The Location and Nature of Accumulated Phosphorus in Seven Sludges from Activated Sludge Plants which Exhibited Enhanced Phosphorus Removal*

L. BUCHAN
Laboratory and Technical Services, City Health Department, PO Box 1477, Johannesburg 2000

Abstract

Electron microscopy combined with the energy dispersive analysis of X-rays (EDX) has been used to examine the nature of the phosphorus accumulated in sludges from seven activated sludge plants exhibiting enhanced phosphorus removal.

Large phosphorus accumulations were located in identical structures in the sludges examined. The phosphorus was located in large electron-dense bodies, within large bacterial cells which were characteristically grouped in clusters. The calcium:phosphorus ratio of these electron-dense bodies precluded them from being any form of calcium phosphate precipitate. Quantitative analysis indicated that the electron-dense bodies contained in excess of 30% phosphorus. The results obtained are supportive of a biological mechanism of enhanced phosphorus uptake in activated sludge.

Introduction

A considerable time has elapsed since 1964 when it was first observed at the Rilling Road plant, San Antonio, Texas, that the activated sludge process could remove significant quantities of phosphorus in excess of normal metabolic requirements (Vacker, Connell and Wells, 1967). A considerable amount of research has since been carried out in many parts of the world to determine the mechanism of this enhanced phosphorus removal.

The purpose of this investigation was to clarify certain aspects of the removal mechanism. For this purpose use was made of electron microscopy combined with energy dispersive analysis of X-rays (EDX) to identify the location and nature of the stored phosphorus.

TABLE 1
SELECTED PHYSICAL AND CHEMICAL PROPERTIES OF THE SLUDGES TAKEN FROM THE SEVEN PLANTS EXAMINED

Activated Sludge Plant	Feed[1] COD: TKN	MLSS[2] mg/ℓ	Phosphorus (mg/ℓ)[3] Influent	Effluent
Laboratory Scale Plant Biochemistry 1	15:1	2 100	9	<1
Laboratory Scale Plant Biochemistry 11	15:1	1 800	9	<1
Brits	—	3 950	4,7	<1
Goudkoppie 1[4]	8:1	2 700	6,6	1,7
Goudkoppie 11[4]	8:1	3 100	6,6	1,1
Northern Works[4]	7,5:1	1 300	7,1	6,4
Umhlatuzana	15:1	6 303	9,0	<1

(1) COD: Chemical Oxygen Demand
TKN: Total Kjeldahl Nitrogen
(2) MLSS: Mixed Liquor Suspended Solids
(3) The figures for the laboratory scale plants and Brits were for orthophosphate, all the other values were for total phosphorus as P
(4) Plants operated by the Johannesburg City Council

Materials and Methods

Sludge Samples

Samples of activated sludge initially examined were drawn from the 4,8 Mℓ/d Umhlatuzana treatment plant at Pinetown. By manipulation of the aeration facility exceptionally good removal of both phosphorus and nitrogen was achieved in this plant (Kerdachi, 1979; Kerdachi and Roberts, 1980).

Subsequently, samples were also obtained from a works operated by the Town Council of Brits, from two laboratory scale plants operated by the Biochemistry Department at the University of Pretoria and three plants operated by the City Council of Johannesburg. Some characteristics of these plants are recorded in Table 1.

Electron Microscopy

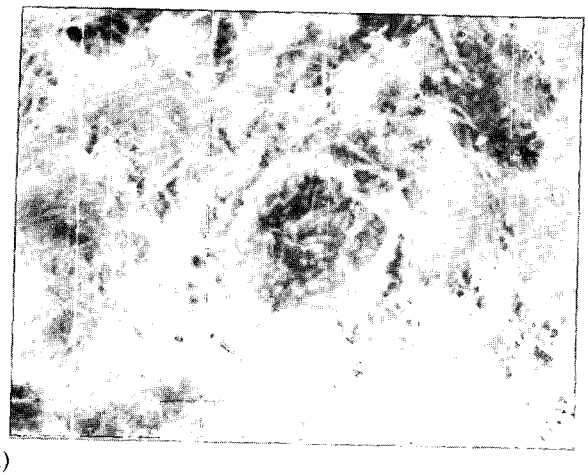
Preparation of Material

Samples used for scanning electron microscopy were prepared by fixation in gluteraldehyde, dehydrated and critical point dried.

