J. Phycol. **15,** 316–321 (1979)

A MORPHOMETRIC STUDY OF LEAD AND COPPER EFFECTS ON *DIATOMA TENUE* VAR. *ELONGATUM* (BACILLARIOPHYTA)[1]

Linda Sicko-Goad[2] *and Eugene F. Stoermer*

Great Lakes Research Division, The University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

Polyphosphate bodies containing lead were induced in laboratory cultures of Diatoma tenue *var.* elongatum *Lyngb. by the addition of phosphorus and 0.05 μg-atoms/l Pb to P deficient medium. Morphometric analysis of cells exposed to Pb showed a significant decrease in number of mitochondria with a concomitant increase in their volume and an increase in membranous organelles in the vacuole compared to phosphorus starved and phosphorus sufficient controls. Exposure of cultures to 0.08 μg-atoms/l copper resulted in reduction of the number of polyphosphate bodies formed during luxury uptake but no other significant morphological changes in cellular organelles. Ecological implications of the interactions between nutrients and low level trace metal contamination are discussed.*

Key index words: copper; Diatoma; *diatoms; diatom morphometry; lead; phosphate; polyphosphate bodies*

During a morphometric study of cell volume components of naturally-occurring phytoplankton (Sicko-Goad et al. 1977) polyphosphate (poly-P) bodies were observed in the vacuole of the diatom *Fragilaria capucina* Desm. These poly-P bodies, subsequently analyzed by X-ray energy dispersive analysis, were found to contain lead, in addition to phosphorus and calcium. Although P and Ca are common components of poly-P bodies (Sicko-Goad et al. 1975, Stewart 1977), there have been few reports of their association with heavy metals.

To study this phenomenon more carefully, poly-P bodies were induced in *Diatoma tenue* var. *elongatum* Lyngb., a diatom isolated from the Great Lakes (USA) and kept in culture in our laboratories. Preliminary results demonstrated it was capable of forming numerous small poly-P bodies in the vacuole and of similar morphology to those in blue-green algae (Jensen and Sicko 1974, Jensen et al. 1977) after a regime of 3 days of phosphate starvation and 2 h PO_4^{3-} uptake. During the poly-P-induction period, 0.05 μg-atoms/l Pb (as lead nitrate) or 0.08 μg-atoms/l Cu (as copper nitrate) was introduced into the culture medium. The subsequent incorporation of lead into the poly-P bodies, and the overall effects of these heavy metals on the ultrastructure of the cells are discussed in this report.

MATERIALS AND METHODS

A unialgal culture of *Diatoma tenue* var. *elongatum* was isolated from Lake Michigan, and grown in FM medium at 15 C, 200 $\mu Ein \cdot m^{-2} \cdot s^{-1}$ of illumination and on a 16:8 LD cycle (Lin and Schelske 1978). Cells from 4-day old cultures, in logarithmic growth (controls), were washed 2× with sterile distilled water, packed by gentle centrifugation and then inoculated into FM medium made without phosphate salts and incubated under the normal culture conditions for 3 days to produce phosphate starvation. At the end of this period, during hour 4 of the light cycle, the cells were packed by gentle centrifugation and resuspended for 2 h in medium containing one of the following: i) 55 μM PO_4^{3-} (2 × PO_4^{3-} concentration of FM medium); ii) 55 μM PO_4^{3-} + 0.05 μg-atoms/l Pb; iii) 55 μM PO_4^{3-} + 0.08 μg-atoms/l Cu.

Samples for transmission electron microscopy (TEM) were taken from control, phosphate-starved, and phosphate-uptake cultures. Cells were fixed 1 h with 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4 C followed by 1 h post-fixation in 1% OsO_4. The osmolality of the glutaraldehyde fixative was 387 mosmol. Cells were dehydrated in a graded ethanol-propylene oxide series and embedded in Epon (Luft 1961).

Thin sections were cut with a diamond knife, collected on copper grids and stained with uranyl acetate (Stempak and Ward 1964). The cells were examined at a standard magnification of 8,500× using a Zeiss EM 9S-2 electron microscope. Magnification calibrations of the microscope were made by use of a grating replica to determine the stability of the standard magnification. The variation was usually less than 2%.

