APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1976, p. 119-126
Vol. 31, No. 1

Printed in U.S.A.

Photooxidation of Cyanobacteria in Natural Conditions

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Received for publication 25 August 1975

Photodynamic effects were demonstrated and assayed under field conditions in a number of different laboratory strains and pond isolates of cyanobacteria; parameters assayed for resistance to photooxidation were viable count, turbidity of the cyanobacterial suspension, and protein and pigment contents. The effects of density, colonial structure, and internal gas vacuoles on the lethal outcome were investigated. The stability and formation of superoxide dismutase under photooxidative conditions in the field and laboratory were studied in the different strains. An isolate of *Microcystis* from blooms in ponds exhibited extremely high resistance to photooxidation, which was abolished by exposure to chloramphenicol.

Previous work in our laboratory showed that CO_2 depletion sensitizes certain laboratory strains of cyanobacteria ("blue-green algae") to photooxidative conditions (2). This demonstration of photodynamic effects under laboratory conditions led to the speculation that photooxidation might be involved in the sudden die-off observed in cyanobacterial blooms in nature. This die-off occurs when the blooms are at peak development and when the organisms are concentrated in heavy scums at the water surface where they are lifted by internal gas vacuoles, a typical feature of many cyanobacteria. In this way a large portion of the cyanobacterial community is exposed to light for many hours. This illumination can be very intense in many of the areas of the world where cyanobacteria thrive. Under such conditions of intensive photosynthetic activity, dissolved oxygen content in the daytime can reach values up to 200 to 300% saturation, whereas CO_2 is depleted rapidly. Although the considerable damages caused by the die-off through release of noxious substances make it important to understand the factors controlling this phenomenon, no satisfactory explanation for the frequent, unpredictable die-off of water blooms is yet available.

Conditions for photooxidative effects exist in Israeli fishponds (2), so we were able to assay photooxidative death and related parameters in these natural conditions. Using a wide range of different cyanobacterial types (unicellular, filamentous, and colonial) and species, as well as samples from natural blooms, various levels of sensitivity were detected and are described.

With onset of photooxidation in the cyanobacteria, the activity of superoxide dismutase (SOD) drops rapidly (1). This enzyme has been associated with protection against the toxic effects of oxygen in all aerobic organisms (3, 5, 6). The correlation of SOD activity and the sensitivity to photooxidation also held for strains tested under field conditions. Possible mechanisms underlying the varying levels of resistance to photooxidation and involving the factors leading to photooxidative death are discussed here in an attempt to clarify the relation of the photooxidation effects to the natural die-off phenomenon.

MATERIALS AND METHODS

Cyanobacterial strains and culture conditions. The axenic strain 7005 (identified as *Microcystis aeruginosa* in the Göttingen collection) and *Synechococcus* sp. 6311 (previously called *Anacystis nidulans* [1, 2]) and *Aphanocapsa* sp. 6714 were kindly supplied by R. Y. Stanier (Institut Pasteur). Unialgal *Anabaena* sp. B-381 and *Plectonema boryanum* 594 were from the Indiana University Culture Collection, and the *Nostoc* sp. was from the collection of the Department of Microbiology, University of California at Berkeley. *Microcystis* sp. NRC-1 (from P. R. Gorham, Research Council of Canada) and *Microcystis* sp. Bethulie strain (obtained from a dam near Bethulie, South Africa) were from the collection of J. Eloff.

Plectonema boryanum was grown on modified Chu no. 10 medium (10). The other cyanobacteria were grown in modified Zehnder and Gorham medium no. 11 (2) modified by doubling the citrate concentration and using 0.006 g of $FeSO_4$ per liter instead of ferrous citrate. The cultures were grown in 100 ml of medium in 250-ml flasks at 26 C

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(except for *Synechococcus* sp., which was grown at 35 C) in continuous fluorescent light (5×10^3 ergs/cm^2 per s) without shaking.

