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Evaluation of toxical effects of heavy metals to unicellular algae. I - The influence of inoculum concentration on the evaluation of toxicity.**

Any experiment aiming at evaluating the toxicity of a chemical element should take into consideration the possible presence of variable factors during the experiment itself; the most obvious way of facing this problem consists in keeping the various parameters at a constant level. This can be easily obtained as far as temperature, light, and up to a certain extent the composition of the medium, etc are concerned.

Other parameters, on the contrary, are extremely variable, expecially the « concentration of algae ». In order to keep this factor constant it is necessary to carry out continuous cultures, but one has to employ complex instruments, and this circumstance entails serious problems of room and time.

As these are often routine works, if one proposes to do without maintaining this factor (concentration of algae) constant, in analysing the experimental results it should be kept in mind that it is variable in the course of the whole experiment.

If the concentration of algae turns out to exert an influence on experimental results, we may assume that the inoculum has a decisive importance. The influence of such a factor on the

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results of toxicity tests is well known (BLANKLEY, 1973); such influence was further pointed out by some other authors (SHIEH, BARBER, 1973; WIUM-ANDERSEN, 1974; BEN-BASSAT, MAYER, 1975) as far as particular heavy metals are concerned. But these authors did not emphasize this factor; this phenomenon is usually explained by assuming that the algae take the metal away, as it has been shown in some cases (MC BRIEN, HASSAL, 1965).

This article intends to make clear how far the concentration of the inoculum is liable to influence the evaluation of toxicity of a chemical element and whether it is possible to generalize this phenomenon.

MATERIALS AND METHODS

We have employed the algae *Cyanidium caldarium*, strain 001 from our collection (from the Pozzuoli Solfatara, near Naples), and *Chlorella saccharophila*, strain 211-9a Gö from the Culture Centre of Algae and Protozoa, Cambridge.

These algae were chosen for the following reasons: they grow easily and stand environmental variations; they are small in size and consequently remain easily suspended when gently shaken; they are resistant to acids (in particular *C. caldarium* is a tipically acidophilic alga): this characteristic allows us to grow them in low-pH media, in which the emergence of fungi and bacteria is contained within reasonable limits and can be considered as irrelevant even after several days.

The enrichment cultures and the tests for *C. caldarium* had been carried on in a liquid medium with the following composition *:

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

$(NH_4)_2SO_4$	1320	mg	$ZnCl_2$	1.4	mg
$MgSO_4 \cdot 7H_2O$	300	mg	$CuSO_4 \cdot 5H_2O$	1.0	mg
KH_2PO_4	300	mg	$Na_2MoO_4 \cdot 2H_2O$	0.5	mg
NaCl	100	mg	$CoCl_2 \cdot 6H_2O$	0.5	mg
$CaCl_2$	20	mg	H_2O	1000	ml
H_3BO_3	6.2	mg	H_2SO_4	3	ml
$FeCl_3 \cdot 6H_2O$	5.4	mg			
$MnCl_2 \cdot 4H_2O$	2.0	mg	pH = 1.5		

The enrichment cultures and the tests for *C. saccharophila* had been carried on in a liquid medium with the following composition:

KNO_3	1010	mg	$MnCl_2 \cdot 4H_2O$	2.0	mg
$MgSO_4 \cdot 7H_2O$	123	mg	$ZnCl_2$	1.4	mg
KH_2PO_4	122.4	mg	$CuSO_4 \cdot 5H_2O$	1.0	mg
K_2HPO_4	17.4	mg	$Na_2MoO_4 \cdot 2H_2O$	0.5	mg
NaCl	10.0	mg	$CoCl_2 \cdot 6H_2O$	0.5	mg
$CaCl_2$	11.0	mg	H_2O	1000	ml
H_3BO_3	6.2	mg			
$FeSO_4 \cdot 7H_2O$	5.6	mg	pH = 6.0		

The tests were done in tubes 14 mm wide and 140 mm high, plugged with parchment paper.

The tubes were placed on a plexiglas shaking apparatus which held them at a 71° angle and rocked them with an amplitude of 11 cm at the rate of 62 cycles per minute (cfr. SHIHIRA, KRAUSS, 1965). The shaker was illuminated from below by lamps Philips TLD 30 W/55 (6000 lux). The ambient temperature was maintained at 37°C for *C. caldarium* and 23°C for *C. saccharophila.*

The enrichment cultures were prepared in 1 liter bottles containing circa 350 ml of algal suspension; these algae were centrifuged, again suspended in a new culture medium and after seven days were employed for the experimental tests (all the cultures were in exponential phase).

