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Hydrogen peroxide induces apoptotic-like cell death in *Microcystis aeruginosa* (Chroococcales, Cyanobacteria) in a dose-dependent manner

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We investigated the capability of *Microcystis aeruginosa* to cause apoptosis by pursuing morphological, molecular and physiological characteristics after exposure to H_2O_2. *Microcystis* proliferation was only weakly affected after exposure to 150 μM H_2O_2 but cell numbers decreased dramatically after exposures of 250 and 325 μM H_2O_2. Cells exposed to 250 and 325 μM H_2O_2 were examined using transmission electron microscopy, and they exhibited membrane deformation and partial disintegration of thylakoids. Correspondingly, fluorescence imaging of DNA by Hoechst 33342 staining revealed the condensation of nucleoid chromatin. Moreover, cellular injury was concomitant with dramatic decreases in photosynthetic efficiency (ratio of variable fluorescence to maximum fluorescence [Fv/Fm], maximum electron transport rate [ETRmax]) and elevated caspase-3–like activity after exposure of 250 and 325 μM H_2O_2. Terminal deoxynucleotidyl transferase Deoxyuridine 5-triphosphate nick end labelling (TUNEL) positive staining appeared in cells exposed to 250 μM and 325 μM H_2O_2, and the percentage staining increased with increasing H_2O_2 concentration. These data suggested that *M. aeruginosa* exposed to H_2O_2 underwent an apoptotic event. Additionally, cells exposed to H_2O_2 had increased cytoplasmic vacuolation and nontypical DNA laddering. Increased caspase-3–like activity was not inhibited in the presence of the synthetic caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone. Therefore, H_2O_2 induced apoptotic-like cell death in a dose-dependent manner. Taken together, our results provided a novel mechanism for explaining cyanobacterial bloom dynamics in response to environmental stress. The results also contributed to the understanding of the origin and evolution of programmed cell death.

Key Words: Hydrogen peroxide, *Microcystis*, Apoptotic-like cell death, Hoechst 33342, Caspase-3–like activity, TUNEL

INTRODUCTION

Traditionally, phytoplankton were considered immortal unless eaten by predators or sank irreversibly (Kirchman 1999). There is recent evidence, for both marine and freshwater species, that phytoplankton can undergo programmed cell death (PCD) in response to environmental stress (Bidle & Falkowski 2004). PCD is an irreversible, genetically controlled form of cell suicide that is essential for the proper development, function and ultimate survival of multicellular organisms (Ameisen 2002). A well-known form of PCD is apoptosis, which is accompanied by plasma membrane blebbing, chromatin condensation, involvement of cysteine proteases (caspases) and fragmentation of DNA (Kerr *et al*. 1972). Under diverse environmental stresses, the filamentous marine cyanobacterium *Trichodesmium* sp. IMS101 initiates PCD with an increase in caspase activity (Berman-Frank *et al*. 2004). DNA fragmentation occurs in the unicellular *Microcystis aeruginosa* (Kützing) Kützing when it is placed in darkness or is exposed to elevated temperatures (Bouchard & Purdie 2011). Various types of PCD, with overlapping morphological and physiological hallmarks, have been described in unicellular organisms (Madeo *et al*. 2002; Herker *et al*. 2004; Kroemer *et al*. 2009; Reape & McCabe 2010). Other cell death types termed 'paraptosis', 'aponecrosis' and 'autophagy', which are fundamentally different from apoptosis, have also been described (Jimenez *et al*. 2008, 2009). However, few studies have dealt with PCD in cyanobacteria. PCD may be ecologically relevant for unicellular algae because it may control massive cell lysis of phytoplankton in nature. Furthermore, PCD investigations can contribute to our understanding of the evolutionary origin of cell death processes. Among the toxic cyanobacteria, *M. aeruginosa* commonly occurs in highly eutrophic lakes, and it often dominates the phytoplankton in eutrophic lakes and ponds (Yamamoto & Nakahara 2009). *Microcystis aeruginosa* produces the hepatotoxin microcystin, which is a threat to human and environmental health (Babica *et al*. 2006). Recently, it has been reported that *M. aeruginosa* NIES 843 and PCC 7806 harbour genes for PCD that may be related to the rapid collapse of *Microcystis* blooms (Kaneko *et al*. 2007; Frangeul *et al*. 2008). Therefore, *M. aeruginosa* is an ideal organism for investigating cellular mechanisms under specific environmental conditions involved in PCD.

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PCD is induced by various stresses, such as viral infection, heat, ultraviolet (UV) irradiation, nutrient deprivation, salt stress, extreme temperature or oxidative stress (Bidle & Falkowski 2004; Bidle *et al.* 2007). Oxidative stress is among the most frequent causes of PCD induction. For example, UV-B radiation generates high amounts of reactive oxygen species (ROS) in phytoplankton (Chen *et al.* 2009). ROS are inevitably generated in processes such as respiration and photosynthesis (Apel & Hirt 2004) when the production of reactive oxidants exceeds the cellular antioxidants. Cyanobacteria are often exposed to changing conditions, including drastic fluctuations in light intensity and temperature, and therefore ROS are easily produced. Common ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). The production of H_2O_2 within the cells is a key element in both biotic and abiotic stresses and PCD (Tsanko & Jacques 2005). For example, the production of ROS in *Microcystis aeruginosa* is significantly increased under exogenous H_2O_2 conditions (Bouchard & Purdie 2011). On the other hand, H_2O_2 may act as an algicide because of its rapid decomposition into oxygen and water. Qian *et al.* (2010) reported that H_2O_2 inhibited algal growth by blocking transcription of photosynthesis-related genes or by destroying photosynthetic pigments. Ross *et al.* (2006) established that the addition of H_2O_2 elicited caspase activity in *M. aeruginosa* in the environment but they did not provide detailed descriptions of other biochemical and cellular parameters. Therefore, we asked the question: Does an apoptotic form of PCD exist in cyanobacteria and can H_2O_2 induce this type of PCD? We investigated whether *M. aeruginosa* is capable of executing apoptosis when exposed to H_2O_2 by studying the morphological, molecular and physiological features associated with this process.

