

Differential action of Hg^{2+} and Cd^{2+} on the phycobilisomes and chlorophyll *a* fluorescence, and photosystem II dependent electron transport in the cyanobacterium *Anabaena flos-aquae*

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Received 26 November 1992; accepted for publication 8 January 1993

The effect of equimolar concentrations of Hg^{2+} and Cd^{2+} on the whole cell absorption spectra, absorption spectra of the extracted phycocyanin (PC) and fluorescence emission spectra of phycobilisomes (PBS) was investigated in the cells of *Anabaena flos-aquae*. The PC component of the PBS was found to be extremely sensitive to the Hg^{2+} rather than the Cd^{2+} ions. Further, the results showed that Hg^{2+} and Cd^{2+} induced decrease in the rate of Hill activity ($\text{H}_2\text{O} - \text{DCPIP}$) was partially restored by the electron donor NH_2OH , not by the diphenyl carbazide. Similarly, chlorophyll *a* fluorescence emission in the presence of metals showed that addition of NH_2OH could effectively reverse the metal induced alterations in the fluorescence emission intensity. These results, together, suggested that Hg^{2+} and Cd^{2+} caused damage to the photosystems (PS) II reaction center. However, a relatively higher stimulation of the chlorophyll *a* emission at 695 nm with a red shift of 4.0 nm in the presence of Hg^{2+} , and Cd^{2+} induced preferential decrease in the emission intensity at 676 nm as compared with the peak at 695 nm were indicative of the differential action of Hg^{2+} and Cd^{2+} on the PS II.

Keywords: *Anabaena flos-aquae*, chlorophyll *a*, fluorescence emission, heavy metals, Hill activity, photosystem II, phycobilisomes

Introduction

Heavy metals are important constituents of many commercial grade biocides used on a large scale to prevent the growth of water weeds and algal blooms (Rai *et al.* 1981). The metals Hg^{2+} and Cd^{2+} are potentially very toxic and are known to interfere with a variety of photosynthetic functions (Clijsters & Van Assche 1985). However, the manner in which these metal ions bring about the inactivation of the photosynthetic properties seems to differ from one metal to another. Mercury has been shown to interrupt the flow of electrons through photosystem (PS) I in both the chloroplasts (Kimimura & Katoh 1972, Globeck *et al.* 1977) and cyanobacterial systems (Kojima *et al.* 1987, Singh *et al.* 1989). However, cadmium at lower concentrations interferes with the electron transfer by PS II in both

prokaryotes and eukaryotes (Li & Miles 1975, Weigel, 1985, Singh & Singh 1987, Atal *et al.* 1991). Further, it has been proposed that the effects of heavy metals arise either from the interaction of these metals with sulphhydryl groups of the electron carrier proteins (Passow *et al.* 1961) or from the replacement of essential trace elements by the heavy metals in the electron transport chain (Haberman 1969, Samuelsson & Oquist 1980). Since the cyanobacterial pigment antenna is mainly comprised of proteins due to the abundance of phycobilisomes (PBS), it seems interesting to study the effect of both Hg^{2+} and Cd^{2+} on the PBS, i.e. the large pigment-protein complexes bound on the protoplasmic surface of the thylakoid membrane (Gantt 1981). The present investigation on the effect of Hg^{2+} and Cd^{2+} in the bloom forming cyanobacterium *Anabaena flos-aquae* is an attempt to characterize the differential mode of Hg^{2+} and Cd^{2+} induced damage to the photosynthetic apparatus. Despite similarities between Hg^{2+} and Cd^{2+} with respect to the ionic

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charges, metal concentrations and chemical properties, the metals are found to cause damage to the photosynthetic apparatus in a different manner.

Materials and methods

Organism and growth condition

The culture of *A. flos-aquae* was isolated in this laboratory. Batch cultures of *A. flos-aquae* were grown in modified Chu-10 medium (Gerloff *et al.* 1950) with trace metals (Allen & Arnon 1955) at $25 \pm 1^\circ\text{C}$ in a culture room. The cultures were illuminated for 14 h per day with cool white fluorescent tubes with an approximate light intensity of 10 W m^{-2} .