Pond samples were collected in large pails from predominantly *Microcystis* blooms of several fishponds in the Beisan Valley during July to October on the day preceding experiments; the raw pond sample was filtered through 225-μm mesh aperture, nylon high-capacity sifting fabric (Zurich Bolting Cloth Manufacturing Co., Ruschlikon, Switzerland) and centrifuged at low speed for 5 min and at 2,000 $\times$ *g* for 10 min to precipitate cell debris and foreign matter. The floating cells were collected with a pipette for the tests and diluted in modified no. 11 medium.

Field photooxidation experiments. Field tests were carried out in ponds at the laboratory in Kibbutz Nir David. For the field test, dialysis tubing with an inflated diameter of 6.4 mm was cut in 1-m lengths, rinsed in distilled water, knotted at one end, and autoclaved to sterilize and remove traces of glycerol present in the tubing; the tubing was never allowed to dry. For the test the tubing was filled in the laboratory with 20 ml of cyanobacterial culture or pond sample and knotted in such a way that no air bubbles were trapped inside; the filled tubes were suspended in a dark cylinder in medium or pond water for transfer to the pond where the tube was suspended with cotton thread from four poles, fixed in the pond bottom and joined by wire so that the tube was within the surface centimeter of water and not shaded. In dark control tests, the tubes were suspended in the pond within a black polythene sleeve. The medium in the dialysis bag equilibrated with the pond water within 30 min.

Physical parameters of the pond water were measured in situ: pH values ranged between 7.35 and 8.25 (Knick portable battery pH meter; Portamess 902); oxygen concentration ranged from 22% to more than 200% oxygen saturation, temperature ranged between 26 and 32 C (measured with an Electronic Instruments Limited portable dissolved oxygen meter model 1520 with a 1521 electrode); and light intensity around midday was 7×10^5 to 8×10^5 ergs/cm^2 per s (measured with a Kettering Radiometer model 65, Yellow Springs Instrument).

Laboratory photooxidation experiments. Cells were washed on a membrane filter (average pore size, 3 μm) and then suspended in the suitable medium prepared without carbonate or bicarbonate. Cell suspensions were placed in Klett test tubes, incubated at 35 C, and illuminated by a 1000-W iodine quartz lamp with a tungsten filament producing an incident light intensity of 10^6 ergs/cm^2 per s. Oxygen was continuously bubbled through the incubation tubes. Tests of pond *Microcystis* were carried out with 20-ml samples in glass tubes (16 mm in diameter) incubated at 30 C.

Parameters measured. Viable counts were determined by serial dilutions (0.5 ml of suspension in liquid medium in 16-mm-diameter glass test tubes) by recording the highest dilution in which growth is observed (8) during a 5-week incubation with shaking in a New Brunswick Psychrotherm at 30 C. The accuracy of this method is somewhat limited by the filamentous or colonial structures of certain cyanobacteria. Turbidity of cell suspensions was determined with the Klett-Summerson colorimeter using a 540 nm filter. Phycocyanin and chlorophyll *a* contents in field tests were determined by measuring the absorption spectra of cell suspensions in a Cary 14 spectrophotometer using an opaque paper filter to minimize the effects of scattering (11). The absorption at 550 nm, where the spectrum had a plateau and hardly any absorption of chlorophyll *a* or phycocyanin takes place, was used for determining the relative pigment contents. The differences between this value and the 660 to 680 nm and the 620 to 630 nm peaks were used to determine the chlorophyll *a* and phycocyanin contents, respectively. In laboratory tests (see Table 2) the chlorophyll content was determined directly from the optical density at 680 nm wavelength in a Bausch & Lomb spectrophotometer.

To prepare cell-free extracts for determinations of SOD activity and protein content, cell samples were washed with 0.1 M potassium phosphate buffer (pH 7) on a membrane filter (average pore size, 0.45 μm). Strain 7005 and *Synechococcus* 6311 were first centrifuged (12,000 $\times$ *g*, 5 min) twice in the same medium. Suspensions (3 ml) of the washed cells in 0.01 M potassium phosphate buffer (pH 7) were disintegrated in a Nossal cell disintegrator with 3-g glass beads (0.10 to 0.11 mm in diameter) for 30 s at 4 C. The supernatant obtained after centrifugation (12,000 $\times$ *g*, 10 min, 4 C) was assayed for SOD activity and protein content.

