RESEARCH ARTICLE

A newly isolated *Chlorella* sp. from desert sand crusts exhibits a unique resistance to excess light intensity

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Abstract

We recently isolated a small green alga from a biological sand crust (BSC) in the NW Negev, Israel. Based on its 18S rRNA and rbcL genes, it is a close relative of *Chlorella sorokiniana* and of certain strains of *C. vulgaris* and *C. variabilis*, but differs substantially in many aspects from *C. sorokiniana.* Because the classification of *Chlorellales* is still not resolved, we designated this species as *C. ohadii* (*Trebouxiophyceae*) in honor of Professor Itzhak Ohad. Under controlled laboratory conditions, *C. ohadii* showed marked structural and photosynthetic performance changes, depending on the carbon source used during growth, as well as remarkable resistance to photoinhibition. CO_2-dependent O_2 evolution was not affected even when exposed to a light intensity of 3500 μmole photons m^{-2} s^{-1}, over 1.5 times the maximal intensity reached at the BSC surface, whereas the variable fluorescence declined sharply. We briefly discuss the use of fluorescence to assess photosynthetic rate and the implications of this finding for the assessment of global BSCs activity.

Introduction

Biological sand crusts (BSCs) play an important role in stabilizing sandy desert areas by reducing wind erosion (Belnap & Gillette, 1998) and by their impact on biotic compositions (Eldridge & Greene, 1994; Prasse & Bornkamm, 2000; Eldridge & Leys, 2003; Belnap *et al.*, 2004). Destruction of these crusts is considered an important promoter of desertification in arid and semi-arid regions. The crusts are formed by adhesion of the soil particles to extracellular polysaccharides excreted mainly by filamentous cyanobacteria, the main primary producers in desert crusts. Other microorganisms, including fungi, microalgae, lichens, and bacteria, are also abundant, particularly in humid areas that are often covered by a thick crust [(Büdel, 2002; Büdel & Veste, 2008; Bates *et al.*, 2012) and references therein].

biological sand crusts represent one of the harshest environments in nature. Organisms inhabiting this ecosystem face frequent hydration/dehydration cycles, extreme light intensities, temperature amplitude from subfreezing during winter nights but up to 60 °C in mid-summer days, and vast osmotic potential changes from close to pure rainwater to salt crystals on the crust's upper layer (Büdel & Veste, 2008). To cope with such conditions, the organisms inhabiting the BSCs likely possess survival mechanisms, the nature of which remains largely unknown (Potts, 2001; Harel *et al.*, 2004; Ohad *et al.*, 2005; Wright *et al.*, 2005). Crucial for survival is the ability to sense diurnal changes in environmental conditions and rapidly activate metabolism and growth in the short periods when water and sufficient light intensity are available, but to turn metabolism off during desiccation (Potts, 2001).

Desert crusts are liable to experience rapid pH rise from around 8 and up to 10.5 (Garcia Pichel & Belnap, 1996) consequent on the photosynthetic activity, thereby reducing the dissolved CO_2 level within the crusts. To cope with the limiting CO_2 level, as compared with the high $K_{1/2}(CO_2)$ of the carboxylating enzyme ribulose-1,5 bisphosphate carboxylase/oxygenase (RubisCO), a CO_2 concentrating mechanism (CCM) (documented in many photosynthetic microorganisms) is likely activated to

enable efficient CO_2 fixation (Palmqvist *et al.*, 1994; Price *et al.*, 1998; Kaplan & Reinhold, 1999; Lange *et al.*, 1999; Beardall *et al.*, 2005; Giordano *et al.*, 2005; Kaplan *et al.*, 2008; Spalding, 2008; Raven, 2010; Fukuzawa *et al.*, 2012; Matsuda *et al.*, 2011). Indeed, a CCM was described in several crust cyanobacteria (Lange *et al.*, 1998; de Araujo *et al.*, 2011). The large fluxes of inorganic carbon and protons associated with CCM activity affect the pH homeostasis of the cells (Kaplan & Reinhold, 1999) and may help dissipate excess light energy (Fukuzawa *et al.*, 2012).

