Antagonistic effect of manganese to cadmium toxicity in the alga *Dunaliella salina*

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**ABSTRACT:** The unicellular halotolerant alga *Dunaliella salina* was exposed to different concentrations of cadmium in the range 9 to 135 μM. Chlorosis was observed at 25 μM cadmium, and inhibition of cell division was established at 45 μM cadmium. Biosynthesis of the cellular osmoregulator, glycerol, was not affected by cadmium toxicity throughout the concentration range. Cadmium toxicity was found to be antagonized by low concentrations of manganese. Michaelis-Menten analysis of substrate (Mn\(^{2+}\))-inhibitor (Cd\(^{2+}\)) interactions showed competitive inhibition with the following K\(_s\) values at the various concentrations of Cd\(^{2+}\): K\(_s \[Cd = 0]\) = 0.175, K\(_s \[Cd = 45 \mu M]\) = 0.175, K\(_s \[Cd = 90 \mu M]\) = 0.126, K\(_s \[Cd = 135 \mu M]\) = 0.10. Ki of cadmium was 0.01. Results indicate a specific hyperbolic competitive cadmium–manganese interaction on one or both of 2 possible sites: on the cell membrane, or intracellularly on a specific metalo-enzyme. Results imply a possible protection of primary phytoplankton producers by nutrient trace metals against toxic heavy metals present in the oceans.

**INTRODUCTION**

In some single-celled algae, the toxic effect of cadmium and other heavy metals is associated with depression of cell division rates and consequent increases in cell size (see Fisher et al. 1981 for complete reference list). Since an increase in cell volume reflects enhancement of cellular osmotic components and water content, *Dunaliella* provides a suitable model to follow the cellular changes of glycerol, the major osmoticum of these halotolerant biflagellates (Ben-Amoth \\ Avron 1978).


Cadmium uptake has been reported in *Dunaliella* (Jennings \\ Rainbow 1979, Rebhun \\ Ben-Amotz 1986), but the exact location of cadmium in the cell was not resolved. Our recent paper (Rebhun \\ Ben-Amotz 1986) showed that the positively charged Cd\(^{2+}\), CdCl\(^+\) and possibly the neutral CdCl\(_2\)(aq) are toxic to *Dunaliella*.

There has been a long debate on the actual influence on primary productivity of toxic trace metals commonly found in aquatic environments. In nature, the variation of trace metal concentration in seawater is very wide and is affected by many environmental and ecological factors. Marine phytoplankton have shown adaptations enabling them to grow close to industrial polluted areas where the concentration of toxic trace metals is high and variable.

Recent interest in algal biotechnology in general, and in *Dunaliella* specifically, as a source of natural beta-carotene and other chemicals (Parkinson et al. 1987), has raised the question of the content in algae of heavy metals, with emphasis on toxic metals. The growth of *Dunaliella* in open salt water ponds and its relatively high resistance to heavy metals may be related to membrane impermeability or to specific cation interactions.

In an attempt to identify the growth requirements of *Dunaliella salina*, we came across a specific manganese-cadmium interaction which may explain the adap-
tation of *Dunaliella* and probably other algae to survival in trace-metal polluted areas.

The present study is based on experimental observations on the manganese-cadmium interaction in *Dunaliella salina*. The antagonistic mode effect of manganese on cadmium toxicity and uptake is described.

**MATERIALS AND METHODS**

**Algae.** *Dunaliella salina* (Dunal) Teod. UTEX # LB 200.

**Growth medium.** An artificial medium (pH 7.4) was used, containing: 0.75 M NaCl, 5 mM MgSO₄, 0.3 mM CaCl₂, 5 mM KNO₃, 0.2 mM KH₂PO₄, 1.5 μM FeCl₃, 6.0 μM EDTA, 50 mM NaHCO₃ and the trace elements: 0.4 μM Cu²⁺, 0.8 μM Zn²⁺, 0.4 μM Co²⁺, 7.0 μM Mn²⁺, 0.3 μM Mo⁴⁺ and 185 μM H₃BO₃. The concentration of EDTA as iron chelator was kept constant at 6.0 μM throughout all the growth experiments to avoid variation in the trace elements by the chelation level. Measurements of the concentration of EDTA, not complexed by iron, showed that the concentration of free EDTA in the medium was about 1 μM. This low level of free EDTA is unlikely to have interfered with the action of cadmium on *Dunaliella salina*.

All chemicals used were analytical grade. Modifications of the growth medium composition are indicated in the text.

