Light Acclimation of the Colonial Green Alga
Botryococcus braunii Strain Showa¹[OPEN]

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In contrast to single cellular species, detailed information is lacking on the processes of photosynthetic acclimation for colonial algae, although these algae are important for biofuel production, ecosystem biodiversity, and wastewater treatment. To investigate differences between single cellular and colonial species, we studied the regulation of photosynthesis and photoprotection during photoacclimation for the colonial green alga Botryococcus braunii and made a comparison with the properties of the single cellular species Chlamydomonas reinhardtii. We show that B. braunii shares some high-light (HL) photoacclimation strategies with C. reinhardtii and other frequently studied green algae: decreased chlorophyll content, increased free carotenoid content, and increased nonphotochemical quenching (NPQ). Additionally, B. braunii has unique HL photoacclimation strategies, related to its colonial form: strong internal shading by an increase of the colony size and the accumulation of extracellular echinone (a ketocarotenoid). HL colonies are larger and more spatially heterogeneous than low-light colonies. Compared with surface cells, cells deeper inside the colony have increased pigmentation and larger photosystem II antenna size. The core of the largest of the HL colonies does not contain living cells. In contrast with C. reinhardtii, but similar to other biofilm-forming algae, NPQ capacity is substantial in low light. In HL, NPQ amplitude increases, but kinetics are unchanged. We discuss possible causes of the different acclimation responses of C. reinhardtii and B. braunii. Knowledge of the specific photoacclimation processes for this colonial green alga further extends the view of the diversity of photoacclimation strategies in photosynthetic organisms.

Evolutionary differences in microalgae photosynthesis are directed by the local light environment. Variations in light intensity and quality combined with the availability of nutrients have shaped a range of fine-tuned algae, adapted to their environmental niches (Croce and van Amerongen, 2014). Within this range of different algae types, many different shapes and sizes are found, from unicells no larger than 1 μm to multicellular and colonial species of several centimeters (Beardall et al., 2009). While data on unicellular species provide us with an emerging view on the variations of the photosynthetic apparatus and its regulation, we know little about multicellular and colonial microalgae that have to deal with increased light attenuation (within colonies) and decreased diffusion of nutrients (Beardall et al., 2009).

One of these freshwater colonial microalgae is Botryococcus braunii (Trebuoxiphyceae, Chlorophyta; Weiss et al., 2010), found in lakes and ponds throughout different climate zones (Metzger and Largeau, 2005). This alga has been targeted for biofuel production since the 1970s because it produces high-quality long-chain hydrocarbons, which are excreted in the extracellular matrix (botryococccenes; Metzger et al., 1985). The oil content of B. braunii colonies can be very high (30%–40% of its dry weight; Metzger and Largeau, 2005) and makes the colonies float close to the water surface, where they can be subjected to high-light (HL) intensities (Wake and Hillen, 1980). Their growth rates are prohibitively low for commercial utilization, despite numerous studies aimed at optimizing...
growing conditions (Sakamoto et al., 2012; Yoshimura et al., 2013; Kim et al., 2014) and despite the occurrence of natural blooms (Wake and Hillen, 1980). A better understanding of the biology of this alga could identify factors that limit growth and lead to selection of targets for strain improvement (Barry et al., 2015).

Here, we focused on the Berkeley strain (Showa) of B. braunii (Nonomura, 1988) because it is one of the best-studied strains (Race B; Metzger and Largeau, 2005) with one of the highest growth rates, and its genome was recently sequenced (Browne et al., 2017). The growth rate depends on light conditions: upon light intensity rise, the growth rate increases up to a saturation level and then a drop is observed (Sakamoto et al., 2012; Yoshimura et al., 2013; Kim et al., 2014). This drop indicates a decrease in photosynthetic efficiency in HL. Colony size increases in HL (Zhang and Kojima, 1998), possibly as a photoprotective strategy (Khatri et al., 2012; Yoshimura et al., 2013; Kim et al., 2014), and this may be related to the observed decrease in photosynthetic efficiency.

We recently isolated and characterized the light-harvesting antenna complexes (LHC) of B. braunii (van den Berg et al., 2018). LHCs are responsible for most of the light absorption in green algae and transfer the resulting excitation energy to the reaction centers of PSI and PSII, where it initiates electron transport coupled with the formation of a transmembrane proton gradient (light reactions). These electrons and protons are used by the ferredoxin-NADP+ reductase and the ATPase to produce the NADPH and ATP necessary for carbon fixation (dark reactions). Some LHCs are specifically associated with a single photosystem forming supercomplexes, such as LHCl in PSI-LHCl (e.g. Chlamydomonas reinhardtii and plants). The major antenna (LHCCI) can be associated with both photosystems (Wientjes et al., 2013a; Drop et al., 2014). General LHC features such as the oligomerization state and chlorophyll (Chl) a/b ratio are conserved in B. braunii, but the carotenoid composition and the stability of the trimers are different from that of the LHCs of C. reinhardtii (van den Berg et al., 2018).

Photosynthetic organisms respond to fast changes in the local light environment by activating photoprotective mechanisms (for review, see Wobbe et al., 2016). A central process of photoprotection is non-photochemical quenching (NPQ) of excitation energy. Regulated NPQ is activated by the acidification of the lumen and reduces the formation of reactive oxygen species by the reduction of the lifetime of the Chl excited state. Multiple components are involved in NPQ in different organisms and act on different time scales (Quaas et al., 2015; Christa et al., 2017; Kress and Jahns, 2017; Farooq et al., 2018). State transitions, in which LHCs redistribute between PSI and PSII (Goldschmidt-Clermont and Bassi, 2015), take several minutes and also may be photoprotective in HL in algae (Alloret et al., 2013). Sustained changes in light conditions lead to long-term (up to several generations) photoacclimation. Photoacclimation induces changes in the photosynthetic machinery that take place on a time scale of hours to days. Such changes concern (1) the amount of photosynthetic protein per cell (e.g. modulation of antenna size and composition and change of the PSI/PSII ratio; Anderson and Andersson, 1988); (2) the amount and content of proteins involved in NPQ (Peers et al., 2009) and carbon-concentrating mechanisms (Yamano et al., 2008); and (3) free carotenoid content (not bound by protein; Ben-Amotz and Avron, 1983). Together, these changes balance light absorption and electron transport to optimize photosynthesis under new sustained light conditions.

