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Femtosecond Laser Disruption of Filamentous Cyanobacteria Unveils Dissimilar Cellular Stability Between Heterocysts and Vegetative Cells

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ABSTRACT

Filamentous cyanobacteria develop heterocysts in response to deprivation for combined nitrogen under aerobic conditions. The most prominent structural change in heterocysts is the biosynthesis of an envelope that restricts gas permeability, providing an appropriate microoxic environment for \( N_2 \) fixation inside. The additional thickness of the differentiated cells, when compared to vegetative cells, makes filamentous cyanobacteria an attractive biological system to investigate cellular response against femtosecond laser processing. By irradiating the cyanobacterial filaments with 120-fs, 795-nm, 1-kHz pulses focused through a 100× microscope objective with a numerical aperture of 0.85, we have determined that the pulse energy threshold for an apparent disruption of the cell wall of vegetative cells is \( 13\pm4 \) nJ/pulse. A further increase in the pulse energy to \( 43\pm13 \) nJ causes the complete removal of vegetative cells. In contrast, the pulse energy threshold has to be augmented about three-fold for heterocyst envelope disruption or two-fold for complete removal of heterocysts. We propose that the singular cross linked structure of the glycolipid multilayer of the envelope, required to restrict gas permeability, accounts for the remarked difference in the ablation energy threshold between vegetative cells and heterocysts.
INTRODUCTION

Femtosecond laser disruption of cells or subcellular organelles has become an effective technique with very innovative experimental applications in cell biology (1). The underlying principles for femtosecond disruption of biological materials are the same as for any transparent dielectric. When ultrafast pulses are focused on the sample, the huge intensity reached in the focal volume leads to the formation of quasi free-electron plasma (2,3). A process that in brief—step 1—begins with the non-linear absorption of photons by the transparent target (multi-photon ionization or tunnelling ionization),—step 2—continues with the absorption of photons by a few free electrons followed by impact ionization with other electrons with low kinetic energy that undergo further absorption of photons, yielding more electrons with high kinetic energy, and—step 3—ends up with an avalanche growth in the number of free electrons after the recurring sequence of step 2. The resulting high electron density is responsible for the localized optical breakdown (or ablation) of materials, a process that takes place at a time scale much longer than the duration of a single pulse. As for other applications, the use of ultrafast pulses for biological material surgery shows several advantages, when compared to nanosecond or picosecond pulses (4,5). First, the highly non-linear nature of the strong-field ionization mechanisms enables one to reduce the size of the processed region to even less than the focal volume; second, the short time duration of the laser-matter interaction makes thermal effects virtually negligible, so that the region surrounding the exposed volume remains unaffected; and third, there exists a strong confinement of the supersonic expansion after optical breakdown.

The use of femtosecond laser micro- or nanosurgery has found important applications in cell biology. Femtosecond laser pulse nanosurgery has been used successfully to deliver foreign DNA into cells by producing tiny, localized perforations in cytoplasmic membranes of
mammalian cells (6). Axotomy by femtosecond laser pulses and the time-course analysis of axon regeneration have unveiled new strategies to investigate molecular mechanisms that affect nerve development (7). Photodisruption of cellular nuclei or organelles has also been successfully achieved by femtosecond laser pulses and used to determine cellular viability (8,9). In addition, high energy femtosecond laser-induced shockwaving has shown to be of interest to determine the force required to detach cells adhering to an extracellular matrix (10).