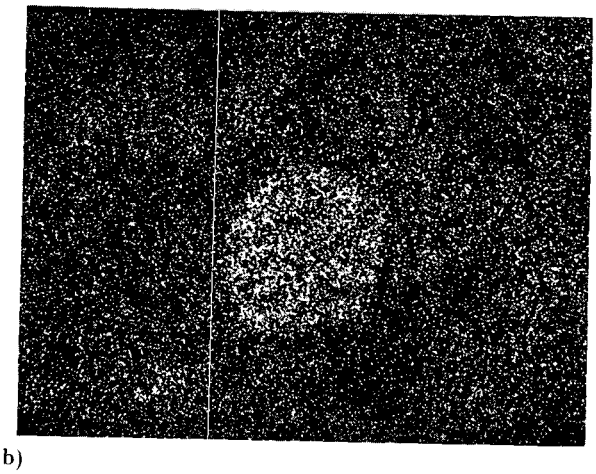
Samples used for thin sections were fixed with gluteraldehyde, followed in certain cases with osmium tetroxide (when elemental analysis was not performed), dehydrated and embedded in Spurrs resin (Spurr, 1969). Areas which stained metachromatically with methylene blue in 1 μm sections were located by floating 1 μm sections onto beryllium grids, staining with 1% methylene blue, rinsing in double-distilled water and examination by light microscopy.

*Data from a thesis being submitted to the Department of Microbiology, Faculty of Agricultural Sciences, University of Pretoria.

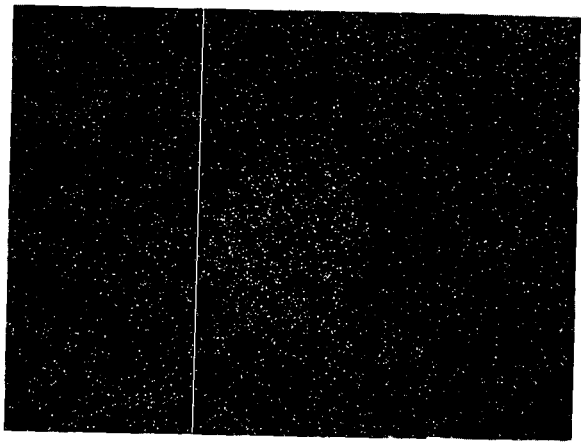
Some sections were stained with uranyl acetate and lead citrate, or with 1% potassium permanganate. Sections used for electron diffraction and energy dispersive analysis of X-rays were examined unstained. Samples used for scanning electron microscopy were carbon-coated.



(a)



(b)



(c)

Figure 1

(a) The morphology of a sample of activated sludge from the Umhlatuzana works as observed in the SEM X 1250.

(b,c) The distribution of phosphorus (b) and calcium (c) for the same sample.

The high concentration of phosphorus associated with the cluster of large cells is clearly visible.

Instrumentation

The external morphology and elemental distribution of calcium and phosphorus in the mixed liquor was investigated with a Philips PSEM 500 scanning electron microscope (SEM) with an EDX attachment.

A Philips 301 transmission electron microscope (TEM) was used to investigate the detail of thin sections (0,1 μm – 1,0 μm) and a Jeol JSM u3 scanning transmission electron microscope (STEM) with an EDX attachment was used to determine the presence and ratio of various chemical elements with atomic numbers larger than 11. Sections were prepared on a Reichert OMU-3 ultra microtome and light microscopy was performed with a Reichert-Univar research microscope.

Energy dispersive analysis of X-rays

Elemental atomic ratios were determined according to the ratio method developed by Duncomb (1968), Russ (1973) and Russ (1974). The P-values were obtained with a computer program published by Russ (1975).

For quantitative analyses, use was made of a 0,5%, 0,1 μm calcium standard embedded in araldite (Agar Aids). The method used was an adaptation of the peak to background method developed by Hall (1971).

Results and Discussion

Investigations Using the SEM-EDX-System

It was found that areas of high phosphorus concentration were associated with what appeared to be clusters of large bacterial cells (Fig. 1).