Permeabilization of the cells

Permeaplasts were prepared as described by Wards & Myers (1972). The exponentially growing cultures were harvested by centrifugation ($400 \times g$, 10 min) and were washed twice with the glass distilled water. The washed pellet was suspended in sodium phosphate buffer (pH 7.0, 20 mM) containing 0.5% (w/v) lysozyme. The rest of the experimental conditions were as described earlier (Singh *et al.* 1989) except that EDTA was omitted from the reaction mixture. The reaction mixture was incubated for 30 min at room temperature. Finally, the permeaplasts were washed with the same buffer to remove the excess lysozyme. There was no measurable release of phycocyanin (PC).

Measurement of absorption spectra

A homogenous cell suspension of *A. flos-aquae* in the growth media was supplemented with the graded concentration ($1\text{--}5\text{ }\mu\text{M}$) of HgCl_2 and CdCl_2 . They were light incubated in the growth room for 48 h before recording of the absorption spectra. The metal treated cells were scanned (400–750 nm) in a DU-64, UV-visible spectrophotometer (Beckman, Switzerland) by using a light path of 1.0 cm. The volume of each sample was 3.0 ml.

Absorption spectra of PC

After extraction of the acetone (80% v/v) soluble pigments, cells of *A. flos-aquae* were suspended in phosphate buffer (pH 7.0, 20 mM) and were sonicated in MSE-soni-prep (UK) at an amplitude of 10 mA for 5 min by using one cycle per minute with an interval of 30 s. The temperature of the sample was maintained at 0°C by keeping it in ice. Total PC was extracted by repeated freezing and thawing. The extracted pigment was supplemented with equimolar concentrations ($5\text{--}20\text{ }\mu\text{M}$) of Hg^{2+} and Cd^{2+} , and was incubated for 5 min at room temperature. Thereafter, the absorption spectrum of the PC was recorded in a DU-64, UV-visible spectrophotometer (Beckman) in the range of 520–700 nm by using a light path of 1.0 cm. The absorption spectrum of PC obtained without addition of the metals served as a control.

Measurement of fluorescence emission spectra

The fluorescence emission spectra (600–750 nm) of *A. flos-aquae* were recorded in a spectrofluorimeter RF-540 (Shimadzu, Japan) at room temperature by using slit widths of 10 nm for excitation and 5.0 nm for emission spectra. The excitation wavelengths used for the measurement of relative fluorescence emission from chlorophyll *a* and PBS were 435 and 570 nm, respectively. The exponentially growing cells of *A. flos-aquae* were harvested and washed with glass distilled water. Then the cells were homogenized in a glass homogenizer and were suspended in phosphate buffer (pH 7.0, 20 mM). Cell suspensions supplemented with Hg^{2+} and Cd^{2+} ($5\text{--}20\text{ }\mu\text{M}$, each) were incubated in the light for 30 min before the measurement of the relative fluorescence emission. The concentration of chlorophyll *a* was approximately $5.0\text{ }\mu\text{g ml}^{-1}$. The value of the relative fluorescence emission was not corrected for the sensitivity of the photomultiplier.

Measurement of 2,6-dichlorophenol-indophenol (DCPIP) photoreduction

The rate of DCPIP photoreduction was measured as described by Holt & French (1948). Permeabilized cells suspended in phosphate buffer (pH 7.0, 20 mM) were supplemented with Hg^{2+} and Cd^{2+} (5 and 10 μM , respectively), and incubated in the light for 15 min. Thereafter, a concentration of 25 μM of DCPIP was added in the reaction mixture to initiate the photoreduction of dye. The absorbance of dye (DCPIP) was measured at 600 nm in a spectronic-20 spectrophotometer (Systronics, India). EDTA (1.0 mM) washing of the metal treated cells was carried out after incubating the cells with metals for 15 min. Then the cells were centrifuged ($3000 \times g$, 5 min) and the pellet was suspended in EDTA (1.0 mM) solution for 5 min. Thereafter, the cells were recentrifuged and washed with the same phosphate buffer to remove the EDTA. The pellet resuspended in the buffer was used for the measurement of Hill activity.