To cope with the combination of declining water availability and rising light intensity to levels much higher than that required to saturate photosynthetic demand, photosynthetic organisms inhabiting the BSCs must possess efficient mechanisms to dissipate excess light energy (Heber, 2008). As an example, exposure of BSC-inhabiting cyanobacterium *Microcoleus* sp. to excess light led to a remarkable light energy quenching while retaining a maximal oxygen evolution rate (Ohad *et al.*, 2010, 2011).

In search of photosynthetic organisms able to withstand harsh environmental conditions in the BSCs, we have isolated a new strain of *Chlorella* sp., a close relative of *C. sorokiniana* (Sorokin, 1959), and we describe here some of its remarkable characteristics.

Materials and methods

Cell isolation and cultivation

A green alga was isolated from a BSC sample taken near the Nizzana sand-dune field station of the Hebrew University Arid Ecosystems Research Centre (AERC), situated close to the Israeli-Egyptian border (30°56′N, 34°23′E, elevation 190 m m.a.s.l.; annual average rainfall about 100 mm). Crust samples (Fig. 1a) were placed in medium BG11 (Stanier *et al.*, 1971), typically used to grow cyanobacteria, in an attempt to raise axenic cultures of the filamentous cyanobacteria inhabiting the crust. Within the developing cultures, mostly consisting of *Microcoleus* sp. and *Leptolyngbia* sp., we observed a small green alga that was isolated and grown in a TAP (Tris, acetate, phosphate buffer) medium (Zito *et al.*, 1997) at 30 °C under continuous fluorescent light (200 μmol photons m^{-2} s^{-1}). Following several dilution\inoculation cycles and resuspension on agar plates containing TAP, we isolated an axenic culture of a small, about 2 μm in diameter, green alga shown in Fig. 1b. Growth experiments on various inorganic carbon concentrations were carried out using TAP medium without acetate with bubbling of air, or CO_2-enriched air (5% CO_2 in air), or with no forced aeration.

TEM imaging

Samples were centrifuged at 6000 ***g*** for 5 min in Eppendorf tubes. Pellets were fixed in 0.5 mL fixative (2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer 0.1 M, pH = 7.4) overnight. Pellets were then centrifuged at 10 000 ***g*** for 2 min, and the fixative was discarded, followed by incubation of the pellets in 0.5 mL fixative (1% OsO_4 in 0.1 M cacodylate buffer + 1.5% potassium ferricyanide) for 1 h. Samples were then incubated in ascending concentrations of ethanol for dehydration, embedded in resin molds, and baked at 60 °C for 48 h. Blocks were cut using LKB 8800 Ultrotome 3, and 70–90 nm sections were collected on 200-nm thin bar copper grids and then stained with 5% aqueous uranyl acetate solution and lead citrate. Thin sections were observed with a Tecnai 12 electron microscope (FEI Phillips, Eindhoven, the Netherlands) equipped with a Mega-View 2 CCD camera and Analysis version 3.0 software (Soft Imaging System GmbH, Münster, Germany).

