THE LOCATION AND NATURE OF ACCUMULATED PHOSPHORUS IN ACTIVATED SLUDGE

by

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PRETORIA

I certify that the thesis hereby submitted to the University of Pretoria for the degree of D Sc (Agric) has not been previously submitted by me in respect of a degree at any other University

SAMEVATTING

DIE LOKALITEIT EN AARD VAN GEAKKUMULEERDE FOSFAAT IN GEAKTIVEERDE SLYK

KANDIDAAT: Leon Buchan

LEIER : Prof P L Steyn

(Departement: Mikrobiologie en Plantsiektekunde)

GRAAD : D Sc (Agric)

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etlike teenstrydige hipoteses aangaande die basiese meganisme betrokke by hoë fosfaatopname.

Omdat daar nie eenstemmigheid was oor sekere basiese aspekte van die verskynsel nie, is besluit om die lokaliteit en aard van die geakkumuleerde fosfor in geaktiveerde slyk te ondersoek. Die tegniek wat gebruik was, was elektronmikroskopie gekombineer met mikrosonde analise.

Om die nodige tegnieke te ontwikkel is aanvanklik gebruik gemaak van 'n geaktiveerde slyk met 'n hoë kapasiteit vir fosfaatopname. Die lokaliteit van die fosforakkumulasies in die slyk is vasgestel en analise van hierdie akkumulasies het daarop gedui dat dit intrasellulêre polifosfate was.

Sewe slyke met verskillende kapasiteite t.o.v. fosfaatopname is toe ondersoek. Dit was duidelik dat die fosforakkumulasies in al die slyke geleë was in een spesifieke morfologiese tipe bakterie. Hierdie bakterieë was kenmerkend in trosse gegroepeer. Dit was ook duidelik dat in die meer doeltreffende slyke, die seltrosse groter en meer talryk was.

Die samestelling van die heterotrofiese bakteriese bevolkings van vier van die slyke is ondersoek, en hieruit is vasgestel dat *Acinetobacter* spp die grootste deel van die heterotrofiese bevolkings uitgemaak het.

Die intrasellulêre fosfor-ryke insluitsels in die verskillende slyke is ontleed en dit is vasgestel dat die verhouding van kalsium tot fosfor in die strukture baie ooreengekom het. Kwantitatiewe analise van hierdie fosfor-ryke insluitsels het daarop gedui dat die strukture fosforkonsentrasies van meer as 30% bevat het.

Die gevolgtrekking is toe gemaak dat hierdie organismes hoogs waarskynlik verantwoordelik is vir verhoogde fosfaatopname deur geaktiveerde slyk.

Die akkumulasie van die fosfor-ryke insluitsels is geïnduseer in 'n reinkultuur van *Acinetobacter* en in 'n slyk met 'n lae kapasiteit vir fosfaatopname. Hierdie ondersoeke het daarop gedui dat verhoogde fosfaatopname deur geaktiveerde slyk afhanklik is van die voorsiening van geskikte substrate aan die betrokke organismes.

ABSTRACT

THE LOCATION AND NATURE OF ACCUMULATED PHOSPHORUS IN ACTIVATED SLUDGE

CANDIDATE: Leon Buchan

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Department: Microbiology and Plant Pathology

DEGREE: D Sc (Agric)

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The removal of phosphorus from sewage works effluents is considered desirable in order to limit the eutrophication of lakes and impoundments.

It has been observed that activated sludge systems are capable of producing effluents with extremely low phosphorus contents. Lack of understanding of the basic principles involved has been responsible for the inability to control the erratic behaviour of such systems.

Because of the complexity of the activated sludge ecosystem, the elucidation of the mechanism involved in enhanced phosphorus-uptake has been slowly forthcoming with many controversial hypotheses being formulated. It was therefore

decided to determine the location and nature of the accumulated phosphorus in activated sludge. The technique used was that of electron microscopy combined with the energy dispersive analysis of X-rays (EDX).

To develop the techniques required for this investigation, use was made of a sludge which had exhibited enhanced phosphorus removal over an extended period of time. The location of the phosphorus accumulations were determined and analyses of these accumulations indicated that they were composed of polyphosphates.