Detailed knowledge of photoacclimation strategies is limited to a few single cellular algae (Meneghesso et al., 2016; Polukhina et al., 2016), and these strategies depend on the environment from which the alga was isolated; thus, our view of the diversity in photoacclimation strategies could be limited. Moreover, recent results show that the diversity of photoprotective mechanisms in green algae is even larger than previously thought (Peers et al., 2009; Tibiletti et al., 2016; Christa et al., 2017). Therefore, detailed studies of algae ranging from unicellular to colonial and multicellular, obtained from multiple environments, are essential to understand photoacclimation strategies. In B. braunii, this may help increase growth rates by light control and strain selection.

We measured pigment content, colony size distributions, and NPQ combined with time-resolved fluorescence and microscopy during photoacclimation to different light intensities (low [LL], medium [ML], and HL). We conclude that B. braunii employs numerous general HL acclimation strategies, such as the reduction of Chl content and the increase of NPQ in combination with additional unique strategies. Colony size increase and accumulation of extracellular echinonene (Echi) content, unique HL acclimation strategies of B. braunii, reduce the light intensity experienced by the majority of the cells, leading to lateral differentiation in Chl content and antenna size in the colony. Such an acclimation strategy could possibly be more common for nonmotile colonial algae that live in environments where shade is minimal.

RESULTS

Growth Kinetics

The effect of acclimation to different growth light intensities on biomass production was assessed by measuring the dry weight of the cultures. A culture grown for 30 d at ML was split into five daughter cultures (day 0), which were then each grown under different light intensities: three times lower (LL; two cultures), three times higher (HL; two cultures), or unchanged (ML; one culture). The specific growth rate in the exponential growth phase (days 0–18) was determined by the best fit with an exponential growth function (Fig. 1A). Surprisingly, no lag phase was observed for LL and HL. The growth rates (Fig. 1B) for the
exponential growth phase were similar for all light intensities, meaning that the extra light in ML and HL was not effectively used for growth.

Colony Size

Colony size distributions at day 0 and 20 d after the change in light intensity (Fig. 2) showed that acclimation to LL leads to smaller colonies compared with ML and HL colonies. For LL acclimation, the volume distribution of the diameter of the colony shows two maxima: one at a similar position as for the ML starting culture and one at 5 to 6 times smaller diameter. This indicates the presence of a fraction of the original colonies together with newly budded smaller colonies. An independent longer experiment (34 d of acclimation in the three different light conditions) showed a single distribution for LL colonies with the maximum around 100 μm, confirming that LL acclimation leads to the formation of smaller colonies (Supplemental Fig. S1).

Pigment Content

Light stress and photoacclimation in algae and plants affect pigment content as a result of changes in the composition of the photosynthetic complexes in the membrane and of the synthesis of new carotenoids (Ballottari et al., 2007; Bonente et al., 2012; Wientjes et al., 2013b; Meneghesso et al., 2016; Polukhina et al., 2016).

The carotenoid content was monitored at four time points (5, 14, 21, and 41 d) during acclimation in all light conditions. All cells contained neoxanthin (Neo), lорoroxanthin (Lor), violaxanthin (Vio), lutein (Lut), zeaxanthin (Zea), Eχи, and β-carotene (β-Car) at all stages, although their relative abundance varied. The carotenoid composition did not change during the exponential growth phase (0–18 d) in ML (Supplemental Table S1).

In LL, the amounts of Neo+Lor, Vio, and β-Car normalized to Chl are higher, and Lut, Eχи, and Zea are lower, than in ML. The most substantial changes occur in the first 5 d of acclimation (Table 1). The Eχи/Chl ratio decreases sharply and remains stable during exponential growth (0–18 d). At the beginning of growth limitation, toward the stationary phase, it increases again. This is in good agreement with the reported increase of Eχи/total carotenoids in the intermediate or early stationary phase (Grung et al., 1989).

In HL, the Zea content increases substantially in the first 5 d of acclimation, while both the Neo+Lor and Vio contents remain as in ML and decrease only after the exponential growth phase. The Eχи content increases within 5 d, remains constant during the exponential growth phase, and increases further in the stationary phase (day 41).

In summary, two phases can be observed: the acclimation to the new light condition, leading to changes in
pigeon composition within 5 d, and the transition toward the stationary phase, leading to further changes in pigment composition when growth rate decreases.

The pigment content of the acclimated cultures in fed-batch is presented in Table 2. The Chl-to-dry weight ratio was ~2 times higher in LL-acclimated cells than in ML-acclimated cells, but it was only slightly lower in HL-acclimated cells than in ML-acclimated cells. This suggests an increase in the number of photosynthetic complexes in LL and a decrease of photosynthetic complexes in HL. The Chl-to-carotenoid ratio (Chl/Car) was in all cases below 2.3, which is lower than in most photosynthetic complexes of algae and plants (Nicol and Croce, 2018). This is most probably due to the presence of free carotenoids such as Echi, which is located in the extracellular matrix (Grung et al., 1994). The relative amount of Echi increases even more in HL, further decreasing the Chl/Car ratio (Table 1). The change in Chl $a/b$ ratio upon acclimation to LL or HL is small.

**Maximum Quantum Yield of PSII and NPQ**

Next, we measured maximum quantum yield of PSII ($F_{v}/F_{m}$) and NPQ values (Table 3) to assess how light acclimation affects photosynthetic performance. These values mainly represent surface cells because the measuring light (460 nm) does not penetrate deep into the colonies, especially for ML and HL cells, which contain large amounts of Echi (see Supplemental Material). $F_{v}/F_{m}$ was 0.68 for the LL culture, close to the values reported for other algae (Parkhill et al., 2001; Bonente et al., 2012; Meneghesso et al., 2016; Polukhina et al., 2016), and it decreased with increasing growing light intensity (LL > ML > HL), which may indicate photoinhibition. Both $F_{v}/F_{m}$ and the value of NPQ were similar at all measurement time points during acclimation in all cultures, indicating that they reach a new constant value within 5 d after the transition from ML.