**Growth conditions.** Late logarithmic phase algae were inoculated in fresh medium to an initial concentration of 10⁵ cells ml⁻¹ and were grown at 22 ± 2°C under cool white fluorescent continuous light of 80 μE m⁻² s⁻¹ with slow shaking. All cultures were aseptic and bacteria free.

**Cd toxicity.** Whenever indicated, Cd(NO₃)₂ atomic absorption grade (Merck) was introduced into the medium in concentrations from 0 to 135 μM. Algae were grown in the cadmium-enriched medium from the onset of the experiment for a complete growth cycle of about 6 d. In order to identify the ion antagonistic to cadmium toxicity, cultures of *Dunaliella salina* were grown in the presence of 4 out of 5 routinely added trace elements, i.e. in each culture a different element was missing.

**Assays.** Algae were assayed daily for cell number, chlorophyll and glycerol. Cells were counted with a Coulter Counter Model ZB. Algal volume was assayed microscopically. Chlorophyll was assayed spectrophotometrically following extraction with 90% acetone (Jensen 1978). Glycerol was assayed by the acetyl-acetone method (Ben-Amotz & Avron 1978). Cadmium was assayed in the algae by centrifuging samples and transferring the algal pellet with a small amount of water into pre-weighed crucibles for heating and ashing at 480°C. The inorganic residue was completely dissolved in acidified distilled water and assayed for cadmium with an atomic absorption spectrophotometer Model IL 900 with an air-acetylene flame. Results were corrected for medium residual Cd.

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**Fig. 1.** *Dunaliella salina*. Effect of Cd²⁺ on growth in the presence and absence of trace elements (TE). Left: cells; right: chlorophyll. Growth conditions as described in Materials & Methods.
and for the effect of salts on the cadmium assay by the atomic absorption spectrophotometer.

**Statistics.** The significance of all growth differences was determined by t-test.

**RESULTS**

**Cadmium toxicity and trace element antagonism**

*Dunaliella salina* was found to grow well in the synthetic medium containing 0.75 M NaCl with no addition of the classical supplemented trace elements Cu²⁺, Zn²⁺, Co²⁺, Mn²⁺, and Mo²⁺ (Fig. 1). Most probably the trace elements in the NaCl supplied the basic needs of the algae for normal cell division and growth, since the addition of trace elements did not produce enhanced cell growth rate nor increased in cell density. However, comparable growth with the addition of cadmium showed cadmium toxicity in the absence of supplemented trace elements. Less than 45 μM Cd²⁺ inhibited growth of trace-element-deficient algae but had no effect on trace-element-enriched algae. The trace-element-enriched algae were inhibited by concentrations of cadmium exceeding 90 μM.

The general phenomenon of cadmium toxicity was initially reflected by partial inhibition of chlorophyll biosynthesis and chlorosis. The pigment content of trace-element-enriched algae decreased from 0.75 to 0.25 pg chlorophyll cell⁻¹ in *Dunaliella salina* grown in a Cd²⁺ concentration of 45 μM.

The partial inhibition of chlorophyll biosynthesis by cadmium was not reflected in the biosynthesis of glycerol, which is the osmoregulator in *Dunaliella*. The algae synthesized glycerol at a normal photosynthetic rate irrespective of chlorosis and even under conditions of cadmium inhibiting cell division. As a consequence, the content of glycerol per chlorophyll increased (Fig. 2) in parallel to cell volume enlargement (not shown).

**Effect of manganese**

Out of the 5 trace elements routinely added to the medium – Cu²⁺, Zn²⁺, Co²⁺, Mn²⁺ and Mo²⁺ – only manganese antagonized the toxic effect of cadmium (Fig. 3). The other trace elements had no effect on cadmium toxicity at nutrient level or at up to 10 times the nutrient concentration. Unimpaired glycerol synthesis and a significant drop in chlorophyll content produced a high glycerol-to-chlorophyll ratio in cadmium-toxicated cells and a normal ratio in cadmium-

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**Fig. 2. Dunaliella salina.** Effect of Cd²⁺ on glycerol content in the presence and absence of trace elements (TE). Growth conditions as described in ‘Materials and Methods’

**Fig. 3. Dunaliella salina.** Effect of trace elements on growth in the presence of Cd²⁺. Algae were exposed to 45 μM Cd²⁺ in the presence of (A) all trace elements, or (B) 4 trace elements out of 5, to determine the antagonistic metal to Cd²⁺ toxicity.
Dunaliella salina. Effect of trace elements on the glycerol-to-chlorophyll ratio. Conditions were as described in Fig. 3. Increase of glycerol-to-chlorophyll ratio from the normal level of around 6 to the elevated level of above 11 always indicated Cd$^{2+}$ toxicity in Dunaliella salina grown under the specified conditions.

