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Growth and Adaptation of Estuarine Unicellular Algae in Media with Excess Copper, Cadmium or Zinc, and Effects of Metal-Contaminated Algal Food on *Crassostrea virginica* Larvae*

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ABSTRACT: Responses of the estuarine unicellular algae *Monochrysis lutheri, Isochrysis galbana, Dunaliella euchlora,* and *Phaeodactylum tricornutum* to excess $CuCl_2$, $CdCl_2$, and $ZnCl_2$ in growth media were determined. Tolerance to metals in artificial seawater was much greater than in enriched natural seawater, presumably because of the higher concentration of metal ligands in the former. Growth differences between species in media containing excess Cu or Cd were observed in both media tested. Growth of *M. lutheri* and *I. galbana* was inhibited considerably more than that of the other test organisms. This difference did not occur in media with excess Zn. After extended periods of subculture in sublethal concentrations of Cu and Cd, algae developed tolerances to metal concentrations that were inhibitory upon initial exposure. Strains of *I. galbana* adapted to grow in a medium with 10 mg% $CuCl_2$ (47.3 ppm Cu) or 2.5 mg% $CdCl_2$ (15.3 ppm Cd) were fed to laboratory-reared veliger larvae of the oyster *Crassostrea virginica.* These algal foods induced poor growth and high mortalities in grazing larvae. We conclude that pollution of estuarine waters with high concentrations of metals can decrease primary productivity and alter algal species dominance. With continued exposure to sublethal concentrations, phytoplankters can exhibit a limited increase in tolerance and adaptation to the metals. These populations are then potentially toxic for grazing species at higher trophic levels.

INTRODUCTION

An increasing body of knowledge is revealing detrimental effects of waste materials on marine life (e.g. Bryan, 1971; Korringa, 1971; Friberg et al., 1974; Cole, 1979). In recent years, the ubiquitous use of metals throughout the world has resulted in the production of highly concentrated metallic wastes which are often released directly into the marine environment or eventually find their way into coastal waters from a variety of dump sites. Metal pollution is a worldwide problem. Abnormally high concentrations of metals have been reported from coastal waters of the southeastern United States (Windom and Smith, 1972), the Japanese coast (Ikuta, 1968), the coast of Spain (Establier and Pasqual, 1974), the British coast (Boyden, 1975), New Zealand (Nielsen, 1975), Long Island Sound (Greig et al., 1977), the coast of Israel (Amiel and Navrot, 1978), and the St. Lawrence River estuary (Cossa and Poulet, 1978).

Bivalves concentrate some required metals to levels in excess of those found in metalloenzymes (Wolfe, 1970; Coombs, 1972, 1974), as well as other metals for which no requirement has been demonstrated (Pringle et al., 1968; Feng and Ruddy, 1974; Valiela et al., 1974). A data bibliography compiled by Kidder (1977) lists 189 published articles reporting concentrations of heavy metals found in bivalves collected throughout the world. Although the latter publication indicates that much research has been conducted to determine the concentration of heavy metals stored in adult bivalves, with the exception of the study of Calabrese et al. (1977) on larvae of the American oyster *Crassostrea virginica* and the hard clam *Mercenaria mercenaria*, little interest has been shown in determining the tolerance to metals of the more fragile larval stages.

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Some micro-algae have been shown to tolerate moderately high concentrations of heavy metals (Cossa, 1976; Braek et al., 1980). This tolerance suggests the possibility that metals may accumulate in phytoplankters and can be transferred from foods to grazing species; thus, metals can reach the higher trophic levels. Concern over this accumulation of metals by commercially marketed marine species has been voiced in the public press (e.g. The New York Times, 1978, 1980), as well as the scientific literature (e.g. Hall et al., 1976). In this latter study, cadmium concentrations up to 20 ppm were found in some fish products intended for human consumption.

For purposes of ecological management it is important to gain an understanding of the routes by which high concentrations of metals are accumulated in fish and shellfish. The present study investigates (1) effects of the heavy metals copper, cadmium and zinc on growth rates of 4 estuarine unicellular algae in controlled culture media containing different concentrations of chelate; (2) the potential of the algae for adaptation to excess metal concentrations in growth media; (3) the effects of algal food cultured in high metal concentrations on grazing *Crassostrea virginica* larvae.

MATERIALS AND METHODS

Experiments were conducted with axenic cultures of the following algae: the flagellates *Monochrysis lutheri* Droop, *Isochrysis galbana* Parke, *Dunaliella euchlora* Lerche (the latter strain identified by Dr. R. A. Lewin but referred to as *D. tertiolecta* by McLachlin, 1960), and the diatom *Phaeodactylum tricornutum* Bohlin. These algal strains have been maintained in the Milford Laboratory Collection for many years as axenic cultures in media identical to those utilized in these experiments.

Algae were cultured in Pyrex screw-capped test tubes (19 mm × 150 mm, with liners in caps removed) and Erlenmeyer screw-capped flasks when larger volumes of culture were required. Glassware was washed in detergent (Alco-Jet), rinsed with tap and distilled water, and immersed for at least 1 h, twice in boiling glass-distilled water.

Seawater from the Milford Long Island Sound estuary (salinity: 27–28 ppt) was stored in a 10,000-gallon (ca. 40,000 l) fiberglass-lined attic tank and brought into laboratories by gravity flow. Seawater was filtered through 10 and 1 µm polypropylene filter cartridges (Filterite Corp., Maryland, USA), irradiated by twin 18-inch ultraviolet lights (design description in Loosanoff and Davis, 1963), and finally passed through an activated charcoal filter cartridge (Barnstead). Two different media formulations were used: an enriched natural seawater medium, E (Ukeles, 1973) and an artificial seawater medium, ASP_2 (Provasoli et al., 1957) (Table 1). Concentrations of tested metal salts are expressed in mg%, i.e. mg weight per 100 ml solution, because this is the usual method of expres-

Table 1. Algal culture media (concentrations l^{-1} •)

Artificial seawater medium (Provasoli et al., 1957)			
NaCl	18 gm	Vitamin mix S_3••	10 ml
$MgSO_4$	5 gm	Na_2EDTA	30 mg
KCl	0.6 gm	$FeCl_3 \cdot 6H_2O$	0.829 mg
$CaCl_2 \cdot 2H_2O$	101 mg	$ZnCl_2$	144 µg
$NaNO_3$	50 mg	$MnCl_2 \cdot 4H_2O$	1.25 mg
K_2HPO_4	5 mg	$CoCl_2 \cdot 6H_2O$	3.1 µg
$Na_2SiO_3 \cdot 9H_2O$	150 mg	$CuCl_2$	1.18 µg
'THAM'•••	1 gm	H_3BO_3	6.16 mg
Vitamin B_{12}	2 µg		
Enriched natural seawater medium (Ukeles, 1973)			
Seawater	500 ml	'THAM'•••	1.0 gm
KH_2PO_4	20 mg	$CuSO_4 \cdot 5H_2O$	0.0098 µg
$NaNO_3$	300 mg	$ZnSO_4 \cdot 7H_2O$	0.022 µg
NaFe Sequestrene	5 mg	$CoCl_2 \cdot 6H_2O$	0.013 µg
Vitamin B_{12}	0.003 mg	$MnCl_2 \cdot 4H_2O$	0.180 µg
Thiamine HCl	0.3 mg	$Na_2MoO_4 \cdot 2H_2O$	0.0063 µg

• Solutions and media are brought to volume with double glass-distilled water

•• Vitamin mix S_3 is prepared by dissolving the following in 100 ml double glass-distilled water: 30 mg thymine, 5 mg thiamine HCl, 1 mg nicotinic acid, 1 mg Ca pantothenate, 0.1 mg para-amino benzoic acid, 0.01 mg biotin, 50 mg inositol, and 0.02 mg folic acid

••• Tris(hydroxymethyl)aminomethane

sing concentrations of nutrients in phycological studies. Retaining this convention facilitates comparisons between experimental metal salt concentrations and those concentrations used in normal growth media. Where appropriate, concentrations of metals are also expressed as mg metal ion per liter (ppm), the designation most often used for water quality criteria. It should be noted that these values do not represent ionic concentrations available to the algae as a large percentage of the metal ions are bound by chelating agents in the growth media. Copper, as well as zinc and cadmium, was tested as sulfate salt in the natural seawater medium since $CuSO_4$ and $ZnSO_4$ are routinely used as trace metal components. Metals were added as chloride salts in experiments with artificial seawater medium as chloride is the anion of the copper and zinc salts in this formulation.

The maximum concentrations of the metals that could be tested were limited to those which remained soluble in the alkaline growth media.

All algal culture media were steam-sterilized in a Castle autoclave for 20 min at 15–17 lb atm pressure and allowed to equilibrate at least 24 h between sterilization and inoculation. Stock cultures of each organism were maintained in test tubes containing 10 ml of ASP_2 or E medium and subcultured routinely every 14 d. Experimental cultures were inoculated aseptically with 0.5 ml of a 14-d stock culture, and each test was conducted in triplicate. Algal cultures were incubated in a Sherer Series RI-LTP Lighted Bioincubator equipped with cool-white fluorescent lights. Illumination was about 500 ft C on a 12/12 h light/dark cycle and temperature was maintained at 20 °C (± 1 C°). Cell counts of culture samples were made in an Improved Neubauer Hemacytometer (Bright-Line). Culture density was determined in matched calibrated test tube cuvettes, using standard blanks of uninoculated medium for each metal concentration, in a Bausch and Lomb Spectronic 20 Colorimeter-spectrophotometer at 520 nm. Experimental results of algal growth studies were expressed as the ratio of growth in excess metals to that of control cultures; this ratio was converted to a percent value.

