# Effects of Sub-Lethal Doses of Copper Sulphate and Lead Nitrate on Growth and Pigment Composition of *Dunaliella salina Teod.*

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Dunaliella salina is a marine alga which can be quite easily maintained in culture (Ryther, 1959; Marre' et al., 1959; Epply et al., 1966; Drokova et al., 1974). The fact that the alga is extremely euryhaline and eurythermal (Kanwisher, 1957) makes it a highly relevant alga for heavy metal pollution studies, because of its wide ecological distribution. Very little work has so far been done on the effects of pollutants on the marine green flagellate Dunaliella salina (Teod). Walsh et al.(1971) found that Dunaliella, under treatment with urea herbicides, reduced its carbohydrate content, although the alga seemed to be the most resistent of the six marine algae that had been studied. Saraiva (1973) used the algae Dunaliella and Lebestes to analyse the toxicity of several heavy metals to oxygen production. Saraiva et al. (1975) have also studied the uptake of $^{51}Cr$ and $^{109}Cd$ by Dunaliella bioculata. Overnell (1975) has studied the effects of various heavy metals on photosynthesis and loss of cell potassium in Dunaliella tertiolecta. Herein we report our observations on the effects of copper and lead on growth and pigment composition of this alga.

## Materials and Methods

Dunaliella salina was maintained in culture at a constant temperature (22±1°C) under continuous white light of 2500 ± 200 lux from fluorescent tubes type Philips Th. 30 W/33RS. The culture medium consisted in an artificial sea-water (NaCl 26.518, $MgCl_2$ 2.447, $MgSO_4$ 3.305, $CaCl_2$ 1.141, KCl 0.725, $NaHCO_3$ 0.202, NaBr 0.083 in grammes per kilogramme of deionised water) enriched with nitrates and phosphates; (0.1 gm.$NaNO_3$ and 0.02 gm. $Na_2HPO_4$ per kilogramme). To ensure rapid growth, air enriched with carbon dioxide (5-7%) was



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bubbled through the culture flasks after being passed through a sterile cotton filter. Flasks used for the cultures were sterilised in an autoclave for 20 minutes at 120°C. To prevent precipitation of salts the culture medium and the heavy metal salt were introduced through millipore filters after autoclaving of the containers.

Measurements of cell concentrations were performed using a colorimeter type Klett-Sommerson Model 900-3 which had previously been calibrated using a Thomas haemocytometer. For the extraction of pigments, 5mls of culture were centrifugated for 10 minutes. The supernatant was discarded and 5mls of ethyl alcohol added. The extract was left for 2 hours in complete darkness and then recentrifugated. Absorption spectra were registered on an automatic spectrophotometer SP 700 A from 300 mμ to 700 mu. The formulae suggested by Senger (1970) and Metzner (1965) were then used to calculate the concentration of chlorophylls and carotenoids in the alcoholic extacts. From these values, the pigment content per cell was then evaluated.

For both experiments using $CuSO_4$ and $Pb(NO_3)_2$ as the heavy metal salts, 15 mls of culture containing $5.6 \times 10^6$ cells per ml were inoculated in 800 mls of artificial enriched sea-water. Copper sulphate was added to give concentrations of 0.5 ppm, 1.25 ppm, 2.5 ppm, 5 ppm, and 15 ppm of $Cu^{2+}$ ions. Lead nitrate was added to another set of identical cultures to give concentrations of 0.3 ppm, 0.9 ppm, 4.5 ppm, and 15 ppm of $Pb^{2+}$ ions, in the culture medium. During the sampling of the cultures, cell counts and mass extraction of pigments were performed in both experiments using the method already described.

## Results and Discussion

Cell growth was followed for 31 days to the stationary phase. In figures 1 and 2 are represented the growth curves obtained for the control cultures and those polluted with copper and lead respectively. For simplification, the growth curve for 15 ppm of lead has not been reproduced in figure 2 since it is extremely similar to that obtained for 4.5 ppm of lead. Tables I and II show the results obtained from pigment analysis of the cultures containing copper sulphate. Table I shows the values of the total pigment extracted measured in mgms per unit volume of culture; Table II shows the

pigment content per cell during the growth period. Correspondingly, Tables III and IV are the results obtained from the analysis of the cultures containing lead. In Table IV are shown the values of the carotenoid content in every cell, expressed as a percentage of the total cellular pigment.