The capacity of NPQ was assessed during light acclimation using blue (460 nm, 520 μmol photons m$^{-2}$ s$^{-1}$) and red (635 nm, 550 μmol photons m$^{-2}$ s$^{-1}$) actinic light (AL). In both conditions, NPQ was fully reversible in all cultures, indicating that no photoinhibition occurred within the 12 min of light exposure (Fig. 3). A total of 550 μmol photons m$^{-2}$ s$^{-1}$ red light was sufficient to induce the maximum NPQ in all cultures (Supplemental Fig. S2A), although the level was higher in HL (1.8) than in ML (1.5) and LL (1.3). In contrast, the induction kinetics of NPQ were similar in all cultures at the same AL intensity (Supplemental Fig. S2B). The contributions of the xanthophyll cycle and state transitions to the NPQ induction curves are possibly small or absent because the fast phase is much larger than the slow phase (Fig. 3).

The level of NPQ induced by blue light was far smaller than by red AL (Fig. 4) in ML and HL cultures but not in LL cultures. The induction kinetics were also different. This can be explained by the presence of pigments (e.g. Echi) that absorb blue light (but not red) and do not transfer energy to the photosynthetic apparatus, effectively acting as a sunscreen. This is qualitatively confirmed by the difference between the fluorescence excitation spectra of the acclimated cultures (Fig. 4, right). An effect from the changes in scattering can be excluded because the absorption spectra of the cultures measured in an integrating sphere (Supplemental Fig. S3) show increased absorption < 550 nm in HL compared with LL, in line with the pigment composition.

In summary, the ML- and HL-acclimated cultures increased their NPQ capacity and displayed a sign of light stress, namely, reduced $F_{v}/F_{m}$ after dark acclimation. Echi accumulated in HL and had a screening function for high-energy photons.

**Time-Resolved Fluorescence**

The small changes in the Chl $a/b$ ratio observed during light acclimation (Table 2) can be explained in three ways: (1) the change in antenna size and/or PSI/PSII ratio is small, as is the case in *C. reinhardtii* when grown photoautotrophically (Polukhina et al., 2016); (2) the change of the antenna size is compensated for by a change of the PSI/PSII ratio (PSI has a higher Chl $a/b$ ratio than PSII in all organisms analyzed so far); and (3) the

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**Table 1. Changes in carotenoid content (relative to 100 Chls) during light acclimation to LL or HL during the culture period**

ML is the aggregate result of measurements at different days (Supplemental Table S2). Error margins represent the SD of n (first column) technical repetitions in two biological replicas. Neo and Lor as well as $\beta$-Car and $\beta$-E-car were not separated in the chromatogram and are therefore quantified together.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Neo+Lor</th>
<th>Vio</th>
<th>Lut</th>
<th>Zea</th>
<th>$\beta$-Car</th>
<th>Echi</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML day 0–42 (n = 8)</td>
<td>6.5 ± 0.4</td>
<td>2.6 ± 0.2</td>
<td>21.0 ± 1.0</td>
<td>2.3 ± 0.3</td>
<td>4.0 ± 1.0</td>
<td>34.0 ± 5.0</td>
</tr>
<tr>
<td>HL day 5 (n = 3)</td>
<td>5.6 ± 0.4</td>
<td>2.6 ± 0.2</td>
<td>23.3 ± 0.2</td>
<td>4.0 ± 0.4</td>
<td>5.6 ± 0.1</td>
<td>48.0 ± 2.0</td>
</tr>
<tr>
<td>HL day 14 (n = 3)</td>
<td>6.9 ± 0.7</td>
<td>3.0 ± 0.2</td>
<td>25.0 ± 2.0</td>
<td>2.9 ± 0.8</td>
<td>3.6 ± 0.1</td>
<td>48.0 ± 4.0</td>
</tr>
<tr>
<td>HL day 21 (n = 3)</td>
<td>5.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>22.5 ± 0.2</td>
<td>4.5 ± 0.4</td>
<td>5.5 ± 0.1</td>
<td>49.0 ± 0.9</td>
</tr>
<tr>
<td>HL day 41 (n = 4)</td>
<td>4.2 ± 2.7</td>
<td>1.6 ± 0.0</td>
<td>24.7 ± 0.3</td>
<td>5.3 ± 0.4</td>
<td>5.2 ± 0.4</td>
<td>62.0 ± 1.0</td>
</tr>
<tr>
<td>LL day 5 (n = 3)</td>
<td>11.4 ± 0.1</td>
<td>3.9 ± 0.0</td>
<td>13.0 ± 2.0</td>
<td>0</td>
<td>4.4 ± 0.2</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>LL day 14 (n = 4)</td>
<td>10.8 ± 0.4</td>
<td>4.6 ± 0.1</td>
<td>10.0 ± 0.8</td>
<td>0</td>
<td>5.6 ± 0.3</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>LL day 21 (n = 4)</td>
<td>10.0 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td>10.4 ± 0.5</td>
<td>0</td>
<td>6.1 ± 0.6</td>
<td>14.0 ± 2.0</td>
</tr>
<tr>
<td>LL day 41 (n = 4)</td>
<td>8.1 ± 0.4</td>
<td>3.7 ± 0.1</td>
<td>14.6 ± 0.5</td>
<td>0</td>
<td>6.1 ± 0.2</td>
<td>25.7 ± 0.8</td>
</tr>
</tbody>
</table>
antenna size and/or PSI/PSII ratio varies within a colony for cells at different positions, as was observed in colonies of the cyanobacteria *Nostoc sphaeroides* (Deng et al., 2008). To be able to discriminate between these possibilities, we measured time-resolved fluorescence by time-correlated single-photon counting (TCSPC) and fluorescence lifetime imaging microscopy (FLIM).

**TCSPC**

TCSPC was measured on colonies with open (F0) or closed (Fm) PSII reaction centers, with preferential excitation of LHCs (650 nm, Chl b) or photosystem cores (662 nm, Chl a) and with detection at 685, 700, and 715 nm. The PSII reaction center state (fully open [F0] or fully closed [Fm]) was confirmed by the lack of changes in the fluorescence decay curve at different excitation powers above and below the set values (example in Supplemental Fig. S4) and by comparing F0/Fm calculated from TCSPC (Table 4) with the values measured by pulse-amplitude modulated fluorometry (Table 3).