SOD was assayed according to Misra and Fridovich (9), using a technique based on the ability of SOD to compete with ferricytochrome C for superoxide anions generated by the aerobic xanthine oxidase system. One unit of SOD is defined as the amount of enzyme required to inhibit the rate of ferricytochrome *c* reduction by 50% at 30 C under the described conditions. The assay was performed with a Gilford spectrophotometer (model 2000) with absorbance indicator equipped with a thermostat sample compartment. Protein concentrations in the cell-free extracts were determined by the method of Lowry et al. (4).

Materials. Xanthine oxidase from buttermilk (grade II), xanthin, and cytochrome *c* from horse heart type III were obtained from Sigma. Chloramphenicol was from Abic, Netanya, Israel. All other reagents were of analytical grade.

RESULTS

Field tests of a number of different laboratory strains of cyanobacteria, including species of *Microcystis, Synechococcus, Plectonema,* and *Nostoc,* showed that conditions for photooxidative death exist in "blooming" ponds and that the cellophane bag method provides a suitable and rapid way to determine such conditions. Marked differences were observed among the different strains in their resistance to photo-

oxidation (Table 1). These field results were very similar to results obtained in photooxidative laboratory conditions (Table 2).

Various parameters (viable count, SOD activity, protein content, turbidity, and chlorophyll *a* and phycocyanin contents) of the cyanobacterial populations exposed to photooxidative field conditions were measured and correlated. Among the strains selected were two strains identified as *Microcystis*, a genus frequently encountered in freshwater blooms; these were the unicellular *Microcystis* sp. NRC-1 (which forms gas vacuoles) and the axenic strain 7005 (which does not produce gas vacuoles in its present form). As an example of results obtained, Fig. 1 shows the test parameters for three strains of different cyanobacterial genera: strain 7005 (Fig. 1A), *Synechococcus* 6311 (Fig. 1B), and *Nostoc* sp. (Fig. 1C). It is clear that photooxidative death, as expressed in decreased viable count, occurred in all cases. Similarly, all the other parameters decreased rapidly in all the strains tested (see Fig. 4E-H).

The correlations established among the different parameters tested in these different species indicate that the decrease in turbidity can serve as a convenient and reliable indicator of the decrease in viable count. Considering the relative simplicity and rapidity of this measurement, it seems useful to adopt turbidity

TABLE 1. *Photooxidation of different cyanobacteria in field tests*[a]

Cyanobacterial strains	Time (h) to decline to:			SOD level (units/mg of protein)		
	1% of original viable count	50% of difference of original and final levels of:				
		Turbidity	Chlorophyll	Initial	After 2 h	After 8 h
Pond *Microcystis*	—[b]	—	3.5	15	15	15
Synechococcus sp. 6311	8	4	3.5	12.4	7.5	2.5
Plectonema boryanum	2	3.5	2.5	14	0	0
Anabaena B-381	2	3.5	3	5.2	0	0
Nostoc sp.	1.3	2.5	2.8	25	1.5	0
Microcystis NRC-1	1.3	3	1.2	11	NT[c]	NT
Strain 7005	1.25	3	2.7	13	0	0

[a] Cultures were harvested towards the end of their logarithmic growth phase and tested in field tests. Turbidity measurements were conducted up to 8 h in field tests.
[b] —, No significant change observed during 8-h field test.
[c] NT, Not tested.

TABLE 2. *Photooxidation of different cyanobacteria under laboratory conditions*[a]

Cyanobacterial strains	Time (h) to decline to:			SOD level (units/mg of protein)	
	1% of original viable count	50% of difference of original and final levels of:			
		Turbidity	Chlorophyll	Initial	After exposure[b]
Pond *Microcystis*	NT[c]	—[d]	NT	15	15 (12)
Pond *Microcystis* + chloramphenicol	NT	6	NT	15	3.8 (12)
Aphanocapsa sp.	6	2.5	2.7	16	NT
Synechococcus sp. 6311	2.5	2.5	2.3	12.4	NT
Plectonema boryanum	0.5	2.2	2.1	14	0 (4)
Nostoc sp.	0.4	1.8	1.8	25	0 (4)
Strain 7005	0.5	1.4	1.5	13	0 (4)
Microcystis bethulie strain	0.25	2.0	2.0	9	0 (4)
Microcystis sp. NRC-1	0.2	1.8	1.7	11	0 (4)

