Preprint: Paper presented at the Institute of Gas, Technology Symposium on gas, oil and Environmental Biotechnology. Nov. 29-Dec. 1 Colorado Springs, CO.

METAL CONTENT OF MICROBES AND INORGANIC PARTICLES IN NATURAL AQUATIC ENVIRONMENTS: AN ANALYTICAL ELECTRON MICROSCOPIC STUDY

William A. Corpe, Ph.D.
Department of Biological Sciences
Columbia University
New York, New York 10027

Thomas E. Jensen, Ph.D.
Department of Biological Sciences
Lehman College, CUNY
Bronx, New York 10468

ABSTRACT

Natural populations of microorganisms are known to immobilize trace and heavy metals from their environments. An electron microscope (TEM) with a scanning transmission mode (STEM) in conjunction with an energy dispersive x-ray spectrometer (EDX) was used to determine the relative abundance of numerically important, metal bearing, cell and particle morphotypes. Metal ions may become bound to microbial cell surfaces, accumulate in cytoplasm or in cytoplasmic inclusions of the various cell types contained in biomass samples recovered from waters of lakes, rivers and streams. Metal bearing non-living particles recovered with the biomass fractions were also examined. Several examples will be shown of how the analytical system can be used to direct a search for important metal binding microbes and to facilitate the search for specific metals in water and sediments. The advantages and disadvantages of the system will be explained.

METAL CONTENT OF MICROBES AND INORGANIC PARTICLES IN NATURAL AQUATIC ENVIRONMENTS: AN ANALYTICAL ELECTRON MICROSCOPIC STUDY

INTRODUCTION

Microorganisms have evolved a number of ways to tolerate environments rich in heavy metals. Many of such microbes have been isolated from polluted sites [10], [19], [24]; many others have been cited in the book edited by Ehlich and Brierley [7]. The finding that such organisms may be able to accumulate heavy metals has encouraged surveys of polluted sites seeking to isolate new species. Balkwill and associates [2] carried out an extensive study of bacteria isolated from 21 contaminated sites. They isolated 626 distinct types in 1,112 isolates, based on growth in different media. In isolation studies one can not know with certainty that the most metal tolerant forms or the most efficient metal concentrating microbes have been isolated. Very little information however about the location of the accumulated metal in such cells is available. Work of this kind generally requires an intensive laboratory investigation.

Recent work in our laboratory has centered on a study of individual cells contained within biomass fractions collected from various fresh water samples as well as activated sludge. The approach is one which employs transmission electron microscopic (TEM) techniques in conjunction with an energy dispersive x-ray spectrometer [6], [14], [15].

In this paper we present some data which demonstrate how the TEM-EDX system can be used to evaluate metal accumulating cells and particles from aquatic environments.

MATERIALS AND METHODS

Samples

Water samples were collected from the Hudson River at Nyack, N.Y. and from Arthur Lake in Black Rock Forest, near Cornwall, N.Y. Glutaraldehyde was added to the 2 liter samples to give a final concentration of 3% (w/v). The fixed samples were filtered through a 20um Nytex screen and then submitted to high speed centrifugation at 14,000 xg for 2 hrs. The biomass was washed with distilled water two times, resuspended in 5 ml of distilled water and refrigerated until used. Some samples were processed as described above but without fixation.

Water samples were collected from streams containing run-off from an abandoned (Argo Mine, Idaho Springs, Co.) gold mine and mine tailings. The water samples examined were acid (pH 2.0 - 2.5) and found to contain, by enrichment culture, Thiobacillus ferrooxidans and T. thiooxidans (Corpe, Jawetz, Hazen and Jensen unpublished work). The stored samples (100-150 ml) were fixed with 3% (W/V) glutaraldehyde and then centrifuged 5,000 xg for 1 hour. The material sedimented from each sample (less than 10 mg wet weight) was refrigerated until used. Five rock samples (tailings) were ground to yield a finely divided powder. The powder was mixed with 2 N H_2SO_4 and shaken in a conical centrifuge tube over a period of 4 days at room temperature. Most of the insoluble material was removed by centrifugation at 2000 xg and discarded. The lightly turbid supernatent fluid was neutralized with 2N NaOH and concentrated to dryness in a stream of filtered air. The residue was "worked up" in 0.1 ml of glass distilled water with a glass rod, and stored in a refrigerator until used.

A two liter sample of activated sludge, provided by the staff of the Bergen County (N.J.) Sewerage Authority, was fixed with glutaraldehyde (3% W/V) overnight and centrifuged 10,000 xg for 30 min., washed once with distilled water and stored in a freezer until used.

A second two liter sample was not fixed but rather was used in an experiment, designed to study uptake and accumulation of heavy metals by the living microbes. Freshly collected activated sludge (pH 7.5) 100 ml (2.28 g dry wt.) was poured into each of eight Ehrlenmeyer flasks. They were supplemented with various metals to a final concentration of 100 ppm each: (1) Pb, AL, Zn, Cu, Cd (2) Cu and Cd (3) Cd (4) Cu (5) Pb (6)Al (7) Zn (8) un-supplemented control. The interactions were allowed to proceed at 25 C for 3 hours on a rotary shaker at 100 rpm. At the end of the incubation the pH was unchanged. The flasks were chilled and centrifuged to remove the biomass which was washed 5x with distilled water. The cells were suspended in water and mounted onto formvar coated Nylon, Cu, or Ni grids and air dried at room temperature overnight and then examined in the analytical electron microscope.

Electron Microscopy

Portions of the concentrated biomass were stained with osmium [20] and embedded in Epon [18]. Thin sections cut with a diamond knife were post stained in uranyl acetate [23] and then examined in the TEM.

X-ray Analysis

For X-ray analysis air-dried cells or other particles were first located using the TEM Mode of the Hitachi-H7000 operating at 75 kV. The microscope was then switched to the Scanning Transmission Electron Microscopic Mode (STEM Mode). Analysis was carried out in the SPOT Mode of the STEM. The SPOT diameter at 100,000 X is approximately 20 nm. The objective lens current was adjusted so a total x-ray count of 600 to 1000 CPS was attained. Count time was 100 sec. Spectra were collected on a PGT System 4 plus energy dispersive x-ray spectrometer (EDX) from cells or other particles and corrected for background.