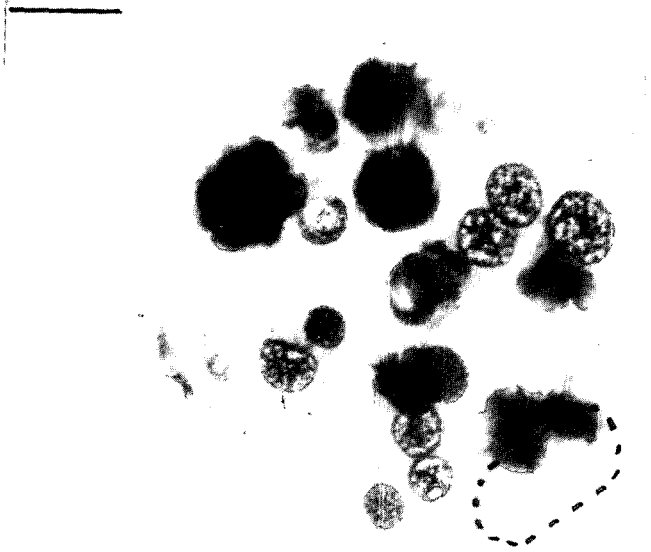


Figure 2

A cluster of large cells as observed in the TEM. The outline of one cell is marked with the dotted line. Bar represents 1 μm.

Investigations Using the TEM and STEM-EDX-System

Examination of metachromatic areas in 1 μm sections with the TEM revealed that they were clusters of relatively large bacterial cells, characterised by the presence of large intracellular electron-dense bodies and by areas within the cells where their contents appeared to have been torn out during sectioning (Fig. 2). The cells which contained these electron-dense bodies were larger than any other of the bacterial cells observed in the specimens.

STEM-EDX analyses of the cell clusters indicated that the electron-dense bodies were composed almost entirely of phosphorus and calcium with traces of magnesium, chlorine and potassium also occurring.

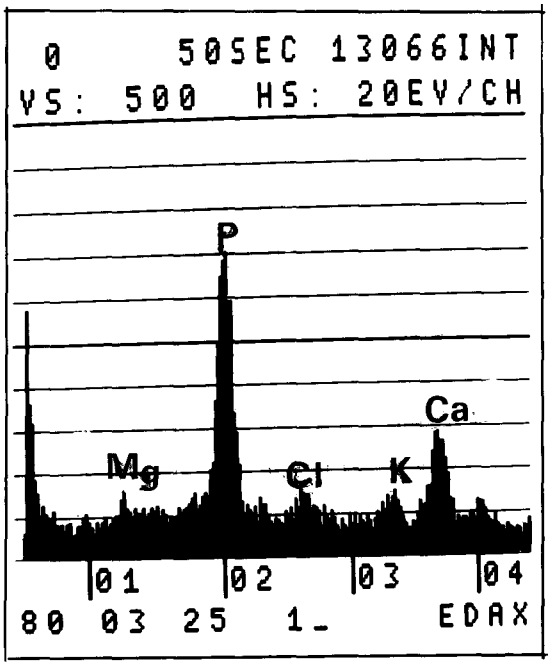


Figure 3
The X-ray energy dispersive spectrum generated by an electron-dense body in a 1 μm section of umhlatuzana sludge

Random micro-analyses over the surface area of the dense bodies produced identical spectra, indicating a uniform composition.

The calcium:phosphorus mass ratio for 25 analyses performed on different electron-dense bodies was consistently in the order of 0,254. These data indicated that the composition of the electron-dense bodies did not conform to the composition of a calcium phosphate species.

The electron-dense bodies were observed to undergo degradation when the electron beam was focused on them. This degradation led to the loss of the electron density of these bodies (Fig. 4).

The dense bodies did not exhibit electron diffraction patterns, but after degradation the remaining shell was scattered with crystals and a diffraction pattern was discerned (Figs. 5a and b).

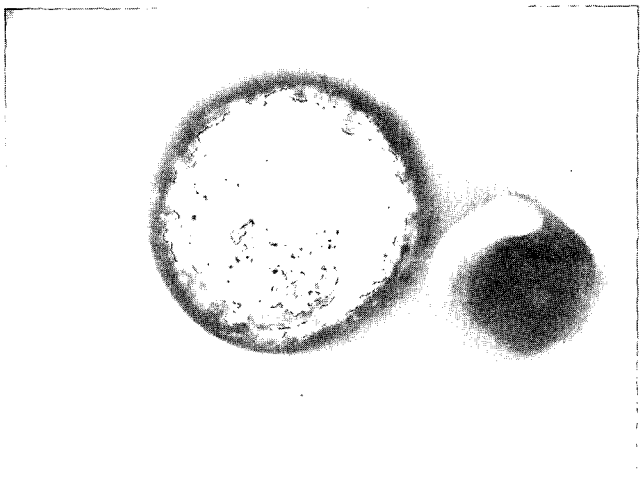


Figure 4
A dense body which had lost its electron density after degradation was induced by the electron beam. Bar represents 1 μm.