MATERIAL AND METHODS

Unicellular *Microcystis aeruginosa* strain FACHB-905 was cultured in BG-11 medium at 25°C under 20–25 μmol photons m^{-2} s^{-1} illumination (Li-Cor LI-185B, USA) with a 12:12 h light:dark cycle. Cultures were grown for 7 to 12 days after inoculation into 250 ml flasks. Cells were harvested by centrifugation and washed three times with sterilized distilled water; the pellets were inoculated into 50 ml flasks. The cell concentration was approximately 8.0 $\times$ 10^6 cells ml^{-1}. The effects of H_2O_2 were tested at concentrations 150, 200, 250 and 325 μM.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma, USA) was prepared in phosphate-buffered saline (PBS; pH 6.8) at a concentration of 0.5 mg ml^{-1} with 0.1 mol l^{-1} sodium succinate. The stock MTT solution was stored at 4°C. A 250 μl sample was used for MTT staining. Samples were washed and suspended in 250 ml BG-11 medium before staining. After incubation, the dye was removed by centrifugation at 5,939 $\times$ *g* for 3 min. The pellets were resuspended in 250 ml distilled water. The 250 ml samples were combined with 100 μl MTT stock solution and incubated at 35 $\pm$ 1°C for 1.5 h; 8 μl of suspension was examined with a hemacytometer using light microscopy (Olympus CX41, Japan). From each sample at least 300 cells were analyzed.

For transmission electron microscopy (TEM), cells were harvested 24 h after exposure to 250 and 325 μM H_2O_2. Cells were washed three times with PBS, fixed with 2.5% glutaraldehyde and then placed in 1% OsO_4 for 2 h at room temperature. After graded ethanol dehydration, samples were embedded in Epoxy epon-812 and polymerized at 70°C for 8 h. Sections were cut, stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 electron microscope.

For Hoechst staining, cells were harvested after 24 h exposure to 250 and 325 μM H_2O_2. Cells were washed three times with PBS and fixed by addition of formaldehyde (final concentration 4% vol/vol), and then drops of the cell suspension were placed on polylysine-coated slides for 90 min. Slides were washed in PBS to remove formaldehyde. To stain DNA with the fluorescent DNA binding probe, cells were incubated with Hoechst 33342 at 10 μg ml^{-1} for 1 h. Cells were photographed with a digital camera and a fluorescence microscope (Olympus BX51).

Chlorophyll fluorescence parameters were measured with a pulse-amplitude–modulated fluorescence monitoring system (PAM, Walz, Effeltrich, Germany). The values of Fv/Fm (ratio of variable fluorescence to maximum fluorescence; the maximum effective quantum yield of photosystem II) and ETRmax (the maximum electron transport rate) were recorded.

The CaspGlow Fluorescein Active Caspase-3 Staining Kit (BioVision, USA) was used to quantify caspase-3–like activity in cells. The assay utilized the fluorescein isothiocyanate (FITC)-labelled Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone, which was cell permeable and irreversibly bound to activated caspase-3 in apoptotic cells. The kit was used according to the manufacturer's instructions. After labelling, cells were washed twice in buffer to remove background fluorescence. For analysis with a fluorescence plate reader, each cell sample was resuspended in 100 μl wash buffer and then transferred into a well of a black microtiter plate. The fluorescence intensity was measured at 485 nm (excitation) and 535 nm (emission). We used nontreated cells that were exposed to FITC-DEVD-FMK as the control. The protein content was estimated by the Bradford Method (Bradford 1976). The caspase activity was expressed as the percentage enzyme activity compared to the control group.

DNA was extracted according to a previously described method (LoSchiavo *et al.* 2000), with the following modifications. Briefly, after exposure to H_2O_2 for 24 or 48 h, the cells were collected, washed once with PBS and lysed in the buffer (10 mM Tris; 1 mM ethylenediaminetetraacetic acid [EDTA]; 100 mM NaCl; 100 μg ml^{-1} lysozyme, pH 9.0) at 37°C for 30 min. Thereafter, 20 mg ml^{-1} proteinase K and 10% sodium dodecyl sulfate were added. After incubation at 55°C for 90 min, DNA was extracted with phenol-chloroform and centrifuged at 13,362 $\times$ *g* for 8 min at room temperature. The upper aqueous phase was mixed with cold ethanol and 10 M ammonium acetate (1/10), precipitated at −20°C for at least for 1 h and centrifuged at 13,362 $\times$ *g* for 20 min. The nucleic acid pellet was washed with 70% vol/vol ethanol, air

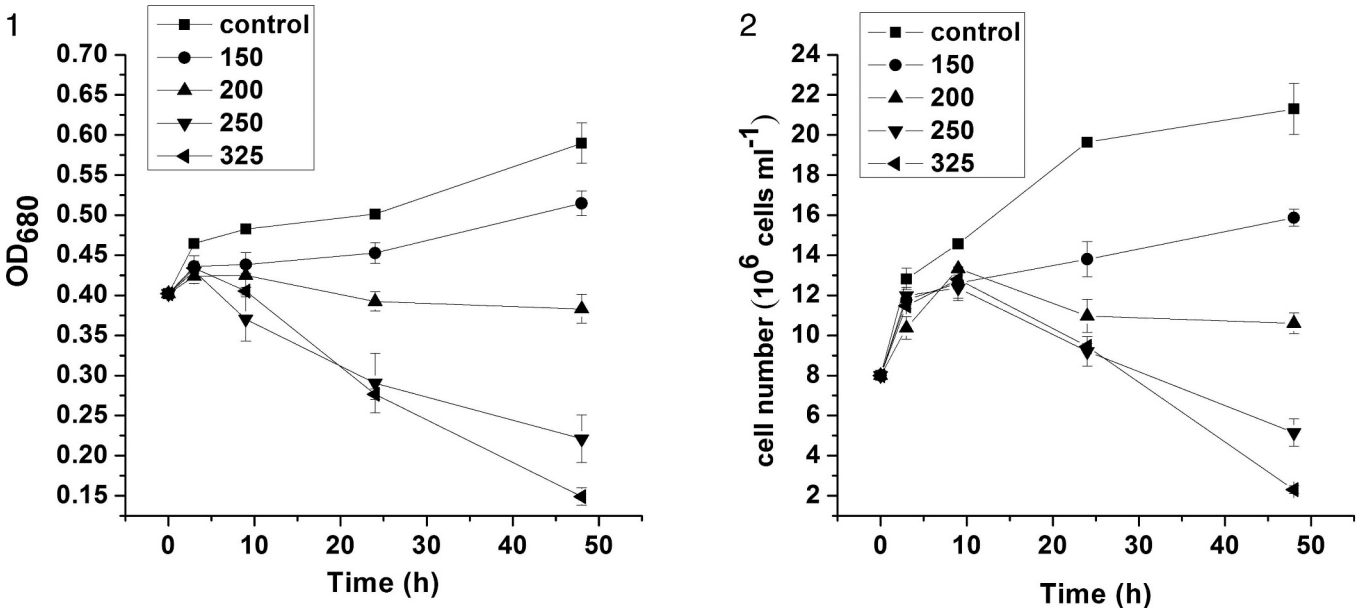


Fig. 1. Growth curves for cells exposed to 150, 200, 250 and 325 μM H_2O_2. Control represents untreated cells.
Fig. 2. Changes in cell number for cells exposed to 150, 200, 250 and 325 μM H_2O_2. Control represents untreated cells.

dried, resuspended in Tris EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and incubated with RNase A (20 μg ml^{-1}) at 37°C for 30 min. DNA fragmentation and standard DNA markers were analyzed by electrophoresis using 1.6% agarose gel at 100 V for 55 min, and the bands were stained with ethidium bromide (0.5 mg l^{-1}) in preparation for UV light visualization using the BioSpectrum Imaging System (UVP, CA, USA).