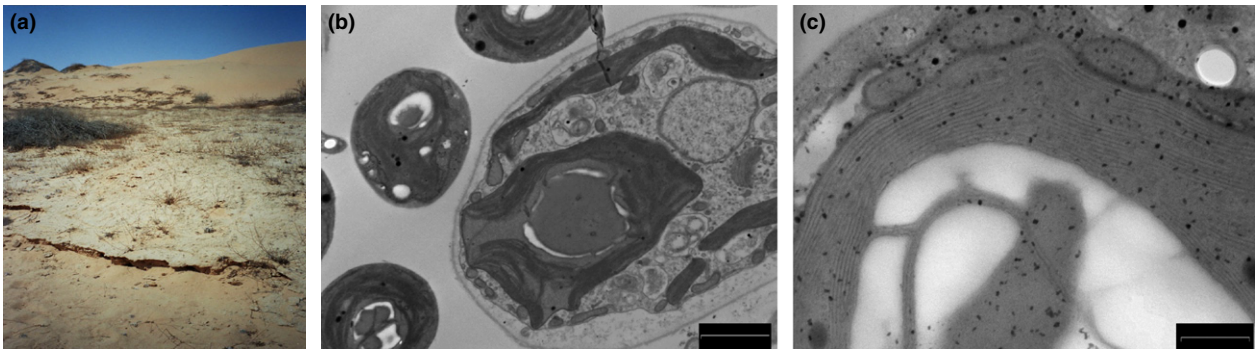

Fig. 1. The site where *Chlorella ohadii* was isolated from (a) and its appearance using electron microscopy (EM) (b and c). (a) A typical crust from which *C. ohadii* was isolated. (b) A general EM picture showing the *C. ohadii* cells next to *Chlamydomonas reinhardtii*. Note the difference in size. *Chlorella ohadii* diameter is *c.* 2 microns, while *C. reinhardtii* is about 7 microns. The bar represents 1 μm. (c) A closer look at *C. ohadii*, emphasizing the pyrenoids and the thylakoid layers. This picture shows cells grown under photoautotrophic conditions in the absence of acetate. The bar represents 0.2 μm.

Phylogenetic analyses using 18S rRNA gene and *rbcL* sequences

As part of a genomic project on *C. ohadii*, in progress, DNA was extracted, purified, and sequenced using 454 and Illumina technologies. The sequences of the 18S rRNA gene and of *rbcL* (a single copy on the chloroplast genome) encoding the large subunit of RubisCO were first compared with known sequences through the NCBI-BLAST alignment tool with a standard nucleotide collection (nr/nr) MEGABLAST query that identified it as a close relative to *Chlorella* species. Relevant gene sequences of the genus *Chlorella* were obtained from the Silva comprehensive ribosomal RNA database (Quast *et al.*, 2013). 18S rRNA gene sequences were aligned using the SINA aligner (Pruesse *et al.*, 2012) and rejected sequences below 70% identity. The *rbcL* (encoding the large subunit of RubisCO) sequences were aligned using T-Coffee (http://www.tcoffee.org) with default parameters. Maximum-likelihood phylogenetic trees were constructed using the MEGA5 software package with default parameters, 1000 bootstrap iterations, and the relevant *Chlamydomonas reinhardtii* genes as outgroup.

CO_2-dependent O_2 evolution

This was measured using a Clark-type O_2 electrode (PS2108, Passport dissolved O_2 sensor Roseville, CA) in a temperature-controlled perspex holder (optical path 0.7 cm, 30 °C). Light was provided to the cell suspension using an LED array (warm white, 3000K) of surrounding temperature-controlled glass columns (optical path 1.9 cm, 30 °C). CO_2 curves were established using ascending concentrations of sodium bicarbonate.

Fluorescence measurements

Light-induced fluorescence parameters were performed using an FL3000 fluorimeter (PSI, Brno, Czech Republic) as described in the study by Ohad *et al.* (2010). Cells corresponding to 0.8–0.9 $OD_{678\ nm}$, 5-mm optical path were used, and the light excitation intensity was 1800 μmol photons m^{-2} s^{-1} for 30 s.

Excess light treatments

These were carried out with cell suspension in a special chamber with a water jacket maintained at 30 °C. The cells were illuminated at the desired light intensity for various durations. Aliquots were withdrawn and placed in a chamber where O_2 exchange and chlorophyll fluorescence kinetics were measured.