[a] Cultures were harvested in the logarithmic growth phase. Final turbidity measurements were determined for each species at the time when turbidity no longer decreased.
[b] Time (hours) after exposure given in parentheses.
[c] NT, Not tested.
[d] —, No significant change observed during 12-h test period.

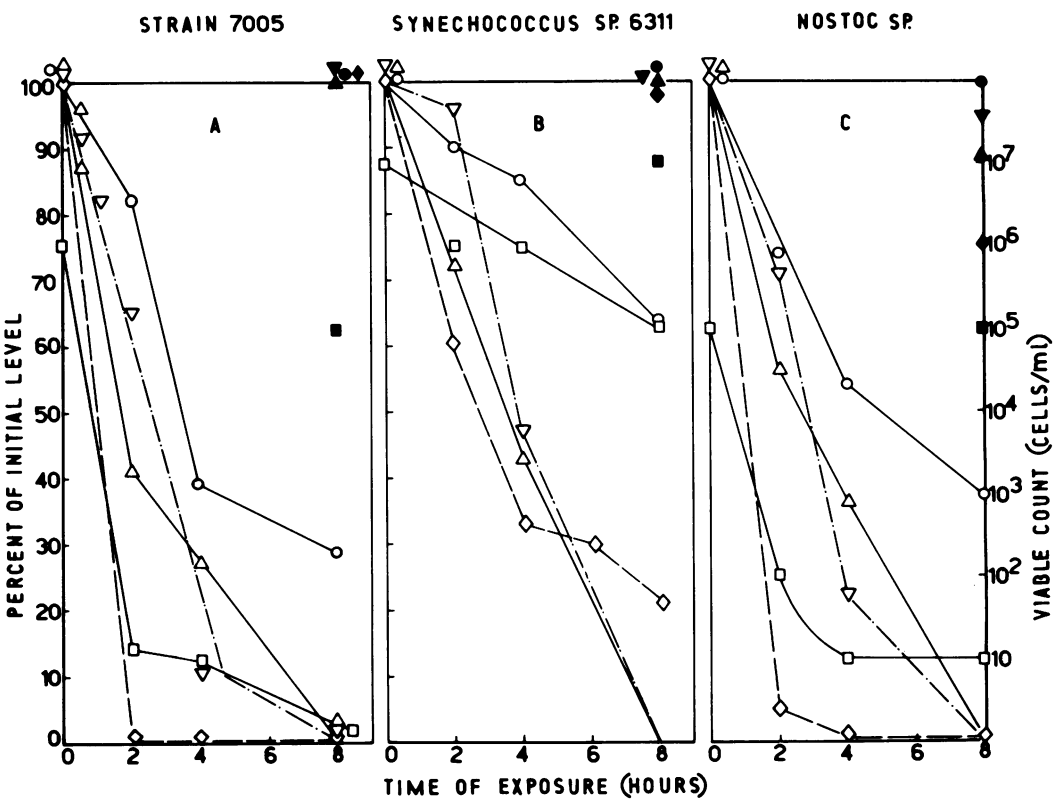

FIG. 1. *Effect of exposure to photooxidative field conditions on viable count, turbidity, SOD activity, and cellular chlorophyll and phycocyanin of suspensions of strain 7005 (A), Synechococcus sp. 6311 (B), and Nostoc sp. (C). Cyanobacterial suspensions were prepared and exposed to pond conditions as described; tests were made in fishponds in summer months (July to October) under conditions detailed in Materials and Methods. All parameters, except viable count, are expressed as the percentage of initial (100%) values. Symbols: □, viable count; ○, turbidity; ▽, cellular chlorophyll; △, cellular phycocyanin; ◇, SOD activity; closed symbols, dark control.*

measurements for field tests.