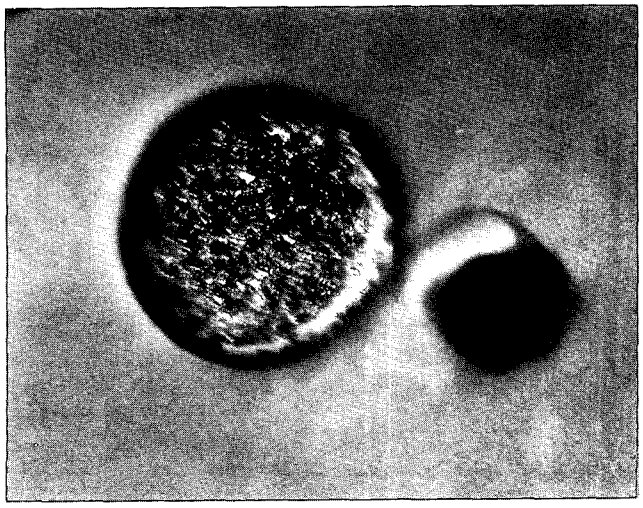


Figure 5a
A dark field image of a dense body after degradation, showing the presence of scattered crystals. Bar represents 1 μm.

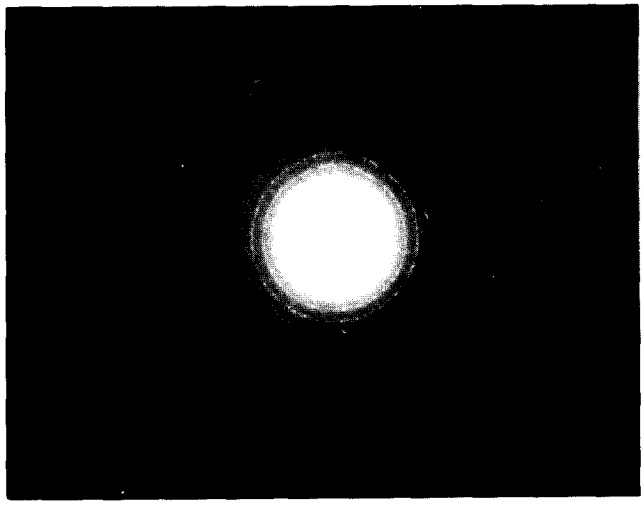


Figure 5b
The electron diffraction pattern obtained after degradation of a dense body, as illustrated in Figures 4 and 5a.

The data indicated that the electron-dense bodies were probably polyphosphates which could undergo degradation when bombarded with an electron beam. The calcium:phosphorus X-ray intensity ratio, as determined at various points across the diameter of an electron-dense body after degradation, were 0,50; 0,278; 0,669; 1,342; 1,05 and 0,72 respectively.

From this data it was evident that after degradation certain regions exhibited X-ray intensity ratios which could be related to some form of calcium phosphate precipitate.

Quantitative analyses of 12 electron-dense bodies indicated that they contained phosphorus concentrations in excess of 30% (m/m), and calcium concentrations in excess of 10% (m/m). Considering Figure 6 it would be conservative to estimate the electron-dense bodies as occupying 60% of the total cell volume. This would imply an intracellular concentration of phosphorus (in the form of polyphosphates) in excess of 18%. Because of the relatively high density of these bodies this figure is probably an underestimation.

In the many reports on polyphosphate accumulation in bacteria, no organisms have been cited which contain such high concentrations of phosphorus. The importance of calcium as a stabilizing cation for these polyanions has also been reported in only a few organisms. It is significant that in *Micrococcus lysodeikticus*, which accumulates polyphosphates on a complete growth medium (Friedberg and Avigad, 1968), calcium was also found to be an important stabilizing cation.

The density of the polyphosphate granules in *M. lysodeikticus* was determined to be 1,23 g/cm^3 by Friedberg and Avigad (1968). The density of these bodies could be the reason why they were sheared out of the cells during sectioning.

The dense bodies seemed to be arranged around a central spherical body, perhaps a proteinaceous structure (Fig. 6).