The sampling scheme employed for the morphometric analysis is outlined in Table 1. Fifty micrographs were examined for each experimental treatment. A transparent 12.5 mm square sampling lattice was superimposed over the micrographs for quantitative measurements. Although several sections were collected on one grid, only 1 section/grid was used for the analysis. Individual cells were photographed, including small grazing tip sections. Blocks were retrimmed after each series of sections was cut in order to avoid repeated sampling of adjacent material within the same organism (Sicko-Goad et al. 1977). When cells were connected in a filament, only 1 cell/filament was sampled.

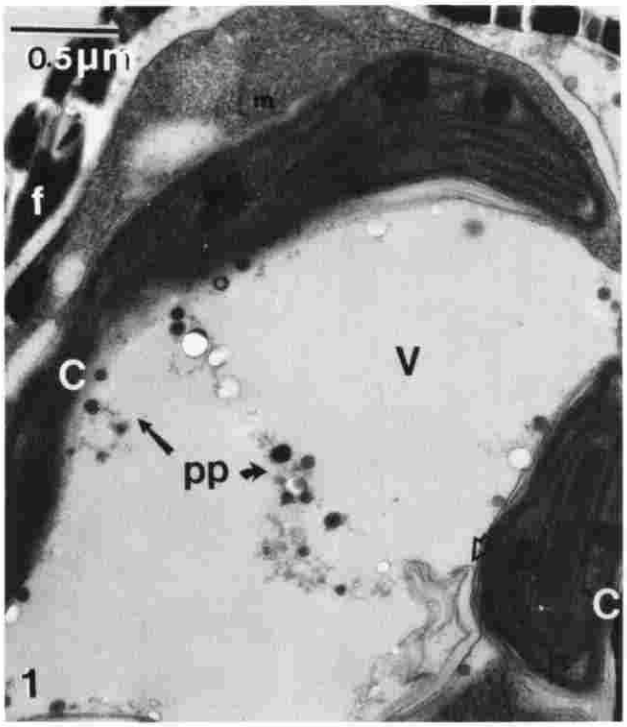
Estimates of volume density, i.e., the fractional volume of a cellular component related to its containing volume, were obtained using the grid point-counting technique (Glagoleff 1933, Chalkley 1943). The point-counting method is an extension of the Delesse principle (Delesse 1847) which states that the areal density of profiles on "two-dimensional" sections is an unbiased estimate of the volume density of the corresponding structures within the tissue, i.e.,

$$\frac{V_i}{V_T} = \frac{A_i}{A_T} = \frac{P_i}{P_T}$$

where: V_i = volume of a component i, A_i = area of i in a section, P_i = number of points falling within the boundary of i, V_T total containing volume, etc. (notation and definitions as used by Weibel and Bolender 1973). Thus, by counting points of a sampling

[1] *Accepted: 8 March 1979.*

[2] Address for reprint requests.



Note: Abbreviations used in figures: *c* = chloroplast; *f* = frustule; *m* = mitochondria; *pP* = polyphosphate bodies; *s* = spheroids; *v* = vacuole

FIG. 1. Control cell of *Diatoma tenue* var. *elongatum* grown 4 days in FM medium containing 55 µM PO_4^{3-}: note numerous small poly-P in vacuole, and membranous organelles (open arrow) near chloroplast.

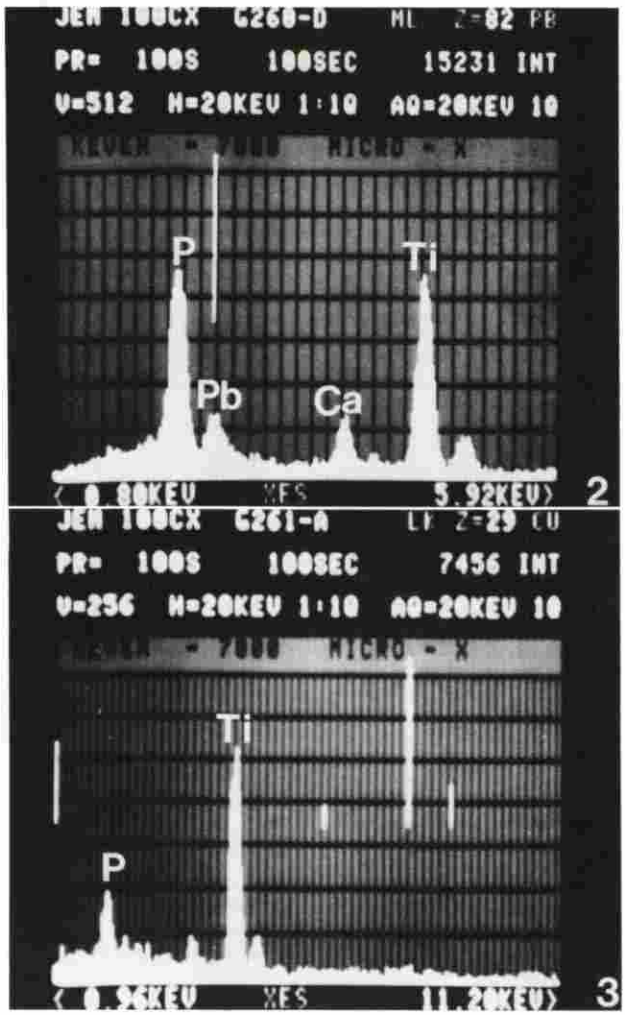
grid striking component *i*, an unbiased estimate of the volume fraction of component *i* in the original tissue can be obtained.

