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Evaluation of toxic effects of heavy metals on unicellular algae
III - Subtraction of the toxic element from the medium by the cells**

A study of the toxic effect of heavy metals on the growth of unicellular algae can encounter difficulties on account of variation during the course of experiments of the quantity of algae and of the concentration of the toxic element.

As far as the concentration of algae is concerned, it has already been demonstrated that an evaluation of toxicity can reveal values which vary considerably according to variation of the initial inoculum (ALBERTANO, PINTO, TADDEI, 1979).

With regard to the variations of the concentration of the toxic element, numerous authors have demonstrated the existence of an active subtraction of the toxic element by the algae. In most cases, the element is incorporated by the cells, as deduced by chemical analysis (NAKANO et al., 1978; MC BRIEN, HASSAL, 1965) and by means of ultrastructural observation: for example, an accumulation of cadmium in mitochondria has been demonstrated (SILVERBERG, 1975, 1976). In rarer cases the element subtracted from the medium is not completely incorporated: mercury can, in fact, be volatilised in part (BEN-BASSAT, MAYER, 1975, 1978).

The subtraction of the toxic element by the algae is not necessarily a phenomenon connected to the vitality of the cells, but can be effected equally by dead cells (MATZKU, BRODA, 1970).

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In every case it is clear that the algae are able to effect a subtraction of the metal from the culture medium and that therefore the concentration of the metal is subjected to changes in the course of the experiments. That this phenomenon must be taken account of has already been underlined by WHITTON & SAY (1975): « Data about toxic concentrations should in particular always be treated with caution. Many toxicity tests have been carried out by adding metals at various levels without making any measurements of the levels of metal in solution at the commencement or during the experiment ».

In the course of the experiments, therefore, there can occur considerable variations in some fundamental parameters, namely in the quantity of algae and the concentration of heavy metal: these variations can then make their effect felt on the growth-curve of the algae. This phenomenon has already been demonstrated by BLANKLEY (1973) and analysed by the present authors in a previous article (ALBERTANO, PINTO, TADDEI, 1980).

The scope of the present article is the demonstration of the role played by the cells (living or dead) in the subtraction of the metal from the culture medium.

MATERIALS AND METHODS

We employed the alga *Chlorella saccharophila*, strain 211.9a Gö from the Culture Centre of Algae and Protozoa at Cambridge *. The enrichment cultures and the experimental tests were carried out in the liquid medium already utilised in one of our previous experiments ** (ALBERTANO, PINTO, TADDEI, 1979).

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* Since in the present experiment it was necessary to effect a vital colouration of the vacuole, the alga *Cyanidium caldarium*, utilised in the previous two investigations in this series, was not employed, in view of the absence of a vacuole in this alga.

** All the reagents employed in this work were chemically pure and produced by Riedel - De Haën.

The experimental tests were conducted in test-tubes 14 mm x 140 mm, sealed with vellum paper; all the culture conditions were identical to those described in the aforementioned article.

The enrichment cultures were conducted in 1-litre Erlenmeyer flasks holding approximately 350 ml of algal suspension; for the purposes of the experimental tests the algae were centrifuged and re-suspended in renewed culture medium some days before each experiment; in this way the inoculum was always effected with algae in a fully exponential phase.

Tests were conducted on the following metals *:

beryllium	as $Be(NO_3)_2 \cdot 4H_2O$	manganese	as $MnCl_2 \cdot 4H_2O$
cadmium	as $CdCl_2 \cdot H_2O$	mercury	as $HgCl_2$
chromium **	as $K_2Cr_2O_7$	molybdenum	as $Na_2MoO_4 \cdot 2H_2O$
cobalt	as $CoCl_2 \cdot 6H_2O$	nickel	as $NiSO_4 \cdot 6H_2O$
copper	as $CuCl_2 \cdot 2H_2O$	thallium **	as Tl_2SO_4

The experiment was articulated into two phases, as is made clear in the diagram on the following page: in the *first phase* a quantity of living algae was suspended in 6 ml of liquid medium containing a metal of a known concentration. The concentration of the algae was such as to allow a colorimeter-reading of 0.4 units of absorbance to be registered; the same process was carried out with an identical quantity of dead algae, killed by means of heat (heated for 5 mins at 70°C).