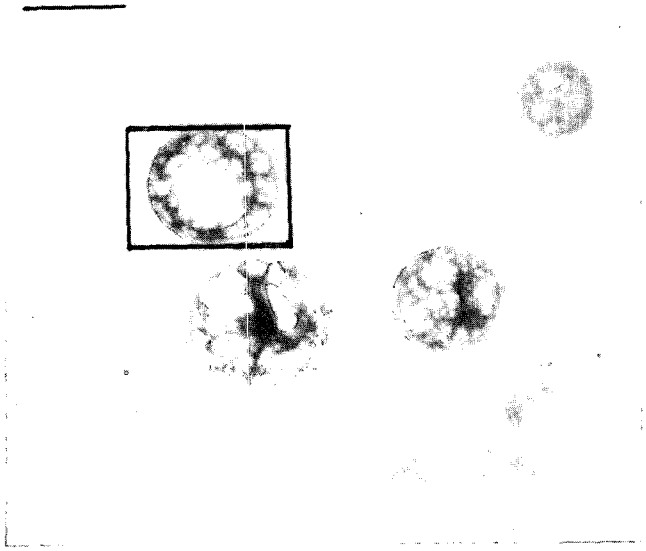


Figure 6
An electron-dense body, illustrating the central spherical body (framed area). The cell volume which the electron-dense bodies can occupy is visible in the cell below the framed area. Bar represents 1 μm.

To avoid excessive shearing from ultra thin sections, specimens were mostly prepared in the range 0,4 μm to 1,0 μm. Figure 7 illustrates the structure of phosphorus accumulating cells in a 0,1 μm section.

In all the activated sludge specimens examined, it appeared as if large accumulations of phosphorus were associated with tight clusters of relatively large cells. The variation in the performance of the sludges in the uptake of phosphorus seemed to be related to the number of large phosphorus accumulating cells, the size of the cell clusters and the size of the dense bodies within the cells.

It was also apparent that the only other cells which contained polyphosphate inclusions were smaller and closely associated with the large polyphosphate-containing cells. The im-

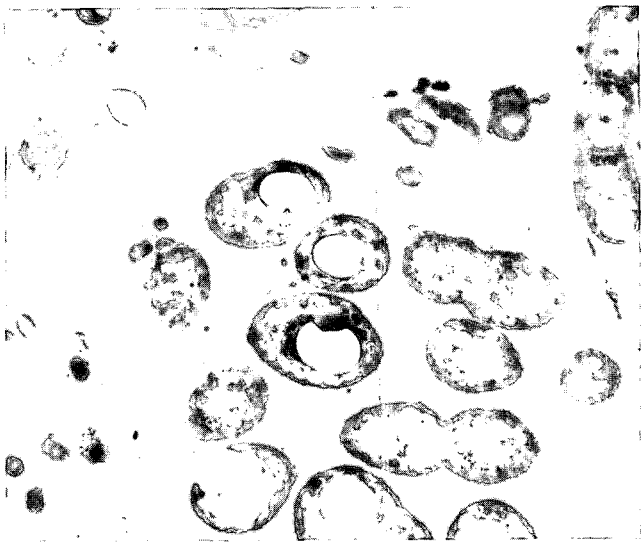


Figure 7
Phosphorus-accumulating cells as observed in a 0,1 μm section. Note the holes torn in the cells. Bar represents 1 μm.

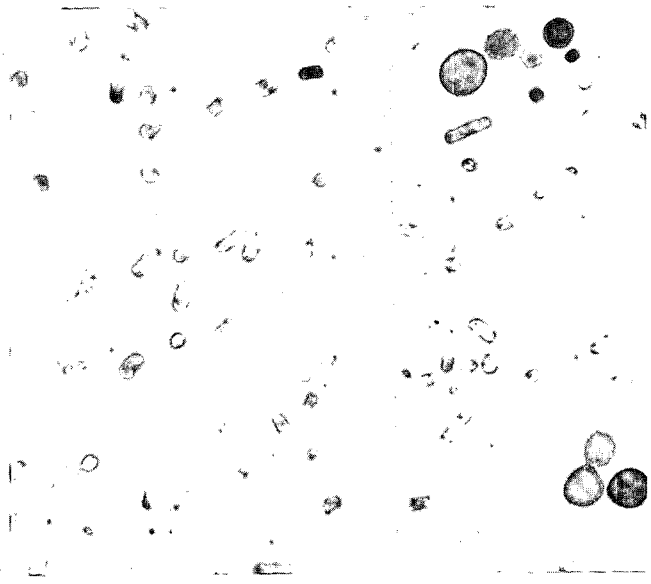


Figure 8
Acinetobacter calcoaceticus *grown on acetate enrichment agar (Fuhs and Chen, 1975). The large electron-dense cells contained numerous polyphosphate inclusions. Bar represents 1 μm.*

pression gained was that these cells were undeveloped forms of the larger cells. That the increase in the size of the cells was associated with increased polyphosphate accumulation was confirmed in another investigation with a pure culture of *Acinebactor calcoaceticus* isolated from a sewage works. From this investigation it was apparent that the organisms in the culture which accumulated polyphosphates were considerably larger than the other cells (Fig. 8). The results of this investigation will be reported at a later date.