Cells from all treatments were stained for poly-P by the method of Ebel et al. (1958) and examined in a light microscope. Actual cell volume estimates (μm^3) were obtained from light microscope examination of epoxy mounts of the same material as used for the quantitative stereologic analysis. Estimates of cell volume are based on ten independent measurements for each treatment, and assume a regular geometric shape (Volume = length ·breadth·height; $V = l \cdot b \cdot h$).

Sections ca. 60 nm thick were cut with a diamond knife and mounted on either 300 mesh copper or 75 × 300 mesh titanium grids for X-ray analysis. They were examined at 100 KV in the STEM mode in a JEM 100C electron microscope equipped with a Kevex series 7000 energy dispersive X-ray analysis system. The

TABLE 1. *Sampling scheme for quantitative electron microscopy (50 photographs examined for each treatment at a magnification of ×25,800).*

	Treatment				
			Uptake: PO_4 +		
	Control	PO_4 starved	O	Pb	Cu
Total points counted	5,053	4,966	5,101	5,179	5,076
Total area examined (μm^2)	1,162	1,142	1,174	1,191	1,167
Total cell volume (μm^3) (light microscope)	280	250	220	220	230



FIGS. 2, 3. X-ray spectrum of poly-P bodies: FIG. 2, phosphate + Pb uptake: FIG. 3, phosphate + Cu treatment: no Cu detectable in poly-P or other areas of cell. Peaks present in Kev: P 2.01; Cl 2.62; Ca 3.69, 4.01; Ti 4.51; Pb 2.34.

specimen was tilted 30° toward the detector, with a specimen distance of 18 mm. The vacuolar inclusions were subjected to spot analysis, with a spot size of 5 nm for a preset time of 100 s.

RESULTS

The experimentally-induced poly-P bodies in *Diatoma* were ca. 75 nm diam and located in the vacuole (Fig. 1). Addition of lead to the uptake medium resulted in Pb incorporation in the poly-P bodies (Fig. 2). At the treatment concentration used, copper was neither incorporated into the poly-P bodies (Fig. 3), nor could it be detected at any other intracellular site.

In addition to determining if there were any intracellular heavy metal concentration sites, morphometric studies were conducted to quantitatively determine what effects the phosphate treatments and heavy metals exerted on the cells. On a relative volume basis, the cellular categories most affected by changes in nutrient conditions were vacuole, cyto-

TABLE 2. *Morphometric results of nutrient treatments: results are mean ± 1 S.E.*

	Treatments				
	Control	PO_4-starved	PO_4 uptake	PO_4 + Pb uptake	PO_4 + Cu uptake
Frustule (V_v)[a]	17.8 ± 2.12	18.5 ± 1.66	18.0 ± 1.06	18.0 ± 1.34	17.7 ± 0.82
Chloroplast (V_v)	16.4 ± 1.08	15.5 ± 0.96	15.1 ± 0.67	15.5 ± 0.96	15.7 ± 0.94
Mitochondria (V_v)	3.07 ± 0.27	2.5 ± 0.27	2.7 ± 0.27	2.9 ± 0.27	3.0 ± 0.27
Mitochondria (N_v)[b]	0.23/μm^3	0.21/μm^3	0.23/μm^3	0.17/μm^3	0.22/μm^3
average volume	0.13μm^3	0.12μm^3	0.12μm^3	0.17μm^3	0.14μm^3
number/cell	64.4	52.5	50.6	37.4	50.6
Residual bodies (V_v)	1.4 ± 0.25	2.0 ± 0.33	2.0 ± 0.28	2.1 ± 0.34	2.0 ± 0.25
Residual bodies (N_v)[c]	0.08/μm^3	0.09/μm^3	0.11/μm^3	0.22/μm^3	0.12/μm^3
average volume	0.18μm^3	0.22μm^3	0.18μm^3	0.10μm^3	0.17μm^3
number/cells	22.4	22.5	24.2	48.4	27.6
Vacuole (V_v)	34.1 ± 1.68	42.8 ± 1.89	40.8 ± 1.56	42.1 ± 1.55	41.4 ± 1.47
Cytoplasm (V_v)	24.3 ± 1.34	18.8 ± 1.28	21.5 ± 1.32	19.3 ± 2.30	20.2 ± 1.09
Storage (V_v)	2.91 ± 0.58	0	0	0	0
Poly-P bodies[d]					
μm^3 vacuole	0.9	0.5	7.6	7.9	3.6