Tests were made at different algal concentration in the presence of 12 heavy metals at different concentrations; the following elements were employed:

beryllium	as $Be(NO_3)_2 \cdot 4H_2O$	mercury	as $HgCl_2$
cadmium	as $CdCl_2 \cdot H_2O$	molybdenum	as $Na_2MoO_4 \cdot 2H_2O$
chromium	as $K_2Cr_2O_7$	nickel	as $NiSO_4 \cdot 6H_2O$
cobalt	as $CoCl_2 \cdot 6H_2O$	thallium	as Tl_2SO_4
copper	as $CuCl_2 \cdot 2H_2O$	tungsten	as $Na_2WO_4 \cdot 2H_2O$
manganese	as $MnCl_2 \cdot 4H_2O$	zinc	as $ZnCl_2$

Various concentrations have been employed by us both for algae and heavy metals, with intervals of 0.3 log units (i.e. with a circa 1:2 ratio between two successive concentrations).

The concentrations of the elements have been expressed in figs 1 and 2 as the log of molarity.

The highest concentration of algae (i.e. the one from which we started for further dilutions) was read as 0.7 absorbance units at colorimeter, which corresponds to the values of fresh weight, dry weight, and number of cells as indicated in table 1; this concentration, taken as the unit, has been indicated with the value 0.0, as expressed in logs, in the graphs of figs 1 and 2.

	fresh weight mg/ml	dry weight mg/ml	number of cells x 10^5/ml
Cyanidium caldarium	1.13	0.41	90.0
Chlorella saccharophila	1.87	0.70	18.3

Table 1. Correspondence among fresh weight, dry weight and number of cells in a suspension of algae (*Cyanidium caldarium* and *Chlorella saccharophila*) having an optical absorbance of 0.7 units.

All the operations concerning the stock cultures were made aseptically; on the contrary the experimental tests were carried out in semi-aseptic conditions because, as we have already pointed out, the growth of fungi and bacteria could be considered negli-

gible. This procedure not only made the operations easier, but also prevented the substances employed from altering during the sterilizing operations.

Recording of results

The growth of the algae was controlled by means of readings at the Bausch & Lomb Spectronic 20 colorimeter at a wave-length of 550 nm, made every 3 days after adding H_2O up to the initial level. The readings could be made without drawing any samples, since the outer diameter of tubes (14 mm) fits well into the hollow of the instrument. The presence of algae was evident upon visual inspection even in the cultures with the lowest inoculum.

The experiment was carried on for 12 days: the readings made at the beginning and after 12 days gave us information about the growth, non-growth, or death of the algae. Readings were made also after 3, 6, and 9 days as a control during the experiment and a confirmation of the final result. We took a term of 12 days because preliminary tests had shown us that the results underwent no major variations when the experiment was protracted.

Results

The results of the tests are recorded in figs 1 and 2. Each of the 12 graphs in these figures contains the results relevant to one of the 12 elements employed; the logs of the algal concentrations are recorded as abscissae, the logs of the chemical elements molarity are recorded as ordinates.