Tests of this kind were carried out in different concentrations for each of the heavy metals indicated above (see tabs. 1-10). Various series of test-tubes were prepared (each series as indicated above) and each series was maintained under the

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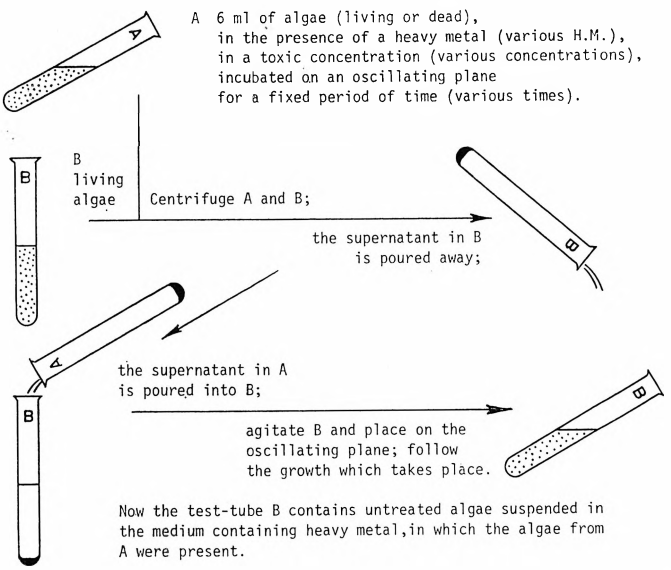
* Tungsten and zinc, investigated in the previous two research articles in this series, have not been analysed here on account of their insolubility in the concentrations required for this experiment.

** In the case of chromium and thallium the molarity indicated in the relevant figures and tables refers to the element and not to its salt.

aforementioned conditions, on an oscillating plane, for one of the following periods of time, namely 0, 6, 24, 48, 72, 120 hours *.

The contents of each test-tube was then centrifuged for 7' at 3000 r/m; the algae were removed and to the supernatant were added new living algae (preventatively centrifuged) in equal quantity to those used during the first phase. Thus began the *second phase* and the test-tubes were once again placed on the oscillating plane.



From this point onward the growth of the algae was followed by means of readings on a Bausch & Lomb Spectronic 20 colorimeter at a wavelength of 550 nm: every two days for 12 days readings were made. At the close of the experiment we registered the algal growth which had taken place, or their death, according to the case (or the commencement of a clear phase of decrease in growth).

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* Times measured at the end of the centrifugation, of which it will be necessary to speak further on. The test indicated as 0 hours was carried out by eliminating the first phase and passing directly to the second phase.

Further, the percentage of living and that of dead algae was controlled at the close of the first phase. To this end the « living » * algae obtained by the centrifugation of the test-tubes at the close of the first phase were re-suspended in 6 ml of culture medium in the absence of any heavy metal, to which was added 1 ml of an aqueous solution of neutral red (1 : 1000); after approximately 30 minutes the algae were observed under the microscope with the aim of evaluating, at least in an approximate fashion, the percentage of dead algae and that of algae which had survived; the colouration by the neutral red of the vacuole in the living algae rendered this examination possible.

All the operations relating to the enrichment cultures were carried out under sterile conditions. The experimental tests, on the other hand, were conducted under semi-sterile conditions, since the development of fungi and bacteria could be considered negligible: by proceeding in this fashion, quite apart from accelerating the operations, eventual alterations of the substances employed during the sterilisation process could be prevented.

Results

Our results are given in figs. 1 - 10 and in tabs. 1 - 10; the number given to each metal is the same in both figures and tables. In the figures we have given the percentage of algae which had survived the effects of the various heavy metals (in various concentrations) at the close of the first phase of our experiment.

The results, largely foreseeable as they were, show that the metal determines the death of the cells to a greater extent the greater the concentration of the metal itself and the longer the algae remain in the metal's presence.