Experiments were designed to determine levels of lethal, sublethal, and non-inhibitory concentrations of Cu, Zn, and Cd in natural and artificial seawater media. Species were cultured in sublethal concentrations of copper and cadmium at different initial cell concentrations to determine if cell density influenced metal tolerance. Capacities of algal species to adapt to metals were investigated by subculture every 2 w in ASP_2 *medium containing sublethal metal concentrations*. Culture densities were determined spectrophotometrically. At subculture 11, living cells from control cultures and from the highest concentration of each metal tolerated were examined microscopically for gross morphological changes.

Adult oysters *Crassostrea virginica* were induced to spawn in the laboratory by warm water stimulation (Loosanoff and Davis, 1963). Fertilized eggs were collected and washed on Nitex (monofilament nylon) screening and suspended at a concentration of 15 ml^{-1} in 26 °C seawater for 48 h. Fully developed veliger larvae were washed on a 36-mesh Nitex screen, resuspended, and counted in a Sedgwick-Rafter cell. Larvae were suspended to yield a concentration of 20 larvae ml^{-1} in 600 ml of seawater in polypropylene beakers that were incubated on a water table maintained at 26 °C.

Isochrysis galbana was studied in the larval feeding experiments because it is rapidly utilized and known to have high nutritional value for larvae (Davis and Guillard, 1958; Walne, 1963; Ukeles, 1971). In preparation for feeding of larvae, algae were concentrated by centrifugation at 1500 RPM (276 G) in an IEC International Centrifuge (Model PR_2) for 8 min at 5 °C. The supernatant medium was decanted, and the cells were resuspended in 40 ml of filter-sterilized seawater. This washed cell suspension was added to the larval culture as food source. *I. galbana* were fed at a concentration of 16.5×10^3 cells ml^{-1} of larval culture. Investigations of larval growth were conducted on the following feeding regimes: (1) unfed larvae; (2) unfed larvae, with solutions of $CuCl_2$ or $CdCl_2$ added to the culture; (3) larvae fed algae cultured in ASP_2 growth medium and, also, solutions of $CuCl_2$ or $CdCl_2$ added to the larvae; (4) larvae fed algae adapted to grow in ASP_2 growth medium containing high concentrations of $CuCl_2$ or $CdCl_2$.

At 24-h intervals, starting 48 h after fertilization, larvae from each beaker were collected on a 36-mesh Nitex screen, washed with a pressurized stream of seawater, and resuspended in beakers containing 500 ml of seawater A 20-ml sample was taken from each beaker and preserved in 4 % formalin. Additions of food and metals were again made, the volume brought to 500 ml with seawater, and the beakers reincubated.

Daily microscopic examinations were conducted to observe the qualitative appearance of the living larvae, and 1-ml fixed samples were counted in a Sedgwick-Rafter chamber to determine dead/live ratios. For growth determinations, 50 larvae were measured along the widest dimension of their shell parallel to the straight-hinge, using a Bausch and Lomb Balplan microscope with a calibrated eyepiece scale.

Samples of larvae from each feeding regime were taken 1 h after the 9th feeding for observation of feeding behavior by epifluorescence microscopy, as described by Babinchak and Ukeles (1979). Larvae

were examined for fluorescence of algae within the gut with a Bausch and Lomb Balplan fluorescence microscope (using the incidence fluorescence mode) fitted with an ABO-50 W mercury vapor lamphouse power supply. A Bausch and Lomb 35-mm camera with a Bausch and Lomb AX-1 Automatic Exposure Controller was used for photographing samples on Kodak Tri-X pan film.

RESULTS

Responses of Algae to Metals in Artificial Seawater

Above a threshold value, there was a direct relationship of metal concentration to growth inhibition. The response to metals varied between species. *Monochrysis lutheri* and *Isochrysis galbana* demonstrated very similar responses and were considerably more sensitive to the metals than were *Dunaliella euchlora* or *Phaeodactylum tricornutum*.

No significant growth inhibition in the artificial seawater medium was observed in $CuCl_2$ concentrations below 5×10^{-1} mg% (2.37 ppm Cu). Partial inhibition of *Isochrysis galbana* occurred in 5 mg% $CuCl_2$, reducing growth in the metal to 73.6 % of the control. The other species were less affected at this concentration: 81.4 % for *Monochrysis lutheri*, 89.5 % for *Phaeodactylum tricornutum*, and 93.7 % for *Dunaliella euchlora*. These relations in growth rate were repeated at 10 mg% and 25 mg% $CuCl_2$. At the latter concentration, growth of *M. lutheri* and *I. galbana* was only 11.2 % and 14.3 %, respectively, of that in the controls. However, *P. tricornutum* populations increased to 59.2 % of the control and *D. euchlora* was only slightly inhibited (Table 2).

$ZnCl_2$, at or less than 1 mg% (4.80 ppm Zn), caused no significant inhibition of any of the algae tested. In medium with 3 mg% $ZnCl_2$, all species were similarly reduced in growth to between 74–79 % of the control. In media with 5 mg% and 7 mg%, somewhat more inhibition was observed (Table 2).

The most pronounced differences in response between species to a metal salt were observed in experiments testing effects of $CdCl_2$. Although no significant growth inhibition occurred in less than 2.5 mg% $CdCl_2$ (15.3 ppm Cd), at this concentration growth of *Monochrysis lutheri* and *Isochrysis galbana* was almost completely inhibited. In 5 mg% $CdCl_2$, growth of *M. lutheri* and *I. galbana* ceased; whereas, *Dunaliella euchlora* and *Phaeodactylum tricornutum* achieved densities of 64.3 % and 80.1 %, respectively, of the controls. At 20 mg% $CdCl_2$, growth of *D. euchlora* was severely reduced, but 50 mg% was required to cause this drastic reduction in *P. tricornutum* (Table 2).

Table 2. Growth of algal populations exposed to metals in artificial seawater medium (ASP_2), observed after 12 d; growth expressed as percentage of that obtained in absence of metal. Dash: no experiment

Concentration				Algal species			
	Metal salt (mg%)		Metal ion (ppm)	*Monochrysis lutheri*	*Isochrysis galbana*	*Dunaliella euchlora*	*Phaeodactylum tricornutum*
$CuCl_2$	0.005	Cu	0.0237	119.3	99.0	103.7	104.9
	0.01		0.0473	100.2	99.0	99.4	103.7
	0.1		0.473	94.0	97.5	97.1	100.4
	0.5		2.37	92.8	101.9	94.1	98.4
	5.0		23.7	81.4	73.6	93.7	89.5
	10.0		47.3	71.9	66.1	96.4	74.8
	25.0		118	11.2	14.3	92.0	59.2
$ZnCl_2$	0.025	Zn	0.120	99.0	98.9	98.0	98.1
	0.1		0.480	97.2	98.8	90.9	99.2
	1.0		4.80	94.5	93.4	89.9	98.8
	3.0		14.4	76.6	76.3	78.7	74.2
	5.0		24.0	71.7	75.6	68.6	62.7
	7.0		33.6	64.7	50.9	60.4	59.6
$CdCl_2$	0.01	Cd	0.0613	97.2	92.8	95.6	100.9
	0.1		0.613	95.6	95.5	92.5	103.9
	1.0		6.13	92.8	92.8	97.2	103.0
	2.5		15.3	13.6	20.3	–	–
	5.0		30.7	6.2	8.3	64.3	80.1
	10.0		61.3	4.9	5.6	27.3	76.2
	20.0		123	4.9	4.7	5.6	52.3
	25.0		153	–	–	5.6	37.9
	50.0		306	–	–	5.0	9.2

Table 3. Growth of algal populations exposed to metals in enriched natural seawater medium (E), observed after 12 d; growth expressed as percentage of that obtained in absence of metal

	Concentration			Algal species			
	Metal salt (mg%)		Metal ion (ppm)	*Monochrysis lutheri*	*Isochrysis galbana*	*Dunaliella euchlora*	*Phaeodactylum tricornutum*
$CuSO_4$	0.119	Cu	0.473	98.3	94.9	88.6	94.6
	1.19		4.73	91.7	92.8	86.4	92.8
	11.9		47.3	10.3	7.4	81.2	85.8
$ZnSO_4$	0.118	Zn	0.480	93.8	90.8	88.7	91.0
	1.18		4.80	90.7	91.8	92.1	106.7
	11.8		48.0	36.5	36.8	38.4	25.8
$CdSO_4$	0.0114	Cd	0.0613	108.6	90.6	96.0	96.3
	0.114		0.613	107.3	90.7	99.4	101.9
	1.14		6.13	10.9	7.7	55.2	94.5
	11.4		61.3	10.0	7.7	7.3	43.1

Responses of Algae to Metals in Enriched Natural Seawater

Differences in species tolerances to metal salts were similar to those observed in the artificial seawater medium although it soon became apparent that algae were less tolerant of cadmium and copper in the enriched natural seawater than in the artificial seawater medium. In contrast, tolerances of zinc were similar in both media.