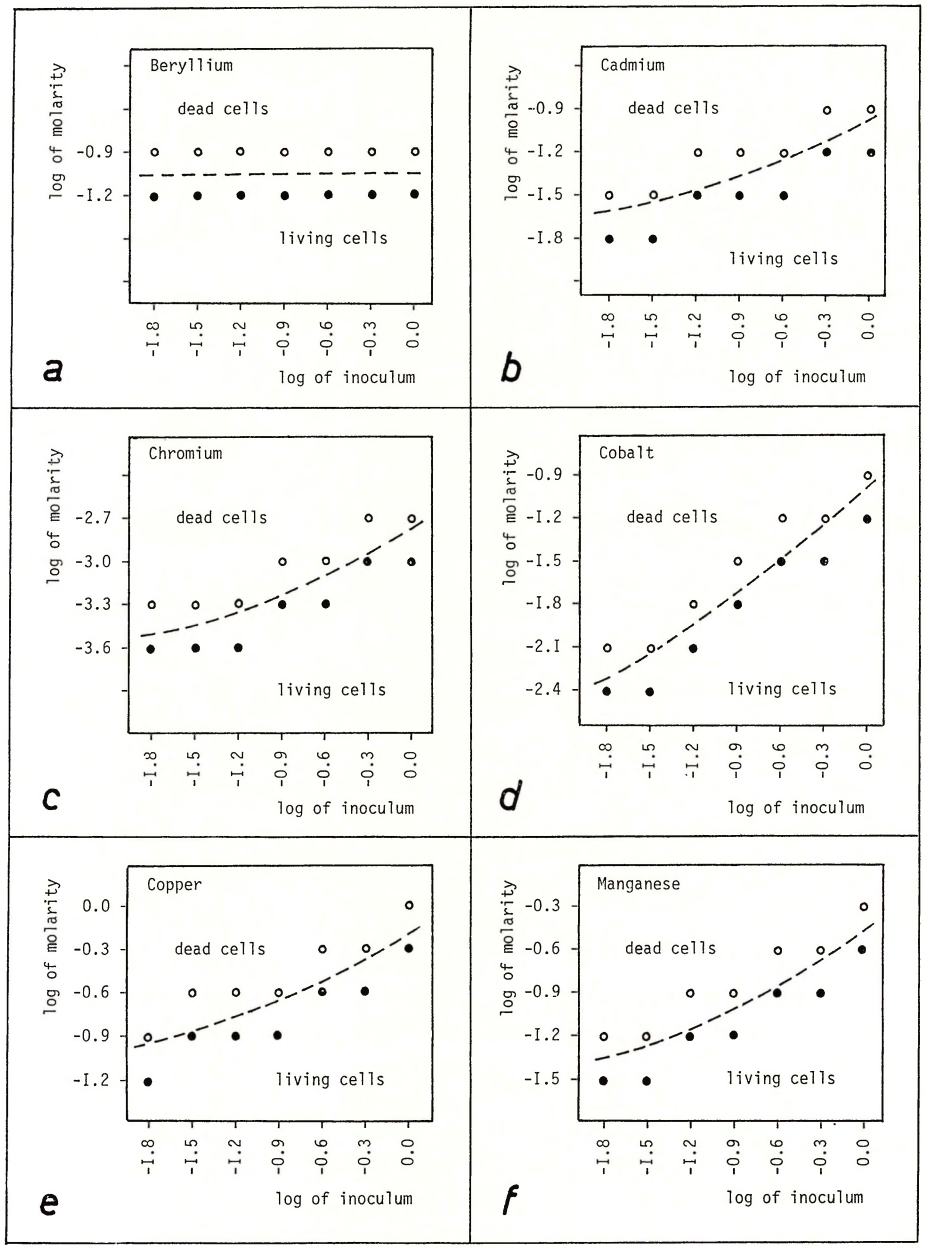
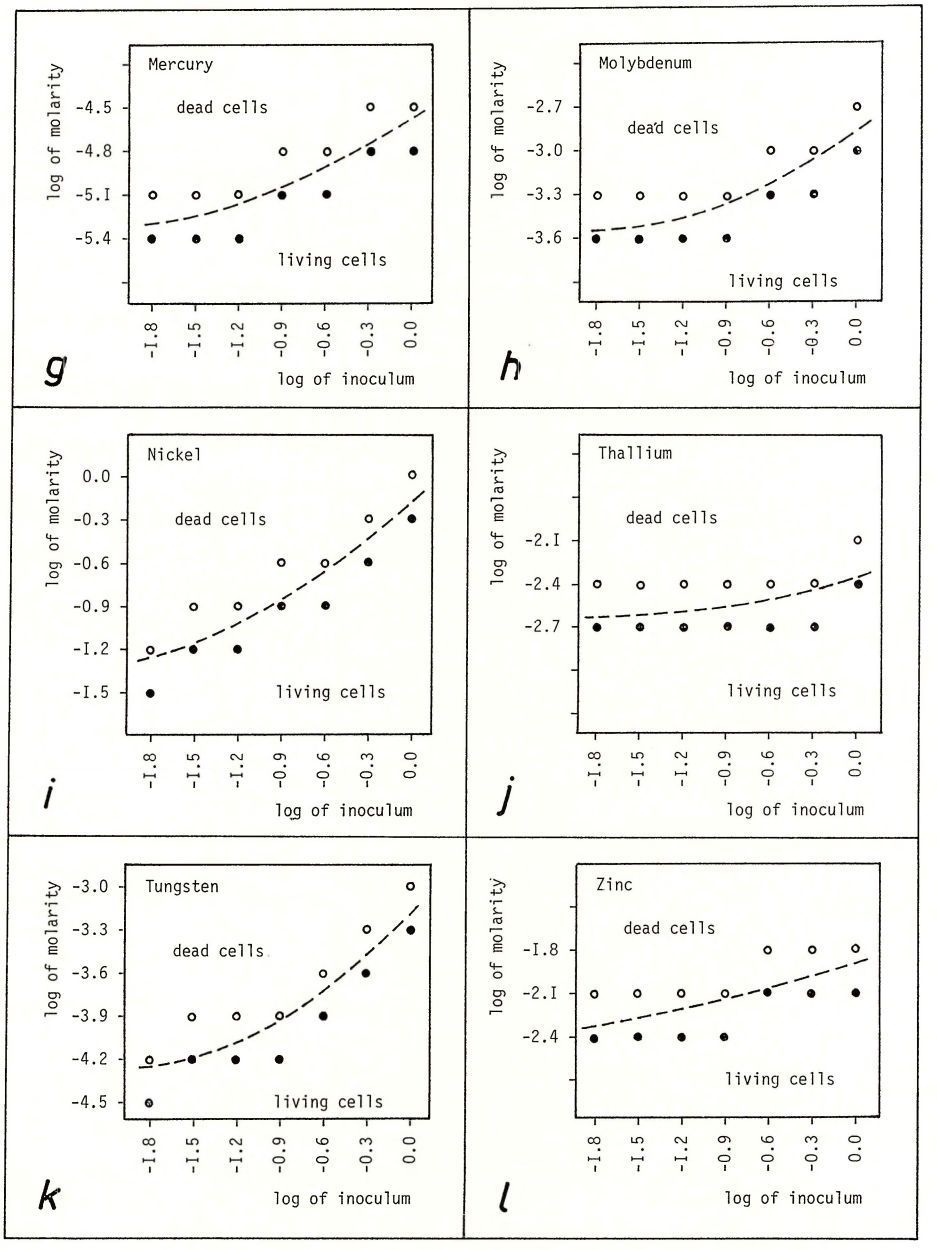