[a] V_v = relative volume.

[b] $N_v = \frac{K}{\beta} \frac{N_a^{3/2}}{V_v^{1/2}}$ (Weibel and Gomez 1962), where K is assumed to be 1.07 and $\beta = 2.25$. (K = coefficient dependent on relative size distribution of particles: $K > 1$ if size not uniform. β = shaping constant. N_a = number/area.)

[c] $K = 1.07$, $\beta = 1.44$.

[d] Calculated by the formula $N_v = \frac{N_a}{D - 2p + T}$ (Underwood 1970), using $\bar{D} = 0.075\mu m$, $p = .005\mu m$, $T = .062\mu m$; T measured by the method of Small (1968). ($\bar{D}$ = mean diameter; p = beam penetration factor; T = section thickness.)

plasm (excluding mitochondria and chloroplasts) and storage products (Table 2). The relative volume of vacuole increased after phosphate starvation and remained high after phosphate uptake in comparison with the controls. The cytoplasm decreased after phosphate starvation, but returned to normal relative volumes after inoculation into culture medium containing phosphate. Storage products were found only in control cultures.

Analysis of variance of phosphate uptake vs. phosphate uptake plus heavy metals demonstrated no statistically significant difference between treatments in all cytoplasmic compartments examined, indicating that at the levels of lead and copper used in the experiment, no additional effects were exerted by the heavy metals on the relative volumes of the components tested. The changes in relative volumes of cytoplasm and vacuole of normal and phosphate starved cells were significant at $P = 0.001$.

In addition to Pb incorporation into poly-P, changes were also observed in the numbers/volume (N_v = numerical density) and volume/organelle of both mitochondria and residual bodies (a category of membranous organelles labelled residual bodies for descriptive purposes; Figs. 1, 4, 5). Although the relative volumes of these categories remained relatively constant in all treatments, mitochondria decreased in number and increased by ca. 50% in volume, while the membranous organelles increased 100% in numbers and decreased by the same percentage in volume when Pb was added to the uptake medium.

The membranous organelles described in Table 2 were present in control cells as well as all other treatments. In most cells they were located in the vacuole (Figs. 1, 4, 5). They were often associated with the vacuolar membrane (Fig. 5) and were adjacent to the chloroplast (Figs. 1, 5). There was no evidence that these membranes were derived from the plasma membrane.

The number of poly-P bodies/unit volume of vacuole also changed significantly with the various treatments. As would be expected, the control cells contained a small number of polyphosphate bodies (Table 2). This number decreased by ca. 50% during the phosphate starvation period, and increased more than seven-fold during phosphate uptake.

Lead did not decrease the number of poly-P bodies during uptake. However, Cu decreased their number by ca. 50%.

DISCUSSION

The results obtained in this study suggest that heavy metals may be incorporated into poly-P bodies in concentration ranges that occur in the natural environment. Such an association may be a means of reducing toxic heavy metal effects, because poly-P bodies are osmotically inert inclusions. Since poly-P is believed to be a phosphate storage form that can be degraded under conditions of phosphate limitation, only phosphate limitation could change the availability of phosphate and heavy metals to intracellular sites.