Inhibition did not occur at or less than 1.19 mg% $CuSO_4$ (4.73 ppm Cu), but at 11.9 mg% population increases of *Monochrysis lutheri* and *Isochrysis galbana* were almost completely inhibited. In contrast, at this latter concentration, *Dunaliella euchlora* and *Phaeodactylum tricornutum* displayed growth 81.2 % and 85.8 % of that in the controls (Table 3).

Growth was not inhibited in 0.118 mg% and 1.18 mg% $ZnSO_4$; in 11.8 mg% $ZnSO_4$ (48.0 ppm Zn) very similar growth responses between 25.8 % and 38.4 % of the controls were observed in all species (Table 3).

None of the tested species were inhibited in 0.114 mg% $CdSO_4$, but in 1.14 mg% $CdSO_4$ (6.13 ppm Cd) growth of *Monochrysis lutheri* and *Isochrysis galbana* virtually ceased. In contrast, *Dunaliella euchlora*

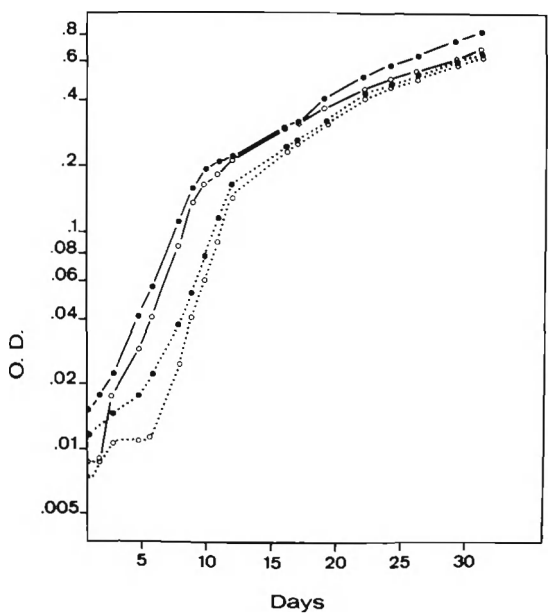

Fig. 1. *Isochrysis galbana*. Growth in ASP_2 medium (solid lines) and in ASP_2 with the addition of 0.1 mg% $CdCl_2$ (0.613 ppm Cd) (dotted lines) at 2 initial cell populations, 9.2×10^4 cells ml^{-1} (solid circles) and 3.5×10^4 cells ml^{-1} (open circles)

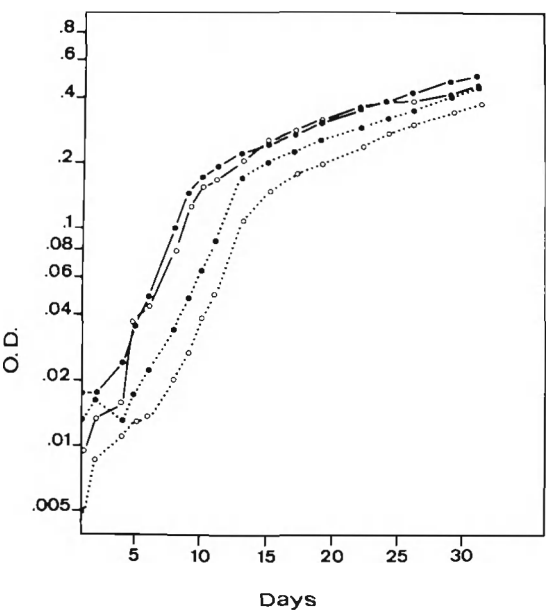

Fig. 2. *Monochrysis lutheri*. Growth in ASP_2 medium (solid lines) and in ASP_2 with the addition of 0.1 mg% $CdCl_2$ (0.613 ppm Cd) (dotted lines) at 2 initial cell populations, 5.7×10^4 cells ml^{-1} (solid circles) and 1.5×10^4 cells ml^{-1} (open circles)

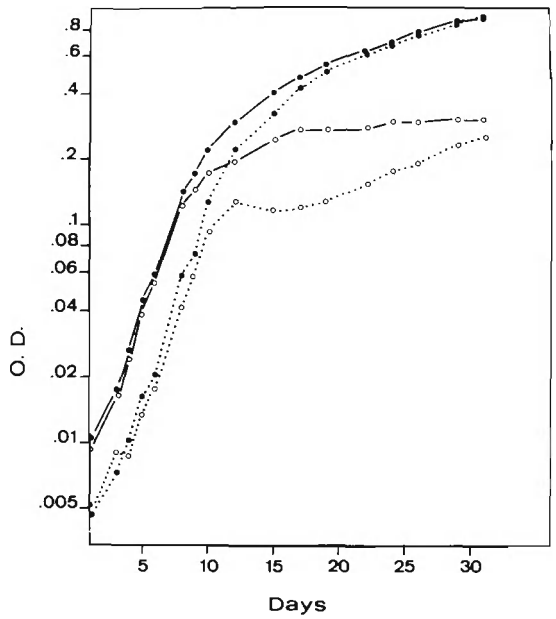

Fig. 3. *Dunaliella euchlora*. Growth in ASP_2 medium (solid lines) and in ASP_2 with the addition of 1.0 mg% $CdCl_2$ (6.13 ppm Cd) (dotted lines) at 2 initial cell populations, 4.1×10^4 cells ml^{-1} (solid circles) and 4.9×10^3 cells ml^{-1} (open circles)

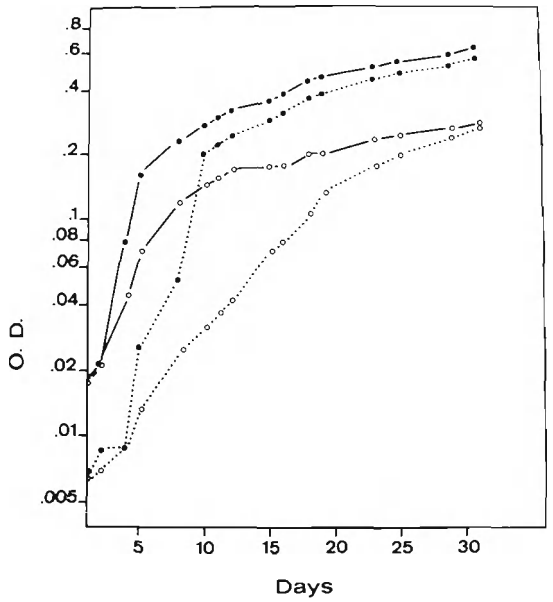

Fig. 4. *Phaeodactylum tricornutum*. Growth in ASP_2 medium (solid lines) and in ASP_2 with the addition of 10 mg% $CdCl_2$ (61.3 ppm Cd) (dotted lines) at 2 initial cell populations, 2.4×10^5 cells ml^{-1} (solid circles) and 2.0×10^4 cells ml^{-1} (open circles)

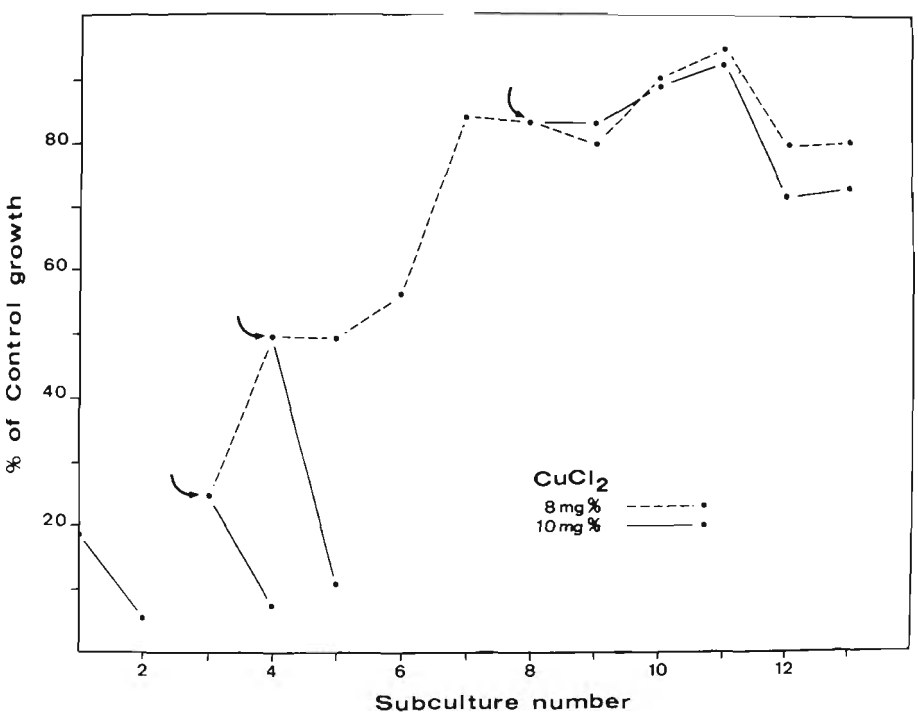

Fig. 5. *Monochrysis lutheri*. Growth during extended exposure to copper by periodic subculture in ASP_2 medium containing added $CuCl_2$ (8 mg% $CuCl_2$ = 37.8 ppm Cu; 10 mg% $CuCl_2$ = 47.3 ppm Cu). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CuCl_2$. At the 3rd subculture the stock strain growing in ASP_2 was transferred into 8 mg% $CuCl_2$

and *Phaeodactylum tricornutum* were fairly tolerant; their populations increased to 55.2% and 94.5%, respectively, of the controls. Similarly, in 11.4 mg% $CdSO_4$ *P. tricornutum* was more resistant than *D. euchlora* (Table 3).