Fig. 1 — Results of the toxicity tests of twelve heavy metals on the alga *Cyanidium caldarium*. In each graph are shown, for every concentration of algae, only the results obtained with the minimum algicidal concentration of heavy metal (MAC), indicated by the



symbol ○, and those obtained with the maximum concentration at which the algae survive, indicated by the symbol ●. The dashed line shows the approximate boundary between life and death.

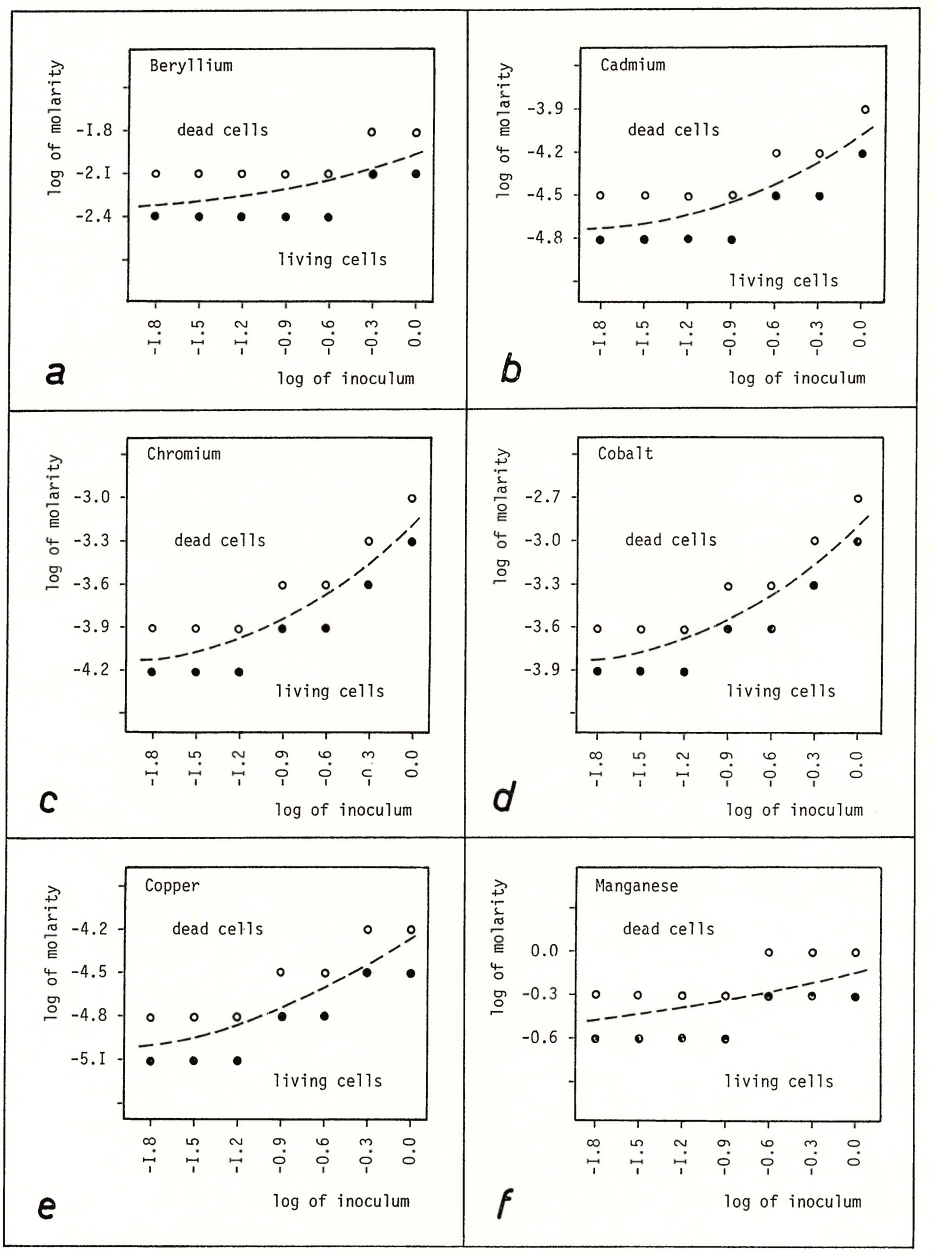
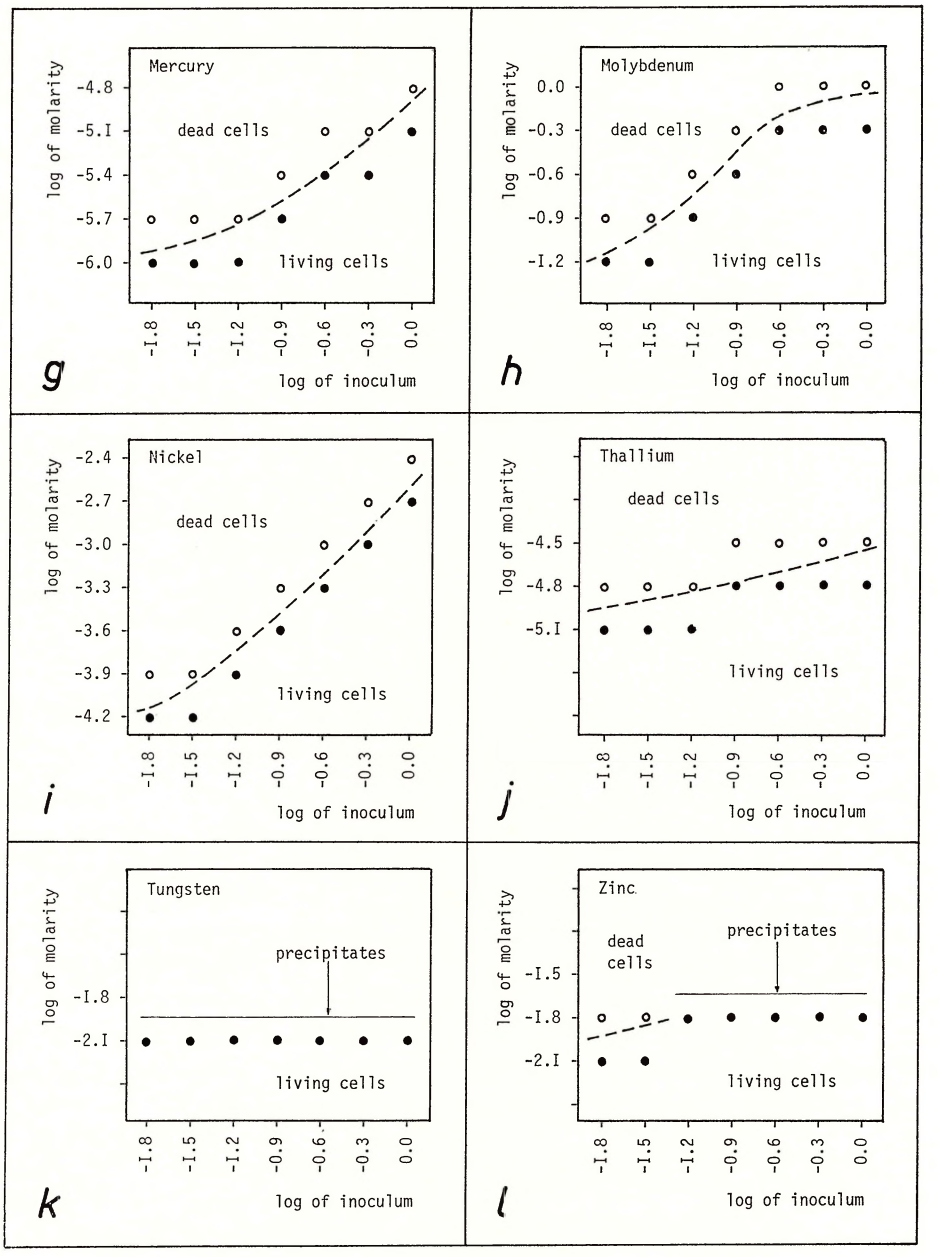


