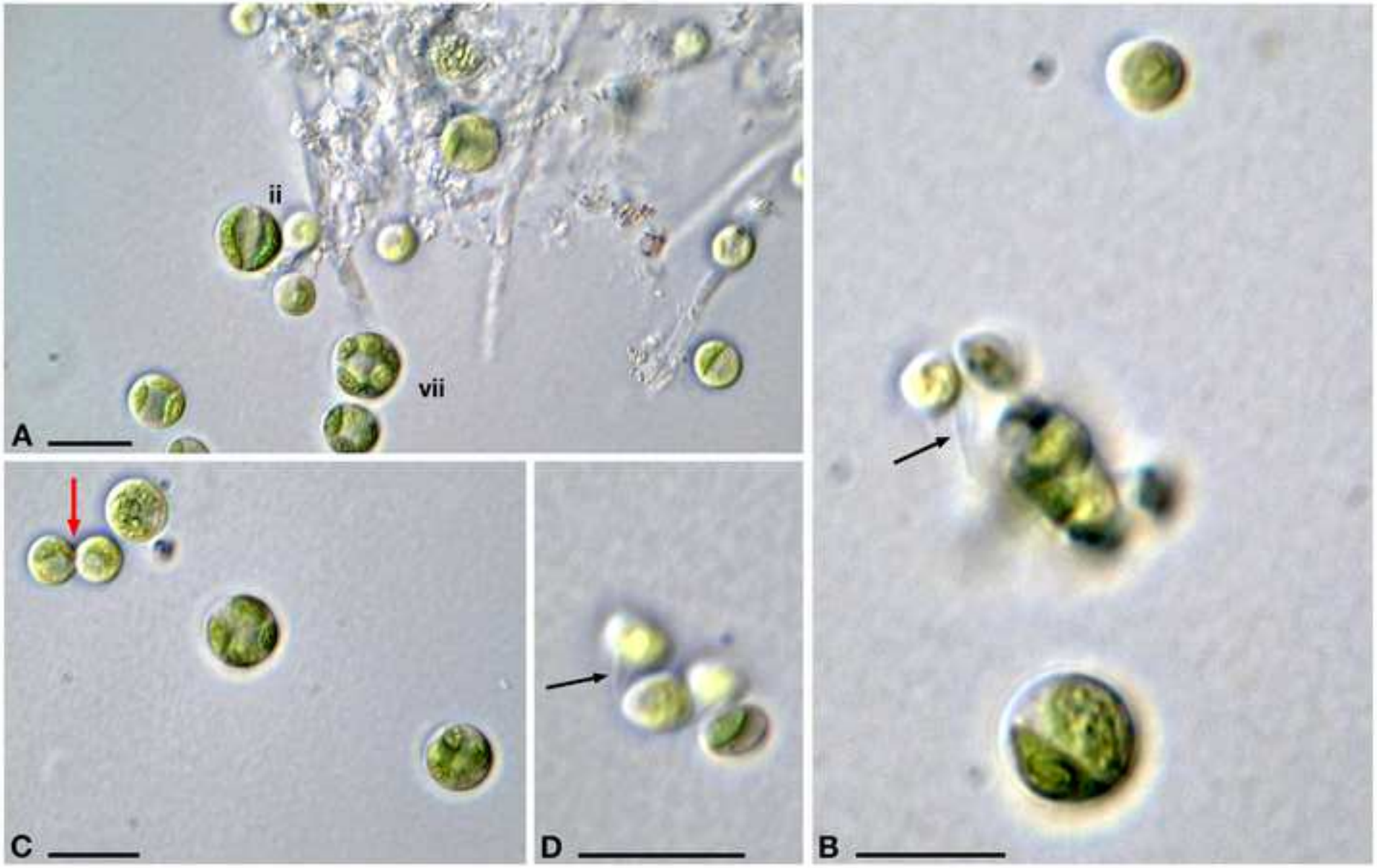


Figure 4

Click here to access/download;Figure;Fig. 4.tif

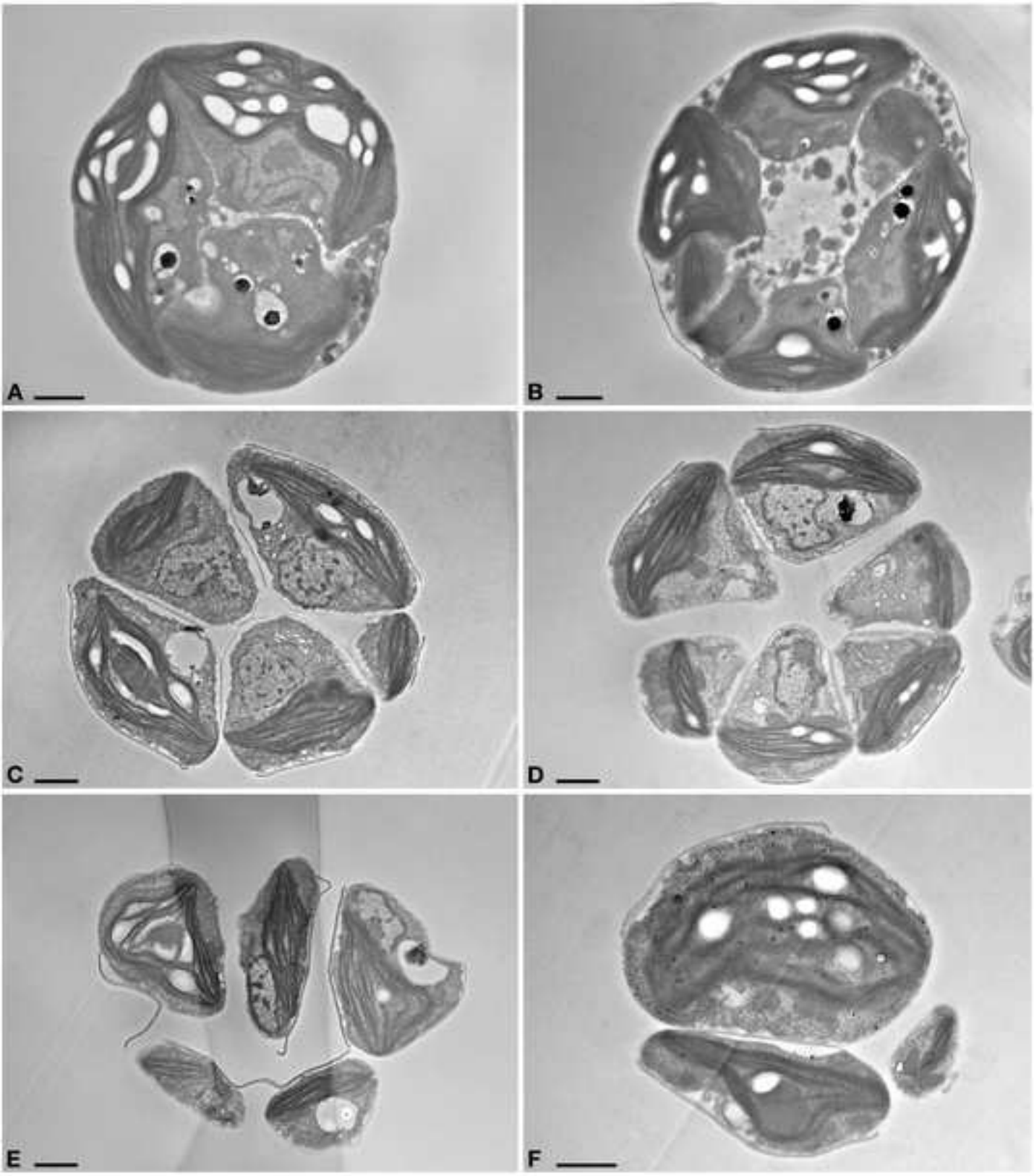


Figure 5

Click here to access/download;Figure;Fig. 5.tif

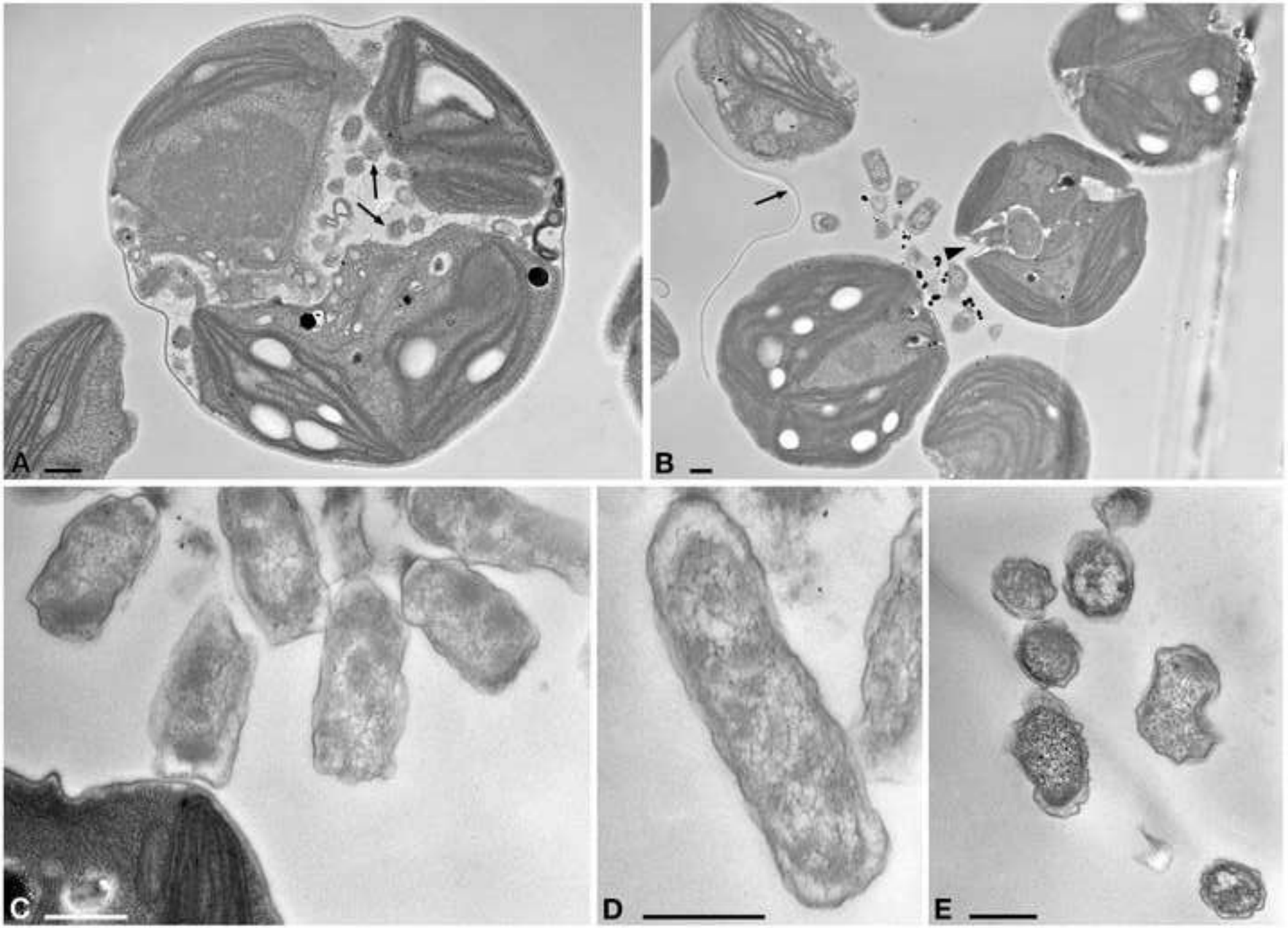


Figure 6