Algal density affected the response to sublethal cadmium concentrations in different ways. In media with cadmium, *Monochrysis lutheri* and *Isochrysis galbana* at lower initial cell densities had a longer lag period than the controls or cultures in cadmium with higher initial cell density. The 2 initial *I. galbana* densities attained the same maximal populations, but *M. lutheri*

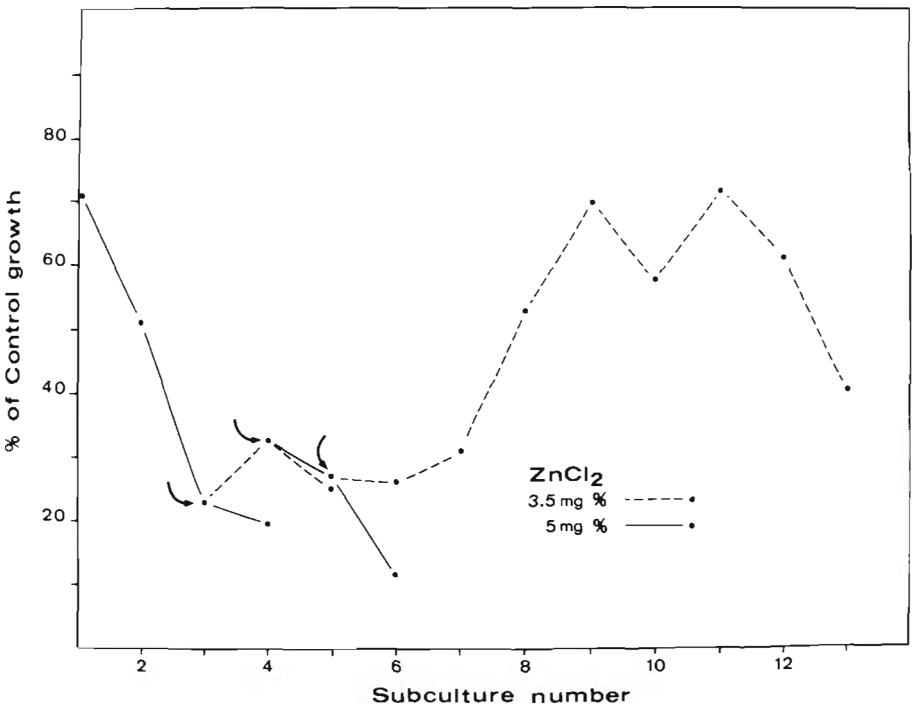

Fig. 6. *Monochrysis lutheri*. Growth during extended exposure to zinc by periodic subculture in medium containing added $ZnCl_2$ (3.5 mg% $ZnCl_2$ = 16.8 ppm Zn; 5 mg% $ZnCl_2$ = 24.0 ppm Zn). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $ZnCl_2$

from the lower initial population density lagged behind in maximal cell numbers (Figs. 1, 2). These results seem to be related to the quantitative variations of the populations at the start of the experiment. With *I. galbana*, one population was higher than the other by a factor of 2.6; with *M. lutheri*, by a factor of 3.8. A still greater difference, i.e. by a factor of 8.5 in the initial populations of *Dunaliella euchlora*, decreased the growth rate of this species towards the end of the log phase of the growth curve (Fig. 3). The greatest effect of metal concentration upon culture response from 2 different initial populations was observed in the logarithmic phase of *Phaeodactylum tricornutum* (Fig. 4); in this species initial culture densities differed by a factor of 12.

Adaptation to Metals in Artificial Seawater

Upon prolonged exposure, the algae demonstrated varying capacities to adapt to inhibitory metal concentrations. *Monochrysis lutheri* in artificial seawater with 10 mg% $CuCl_2$ (47.3 ppm Cu) was severely reduced to 5.6 % of the control values, but in 8 mg%, growth was more successful and population density increased from 25 % to over 90 % of the control during 9 subcultures. This strain in the 8th subculture was then inoculated into medium with 10 mg% $CuCl_2$. This time the strain yielded growth that was between 72 % and 94 % of the control population (Fig. 5).

Monochrysis lutheri in medium containing 5 mg%

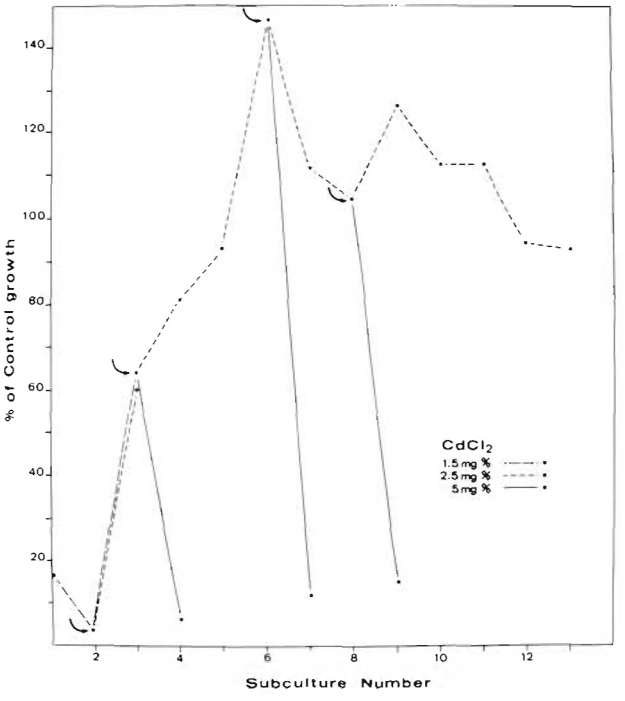

Fig. 7. *Monochrysis lutheri*. Growth during extended exposure to cadmium by periodic subculture in medium containing added $CdCl_2$ (1.5 mg% $CdCl_2$ = 9.20 ppm Cd; 2.5 mg% $CdCl_2$ = 15.3 ppm Cd; 5.0 mg% $CdCl_2$ = 30.7 ppm Cd). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CdCl_2$

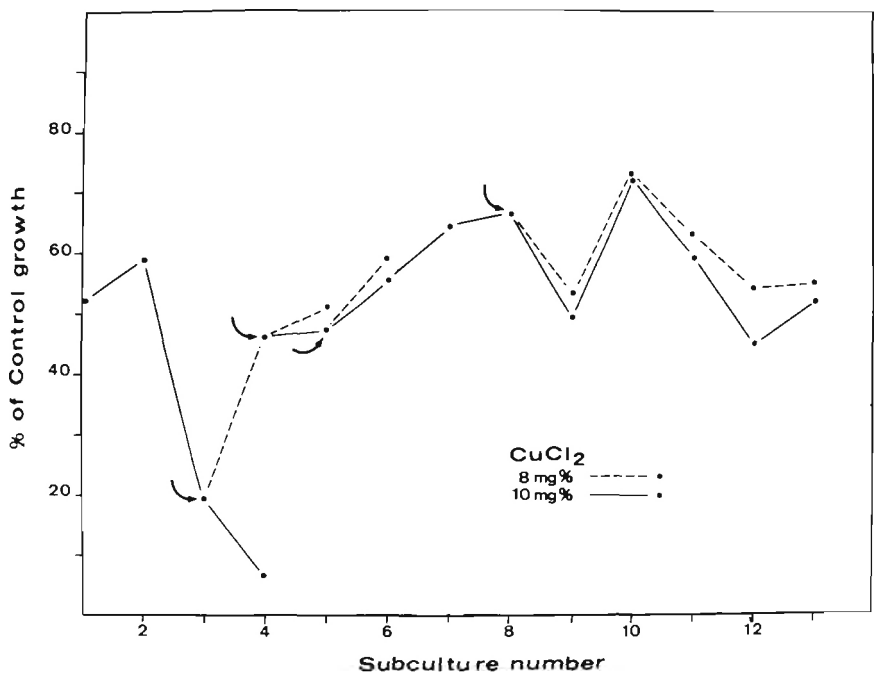

Fig. 8. *Isochrysis galbana*. Growth during extended exposure to copper by periodic subculture in ASP_2 medium containing added $CuCl_2$ (8 mg% $CuCl_2$ = 37.8 ppm Cu; 10 mg% $CuCl_2$ = 47.3 ppm Cu). Arrows: subcultures at which cells growing in one concentration were continued in this medium and also transferred into an additional higher or lower concentration of $CuCl_2$

$ZnCl_2$ decreased in growth through 4 subcultures. At subculture 3 the viable cell population was transferred into medium with 3.5 mg% $ZnCl_2$ (16.8 ppm Zn). Cultures slowly increased in density, reaching a maximum of 71 % of the control at Subculture 11. The appearance of a subsequent reduction in population at Subcultures 12 and 13 may be, in reality, only a reflection of an unusually dense growth in the control cultures rather than an actual decrease in adaptation (Fig. 6).