RESULTS

Cells and non-living particles from lakes and the Hudson River

Our earlier studies had shown that about 50% of the bacteria-sized planktonic organisms possessed one or more intracellular polyphosphate body [6]. PPBs have as one of their properties the ability to immobilize cations [3]. Most of the lake bacterial PPBs (Figure 1a) contained P, Mg, Ca, and K as shown in Figure 1b. In addition all show detectable C and O emissions. In some of the bodies no K was detected. In about 25% a significant S and Fe peak were also present. Fifty percent of the PPBs showed the presence of small amounts of Cl and Na.

In three microbial morphotypes with PPBs, a significant Al peak was found. The presence of Al in PPBs of these organisms, demonstrates that metals can be selectively taken up by different species in nature [14].

In addition to polyphosphates other cell parts are known to be involved in the accumulation of metal ions by cells. They are, cell envelopes which include extracellular polymer and cell walls [6].

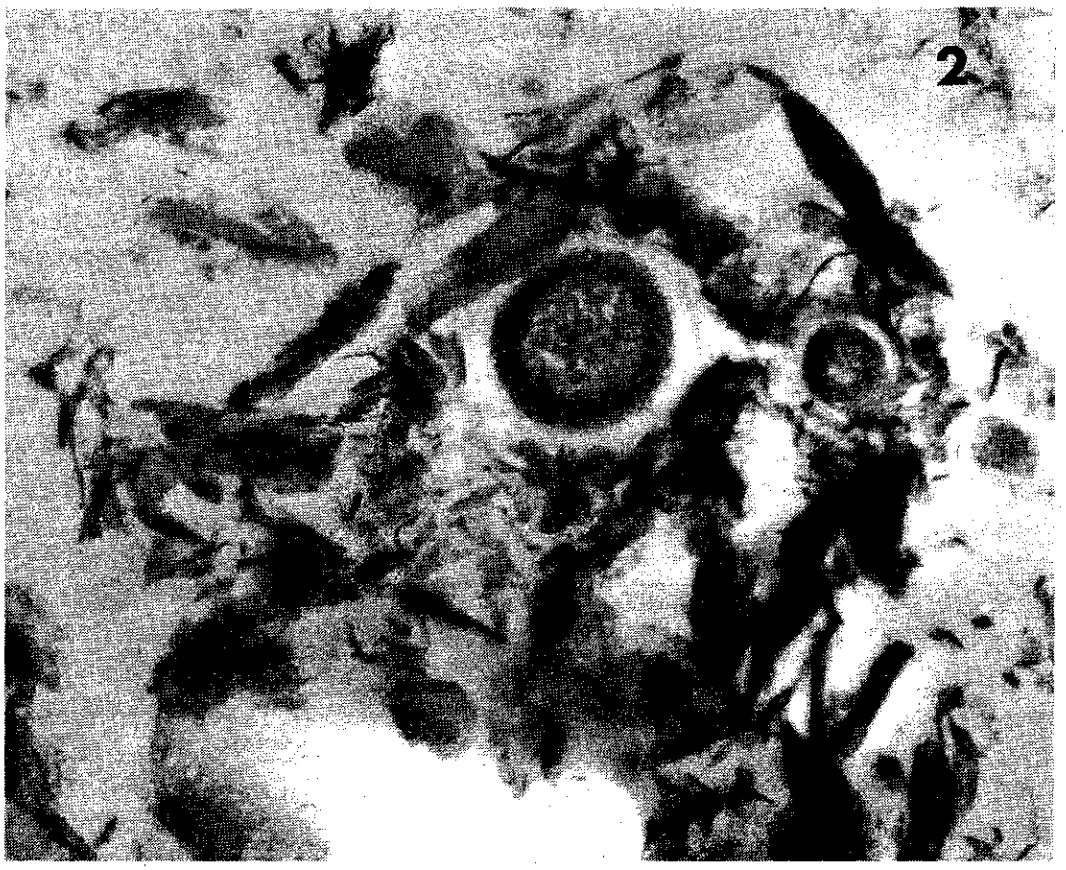

Figure 2. A small Hudson River bacterium with a capsule to which jagged particles are attached. The particles were organic. X 58,000.

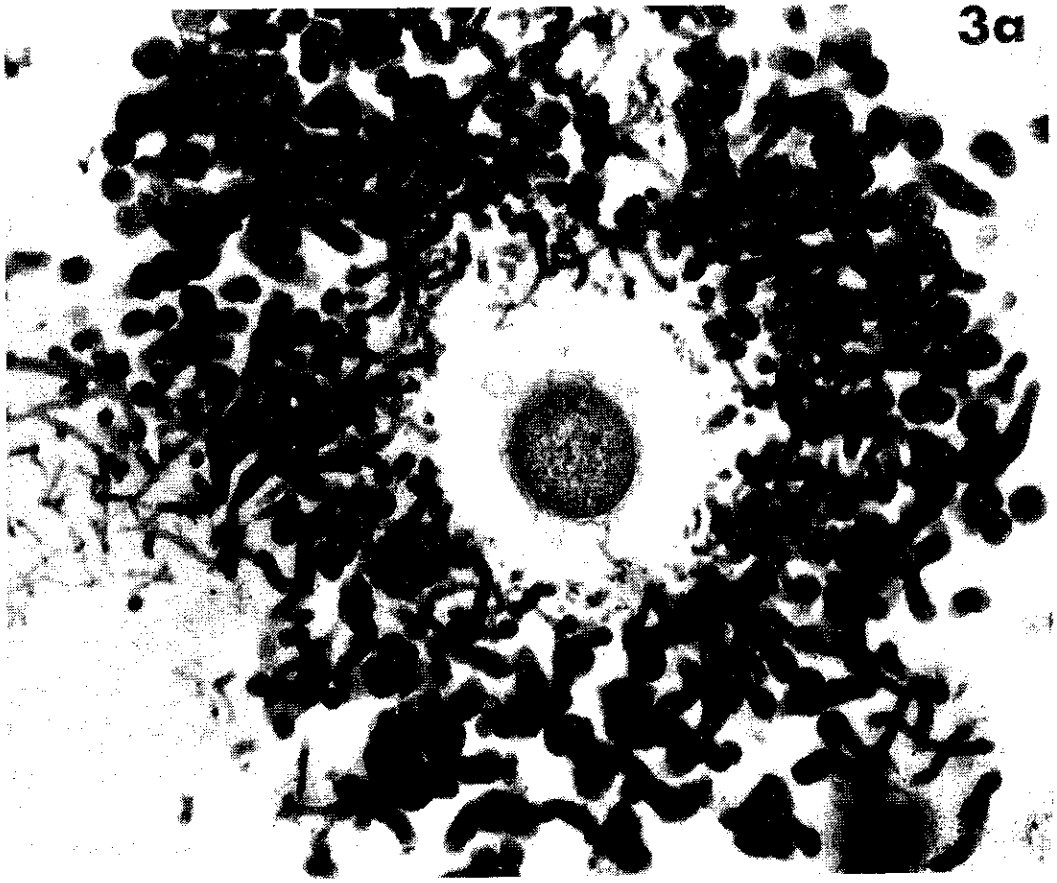

Figure 3a. A thin section of a capsulated, Hudson River bacterium surrounded by iron particles. X 60,000.