Fluorescence kinetics were fitted globally with a sum of exponential decays while linking lifetimes between multiple decay traces in F0 (see “Materials and Methods” and Supplemental Methods). Examples of decay traces are shown in Figure 5, demonstrating the slower decay in Fm compared with F0 and the differences between HL and LL. All decay traces and fit residuals are shown in Supplemental Figures S5 and S6, together with the decay-associated spectra (DAS; Supplemental Figs. S7 and S8). The results are shown in Supplemental Table S2.

The multiwavelength approach enables separation of the decay components associated with PSI and PSII thanks to spectral differences between the two complexes (van Oort et al., 2010; Ünlü et al., 2014). The lifetime of the first component is approximately 0.11 ns in all experiments. The corresponding DAS (DAS1; Fig. 6) increased toward longer wavelengths in all conditions, and the amplitude was lower for 650 nm than for 662 nm excitation. Together, these results indicate that DAS1 mainly originates from PSI, as was observed before in plants and *C. reinhardtii* (van Oort et al., 2010; Ünlü et al., 2014), with possibly a minor contribution of PSII at F0. The DAS1 amplitudes are similar for HL and LL colonies, showing that the PSI/PSII excitation ratio is the same in both samples (average difference in ratio DAS1/total between HL/LL in the same condition is 7% ± 4%) and suggesting that light acclimation does not affect the excitation ratio of PSI and PSII in *B. braunii*.

The PSII antenna size was previously assessed from the lifetimes and DAS attributed to PSII in F0 and their dependence on excitation wavelength (van Oort et al., 2010; Ünlü et al., 2014). However, the likely presence of photoinhibition complicates this analysis in *B. braunii* cultures. Indeed, the average decay times (\(<\tau>\)) of PSII (greater than 0.12 ns and less than 3 ns) detected at 685 nm, where PSII emission dominates, are counterintuitive (Table 4): \(<\tau>\) is longer for HL than for LL colonies, which would correspond to a larger antenna size in HL colonies, whereas the antenna size is typically larger for LL-grown plants or algae (Falkowski and Chen, 2003; Wientjes et al., 2013b). At Fm, \(<\tau>\) is longer for LL than for HL colonies. Thus, it appears that the HL colonies contain fluorescent species with less (or none) variable fluorescence and a lifetime longer than that of PSII at F0 but shorter than PSII in Fm. Possible candidates for such species are quenched antennas disconnected from the photosystems and photoinhibited photosystem. The difference between \(<\tau>\) at F0 and Fm between LL and HL is smaller for 650 nm than for 662 nm, indicating that the species is not likely to be an antenna.

In summary, the results suggest that the excitation ratio of PSI and PSII (including species without variable fluorescence) is the same for HL- and LL-acclimated colonies and that photoinhibited PSII is present in HL colonies.

### Table 2. The pigment content of fed-batch cultures fully acclimated to different light intensities

<table>
<thead>
<tr>
<th>Light</th>
<th>Chl (% dry weight)</th>
<th>Car (% dry weight)</th>
<th>Chl a/b</th>
<th>Chl/Car</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>1.3 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>ML</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>HL</td>
<td>0.5 ± 0.2</td>
<td>0.45 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

Errors represent the sd of two biological replicas with five technical replicas each.

### Table 3. F0/Fm and maximum NPQ of cultures in the different conditions

<table>
<thead>
<tr>
<th>Culture</th>
<th>LL</th>
<th>ML</th>
<th>HL</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0/Fm</td>
<td>0.68 ± 0.02 (n = 18)</td>
<td>0.65 ± 0.02 (n = 12)</td>
<td>0.59 ± 0.03 (n = 20)</td>
</tr>
<tr>
<td>Blue/red excitation ratio</td>
<td>1.16 ± 0.01 (n = 2)</td>
<td>0.87 ± 0.04 (n = 2)</td>
<td>0.63 ± 0.02 (n = 2)</td>
</tr>
<tr>
<td>Maximum NPQ (red AL, 550 μE m² s⁻¹)</td>
<td>1.3 ± 0.1 (n = 10)</td>
<td>1.5 ± 0.2 (n = 6)</td>
<td>1.8 ± 0.4 (n = 8)</td>
</tr>
<tr>
<td>Maximum NPQ (blue AL, 520 μE m² s⁻¹)</td>
<td>1.1 ± 0.3 (n = 4)</td>
<td>0.9 ± 0.3 (n = 2)</td>
<td>0.5 ± 0.1 (n = 4)</td>
</tr>
</tbody>
</table>

These values mainly represent surface cells (see text). The values of F0/Fm and maximum NPQ did not change with time, so we report averages of measurements at days 5, 13, 20, 22, and 29. Errors indicate the sd of n technical replicas in two (LL and HL) or one (ML) biological replicas at these five time points. Blue/red excitation ratio indicates the ratio of amounts of excitation by the blue/red LEDs, calculated as the ratio of the overlap integral of each LED spectrum and the fluorescence excitation spectrum (Fig. 4, right).
Confocal and Reflection Microscopy on the Largest of the HL Colonies

Fifteen of the largest (approximately greater than 1 mm) HL colonies were cut in half to study heterogeneity by optical reflection and confocal fluorescence microscopy. All colonies showed the same multilayer structure (Fig. 7). Each colony consisted of a hollow cavity surrounded by an orange and then a green domain (Fig. 7A). The orange domain shows no Chl autofluorescence (Fig. 7B). Green autofluorescence is seen throughout the colony (Fig. 7C) and originates from the extracellular matrix (Bachofen, 1982). The lifetime of the green autofluorescence was extremely short (less than 5 ps; Supplemental Fig. S9), suggesting that it could originate from Echi. The orange domain does not contain cells, as evidenced by the lack of SYTOX Green fluorescence (see “Materials and Methods”). Instead, the green outer domain is composed mainly of living cells containing Chl (Fig. 7B; see also FLIM results below).