Bearing in mind the applications that femtosecond laser processing might offer, we have investigated cellular disruption of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, a multicellular prokaryotic oxyphototroph whose singular cellular differentiation is of interest in research fields of photosynthesis and atmospheric N₂ fixation. Here we describe some of the physiological features of heterocyst-forming cyanobacteria that are pertinent to a better understanding of the motives for our investigation. In response to deprivation for combined nitrogen under aerobic conditions, many filamentous cyanobacteria, as *Anabaena* sp. strain PCC 7120, develop specialized cells; where atmospheric N₂ is fixed (11,12). The differentiation of N₂ fixing cells, the so-called heterocysts, at semiregular intervals between vegetative cells, where O₂ is produced as a by-product of photosynthesis, converts heterocyst-containing filamentous cyanobacteria into a true multicellular organism with one of the simplest patterns in developmental biology (13). The compartmentalization of N₂ and O₂ in the interdependent cells of filamentous cyanobacteria, *i.e.* N₂ fixation in heterocysts and O₂ evolution in vegetative cells, is anything but simple. Nitrogenase, the enzyme that catalyzes the reduction of N₂ to ammonia in heterocysts, is very sensitive to O₂ and is rapidly and irreversibly inhibited under aerobic conditions (14). Because of O₂ poisoning, physical and biochemical barriers are built up in heterocysts to diminish the intracellular partial pressure of O₂ and to maintain a microoxic environment for optimal nitrogenase activity. As a physical barrier, heterocysts have—Together
with a cell wall similar, to a certain extent, to that of vegetative cells (15)—a thick envelope consisting of an inner laminated layer of glycolipids and an outer homogenous layer of polysaccharides that protects the former (11,16). The glycolipid layer is proposed to form a hydrophobic barrier that significantly restricts gas permeability (17,18). The biochemical barriers are inside the heterocyst: heterocyst respiration is very active and consumes O₂ that might diffuse either from the extracellular milieu or from adjoining vegetative cells through the connecting polar channel; the activity of enzymatic scavengers of reactive oxygen species responds quickly to intracellular O₂ fluctuations; and heterocyst thylakoids are devoid of the endogenous source of O₂ (i.e. Photosystem II).

In this work, we have paid special attention to the physical barrier (i.e. the thick envelope) of heterocysts and whether there is any difference in cellular disruption between heterocysts and vegetative cells of filamentous cyanobacteria by femtosecond laser processing. Our results show that heterocysts are more resilient to femtosecond laser pulses than vegetative cells. Chlorophyll fluorescence emission vanishes irreversibly in vegetative cells at pulse energies that do not affect the cell morphology, indicating that thylakoid membranes in cyanobacteria are far more sensitive to femtosecond irradiation than the vegetative cell wall or the heterocyst envelope.

MATERIAL AND METHODS

Growth conditions of Anabaena sp. strain PCC 7120 culture. Anabaena sp. strain PCC 7120 was grown autotrophically on agar solid medium under combined nitrogen free conditions using the BG11₀ culture medium (19). Cyanobacterial culture was routinely maintained at 34°C under continuous light provided by an 11-W fluorescent lamp. Heterocyst frequency was determined in light micrographs obtained with the optical microscope Nikon Eclipse E800 (Nikon Corporation,
Kanagawa, Japan). One day before femtosecond laser processing, *Anabaena* sp. strain PCC 7120 was gently striated on 1-mm-thick layer of agar solid medium (BG11o) deposited on a glass-bottom culture dish (FluoroDish™, World Precision Instruments, Inc. Sarasota, Florida).

**Experimental setup for femtosecond laser processing.** A commercial (Spectra Physics Inc, Mountain View, California) chirped pulse amplification laser system was used to produce linearly polarized 120-fs pulses at 795 nm with a repetition rate of 1 kHz. The maximum pulse energy delivered by the system was 1.0 mJ and the diameter of the laser beam was 10 mm at 1/e² intensity. The culture dish, containing the cyanobacterial filaments, was placed on a computer-controlled motorized XYZ translation stage with a precision of 1 μm along the three axes. The laser beam was focused on the sample with a microscope objective (Leica N-Plan-Epi 100× magnification, numerical aperture (NA) of 0.85, Leica Microsystems, Wetzlar, Germany). The aperture diaphragm of the objective reduced the beam size to 5.6 mm. The calculated spot size in the focus at the central maximum of the Airy disc was about 1 μm in diameter (or < 1 μm² in surface extension), giving enough spatial resolution to irradiate different regions of the same cyanobacterial cell. A home-built optical microscope was arranged for *in situ* visualization of the samples. Cyanobacterial filaments were back illuminated (in transmission) with a white light-emitting diode and imaged on a charge-coupled device (CCD) through the 100× microscope objective. To monitor chlorophyll fluorescence emission, the antenna complexes of cyanobacteria (*i.e.* phycobilisomes) were excited by means of a continuous solid state laser emitting at 532 nm with a maximum power of 5 mW and without focusing optics. The green beam was directed with no focusing lens to filamentous cyanobacteria. The emitted fluorescence was CCD-monitored with the same imaging system as described above. A long pass filter (Chroma E590lp, Chroma Technology Corp, Rockingham, Vermont, USA) was placed before
the CCD to block unwanted green radiation. A scheme of the experimental setup is shown in Fig. 1.