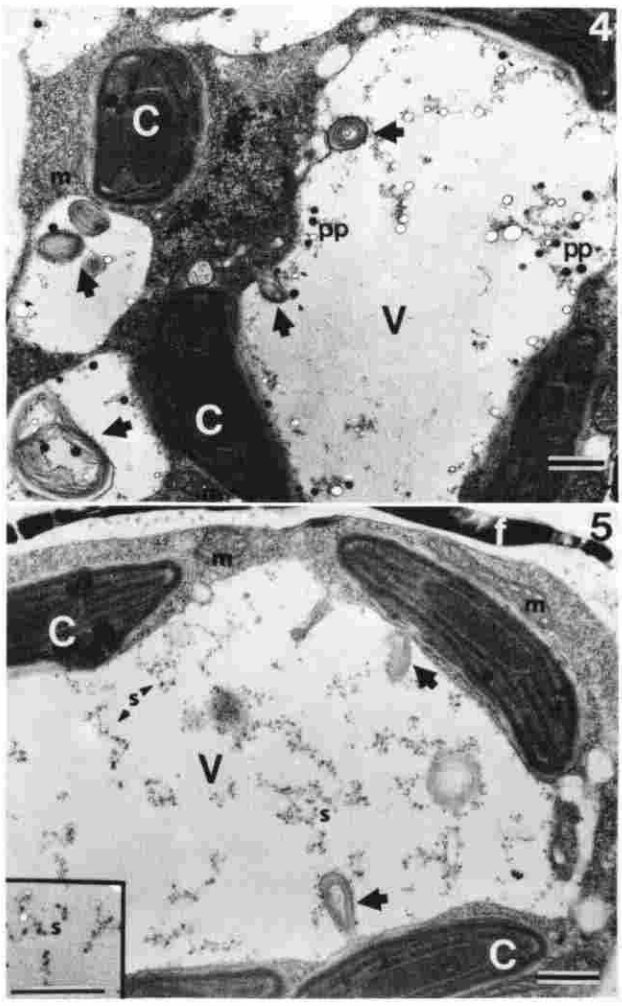
Our results are in agreement both with results obtained by Crang and Jensen (1975) and with observations made in our laboratory with naturally-occurring phytoplankton assemblages. Crang and Jensen (1975) demonstrated titanium (Ti) incorporation in poly-P bodies in the cultured bluegreen *Anacystis nidulans* (Richt.) Drouet & Daily. They found that increasing Ti concentrations in the growth medium resulted in greater X-ray microanalysis peak-to-

background ratios of Ti in the poly-P bodies and suggested that the algae may accumulate and store non-essential cations. Similarly, we have observed the incorporation of Pb in poly-P bodies of the diatoms *Fragilaria capucina* Desm., *Stephanodiscus alpinus* Hust. and *S. niagare* Ehr. which were collected in natural phytoplankton assemblages from another Great Lake (Saginaw Bay of Lake Huron). These observations support the theory that such a binding mechanism of heavy metals does indeed occur in naturally-occurring algal species.

Analysis of variance between phosphate uptake and phosphate uptake plus heavy metal treatments indicated that there were no significant differences between the relative volumes of all other cytological components tested. That is, no additional cytological volume changes occurred after exposure to the heavy metals tested at low concentrations. The only detectable changes occurring with the Pb treatment were to decrease the number of mitochondria per cell and increase the number of membranous organelles, with the relative volumes of both organelles remaining constant.

These results are interesting in respect to previous reports of heavy metal effects on algae. Silverberg (1975) demonstrated Pb accumulation in the green alga *Stigeoclonium tenue* Kütz. on the cell wall in the peripheral vacuole. He noted the Pb was in the vacuole in the form of spheroids that were often inside pinocytotic vesicles or multivesicular bodies in the larger peripheral vacuole. He observed no effects on mitochondria, plastids, or nuclei. In 1976 he also observed that cadmium induced the formation of dense intramitochondrial granules in three green algae, and reported that early symptoms of cadmium toxicity included swelling and vacuolization in the mitochondria.

Our studies indicated that at concentrations of 0.05 μg-atoms/l of Pb, the mitochondria were affected in that their numbers were reduced while their volume increased. At the same time, membranous organelles resembling residual bodies and mutivesicular bodies increased. Although we have no concrete evidence that these organelles are the result of autophagocytic activity, there appears to be a direct correlation between the increase in membranous organelles and a decrease in the number of mitochondria. These effects were not observed with phosphate starvation or uptake, or in Cu treatment. The spheroids described by Silverberg (1975) were also observed in all our samples. X-ray analysis of vacuolar regions containing these spheroids did not reveal accumulations of any elements that are readily detectable by energy dispersive analysis, i.e., $Z \geq 11$. We speculate that they are organic in nature. The only detectable Pb accumulation in the cell was in the poly-P bodies. In addition to X-ray analysis, the presence of clusters of the small poly-P bodies was confirmed by light microscopy using the staining technique of Ebel et al. (1958). The Pb exerted