Table 1. Effect of Mn$^{2+}$ on uptake of Cd$^{2+}$

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**Table 1.** *Dunaliella salina.* Effect of Mn$^{2+}$ on uptake of Cd$^{2+}$

<table>
<thead>
<tr>
<th>Initial Cd$^{2+}$ concentration</th>
<th>Cd$^{2+}$ uptake $\mu g [mg$ organic weight$]^{-1}$</th>
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<tr>
<td>9</td>
<td>0.3</td>
</tr>
<tr>
<td>45</td>
<td>1.0</td>
</tr>
<tr>
<td>90</td>
<td>1.4</td>
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**DISCUSSION**

Low concentrations of cadmium caused partial chlorosis and low chlorophyll content per cell in a culture of *Dunaliella salina* presenting normal cell division. Above 45 $\mu M$ cadmium, cell division was impaired, but since the biosynthesis of the osmoregulator glycerol and probably other carbohydrates was not impaired, and their synthesis continued at the optimal rate, the volume of the algae increased. It was interesting to note that cadmium toxicity did not affect the biosynthesis of glycerol and that *D. salina* could maintain its osmoregulation under conditions where the culture was heavily toxicated by cadmium. High resistance of the glycerol biosynthetic machinery in *Dunaliella* to protein biosynthetic inhibitors and the lack of specific inhibitor enzymes in the glycerol cycle (Ben-Amotz & Avron 1978) have already pointed to the independence of glycerol metabolism in this halotolerant alga. Cadmium toxicity in *D. salina* is, therefore, metabolic specific, inhibiting initially chlorophyll formation, subsequently (at higher concentrations of cadmium) protein biosynthesis, and finally cell division. This cell swelling has some similarity to results from the work of Fisher et al. (1981) on the effect of elevated
Fig. 5. *Dunaliella salina*. Inhibitory effect of Cd$^{2+}$ in the presence of different concentrations of Mn$^{2+}$. Upper: cells; lower: chlorophyll. Growth conditions as described in 'Materials and Methods'.
levels of copper and zinc on the growth, morphology and metabolism of *Asterionella japonica*, as expressed by reduction of cell division and increase in cell size. Increase in cell size is presumably dependent, however, on the continuous production of cellular soluble osmotic components which will in turn cause water influx and cell size enhancement.

Previous studies by Sunda et al. (1981) showed antagonistic interaction between copper toxicity and manganese in marine phytoplankton, and that a combination of low manganese concentrations and high cupric ion activities may limit the growth of phytoplankton in upwelling seawater. Our study of *Dunaliella salina* growth in the presence of different trace elements clearly identifies manganese as the antagonist cation to cadmium. The presence of manganese in cultures of *D. salina* exposed to cadmium at the investigated levels is essential for normal chlorophyll synthesis.

The increased cadmium uptake in the absence of manganese or of all 5 trace elements confirmed our conclusion that cadmium interferes with the process of pigment biosynthesis in *Dunaliella salina*. Manganese deprivation in algae is known to cause chlorosis and to interfere with the maintenance of the chloroplast membrane structure and with the electron transport of Photosystem II (L. Kelley 1974, Rains 1976). Cadmium toxicity as analysed by competitive inhibition with manganese showed a similar effect on *D. salina*: chlorosis and cell death. The sequence of events which caused the inhibition of cell growth may be hypothesized as indicating a series of events starting with cadmium competition with manganese, continuing via manganese deprivation, disintegration of the chloroplast membrane, and chlorosis, and eventually leading to interference of cell division.

To explain the relation between the effect of cadmium, manganese and the growth of *Dunaliella salina*, we have used the classical enzyme kinetics model where manganese functions as a substrate (S), cadmium as an inhibitor (I), the alga as an enzyme (E), and the growth rate as velocity (V). The kinetics of most enzymic reactions in the presence of varying concentrations of inhibitors are characterized by linear double reciprocal plots of $1/V$ versus $1/S$, but the slope, the intercept, or both are altered by factors $(1+i/k_i)$ where $i$
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Fig. 7. Dunaliella salina. Kinetic diagnostic for competitive inhibition of Mn$^{2+}$ to Cd$^{2+}$ toxicity. Data illustrated were derived from Fig. 6

is the concentration of the inhibitor and $k_{ij}$ is a characteristic constant. Plots of $V$ vs $i$ as well as secondary replots of slopes or interceptors versus $i$ may or may not be linear because the effect on the steady-state rate equation itself is that certain denominator terms are multiplied by the same factor $(1+i/k_{ij})$ (Mahler & Con des 1966).