Results and discussion

Phylogeny and growth

The presence of green algae together with cyanobacteria, bacteria, lichens, and even mosses in BSCs has long been documented (Danin *et al.*, 1992; Belnap *et al.*, 2004; Gundlapally & Garcia-Pichel, 2006; Langhans *et al.*, 2009). A small green alga (shown here next to a *Chlamydomonas reinhardtii* cell, Fig. 1b) was isolated and grown in axenic cultures. DNA was isolated and sequenced using 454 and Illumina technologies, and the sequences of 18S rRNA gene and *rbcL* (encoding the large subunit of RubisCO were examined and compared with those available from databases (Fig. 2). The 18S rRNA gene analysis (Fig. 2a) revealed close similarity to *Chlorella sorokiniana* (formerly *C. pyrenoidosa*) (Sorokin, 1959) but also to *C. vulgaris* and *C. variabilis*. Generally, the analysis based on the *rbcL* sequences showed larger phylogenetic span, actually suggesting three subgroups among the *Chlorella* species examined here. With the classification of Chlorellales still not fully resolved (Wolf *et al.*, 2003; Krienitz *et al.*, 2004; Marin, 2012) and in view of our phylogenetic analyses (Fig. 2), we designated the newly isolated species as *Chlorella ohadii* (*Trebouxiophyceae*) in honor of Prof. Itzhak Ohad who has made an enormous contribution to our understanding of photosynthetic machinery and was the first to isolate and study this organism.

Despite the homology, in many aspects, *C. ohadii* can be easily distinguished from *C. sorokiniana* strains UTEX1663 and SAG 211-8k obtained from culture collections. Below are a few examples. Cells of *C. ohadii*, about 2 μm in diameter, are smaller than those of *C. sorokiniana*, *C. vulgaris*, or *C. variabilis* and possess far less starch bodies than those observed in *C. sorokiniana* (Rabinowitch *et al.*, 1983; de-Bashan *et al.*, 2002). When placed in glass tubes without agitation, *C. sorokiniana* sedimented much faster in the water column than *C. ohadii* (Fig. 3), possibly as a consequence of the larger amount of starch bodies in the former. Taken together – the size, appearance, stability in the water column, and other physiological parameters – it is clear that the two closely related *Chlorella* sp. strains differ from one another.

CO_2 fixation and photoinhibition

Inspection of photoautotrophic growing *C. ohadii* cells aerated with air levels of CO_2, as in Fig. 1b and c, showed two properties of this organism that likely affect its photosynthetic performance: very large pyrenoids and highly condensed thylakoids. The pyrenoids play an important role in the algal CO_2 concentrating mechanism (CCM), a process that enables efficient photosynthetic