The self-shading effect of dense algal blooms might be a factor in protecting the microorganisms against photooxidative effects. It was therefore important to determine the extent to which the cell density in test suspensions in our experimental system influences sensitivity to photooxidative conditions. Three different concentrations of a strain 7005 culture (4 days old) were exposed to photooxidative pond conditions (Fig. 2). It is clear that bleaching occurred in all cases and that cell numbers were reduced, although less rapidly in the more concentrated suspensions.

No effect of culture age on the relative sensitivity to photooxidation was detected in comparative field tests of early (3.5 days) and late (8 days) logarithmic and stationary (34 days) phase cultures of strain 7005.

We compared the level of SOD activity with the degree of sensitivity to photooxidative conditions in the various species and strains of cyanobacteria tested (Tables 1 and 2). Similar to our earlier experiments (1), SOD levels dropped drastically in all laboratory strains upon the onset of photooxidation. Even strains with extremely high initial levels, such as *Nostoc,* lost all their SOD activity within a short time. The pond *Microcystis* showed extremely high resistance, expressed in all tested parameters; its initial SOD level was low compared to *Nostoc* but was not affected by exposure to photooxidative conditions. Among all the strains tested, the pond sample was the only form that retained all the initial SOD activity after exposure to photooxidative pond conditions.

These results indicate that there is a remarkable difference in the sensitivity to photooxidation between the laboratory strains and the

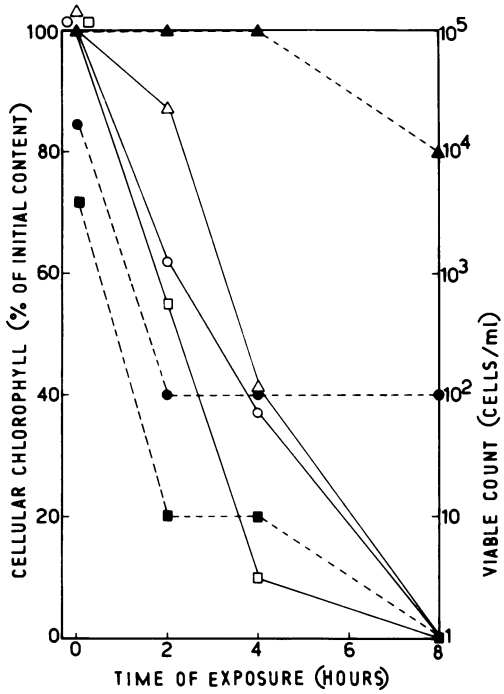

FIG. 2. *Dynamics of viable count and cellular chlorophyll content in strain 7005 suspensions of varying density exposed to photooxidative field conditions. Pond conditions at time of test were 50% oxygen saturation, pH 7.35, and 29.5 C. Chlorophyll content (in percentage of initial value) is shown by open symbols; viable count (cells/milliliter) is shown by closed symbols: △, fourfold concentrated suspension (10^5 cells/ml); ○, initial suspension (2.5×10^4 cells/ml); □, fourfold diluted suspension (6.25×10^3 cells/ml).*

pond sample. The natural pond *Microcystis* not only showed no decrease in enzymatic activity after 8 h of exposure to photooxidative conditions, but actually some increase in SOD activity was observed (Fig. 3).

The relation of SOD production and the level of resistance to photooxidative conditions in the pond *Microcystis* was tested by exposing this form to light or dark in the presence of the protein synthesis inhibitor chloramphenicol. Such a laboratory test with the pond sample gave the results shown in Fig. 3 and Table 2. In the control series, the pond sample was as resistant to photooxidation as under field conditions. However, the SOD level in suspensions exposed to light and chloramphenicol decreased after the first 5 h; turbidity also decreased but, together with the cell protein, lagged behind the SOD drop (Fig. 3). Morphological observations of these suspensions showed that the cells were bleached and lysed (Fig. 4A-D).