FLIM on the Largest of the HL Colonies

The structural differences from the surface toward the inside in the largest of the HL colonies observed by confocal and reflectance microscopy could indicate functional heterogeneity. To test this, we used FLIM to compare the time-resolved Chl fluorescence at different distances (depths) from the colony surface. Fluorescence decay curves were measured in each three-dimensional pixel (voxel) and fitted by a sum of exponentials (Fig. 8B). FLIM was measured on 100 cells close to the colony surface (outer cells) and on 80 cells far from the colony surface (inner cells) in two of the largest of the HL-acclimated colonies. For a better signal-to-noise ratio, the fluorescence decay traces of all voxels belonging to each individual cell were summed. Dead cells marked by SYTOX Green fluorescence were low in number (Supplemental Fig. S10) and were excluded from the analysis. From the resulting traces, we determined the following for each cell: (1) the peak intensity of the decay trace, which is approximately proportional to the number of contributing pigments; and (2) the average lifetime (t).

Table 4. Average fluorescence lifetimes (ns) of LL and HL colonies at F0 or Fm excited at 650 or 662 nm and detected at 685 nm

<table>
<thead>
<tr>
<th>λexc</th>
<th>LL (F0)</th>
<th>LL (Fm)</th>
<th>HL (F0)</th>
<th>HL (Fm)</th>
<th>F0/Fm LL</th>
<th>F0/Fm HL</th>
</tr>
</thead>
<tbody>
<tr>
<td>650 nm</td>
<td>0.618</td>
<td>0.710</td>
<td>1.795</td>
<td>1.729</td>
<td>0.66</td>
<td>0.59</td>
</tr>
<tr>
<td>662 nm</td>
<td>0.584</td>
<td>0.683</td>
<td>1.894</td>
<td>1.584</td>
<td>0.69</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Instead, the green outer domain is composed mainly of living cells containing Chl (Fig. 7B; see also FLIM results below).

Figure 3. NPQ induction curves (averages of days 5, 13, 20, 22, and 29). LL (red-pink, triangles; two biological replicas), ML (black, crosses), and HL (blue-green, squares; two biological replicas) colonies are shown. The AL was red (550 μmol photons m−2 s−1). The shaded area represents the SD from nine to 12 technical replicas. The differences between all light conditions are significant as judged by a double-sided Student’s t test (P < 0.05). The spread of NPQ in time was the largest for HL, possibly because the error is larger due to the lower Chl content, larger colony size (and size variation), and higher Echi content of HL colonies.

Figure 4. Screening effect of blue photons in HL-acclimated conditions. Left, One example of NPQ induction curves at day 22 of LL (red triangles), ML (black crosses), and HL (green squares) cultures measured with red AL (RL; 550 μmol photons m−2 s−1; red curves) or blue light (BL; 520 μmol photons m−2 s−1; blue curves). Right, Excitation spectra of Chl fluorescence (735 nm detection) at room temperature of LL (red), ML (black), and HL (green) cultures (spectra are normalized to the peak around 670 nm). Excitation spectra are among others distorted by absorption flattening. Light blue and light red traces represent the emission spectra of the pulse-amplitude modulated AL light-emitting diodes (LEDs) normalized to their relative intensities.
The average lifetime of outer cells (850 ± 160 ps) was significantly shorter than that of inner cells (1,040 ± 160 ps; Fig. 8C; P < 0.001). In contrast, cells in the smaller LL colonies had a homogenous distribution of the fluorescence lifetime throughout the colony (Supplemental Fig. S11). The longer average lifetimes of the outer cells in the FLIM measurements compared with the TCSPC measurements can be explained by a higher level of photoinhibition in FLIM, since it focuses exclusively on the outermost layer of cells. Together, the FLIM results show a spatial variation of the functional composition of chloroplasts throughout the largest of the HL-acclimated colonies.

DISCUSSION

Photoacclimation strategies in colonial algae are largely unknown. Here, we used a combination of biochemical and spectroscopic techniques to study the photoacclimation of B. braunii grown in photoautotrophic conditions under continuous light in a bubble column photobioreactor. The photoacclimation strategies of B. braunii partly overlap with those of single cellular algae but are also in part unique to this alga, and possibly more generally for colonial photosynthetic species. We will first address the unique strategies of B. braunii, followed by those that are similar in B. braunii and other algae, and finish with a discussion on the effects of HL on Fv/Fm, colony size, and growth rate. Our comparison will focus on C. reinhardtii because it is the best-studied green alga.

Unique HL Acclimation Strategies of B. braunii: Shading and Sunscreen

The function of B. braunii colony size changes and the relation with light intensity are unclear (Zhang and Kojima, 1998; Khatri et al., 2014; García-Cubero et al., 2018). However, previous studies were performed in the presence of high CO2 concentration, which does not correspond to physiological conditions. Here, we show that in carbon limitation (ambient CO2, a condition that activates carbon-concentrating mechanisms already at low light; Suzuki et al., 2013), colony size is increased at light intensities of 150 and 450 μmol photons m−2 s−1. This suggests that the increase in colony size, which increases shading, is a photoprotective strategy, corroborating an earlier hypothesis by Khatri et al. (2014).

Additional photoprotection in HL arises from Echi (Table 1), which accumulates in the extracellular matrix (Grung et al., 1994) and blocks approximately 50% of the incident light between 350 and 550 nm (compare red and green spectra in Fig. 4, right). Photoprotection by optical screening by ketocarotenoids (but not Echi) is observed in many other green algae under stress conditions (Solovchenko, 2013).

The decrease of the Echi/Chl ratio upon transition to LL (34 [ML] to 4 [LL]; Table 1) suggests that the cells may be actively degrading Echi, raising the question whether B. braunii can catabolize extracellular Echi. Echi/Chl also decreases by increasing the Chl content per cell (2.6×; Table 2) and by dilution by growth (~1.33×; Fig. 1), but these two effects led to a decrease of Echi/Chl to 10 ± 4, considerably higher than the experimental 4 ± 1 (Table 1). Thus, it appears that Echi is indeed catabolized to some extent.

