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Effect of Cadmium and Zinc on Respiration and Photosynthesis in Suspended and Immobilized Cultures of *Chlorella vulgaris* and *Scenedesmus acutus*

K. Ilangovan,[1] R. O. Cañizares-Villanueva,[2] S. González Moreno,[3] D. Voltolina[4]

[1] Instituto de Ingeniería, UNAM, Apdo Postal 70-472, México D.F. 04510, Mexico
[2] Department of Biotechnology and Bioengineering, Center of Advanced Research Studies, Institute of National Politechnic, Post Box 14-740, Mexico D.F. 7300, Mexico
[3] National University of Mexico (UNAM), Biochemistry Laboratory, Post Box 314, Tlalnepantla, State of Mexico, Mexico
[4] Department of Agriculture, CICESE, B.C. Post Box 2732, Ensenada, B.C., México

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Resistance to metal ions has been observed in several microorganisms. Microalgae have been used to remove heavy metals from aqueous systems since they have a high capacity to accumulate dissolved metals (Ting *et al.*, 1989; Wilde and Benemann, 1993). At the cellular and molecular level, toxicity of heavy metal is not only related to their interaction with thiol groups of proteins (Smith *et al.*, 1987; Chávez and Holguín, 1988; Kone *et al.*, 1990, Ilangovan *et al.*, 1992;), but also to their ionophoretic properties (Gutknecht, 1981) and their ability to generate free radicals (Stadman and Oliver, 1991). Suspended and immobilized cultures of microlgae have shown that their cell response depends mainly on the metal (type, concentration and activity), on the species used. Immobilization process may increase tolerance to heavy metals (De la Noüe *et al.*, 1990; Leite *et al.*, 1993).

Zinc and cadmium are the main transition heavy metals in the sewage of Mexico City (Ilangovan *et al.*, 1992). Cadmium has been recognized as a hazardous environmental pollutant (Hiatt and Huff, 1975) and its uptake by phytoplankton, the first level of the food chain, might be of ecological significance. It has a cytotoxic effect in freshwater green algae through specific structural alterations and inhibition of several enzyme activities in the functional mitochondrial system (Silverberg, 1976). Zinc is an essential element for carbonic anhydrase activity, increasing CO_2 and HCO_3^- availability to ribulose biphosphate carboxylase (RUBP- Case) and phoshoenol pyruvic acid carboxylase (PEP-Case), respectively and It has been reported that Zn^{+2} could reduce the effect of Cd^{2+} in *Chlorella pyrenoidosa* (Ilangovan *et al.*, 1992). The aim of this study was to evaluate the effects of Cd^{2+} and of a mixture of Cd^{2+} - Zn^{2+} on Photosystem II (PSII), rate of respiration and photosynthesis in suspended and in immobilized cultures of *Chlorella vulgaris* and *Scenedesmus acutus.*

MATERIALS AND METHODS

The algae used in this study were *Chlorella vulgaris* IAM-C30 and *Scenedesmus acutus* IAM-C64, both obtained from the collection of the Institute of Applied Microbiology, University of Tokyo, Japan. Algae inoculum were obtained from

Correspondence to: K. Ilangovan

120 hr old slants and grown at 25 ± 3°C under continuous illumination with fluorescent lamps (22 μE $m^{-2} s^{-1}$) and aeration (approx. 100 ml air/min.) and were used to inoculate cultures of increasing volumes to a final volume of 1000 ml. The standard inoculum for all experiments was 10% (v/v) of a 120 h culture, supposed to be in near-exponential growth phase.

For the immobilized cultures 1 L samples of exponentially growing cells were vacuum filtered on Millipore membranes HAWP (0.45 μm). The cells were washed three times with deionized water and a suspension was prepared as inoculum for the immobilization experiments.

Formulation of synthetic media for the culture of both microalgae were provided for the Institute of Applied Microbiology, Universty of Tokyo, Japan. C-30 medium was used for the culture of *Chlorella vulgaris* and contained (in gl^{-1} distilled water) KNO_3, 5.0; $MgSO_4 \cdot 7H_2O$, 2.5., KH_2PO_4, 1.25., K_2HPO_4, 0.10; $F_eSO_4 \cdot 7H_2O$, 0.0028 and also lml of Arnon 5 solution containing (in gl^{-1} distilled water): H_3BO_3, 2.86; $MnC_2 \cdot 4H_2O$, 1.81; Zn $SO_4 \cdot 7H_2O$, 0.22; Cu $SO_4 \cdot 5H_2O$, 0.008; $Na_2M_oO_4$, 0.021. *Scenedesmus acutus* was cultivated using modified Bristol medium (in gl^{-1} distilled water): KNO_3, 0.25; Mg $SO_4 \cdot 7H_2O$, 0.075; NaCl, 0.025; $CaCl_2 \cdot 2H_2O$, 0.01 and also 1 ml of Arnon 5 solution and 1 ml Fe-solution containing $FeSO_4 \cdot 7H2O$, 2.0; and 2 drops of H_2SO_4; pH 6.0. Growing cultures were aerated by filtered air bubbling. Medium without metal served as blank. Exponentially growing cultures harvested by centrifugation (10 x 10^3 rpm during 15 min.) were used for the experiments.