For femtosecond laser processing of cyanobacteria, the 795-nm laser beam was focused on the upper hemisphere of cells and series of 200 or 2000 pulses at a repetition rate of 1 kHz were used. At this repetition rate, the mechanical and thermal events induced by subsequent pulses are largely independent (1). Both the visible and fluorescence images were recorded before and after femtosecond laser irradiation. The optimum focusing conditions for the femtosecond laser beam in the cells were achieved for z-axis positions that slightly differed from the z-axis positions to maximize the fluorescence signal, so slight changes in the overall fluorescence intensity could sometimes be observed in fluorescence micrographs before and after laser irradiation. The pulse energy was fine-tuned with a λ/2 plate and a linear polarizer. Neutral density filters were placed after the polarizer. The pulse energy reaching the microscope objective was determined by knowing the mean beam power measured with a bolometer (Spectra Physics, 407-A) before the neutral density filters and the combination of filters used. The experimental error in transmittance measurements of the high density filters gives rise to large standard deviations for the energy (and fluence) values reported in this work. The laser system compressor was adjusted before femtosecond laser irradiation to minimize the length of the autocorrelation trace after the polarizer. To this end we used the measurements obtained with a single-shot autocorrelator (Spectra Physics, SSA).

RESULTS

Development of heterocysts on agar solid BG110 culture medium
Anabaena sp. strain PCC 7120 was grown autotrophically on agar solid BG11\textsubscript{0} culture medium to induce cellular differentiation. Heterocyst development was examined under the optical microscope Nikon Eclipse E800 and the differentiated cells could be distinguished in terminal or central positions of the filamentous cyanobacteria by their dim chlorophyll fluorescence (data not shown). The heterocyst frequency was 5–7\% of total cells. The main advantage of the agar solid BG11\textsubscript{0} culture medium was that the filamentous cyanobacteria were fixed to the medium matrix, making easier cell focusing when undertaking femtosecond laser processing. Femtosecond laser processing was performed in several cell batches of Anabaena sp. strain PCC 7120 during different days. The pulse energies we report below were independent of the selected batch and were reproducible within experimental error.

**Thylakoid membrane and cell wall disruption of vegetative cells**

Chlorophyll fluorescence emitted by vegetative cells of cyanobacterial filaments was monitored while exciting at 532 nm. Thylakoid membranes of vegetative cells contain photosystem II and photosystem I. They both are the main source of red fluorescence, although photosystem I has a very small contribution at room temperature. The fluorescence intensity was inhomogeneous from one cyanobacterial filament to another or even among vegetative cells in the same cyanobacterial filament. Figure 2a shows an optical field where a cyanobacterial filament, containing three vegetative cells with similar fluorescence intensity (see Fig. 2b), is selected to perform femtosecond laser processing. The experiment started by irradiating the upper part of the target vegetative cell (indicated with a white arrowhead) with a series of 200 pulses with the energy of 9±3 nJ/pulse. After femtosecond laser irradiation, the fluorescence intensity of the target vegetative cell did not change significantly and damage in the cellular morphology was not
apparent (data not shown). On increasing the number of pulses to 2000 while keeping constant 
the energy per pulse, the femtosecond laser irradiation caused a partial damage in the target 
vegetative cell, which manifested itself as a loss of fluorescence in the irradiated region (Fig. 2c).

To confirm that the disappearance of chlorophyll fluorescence after femtosecond laser 
processing was due to the structural disruption of thylakoid membranes, rather than 
photochemical disruption, the irradiated vegetative cell was kept under optical focusing for a 
time. In the event that femtosecond laser irradiation had only caused a photochemical disruption, 
subsequent recovery of chlorophyll fluorescence in the laser exposed region would have 
occurred in a few seconds or minutes due to inward diffusion of phycobilisomes or in situ processes (20,21). However, fluorescence photobleaching recovery was not observed even after 
4 h, pointing to an irreversible structural damage in thylakoid membranes. In spite of the 
irreversible loss of fluorescence emission, the light micrograph shows that the wall of the 
irradiated vegetative cell did not display any discontinuity (Fig. 2d). The energy per pulse was 
increased step by step, while maintaining trains of 200 pulses, until a discernible damage could 
be observed in the cell wall of vegetative cells. Each trial was performed in a fresh cell to ensure 
no damage induced by the accumulative effect of femtosecond pulse irradiation. On reaching the 
pulse energy of 13±4 nJ, changes in the shape of the cell morphology and partial disappearance 
of biomaterial could be tracked down (data not shown). This pulse energy was taken as the 
ablation energy threshold for the cell wall of vegetative cells, and it corresponded to a peak 
fluence of 1.2±0.4 J/cm² under our experimental conditions.