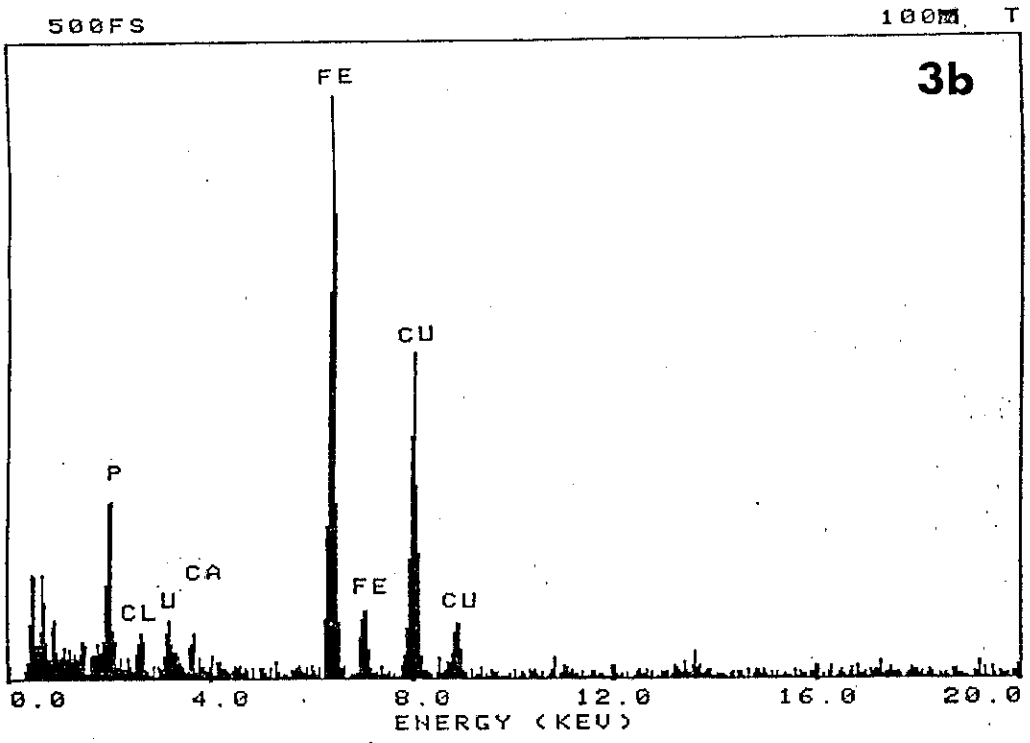

Figure 3b. EDX spectrum of iron particles. The section was mounted on a copper grid.

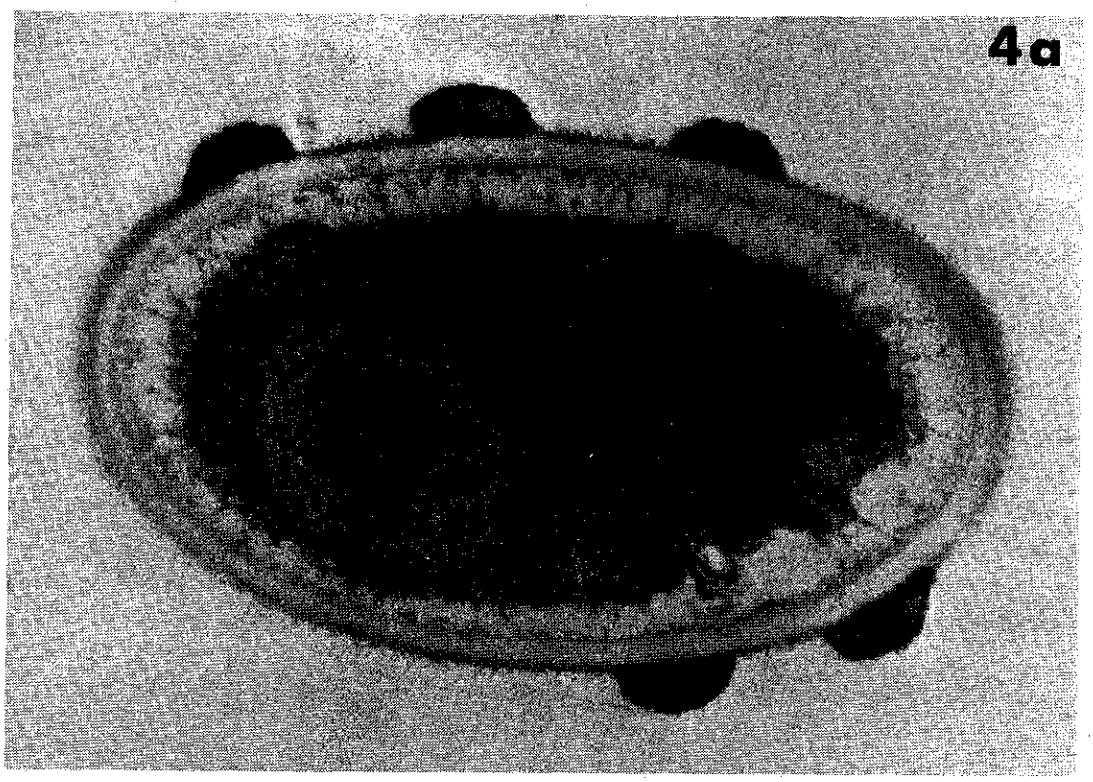

Figure 4a. A thin sectioned eukaryotic cell showing "mounds" of dense material attached to the outer cell wall surface. X 42,000.