Samples of predominantly *Microcystis* blooms in four different localities in the Beisan Valley were also tested for their sensitivity to photooxidation. These samples included both sheathed large-celled types and nonsheathed small-celled types similar to *M. aeruginosa* (Fig. 4A and C). In all these samples essentially

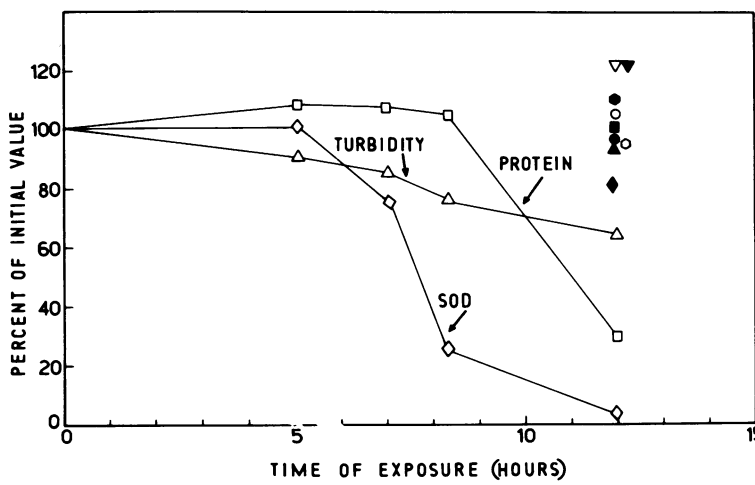

FIG. 3. *Effects of photooxidative conditions and chloramphenicol on SOD activity of the pond isolate (Microcystis sp.). Pond samples were prepared as described. Light tests are shown by open symbols; dark controls are shown by closed symbols. Cells suspended in medium alone: ○, turbidity; ▽, SOD activity; ⬡, protein. Cells suspended in medium with chloramphenicol (50 μg/ml, except 75 μg/ml, in dark control for SOD activity at 8 h: △, turbidity; ◇, SOD activity; □, protein in cell-free extract.*