The pulse energy was further increased in steps of about 4 nJ up to 43±13 nJ. At this pulse 
energy—nearly 3 times the ablation energy threshold for the cell wall—, the complete removal 
of a 3-µm vegetative cell was monitored without observing an apparent damage in the adjoining 
cells. The enlargement of the above-threshold focal volume, when working several times above
the ablation threshold, caused the ablation of a substantial region of the cell wall; however, other processes like mechanical or shock waves have to come into play to explain the complete removal of the vegetative cell, see for instance (22). The measured pulse energies for the ablation of the cell wall or the complete removal of vegetative cells were used as a reference to establish whether the thick envelope of heterocysts would give further strength against femtosecond laser disruption.

**Cellular disruption of heterocysts**

Figure 3a shows an optical field of several cyanobacterial filaments, where one of them, occupying a central position in the light micrograph, contains a target terminal cell indicated with an arrowhead. To establish whether the terminal cell was a vegetative cell or a heterocyst, the emitted chlorophyll fluorescence of the cyanobacterial filaments was monitored while exciting phycobilisomes at 532 nm. The fluorescence micrograph revealed that the target terminal cell emitted no fluorescence, whereas the adjoining cell displayed intensive chlorophyll fluorescence emission (Fig. 3b). It indicated that the target terminal cell was in fact a heterocyst. Hence, the criteria we followed to search for heterocysts in optical fields were three: (i) the cell had to be in a terminal position of the cyanobacterial filament, (ii) the cell had to display a complete cellular development, and (iii) the cell had to emit no (or very dim) fluorescence when exciting at 532 nm.

To determine the energy threshold for the femtosecond laser disruption of heterocysts, we started by using trains of 200 pulses with the energy of 9±3 nJ/pulse. Under these conditions, changes in the heterocyst morphology or disappearance of biomaterial were not observed. Further laser exposure under the former conditions or any increase in the number of pulses per
series from 200 to 2000 did not bring any accumulative effect that could result in an apparent damage in the heterocyst envelope. Neither the use of the same pulse energy (13±4 nJ), which caused the ablation of the cell wall of vegetative cells, showed any apparent effect on heterocysts. To observe a cellular disruption, the energy per pulse was increased in steps of about 4 nJ. The increase in the energy per pulse did not cause any significant morphological change until a value of about 43±13 nJ/pulse was used for processing. At this particular stage, laser exposure using trains of 200 pulses caused a prominent crater in the heterocyst envelope and expulsion of the biomaterial from the ruptured heterocysts into the extracellular matrix could be observed; a phenomenon we ascribed to the turgor pressure inside the heterocyst. The search for other terminal heterocysts in other optical fields confirmed that the pulse energy threshold of about 43±13 nJ was required for the partial disruption of the heterocyst envelope. Further increase in the energy per pulse up to 68±21 nJ caused the complete removal of heterocysts due to the severe breakdown of cellular material after processing (Fig. 3c). A fluorescence micrograph of the cyanobacterial filament after heterocyst ablation displays how the chlorophyll fluorescence emission in the adjoining vegetative cell was not affected (Fig. 3d), indicating that there were no thermal effects beyond the region of energy deposition when using pulse energies of even 68±21 nJ at a repetition rate of 1 kHz. The laser pulse energies for the ablation of central heterocysts were similar to the ones for terminal heterocysts (data not shown); however, we did not include them in the statistical analysis because very few were processed. Table 1 summarizes the energy per pulse and peak fluence used while processing heterocysts and vegetative cells of Anabaena sp. strain PCC 7120.

DISCUSSION
Femtosecond laser processing of cyanobacterial filaments has proven that heterocysts and vegetative cells have different energy thresholds for partial disruption and complete cellular ablation. On the basis of the present study, the dissimilar femtosecond pulse energy dependence for both types of cells is explained by the morphological changes that vegetative cells undertake under combined nitrogen deprivation conditions. The differentiated cells (i.e. heterocysts) develop a thick envelope that restricts gas permeability. This biological sheath gives further strength to heterocysts.