Similar HL Acclimation Strategies in B. braunii and Other Green Algae: Decreased Chl Content, Increase of Free Carotenoids, Increase of NPQ, and Antenna Size Modification

Decreased Chl content and increased carotenoid content upon HL acclimation in B. braunii (Table 1) are general strategies observed in algae and plants, although the nature of the carotenoid varies between species (Ballottari et al., 2007; Bonente et al., 2012;
Meneghesso et al., 2016; Polukhina et al., 2016). The increase in Zea/Chl ratio in *B. braunii* in HL (Table 2) might suggest an involvement of this xanthophyll in NPQ as in plants (Demmig-Adams, 1990) and some algae (Quaas et al., 2015; Christa et al., 2017). However, the presence of Zea is not necessarily evidence of its involvement in NPQ, since a similar increase of Zea content and NPQ capacity in HL also occurs in *C. reinhardtii*, where Zea does not play a role in NPQ (Bonente et al., 2012; Quaas et al., 2015).

The NPQ capacity in LL in *B. braunii* is considerable (1.3; Fig. 3) when compared with that of *C. reinhardtii* (0.3–1; Bonente et al., 2011, 2012; Quaas et al., 2015; Polukhina et al., 2016), and it is similar to that of four biofilm-forming algae (1–1.2; Quaas et al., 2015). In *C. reinhardtii*, NPQ capacity increases to greater than 3 in HL and requires an inducible actor (LHCSR3; Peers et al., 2009). In *B. braunii*, the increase in HL is smaller (1.9 times), suggesting that such an actor (e.g. LHCSR or PSBS, both are present in the genome; Browne et al., 2017) may only be weakly inducible in *B. braunii*.

HL acclimation can affect the antenna size of PSII and PSI but generally not the PSI/LHCI ratio (Bailottari et al., 2007; Bonente et al., 2012; Wientjes et al., 2013a). The PSI excited state lifetimes are similar in LL and HL (Fig. 6), suggesting that in *B. braunii*, HL acclimation...
does not affect the PSI antenna size, since a larger PSI antenna would result in longer average lifetimes (Le Quiniou et al., 2015). It is interesting that the PSI lifetime of \textit{B. braunii} is \(\sim 110\) ps, which is longer than in \textit{Arabidopsis} (\textit{Arabidopsis thaliana}; Wientjes et al., 2013b) and \textit{C. reinhardtii} (Unlü et al., 2014), suggesting a larger PSI antenna.

Assessing PSI antenna size is complicated in HL colonies, which are functionally heterogenous, as observed by FLIM (Fig. 8). However, although the likely presence of photoinhibition does not permit the acquisition of quantitative data, within large HL colonies, cells at the colony surface have shorter excited state lifetimes than those deeper inside (Fig. 8C), suggesting smaller PSI antenna size at the surface. Such variations between cells at different depths were observed before in the large colonies of the cyanobacterium \textit{N. sphaeroides} (Deng et al., 2008) and in leaves of \textit{Arabidopsis}, in which the PSI antenna size was found to be smaller on the sun side (Iermak et al., 2016).

Effects of HL on Fv/Fm, Colony Structure, and Growth Rate

LL colonies are small (80 \(\mu\)m) and homogenous, whereas the ML and HL colonies are larger (\(\sim 400\) \(\mu\)m), and the largest HL colonies are structurally heterogenous. In the largest colonies, the cell density decreases with increasing distance from the colony surface until the central domain, where there are no more cells. A decrease in cell density was also found in large colonies (100–2,000 \(\mu\)m) from a bloom of \textit{B. braunii}, with a green surface layer of cells of approximately 20 \(\mu\)m (Wake, 1983), similar to the 20 to 60 \(\mu\)m found for \textit{B. braunii} biofilms (Wijihastuti et al., 2016). Additionally, the long fluorescence lifetimes for inner cells may be indicative of nutrient starvation (no repair of photodamaged PSII). Together, this suggests that deeper inside the colony (\(\pm 200\) \(\mu\)m; Fig. 2), light and/or nutrients become depleted, leading to cell death. Cell death and subsequent degradation may have caused the cavity in the center of these colonies. Diffusion of nutrients and light attenuation can limit the viable region within colonies (Ploug et al., 1999) and biofilms (Stewart, 2003). Moreover, diffusion was recently shown to be low in the oil-rich parts of the colony (van Schadewijk et al., 2018), making this scenario very plausible.

Although the largest of the HL colonies have decreased viability deep inside compared with small LL colonies, this detrimental effect is apparently outweighed by other advantages (Beardall et al., 2009), including increased mutual shading of cells. Moreover, the viable region is \(\sim 200\) \(\mu\)m thick (Fig. 7), meaning that most HL colonies (typically 400 \(\mu\)m; Fig. 2) fully sustain living cells and lack the hollow cavity (this is corroborated by functional magnetic resonance imaging studies; van Schadewijk et al., 2018). Additionally, the possibility of programmed death of inner cells could lead to nutrient recycling that could benefit other nutrient-limited inner cells (Durand et al., 2016), and indications of remnants from cell death were identified in the \textit{B. braunii} extracellular matrix (Weiss et al., 2012).

The HL acclimation of surface cells leads to lower pigmentation, increased NPQ, and Echi shading, thus largely diminishing PSII damage. The Fv/Fm of HL surface cells is similar to that of faster growing species such as \textit{C. reinhardtii} (Bonente et al., 2012; Polukhina et al., 2016), suggesting that inner cells are over-protected by the additional shading by layers of cells and extracellular Echi. Perhaps the increased shading for inner cells in the HL colony is a more economical way of photoprotection: the one-time cost of extra photosynthetic complexes (to adapt to lower local light intensity) may be less than the increased cost of repair that would be required in the absence of shading. Moreover, the additional shading of blue photons will decrease the photodamage caused by direct absorption of these blue photons by the manganese cluster (Tyystjärvi, 2008), a source of photodamage upon which NPQ has no effect.