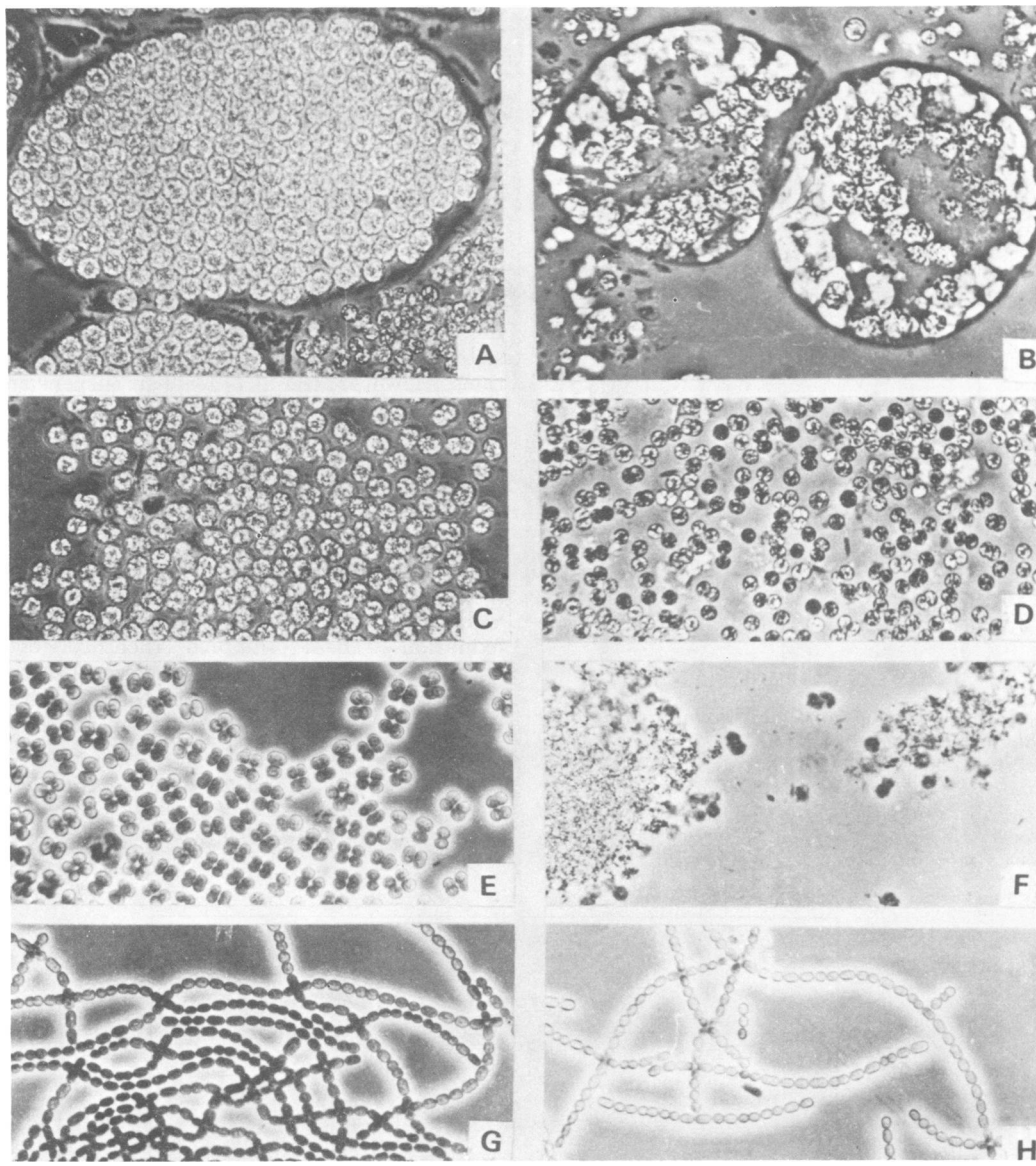

FIG. 4. *Comparison of different cyanobacterial cultures under different conditions. (A) Undamaged, sheathed colonies of pond strain of Microcystis after 8 h of exposure to photooxidative conditions; (B) completely destroyed colonies of pond strain of Microcystis after 8 h of exposure to photooxidative conditions in the presence of 100 μg of chloramphenicol per ml; (C) undamaged, nonsheathed pond strain of Microcystis under same conditions as in (A); (D) damaged, nonsheathed pond strain of Microcystis under same condition as in (B); (E) strain 7005, dark control; (F) strain 7005 after 6 h under photooxidative conditions; (G) Nostoc sp., dark control; (H) Nostoc sp., damaged after 6 h under photooxidative conditions. Phase-contrast micrograph. ×1,050.*

the same results of high resistance and no loss of SOD activity were obtained.

Since cell density was shown to influence the reaction to photooxidative conditions, it seemed possible that the colonial organization of the pond *Microcystis* might provide protection in comparison to the single-cell growth habit, as in strain 7005. In one experimental series (Fig. 5) the colonies were broken up by a 30-s mechanical shaking (without glass beads) in a Nossal

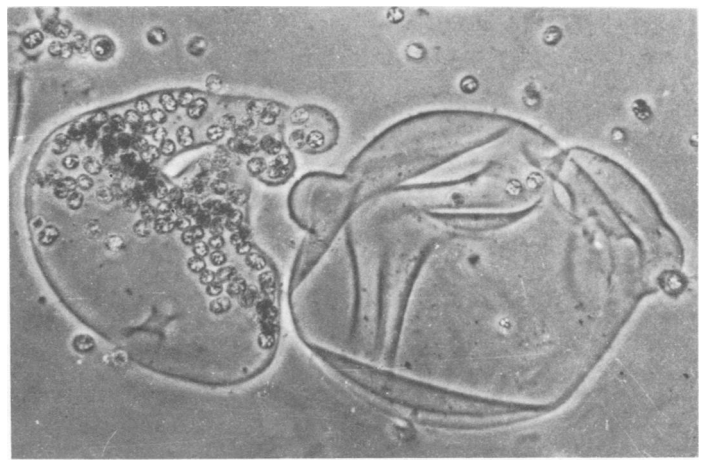

FIG. 5. *Ruptured, sheathed pond Microcystis colonies. Single Microcystis cells can be seen released in the medium; some cells remain within the sheath. Phase-contrast micrograph. ×320.*