Watanabe et al. (9) performed femtosecond laser disruption of mitochondria of HeLa cells by focusing trains of 250 laser pulses of 150 fs, 800 nm and 1 kHz through objectives with an NA of 1.2 or 1.4. They concluded, first, that the energy threshold required for the disruption of individual mitochondria of 1-µm with their experimental setup was about 2–4 nJ/pulse and, second, that the disappearance of the fluorescence emitted by a fusion protein containing an enhanced yellow fluorescence protein and a mitochondria-targeted sequence of cytochrome c oxidase was not simply due to a fluorescence photobleaching of the chimeric protein, but to the disruption of the mitochondrial membrane(s). Using a very similar strategy with laser pulses of 120 fs, 795 nm and 1 kHz focused through an objective with an NA of 0.85, we observed that the loss of red fluorescence in vegetative cells of filamentous cyanobacteria occurred when using trains of 2000 pulses with the energy of 9±3 nJ/pulse. If photochemical disruption had been the only event responsible for the loss of red fluorescence, the recovery of fluorescence would have been monitored after several tens of seconds or a few minutes after inward diffusion of phycobilisomes \( (D = 0.5–5\times10^{-10} \text{ cm}^2/\text{s}) \) or in several tens of minutes after in situ processes (20,21). However, inward red fluorescence in the femtosecond laser irradiated region of the vegetative cell did not recover even after 4 h. Therefore, we concluded that the loss of red fluorescence was not simply due to the photochemical disruption, but to the structural disruption
of thylakoid membranes. The work by Watanabe et al. (9) and ours share together that the energy per pulse used for subcellular processing causes a clear structural disruption of mitochondrial and thylakoid membranes. On the basis that both thylakoid and mitochondrial membranes have some similarities, for example, the high content of embedded proteins (about 50% and 60–80%, respectively), it would have been expected that the energy threshold for femtosecond laser disruption of both types of membranes had been similar (or at least closer). There is no doubt that other biological reasons might escape our knowledge to explain the difference in femtosecond pulse energy for processing both types of membranes, but the most evident technical difference between our study and the one by Watanabe et al. (9) is the focusing lens. The 100× objective we use has a small NA (0.85), in comparison with the one used by Watanabe et al. (9) (NA 1.2 or 1.4). As a consequence, the peak fluence at the focus has to be lower in our study. Furthermore, our focusing lens limits the beam size to 5.6 mm and thus reduces the pulse energy transmitted to the focus. These considerations might account for the different energy thresholds found in both studies. Unfortunately, Watanabe et al. (9) did not report an estimation of the peak fluence at the focus when disrupting mitochondria of HeLa cells, which limits the comparison between both studies.