Chlorophyll *a* was measured by the method of MacKinney (1941). Protein was measured by the folin-phenol reagent (Lowry *et al.* 1951) by using lysozyme (Sigma, St Louis, MO) as a standard.

Chemicals

All chemicals were purchased from Sigma except the chloride salts of metals which were obtained from BDH (India).

Results

Effect of Hg^{2+} and Cd^{2+} on the whole cell absorption spectra

Absorption spectra of *A. flos-aquae* (Figure 1A and B) were obtained after incubation of the cells in the presence of graded concentrations ($1\text{--}5\text{ }\mu\text{M}$) of Hg^{2+} and Cd^{2+} ions. The results showed a metal induced

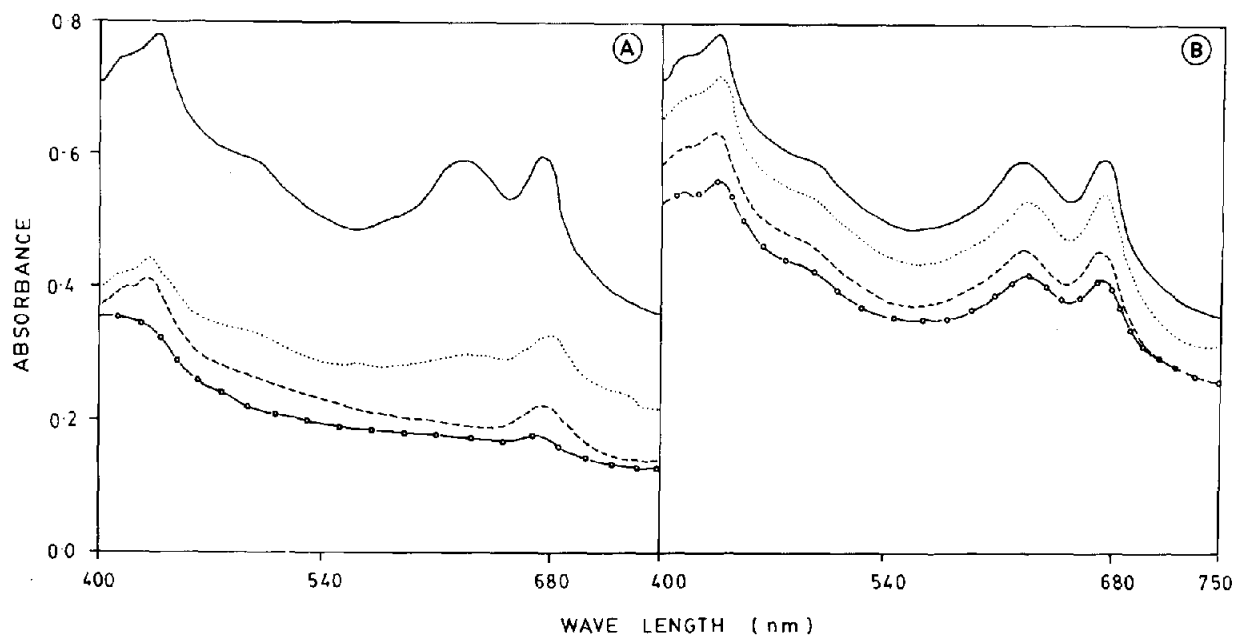


Figure 1. Whole cell absorption spectra (400–750 nm) of *A. flos-aquae* treated with equimolar concentrations of the Hg^{2+} (A) and Cd^{2+} (B) for 48 h. The cells without addition of metal served as control (—). The concentrations of each metal used in the experiment were $1 \mu\text{M}$ (.....), $3 \mu\text{M}$ (---) and $5 \mu\text{M}$ (—○—).

general decrease in absorbance by pigments like chlorophyll *a* (675 nm), β -carotenes (480 nm) and PBS (620 nm). However, the effect of Hg^{2+} at all concentrations was found to be relatively higher than Cd^{2+} ions, particularly at 620 nm. Since the absorbance peak observed at 620 nm was due to the PC component of the PBS, efforts were made to see the effect of heavy metals on PC *in vitro*.