Terminal deoxynucleotidyl transferase labelling (TUNEL) assays were carried out with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Cat. No. 11 684 795 910, Mannheim, Germany). The samples were harvested after 24 h and 48 h exposure to H_2O_2. Cells were fixed at room temperature for 1.5 h with 2% paraformaldehyde in PBS and then washed with PBS. Cells were permeabilised for 20 min at 4°C in solution containing 0.1% Triton X-100 and 0.1% sodium citrate. The labelling and signal conversions were carried out according to the manufacturer's instructions. Finally, samples were analyzed under a fluorescence microscope (Olympus BX51) using an excitation wavelength in the range of 450–500 nm, and results were processed using the Image-Pro Express 6.0 software program. Representative images were taken after analysis of at least 500 cells per sample.

All experiments were performed in triplicate; data were presented as means ± standard deviations (*s*) and analyzed using Microcal Origin Software (Version 8.0, Microcal Software Inc. Northampton, MA, USA).

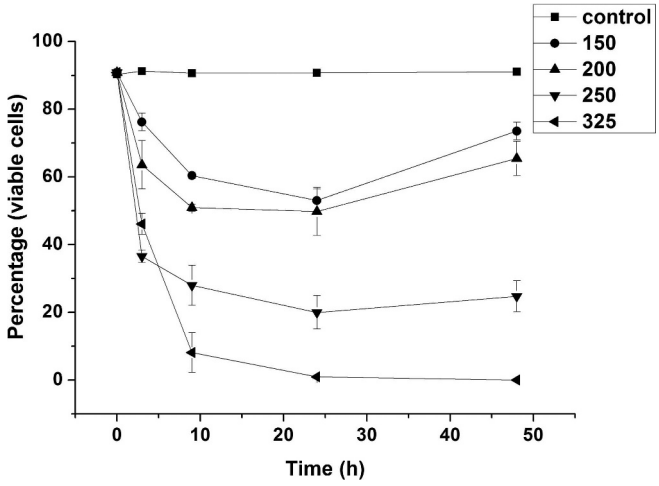


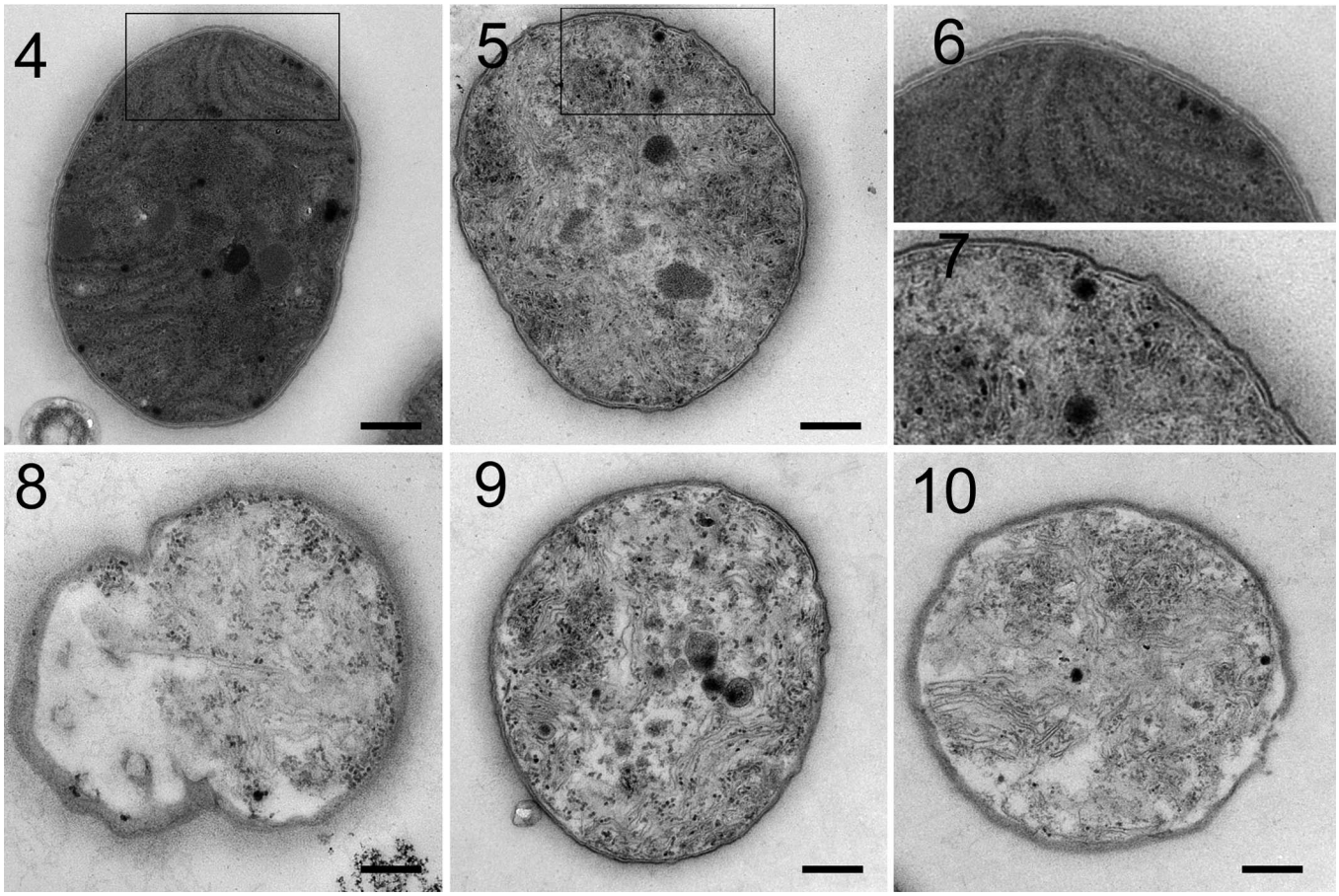
Fig. 3. Cell viability determined by MTT assay after exposure to 150, 200, 250 and 325 μM H_2O_2. Control represents untreated cells.