Click here to access/download;Figure;Fig. 6.tiff

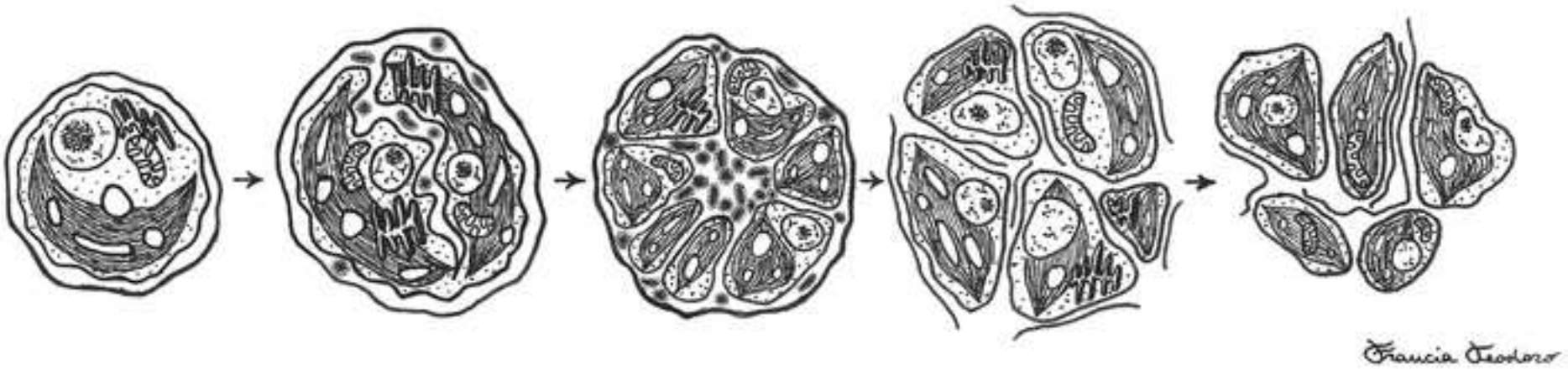
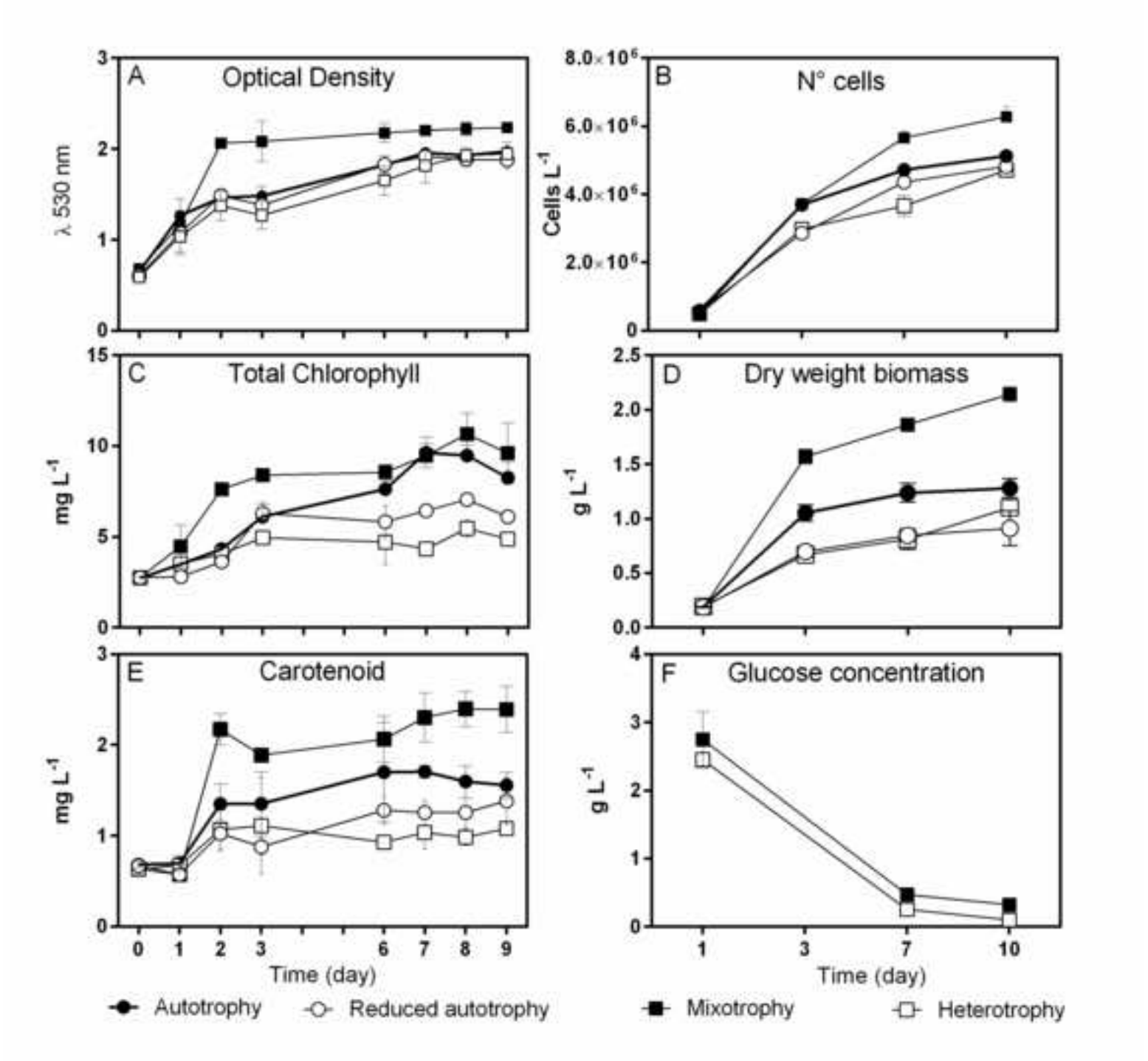


Figure 7

Click here to access/download;Figure;Fig. 7.tif



Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

Supplementary Fig S1

Click here to access/download

Supplementary Material

Fig. S1.tiff

Supplementary Fig S2

Click here to access/download
Supplementary Material
Fig. S2.tif

Supplementary Fig S3

Click here to access/download
Supplementary Material
Fig. S3.tif

Click here to access/download
Supplementary Material
Fig. S4.tif

Supplementary Fig S5

Click here to access/download

Supplementary Material

Fig. S5.tif

Supplementary Fig S6

Click here to access/download
Supplementary Material
Fig. S6.tif

Supplementary Fig S7

Click here to access/download

Supplementary Material

Fig. S7.tif