Concentrations above 5 ppm of $Cu^{2+}$ are lethal to Dunaliella salina. In fact under treatment with 5 ppm of copper ions, no growth of culture was recorded. At lower concentrations of copper, cell concentrations obtained

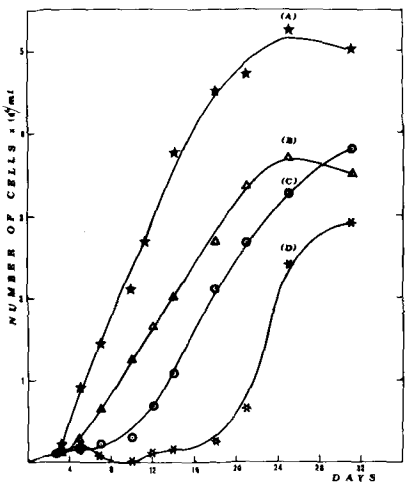


Figure 1: Growth curves of Dunaliella salina treated with different concentrations of $Cu^{2+}$.
(A) control
(B) 0.5 ppm
(C) 1.25ppm
(D) 2.5 ppm

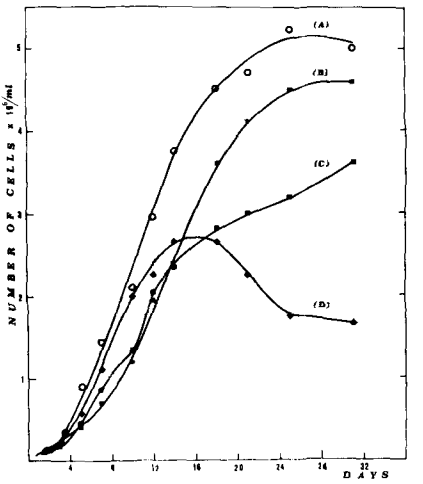


Figure 2: Growth curves of Dunaliella salina treated with different concentrations of $Pb^{2+}$.
(A) control
(B) 0.3 ppm
(C) 0.9 ppm
(D) 4.5 ppm

were always lower than control values. The presence of sub-lethal concentrations of copper seems to retard the onset of the logarithmic phase of growth. In fact on treatment with 2.5 ppm of copper, the growth curve arrives at this phase only after 18 days from the inoculum date.

Control cultures reach the logarithmic phase only after a short period of 3 days. Treatment with 0.5 ppm of $Cu^{2+}$ ions seems to increase the amount of pigment which can be extracted from the cultures from the 14th day onwards. This is quite evident from Table I. In the case of the use of higher concentrations of copper (1.25 ppm and 2.5 ppm), the pigment content of the cultures was markedly reduced.

Cells which are not treated with copper sulphate, undergo a normal reduction in their pigment content (Chlorophylls and carotenoids) during the logarithmic phase of rapid growth. This is also accompanied by a relative increase in the carotenoid content. Such an effect is probably due to a reduction in nitrates and phosphates in the culture medium (Ketchum et al. 1958). In cells treated with copper, which grow at a slower rate, the

TABLE I

Total pigment content of cultures expressed in mgms per litre as a function of age of culture and the concentration of $Cu^{2+}$.

| age of culture in days | control | $Cu^{2+}$ | | |
|---|---|---|---|---|
| | | 0.5ppm | 1.25ppm | 2.5ppm |
| 3 | | | 0.690 | |
| 7 | | | 0.742 | 0.397 |
| 10 | | | 1.314 | 0.264 |
| 12 | 6.11 | 3.44 | 1.93 | 1.81 |
| 14 | 5.87 | 6.37 | 3.29 | |
| 21 | 6.40 | 7.02 | 2.62 | |
| 25 | 5.5 | 6.97 | 4.25 | 3.13 |

TABLE II

Pigment content per cell expressed in mgms ($x10^{-9}$) as a function of the age of culture and the concentration of $Cu^{2+}$.

| age of culture in days | control | $Cu^{2+}$ | | |
|---|---|---|---|---|
| | | 0.5ppm | 1.25ppm | 2.5ppm |
| 3 | | | 3.17 | |
| 7 | | | 3.75 | 4.97 |
| 10 | | | 4.38 | 13.3 |
| 12 | 2.07 | 2.08 | 2.75 | 14.9 |
| 14 | 1.56 | 3.18 | 2.99 | |
| 21 | 1.36 | 2.09 | 1.74 | |
| 25 | 1.05 | 1.95 | 1.31 | 1.30 |

pigment content per cell, reaches higher values and hence the reduction in total pigment and the relative increase in carotenoid content, obtained during the logarithmic phase is much less marked. In fact for non-treated cells, cell carotenoids increase by about 22% from the 12th to the 25th day whilst during this same period, the increase of carotenoids in cells treated with 0.5, 1.25, and 2.5 ppm of $Cu^{2+}$, is only 5.4%, 7.6%, and 8.8% respectively.