RESULTS

The effects of H_2O_2 on the growth of *Microcystis aeruginosa* within a period of 48 h were shown in Figs 1, 2. Compared to the control, cells exposed to 150 μM H_2O_2 were only weakly affected. However, the optical $density_{680}$ of the sample exposed to 325 μM H_2O_2 decreased from 0.402 to 0.149 within 48 h, while the cell number decreased from 8×10^6 cells ml^{-1} to 2.3×10^6 cells ml^{-1}.

As shown in Fig. 3, the exposure to H_2O_2 for 0, 3, 9, 24 and 48 h resulted in a dose-dependent decrease in viability compared with control cells. More than 60% of the cells were alive after 3 h of exposure to 150 and 200 μM H_2O_2. After 24 h, 50% of the cells were dead at both concentrations. However, only 20% of cells were alive after 24 h when the cells were exposed to 250 μM H_2O_2, and only 1% were alive after 48 h when exposed to 325 μM H_2O_2. Therefore, control cells and cells exposed to 250 and 325 μM H_2O_2 were used for TEM examination and Hoechst 33342 staining experiments.

The control cells showed normal nucleoid zone, dense cytoplasm, well-defined thylakoid and intact plasma membranes (Fig. 4). Cells exposed to H_2O_2 had less dense



Figs 4–10. Various stages of the ultrastructural changes induced by H_2O_2. Scale bars = 500 nm.
Fig. 4. Ultrastructure of a control cells.
Figs 5, 8. Ultrastructural changes of cells after exposure to 250 μM H_2O_2 for 24 h.
Figs 6, 7. Enlarged views of designated windows in 4 and 5, respectively.
Figs 9, 10. Ultrastructural changes of cells after exposure to 325 μM H_2O_2 for 24 h.

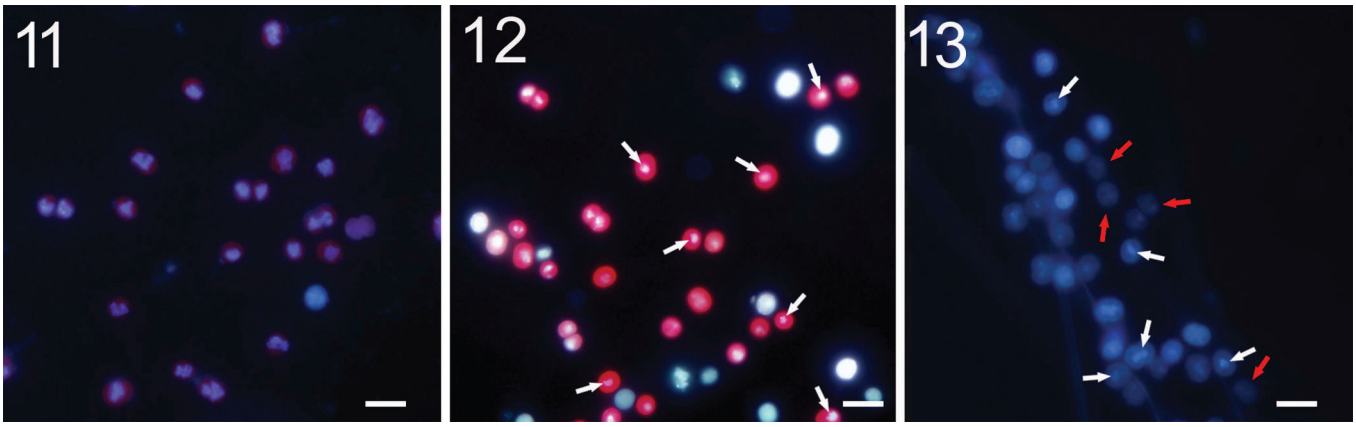
stroma, and membrane deformation was observed (Figs 5, 8–10). And Figs 6, 7 clearly showed that the wavy folds of outer cells became irregular. Deformation, partial disintegration of thylakoids and cytoplasmic vacuolation were observed after exposure. Figs 8–10 show a progressive amplification of these changes, especially the increasing cytoplasmic vacuolation.

Control cells exhibited more diffuse chromatin and an intact cell membrane (Fig. 11). Cells exposed to H_2O_2 showed highly condensed chromatin, an early apoptotic character (Figs 12, 13 white arrows). The loss of chromatin architecture or even a completely lysed nucleoid zone, characteristic of late apoptosis, was observed in cells exposed to 325 μM H_2O_2 (Fig. 13, red arrows). Moreover, apoptotic-like cells were more permeable to Hoechst 33342, causing a brighter blue fluorescence.

To determine the effect of H_2O_2 on photosynthesis, the maximum photochemical yield (Fv/Fm) and the maximum ETR (ETRmax) of photosystem II were measured (Figs 14, 15). Within 3 h of exposure, the Fv/Fm values of cells exposed to H_2O_2 were all rapidly decreased comparable to control cells. The ETRmax of cells exposed to 250 and 325 μM H_2O_2 decreased nearly to zero at 9 h, while the Fv/Fm value decreased to zero at 24 h.

After exposure to 250 and 325 μM H_2O_2, the caspase-3–like activity increased gradually from 2 h to 8 h compared to controls (Fig. 16). At 8 h, the caspase-3–like activity of cells exposed to 325 μM H_2O_2 reached the peak, 33-fold compared with the control. Caspase-3–like activity showed a statistically significant decrease after 24 h exposure ($P < 0.05$). Caspase-3–like activity of cells exposed to 250 μM H_2O_2 for 24 h was not statistically significantly different from the activity detected at 8 h, and activity was still on average six times higher than that of the control cells ($P < 0.05$). When the cells were preincubated with the caspase inhibitor Z-VAD-FMK, caspase-3–like activity was not inhibited (data not shown).

To discern whether the degradation of chromatin was accompanied by characteristic DNA fragmentation associated with PCD, we analyzed the DNA by adopting classical methods, i.e. with detection of DNA laddering in agarose gels and with the TUNEL assay. Agarose gel analysis of genomic DNA isolated from control cells and from cells exposed to increasing amounts of H_2O_2 at 24 h and 48 h was used to determine whether H_2O_2 stress could induce DNA fragmentation (Fig. 17). DNA was extracted from the cells after 24 h of H_2O_2 exposure, when caspase-3–like activity was increased (Fig. 16). The DNA was slightly



Figs 11–13. Cells stained with fluorescent dye Hoechst 33342 at 24 h after H_2O_2 exposure. White arrows = chromatin condensation; red arrows = loss of chromatin architecture. Scale bar = 5 μm.
Fig. 11. Untreated cells.
Fig. 12. Cells exposed to 250 μM H_2O_2.
Fig. 13. Cells exposed to 325 μM H_2O_2.

degraded and appeared as a faint smear on the gel (Fig. 17, lane 3). However, at 48 h of 250 μM H_2O_2 exposure, diffuse low molecular weight bands were visible (Fig. 17, lane 4). Such fragmentation was not observed in the cells exposed to 325 μM H_2O_2, and only a smear was detectable on the gel after 24 h (Fig. 17, lane 5). No DNA laddering was detected in the controls.