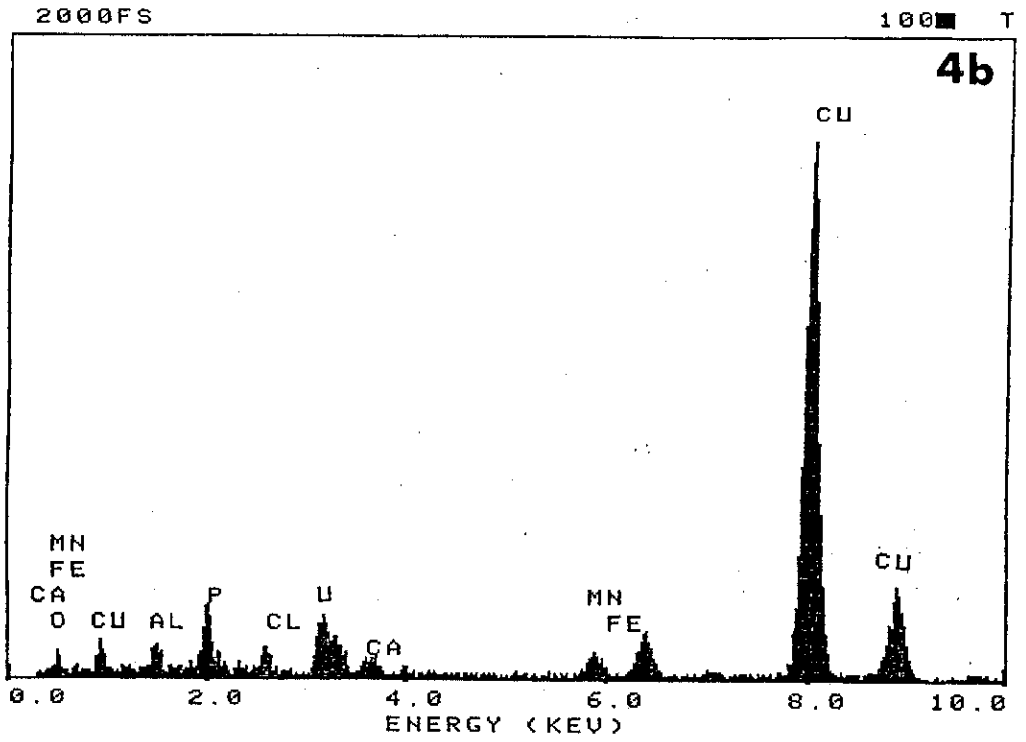

Figure 4b. EDX spectrum of dense mounds attached to the eukaryotic cell surfaces, that show the presence of Mn, Fe, Ca, P, Cl and Al. The section was stained with uranyl acetate and mounted on a copper grid.

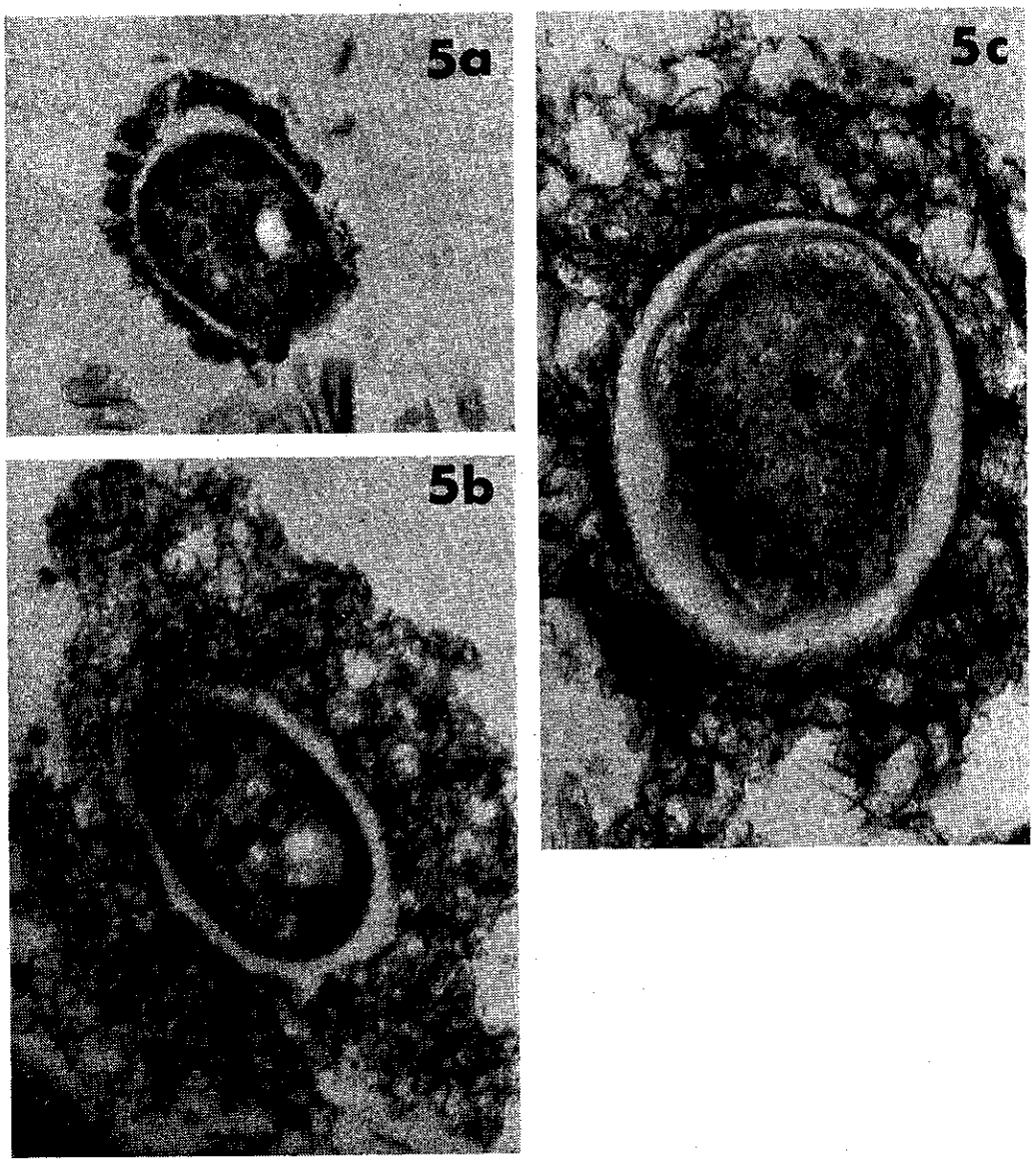

Figure 5. Three capsulated bacteria from Hudson River waters that show no tendency to form aggregate with particulate matter. (5a X 1000,000 5b X 60,000 5c X 60,000).