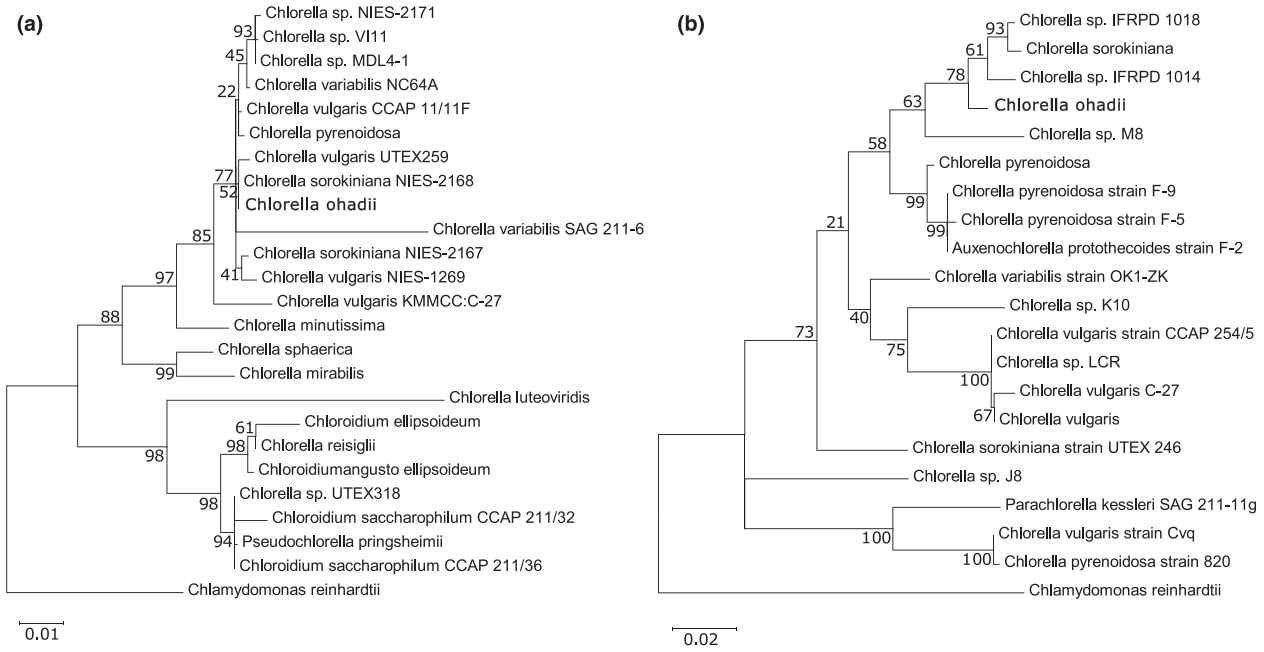

Fig. 2. Phylogenetic analyses based on *Chlorella* 18S rRNA gene (a) and *rbcL* (b) sequences, with *Chlamydomonas reinhardtii* used as an outgroup. Bootstrap consensus trees are shown with support values provided for each node.

CO_2 utilization (Kaplan & Reinhold, 1999; Giordano *et al.*, 2005; Spalding, 2008). In *Chlamydomonas reinhardtii*, often used as a model system to study photosynthesis and where this aspect was intensively investigated (Rawat *et al.*, 1996), most of the carboxylating enzyme, RubisCO, is located in these bodies (Smith & Griffiths, 1996; Kaplan & Reinhold, 1999; Moroney & Somanchi, 1999; Raven *et al.*, 2002; Hanson *et al.*, 2003; Yang & Gao, 2003; Giordano *et al.*, 2005; Xia & Gao, 2005; Ma *et al.*, 2011; Meyer *et al.*, 2012). The thylakoids traversing the pyrenoids bear carbonic anhydrase (Duanmu *et al.*, 2009), which facilitates the formation of CO_2 from bicarbonate in close proximity to RubisCO, thus leading to an elevated apparent photosynthetic affinity for extracellular CO_2 (Raven *et al.*, 2002). Indeed, analyses of the CO_2 response curves (Fig. 4) showed a very high apparent photosynthetic affinity for external inorganic carbon, $K_{1/2}$ (HCO_3^-) = 12 μM. Given that the pH in the upper crust layer during photosynthesis is 8.5 or higher [see (Garcia Pichel & Belnap, 1996), consistent with our own measurements] and assuming equilibrium between the various inorganic carbon species, this is equivalent to *c.* 100 nM CO_2, a higher apparent photosynthetic affinity than that observed using *C. reinhardtii* (Badger *et al.*, 1980) or various *Chlorella* sp. (Beardall *et al.*, 1991; Rotatore & Colman, 1991; Matsuda & Colman, 1995; Badger *et al.*, 1998; Spijkerman, 2011). The photosynthetic Vmax (the maximal rate of photosynthesis under light and CO_2 saturating conditions and optimal temperature) observed here is, to the best of our knowledge, among the highest ever reported in phytoplankton.