The TUNEL assay was performed to corroborate the DNA fragmentation (Figs 18–24). Free 3′OH ends of DNA, generated by activation of endonuclease activity in dying cells, were fluorescently labelled in the TUNEL assay. The percentage of TUNEL-positive cells after exposure to H_2O_2 increased gradually from 2 h to 48 h (Fig. 18). In negative controls without the terminal deoxynucleotidyl transferase enzyme, no TUNEL-positive cells were observed (Fig. 19). The TUNEL-positive signal was only found in H_2O_2 treated cells, while the control cells showed no positive signal, apparently because of the absence of DNA free ends that allow the incorporation of fluorescein labelled dUTP (Fig. 20). When cells were treated with 250 μM H_2O_2 at 24 h, 51% of the cells were TUNEL-positive (Fig. 21). When cells were treated with 325 μM H_2O_2 at 48 h, 92% of cells were TUNEL-positive (Fig. 24).

DISCUSSION

We found several classic parameters associated with apoptosis in *Microcystis aeruginosa* cells exposed to 250 and 325 μM H_2O_2, e.g. membrane deformation, cytoplasmic vacuolation, chromatin condensation, DNA fragmentation and increasing caspase-3–like activity. Caspases have only been isolated from multicellular animals (sponges to

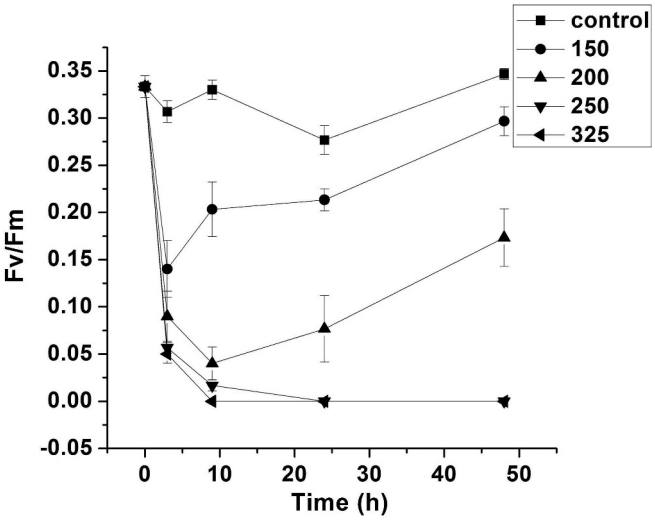


Fig. 14. Ratio of variable fluorescence to maximum fluorescence (Fv/Fm) for cells exposed to 150, 200, 250 and 325 μM H_2O_2. Control represents untreated cells.

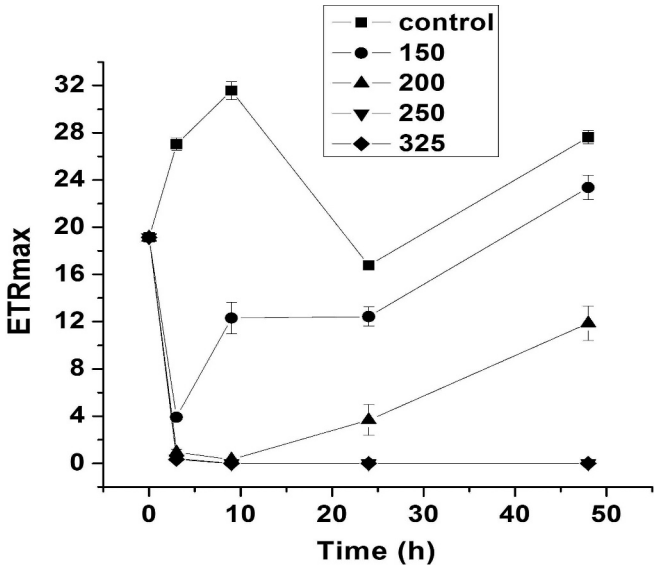


Fig. 15. Electron transport rate maximum (ETRmax) for cells exposed to 150, 200, 250 and 325 μM H_2O_2. Control represents untreated cells.

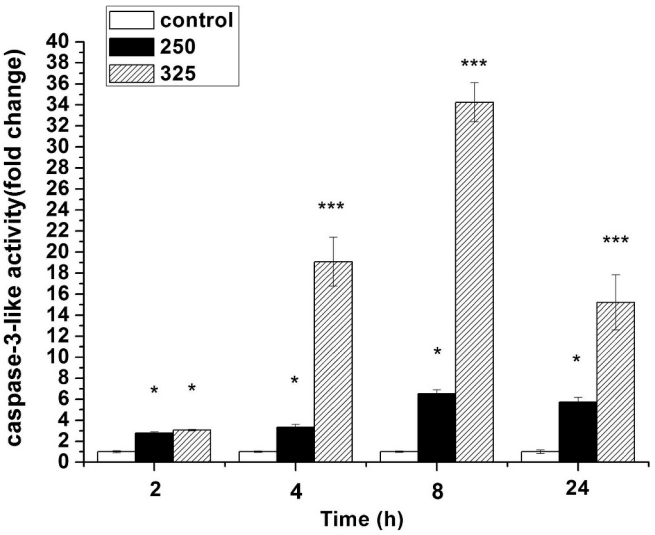


Fig. 16. Caspase-3–like activity measured by fluorescence intensity at 485 nm (excitation) and 535 nm (emission). * $P < 0.05$, *** $P < 0.001$.

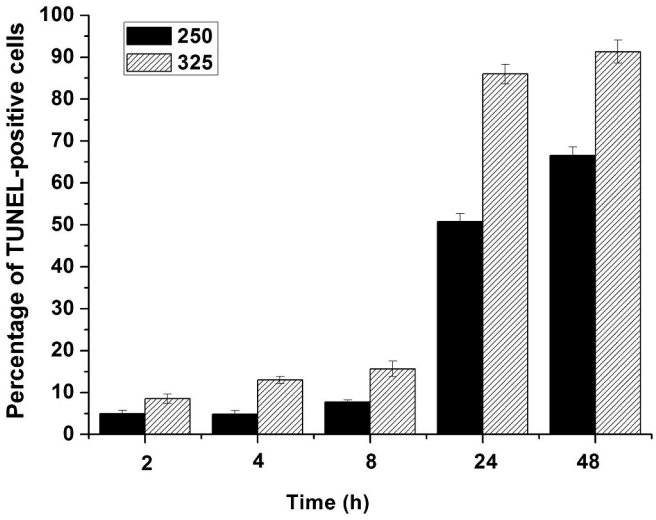


Fig. 18. The percentage of TUNEL-positive cells exposed to 250 and 325 μM H_2O_2 for 2, 4, 8, 24 and 48 h.