Fig. 2 — Results of the toxicity tests of twelve heavy metals on the alga *Chlorella saccharophila*. In each graph are shown, for every concentration of algae, only the results obtained with the minimum algicidal concentration of heavy metal (MAC), indicated



by the symbol ○, and those obtained with the maximum concentration at which the algae survive, indicated by the symbol ●. The dashed line shows the approximate boundary between life and death.

The method employed by us for quantifying our results is substantially based upon the analysis of the « minimal algacidal concentration », usually named MAC (cfr. LEE et Al., 1975). We have indeed indicated with the symbol ● the maximum concentration of the element at which the algae grow (or at least survive) and with the symbol ○ the minimum concentration at which the algae die (MAC).

Within these two series of points we may imagine the existence of a life-death line, which synthetically visualizes the course of the phenomenon (dashed line in figs 1 and 2).

Every experimental test was carried out twice and the results were always identical. Further tests carried out with cultures not in exponential phase yielded slightly different results, but the phenomenon was confirmed.

DISCUSSION AND CONCLUSION

The experimental tests (figs 1 and 2) demonstrate that changing the initial concentration of the inoculum, the minimum lethal concentration of the heavy metal also changes.

This is particularly clear in the case of some of the heavy metals, such as nickel for the alga *C. saccharophila* (see fig. 2,i) in which the minimum algicidal concentration goes from $10^{-2.4}$M for a unitary algal concentration to $10^{-3.9}$M (i.e. about 1/30 of it) for an algal concentration 1/64 of the unitary one. On the contrary the life-death line is practically horizontal in the case of other elements (see e.g. fig. 2,a).

Since both these two algal concentrations (the minimum and the maximum ones employed by us in this work) could be used as initial inoculum in toxicity experiments, it is necessary

to take precautions by stating the concentrations of the inoculum; it is essential to verify that the limits of toxicity are not susceptible of variations according to the variations of inoculum.

Whenever they are not (e.g. fig. 2,i), i.e. when the response varies following the variations of inoculum, we have to ask these questions: does the chemical element really exert a different toxical influence (as it seems) when it is in the presence of different quantities of algae? or is rather its toxical influence actually independent of the quantity of algae? in the latter case, which one of those recorded should be chosen as toxicity index?

The answer to these questions obviously requires a study of the causes of the phenomenon we have pointed out in the present paper. This analysis should hold in due consideration the change of the concentration of heavy metal during the experimental tests (cfr. WHITTON, SAY, 1975).

There is a connection between these changes and the activity of the cells (cfr. BEN-BASSAT, MAYER, 1975), but is possible that the changes depend also on the presence of dead cells, that can store up the heavy metals by adsorption or by mechanisms of chemical combination (cfr. MATZKU, BRODA, 1970).

SUMMARY

The evaluation of the toxicity of heavy metals on unicellular algae may show very different values (even of about 1:30) according to the variations of the initial inoculum.

The authors suggest that this effect is taken into due consideration and the quantity of inoculum is specified in the publication of each experiment.

RIASSUNTO

La valutazione della tossicità dei metalli pesanti sulle alghe unicellulari può presentare valori molto diversi (anche dell'ordine di 1:30) al variare dell'inoculum iniziale.

Gli autori raccomandano di tenere nel debito conto questo effetto e che la quantità dell'inoculum sia sempre specificata nella pubblicazione di ogni esperimento.

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