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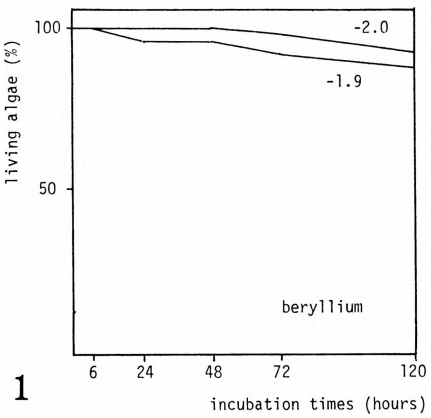
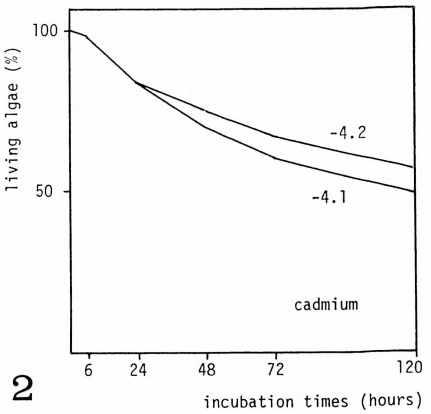
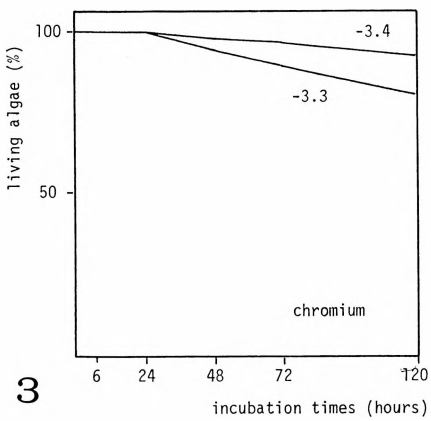
* Algae which were at least initially « living », namely at the time of their insemination at the beginning of the first phase.


100
50
living algae (%)
-2.0
-1.9
beryllium
6
24
48
72
120
incubation times (hours)

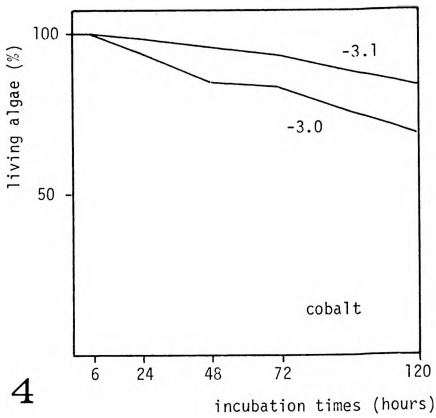
1


100
50
living algae (%)
-4.2
-4.1
cadmium
6
24
48
72
120
incubation times (hours)

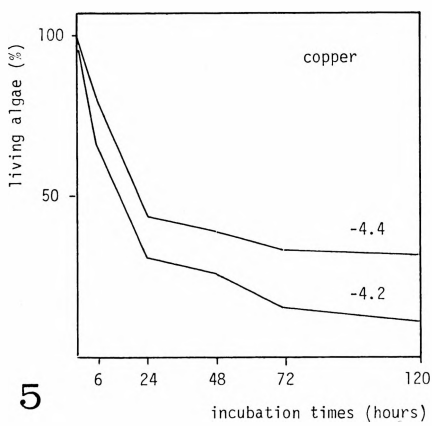
2


100
50
living algae (%)
-3.4
-3.3
chromium
6
24
48
72
120
incubation times (hours)