Seven sludges with varying phosphorus-uptake capabilities were then examined. It was evident that the phosphorus accumulations in all the sludges were located in one specific morphological bacterial type, the cells of which were characteristically grouped in clusters. It was also apparent that the more efficient sludges contained very large and more numerous clusters of these cells.

Bacterial population analyses of four of the sludges indicated that *Acinetobacter* spp predominated.

The phosphorus-rich inclusions in all the sludges exhibited similar elemental ratios, which indicated similarity in composition. Quantitative analyses of these phosphorus-rich inclusions indicated that they contained phosphorus

concentrations in excess of 30%. It was consequently concluded that these organisms can account for enhanced phosphorus removal by activated sludge.

Accumulation of phosphorus-rich inclusions was induced in a pure culture of *Acinetobacter* and in a sludge which had poor phosphorus-uptake capacity. From these investigations it is suggested that enhanced phosphorus removal in activated sludge is dependent on the supply of suitable substrates to the organisms involved.

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CHAPTER 1

INTRODUCTION

Because of the contribution of phosphorus-containing sewage effluents to the eutrophication of lakes and impoundments, a great deal of research has been conducted in efforts to find the most efficient and acceptable method of minimising the discharge of this element from sewage purification works.

Two methods are employed for reducing the phosphorus content of sewage effluents, namely, precipitation of effluent phosphates, or by increasing the phosphorus-uptake capacity of the sludge by manipulating the activated sludge process.

Removal of phosphorus by a modified activated sludge process would be the most acceptable method. Poor understanding of the basic mechanisms involved have, however, made it difficult to control the erratic behaviour of such systems. Since the early reports of enhanced phosphorus removal by activated sludge (Srinath, Sastry and Pillai, 1959), many reports have been published describing the occurrence of this phenomenon in full scale plants.

Various contradictory hypotheses have been formulated to explain the basic mechanisms involved. Many conclusions have

been drawn from the physical and chemical monitoring of full scale plants, and also from investigations conducted under controlled artificial conditions which were distinct from the natural environment of the sludges or organisms examined.

The physico-chemical micro-environment of the micro-organisms in activated sludge is probably different from the properties of the bulk mixed liquor such as pH and dissolved oxygen, etc. Therefore the macro-environment does not necessarily reflect the micro-environment in which the organisms perform their physiological functions.

In laboratory studies the conditions imposed on the organisms may differ vastly from their natural environment where they are continually exposed to changes in critical factors such as the nature and concentration of substrates. Lighthart and Loew (1972) found that the group prevalence of the organisms changed continually with the diurnal loading cycle, their location in the plant and also from season to season.

At the beginning of this study basic concepts of the problem were still controversial in spite of prolonged world-wide research. From literature it appeared as if many of the investigations conducted were matched to preconceived hypotheses, i.e. theories were developed and extended upon, on what investigators thought took place during enhanced phosphorus removal.

It was therefore considered necessary to adopt a different approach in investigating this complex and incompletely understood phenomena with regard to the following:

1) Is the process due to chemical precipitation or to biological accumulation?
2) If it is a biological process,
   (i) is the phosphorus accumulated extracellularly in the slime or capsular material or intracellularly as polyphosphate inclusions?
   (ii) can accumulation be ascribed to a specific type of organism?
3) What is the origin of phosphorus released during subjection of phosphorus-rich activated sludge to anaerobiosis?
4) Does oxygen deprivation and/or substrate quality induce phosphorus accumulation?

The following report outlines the approach followed to resolve these questions, the results obtained and conclusions drawn.

CHAPTER 2

LITERATURE REVIEW

2.1 ENHANCED PHOSPHORUS REMOVAL

The enhanced uptake of phosphorus by activated sludge has been observed in many activated sludge systems (Barnard, 1976). Suggestions referring to the mode of operation required to achieve enhanced phosphorus removal, obtained from experience with full scale plants, are speculative, confusing and contradictory. This survey is largely confined to investigations which were aimed at clarifying the basic mechanisms involved in the phenomenon.

After many years of research two seemingly viable opposing basic theories remain. There are the proponents of a biological uptake theory and opposed to this are the proponents of a chemical precipitation theory. Both groups generally agree on three requirements for the phenomenon to occur, i.e. the presence of anaerobic conditions at some stage in the process, the release of phosphorus from the sludge during this phase and the subsequent aeration of the sludge (Barnard, 1975).