Effect of Hg^{2+} and Cd^{2+} on the absorption spectra of extracted PC

The extracted PC in the phosphate buffer (pH 7.0, 20 mM) was incubated for 5 min in the presence of graded concentrations (5–20 μM) of Hg^{2+} and Cd^{2+} ions. The results (Figure 2A and B) exhibited a Hg^{2+} induced loss of absorbance at 620 nm which was several fold higher than that observed at corresponding concentrations of Cd^{2+} ions. A concentration of 20 μM of Hg^{2+} reduced the absorbance peak by 70% of the control, while the same concentration of Cd^{2+} resulted in a decrease of the absorbance by 25% of the control (100%, without metal).

Effect of Hg^{2+} and Cd^{2+} on fluorescence emission from PBS

Since PBS are an integral component of PS II, a study on the relative fluorescence emission from

PBS in the presence of Hg^{2+} and Cd^{2+} can be a good indicator of metal induced alterations in its energy transfer efficiency. The results (Figure 3A and B) showed a metal induced decrease in the relative intensity of fluorescence emission (638 nm) from PBS at all the concentrations (10–20 μM) of Hg^{2+} and Cd^{2+} . However, the effect of Hg^{2+} was found to be several fold higher than that observed at corresponding concentrations of Cd^{2+} ions.

Effect of Hg^{2+} and Cd^{2+} on the photoreduction of DCPIP

The rates of dye reduction (Table 1) supported by various exogenous electron donors were measured in the presence of Hg^{2+} and Cd^{2+} to delineate the action sites of these metals on the electron transport chain. The results showed that the Hg^{2+} (10 μM) and Cd^{2+} (20 μM) induced decreases in the rate of Hill activity were 39.2 and 42.9%, respectively, as compared with the control (100%, without metal). Addition of 50 μM of diphenyl carbazide (DPC), which donates electrons on the oxidizing side of PS II (Yamashita & Butler 1969), did not bring about any change in the extent of metal induced inhibition of the Hill activity. However, inclusion of NH_2OH (100 μM), which donates electrons close to the PS II reaction centre (Hauska 1977) resulted in partial recovery of the Hg^{2+} and Cd^{2+} induced inhibition to