Growth of *Monochrysis lutheri* in artificial seawater with 1.5 mg% $CdCl_2$ was limited to a population of only 5 % of the control in the 2 subcultures, but viable cells were available to be again transferred to media with 1.5 mg%, as well as 2.5 mg% $CdCl_2$ (15.3 ppm Cd). The strain in 2.5 mg% increased to well over 100 % of the control levels between Subcultures 4 and

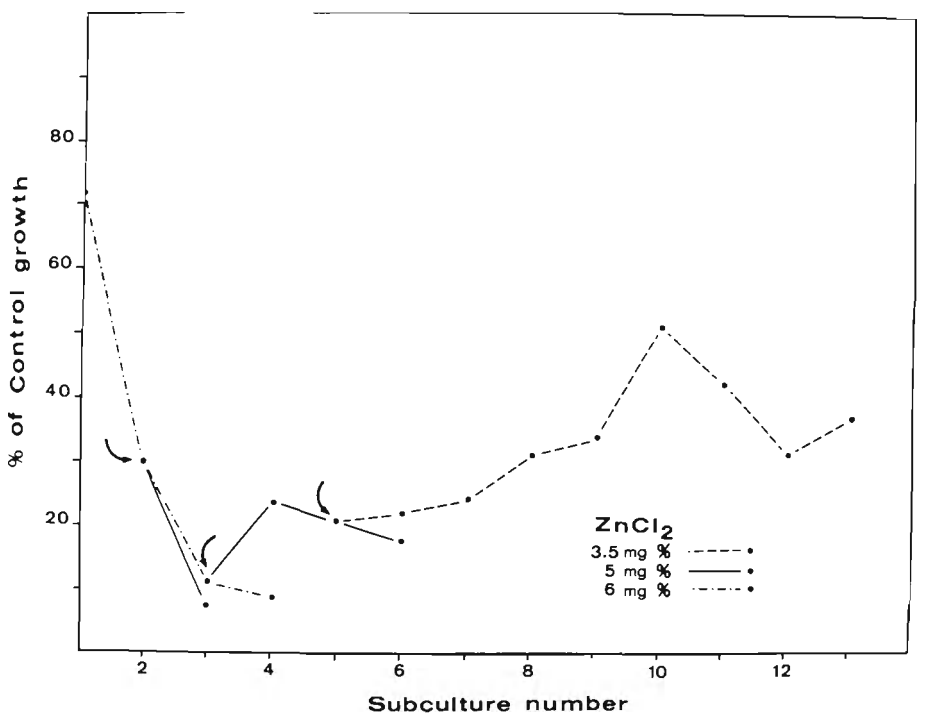

Fig. 9. *Isochrysis galbana*. Growth during extended exposure to zinc by periodic subculture in medium containing added $ZnCl_2$ (3.5 mg% $ZnCl_2$ = 16.8 ppm Zn; 5 mg% $ZnCl_2$ = 24.0 ppm Zn; 6 mg% $ZnCl_2$ = 28.8 ppm Zn). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $ZnCl_2$

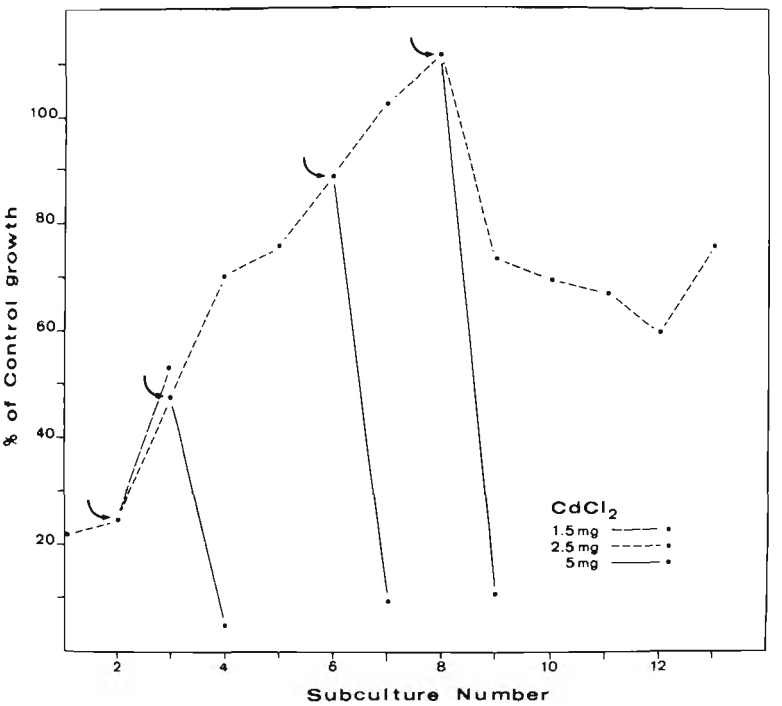

Fig. 10. *Isochrysis galbana*. Growth during extended exposure to cadmium by periodic subculture in medium containing added $CdCl_2$ (1.5 mg% $CdCl_2$ = 9.20 ppm Cd; 2.5 mg% $CdCl_2$ = 15.3 ppm Cd; 5.0 mg% $CdCl_2$ = 30.7 ppm Cd). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CdCl_2$

11. However, repeated attempts to grow this species in 5 mg% $CdCl_2$ medium failed (Fig. 7).

Growth of *Isochrysis galbana* in medium with 10 mg% $CuCl_2$ (47.3 ppm Cu) was greatly reduced after 4 subcultures. Inoculation of 8 mg% $CuCl_2$ medium with viable cells from the 10 mg% culture yielded populations that were judged to be sufficient for the next transfer to be again in the higher concentration of $CuCl_2$. Growth in 10 mg% was now satisfactory, and the strain was continued. Algae from the latter strain were used to re-inoculate 8 mg% $CuCl_2$ medium to determine if this strain would show an improved growth density in the lower copper concentration. In fact, this did not occur, and both strains in 8 mg% and 10 mg% $CuCl_2$ demonstrated a reasonably steady growth that remained between 50 % and 70 % of the control for the duration of the experiment (Fig. 8).

Isochrysis galbana in 5 mg% and 6 mg% $ZnCl_2$ media was depressed. At Subculture 5, the declining 5 mg% $ZnCl_2$ strain was used to inoculate medium containing less $ZnCl_2$, 3.5 mg% (16.8 ppm Zn). The latter strain gradually increased to 50 % of the control between Subcultures 6 and 10 (Fig. 9).

Isochrysis galbana inoculated into medium with 2.5 mg% $CdCl_2$ (15.3 ppm Cd) resulted in only a little over 20 % of control growth, but subsequently showed a steady increase to over 100 % in 8 subcultures. Repeated attempts to grow this strain in 5 mg% $CdCl_2$ medium were not successful (Fig. 10).

Dunaliella euchlora proved to be very resistant to copper, growing above 80 % of the control levels in medium with 25 mg% $CuCl_2$ (118 ppm Cu). Growth was further stimulated to 120 % of the controls after 6 subcultures and subsequently remained between 80 % and 100 % of the controls (Fig. 11). *D. euchlora* in 6 mg% $ZnCl_2$ decreased in growth with the first 3 subcultures but, at the 2nd subculture, a strain in 5 mg% $ZnCl_2$ (24.0 ppm Zn) was started and density in this concentration eventually increased. This strain was again put into 6 mg% $ZnCl_2$ where it then appeared to tolerate temporarily the higher metal concentration. At the 9th subculture the declining strain in 6 mg% was transferred to medium with 5 mg% $ZnCl_2$, and growth continued to improve (Fig. 12).

Growth of *Dunaliella euchlora* in 10 mg% $CdCl_2$

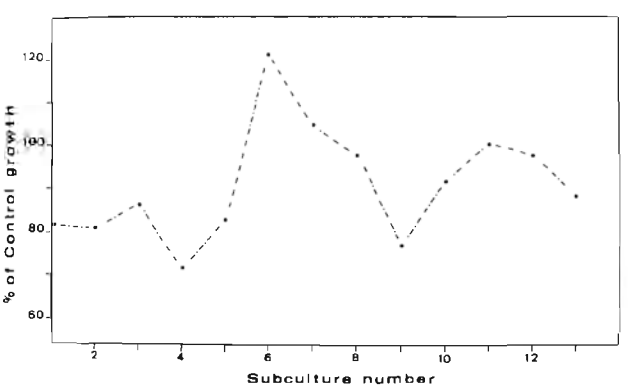

Fig. 11. *Dunaliella euchlora*. Growth during extended exposure to copper by periodic subculture in ASP_2 medium containing added $CuCl_2$ (25 mg% $CuCl_2$ = 118 ppm Cu)