In our study the energy per pulse has been increased up to 13±4 nJ to observe partial loss of biomaterial in vegetative cells. This is considered to be the ablation threshold for the cell wall. A survey in the literature shows that there are very few studies of femtosecond laser processing performed in organisms containing cell walls. Tirlapur and Köning (23) achieved non-invasive intra-tissue nanodissection of cell walls of the aquatic plant Elodea densa by focusing laser pulses of 170 fs, 740 nm and 80 MHz through an objective with an NA of 1.3. They reported ultraprecise cuts in the cell wall with widths <400 nm that required pulse energies of only ~0.6 nJ. Such a small value for the pulse energy suggests a small ablation threshold for plant cell
walls. In fact, the energy threshold they reported is about 20 times smaller than the one we have determined for the vegetative cell walls of filamentous bacteria. The utmost differences between both procedures are the very large repetition rate and laser exposure time used by Tirlapur and Köning (23), together with the different focusing optics, which results in the use of trains of several thousand pulses, while we have used trains of only 200 pulses at 1 kHz. These technical differences can be the reason for such a discrepancy between the ablation thresholds of both types of cell walls under investigation. In another study, Assion et al. (22) investigated the ablation process in the outer epidermal wall of the sunflower seedling (*Helianthus annuus*) stem with amplified laser pulses of 30 fs, 790 nm and 1 kHz focused through a microscope objective with NA 0.4. Our experimental approach for femtosecond laser processing is closer to that described by Assion et al. (22); in particular they both use amplified laser pulses at low repetition rate. The ablation threshold of the wall of a sunflower stem reported by Assion et al. (22) is, however, very high, 11±1 J/cm², if compared with the ablation threshold of the cell wall of vegetative cells reported in our work (1.2±0.4 J/cm²). We ascribe this divergence to the smaller number of pulses used by Assion et al. (22) to determine the ablation threshold in the plant cell wall. It is well known that the ablation threshold is much higher for a single pulse (or for a few pulses) than for a few hundred pulses due to incubation effects in the material (24). Assion et al. (22) used only five femtosecond laser pulses to identify the ablation region in the cell wall and it could explain why the peak fluence in their study is higher than the one we report. The two attempts with cell walls of higher plants and the one we have carried out with the cell wall of cyanobacterial vegetative cells might not be directly comparable to our approach (*i.e.* different organisms and technical approaches); however, to the best of our knowledge, they represent the only femtosecond laser processing studies carried out with cell wall containing organisms.
It is evident that the role of the cell wall of vegetative cells of filamentous cyanobacteria and of plants is the same (i.e. to withstand the outward force generated by the cell turgor pressure). The cytoplasmic membranes are mechanically weak. The turgor pressure in plant cells is between 0.3 MPa and 1.0 MPa, depending on species and growing conditions, and is about 0.4 MPa in cyanobacteria, although it can also increase under high photon irradiance (25). Although the structural composition of plant cell walls (comprised of hemicellulose, pectins and phenolic-cross-linked structural proteins) and of cyanobacterial vegetative cell walls (comprised of peptidoglycan layers and an outer membrane) are different, they share together that some of their structural components are cross linked polymers and can bear a large mechanical load. In particular, there is very little information on the mechanical resistance of the polymeric structure of peptidoglycan in vegetative cells of cyanobacteria. A recent atomic force microscopic study has been carried out in the cell walls of the gram-positive bacterium *Lactobacillus helveticus* to peel off a part of the outside surface of the cell wall with the help of the tip, thereby exposing the peptidoglycan strands beneath (26). This experiment shows that the cross linked peptidoglycan can bear the force applied with the tip to remove the smooth outer surface of the cell wall. Although a precise comparison between the tip force and peak fluence is not straightforward, the atomic force microscopic study is a compelling indication that this cross linked polymeric structure in gram-positive bacteria confers resistance to mechanical disruption. Despite the overall gram-negative structure of cyanobacterial cell walls, a high resistance to disruption is also expected considering that the cyanobacterial cell walls are unusually characterized by thick and high cross linked peptidoglycan layers (27).

In a further step we investigated the femtosecond laser processing of terminal heterocysts in filamentous cyanobacteria. Our results have shown that the peak fluence has to increase substantially up to 6.4±1.9 J/cm², if complete removal of individual terminal heterocysts is
attempted. In spite of the high energy fluence, the processing of terminal heterocysts did not affect the fluorescence intensity of adjoining vegetative cells; indicating that the viability of the adjoining vegetative cells were not at risk. The energy fluence required for the heterocyst ablation is comprised of the energy fluence to disrupt (cytoplasmic and thylakoid) membranes, the cell wall and the heterocyst envelope. Zhu et al. (15) proposed that the permeability of the peptidoglycan multilayer of the cell wall of developing heterocysts may be higher to facilitate the transport of glycolipids and polysaccharides to the heterocyst envelope. The perforation of the peptidoglycan multilayer of the heterocysts could possibly affect its mechanical resistance and possibly the energy fluence to process it; however, with the present data, we cannot give any support for or against this possibility. Hydrophobic interactions between lipids in the glycolipid multilayer of the heterocyst envelope are expected to increase its stability; however, this hydrophobic interaction is not expected to be much stronger than the one in (cytoplasmic or thylakoid) membranes of vegetative cells. The glycolipid multilayer of the heterocyst envelope contains boron as a structural element (28). Boron is well known to strengthen the cell wall of plants (29). On one side, boron toxicity is responsible for plant tissues with abnormally resilient walls, but on the other side, boron deficiency results in fragile plant tissues. Boron deficiency results in swelling of the plant cell wall, while the content of rhamnogalacturonan II—a pectic polysaccharide that exists mainly as a dimer that is covalently cross-linked by a borate ester—remains similar irrespective of the presence or absence of boron (30). In contrast to plant cell walls, the changes in the primary structure of the heterocyst envelope are very profound under boron deficiency and notably a drastic decrease in the content of glycolipids is observed in heterocyst envelopes (28). On the basis that boron plays an essential role in keeping both the thickness and the stability of the inner laminated layer of glycolipids by forming diester bridges between cis-hydroxyl groups of glycolipid molecules (31), it is envisaged that boron can be
responsible, at least in part, for the increase in the laser pulse energy threshold when processing heterocysts.