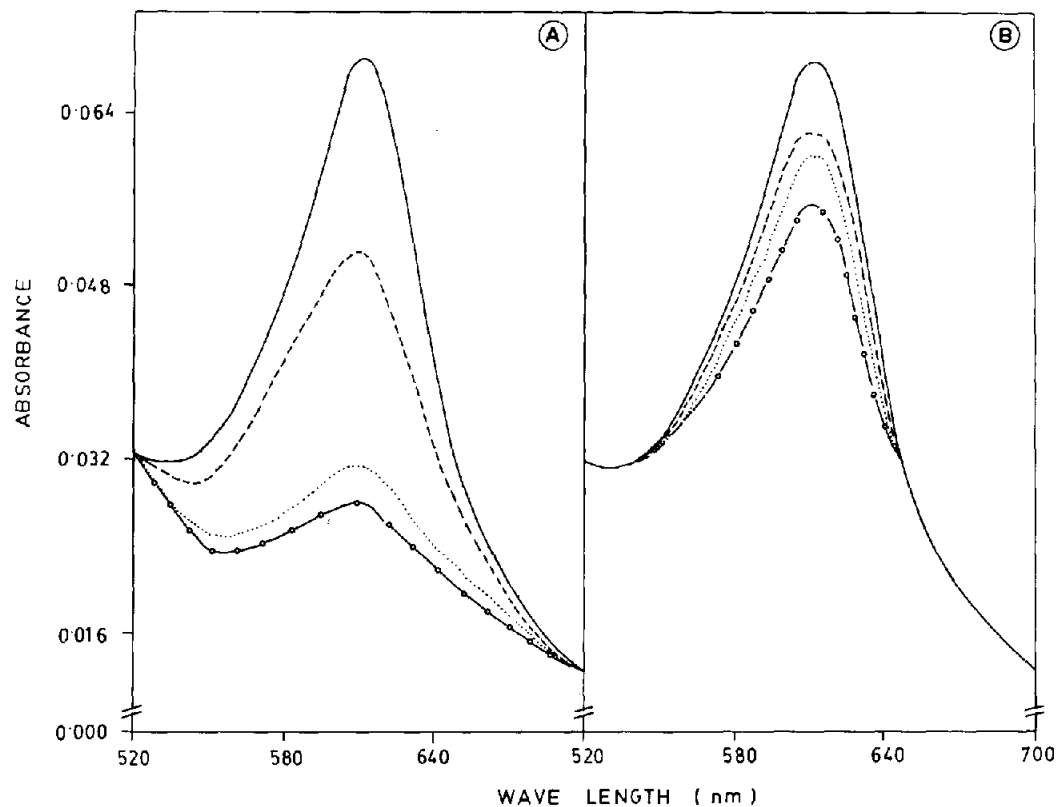


Figure 2. Absorption spectra of the extracted PC pigment in the presence of equimolar concentrations of Hg^{2+} (A) and Cd^{2+} (B). The extracted PC was incubated for 5 min without (—) and with different concentrations of each metal: 5 μM (---), 10 μM (.....) and 20 μM (—○—).

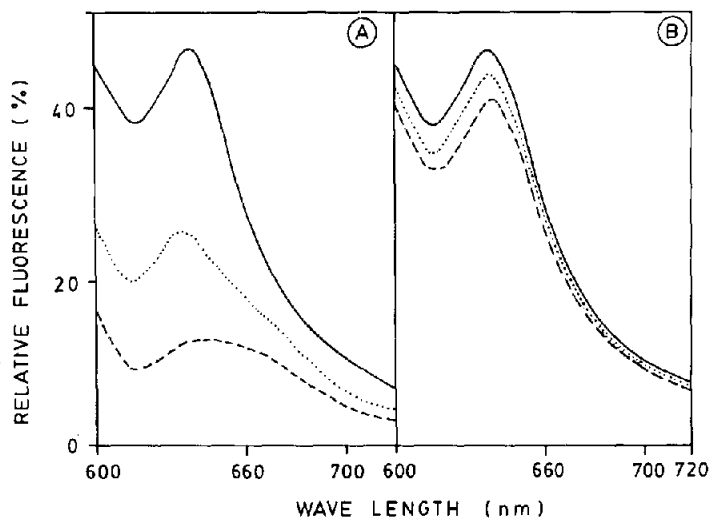


Figure 3. PBS fluorescence emission spectra (600–720 nm) of intact cells of *A. flos-aquae* were recorded in relative units (%) at room temperature. Cells with equimolar concentrations of Hg^{2+} (A) and Cd^{2+} (B) were incubated for 30 min prior to use. The wavelength of excitation was 570 nm. The cells were treated without (—) and with different concentrations of each metal: 10 μM (.....) and 20 μM (---). The concentration of chlorophyll *a* was kept at 5 $\mu\text{g ml}^{-1}$.