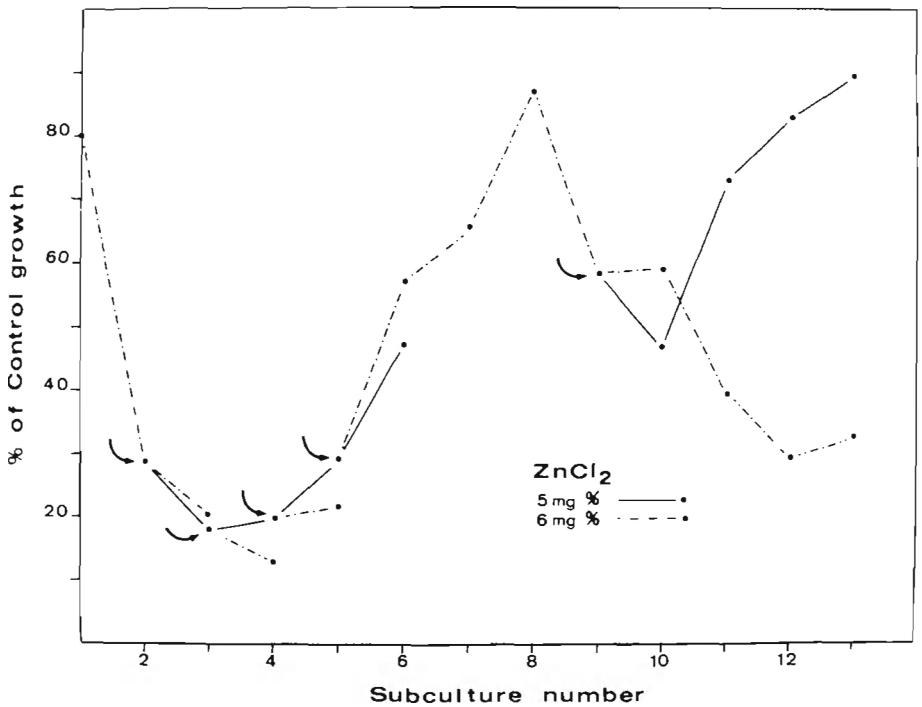

Fig. 12. *Dunaliella euchlora*. Growth during extended exposure to zinc by periodic subculture in ASP_2 medium containing added $ZnCl_2$ (5.0 mg% $ZnCl_2$ = 24.0 ppm Zn; 6.0 mg% $ZnCl_2$ = 28.8 ppm Zn). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $ZnCl_2$

(61.3 ppm Cd) medium increased to a maximum of 100% of the control after 6 subcultures; thereafter, growth varied between 70% and 95% of control

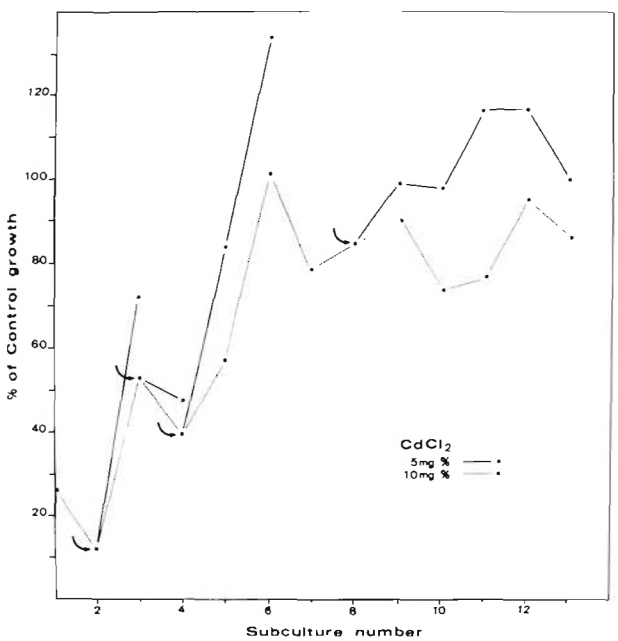

Fig. 13. *Dunaliella euchlora*. Growth during extended exposure to cadmium by periodic subculture in medium containing added $CdCl_2$ (5 mg% $CdCl_2$ = 30.7 ppm Cd; 10 mg% $CdCl_2$ = 61.3 ppm Cd). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CdCl_2$

levels. The 10 mg% $CdCl_2$ strain was inoculated into 5 mg% $CdCl_2$ medium, in which growth was stimulated to 133.3% of the control growth. This procedure was repeated at Subculture 8 and also yielded a culture of high density (Fig. 13).

Medium with 25 mg% $CuCl_2$ severely reduced growth of *Phaeodactylum tricornutum* after 3 subcultures. At Subculture 2, cells from the 25 mg% $CuCl_2$ transferred into 10 mg% $CuCl_2$ (47.3 ppm Cu) medium attained 105% of the control growth at Subculture 5. Attempts to grow the 10 mg% $CuCl_2$ strain in 25 mg% $CuCl_2$ medium were not successful (Fig. 14).

Growth of *Phaeodactylum tricornutum* failed in 6 mg% and 5 mg% $ZnCl_2$. A 3.5 mg% $ZnCl_2$ (16.8 ppm Zn) strain, after an initial decline, eventually grew to population densities of 60 % of the control, but this stimulation was only temporary, and density declined after the 11th subculture (Fig. 15).

Phaeodactylum tricornutum in 20 mg% $CdCl_2$ (123 ppm Cd) medium grew to just over 60% of control levels in the 1st subculture. This strain was continued for 13 subcultures, during which growth was erratic, but the strain remained between 60% and 80% of control levels. At various times the 20 mg% $CdCl_2$ strain was used to inoculate 10 mg% medium, and cells from this culture subsequently were used to inoculate 5 mg% $CdCl_2$ medium. Resulting strains in the 5 mg% and 10 mg% $CdCl_2$ concentrations grew somewhat better than the 20 mg% $CdCl_2$ strain, but there was no evidence of significant stimulation with time (Fig. 16).

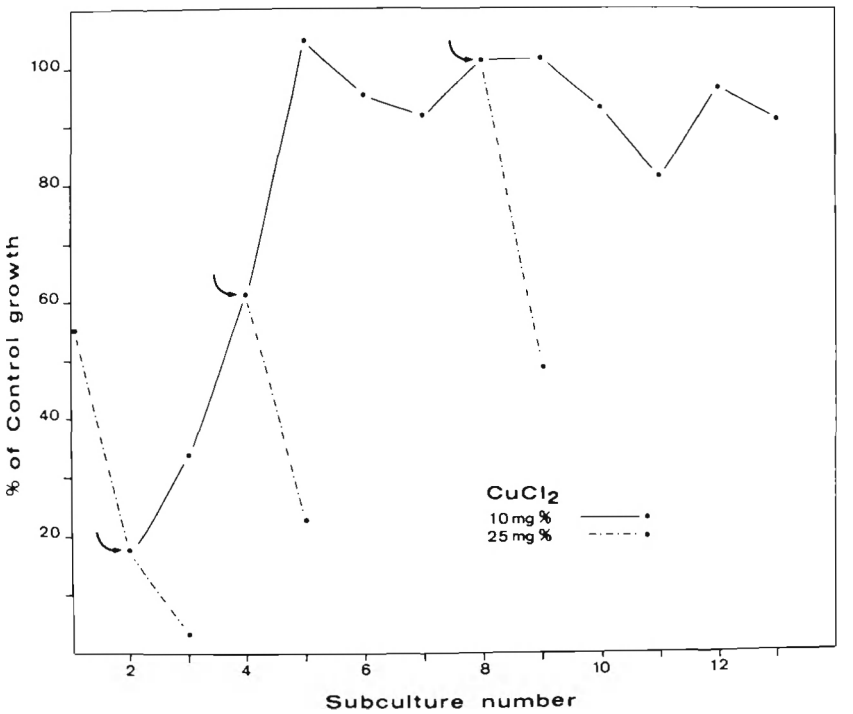

Fig. 14. *Phaeodactylum tricornutum*. Growth during extended exposure to copper by periodic subculture in ASP_2 medium containing added $CuCl_2$ (10 mg% $CuCl_2$ = 47.3 ppm Cu; 25 mg% $CuCl_2$ = 118 ppm Cu). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CuCl_2$

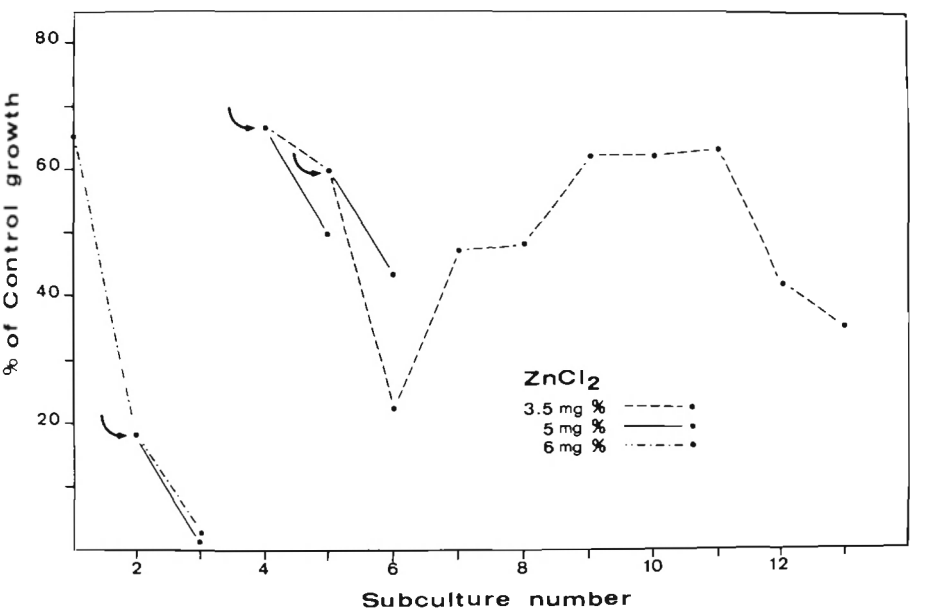

Fig. 15. *Phaeodactylum tricornutum*. Growth during extended exposure to zinc by periodic subculture in medium containing added $ZnCl_2$ (3.5 mg% $ZnCl_2$ = 16.8 ppm Zn; 5.0 mg% $ZnCl_2$ = 24.0 ppm Zn; 6.0 mg% $ZnCl_2$ = 28.8 ppm Zn). Arrows: subcultures at which cells growing in one concentration of the metal were continued in the medium and also transferred into an additional higher or lower concentration of $ZnCl_2$. At the 4th subculture the stock strain growing in ASP_2 was transferred into 3.5 mg% $ZnCl_2$

Microscopic Examination of Adapted Strains

Samples taken from the highest concentration of each metal at Subculture 11 were examined microscopically. No evident morphological anomalies or consistent size differences were observed after extended exposure to the metals.