humans) but their activity has also been reported for vascular plants, yeast and bacteria (De-Jong *et al.* 2002; Madeo *et al.* 2002; Herker *et al.* 2004; Jimenez *et al.* 2008, 2009; Chen & He 2009). Within the caspase family, caspase-3 is believed to be the final executor of apoptotic cell death; it elicits organized degradation of many cellular proteins, and it changes the nuclear morphology (Fischer *et al.* 2003; Rice & Bayles 2008). We found caspase-3–like activity increased over time after exposure to 250 and 325 μM H_2O_2. Similar results have also been reported during PCD for the green algae *Micrasterias denticulata* Brébisson & Godey ex Ralfs (Darehshouri *et al.* 2008), *Dunaliella tertiolecta* Butcher (Segovia *et al.* 2003) and *Chlorella saccharophila* (Krüger) Migula (Zuppini *et al.* 2007) as well as the cyanobacterium *Trichodesmium* sp. IMS101 (Berman-Frank *et al.* 2004). However, our findings show that increased caspase-3–like activity was not inhibited in the presence of the caspase inhibitor Z-VAD-FMK. Therefore, we cannot claim that H_2O_2 triggers a caspase-dependent cell death. The broad-spectrum caspase inhibitor Z-VAD-FMK inhibits caspase-3 in animals and is commonly used in elucidating apoptotic pathways; however, it does not inhibit all caspases in plants (Equils *et al.* 2009; Elodie *et al.* 2010). *Microcystis* is a prokaryote, and Z-VAD-FMK might not bind to the catalytic site of its caspase-3–like proteins. Cyanobacterial caspase-like proteins form several distinct clusters in phylogenetic analyses, showing considerable diversity (Bidle & Falkowski 2004). Perhaps cyanobacterial caspase-like orthologues represent an ancestral core of caspase-like proteins, different from caspases in metazoans.

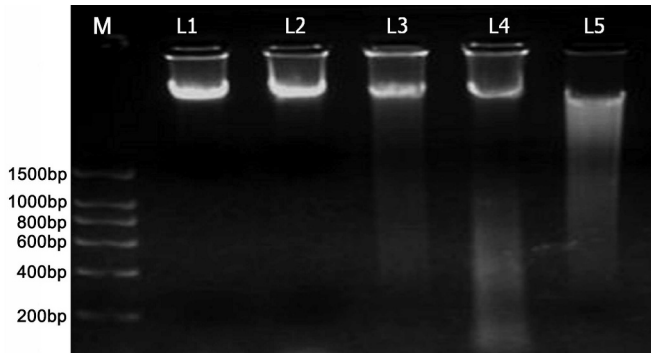
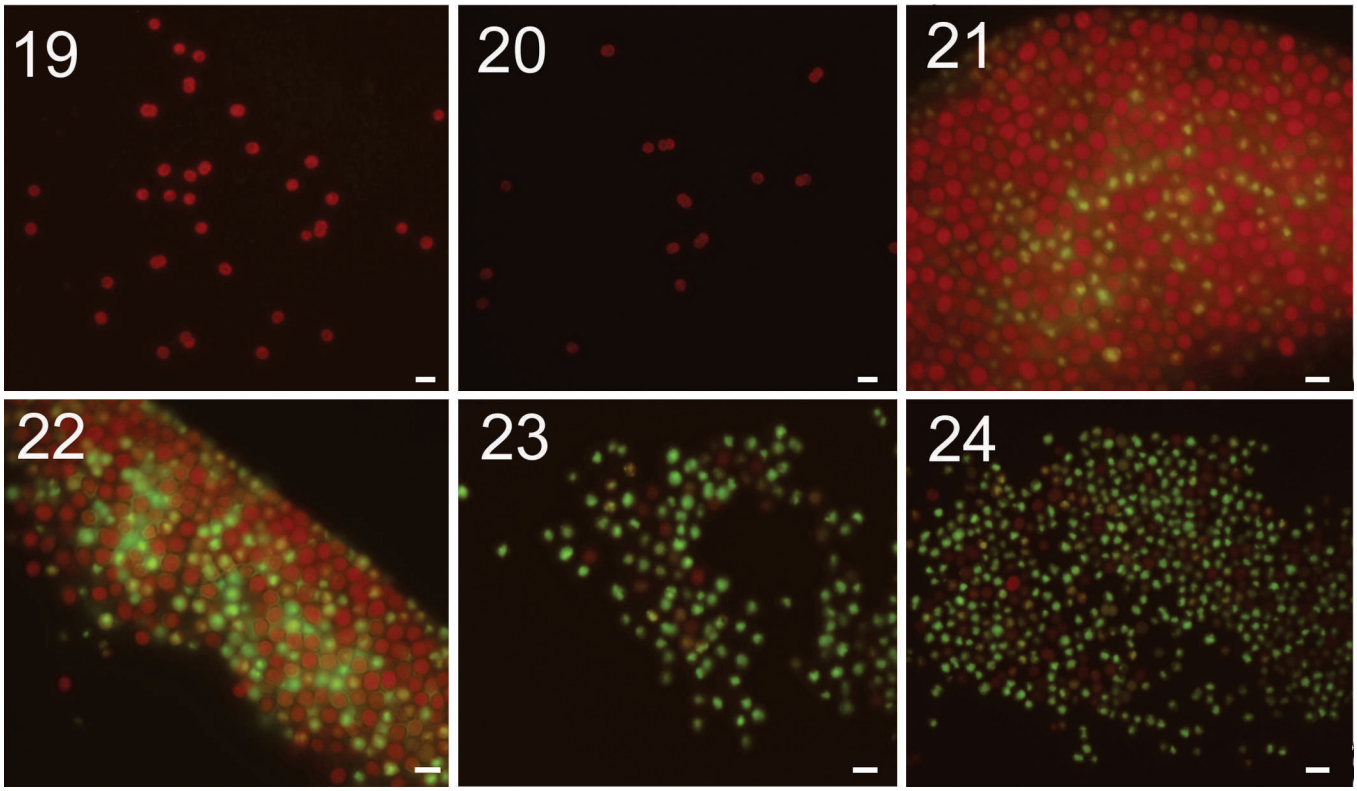


Fig. 17. DNA degradation shown by agarose gel electrophoresis. L1 = control cells for 24 h; L2 = control cells for 48 h; L3 = 250 μM H_2O_2 for 24 h; L4 = 250 μM H_2O_2 for 48 h; L5 = 325 μM H_2O_2 for 24 h; M = molecular markers.