Activated sludge from Umhlatuzana and from the laboratory scale plants at the University of Pretoria, which all removed high levels of phosphorus, contained large clusters of large cells with large dense bodies (Fig. 9). The polyphosphate-containing cells constituted the major portion of the cells present in these sludges.

Activated sludge from the Goudkoppie and Brits plants contained smaller clusters and visibly fewer large cells. The large cells, however, also contained large dense bodies (Fig. 10).

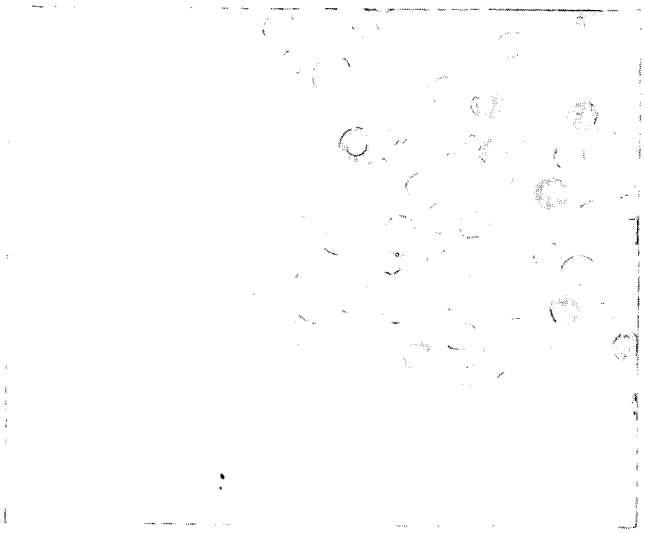


Figure 9a
A cluster of polyphosphate-containing cells as observed in a 0,4 µm section of Umhlatuzana sludge. Note the holes in many of the cells and the smaller cells which also contained polyphosphate inclusions. Bar represents 1,0 µm.

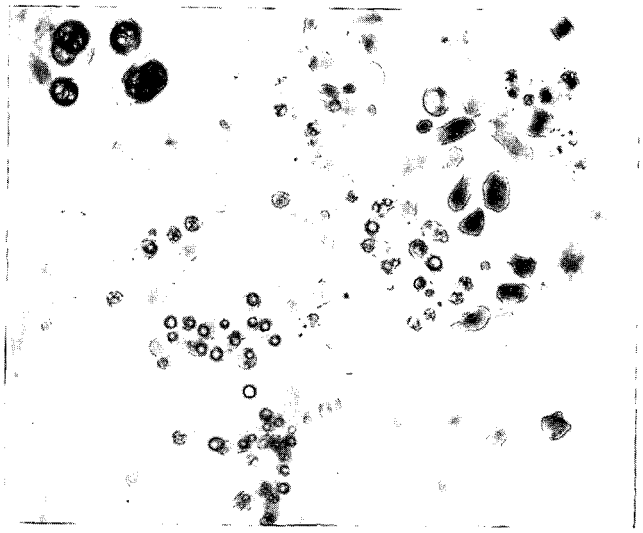


Figure 10a
A 0,4 µm section of activated sludge from the Goudkoppie works. Bar represents 1,0 µm.

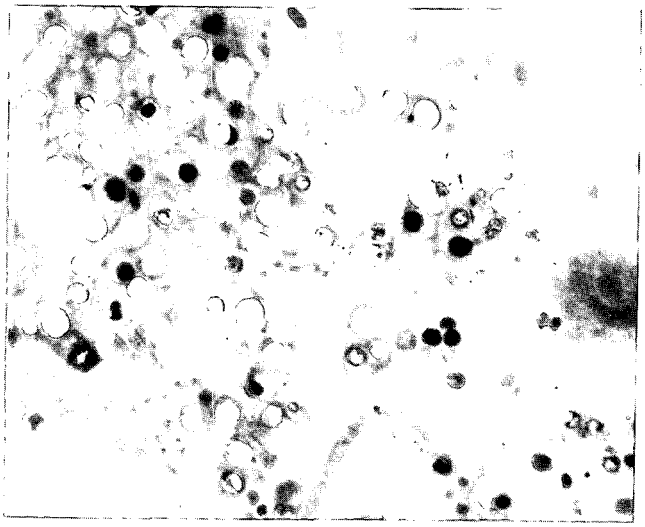


Figure 9b
A cluster of polyphosphate-containing cells in a 0,4 µm section of sludge from the laboratory scale plant at the University of Pretoria. Note that most of the dense bodies had been torn from the cells. Bar represents 1,0 µm.