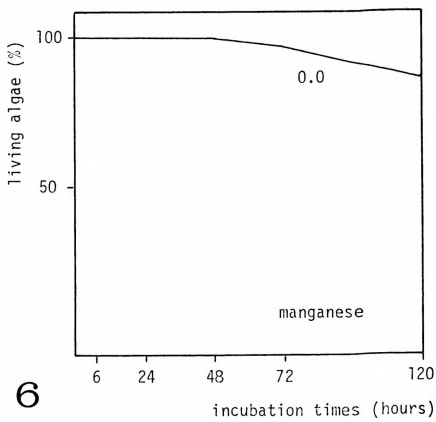
3


100
50
living algae (%)
-3.1
-3.0
cobalt
6
24
48
72
120
incubation times (hours)

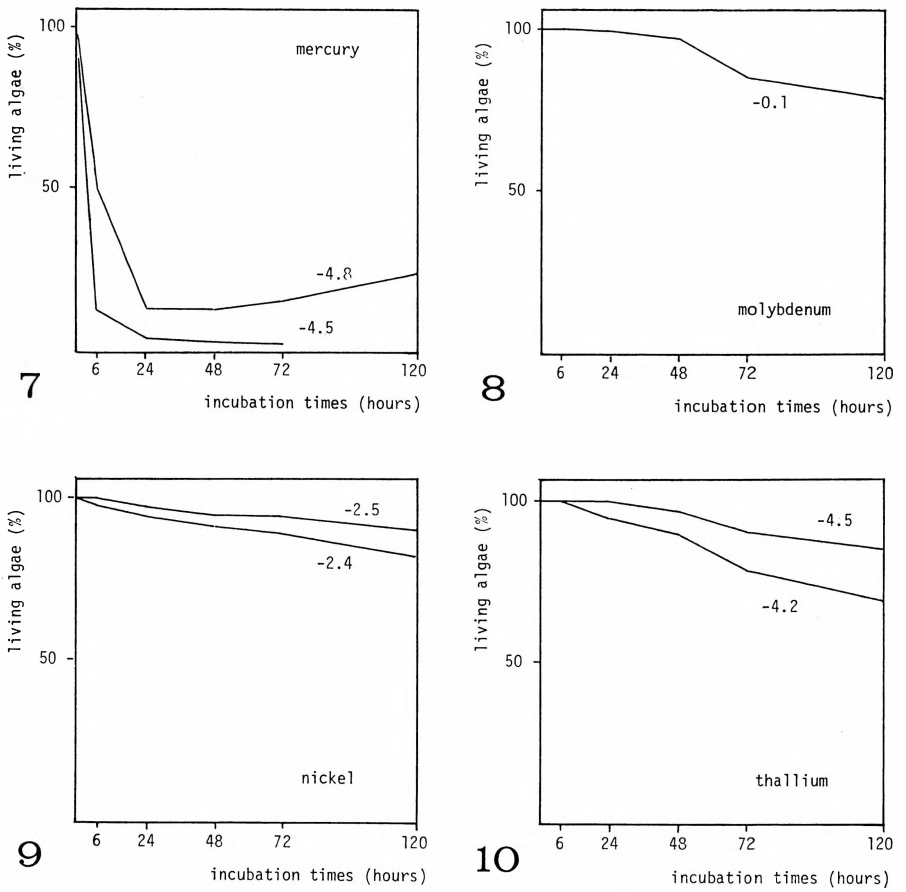
4


100
50
living algae (%)
copper
-4.4
-4.2
6
24
48
72
120
incubation times (hours)

5


100
50
living algae (%)
0.0
manganese
6
24
48
72
120
incubation times (hours)

6



Figs. 1-10 — Percentage of cells still alive after incubation for various lengths of time in culture media containing a concentration of heavy metal superior to that of the least algicide. The concentrations are expressed as logs of molarity.
1) beryllium 2) cadmium 3) chromium 4) cobalt 5) copper 6) manganese 7) mercury 8) molybdenum 9) nickel 10) thallium.

In the tables we have synthesised the results of the experiments: on the left hand side we have indicated for both living and dead algae the initial concentration of the element (expressed in log of the molarity); above this we have indicated the lengths of time during which the algae, living or dead, remained in the presence of the said concentrations. To the media, initially containing the various concentrations of metal as indicated in the table and treated with living or dead algae for the lengths of time shown, were added new living algae, which at the close of the second phase proved to be still alive (symbol +) or to have died (symbol —). Since all the experiments were conducted twice, we used the simple symbol + (and respectively —) whenever the results obtained were identical in the two tests, while the symbol ? was preferred in the case of the two results being contradictory or doubtful.