DNA fragmentation has also been used to diagnose apoptosis in animals and plants (Ameisen 2002; Williams & Dickman 2008). Our TUNEL results demonstrated that *Microcystis aeruginosa* exposed to H_2O_2 can lead to DNA fragmentation, which indicates apoptosis. Similar results are reported for *Anabaena* sp. (Ning *et al.* 2002), *Microcystis aeruginosa* (Bouchard & Purdie 2011), *Dunaliella viridis* Teodoresco (Jimenez *et al.* 2009) and *Skeletonema costatum* (Greville) Cleve (Chung *et al.* 2005). However, we found only diffuse low molecular weight bands on agarose gels at 48 h following 250 μM H_2O_2 exposure, not DNA laddering. Nevertheless, the absence of typical DNA laddering does not rule out the existence of PCD because most unicellular prokaryotic organisms, and even in some eukaryotic algae, don't produce DNA laddering with PCD. For instance, programmed cell death without a DNA ladder has been observed in *Thalassiosira pseudonana* Hasle & Heimdal (Bidle & Bender 2008), *Anabaena* sp. (Ning *et al.* 2002) and *Dictyostelium discoideum* Raper (Cornillon *et al.* 1994). Affenzeller *et al.* (2009) found that DNA laddering only occurred in NaCl-stressed *Micrasterias* cells but not in cells undergoing other types of stress. Therefore, it appears that the mechanism of DNA laddering varies in different species or even as a result of different environmental stresses.

During cell death induced by high concentrations of H_2O_2, there is an increase in cytoplasmic vacuolation, a feature regarded as a cytological hallmark of paraptosis. Cytoplasm vacuolation was also observed during PCD for



Figs 19–24. *In situ* detection of cell death using TUNEL assay; TUNEL-positive cells = green; others = red. Scale bar = 5 μm.
Fig. 19. Negative control without terminal transferase.
Fig. 20. Cells not exposed to H_2O_2.
Fig. 21. Cells exposed to H_2O_2 250 μM H_2O_2 at 24 h.
Fig. 22. Cells exposed to H_2O_2 250 μM H_2O_2 at 48 h.
Fig. 23. Cells exposed to H_2O_2 325 μM H_2O_2 at 24 h.
Fig. 24. Cells exposed to H_2O_2 325 μM H_2O_2 at 48 h.

Anabaena sp. (Ning *et al.* 2002), *Micrasterias denticulata* (Darehshouri *et al.* 2008) and *Amphidinium carterae* Hulbert (Franklin & Berges 2004). However, paraptosis involves mitochondrial swelling and lacks apoptosis characters such as caspase activation and DNA fragmentation. To gain deeper insight, *Microcystis aeruginosa* cells were stained with Hoechst 33342. Under the fluorescence microscope, we observed chromatin condensation in cells exposed to H_2O_2, and this evidence indicates an apoptotic event. There was a complete loss of chromatin architecture or a completely lysed nucleoid zone in cells exposed to 325 μM H_2O_2; this may be due to DNA fragmentation because small DNA fragments are typical during the final stages of apoptotic-like PCD. Overall, our results clearly suggest that H_2O_2 induced apoptotic-like cell death in a dose-dependent manner.

Apoptosis has been mainly studied in metazoans, but the precise subcellular mechanisms and evolutionary drivers are still largely unknown in unicellular organisms. Our study, using a diverse suit of markers with H_2O_2 treatments, contributes to the understanding of PCD models that occur in unicellular phytoplankton and to how PCD events are regulated. Differences between the typical animal apoptotic characteristics and our results are probably due to fundamental differences between metazoa and prokaryotes. For animals and plants, mitochondria and chloroplasts are sources of ROS in stress situations, and the chloroplast plays an important role in plant PCD because the process is light-dependent (Doyle *et al.* 2010; Li & Xing 2011). Given the fact that cyanobacteria do not possess mitochondria, it is likely that their photosynthetic apparatus might have full control over cell death regulation.

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REFERENCES

Affenzeller M.J., Darehshouri A., Andosch A., Lutz C. & Lutz-Meindl U. 2009. Salt stress-induced cell death in the unicellular green algae *Micrasterias denticulata*. *Journal of Experimental Botany* 60: 939–954.

Ameisen J.C. 2002. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. *Cell Death and Differentiation* 9: 367–393.

Apel K. & Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55: 373–399.

Babica P., Blaha L. & Marsalek B. 2006. Exploring the natural role of microcystins—a review of effects on photoautotrophic organisms. *Journal of Phycology* 42: 9–20.

Berman-Frank I., Bidle K.D., Haramaty L. & Falkowski P.G. 2004. The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway. *Limnology and Oceanography* 49: 997–1005.

Bidle K.D. & Bender S.J. 2008. Iron starvation and culture age activate metacaspases and programmed cell death in the marine diatom *Thalassiosira pseudonana*. *Eukaryotic Cell* 7: 223–236.

Bidle K.D. & Falkowski P.G. 2004. Cell death in planktonic, photosynthetic microorganisms. *Nature Reviews Microbiology* 2: 643–655.

Bidle K.D., Haramaty L., Ramos J.B. & Falkowski P. 2007. Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*. *Proceedings of the National Academy of Sciences USA* 104: 6049–6054.

Bouchard J.N. & Purdie D.A. 2011. Effect of elevated temperature, darkness and hydrogen peroxide treatment on oxidative stress and cell death in the bloom-forming toxic cyanobacterium *Microcystis aeruginosa*. *Journal of Phycology* 47: 1316–1325.

Bradford M.M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Chen F. & He Y.Q. 2009. Caspase-2 mediated apoptotic and necrotic murine macrophage cell death induced by rough *Brucella abortus*. *Plos One* 4, (8), 13. e6830. doi:10.1371/journal.pone.0006830

Chen L.Z., Wang G.H., Hong S., Liu A., Li C. & Liu Y.D. 2009. UV-B-induced oxidative damage and protective role of exopolysaccharides in desert cyanobacterium *Microcoleus vaginatus*. *Journal of Integrative Plant Biology* 51: 194–200.

Chung C.C., Hwang S.P.L. & Chang J. 2005. Cooccurrence of ScDSP gene expression, cell death, and DNA fragmentation in a marine diatom, *Skeletonema costatum*. *Applied and Environmental Microbiology* 71: 8744–8751.

Cornillon S., Foa C., Davoust J., Buonavista N., Gross J.D. & Golstein P. 1994. Programmed cell-death in *Dictyostelium*. *Journal of Cell Science* 107: 2691–2704.

Darehshouri A., Affenzeller M. & Lutz-Meindl U. 2008. Cell death upon H_2O_2 induction in the unicellular green algae *Micrasterias*. *Plant Biology* 10: 732–745.