Light acclimation changes are considered to optimize growth of the algae under the new condition. This is achieved by increasing light harvesting under light-limited conditions and by limiting photodamage and optimizing carbon assimilation in light-saturated conditions (MacIntyre et al., 2002; Falkowski and Chen, 2003). Therefore, it was expected that directly after changing the light intensity (before acclimation), the growth rate would decrease, followed by an increase to a new constant rate. Light acclimation is usually faster or equal to the doubling time (Falkowski and Chen, 2003), and the doubling time of \textit{B. braunii} is more than 9 d (Fig. 1). Indeed, changes in pigment composition, Fv/Fm, and NPQ reach a new constant value within 5 d. During this period, the growth rate was thus expected to recover to a new constant rate. However, the dry weight measurements (Fig. 1) gave no indication of such an initial dip in growth rate and were well fitted with a single exponential growth function. This suggests that the acclimation effects on the observed growth rate are small because acclimation is fast compared with the growth rate.

In conclusion, we show that \textit{B. braunii} has a unique strategy for acclimation to HL, strictly correlated with its colonial nature: the accumulation of the extracellular Echi and intracolony shading by photosynthetic pigments. The capacity for NPQ is already substantial in low light, and NPQ kinetics are unchanged upon HL acclimation. This acclimation strategy allows the environment of this flagella-less alga that often floats near the surface of lakes and ponds, where shade is minimal (Wake and Hillen, 1980).

MATERIALS AND METHODS

Strain and Culture Conditions

\textit{Botryococcus braunii} Race B strain Berkeley (Showa; Wolf et al., 1985) was cultivated in a modified CHU-13 medium (composition is provided in Supplemental Table S3; Grung et al., 1989). The cultures were grown in batch or
fed-batch in a bubble column photobioreactor (MC1000; Photon Systems Instruments) in a volume of 75 mL under continuous illumination from a cool-white LED at 26°C ± 1°C. Precultures were inoculated at a biomass concentration of 0.5 g L⁻¹ dry weight (see below) and grown at least 30 d at 150 μmol photons m⁻² s⁻¹. From this preculture, five new cultures were inoculated at 0.3 g L⁻¹ dry weight. Two were grown at 50 μmol photons m⁻² s⁻¹ (LL), two at 450 μmol photons m⁻² s⁻¹ (HL), and one control at 150 μmol photons m⁻² s⁻¹ (ML) for at least 40 d. In fed-batch conditions, 50% (v/v) of the medium was replaced every 5 to 6 d, and cultures were diluted once they reached 1.5 g L⁻¹. Nitrate concentrations were measured with Quantofluor nitrate test sticks (Macherey-Nagel) during cultivation and did not decrease below 125 mg L⁻¹.

Dry Weight Measurements
Dry weight concentrations of batch cultures were determined by vacuum filtration of 3 mL of culture onto a preweighed, predried nitrocellulose filter with a pore size of 8 μm (N4146-100EA; Merck-Millipore) in a filter funnel (DS0315-0047; Nalgene). This pore size allows bacteria that are not associated with the colonies to pass but is too small for B. braunii colonies. Filters with colonies were washed by vacuum filtration with 10 mL of demineralized water in the filter funnel (DS0315-0047; Nalgene), dried in an oven for 16 h at 80°C, and subsequently weighed. All dry weight measurements were done in duplicate.

Colony Size Measurements
Colony size measurements (batch cultures) were performed by laser diffraction of the colonies in liquid culture analyzed with the Mie scattering model (Allen, 2013) with a Mastersizer 2000 (Malvern Instruments) equipped with a 3-mm stirring bar, at a concentration of 0.5 g L⁻¹ dry weight. Two were grown at 50 μmol photons m⁻² s⁻¹ (LL), two at 450 μmol photons m⁻² s⁻¹ (HL), and one control at 150 μmol photons m⁻² s⁻¹ (ML) for at least 40 d. In fed-batch conditions, 50% (v/v) of the medium was replaced every 5 to 6 d, and cultures were diluted once they reached 1.5 g L⁻¹. Nitrate concentrations were measured with Quantofluor nitrate test sticks (Macherey-Nagel) during cultivation and did not decrease below 125 mg L⁻¹.

Optical Reflection and Confocal Microscopy
The largest colonies from fed-batch cultures were cut in half and imaged with a 4X air objective of the BIS00 Microscope (VWR) equipped with an RGB CMOS camera (10MP; Motic). Illumination was performed from the side or top with a Schott KL200 cold light source (Galvoptics) at maximum intensity. Autofluorescence microscopy was performed with a Zeiss LSM510 confocal scanning microscope with a Plan-Neofluar objective (10X, numerical aperture 0.3) with a step size of 2.54 μm, 2.54 μm, and 5 μm (field size 1.303 × 1030 × 495 μm). Excitation was at 488 nm and detection either above 635 nm (red channel) or between 505 and 545 nm (blue-green channel).

FLIM
Time-resolved fluorescence of individual chloroplasts within the largest single HL colonies from fed-batch culture was measured with multiphoton FLIM, with a setup described earlier (Bresso et al., 2009). In brief, two-photon excitation was performed with 860-nm, 15-fs pulses at a repetition rate of 76 MHz (generated with a Titanphoton laser [Mira900, Coherent]). Excitation pulses were focused on the sample with a 60X water-immersion objective lens (CFL Plan Apochromat, numerical aperture 1.2; Nikon). Imaging was done with a Nikon TE300 inverted scanning microscope equipped with an HPM-100-40 detector (Becker & Hickel). chloroautofluorescence was recorded through a 680 ± 20 nm band-pass filter (Semrock), and SYTOX Green fluorescence and auto-fluorescence were recorded through a 525 ± 45 nm band-pass filter (Semrock). Each image consisted of 128 × 128 pixels (153.6 × 153.6 μm, 5 μm step size in z) with each containing a decay trace of 256 time channels (20 ps per channel). Colonies were acclimated to HL for 5 d (fed-batch). Single colonies were dark acclimated for at least 20 min and moved to a four-well microscope chamber (Ibidi) with fresh medium. Dead cells were stained with 5 μM SYTOX Green (Molecular Probes; Sato et al., 2004). This approach was validated with a positive control of cells killed with 96% ethanol (data not shown). Different laser powers (40 μW, 160 μW, 1 mW, and 2 mW at the sample) were tested to identify a power (160 μW) with sufficient excitation for good signal to noise but low enough to prevent singlet-singlet or singlet-triplet annihilation or the closing of PSI reaction centers, ensuring measurements in the F1 state (Supplemental Fig. S13).