In-mobilization of cells were carried out according to the methodology described by Lukavský, 1986.The experiments were run in 3L column acrylic reactors with 2 L sterile culture medium with or without metals and with an inoculum of 200 ml of recently prepared beads, kept in suspension with filtered air bubbling (≅ 500 ml air/min.). Batch cultures were in triplicate and lasted 5 days in all cases. To release the immobilized cells, approximately 150 ml of gel beads were dissolved in a known volume of 0.1M K_2HPO_4 (pH 8.5) magnetically stirred, with 2 mM Na-EDTA for 30 minutes for *S. acutus* and without Na-EDTA for *C. vulgaris.* The suspension was centrifuged and treated with 0.1 M K_2HPO_4 (pH 8.5) then washed with distilled water and centrifuged. Released cells were suspended in lml of distilled water, counted in a Neubauer hemacytometer.

Cadmium sulphate and zinc chloride (Merck) were used as metal sources. Suspended and immobilized *C. vulgaris* cultures were grown in the presence of 1 mg Cd^{2+}/L and in a mixture of 2 mg Cd^{2+}/L - 50 mg Zn^{2+}/L. In the case of *S. acutus* concentrations were 0.5 mg Cd^{2+}/L and 0.5 mg Cd^{2+}/L - 50 mg Zn^{2+}/L for suspended cultures, and 1 mg Cd^{2+}/L and 1 mg Cd^{2+} - 50 mg Zn^{2+} for immobilized cells. The rates of endogenous respiration and phosynthesis (nmol $O_2 min^{-1}$) of cells (48 *x* 10^6 *Chlorella* and 152 x 10^6 *Scenedesmus*) were measured (Delieu and Walker, 1972).

Chlorophyll **a** fluorescence transients were measured by a Plant Efficiency Analyzer (PEA, Hansatech, Ltd. King's Lynn, Norfolk, England). Illumination was provided by an array of 6 light emitting diodes (peak of emission 650 nm) focused on the sample surface contained in a cap of microfuge tube adjusted in the clamp for leaves or disc of leaves. Aliquots of 60 microliters cell suspension in mineral media (specific for each algae, 2.41 x 10^6 *Scenedesmus* cells and 42 x 10^6 *Chlorella* cells) were used and 4 mm sample area was irradiated.

Samples were kept in dark for 15 min. before fluorescence transients monitoring. Chlorophyll **a** fluorescence transients were recorded during 5 sec. The results were calculated on a logarithmic time scale using the software PEA analyzer version 2.04 and summary version 2.02 Hansatech Instruments Ltd. Photochemical (qP) and non photochemical quenching (qN) were determined according to Van Kooten and Snell (1990). $qP = (F'_m-F) / (F'_m-F'_o)$ and $qN = 1 - \{(F'_m-F'_o)/(F_m-F_o)\}$.

RESULTS AND DISCUSSION

As shown in Table 1 cadmium and cadmium-zinc treatments did not affect oxygen liberation in suspended cultures of *Chlorella vulgaris* on cell number basis with respect to their control. Chlorophyll **a** concentration decreased 34 % and 41 % in Cd and Cd-Zn treatments respectively. Results of chlorophyll based oxygen liberation indicate an activation of 34 % by Cd and 52 % by Cd-Zn, probably due to the uncoupling of electron transport chain and a stimulation of Hill's reaction on the activation of another process (release of peroxidases) for oxygen liberation. Respiration was not modified by Cd or Cd-Zn treatments.

TABLE 1. Effect of Cd^{2+} y Cd^{2+}-Zn^{2+} on the oxygen uptake and evolution of *Chlorella vulgaris* suspended cultures.