Table 1. The rate of photoreduction of DCPIP supported by various exogenous electron donors was measured in the presence of Hg^{2+} ($10 \mu M$) and Cd^{2+} ($20 \mu M$)

Sample no.	Electron donation		Rate of DCPIP reduction (mmol DCPIP mg protein ⁻¹ min ⁻¹)		
	from	to	control (without metal)	Hg^{2+} ($10 \mu M$)	Cd^{2+} ($20 \mu M$)
1.	H ₂ O	DCPIP	1.63 ± 0.06 (100)	0.64 ± 0.05 (39.2)	0.70 ± 0.04 (42.9)
2.	DPC ($50 \mu M$)	DCPIP	1.43 ± 0.06 (100)	0.57 ± 0.07 (39.8)	0.69 ± 0.06 (48.2)
3.	NH ₂ OH ($100 \mu M$)	DCPIP	1.21 ± 0.05 (100)	0.79 ± 0.05 (65.2)	0.95 ± 0.06 (78.5)
4.	after EDTA (1.0 mM) washing of the metal treated cells (H ₂ O)	DCPIP	0.88 ± 0.05 (100)	0.42 ± 0.05 (47.7)	0.65 ± 0.05 (73.8)

The reaction was initiated by the addition of $25 \mu M$ of DCPIP to the assay mixture (phosphate buffer, pH 7.0, 20 mM). The rest of the experimental conditions were the same as described in the Materials and methods. The values given in the parentheses denote the percentage activity as compared with the control (without metal, 100%).

levels of 65.2 and 78.5%, respectively, as compared with the control (100%, without metal). The results, thus, indicate that the action sites of both the metals were bypassed by the electron donation site of the NH₂OH. Further, EDTA (1.0 mM) washing of the metal treated cells could alleviate the degree of Cd^{2+} induced inhibition of Hill activity, while the effect of Hg^{2+} on Hill activity was little altered as compared with the results in the EDTA untreated cells.

Effect of Hg^{2+} and Cd^{2+} on chlorophyll *a* fluorescence emission

The fluorescence emission spectra (600–750 nm) of *A. flos-aquae* obtained at room temperature (25 °C) by using an excitation wavelength of 435 nm (Figure 4A–D) showed a typical fluorescence emission pattern with two small humps at 678 and 695 nm. However, treatment of the cells with Hg^{2+} (20 and $50 \mu M$) resulted in relatively higher stimulation of fluorescence emission intensity at 695 nm with a red shift of 4.0 nm as compared with the emission intensity at 678 nm. On the contrary, treatment of the cells with equimolar concentrations of Cd^{2+} brought about a decrease in the fluorescence emission intensity which was rather more pronounced at 678 nm as compared with the intensity of emission at 695 nm. However, addition of $100 \mu M$ of NH₂OH could reverse the effect of both Hg^{2+} and Cd^{2+} on the fluorescence emission intensity of chlorophyll *a*. The results again suggest that both the metals caused

damage to the PS II reaction center, but in a different manner.

Discussion

The absorbance and the fluorescence emission spectra of PBS in *A. flos-aquae* obtained in the presence of equimolar concentrations of Hg^{2+} and Cd^{2+} exhibit a drastic decrease in the absorbance of PC at 620 nm and fluorescence emission from the PBS at 638 nm mainly from the PC component of the PBS. The Hg^{2+} induced damage to the PC component of the PBS is found to be several fold higher than that with Cd^{2+} ions. These results, to some extent, are in agreement with the observations of Pecci & Fujimori (1967) on the mercurial, *p*-chloromercuribenzoate (PCMB) induced damage to the phycoerythrine component of the PBS in *Ceramium rubrum*. Similarly, Murty *et al.* (1989) have also reported a greater sensitivity of PC towards Hg^{2+} ions as compared with the allophycocyanin component of the PBS in the cyanobacterium *Spirulina platensis*. Further, our results on the photoreduction of DCPIP (Hill activity) in the presence of heavy metals have shown that the Hg^{2+} and Cd^{2+} induced decrease in the rate of Hill activity cannot be relieved by the addition of DPC which donates electrons on the oxidizing side of PS II (Yamashita & Butler 1969). However, the effect of both Hg^{2+} and Cd^{2+} on the Hill activity is partially restored by