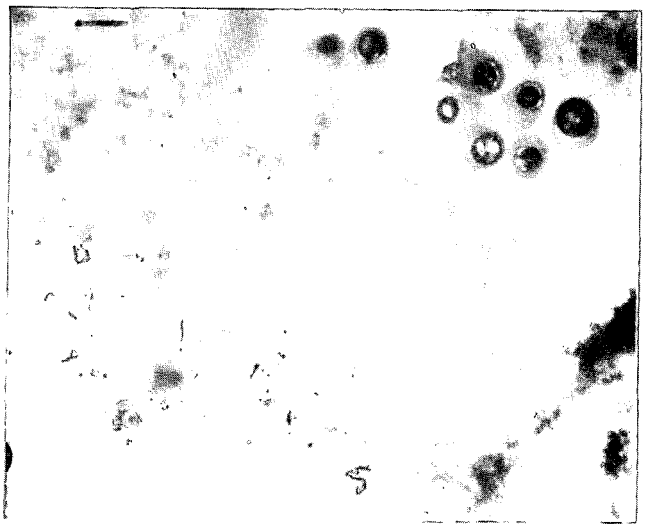


Figure 10b
A 0,4 µm section of activated sludge from the Brits works. Note the small polyphosphate granules in the smaller cells. Bar represents 1,0 µm.

The Northern Works plant which performed poorest of all the plants examined, had a relatively sparse distribution of the large phosphorus-accumulating cells (Fig. 11).

The photo-micrographs obtained with activated sludge from the Goudkoppie, Brits and Northern Works plants, created the impression of cells in various stages of development, culminating in large cells with large phosphorus-rich inclusions. It seems feasible to deduce that these plants possessed the microbial populations necessary for enhanced phosphorus removal, but that the cells needed to be enriched and induced in some way whereby they would increase in size and in the apparently associated phosphorus accumulating properties.

Sludges were also investigated which had been left anaerobic for 24 h, thereby releasing up to 40% of the total phosphorus in the sludge into the supernatant as orthophosphate.

Mixed liquor from the anaerobic zone of the laboratory scale plant was also investigated.

Electron microscopic investigation revealed that the sludges which had been left anaerobic for 24 h had released virtually all the phosphorus from the electron-dense bodies (Fig. 12).

The specimen from the laboratory scale plant revealed that the phosphorus in the electron-dense bodies had been partially released from the cells, the release being preceded by the dispersal of the dense bodies into smaller particles (Fig. 13).

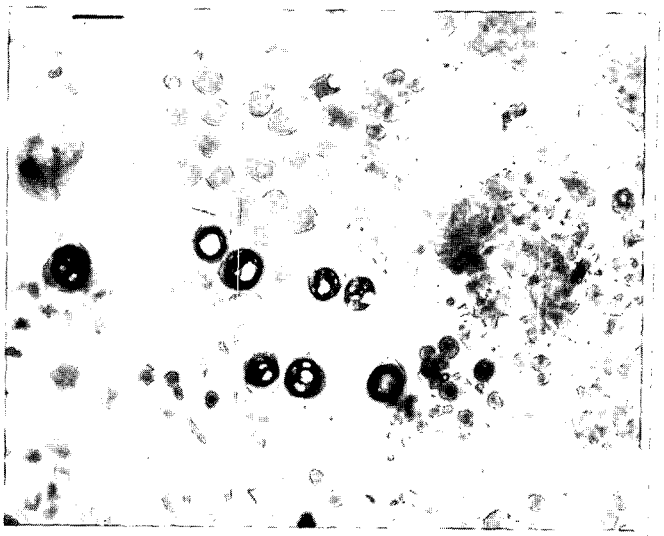


Figure 11
A 0,4 µm section of activated sludge from Northern Works. Bar represents 1,0 µm.