Jenkins, Menar and Ferguson (1971) reviewed various aspects of the chemical precipitation concept. According to this theory, phosphorus-uptake in excess of the normal metabolic requirements of the organisms is caused by chemical precipitation of phosphates by metals in the sludge. These precipitates are then entrapped in the sludge matrix. Compounds such as monetite ($CaHPO_4$), hydroxypatite $\{Ca_5(PO_4)_3OH\}$, tricalcium phosphate $\{Ca_3(PO_4)_2\}$ and octacalcium phosphate $\{Ca_4H(PO_4)_3\}$ have been considered to be precipitated. Morgan and Fruh (1972) also considered magnesium and iron precipitates.

One of the main arguments of the proponents of the chemical precipitation theory is that the carbon to nitrogen to phosphorus ratio (C:N:P) in sewage is unfavourable for a biological mechanism. The biological mechanism is an energy-requiring process and carbon (energy) is the limiting nutrient in sewage (Finstein, 1966; Garber, 1972).

Recently an intermediate theory was formulated (Kerdachi and Roberts, 1980). According to this theory colloidal calcium phosphate crystals are precipitated through mediation of bacterial extracellular enzymes. These crystals are then supposedly chemically bound to an extracellular polymer matrix of microbial origin.

The observation that metabolic inhibitors repress phosphorus removal has frequently been cited as supportive of a

biological mechanism (Levine and Shapiro, 1965; Yall, Boughton, Knudsen and Sinclair, 1970; Carberry and Tenney, 1973; Fuhs and Chen, 1975). According to the proponents of this mechanism, excess phosphorus is stored within the cells as polyphosphates.

There are other mechanisms whereby organisms can accumulate phosphorus which have never been considered applicable to the process occurring in activated sludge. Terry and Hooper (1970) found that cells of *Nitrosomonas europaea* could accumulate a cellular concentration of one molar orthophosphate from a growth solution. They considered the possibility that much of this orthophosphate was bound to the cell surface.

Rorem (1955) found that under certain conditions *Leuconostoc mesenteroides* formed an exopolysaccharide with remarkable phosphate-binding properties.

The biology and functions of polyphosphates were reviewed by Harold (1966), Dawes and Senior (1973) and Kulaev (1975).

Harold (1966) summarised the conditions under which micro-organisms accumulate polyphosphates as follows:

(i) When growth is arrested by the lack of an essential nutrient.

(ii) Upon addition of phosphate after the micro-organisms had been exposed to a phosphorus deficiency.

The excessive uptake of phosphorus due to nutrient limitation other than phosphorus or energy source has been termed 'Luxury Uptake'. The excessive uptake of phosphorus by bacteria after deprivation of this element, was termed 'Uberkompensation' by Liss and Langen (1962), and has been translated by Harold (1963) as the phosphate 'Overplus'.

Researchers into the mechanism of enhanced biological phosphorus removal in activated sludge have generally accepted that this is due to luxury uptake or overplus, and have consequently concentrated their efforts on explaining how one or both of these mechanisms are induced in the process.

Lawson and Tonhazy (1980) isolated several *Acinetobacter* spp from activated sludge and examined their phosphorus-uptake capabilities under various conditions, with emphasis on nutrient limitation. From their work, however, it was apparent that the organisms grown in a shake culture were equally capable of performing uptake in a complete growth medium.

Fuhs and Chen (1975) concluded that the organism which they isolated from activated sludge, performed luxury uptake in a complete growth medium. However, according to the definition of luxury uptake, the accumulation of polyphosphates

under complete growth conditions should not be termed luxury uptake.

Friedburg and Avigad (1968) demonstrated that *Micrococcus lysodeikticus* accumulated polyphosphates during conditions of balanced nutrition. They concluded that the metabolic pathways concerned with polyphosphate biosynthesis and degradation, or the control mechanisms which regulate them, differ in various organisms.

Roinestad and Yall (1970) suggested that enhanced phosphorus uptake appeared to be due to the predominance of certain organisms. They based their conclusions on light microscopic observation of methylene-blue-stained slides of activated sludge. The work of Fuhs and Chen (1975) seemed to confirm that a certain group of organisms, i.e. *Acinetobacter* spp was responsible for phosphate removal in activated sludge.