In its natural habitat, *C. ohadii* is exposed to excess light intensity with rising sunlight concomitant with dehydration. Chlorophyll fluorescence yield (Fv/Fm) is often used to assess the activity of the photosynthetic reaction center II (PSII) and even to calculate a 'relative electron transfer rate', rETR. Fluorescence has also been used to assess the photosynthetic activity in lichens (Heber *et al.*, 2007) and desert soil crusts (Tang *et al.*, 2007; Ohad *et al.*, 2010). Figure 5 shows a typical experiment where the fluorescence parameters and O_2 exchange of *C. ohadii* culture were simultaneously examined during exposure to excess light intensity (3500 μmole photons m^{-2} s^{-1}) in the presence of a saturating Ci level (1 mM $NaHCO_3$, Fig. 4). Following exposure to this excess illumination, the CO_2-dependent rate of O_2 evolution increased significantly, perhaps reflecting the accumulation of a relatively reduced end product. These data clearly indicate that the photosynthetic machinery in *C. ohadii* is not sensitive to excess light as high as used here, close to twice the maximal sun light intensities observed under natural desert conditions. We are not aware of any photosynthetic organism, including various algae such as *C. reinhardtii* (Ohad *et al.*, 2011), *Chlorella pyrenoidosa*, and *Scenedesmus obliquus* (Yang & Gao, 2003), which is able to withstand such illumination without marked photodamage to photosystem II.

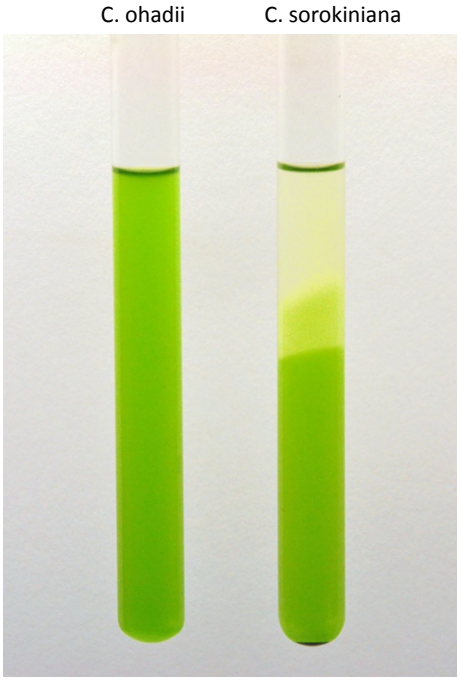

Fig. 3. Sedimentation in the water column. Cells of *Chlorella sorokiniana* (left) and *C. ohadii* (right) were grown under identical conditions (TAP medium, 30 °C, agitation at 100 r.p.m., light intensity 200 μmole photons m^{-2} s^{-1}) and then diluted to the same optical density (1.9 $OD_{750\ nm}$) and transferred into the glass tubes. The picture, taken 24 h thereafter, shows that almost all the *C. sorokiniana* cells are at the bottom of the tube, unlike *C. ohadii*.

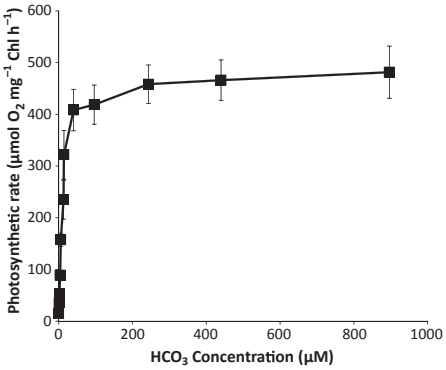

Fig. 4. The rate of photosynthetic O_2 evolution as a function of the inorganic carbon concentration experienced by the cells. Consecutive injections of $NaHCO_3$ were provided to *C. ohadii* cells grown in a TAP medium without acetate and aerated with air. Temperature was 30 °C, and light intensity, provided with quartz halogen white light, was 1300 μmole photons m^{-2} s^{-1}.