De-Jong A.J., Yakimova E.T., Kapchina V.M. & Woltering E.J. 2002. A critical role for ethylene in hydrogen peroxide release during programmed cell death in tomato suspension cells. *Planta* 214: 537–545.

Doyle S.M., Diamond M. & McCabe, P.F. 2010. Chloroplast and reactive oxygen species involvement in apoptotic-like programmed cell death in *Arabidopsis* suspension cultures. *Journal of Experimental Botany* 61: 473–482.

Elodie L., Delphine M., Justin T., Nicole T., Nathalie A.A., Thierry L., Herve B. & Bruno S. 2010. Caspase-10-dependent cell death in Fas/CD95 signalling is not abrogated by caspase inhibitor zVAD-fmk. *Plos One* 5, (10), e13638. doi:10.1371/journal.pone.0013638.

Equils O., Moffatt-Blue C., Ishikawa T., Simmons C.F., Ilievski V. & Hirsch E. 2009. Pretreatment with pancaspase inhibitor (Z-VAD-FMK) delays but does not prevent intraperitoneal heat-killed Group B Streptococcus-induced preterm delivery in a pregnant mouse model. *Infectious Diseases in Obstetrics and Gynecology* 2009: 749432.

Fischer U., Janicke R.U. & Schulye-Osthoff K. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death and Differentiation* 10: 76–100.

Frangeul L., Quillardet P., Castets A.M., Humbert J.F., Matthijs H.C.P., Cortez D., Tolonen A., Zhang C.C., Gribaldo S., Kehr J.C., Zilliges Y., Ziemert N., Becker S., Talla E., Latifi A., Billault A., Lepelletier A., Dittmann E., Bouchier C. & Tandeau de marsac, N. 2008. Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. *BMC Genomics* 9: 274. doi:10.1186/1471-2164-9-274.

Franklin D.J. & Berges J.A. 2004. Mortality in cultures of the dinoflagellate *Amphidinium carterae* during culture senescence and darkness. *Proceedings of the Royal Society of London, Biological Series* 271: 2099–2107.

Herker E., Jungwirth H., Lehmann K.A., Maldener C., Frohlich K.U., Wissing S., Buttner S., Fehr M., Sigrist S. & Madeo F. 2004. Chronological aging leads to apoptosis in yeast. *Journal of Cell Biology* 164: 501–507.

Jimenez C., Capasso J.M., Edelstein C.L., Rivard C.J., Lucia S., Breusegem S., Berl T. & Segovia M. 2009. Different ways to die: cell death modes of the unicellular chlorophyte *Dunaliella viridis* exposed to various environmental stresses are mediated by the caspase-like activity DEVDase. *Journal of Experimental Botany* 60: 815–828.

Jimenez V., Paredes R., Sosa M.A. & Galanti N. 2008. Natural programmed cell death in *T. cruzi* epimastigotes maintained in axenic cultures. *Journal of Cellular Biochemistry* 105: 688–698.

Kaneko T., Nakajima N., Okamoto S., Suzuki I., Tanabe Y., Tamaoki M., Nakamura Y., Kasai F., Watanabe A., Kawashima K., Kishida Y., Ono A., Shimizu Y., Takahashi C., Minami C., Fujishiro T., Kohara M., Katoh M., Nakazaki N., Nakayama S., Yamada M., Tabata S. & Watanabe M.M. 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES–843. *DNA Research* 14: 247–256.

Kerr J.F.R., Wyllie A.H. & Currie A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26: 239–257.

Kirchman D.L. 1999. Oceanography—phytoplankton death in the sea. *Nature* 398: 293–294.

Kroemer G., Galluzzi L., Vandenabeele P., Abrams J., Alnemri E.S., Baehrecke E.H., Blagosklonny M.V., El-Deiry W.S., Golstein P., Green D.R., Hengartner M., Knight R.A., Kumar S., Lipton S.A., Malorni W., Nuñez G., Peter M.E., Tschopp J., Yuan J., Piacentini M., Zhivotovsky B. & Melino G. 2009. Classification of cell death: recommendations of the Nomenclature Committee on cell death. *Cell Death and Differentiation* 16: 3–11.

Li Z. & Xing D. 2011. Mechanistic study of mitochondria-dependent programmed cell death induced by aluminium phytotoxicity using fluorescence techniques. *Journal of Experimental Botany* 62: 331–343.

Loschiavo F., Baldan B., Compagnin D., Ganz R., Mariani P. & Terzi M. 2000. Spontaneous and induced apoptosis in embryogenic cell cultures of carrot (*Daucus carota* L.) in different physiological states. *European Journal of Cell Biology* 79: 294–298.

Madeo F., Herker E., Maldener C., Wissing S., Lächelt S., Herlan M., Fehr M., Lauber K., Sigrist S.J., Wesselborg S. & Fröhlich K.U. 2002. A caspase-related protease regulates apoptosis in yeast. *Molecular Cell* 9: 911–917.

Ning S.B., Guo H.L., Wang L. & Song Y.C. 2002. Salt stress induces programmed cell death in prokaryotic organism *Anabaena*. *Journal of Applied Microbiology* 93: 15–28.

Qian H.F., Yu S.Q., Sun Z.Q., Xie X.C., Liu W.P. & Fu Z.W. 2010. Effects of copper sulfate, hydrogen peroxide and N-phenyl-2-naphthylamine on oxidative stress and the expression of genes involved photosynthesis and microcystin disposition in *Microcystis aeruginosa*. *Aquatic Toxicology* 99: 405–412.

Reape T.J. & Mccabe, P.F. 2010. Apoptotic-like regulation of programmed cell death in plants. *Apoptosis* 15: 249–256.

Rice K.C. & Bayles K.W. 2008. Molecular control of bacterial death and lysis. *Microbiology and Molecular Biology Reviews* 72: 85–109.

Ross C., Santiago-Vazquez L. & Paul V. 2006. Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. *Aquatic Toxicology* 78: 66–73.

Segovia M., Haramaty L., Berges J.A. & Falkowski P.G. 2003. Cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the evolution of apoptosis in higher plants and metazoans. *Plant Physiology* 132: 99–105.

Tsanko S.G. & Jacques H. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. *The Journal of Cell Biology* 168: 17–20.

Williams B. & Dickman M. 2008. Plant programmed cell death: can't live with it; can't live without it. *Molecular Plant Pathology* 9: 531–544.

Yamamoto Y. & Nakahara H. 2009. Seasonal variations in the morphology of bloom-forming cyanobacteria in a eutrophic pond. *Limnology* 10: 185–193.

Zuppini A., Andreoli C. & Baldan B. 2007. Heat stress: an inducer of programmed cell death in *Chlorella saccharophila*. *Plant and Cell Physiology* 48: 1000–1009.

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