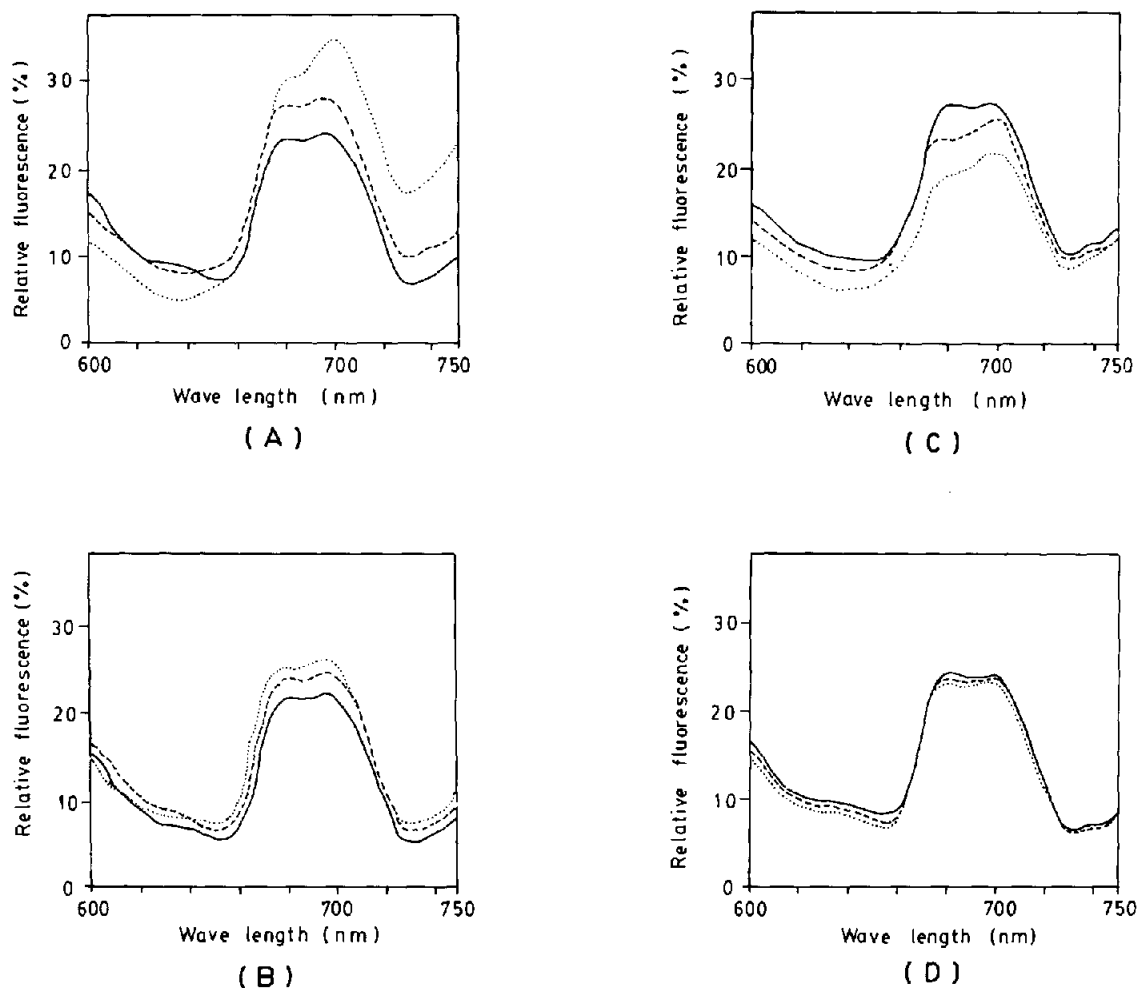


Figure 4. Chlorophyll *a* fluorescence emission spectra (600–750 nm) of intact cells of *A. flos-aquae* were recorded in relative units (%) at room temperature (25 °C). The cells were supplemented with Hg^{2+} alone (A) and Hg^{2+} plus 100 μM of NH_2OH (B), and were light incubated for 30 min. The wavelength of excitation was 435 nm prior to use. The cells were treated without (—) and with different concentrations of Hg^{2+} : 10 μM (---) and 20 μM (.....). Chlorophyll *a* fluorescence emission spectra (600–750 nm) of the intact cells of *A. flos-aquae* were recorded in relative units (%) at room temperature (25 °C) in the presence of Cd^{2+} alone (A) and Cd^{2+} plus 100 μM of NH_2OH (B). The wavelength of excitation was 435 nm. The cells supplemented without (—) and with 10 μM (---) and 20 μM (.....) of Cd^{2+} were light incubated for 30 min prior to use.