TABLES 1 - 10

The culture medium containing a heavy metal (diverse metals and diverse concentrations) was treated for various lengths of time with living algae or with dead algae. At the close of the incubation the algae were removed for centrifugation and a renewed insemination of living algae was made. After twelve days these algae were in a clearly decrescent phase (—) or were entering a phase of more or less active growth (+). Occasionally this process proved inconclusive or non-repeatable (?).
1) beryllium 2) cadmium 3) chromium 4) cobalt 5) copper 6) manganese 7) mercury 8) molybdenum 9) nickel 10) thallium.

1

beryllium	incubation times (hours) 0	6	24	48	72	120	
log of molarity — 1.9	—	—	—	—	—	—	living algae
— 2.0	—	—	—	?	—	?	
— 2.1	+	+	+	+	+	+	
— 1.9	—	—	—	—	—	—	dead algae
— 2.0	—	—	—	—	—	?	
— 2.1	+	+	+	+	+	+	

2 cadmium — incubation times (hours)

log of molarity	0	6	24	48	72	120	
—4.0	—	—	—	—	—	—	living algae
—4.1	—	—	—	—	?	—	
—4.2	—	—	—	?	+	+	
—4.3	+	+	+	+	+	+	
—4.0	—	—	—	—	—	—	dead algae
—4.1	—	?	?	+	+	?	
—4.2	—	+	+	+	+	+	
—4.3	+	+	+	+	+	+	

3 chromium — incubation times (hours)

log of molarity	0	6	24	48	72	120	
—3.3	—	—	—	—	—	—	living algae
—3.4	—	—	—	—	+	?	
—3.5	+	+	+	+	+	+	
—3.3	—	—	—	—	—	—	dead algae
—3.4	—	?	+	?	+	+	
—3.5	+	+	+	+	+	+	

4 cobalt — incubation times (hours)

log of molarity	0	6	24	48	72	120	
—3.0	—	—	—	—	—	—	living algae
—3.1	—	—	—	+	?	+	
—3.2	+	+	+	+	+	+	
—3.0	—	—	—	—	—	—	dead algae
—3.1	—	—	?	—	?	—	
—3.2	+	+	+	+	+	+	

5

copper	log of molarity	incubation times (hours) 0	6	24	48	72	120	
living algae	— 4.2	—	—	—	—	—	?	
	— 4.3	—	—	—	+	+	+	
	— 4.4	—	+	+	+	+	+	
	— 4.5	+	+	+	+	+	+	
dead algae	— 4.2	—	—	—	—	—	—	
	— 4.3	—	+	+	+	+	+	
	— 4.4	—	+	+	+	+	+	
	— 4.5	+	+	+	+	+	+	

6

manganese	log of molarity	incubation times (hours) 0	6	24	48	72	120	
living algae	0.0	—	—	—	—	—	—	
	— 0.1	+	+	+	+	+	+	
dead algae	0.0	—	—	—	—	—	—	
	— 0.1	+	+	+	+	+	+	

7

mercury	log of molarity	incubation times (hours) 0	6	24	48	72	120	
living algae	— 4.5	—	—	—	—	—	—	
	— 4.6	—	—	?	+	?	+	
	— 4.7	—	+	+	+	+	+	
	— 4.8	—	+	+	+	+	+	
	— 4.9	—	+	+	+	+	+	
	— 5.0	+	+	+	+	+	+	
dead algae	— 4.5	—	—	—	—	—	?	
	— 4.6	—	—	—	—	—	+	
	— 4.7	—	+	?	?	+	+	
	— 4.8	—	+	+	+	+	+	
	— 4.9	—	+	+	+	+	+	
	— 5.0	+	+	+	+	+	+	