Yall, Sinclair, Roinestad and Russ (1974) patented a phosphorus-removing process which relies on the presence of sufficient numbers of the organism *Acinetobacter phosphadevorus* in the activated sludge system.

These proposals that only a selected group of organisms are responsible for the phenomenon, gave rise to another controversy. The viable mass fraction of activated sludge has been calculated to be 40 to 60% of the volatile fraction (Marais and

Ekema (1976). Sludges have been reported to contain five to eight percent phosphorus (m/m) of dry sludge (Yall, Boughton, Roinestad and Sinclair, 1972). This would imply that during biological uptake the phosphorus would have to be accounted for by 40 to 60% of the volatile fraction. If only a portion of the viable population were to be responsible for the uptake, it appeared that such organisms would have to contain unrealistically high levels of phosphorus.

Nicholls and Osborn (1978), in pursuance of concepts proposed by Fuhs and Chen (1975), examined enhanced phosphorus-removing sludges. They applied stains to aerobic and anaerobic sludges to examine them for the presence of poly-phosphates and poly-β-hydroxybutyric acid. Their observations also indicated that a certain morphological type of organism, which they presumed to be *Acinetobacter*, was responsible for the accumulation of polyphosphates. They also suggested that the phenomenon was due to a luxury uptake and overplus mechanism.

The role of the anaerobic zone in the process is the subject of much controversy. Fuhs and Chen (1975) attributed two roles to this zone:

(i) The selection of a facultative anaerobic population which would produce certain short-chain carbon compounds.

(ii) The selection of *Acinetobacter* spp which would presumably occur as a consequence of the substrates produced by the facultative anaerobic population.

Kerdachi and Roberts (1980) suggested that the release of phosphorus in the anaerobic zone was necessary in order to create sufficient concentrations of this element in the mixed liquor to initiate nucleation of phosphate precipitates.

Nicholls and Osborn (1978) suggested that this zone induced the overplus phenomenon by depleting the internal phosphorus reserves of the *Acinetobacter* spp. Lawson and Tonhazy (1980) also suggested that the anaerobic zone has a more complex role than merely the selection of certain population groups. They also considered the induction of the overplus phenomenon as possibly occurring in this zone.

In an attempt to determine whether the inclusion of an anaerobic zone exerted a selective influence on the microbial populations in activated sludge, Davelaar, Davies and Wiechers (1978) examined 90 bacterial cultures from pilot plants, with and without anaerobic zones. They concluded that the inclusion of an anaerobic zone did not cause a major change in the population structure. Toerien, Van Vuuren, Sadie and Tracey (1979) grouped the 90 bacterial isolates of Davelaar *et al* (1978) by numerical analysis,

and compared the results with the phosphate-accumulating properties as determined by Davelaar *et al* (1978). They concluded that there was little evidence that the anaerobic zone led to the selection of specific population groups.

There are, however, problems associated with the quantitative and qualitative characterization of microbial populations in activated sludge. Banks and Walker (1976) have shown how the recovery of organisms can vary with the homogenization procedure and culture media used. Roinestad and Yall (1970) indicated that the phosphorus-rich cells were grouped in clusters. It is obvious that such clusters, if not dispersed, would each give rise to single colonies, which would lead to underestimation of their numbers.

Various workers have attempted to determine the amount of polyphosphates in sludges which exhibited enhanced phosphorus removal. Yall *et al* (1970) measured the P^{32} incorporated into various extracts. The polyphosphate fraction contained only ten per cent of the total P^{32} activity. Fuhs and Chen (1975) chemically fractioned a culture of *Acinetobacter lwoffi* which contained 36µgP/mg dry mass before uptake of phosphorus and 49,9µgP/mg after uptake. During this uptake the total phosphorus in the culture increased by 13,9µgP/mg. The acid-insoluble polyphosphates which are the granular storage form of these compounds, however, only increased by 1,5µgP/mg dry mass. Field (1977) chemically fractionated activated

sludge from the Johannesburg Goudkoppie works, and concluded that the figures obtained for the polyphosphate fractions could not support the theories for phosphorus removal by incorporation into polyphosphates.

The quantitative estimation of polyphosphates in activated sludge requires extreme care as these compounds are known to occur in various forms which have different properties and would therefore require different extraction procedures (Kulaev, 1975; Potgieter, 1978).