the addition of NH_2OH —an electron donor to the PS II reaction center (Hauska 1977).

In view of the above observations, it can be said that besides Hg^{2+} induced damage to the PBS, both metals inhibit electron transport supported by PS II at a site close to the PS II reaction center. Our results on Cd^{2+} induced inhibition of the PS II dependent electron transfer are similar to the other findings in both the higher plants as well as cyanobacteria (Li & Miles 1975, Weigel 1985, Singh & Singh 1987, Atal *et al.* 1991). Our observations on the reversal of Hg^{2+} induced inhibition of the Hill activity and chlorophyll fluorescence emission in the presence of NH_2OH are indicative of an action site

for Hg^{2+} close to the PS II reaction center. There seems to be no parallel report on these lines in cyanobacteria as well as in higher plants. A number of investigations have so far demonstrated that Hg^{2+} inhibits the plastocyanin (Kimimura & Katoh 1972), cytochrome *c* reductase (Singh *et al.* 1989), the iron–sulfur center (Golbeck *et al.* 1977) and the reaction center of PS I (Kojima *et al.* 1987). However, Murty *et al.* (1989) have tentatively suggested that Hg^{2+} induced damage to pigment–proteins in the cyanobacterium *Spirulina platensis* may account for the reduced rate of PS II dependent O_2 evolution in the presence of Hg^{2+} ions.

Chlorophyll *a* fluorescence emission in *A. flos-*

aquae shows a typical fluorescence spectrum and is found to be more or less similar to the emission spectrum of PS II enriched particles of the cyanobacterium *Anacystis nidulans* when excited at 435 nm (Pakrasi & Sherman 1984). A similar chlorophyll *a* fluorescence emission spectrum has been recorded in the cells of the red alga *Porphyridium cruentum* when grown in blue and red light conditions (Ley & Butler 1980). Further, it has been suggested that the short (678 nm) and long wavelength (696 nm) emission peaks of chlorophyll *a* are, respectively, associated with the outer core of the pigment antenna, which accepts energy from the accessory pigments and with the inner core of the pigment antenna involving energy transfer in the PS II reaction center (Ley & Butler 1980, Pakrasi & Sherman 1984).

In the light of the above findings, it may be said that the relatively higher stimulation of the fluorescence intensity at 695 nm with a red shift of 4 nm in the presence of Hg^{2+} was due to the binding of Hg^{2+} ions with the inner core of the pigment antenna, whereas the Cd^{2+} induced preferential decrease in the fluorescence emission intensity at 676 nm may be attributed to the binding of Cd^{2+} ions onto the outer core of the pigment antenna. This suggestion is further supported by the experiment on the EDTA washing of the metal treated cells which shows that, unlike Hg^{2+} , the effect of Cd^{2+} on the Hill activity is partially restored by EDTA washing of the metal treated cells. Further, the binding of Hg^{2+} ions with the inner core of the pigment antenna of PS II cannot be ruled out due to its greater penetration power as suggested by Nakada *et al.* (1978) and Nuzzi (1972). Taken together, these results suggest that both Hg^{2+} and Cd^{2+} ions interrupt the photochemical activity of the PS II reaction center, but in a different manner.

Acknowledgments

We are thankful to Professor Sudhakar Bharati, Head, Department of Life Sciences, Indore University, Indore (M.P.) for extending some laboratory facilities. The financial assistance from CSIR, New Delhi, in the form of a research project (9(216)/86-EMR II) is gratefully acknowledged.

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