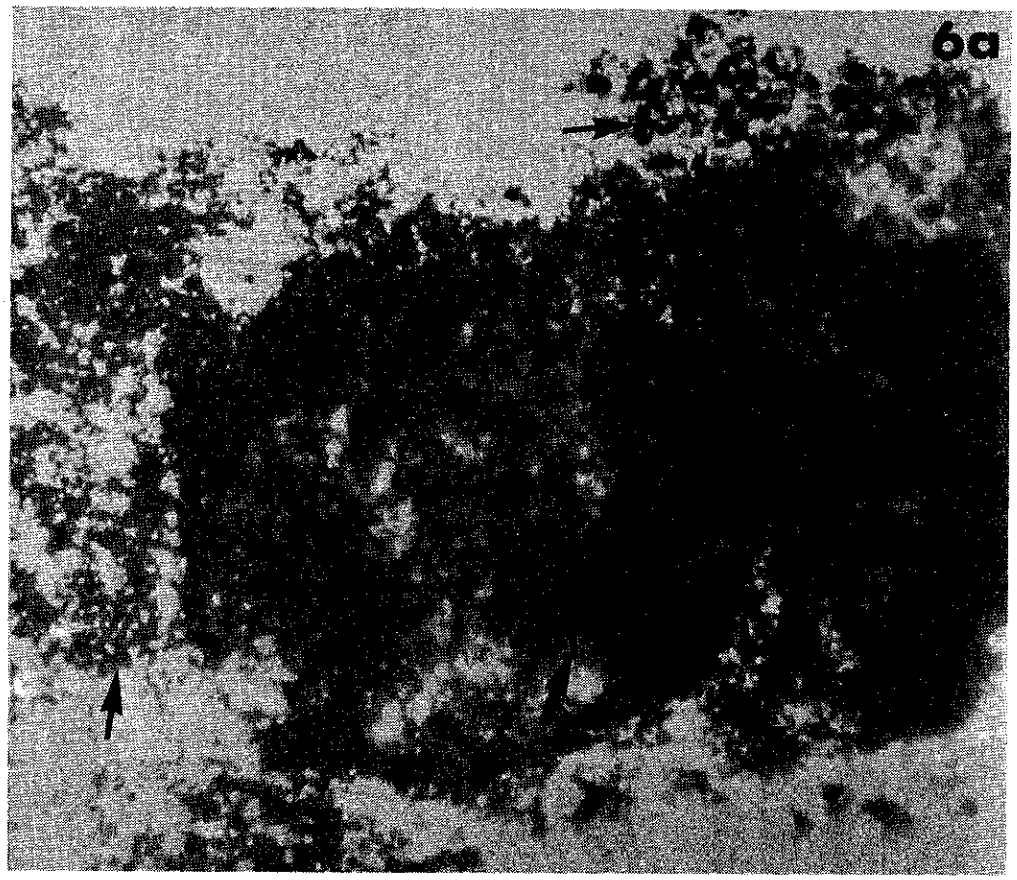

Figure 6a. Sedimentary material in which a gold particle was found, overlain by dense particles containing Fe and P. Large arrows are where probe was placed which showed only Au and small arrows where probe was placed which showed Fe and P. X 18,000.

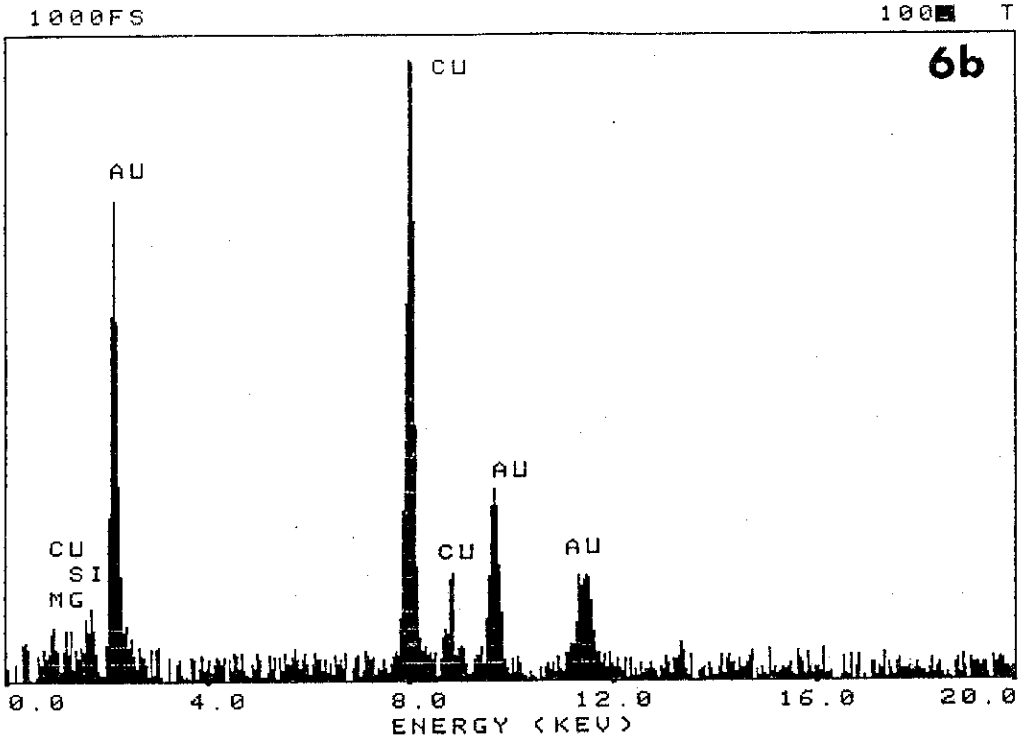

Figure 6b. EDX spectrum of "dense particle free" zone, gray in the electron beam showing three prominent gold peaks. Sample placed on a formvar coated copper grid.