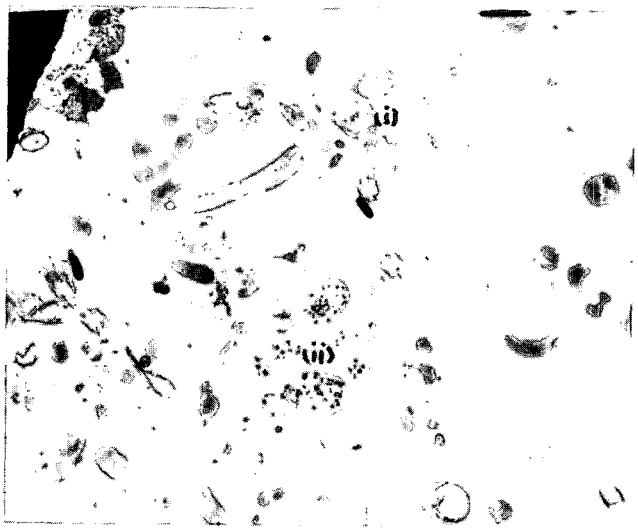


Figure 13a
A 0,4 µm section of activated sludge from the anaerobic zone of the laboratory scale plant at the University of Pretoria. The dense bodies in cluster (i) had released virtually all their phosphorus. In cluster (ii) the dense bodies had dispersed into smaller particles. Bar represents 1 µm.

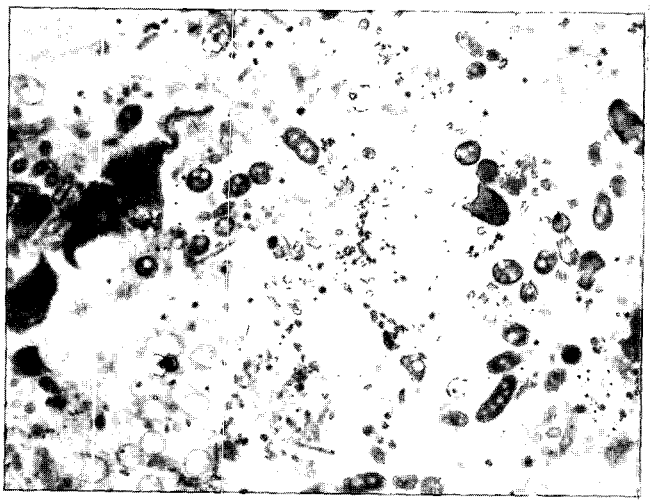


Figure 12
A 0,4 µm section of 24 h anaerobic sludge from the Umhlatuzana Works. Note how the cells had been voided of their dense bodies. Bar represents 1,0 µm.

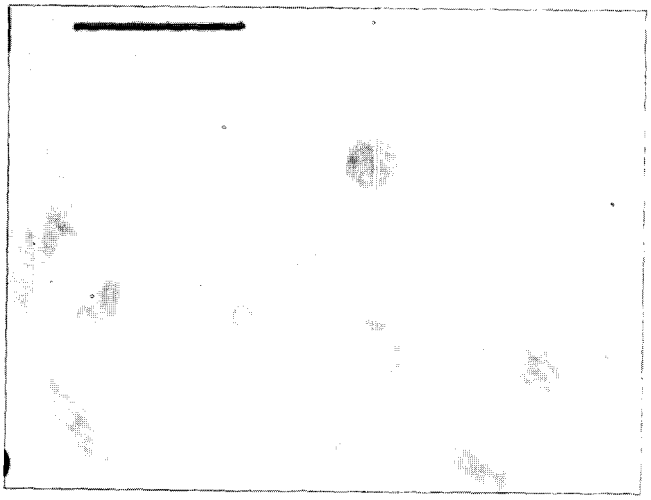


Figure 13b
A 0,4 µm section of activated sludge from the anaerobic zone of the laboratory scale plant of the University of Pretoria. Note how the electron-dense bodies were dispersing into smaller particles. Bar represents 1 µm.

Conclusions

A common mechanism for enhanced phosphorus removal was indicated by the significant fact that the sludges from the seven activated sludge plants investigated had phosphorus accumulations in identical intracellular structures.

The calcium:phosphorus X-ray intensity ratio of the intracellular electron-dense bodies precluded them from being any form of calcium phosphate precipitate.

Extracellular phosphorus-containing precipitates were not located. It is possible that if such precipitates were present that they could have been solubilized during the preparation procedures. However, judging by the number of polyphosphate accumulating cells in the sludges and by the vast quantities of phosphorus within these cells, a biological mechanism of phosphorus uptake by all the sludges was strongly indicated.

The orthophosphate released into the supernatant of mixed liquor during anaerobiosis is derived from the intracellular electron-dense bodies.

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