Treatment	Chlorophyll **a** (mg/48x10^6 cells)	Oxygen evolution (μmol $min.^{-1}$/mg chlorophyll **a**)	(μmol $min.^{-1}$/48x10^6 cells)	Oxygen uptake (μmol $min.^{-1}$/48 x10^6 cells)
Control	0.041 ± 0.007 (100)	2.50 ± 0.143 (100)	0.101 ± 0.008 (100)	0.039 ± 0.005 (100)
1 mg Cd/L	0.027 ± 0.004 (66)	3.35 ± 0.194 (134)	0.090 ± 0.008 (89)	0.028 ± 0.002 (72)
2 mg Cd/L - 50 mg Zn/L	0.024 ± 0.0006 (58)	3.80 ± 0.200 (152)	0.090 ± 0.005 (89)	0.033 ± 0.005 (85)

Values represent mean ± standard error of 5 different experiments.
Values in parenthesis represent percentage control.

Oxygen production in *Chlorella* (on a cell number basis) was stimulated 56 % by Cd and inhibited 50 % by Cd-Zn compared to their controls with immobilized cells. Cadmium stimulated oxygen liberation (on a chlorophyll basis) by a factor of 6

while, Cd-Zn produced 5% inhibition (Table 2). Oxygen liberation was only 38 % of the suspended control by immobilized cells (on a cell number basis), it was 1.6 times greater than suspended control with reference to chlorophyll **a.** Chlorophyll **a** concentration decreased 5 times (control), 13 times (Cd) and 6 times (Cd-Zn) compared to suspended cells in immobilized *Chlorella.* Respiration was activated 40 % by cadmium in immobilized *Chlorella* but Cd-Zn treatment inhibited it to 27 %.

Contrary to *Chlorella,* in suspended *Scenedesmus acutus* cultures oxygen liberation was inhibited 75 % by cadmium and 46 % by Cd-Zn treatments on a chlorophyll **a** basis, but 83 % and 46 % respectively when expressed on the same cell number compared to their respective control. Respiration was inhibited almost 22 % by Cd

TABLE 2. Effect of Cd^{2+} y Cd^{2+}-Zn^{2+} on the oxygen uptake and evolution of *Chlorella vulgaris* immobilized cultures.

Treatment	Chlorophyll **a** ($mg/48x10^6$ cells)	Oxygen evolution ($\mu mol\ min.^{-1}/mg$ chlorophyll **a**)	Oxygen evolution ($\mu mol\ min.^{-1}/48x10^6$ cells)	Oxygen uptake ($\mu mol\ min.^{-1}/48\ x10^6$ cells)
Control	0.008 ± 0.0003 (100)	4.00 ± 0.367 (100)	0.032 ± 0.003 (100)	0.015 ± 0.001 (100)
1 mg Cd/L	0.002 ± 0.0001 (25)	26.17 ± 1.810 (654)	0.050 ± 0.003 (156)	0.021 ± 0.002 (140)
2 mg Cd/L - 50 mg Zn/L	0.004 ± 0.0001 (50)	3.80 ± 0.200 (95)	0.016 ± 0.002 (50)	0.011 ± 0.001 (73)

Values represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

TABLE 3. Effect of Cd^{2+} y Cd^{2+}-Zn^{2+} on the oxygen uptake and evolution of *Scenedesmus acutus* suspended cultures.

Treatment	Chlorophyll **a** ($mg/152x10^6$ cells)	Oxygen evolution ($\mu mol\ min.^{-1}/mg$ chlorophyll **a**)	Oxygen evolution ($\mu mol\ mn.^{-1}/152x10^6$ cells)	Oxygen uptake ($\mu mol\ min.^{-1}/152x10^6$ cells)
Control	0.067 ± 0.002 (100)	2.196 ± 0.120 (100)	0.151 ± 0.008 (100)	0.037 ± 0.003 (100)
0.5 mg Cd/L	0.049 ± 0.004 (73)	0.544 ± 0.120 (25)	0.026 ± 0.006 (17)	0.029 ± 0.002 (78)
0.5 mg Cd/L -50 mg Zn/L	0.069 ± 0.011 (103)	1.176 ± 0.186 (53)	0.081 + 0.012 (54)	0.029 ± 0.005 (78)

Values represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

and Cd-Zn treatments in suspended *Scenedesmus* cultures (Table 3). Oxygen liberation was inhibited 89 % by cadmium in immobilized *Scenedesmus* (on a cell

number basis) but stimulated 39 % if expressed on a chlorophyll **a** basis. Respiration was inhibited 42 % by cadmium (Table 4).

TABLE 4. Effect of Cd^{2+} y Cd^{2+}-Zn^{2+} on the oxygen uptake and evolution of *Scenedesmus acutus* immobilized cultures.