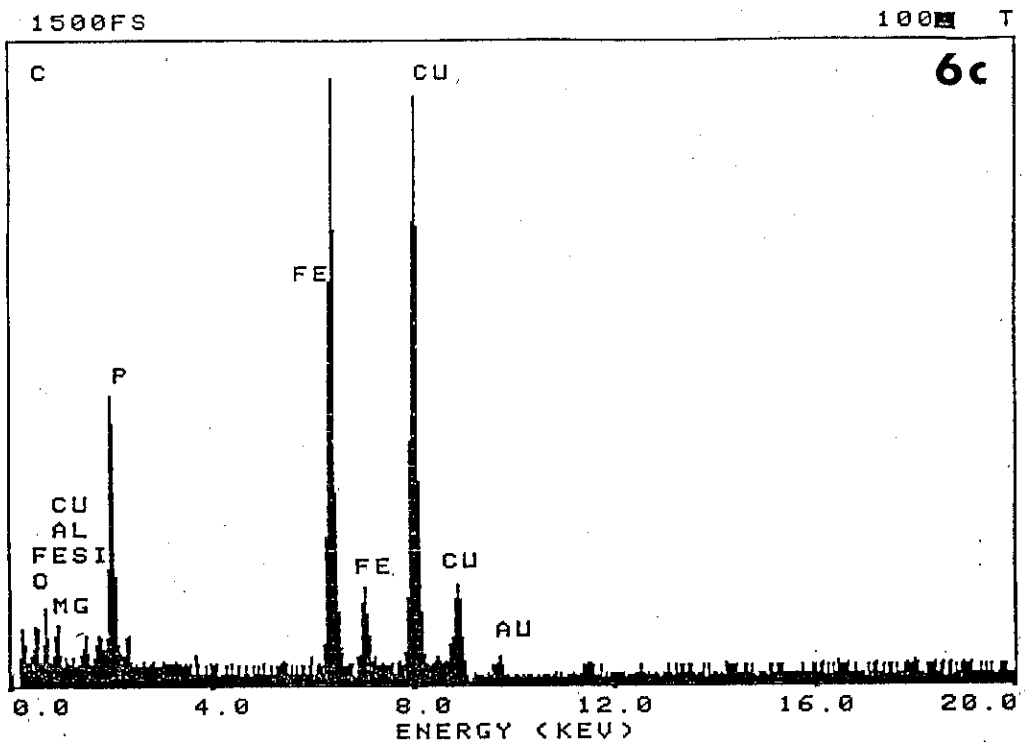

Figure 6c. EDX spectrum of electron dense particles. Note the minor gold peak.

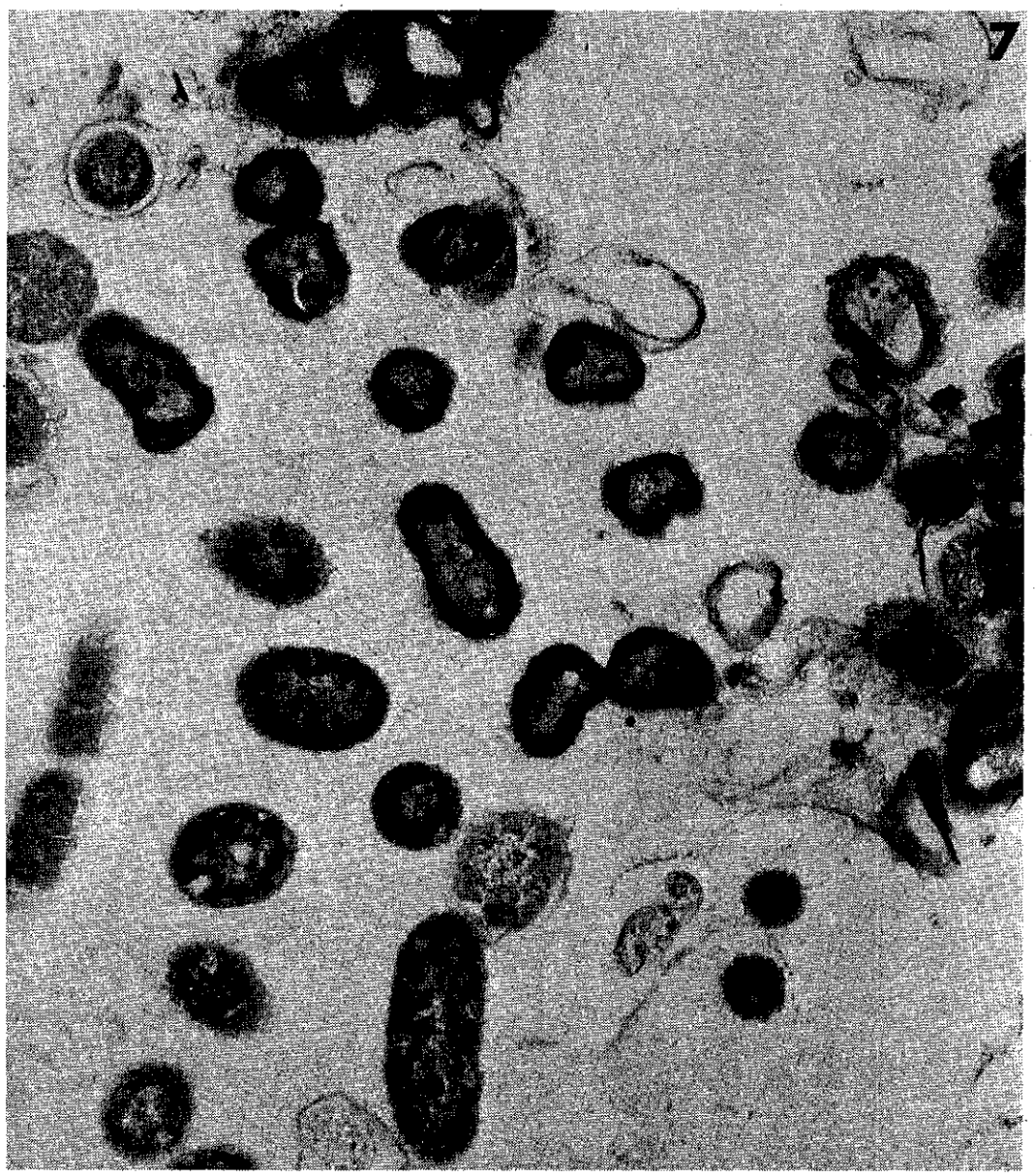

Figure 7. Various organisms found in activated sludge in thin section showing Gram negative, Gram positive cells, capsulated forms, and polyphosphate bodies. X 24,000.