Feeding of *Crassostrea virginica* Larvae with Metal-Adapted Algal Strains

Development at 48 h of at least 75 % of the fertilized egg suspension to normal veliger larvae was used as criterion of a healthy population required for conducting experiments. Average size of veligers at 48 h was

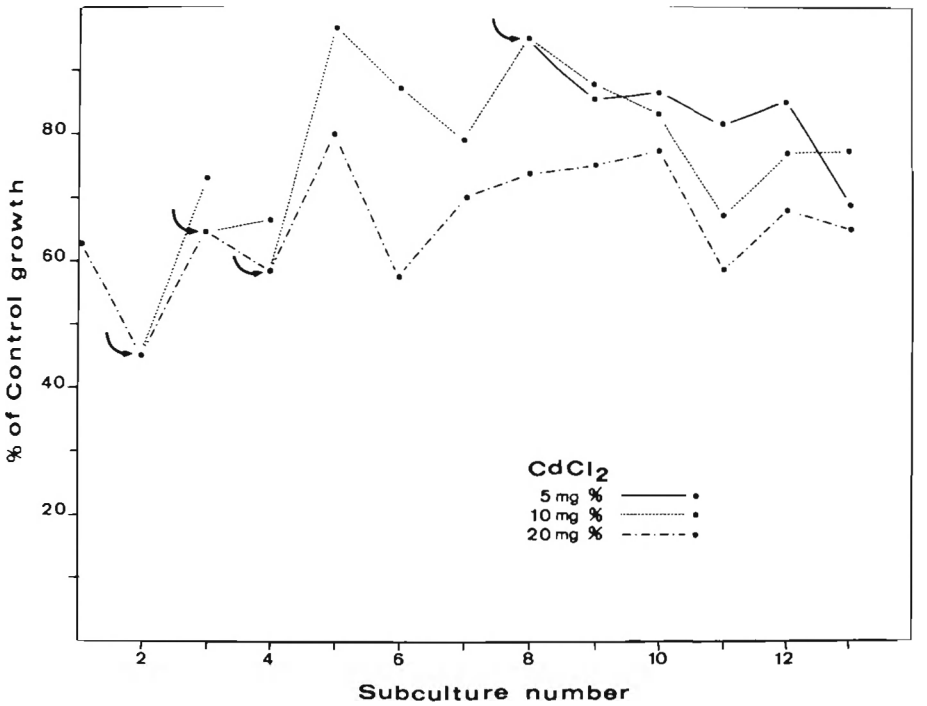

Fig. 16. *Phaeodactylum tricornutum*. Growth during extended exposure to cadmium by periodic subculture in medium containing added $CdCl_2$ (5.0 mg% $CdCl_2$ = 30.7 ppm Cd; 10 mg% $CdCl_2$ = 61.3 ppm Cd; 20 mg% $CdCl_2$ = 123 ppm Cd). Arrows: subcultures at which cells growing in one concentration of the metal were continued in this medium and also transferred into an additional higher or lower concentration of $CdCl_2$

79 µm. On the 5th day of the experiment, unfed larvae averaged 80.6 µm; larvae not fed but treated with 86 µg l^{-1} (ppb) copper ion in solution were 85.6 µm; and larvae not fed but treated with 27 µm l^{-1} cadmium ion in solution, 84.8 µm. In contrast, larvae fed daily with living *Isochrysis galbana* cultured in ASP_2 medium were 120.9 µm in size. The average sizes of larvae fed *I. galbana* plus copper in solution, and larvae fed *I. galbana* plus cadmium in solution were 128.7 µm and 128.8 µm, respectively. Larvae fed *I. galbana* adapted to 2.5 mg% $CdCl_2$ (15.3 ppm Cd) attained 128.9 µm. However, algae adapted to 10 mg% $CuCl_2$ (47.3 ppm Cu), exerted an inhibitory effect upon larval survival and growth. After 5 d of feeding, these larvae averaged only 89.2 µm. Larval growth after 2, 5, and 8 d of feeding is given in Table 4.

All unfed larvae showed high mortality on Days 2 and 5; unfed larvae treated with metals in solution exhibited only slightly higher mortalities. Mortality of larvae fed living *Isochrysis galbana* plus metals in solution was not appreciably higher than that of larvae fed living *I. galbana* alone. Feeding of larvae with copper-adapted *I. galbana* strains resulted in the highest mortality. Mortality of larvae in samples taken on the 2nd, 5th, and 8th days of feeding is listed in Table 4.

Larvae were observed under the fluorescence microscope to ascertain that cells from the various *Isochrysis*

Table 4. *Crassostrea virginica*. Growth and mortality of larvae in 8 feeding regimes. Food organism: *Isochrysis galbana*

Feeding regime	Size of larvae (µm) Day fed			Percent of larvae dead Day fed		
	2	5••	8	2	5	8
Unfed larvae	85.9	80.4 ± 0.39	•	6.8	26.7	•
Unfed larvae + Cu (86 µg l^{-1})	82.8	86.0 ± 0.47	•	11.7	48.1	•
Unfed larvae + Cd (27 µg l^{-1})	84.1	84.8 ± 0.48	•	19.5	35.2	•
Larvae fed *I. galbana*•••	100.0	120.5 ± 0.84	160.0	2.6	8.0	14.4
Larvae fed *I. galbana* + Cu^{++} (86 µg l^{-1})	100.0	128.7 ± 0.78	148.5	2.5	8.6	10.9
Larvae fed *I. galbana* + Cd^{++} (27 µg l^{-1})	98.9	128.8 ± 0.65	150.0	3.4	8.4	9.8
Larvae fed *I. galbana* adapted to 10 mg% $CuCl_2$	83.1	89.0 ± 0.60	104.5	36.2	44.4	61.3
Larvae fed *I. galbana* adapted to 2.5 mg% $CdCl_2$	88.1	128.9 ± 0.76	153.0	18.7	25.7	30.6

• Samples not measured or counted; all larvae dead
•• Probable error calculated for Day 5
••• Number of cells fed = 16.5 × 10^3 cells ml^{-1} of larval culture (containing 20 larvae ml^{-1})

galbana strains were indeed being ingested. Autofluorescence of algae within fed larvae revealed that the larvae were ingesting and lysing *I. galbana* cells, including those adapted to copper and cadmium (Fig. 17). Particulate and partially lysed *I. galbana* were observed in all feeding regimes.

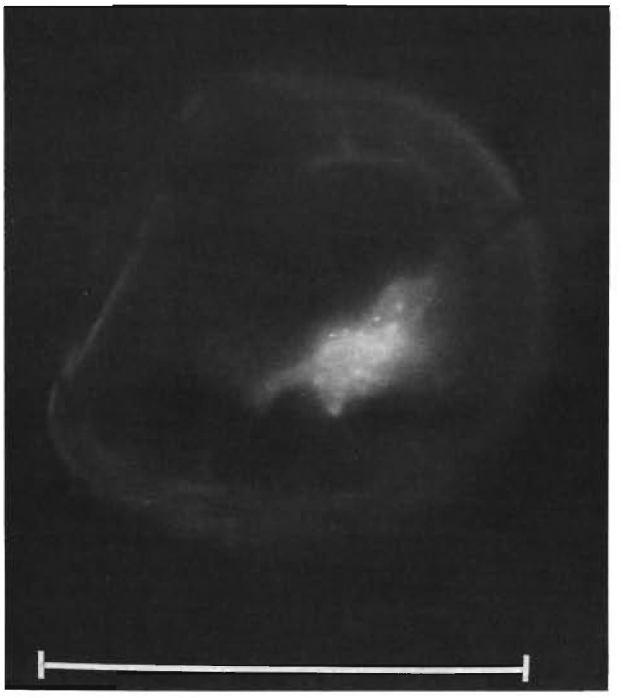

Fig. 17 *Crassostrea virginica*. Straight-hinge-stage larva fed *Isochrysis galbana*, adapted to 10 mg% $CuCl_2$ (47.3 ppm Cu) for 8 d, showing epifluorescence of whole and partially lysed algal cells in the gut. Sample taken 1 h after feeding. Scale line: 100 µm

DISCUSSION

Mineral elements, both major and trace, are essential nutrients for micro-algae (O'Kelley, 1974) but, in excessive concentration or the wrong speciation, can become toxic (Allen et al., 1980). Copper toxicity for micro-algae has been known since early in the century when copper sulphate was used to control nuisance growths of algae in eutrophic lakes (Moor and Kellerman, 1904). Later, oxides of metals were incorporated into boat and gear paints to discourage attachment of algae and other planktonic forms (Orton, 1929–30). Recently, attention is being paid to the role of metals as inhibitors of primary productivity in aquatic environments, both freshwater (Steemann-Nielsen and Wium-Andersen, 1969, 1970; Whitton, 1970) and marine (Davies, 1978; Thomas et al., 1980).