8

log of molarity (molybdenum)	incubation times (hours) 0	6	24	48	72	120	
— 0.1	—	—	—	—	—	—	living algae
— 0.2	+	+	+	+	+	+	
— 0.1	—	—	—	—	—	—	dead algae
— 0.2	+	+	+	+	+	+	

9

log of molarity (nickel)	incubation times (hours) 0	6	24	48	72	120	
— 2.4	—	—	—	?	—	—	living algae
— 2.5	—	—	—	+	+	?	
— 2.6	+	+	+	+	+	+	
— 2.4	—	—	—	—	—	—	dead algae
— 2.5	—	?	—	—	?	—	
— 2.6	+	+	+	+	+	+	

10

log of molarity (thallium)	incubation times (hours) 0	6	24	48	72	120	
— 4.2	—	—	—	—	—	—	living algae
— 4.3	—	—	—	—	—	—	
— 4.4	—	—	—	?	—	+	
— 4.5	—	—	—	+	+	+	
— 4.6	—	—	?	+	+	+	
— 4.7	+	+	+	+	+	+	
— 4.2	—	—	—	—	—	—	dead algae
— 4.3	—	+	?	+	+	+	
— 4.4	—	+	+	+	+	+	
— 4.5	—	+	+	+	+	+	
— 4.6	—	+	+	+	+	+	
— 4.7	+	+	+	+	+	+	

Discussion and conclusions

The examination of the tables relating to the ten metals employed shows that for six of them (Be, Cr, Co, Mn, Mo, Ni) the incubation with algae (living or dead) had no conspicuous effect.

For the remaining four metals (Cd, Cu, Hg, Tl) the incubation effected a detoxification of the culture medium. More particularly we observe that for all four of these metals, the detoxification attains its peak after scarcely 6 hours on the part of the dead cells, while that effected by the living cells occurs after time-spans of various lengths.

We would like to underline further that the toxicity of mercury and of copper (besides being extremely high) begins to manifest itself after only a very short time (figs. 5 and 7): equally brief is the time necessary for the detoxifying action caused by the living algae to begin to develop (tabs. 5 and 7).

Analogously the toxicity of cadmium and thallium (slightly less than that of Cu and Hg) begins to manifest itself after relatively longer periods of time (figs. 2 and 10): equally long are the times required by the living algae in order to unfold their detoxifying effect (tabs. 2 and 10).

This circumstance leads us to hypothesize, that the detoxifying action ought principally to be attributed to the dead rather than to the living algae.

Such a hypothesis is not to be excluded in the case of those metals for which the phenomena described in this article was not established (Be, Mn, Mo, Ni; we will deal with Co and Cr separately further on): we would in fact draw attention to the circumstance that the toxicity of these 4 elements is so slight that the death of the algae occurs in a consistent manner only after 120 hours; further, having had to employ very high concentrations, the subtraction by the algae in all probability must be seen as having a place in an order of greatness inferior to the intervals of concentration of the metal employed by us.

We can assume further, that the phenomenon is the less apparent the smaller the number of sites on the algae to which the metal is capable of attaching itself (PASSOW, 1961). This is probably so in the case of Co and Cr, with which the phenomenon does not manifest itself, regardless of the fact that the metals are present in the culture medium in a relatively small quantity.

Finally we consider it useful to confront the results of this article with the growth-curves of the algae in the presence of various concentrations of heavy metal (ALBERTANO, PINTO, TADDEI, 1980; cf. fig. 3 e, g). It is of interest to observe that copper and mercury are the sole elements which, with *C. saccharophila*, show behaviour of type III (prolongation of the lag-phase).

Such behaviour is dependent on the fact that in the initial phase of the experiment, there is complete inhibition or even death of a part of the cells. The algae (above all those dead) subtract from the culture medium a sufficient amount of heavy metal so as to lower the concentration to non-inhibitory levels. Once this initial phase has been overcome, the surviving algae find themselves in a non-toxic environment and their growth takes place as in the absence of metal.