Treatment	Chlorophyll **a** ($mg/152x10^6$ cells)	Oxygen evolution ($\mu mol\ min.^{-1}/mg$ chlorophyll **a**)	Oxygen evolution ($\mu mol\ mn.^{-1}/152x10^6$ cells)	Oxygen uptake ($\mu mol\ min.^{-1}/152x10^6$ cells)
Control	0.022 ± 0.0009 (100)	3.079 ± 0.386 (100)	0.067 ± 0.008 (100)	0.057 ± 0.006 (100)
1 mg Cd/L	0.0017 ± 0.0008 (45)	4.266 ± 0.315 (138)	0.007 ± 0.0005 (10)	0.033 ± 0.001 (58)

Values represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

Similar to *Chlorella,* the immobilization procedure provoked 55 % inhibition of oxygen liberation in control *Scenedesmus* and 73 % in cadmium treated cells compared to suspended cultures. Chlorophyll **a** concentration also decreased 67 % in the immobilized control and 96 % in cadmium treatment. To check whether or not a component of the immobilization procedure produced alteration of respiratory and photosynthetic proceses in *Chlorella* and *Scenedesmus,* cells were incubated in sodium alginate for 1 h or 0.3 M CaCl, 24 h then washed twice with distilled water and determinations of physiological activities related to carbon assimilation and utilization were done in the same conditions described in Materials and Methods.

Oxygen liberation was reduced (27 % *Chlorella* and 42 % *Scenedesmus*) by sodium alginate similar to respiration (59% *Chlorella* and 27 % *Scenedesmus*). Incubation in 0.3 M $CaCl_2$ reduced oxygen liberation (53 *% Chlorella* and 43 *% Scenedesmus*) and produced 72 % inhibition of respiration in *Chlorella* and 81 % stimulation in *Scenedesmus* (data not shown). Although some components of the immobilization procedure have effect on the oxygen liberation and oxygen uptake, it could not be said that results from five days *Chlorella* and *Scenedesmus* immobilized cultures are consequence of these factors because each component is not free into the matrix in the initial mixture concentration as to exert a direct effect. For instance $CaCl_2$ is considerably eliminated by washing the spheres. On the other hand, data on oxygen liberation and uptake by immobilized *Chlorella* and *Scenedesmus* cells, could be a consequence of the release treatment of the cells besides the specifics or inespecifics effects of cadmium and cadmium-zinc. To determine the effect of Cd and Cd-Zn on photosystem II (PS II) of the algal cultures chlorophyll and fluorescence parameters (Fo, Fm, Fv, Fv/Fm) as well as photochemical and non photochemical quenching and pH gradient were measured. Table 5 shows that cadmium (1 mg/L and 2 mg/L) and Cd-Zn did not affect the photochemical yield (Fv/Fm) in suspended *Chlorella* cultures.

TABLE 5. Chlorophyll fluorescence parameters of *Chlorella vulgaris* in suspended cultures.

Treatment	Fo	Fm	Fv	Fv/Fm	qP	qN
Control	188.00 ± 24 (100)	599.00 ± 62 (100)	410.00 ± 42 (100)	0.689 ± 0.0130 (100)	0.63 ± 0.04 (100)	0.14 ± 0.02 (100)
Cd (1 mg/L)	222.50 ± 46 (118)	687.50 ± 144 (115)	465.00 ± 98 (113)	0.676 ± 0.0005 (98)	0.62 ± 0.06 (98)	0.1 ± 0.06 (71)
Cd (2 mg/L)	115.25 ± 13 (61)	38.40 ± 52 (64)	268.58 ± 39 (65)	0.676.2 ± 0.018 (98)	N.D.	N.D.
Cd- Zn (2 mg/L-50 mg/L)	191.94 ± 17 (102)	639.61 ± 47 (107)	448.00 ± 30 (109)	0.7052 ± 0.006 (102)	0.58 ± 0.04 (92)	0.25 ± 0.14 (178)

Values shown (in relative fluorescence/ 52 x 10E 6) represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

Nevertheless variable fluorescence (Fv) decreased 34 % and maximal fluorescence (Fm) decreased 36 % at Cd concentration of 2 mg/L. Although these diminutions are restored to values slightly higher than those from control. *Chlorella* immobilized cells are more sensitive to the cadmium decreasing effects Fv/Fm (Table 6). Increase in Fo value in the presence of 1 mg/L cadmium could indicate an energy loss during transfer to the PSII reaction center. Although maximal and variable fluorescence are higher than control indicating a possible increase in the electron transfer rate from the reaction center to Q_A and a lower oxidation rate of photosystem I (PSI).