agitator and filtered through a membrane filter (average pore size, 30 μm) to remove unbroken colonies before being tested under field conditions. Experiments with comparable cell concentrations of colonially organized cells or single-cell suspensions were carried out. Still another difference between the two strains was the abundant gas vacuoles in the pond sample, which were practically absent in strain 7005. Thus, in another series of experiments the gas vacuoles were collapsed by sudden application of pressure as described by Walsby (13). In still another series, the colonial pond sample was diluted or concentrated as in previous tests with strain 7005 (Fig. 2). In all these experiments, very little photooxidative death was observed with the pond sample. Decreasing the concentration, breaking colonies, or effecting collapse of the gas vacuoles had no significant effect on the viable count or the turbidity of cell suspensions kept under photooxidative conditions in the field. Indeed there was some increase in turbidity of single-cell suspensions in cases where the colonies were broken up due to clumping of cell observed after 2 h of exposure.

DISCUSSION

The fact that many species of cyanobacteria (e.g., *Gleocapsa* sp., *Oscillatoria redekei*) are sensitive to high light intensities when grown axenically is well known (7, 12). We have observed that our most sensitive strain 7005 was affected even by low light intensities, whereas its growth was enhanced when O_2 was selectively removed using pyrogallol in a closed container; increased CO_2 concentration also protected this strain from photooxidative effects.

The laboratory strains tested under field conditions, including forms similar to those sampled directly from the ponds, showed moderate to high sensitivity to photooxidation; on the other hand, when tested under these same conditions the pond strains exhibited an extremely high resistance which is not related to colonial morphology, sheath protection, gas vacuolation, or physiological age. The very great discrepancies in resistance to photooxidation among the laboratory strains and pond strains tested here once again stresses the danger inherent in extrapolating to natural conditions and drawing ecological conclusions from laboratory tests using strains long kept in culture collections.

The various levels of resistance shown by the different cyanobacterial species to photooxidative effects in the field and laboratory seem to be correlated to the rate of loss of SOD activity on exposure rather than to the initial SOD level. Thus, a species of *Nostoc* with extremely high SOD levels hardly differed in resistance from strains containing less than half this SOD activity. Nevertheless, this does not exclude the possibility that different initial SOD levels produced in the same strain by varying the previous growth history do affect the resistance to photooxidation (1). We were led to speculate whether the SOD per se of the resistant strain is more stable under photooxidative conditions than that of the sensitive strains or whether its SOD-forming system is more resistant. The chloramphenicol test with the pond strain suggests that the preformed enzyme of this strain is labile in photooxidative conditions and that

the enzyme-forming system most probably continues to function under photooxidative conditions.

The various factors comprising photooxidative conditions for cyanobacteria have been defined in laboratory tests where it is possible to control oxygen and CO_2 content, temperature, and light intensity. Such photooxidative conditions are known to exist in the natural milieu, but it is difficult to assess directly the lethal effects in natural blooms. The field assay described here is convenient, cheap, and rapid and may prove useful for assessing photooxidative conditions prevailing in a natural water body. The availability of cyanobacterial strains with different intrinsic resistance to photooxidation, ranging from the highly sensitive strain 7005 to the resistant pond *Microcystis,* allows selecting a bioassay of any desired degree of sensitivity.

The significance of the photodynamic factor in bloom die-off must be reassessed in the light of the finding of resistant strains. Nevertheless, the fact that this resistance could be suppressed by exposure to chloramphenicol raises the possibility that certain yet unidentified factors may render blooms sensitive and cause die-off.

ACKNOWLEDGMENTS

We wish to thank Zeev Mazor for his contribution to this work, Martin Kessel for his help in preparing the photographs, and Binah Golek for preparation of the manuscript. We also wish to thank Shmuel Sarig and his colleagues in the field laboratory at Nir David.

This study was supported by a grant from the Deutsch Forschungsgemeinschaft. One of us (J. N. E.) received financial support from the CSIR, South Africa, and University of the Orange Free State, South Africa.

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