FIGS. 4, 5. Phosphate + Pb or Cu uptake cells of *Diatoma*. Scale = 0.5 μm. FIG. 4, Phosphate + Pb uptake cell showing numerous poly-P bodies in vacuole are more electron dense than in phosphate uptake cells with no added heavy metals: note membranous organelles (arrow) present in several vacuole regions: FIG. 5, phosphate + Cu uptake cell showing numerous organic spheroids containing no heavy metals in vacuole: inset shows enlarged spheroids: note membranous organelles present in vacuole near chloroplast which appear derived from vacuolar membrane.

no other apparent morphological effects on *Diatoma*. Heavy metals have been demonstrated to exert effects on various organelles such as mitochondria (Stuve and Galle 1970, Silverberg 1976, Goyer 1973), chloroplasts (Fujita et al. 1977, Silverberg 1975), and nuclei (McLean and Williamson 1977, Choie and Richter 1972, Skarr et al. 1973), but only minor mitochondrial effects were detected in *Diatoma*. The lead apparently did not affect the phosphate uptake mechanism, since similar numbers of poly-P bodies were encountered both for phosphate and phosphate + Pb treatments.

In contrast to the results with Pb, the only apparent effect of Cu on the cells was a reduction to half the number of poly-P bodies (Table 2). No differ-

ences were observed either in numbers or volumes of mitochondria or other organelles. Copper is known to be a potent algicide (Fitzgerald and Faust 1963, Bartlett et al. 1974) and to have effects on protein synthesis, photosynthesis (Morgan and Lackey 1958, Steeman Nielsen and Wium-Andersen 1971), and silica uptake (Morel et al. 1978). We speculate that at the concentration tested, Cu was exerting an effect on the phosphate uptake mechanism.

There is some indication in the literature that Pb toxicity effects on cells or organelles are directly related to phosphate nutrient status. Koeppe and Miller (1970) demonstrated that, with maize mitochondria, the addition of Pb followed by the addition of phosphate resulted in fewer enzymatic inhibitions than if Pb alone was added to the mitochondrial suspensions. They concluded that Pb effects are minimized when sufficient phosphate is present due to the precipitation of Pb. Similarly, Monahan (1973) found that cells of the green algae *Hormotila blennista* Trainor & Hilton that were phosphate sufficient and capable of undergoing several cell divisions in phosphate-free medium were less susceptible to Pb toxicity than cells which had no stored phosphate. Our results also indicate that phosphate may protect the cells to some extent from Pb effects, by reducing the amount of Pb available to intracellular sites through incorporation into polyphosphate in an energy-requiring reaction. This is not to be confused with Pb phosphate precipitation.

Studies of various heavy metals on a variety of organisms have indicated that several cellular locations are prime sites for incorporation or binding. Many of the studies cited previously were conducted at heavy metal concentrations much higher than are found even in grossly polluted areas. We have demonstrated subtle effects of Cu and Pb at concentrations that are closer to those found in natural ecosystems by the use of quantitative electron microscopy. We feel that in natural ecosystems, at constant low level exposure, some algae may accumulate heavy metals and suffer no apparent adverse effects. In this respect, studies of constant low level exposure and tolerance mechanisms would prove more fruitful than studies of acute toxicity.

The results of this study may also be important in understanding the effects of point sources of contamination on large aquatic systems. A source (e.g., contaminated stream) will often contain both elevated levels of nutrients and toxic trace metals. Our evidence indicates that, in the case of phosphorus and lead, these materials are rapidly sequestered by algal populations in the zone of immediate effect. It is clear that many populations are capable of sequestering phosphorus in quantities greatly in excess of their immediate physiological requirements. Subsequent transport of such populations to a region of the system deficient in P could result in utilization of P bound in poly-P bodies and re-mobilization of the incorporated Pb. By this mechanism the ultimate effect of Pb contamination may be both delayed and removed from the apparent source. Delayed and distant re-mobilization of sequestered Pb could also be effected by consumer organisms grazing phytoplankton developed near sources. In any event, understanding the interactions between nutrient levels and toxic trace metal contamination is important in evaluating their environmental impact.

We wish to acknowledge the technical assistance of Ms. D. Lazinsky. This work was supported by grants R805146020 and R805796-01 from the U.S. Environmental Protection Agency, Contribution Number 249 of the Great Lakes Research Division.

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