It is quite apparent from figure 2, that although the rate of growth depends on the concentration of the

lead salt, the effect is not as clearly marked as that for copper. In fact it seems that such concentrations of lead are not directly toxic to Dunaliella. From the growth curves it seems quite obvious that the effect of lead is more pronounced towards the end of the logarithmic phase of growth, thus giving always lower final values of cell concentrations. However this tolerance for lead nitrate (even at high concentrations) in the first phase of growth was suspected to be due to a masking effect of the nitrates present on growth velocity. It was thus suspected that the addition of nitrates through the pollutant could stimulate growth to such an extent that it could even counteract the noxious effects of the lead ions. To confirm this hypothesis, a series of identical experiments were performed using higher concentrations of lead nitrate (up to 30 ppm) and identical concentrations of $Pb^{2+}$ ions (0.3 ppm, 0.9 ppm, 4.5 ppm and 15 ppm) through the addition of lead chloride.

TABLE III

Total pigment content of cultures expressed in mgms per litre as a function of age of culture and the concentration of $Pb^{2+}$.

| age of culture in days | control | $Pb^{2+}$ | | | |
|---|---|---|---|---|---|
| | | 0.3 ppm | 0.9 ppm | 4.5 ppm | 15 ppm |
| 5 | | 0.87 | | | 0.76 |
| 10 | | 2.9 | | | 3.46 |
| 12 | 6.12 | | 2.62 | 3.03 | 3.84 |
| 14 | 5.87 | 3.19 | 2.88 | 3.02 | 4.49 |
| 21 | 6.40 | 4.74 | 4.68 | 2.67 | 3.76 |
| 25 | 5.50 | 4.74 | 4.75 | 1.94 | 3.33 |

TABLE IV

Carotenoid content of cells expressed as a percentage of the total cellular pigment content, as a function of age of culture and conc. of $Pb^{2+}$.

| age of culture in days | control | $Pb^{2+}$ | | | |
|---|---|---|---|---|---|
| | | 0.3 ppm | 0.9 ppm | 4.5 ppm | 15 ppm |
| 5 | | 52% | | | 45,5% |
| 10 | | 37% | | | 41,8% |
| 12 | 40% | | 52,3% | 54,8% | 50% |
| 14 | 50% | 47,5% | 47,5% | 54,4% | 44% |
| 21 | 52,2% | 50% | 48,1% | 55,0% | 59% |
| 25 | 62,0% | 54,3% | 49,0% | 69,4% | 64,2% |

The results obtained showed that, although the addition of nitrates may give a positive increase in cell growth the effect is only transient, usually disappearing after 5 or 6 days. Moreover the presence of lead chloride as pollulant did not alter the picture obtained for identi-

cal concentrations of lead using lead nitrate instead of lead chloride.

Mass pigment extraction shows that the presence of lead even at relatively low concentrations, tends to reduce the amount of chlorophyll and carotenoid pigments. At least up till the 12th day of growth there is a decrease in the total pigment per cell. The relatively higher values for the same parameter after the 12th day of growth, for the polluted cultures can be explained to be due to a drastic decrease in the rate of cell division (cf. effect of copper). Far more elucidative are the results obtained for the carotenoid content of the cells for the different cultures shown in Table IV. The presence of lead at concentrations of 4.5 and 15 ppm tends to increase the relative amounts of carotenoid pigments with respect to control values. An increase in carotenoids during the stationary phase of cell growth is a normal phenomenon associated with a general decrease of nutrients in the culture medium. A similar result in fact has been obtained by Ketchum et al. (1958) using Dunaliella euchlora. It seems however that the presence of sub-lethal but higher concentrations of lead gives a more rapid and pronounced increase in the ratio carotenoids: chlorophylls. Such changes are obviously reflected in photosynthetic activities. Measurement of the ability of the cultures to develop oxygen under illumination using a Clark electrode showed that oxygen production is depressed when a relative decrease in chlorophyll and increase in carotenoids is obtained in the cultures.

Notwithstanding all these secondary effects lead seems not to be extremely toxic to Dunaliella for the range of concentrations studied. It is perhaps important to note that the negative effect of lead might be expressed more significantly in measurements of cellular absorption and accumulation. The delayed effect of lead on cell growth rate might in fact be explained by the slow rate of accumulation of lead in cells of Dunaliella. Very probably it is only after several cell generations that the cell lead content arrives to toxic levels. This of course does not make lead, a pollutant which is less dangerous than a highly toxic heavy metal like copper.

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