The work of Friedburg and Avigad (1968) indicated that reagents utilised in extraction procedures can destroy the integrity of polyphosphate granules, e.g. detergents and EDTA.

The factors responsible for the release of the accumulated phosphorus as orthophosphate in the anaerobic zone are obscure.

2.2 METACHROMATIC STAINING

The appearance of more than one colour in material stained with a single dye was termed metachromacy by Ehrlich. The groups that allow dyes to bind to certain substrates were termed colligators by Gurr (1965). Methylene blue possesses

basic colligators and is termed a cationic dye. Polyanionic substances such as polyphosphates are strongly ionised above pH 5, and will therefore bind with cationic dyes. The purple colour that results from the binding of polyphosphates with methylene blue, results from the shifting of the absorption spectrum of the pure dye. Feder and Wolf (1965) suggested that the shift in the absorption spectrum of basic dyes is produced by the formation of stacked dye ions bound by neighbouring sites of polyanions. They suggested that, due to the flexible single stranded coils of polyphosphates, these compounds have high stacking coefficients, i.e. these compounds exhibit marked metachromacy when stained with cationic dyes.

2.3 ELECTRON MICROSCOPY COMBINED WITH THE ENERGY DISPERSIVE ANALYSIS OF X-RAYS (EDX)

1) Sample preparation

Electron microscopy combined with X-ray microanalysis is a method of elemental analysis within well-defined regions of a specimen. There are many problems associated with this technique when applied to biological material, as the standard specimen preparation methods for electron microscopy can displace, transform and dissolve many elements within biological samples, which are therefore not representative of the *in vivo* situation. The only preparation method known which would give a reasonable representation of the *in*

vivo situation would be rapid freezing, freeze sectioning and X-ray analysis in the frozen state. This is, however, a complex technique requiring special instrumentation.

2) Energy-dispersive analysis of X-rays; basic theory

The basic principles of X-ray analysis have been comprehensively reviewed by Hall (1971), Reed (1975), Chandler (1979), Russ (1978) and Russ (1979).

High velocity incident electrons cause ionisation of the atoms in a specimen. This ionisation creates a vacancy in one of the energy levels of the atoms, which is almost immediately filled by an electron which was at a higher energy level. As these electrons transfer to the lower energy levels, the excess energy is emitted in the form of X-ray photons. If the vacancy was created in the K-shell it is called a K X-ray. Since an electron from one of several higher energy levels could fill the vacancy, a photon corresponding to one of these transitions is emitted. These are called $K\alpha_1$, $K\alpha_2$, $K\beta$, etc. X-ray photons.

Each element in the periodic table contains electrons in orbits with particular discrete energy levels, and it is on the basis of the differences between these specific energies that each element can be identified. Mosely first demonstrated that X-rays arising from such processes

could be used to characterize each element in the periodic system, and he showed that a simple relationship existed between the frequency (energy) of a K_α photon and the atomic number (Z) of the element, where:

$$\nu = 0,248\ (Z-1)^2 \times 10^{16}$$

The frequency of a photon can be converted to the equivalent energy (eV), in which form the data is produced by the energy dispersive analyser. All the elements in the periodic table have now been characterized and their energy spectra tabulated.

In thin biological specimens, as defined below, the amount of characteristic photons emitted by a particular element in a specimen, is proportional to the number of atoms of that element present in the volume of material excited by the primary electron beam.

In addition to the characteristic X-ray radiation emitted by each element in a specimen, a background radiation is also emitted over the whole energy spectrum up to the energy of the incident electrons. This radiation is known as 'bremsstrahlung' or 'background radiation', and arises from the deceleration of the electrons due to interaction with the nuclei of all the atoms in a specimen.

In the case of thin biological specimens the amount of bremsstrahlung is proportional to the mass of the specimen

through which the electrons pass. The ratio of the characteristic elemental X-rays to the total bremsstrahlung X-rays generated (or 'Peak to Background' ratio - P/B ratio), is thus proportional to the ratio of the elemental mass to the total mass in the excited volume. This relationship is the basis of quantitative analysis in biological specimens.

The bremsstrahlung forms the background underlying the characteristic peaks, (as shown in the energy dispersive spectrum of Fig. 2).