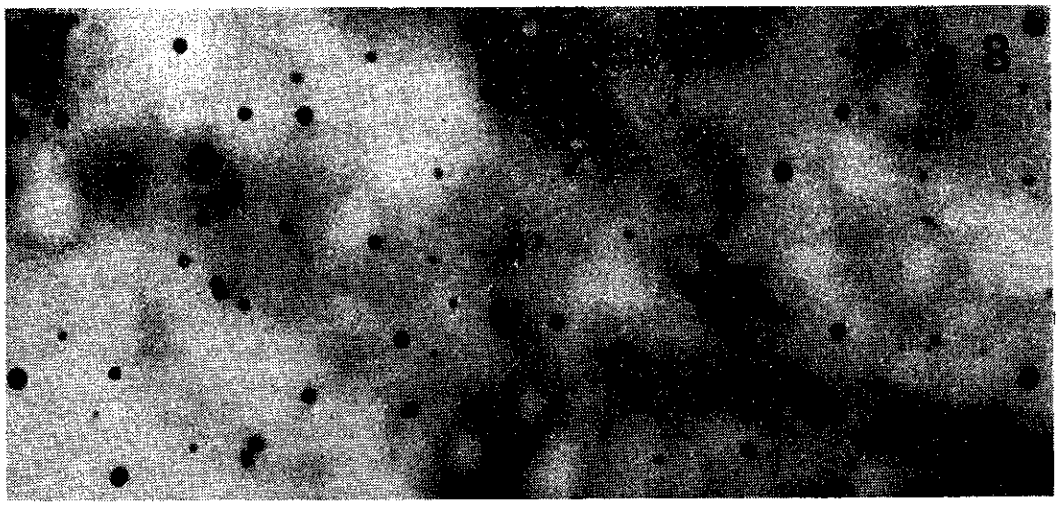

Figure 8. Polyphosphate bodies in the activated sludge biomass. In the air dried preparation the PPBs are dense in the electron beam. X 24,000.

In activated sludge exposed singly to Zn and Pb about 50% of the PPBs generate peaks for the elements. No Zn or Pb was detected in the walls or cytoplasm. In cells exposed singly to Cd, Al and Cu all PPBs had the elements. In addition the cell walls and cytoplasm generated small peaks for the elements. In activated sludge treated with both Cu and Cd all PPBs generated peaks for the elements. The cytoplasm and cell wall also generated a small peak. In addition about 90% of the PPB generated an x-ray peak for Al. In cells exposed to all 5 metals Pb, Al, Zn, Cu and Cd, 60% of the PPBs possessed peaks for all of the elements, 30% had peaks for Pb, Al, Cu and Cd and 10% had peaks for all elements except Zn. The walls and cytoplasm had peaks for Al, Cu and Cd (much smaller peaks). In all PPB the usual elements found in untreated controls were also present.

DISCUSSION

The use of the methods described in this paper allow rapid and accurate determination of elements present in samples. The method of preparation is simple as the sample has only to be mounted on a grid which is coated with a material such as formvar. The contribution of the supporting film can be easily subtracted by making an analysis of the material and then having the computer system subtract these x-rays from the sample of interest. This system also allows the simultaneous display of all the elements present in the sample. To determine which elements are present the systems allow quick and easy determinations. In general peak height is indicative of amount of the element present in the sample. It is not, however, quite so straight forward. Some elements generate x-rays from the various shells quite readily while other elements do not readily generate x-rays. The result is that each element has its own level at which it can be detected. This is generally in the 10^{-18} to 10^{-19} grams per ml range. This means that the system is very sensitive and will detect down to very low levels the elements present in a sample. To determine accurately the amount of metal is much more labor intensive. Standards must be run and entered into the computer. The sample can then

be analyzed using the same parameters. This information can then be used to determine the amount of the different elements in either PPMs or grams per ml [9].

In the work reported here we can see how the method is used. In the case of iron we were able to detect it in cell surface polymers of some bacteria. Many capsule producing bacteria however showed no metal content with the EDX spot mode. This could mean (1) that the capsule in thin section have little density and the amount of metal/spot area is insufficient to generate a spectral peak or (2) the polymers of all the capsulated cells are not invariably the polyanionic type, and do not take up cations.

The minute particles found in the picoplanktonic (0.2-2.0 uM) fractions isolated from water column samples were easily analyzed using our system and were reported in detail [15]. The particles were of several morphotypes each with distinctive composition. Particles of this dimension are generally considered to be soluble [25]. We can see that with our method this definition must be taken to a different level. There were no particles of such small size range that contained only C and O, however other particles larger than 2 uM were certainly present in water samples one of which is shown in Figure 2, aggregated about a capsulated bacterium.

Particles of much larger size were encountered in the samples taken from Idaho Springs and run-off samples, from the Argo mine and its tailings. We could again determine easily the elemental composition of these particles. Suspensions of the rust-red sediment removed by centrifugation was deposited on Formvar coated grids and air dried. The grids were searched for microbial cells and particles that contained spectroscopic evidence of gold which was observed in one sample. The microbial cells encountered but not shown, were probably residue of dead fungi and had no unusual metal content. The small inorganic particles were probably iron hydroxide(Figure 6c). Fairly large particles which appeared grey in the electron beam gave a large peak for gold when analyzed. We interpret this area to be a spherical area where the preparation drop dried. Underneath we envision a thin gold particle with lobes which are seen as the gray area underneath the iron particles (Figure 6a). The particle shown in Figure 6a is vaguely similar to those derived from Welch coal samples by Gayer and Rickard [8]. We could have prepared a cleaner display by removing the iron particles.

Very small peaks for gold were found in 2 of 6 samples of tailings extracted with 2N H_2SO_4. No specific particles could be observed in the background or other debris but the surface of the grid was scanned using the rassetter mode (beam scan of a small area) and a small peak was found. In this case we believe the acid probably decomposed the rock sample releasing small gold particles. We did not attempt to determine if soluble gold salts exist in the water. The gold from the water sample described above is clearly particulate, probably in the water when the sample was taken. They would probably have been more abundant in sediment samples.

Polyphosphate bodies (PPB)

The presence of PPBs was recognized in about half of the thin sections of bacteria sized plankton in an oligotrophic lake [6]. The finding suggests the ability of a large proportion of the cells to store phosphorus in this manner. Accumulations are often correlated with exogenous nutrient limitation and low endogenous rates of metabolism [16]. The conservation of phosphate seems to be one of the functions of insoluble polyphosphate in cells. Work in the Jensen laboratory has shown that in cells of cyanobacteria they function in the immobilization of trace and heavy metals [3], [11], [12], [21], [13], [22]. An examination of a large number of picoplanktonic bacterial

cells from Lake Arthur showed that at least three morphologically distinct organisms in that environment were able to concentrate Al in addition the usual elements, Mg, P, Ca and K in their PPBs [14]. Some of the PPBs also possessed S, Cl, Na and Fe [14].

Aluminum and other metal ions being concentrated in polyphosphate bodies causes one to visualize the potential use of cells rich in PPBs as metal absorbants. Cell bodies of activated sludge fits this requirement as many PPBs are present in the wide variety of cell types in this biomass (Figure 7). The preliminary data presented here indicate that this system may be one which could be adopted using the correct parameters, for metal removal from water.