TABLE 6. Chlorophyll fluorescence parameters of *Chlorella vulgaris* in immobilized cultures.

Treatment	Fo	Fm	Fv	Fv/Fm	qP	qN
Control	280.83 ± 36 (100)	737.00 ± 100 (100)	457.08 ± 65 (100)	0.611 ± 0.014 (100)	0.49 ± 0.05 (100)	0.41 ± 0.09 (100)
Cd (1 mg/L)	410.17 ± 37 (146)	982.33 ± 87 (133)	572.17 ± 51 (125)	0.582 ± 0.011 (95)	0.54 ± 0.06 (110)	0.25 ± 0.10 (61)
Cd (2 mg/L)	153.50 ± 44 (55)	412.50 ± 129 (56)	259.00 ± 85 (57)	0.579 ± 0.046 (95)	N.D.	N.D.
Cd- Zn (1mg/L-50 mg/L)	291.58 ± 46 (104)	811.08 ± 133 (110)	519.50 ± 90 (114)	0.625 ± 0.014 (102)	N.D.	N.D.
Cd- Zn (2 mg/L-50 mg/L)	686.17 ± 56 (244)	1759.00 ± 146 (239)	1072.83 ± 91 (235)	0.609 ± 0.003 (99)	0.69 ± 0.04 (141)	0.39 ± 0.13 (95)

Values shown (in relative fluorescence/ 52 x 10E 6) represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

At cadmium concentrations of 2 mg/L it was observed a decrease of Fm, Fv, and Fv/Fm that could be attributed to an inhibition in the reduction of Q_A, Q_B and PQ

probably due to a lower reduction rate or by the increase in the oxidation rate of PSI. Zinc restored Fv/Fm up although modified the Fo, Fv, Fm parameters in different ways in presence of 1 mg Cd/L or 2 mg Cd/L.

TABLE 7. Chlorophyll fluorescence parameters of *Scenedesmus acutus* in suspended cultures.

Treatment	Fo	Fm	Fv	Fv/Fm	qP	qN
Control	88.5 ± 8 (100)	209.75 ± 20 (100)	121.25 ± 15 (100)	0.5745 ± 0.027 (100)	0.70 ± 0.05 (100)	0.43 ± 0.05 (100)
Cd (0.5 mg /L)	198.42 ± 34 (224)	394 ± 69 (188)	194 ± 35 (160)	0.4923 ± 0.005 (86)	0.31 ± 0.03 (44)	0.26 ± 0.06 (60)
Cd-Zn (0.5mg/L-50mg/L)	123.55 ± 14 (140)	371 ± 46 (177)	247.11 ± 33 (204)	0.6584 ± 0.014 (115)	0.62 ± 0.1 (88)	0.29 ± 0.16 (67)

Values shown (in relative fluorescence/ 2.41 x10E 6) represent mean ± standard error of 5 different experiments, Values in parenthesis represent percentage control.

TABLE 8. Chlorophyll fluorescence parameters of *Scenedesmus acutus* in immobilized cultures.

Treatment	Fo	Fm	Fv	Fv/Fm	qP	QN
Control	90 ± 7 (100)	204 ± 29 (100)	114 ± 23 (100)	0.508 ± 0.039 (100)	0.56 ± 0.01 (100)	0.32 ± 0.10 (100)
Cd (1 mg/L)	183 ± 29 (203)	252 ± 37 (123)	69 ± 9 (60)	0.286 ± 0.013 (56)	0.38 ± 0.15 (68)	0.56 ± 0.12 (175)
Cd- Zn- (1mg/L-50 mg/L)	122 ± 14 (135)	201 ± 24 (98)	79 ± 10 (69)	0.390 ± 0.006 (77)	-	-

Values shown (in relative fluorescence/ 2.41 x10E 6) represent mean ± standard error of 5 different experiments. Values in parenthesis represent percentage control.

Cadmium diminished Fv/Fm in suspended *Scenedesmus* cultures meanwhile Cd-Zn restored it up to a value higher than that from the control (Table 7). Immobilized *Scenedesmus* cells are more sensitive to cadmium effect decreasing Fv/Fm of 0.508 to 0.286 at 1 mg Cd/L (Table 8). In this case the restoring effect of Zn on Fv/Fm of PSII was not observed. The decreased of Fv at 1 mg Cd/L , 0.5 mg Cd/L-50 mg Zn/L or 1 mg Cd/L-50 mg Zn/L and the increase of Fo in presence of 1 mg Cd/L or 1 mg Cd/L-50 mg Zn/L indicate that it is possible that cadmium is diminishing the energy transfer from the antenna to the reaction center.

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