For quantitative analysis of biological specimens, sections are considered to be thin, if:

(i) X-rays emitted by the elements in a specimen are not absorbed significantly by their passage through the specimen.

(ii) the generation of additional X-rays by the process of fluorescence is also negligible.

3) The determination of elemental ratios in thin sections

Russ (1979) reviewed the major methods used for the analysis of elemental ratios. The 'ratio' method relates the relative characteristic X-ray intensity of elements within the same specimen to their elemental ratios,

i.e.

$$\frac{N_1}{N_2} = \frac{I_1}{I_2} \cdot \frac{P_2}{P_1}$$

N denotes the number of atoms of elements 1 and 2 in the analysed volume, I denotes the peak intensities of elements 1 and 2, and P_1 and P_2 are constants of proportionality.

The proportionality constants are the product of the probabilities involved in X-ray excitation, and detection of each particular element and can be calculated from published models describing the process (Russ, 1979).

The relative atomic ratio is converted to the relative mass ratio as follows:

$$\frac{C_1}{C_2} = \frac{N_1}{N_2} \cdot \frac{A_1}{A_2} = \frac{I_1 \; A_1 \; P_2}{I_2 \; A_2 . P_1}$$

C_1/C_2 denotes the relative mass ratio of elements 1 and 2; A denotes the atomic masses of the elements.

A computer program can be used to calculate the relative values of P_1/A_1 and P_2/A_2 (Russ, 1975). (These relative values are denoted P_1' and P_2' in this report.)

The relative mass ratio of the elements is thus:

$$\frac{C_1}{C_2} = \frac{I_1}{I_2} \cdot \frac{P_2'}{P_1'}$$

The ratio method requires no standards, and there is no need to consider bremsstrahlung, because the analysed volume is the same for each element and therefore cancels.

4) Absolute quantitation

In the peak to background method (P/B) the characteristic elemental X-ray intensity (I_P) is used as a measure of the elemental mass, and the background intensity (I_B) in the spectrum is used as a measure of the total mass of the excited volume. The P/B ratio is therefore proportional to the concentration of the element of interest. This concentration can be compared in two samples; the specimen and a standard containing a known concentration of the element of interest, by using the background as a measure of the total mass excited in each sample. The measurement compensates for any differences in sample thickness and density in the two materials. Account is taken of the difference in composition of materials by the work of Kramers (1923), which states that the bremsstrahlung intensity is proportional to the total number of atoms in the analysed volume, weighted by the square of their atomic numbers Z.

This can be expressed in the following form:

$$\frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} = \frac{(N/\sum_j N_j Z_j^2)_{sp}}{(N/\sum_j N_j Z_j^2)_{st}} \quad \text{----------} \quad (1)$$

P and B relate to peak and background respectively; sp and st denote the specimen and standard respectively. The sums are over all the constituent atoms which total j in number.

An equation for calculating the concentration (C) of an element directly can be developed from equation (1), as shown in Appendix I. (An alternative form in which concentration of the elements are used in place of number of atoms (N) is also given).

This equation is:

$$Csp = \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot \left(\frac{N}{\sum_j N_j Z_j^2}\right)_{st} \cdot (\Sigma CZ^2/A)_{sp} \quad \text{------(2)}$$

The quantity $N/\sum_j N_j Z_j^2$ can be calculated from the stoichiometry of the standard.

$\Sigma CZ^2/A$ is normally taken as being 3,28 for soft biological tissue. Based on an assumed average composition for ordinary soft tissue, this value was derived as follows (Henke, 1959):

Element	Mass fraction (C)	Z^2	A	Z^2/A	CZ^2/A
H	0,07	1	1	1	0,07
C	0,50	36	12	3	1,50
N	0,16	49	14	3,5	0,56
O	0,25	64	16	4	1,00
S & P	0,02	240	31,5	7,7	0,15
				$\Sigma CZ^2/A=$	3,28

If $\Sigma CZ^2/A$ does not have a constant known value, an iterative calculation procedure is invoked in which a tentative value of $\Sigma CZ^2/A$ is assumed and concentrations calculated; a new value of $\Sigma CZ^2/A$ is then calculated using these values and concentrations redetermined; the procedure continues until new values of $\Sigma CZ^2/A$ no longer significantly change the calculated concentrations (Barbi, 1979).