In contrast to O_2 evolution, the variable fluorescence declined sharply to 20% of its original value (0.66 ± 0.02) within 20 min, exactly the time required to reach maximal O_2 evolution. Uncoupling of the fluorescence

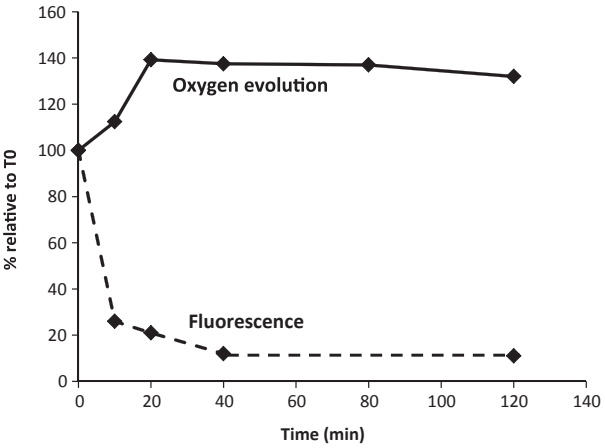

Fig. 5. Photosynthetic O_2 evolution and variable fluorescence (Fv) as affected by the duration of exposure to 3500 μmole photons m^{-2} s^{-1}. The 100% corresponded to Fv = 0.66 ± 0.024 and an O_2 evolution rate of 350 ± 15 μmole O_2 mg^{-1} Chl h^{-1}, n = 6.

yield from O_2 evolution during exposure to stress was observed in several other cases such as the diatom *Phaeodactylum tricornutum* (Eisenstadt *et al.*, 2008), the BSCs-inhabiting cyanobacterium *Microcoleus vaginatus* (Ohad *et al.*, 2010), and *Synechocystis* sp. strain 6803 (Maenpaa *et al.*, 1995) often used as model cyanobacteria. Clearly, at least in some of the phytoplankton species examined so far, the fluorescence yield does not represent the activity of PSII. Uncoupling of O_2 evolution from fluorescence yield may indicate activation of mechanisms that enable dissipation of excess light energy, thereby avoiding damage to the photosynthetic machinery. As indicated, the amount of thylakoids in *C. ohadii* and their stacking are remarkable. Cases where 14–20 thylakoid membranes (stroma lamella) are appressed together are quite common (Fig. 1c), unlike all other green algae that we are familiar with. It is not known whether self-shading may explain why *C. ohadii* is relatively resistant to photodamage by excess light.

Survival and global impact

Many of the algal strains used as model organisms or in various growth facilities such as *Chlamydomonas* sp. or *Chlorella* sp. were originally isolated from soil samples, where they frequently experience large fluctuations in water availability and may even face desiccation. The sand-dune belt where *C. ohadii* was isolated from is one of the harshest environments for algal growth, with frequent hydration (by early morning dew) and desiccation and very high surface temperatures during the summer. The means by which they are able to cope with this environment is poorly understood. However, their ability to

resume activity and perform a high photosynthetic rate and growth during the short time in which both humidity and light are available is likely to affect their survival in this habitat. This may determine the fitness of *C. ohadii* in the BSC and affect its abundance in the field.

Fluorescence parameters are widely used to assess global aquatic and terrestrial productivity (Karnieli, 1997; Karnieli *et al.*, 1999; Grace *et al.*, 2007; Malenovsky *et al.*, 2009; Garbulsky *et al.*, 2011). Based on these analyses, it is widely accepted that BSC contributes little to global productivity, but this may not be the case (Yoshitake *et al.*, 2010; Elbert *et al.*, 2012; Williams & Büdel, 2012). In view of the uncoupling of fluorescence yield from photosynthetic productivity in both *C. ohadii* (Fig. 5) and *M. vaginatus* (Ohad *et al.*, 2010) and other filamentous cyanobacteria inhabiting the BSCs (H. Raanan, preparation), we submit that the contribution of BSC organisms to the global carbon cycle should be re-assessed.

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