In the present study, extensive growth inhibition of *Monochrysis lutheri* and *Isochrysis galbana* became evident in 47.3 ppm Cu in the enriched natural seawater medium, but inhibition occurred at more than twice this concentration, 118 ppm Cu, in the artificial seawater medium. *Dunaliella euchlora* and *Phaeodactylum tricornutum* showed similar responses (about 80 % of control growth) at 47.3 ppm Cu in natural seawater medium. In the ASP_2 medium, however, *D. euchlora* was resistant to 118 ppm Cu; whereas, growth of the diatom was diminished at this concentration. This difference in response of the latter 2 species in the chelated medium suggests that *P. tricornutum* has a far more sensitive metal uptake mechanism than does the flagellate. Davies (1976) demonstrated that the membrane of *D. tertiolecta* was markedly less permeable to mercury than that of *I. galbana*. The latter took up almost 4 times the metal across a unit area of surface in the period of the experiment than did *D. tertiolecta*. The remarkable resistance of *D. euchlora* to copper suggests that a similar difference in the rate of copper uptake exists as that described for mercury by Davies.

Cadmium appeared to be a more potent inhibitor of algal growth than copper. In both media lower concentrations of cadmium inhibited growth of the most sensitive species, *Monochrysis lutheri* and *Isochrysis galbana*, than was the case with copper. Cadmium was also a more effective inhibitor than copper of *Phaeodactylum tricornutum* and *Dunaliella euchlora* in both media. However, it may be noteworthy that, in both media, cadmium inhibited growth of *D. euchlora* more than *P. tricornutum*, which is a reverse of the species response to copper.

Significant growth inhibition occurred in lower concentrations of copper and cadmium in the enriched natural seawater than in the artificial seawater medium. The enriched natural seawater medium (E) contains NaFe Sequestrene, an iron-specific chelator, and the buffer, tris(hydroxymethyl)aminomethane, which has some complexing properties. On the other hand, the artificial seawater medium ASP_2 contains the same concentration of the buffer and, in addition, the strong cationic chelator, disodium ethylenediaminetetracetic acid. The results presented here confirm earlier studies showing that chelation can reduce the toxicity of copper to phytoplankters (Erickson et al., 1970; Steemann-Nielsen and Wium-Andersen, 1970) and that toxicity is related only to the concentration of free cupric ion (Sunda and Guillard, 1976; Anderson and Morel, 1978).

Differences in species sensitivity to metals, such as those observed with copper and cadmium, were not repeated in experiments on tolerance to zinc. In the artificial seawater medium with 33.6 ppm Zn, growth of all species ranged from 50.9 % to 64.7 % of controls. In the natural seawater medium, inhibition in

48.0 ppm Zn also remained within a similar range (25.7 %–38.4 %) for all species. The work of Davies (1973) supports the hypothesis that Zn uptake in *Phaeodactylum tricornutum* is passive. His experiments showed that growing cultures reached a maximum of intracellular zinc in 12–14 h after the metal was introduced and then decreased steadily, although zinc was still available for additional uptake. If such a phenomenon exists in other phytoplankton as well, a limited capacity to build up intracellular concentrations of zinc could explain the relatively low toxicity of this metal and the similarity of the growth response in different species.

The transfer of metals from the aquatic environment to phytoplankton must take place by either or both of 2 mechanisms: (1) penetration of ions through cell membrane barriers by an active or passive diffusion process; (2) adsorption on the cell surface. A negatively charged surface on 3 species of estuarine micro-algae has been demonstrated experimentally (Myers et al., 1975). These negatively charged adhesive sites, normally coupled to seawater cations, may become displaced by high concentrations of cationic pollutants. In such an event, the relatively large surface area of micro-algae available for sorption, together with the high cell concentrations used in laboratory culture experiments or to be found in natural blooms, presents ample opportunity for the transfer of high concentrations of metallic ions to the grazing species of the next trophic level.

Although studies on adaptation of algae to heavy metals in laboratory or natural environments are scarce, there is evidence that such adaptation exists in the natural environment. Populations of the brown alga *Ectocarpus siliculosus* isolated from the hulls of freight liners coated with a copper-based anti-fouling paint were reported more resistant to dissolved copper than populations of this alga isolated from an open, uncontaminated rocky shore (Russell and Morris, 1970). A copper-tolerant population of *E. siliculosus* was shown by Hall (1980) to be co-tolerant to cobalt and zinc in laboratory culture.

Stockner and Antia (1976) criticized much of the literature on phytoplankton pollution stress and bioassay procedures for ignoring the possibility of biological adaptation in these studies. Bioassay studies that measure population growth after exposure of algae to the pollutant for a few days or inhibition of photosynthesis after a brief exposure to the pollutant are not sufficient. According to Stockner and Antia '. . . it is only the long term response that is expected to be ecologically realistic for application to environmental protection against pollution and eutrophication.' In addition, they say that rather than concern over the mechanistic nature of this adaptation, i.e. is it acquired via mutations or physiologically? '. . . it would be more important to know whether such adaptation may impair the food value of phytoplankton to the next trophic level in the aquatic food web.' The present report supports these statements of Stockner and Antia.

All species gave evidence of some capacity for adaptation to metal concentrations that were inhibitory upon initial exposure. There were 3 culture procedures that induced this adaptation: (1) prolonged subculture in a constant sublethal metal concentration, (2) subculture of viable cells from a population diminished by a high metal concentration into a slightly less toxic concentration, and (3) subculture of viable cells to a slightly increased concentration. Each of these procedures or their combinations gave evidence of the potential for adaptation to metals in the tested algae. Although with some cultures no consistent increase in population density was observed with the above procedures, a long-term tolerance or a temporary increase in populations rather than declining growth with time was demonstrated, constituting a type of adaptation.

Tolerance to high metal concentrations could be mediated by developing metabolic pathways as alternatives to those utilizing enzymes sensitive to metallic poisoning or by reducing cell permeability. An alternative adaptation mechanism may be a stimulation of the production of extracellular metabolites that can act as chelators, thereby reducing the effective metal ion concentration. Such extracellular metabolites with chelating potential can be released into the medium, retained intracellularly on non-metabolic binding sites or remain attached to the cell wall (Jackson and Morgan, 1978; Härdstedt-Roméo and Gnassia-Barelli, 1980).

The general binding capacity of compounds, such as protein, provides a method for the storage of metals. Specific storage proteins of the metallothionen type have been discovered in several marine groups, and their synthesis can be induced in the field and laboratory (Bryan, 1979). Whereas the ability of phytoplankters to adapt to metal pollutants is of survival value, this accumulation of metals as storage products possesses a direct threat to the grazing population. Romeril (1971) considered 3 possible methods for accumulation of metals by marine animals: (1) adsorption of ions at membrane/water interfaces; (2) absorption by active and/or passive diffusion of metal ions from seawater across semi-permeable membranes into cell or body fluids; (3) ingestion of ions with food, in combination with particulate matter or mucus, and absorption through the gut wall. Romeril concluded that accumulation by the last of these processes would be small, but an opposite conclusion was reached by Pentreath (1973) from a study conducted on the adult bivalve *Mytilus edulis*. Metal accumulation by way of food was

also shown to be important in marine gastropods (Young, 1977; Klumpp, 1980) and the fish *Pleuronectes platessa* and *Raja clavata* (Pentreath, 1977). Thus, there is some evidence that planktonic algae, the main primary producers in the marine environment, could be a major entry point of heavy metal ions into the marine food web. This hypothesis is also supported by the data of the present study

Oyster larvae fed cultures of *Isochrysis galbana* adapted to 10 mg% $CuCl_2$ (47.3 ppm Cu) responded with a decreased growth rate, as well as a high mortality. Feeding larvae with copper in solution and algae cultured in the absence of excess metal resulted in growth that was similar to that of the controls and a mortality that was well within the normal expected mortality range. Larvae that were fed *I. galbana* adapted to 2.5 mg% $CdCl_2$ (15.3 ppm Cd) or cadmium in solution with normal *I. galbana* were less affected than with copper. Perhaps variation in response to the 2 metals can be attributed to the fact that the algae had been adapted to a lower concentration of cadmium than of copper, thus introducing different amounts of the metals to the larvae. Although growth of larvae was slightly affected by cadmium in solution and by cadmium-adapted algal food, the mortality of larvae fed cadmium-adapted algae was about twice that of larvae fed *I. galbana* not exposed to high levels of metals. This observation implies that larval populations are not uniform in grazing capacity; individuals that grazed very actively had the opportunity to accumulate higher concentrations of metals than normal grazers. Variation among larvae in grazing ability has often been observed in oyster rearing experiments (Landers and Ukeles, unpubl.).

Our experiments clearly demonstrate how pollutants can affect organisms at higher trophic levels by bioaccumulation from primary producers, while toxic concentrations cause mortality of grazers, accumulation of sublethal concentrations can be passed on and concentrated in species still higher up in the trophic pyramid.

The great disparity of phytoplankton response to metals in laboratory experiments suggests that introduction of metal contaminants into a natural assemblage of estuarine micro-algae can shift the species composition of the assemblage. In fact, it has been shown that introduction of copper to an experimental ecosystem with a natural mixed composition of phytoplankton led to the bloom of one copper-resistant diatom species, *Amphiprora paludosa* (Sanders et al., 1981).

Bioassay studies must be evaluated in relationship to a particular ecosystem before extrapolating laboratory studies on acute toxicity levels to field conditions. To establish water-quality standards, the potential for complexing of metallic ions should be determined, as well as the possibility of altering species composition and its immediate and chronic impact on the food web.

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