We examined activated sludge as a rich source of PPBs. We have begun a study of the capacity of those inclusions to take up metals. The preliminary studies reported here found as with other bacteria that polyphosphate bodies take up trace elements. We were moderately surprised that PPB in fresh activated sludge did not also contain, substantial amounts of heavy metal [1]. When the cells were exposed in the laboratory to high concentrations of such metals for a period of three hours, PPBs were found to take up more than enough metal to provide a substantial signal by the EDX method. The preliminary explanation of these observations include the fact that (1) many metals may be absorbed to cell surfaces and not transported or allowed to diffuse into the cytoplasm of living cells (2) high concentrations of soluble salts of heavy metals may denature cell membranes and the Law of Mass action could explain why the metals move into the cytoplasm where they can be sequestered in the PPB.

We can see that the method we have explored TEM-STEM-EDX is a way to easily determine the elemental composition of particulates. It is a method which should find wide use in determining the elemental composition of small particles as well as studies to determine how elements move and are sequestered and then perhaps released into the environment. It may prove an invaluable method in ecological, toxicological and minerological studies.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Mike Baxter, Lise Hazen, Robert Jawetz and Andrei Shtanko in carrying out various aspects of this work. The research was supported by grants from The Black Rock Forest Consortium and The National Science Foundation Grant BC5-91-17165 to W.A. Corpe and T.E. Jensen; and the Faculty Research Award Program (Grants 6-62174 and 6-63161) to T.E. Jensen.

REFERENCES CITED

1. Aitken, M.D., Heck, P.E., Alvarez-Cohen, L., Grimberg, S.J. and Stringfellow, W.T. "Activated sludge," Water Environm. Res. 65,324-345. (1993).

2. Balkwill, D.L., Fredrichson, J.K. and Thomas, J.M. "Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep southeast coastal plain sub-surface sediments.," Appl. and Environment. Microbiol. 55,1058-1065. (1989).

3. Baxter, M. and Jensen, T.E. " Uptake of magnesium, strontium, and manganese by Plectonena boryanum (Cyanophyceae) with special reference to polyphosphate bodies," Protoplasma 104,81-89. (1980).

4. Brierley, C.L. "Microbiological Mining," Sci. Am. 247, (2) 44- (1982).

5. Corpe, W.A. and Jensen, T.E. " Major antigens in Methylobacterium species and their location in cells using immunoelectron microscopic methods," Cytobios 67,117-126. (1991).

6. Corpe, W.A. and Jensen, T.E. "An electron microscopic study of picoplanktonic organisms from a small lake," Microbial Ecol.. 24,81-197. (1992).

7. Ehrlich, H.L. and Brierly, C.L. (Editors) "Microbial Mineral Recovery," N.Y.: McGraw-Hill Book Co., (1990).

8. Gayer, R. and Rickard, "Gold in South Wales Coal." Nature 364, 38 (1993).

9. Hall, T.A. and Gupta, B.J. "EDS quantitation and applications to biology" In: Introduction to Analytical Electron Microscopy, (Editors). Hren, J.J., Goldstein, J.I. and Joy, D.C. N.Y.: Plenum (1979).

10.Horikoshi, T., Nakajima, A. and Jasper, S. "Studies on the accumulation of heavy metal elements in biological systems XIX. Accumulation of uranium in microorganisms." European J. Appl. Microbiol. Biotechnol. 12,90-96. (1981).

11.Jensen, T.E., Rachlin, J.W., Jani, V. and Warkentine, B. "An x-ray energy dispersive study of cellular compartmentalization of lead and zinc in Chlorella saccharophila (Chlorophyta), Navicula incerta, and Nitzachia closterium. (Bacillariophyta)." Environ. and Experimental. Bot. 22,319-328. (1982).

12.Jensen, T.E., Baxter, M., Rachlin, J.W. and Jani, V. "Uptake of heavy metals by Plectomona boryanum (Cyanophyceae) into cellular components, especially polyphosphate bodies: an x-ray energy dispersive study." Environm. Poll. (Series A) 27,119-127. (1982).

13.Jensen, T.E., Baxter, M., Rachlin, J.W., Jani, V. and Warkentine, B.E. "Heavy metal uptake in relation to phosphorus nutrition in Anabaena variabilis (Cyanophyceae)," Environm. Poll. (Series A)42,261-271. (1986).

14.Jensen, T.E. and Corpe, W.A. "Elemental composition of the polyphosphate bodies in microbial cells from a small lake." Arch. Hydrobiol. 127,385-393. (1993).

15.Jensen, T.E. and Corpe, W.A. "Elemental analysis of non-living particles in picoplankton fractions from oligotrophic lake water." Water Res. (In press).

16.Kulaev, I.S. and Vagobov, V.M. "Polyphosphate metabolism in microorganisms." Adv. Microbiol. Physiol. 24,83-171. (1983).

17.Lake, D.L., Kirk, P.W.W. and Lester, J.N. "Fractionation, characterization, and speciation of heavy metals in sewage sludge and sludge-amended soils: A Review." J. Environm. Qual. 13,175-183. (1984).

18.Luft, J.H. "Improvements in epoxy resin embedding methods." J. Biophys. Biochem. Cytol. 9,409-414. (1961).

19.Macashie, L.E. and Dean, A.C.R. "Cadmium accumulation by microorganisms." Environmental Technol. Letters 3,49-56.(1982).

20.Pankratz, H.S. and Bowen, C.C. "Cytology of blue-green algae. I. The cells of Symploca muscorum." Am. J. Bot. 50,387-389. (1963).

21.Rachlin, J.W., Jensen,, T.E. and Warkentine, B. "The toxicological response of the algae Anabaena flos-aquae (Cyanophyceae) to cadmium." Arch. Environm. Contam. Toxicol. 13,143-151. (1984).

22.Rai, L.C., Jensen, T.E. and Rachlin, J.W. "A morphometric and x-ray energy dispersive approach to monitoring pH-altered cadmium toxicity in Anabaena flos-aquae." Arch. Environm. Contam. Toxical. 19,479-487. (1990).

23.Stempak, J.B. and Ward, R.T. "An improved staining method for electronmicroscopy" J. Cell. Biol. 22,697-701. (1964).

24.Trevors, J.T. and Cotter, C.M. "Copper toxicity and uptake in microorganisms." J. Industrial Microbiol. 6,77-84.(1990).

25.Wotten, R.S. The Biology of Particles in Aquatic Systems (Editor Wotten, R.S.) Boca Raton, Fl. CRC Press, (1990).