Each measurement series started with imaging cells on the surface of a dark-acclimated colony. A z-scan, consisting of images at three horizontal planes (vertically separated by 5 μm), was recorded for several colony regions that were in direct contact with the bottom of the sample chamber. Next, cells more buried in the colony were imaged. To this end, the colony was cut in half with a razor blade and the z-scan was repeated. Care was taken that only cells at the surface of the cut were measured to avoid distortion by layers of extracellular matrix or other cells. Dead cells that displayed SYTOX Green fluorescence were quantified and excluded from analysis. Fluorescence decays of individual cells were integrated by selecting all voxels in the cup-shaped chloroplast (using Glotaran 1.5; Snellenburg et al., 2012). The resulting decay traces were fitted with FluoFit software (version 4.6; PicoQuant) with a sum of exponential decays, convoluted with the instrument response function (170 ps) that was measured with the same setup using pinacyanol iodide in methanol (van Oort et al., 2008) in an identical sample chamber.

 qp=25 \quad \text{g} \quad \text{mol}^{-1} \quad \text{photons}^{-1} \quad \text{m}^{-2} \quad \text{s}^{-1} 

\text{NPQ of Chl Fluorescence}

Quenching analyses were performed with a Dual-PAM (Walz) while stirring at 22°C on fed-batch cultures. Cells were dark acclimated for 40 min in 22°C, 495 cm² quartz optical cell equipped with a 3-mm stirring bar, at a concentration of 2 g L⁻¹ dry weight. Each measuring sequence consisted of an initial period with measuring light (460 nm, 5 μmol photons m⁻² s⁻¹) of 5 min with two saturating pulses (635 nm, 180 μmol photons m⁻² s⁻¹), followed by a period of 12 min with the AL (460 nm [500 or 2,090 μmol photons m⁻² s⁻¹] or 635 nm [520 μmol photons m⁻² s⁻¹]; spectra in Fig. 4) switched on with seven saturating pulses (635 nm, 180 μmol photons m⁻² s⁻¹). The saturating pulse light intensity was 12,000 μmol photons m⁻² s⁻¹ with 180-ms duration provided by a 635-nm LED. The estimated Fluorescence (F0) was calculated as F0 = Fl / Fm - NPQ and NPQ was calculated as = Fl / Fm - F0. Fm is the maximum fluorescence during a saturating pulse after dark acclimation, and F0 is the maximum fluorescence during a saturating pulse during or after exposure to the AL. Fm is the minimal fluorescence after dark acclimation.

TCSPC
Time-resolved fluorescence measurements were performed using the time-correlated single-photon counting setup described previously (Müller et al., 1992) with modifications (Tian et al., 2017). Excitation at 650 nm was used to preferentially excite Chl b and 662 nm for Chl a (0.5-mm spot size). Time-resolved fluorescence decay traces were measured at three detection wavelengths (685, 700, and 715 nm), with 4,096 time channels of 2,569-ps width. Colonies from fed-batch culture were placed between two glass plates and mounted in the rotation optical cell (diameter = 10 cm, thickness = 1 mm). The optical cell was rotating at 50 rpm and oscillating sideways (78 rpm). Fluorescence was measured in a front-face arrangement, with PSI in open (Fp) or closed (Fm) state, using the following conditions. (1) For Fm, 50 μM 3(3,4-dichlorophenyl)-1,1-dimethylnaphthalene was added to the growth medium. The excitation laser power was 50 μW, and the repetition rate was 4 MHz. To achieve full closure of the PSI reaction centers during the measurement, an additional blue LED (460 nm, intensity ~40 μmol photons m⁻² s⁻¹) was used to preilluminate the cells just before detection of the signal. (2) For Fp, the sample was in complete darkness during the experiment and dark adapted 20 min before the
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experiment. The excitation power was 7 to 10 µW, and the repetition rate was 0.8 MHz. Checks with different powers and repetition rates showed that the sample was in the F2 state (Supplemental Fig. S4A).

The measurement time at a single wavelength was limited to a maximum of 20 min to avoid changes in the colonies due to prolonged measurement in the rotating optical cell. Each first experiment was repeated at the end to make sure that the fluorescence decay did not change during the measurement (Supplemental Fig. S4B). The decay traces were globally analyzed with TRFA software (Digris et al., 1999). The methodology of global analysis was described previously (van Stokkum et al., 2004). In short, a number of parallel, non-interacting kinetic components were used as a kinetic model, so the total data set was fitted with function \( f(t, \lambda) = \sum_{i=1}^{N} DAS_i(\lambda) \exp (\frac{-t}{\tau_i}) g_i(t, \lambda) \), where DAS, is the wavelength-dependent amplitude factor associated with a decay component \( i \) having a decay lifetime \( \tau_i \), and instrument response function \( g_i(t, \lambda) \) is measured by scattering light. Four to six components were enough to get a good fit of the LL and HL data upon 650- or 662-nm excitation (for residuals and fitted traces, see Supplemental Figs. S5 and S6).

Supplemental Data

The following supplemental materials are available.

- Supplemental Figure S1. Colony size distributions.
- Supplemental Figure S2. NPF induction curves.
- Supplemental Figure S3. Absorption spectra (integrating sphere).
- Supplemental Figure S4. TCSPC: powerseries and control experiments.
- Supplemental Figure S5. TSCPC: decay curves \( F_0 \).
- Supplemental Figure S6. TSCPC: decay curves \( F_\infty \).
- Supplemental Figure S7. TSCPC: DAS \( F_0 \).
- Supplemental Figure S8. TSCPC: DAS \( F_\infty \).
- Supplemental Figure S9. Ultrafast green autofluorescence.
- Supplemental Figure S10. FLIM: HL colony.
- Supplemental Figure S11. FLIM: LL colony.
- Supplemental Figure S12. HPLC chromatogram.
- Supplemental Figure S13. FLIM: powerseries.
- Supplemental Table S1. Carotenoid composition in the ML control culture.
- Supplemental Table S2. TCSPC: global fit results.
- Supplemental Table S3. Medium composition.
- Supplemental Methods. Pigment extraction.

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