The kinetic analysis in our study (Figs. 5 to 7) clearly showed a specific hyperbolic competitive cadmium-manganese interaction. The hyperbolic plot of $1/V$ versus $i$ showed that there is not formation of EI or IES as in pure competitive inhibition, but the effect of cadmium is on the affinity of E for S only. Hyperbolic competition may be regarded as an instance of allosteric inhibition.

Our results do not specify which of the possible competitive inhibitions takes place: cell membranal or intracellular. Both mechanisms would fit the effect of cadmium on the manganese requirement. Cadmium may interfere with manganese uptake and its transfer across the membrane by blocking the manganese channel in the membrane, or by replacing manganese with cadmium to facilitate active uptake of toxic cadmium from the medium into the cell by the manganese carrier. In either case, a phenomenon of manganese deficiency would be shown and the algae would manifest manganese starvation symptoms. The Michaelis-Menten analysis cannot distinguish between these 2 possibilities, as the kinetics equation is similar for nutrient uptake by a membrane carrier and for enzymatic catalysis. The question of the exact site of cadmium interference with manganese utilization in the cell remains open for further studies, but the implication of this study may be that unicellular marine algae can be adapted to survive in trace-metal polluted aquatic environments. Whenever a toxic level of a heavy metal is antagonized by an appropriate level of a nutrient metal, the algal hyperbolic competitive protection mechanism may allow the cell to maintain its biological activity.

LITERATURE CITED


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Results of ecological monitoring of three beaches polluted by the 'Amoco Cadiz' oil spill: development of meiofauna from 1978 to 1984

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ABSTRACT: Following the 'Amoco Cadiz' oil spill, time-series sampling of the meiofauna was carried out from 1978 to 1984 in the intertidal zone of 3 sandy beaches on the northern Finistère coast (Brittany, France). Quantitative analysis documented 2 principal phases in the development of the main taxa (Nematoda and Copepoda). First came a degradation phase leading to impoverishment in density and diversity of the populations. This first phase could be subdivided into several stages corresponding mainly to the toxicity period and, on one beach (Kersaint), to a summer 'bloom'. Then came a recovery phase corresponding to a quantitative and qualitative reconstitution of the meiofauna. Each phase lasted a greater or lesser time according to station exposure and the considered taxon. A qualitative analysis of harpacticoid copepods illustrated the development of population diversity and 'ecological groups'. During the first phase, replacement of the original population by a substitute fauna was observed. Correspondence factorial analysis on the development of harpacticoid communities allowed a better understanding of the main pollution and recovery factors such as toxicity, organic matter, hydrodynamism and zoological groups. Meiofauna, particularly harpacticoid copepods, are significant bioindicators of ecological disturbances.

INTRODUCTION

Few long-term studies on meiofauna exist, particularly any dealing with the consequences of oil pollution (Feder et al. 1976, Boucher 1983, 1985, Elmgren et al. 1983).

Following the wreck of the oil tanker 'Amoco Cadiz' (16 March 1978), time-series sampling of the meiofauna was carried out from 1978 to 1984 in the intertidal zone of 3 sandy beaches on the northern Finistère coast (Brittany, France). Two of these beaches (Brouennou and Corn ar Gazel) are situated on either side of the mouth of the Aber Benoît river; the third (Kersaint) about 4 km south of the wreckage point, near Port;ais (Fig. 1). Preliminary results of this ecological monitoring were presented in 2 previous papers (Bodin & Boucher 1981, 1983) and several reports. The aim of the present paper is to synthesize the final results. Special attention is paid to the 2 principal zoological groups involved: nematodes and harpacticoïd copepods.

MATERIAL AND METHODS

Study sites. Brouennou is a fine-sand beach (median grain size 127 to 160 μm), poorly sorted, containing 1.7 to 6.6 % silt/clay and 0.2 to 0.3 % organic carbon (Table 1, Fig. 2). It faces west, but a rocky point and several small islands shelter it from open sea swells. Its 'morpho-sedimentary vulnerability index' is 6 (Berné & D'Ozouville 1979). On this vulnerability scale, ranging from 1 to 10, low figures mean greater exposure to wave and current action.

Corn ar Gazel is a fine-sand beach (median grain size 122 to 150 μm), well sorted, containing 0.9 to 2.2 % silt/clay and 0.3 to 0.4 % organic carbon. It faces northeast and is under the influence of strong tidal currents as well as wave action. Its morpho-sedimentary vulnerability index is 2.

Kersaint has coarser sand (median grain size 191 to 210 μm), well sorted, containing 0.9 to 2.4 % silt/clay and about 0.1 to 0.3 % organic carbon. It faces north-northwest and, consequently, is exposed to the strong...