We should like to make clear in this respect that the course of the growth-curve in the lag-phase period, registered by us as sub-horizontal, is in reality no more than the result of the superimposition of two opposed phenomena: on the one hand the algal concentration, in fact, decreases due to the death of a part of the cells, while the colorimeter-readings continue to register traces of their presence still some days after their death; on the other hand, the cells which survived, by dividing and multiplying, raise the algal concentration.

SUMMARY

By means of biological tests carried out on a unicellular alga *Chlorella saccharophila*, the authors demonstrate that, at least in a number of cases, the cells subtract heavy metal from the culture medium. Such subtraction is effected predominantly by the dead algae rather than by the living.

The detoxification of the medium is particularly evident in the case of highly toxic metals whose toxicity develops over short periods of time (Hg and Cu).

The authors confront these results with those attained in a previous work of theirs: they draw attention to the process whereby such metals (Hg and Cu) can be identical with those which reveal their inhibitory effects on algal growth by a prolongation of the lag-phase, and interpret this particular type of inhibition by referring to the phenomenon described in the above.

RIASSUNTO

Mediante tests biologici effettuati sull'alga unicellulare *Chlorella saccharophila*, gli Autori dimostrano che, almeno in alcuni casi, le cellule sottraggono metallo pesante dal mezzo di coltura. Tale sottrazione è operata prevalentemente dalle alghe morte, piuttosto che da quelle vive.

La detossificazione del mezzo è particolarmente evidente per i metalli fortemente tossici e la cui tossicità si esplica in tempi brevi (Hg e Cu).

Gli Autori pongono a confronto questi risultati con quelli di un loro precedente lavoro: fanno notare come tali metalli (Hg e Cu) siano gli stessi che manifestano la loro inibizione sulla crescita delle alghe con un prolungamento della lag-fase ed interpretano questo particolare tipo di inibizione con il fenomeno sopra descritto.

LITERATURE CITED

ALBERTANO P., PINTO G., TADDEI R., 1979. *Evaluation of toxical effects of heavy metals to unicellular algae.* I - *The influence of inoculum concentration on the evaluation of toxicity.* Delpinoa, **20**: 75-86.

— —, 1980. *Idem.* II - *Growth curves with different concentrations of heavy metals.* Delpinoa, **21**: 23-34.

BEN-BASSAT D., MAYER A. M., 1975. *Volatilization of mercury by algae.* Physiol. Plant., **33**: 128-132.

— —, 1978. *Light-induced Hg volatilization and O_2 evolution in Chlorella and the effect of DCMU and methylamine.* Physiol. Plant., **42**: 33-38.

BLANKLEY W. F., 1973. *Toxic and inhibitory materials associated with culturing.* In: *Handbook of Phycological Methods* (J. R. Stein, ed.), 207-229, Cambridge.

MATZKU S., BRODA E., 1970. *Die Zinkaufnahme in das Innere von Chlorella.* Planta, **92**: 29-40.

MCBRIEN D. C. H., HASSALL K. A., 1965. *Loss of cell potassium by Chlorella vulgaris after contact with toxic amounts of copper sulphate.* Physiol. Plant., **18**: 1059-1065.

NAKANO Y., OKAMOTO K., TODA S., FUWA K., 1978. *Toxic effects of cadmium on Euglena gracilis grown in zinc deficient and zinc sufficient media.* Agric. Biol. Chem., **42**: 901-907.

PASSOW H., ROTHSTEIN A., CLARKSON T. W., 1961. *The general pharmacology of the heavy metals.* Pharmacol. Rev., **13**: 185-224.

SILVERBERG B. A., 1975. *Ultrastructural localization of lead in Stigeoclonium tenue (Chlorophyceae, Ulotrichales) as demonstrated by cytochemical and X-ray microanalysis.* Phycologia, **14**: 265-274.

— —, 1976. *Cadmium-induced ultrastructural changes in mitochondria of freshwater green algae.* Phycologia, **15**: 155-159.

WHITTON B. A., SAY P. J., 1975. *Heavy metals.* In: *River ecology* (B. A. Whitton, ed.), 286-311, Oxford: Blackwell.