Our main conclusions maybe summed up as follows: femtosecond laser processing of filamentous cyanobacteria has shown that the structural disruption of thylakoid (and cytoplasmic) membranes require a relatively low pulse energy threshold. This pulse energy is in close agreement with other studies where subcellular organelle disruption has been carried out; technical considerations and subtle differences in the membrane structure and composition of organelles or organisms have to be taken into account to explain the pulse energy variation among experiments. When organisms with cell wall are subject to femtosecond laser processing, a substantial increase in the pulse energy is demanded for cellular ablation. The cross linked structure of polymers in plant and cyanobacterial cell wall is here proposed to explain the high pulse energy threshold found in our study and in the one by Assion et al. (22). Finally, the very high pulse energy threshold to ablate heterocysts is ascribed to the presence of both the cell wall and envelope, consisting respectively of highly cross linked peptidoglycan layers and cross linked inner-laminated glycolipid layers that together give rise to further physical resistance.

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FIGURE LEGENDS

Figure 1. Experimental setup for femtosecond laser pulse processing of filamentous cyanobacteria (left panel). Light and fluorescence micrographs of *Anabaena* sp. strain PCC 7120 were obtained by means of a 100× microscope objective imaging onto a CCD camera. A closer inspection of the sample holder and the sample in the glass-bottom culture dish is shown (right panel). Other details are given in the text.

Figure 2. Effect of femtosecond laser irradiation on the chlorophyll fluorescence emitted by vegetative cells of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 in agar solid medium (BG110). Arrowheads in frame a) (light micrograph) and b) (fluorescence micrograph) indicate the position of the target vegetative cell before femtosecond laser irradiation. Arrowhead in frame c) (fluorescence micrograph) points the partial fluorescence photobleaching in the irradiated vegetative cell. Arrowhead in d) (light micrograph) points the irradiated cell displaying neither changes in its morphology nor cell wall disruption. Energy per pulse, 9±3 nJ and 2000 pulses per series. Bar, 10 µm.

Figure 3. Light (frames a) and c)) and fluorescence (frames b) and d)) micrographs of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 in agar solid medium (BG110). Arrowheads in frames a) and b) indicate the position of the target terminal heterocyst at one end of a cyanobacterial filament before femtosecond laser irradiation. The target terminal heterocyst is dimly fluorescence, when compared to vegetative cells of the same cyanobacterial filament. Arrowhead in frame c) points the position of the target heterocyst after femtosecond laser
irradiation and arrowhead in d) points the adjoining vegetative cell, fluorescence emission of which is not affected after heterocyst ablation. Energy per pulse, 68±21 nJ and 200 pulses per series. Bar, 10 µm.
Table 1. Pulse energy dependence for femtosecond laser processing of heterocysts and vegetative cells in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 under combined nitrogen deprivation.

<table>
<thead>
<tr>
<th>Pulse energy (nJ)</th>
<th>Peak fluence (J/cm²)</th>
<th>Pulse series number</th>
<th>Vegetative cells (number of cells, &gt;100)</th>
<th>Heterocysts (number of cells, ~50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9±3</td>
<td>0.8±0.3</td>
<td>200</td>
<td>No damage.</td>
<td>N/A</td>
</tr>
<tr>
<td>9±3</td>
<td>0.8±0.3</td>
<td>2000</td>
<td>Thylakoid membrane disruption without apparent removal of cellular material.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Changes in the shape of cell and partial loss of cellular material.</td>
<td></td>
</tr>
<tr>
<td>13±4</td>
<td>1.2±0.4</td>
<td>200</td>
<td>No damage.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Partial ablation of the cell wall.</td>
<td></td>
</tr>
<tr>
<td>43±13</td>
<td>4.0±1.2</td>
<td>200</td>
<td>Complete cellular ablation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Changes in the shape of the cell and material eruption.</td>
<td>Partial ablation of the envelope.</td>
</tr>
<tr>
<td>68±21</td>
<td>6.4±1.9</td>
<td>200</td>
<td>N/A</td>
<td>Complete cellular ablation.</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3