Hall, Clarke Anderson and Appleton (1973) described the detail of data collection and processing by working through an example of an actual measurement. (In this report the alternative form of the equation used by the aforementioned authors is used, since the composition of the standard was given as a concentration.)

5) Accuracy of quantitation

Russ (1979) concluded that regardless of the details of the procedure, the general accuracy of the method is in the order of 10%, and that in the analysis of biological

thin sections, the greatest, and therefore, limiting errors arise in counting statistics, specimen preparation variables and in the difficulty in determining the density and thickness of specimens.

CHAPTER 3

EXPERIMENTAL PROCEDURE

3.1 SOURCES OF ACTIVATED SLUDGE AND ORGANISMS

3.1.1 Activated sludge

Activated sludge from seven plants exhibiting enhanced phosphorus removal was examined. The plants were situated at diverse geographical locations and varied considerably in their mode of operation and in the nature of their influent sewage. Samples were drawn from the end of the aeration basins, where the phosphorus accumulation of the sludge was maximal. In one plant sludge was also drawn from the anaerobic zone.

Samples from the Umhlatuzana plant were airfreighted from Durban to Johannesburg and prepared for microscopy within three hours of sampling. All other samples were prepared within 30 minutes of sampling. Selected physical and chemical properties of the influent sewages, mixed liquors and the effluents for the various plants are recorded in Table 1. These were the values at the time of sampling.

TABLE 1: RELEVANT PHYSICAL AND CHEMICAL PROPERTIES OF THE INFLUENT SEWAGES, MIXED LIQUORS AND EFFLUENTS OF THE SEVEN ACTIVATED SLUDGE PLANTS[i]

Activated Sludge Plant[ii]	Influent Sewage			Mixed liquor	Effluent
	COD[iii] (mg/ℓ)	COD:TKN[iv]	Phosphorus[v] (mg/ℓ)	Mixed liquor suspended solids (mg/ℓ)	Phosphorus[v] (mg/ℓ)
Laboratory scale plant I	480	15:1	9,0	2 100	<1
Laboratory scale plant II	480	15:1	9,0	1 800	<1
Brits	285	not done	4,7	3 950	<1
Goudkoppie:					
Module I	380	8:1	6,6	2 700	1,7
Module II	380	8:1	6,6	3 100	1,1
Northern works	300	7,5:1	7,1	1 300	6,4
Umhlatuzana	780	15:1	9,0	6 303	<1

(i) Figures are as obtained from the various authorities;

(ii) Laboratory scale plants 1 and 11 operated by the Department of Biochemistry, Faculty of Agricultural Sciences, University of Pretoria. Brits plant operated by the Brits Town Council. Goudkoppie and Northern works plants operated by the Johannesburg City Council. Umhlatuzana plant operated by the Pinetown Town Council;

(iii) COD: Chemical Oxygen Demand; (iv) TKN: Total Kjeldahl Nitrogen; (v) Values for Laboratory scale plants and Brits were for orthophosphate. All other values were for total phosphorus as P.

3.1.2 Organisms

A culture of *Acinetobacter calcoaceticus* (SWIb), isolated by Lawson and Tonhazy (1980) from an activated sludge pilot plant at the Johannesburg Northern works, was maintained by weekly sub-culture onto acetate enrichment agar and incubation at 20°C.

3.2 MEDIA

All prepared media were stored at 4°C.

(1) Glycerol-casitone-yeast medium (GCY Agar)
(Curtis, E.J.C., as cited by Pike, Carrington and Ashburner, 1972).

Casitone	1g
Yeast Extract	1g
Agar	13g
Distilled Water	1ℓ
pH	7,2

Sterilised at 121°C for 15 min

(2) Acetate enrichment medium (acinetobacter-agar)
(Fuhs and Chen, 1975)

Sodium Acetate	5g
$(NH_4)_2SO_4$	2g
$MgSO_4.7H_2O$	0,5g

KH_2PO_4	25g
$CaCl_2.2H_2O$	0,2g
Tap water	1ℓ

pH adjusted to 7,0

Sterilised at 121°C for 15 min

(3) Carbon source medium (modified acinetobacter-agar)

The sodium acetate in the acinetobacter-agar was replaced by ethanol*, sodium propionate, sodium butyrate or sodium iso-butyrate

* Filter sterilised and added after the basic medium had been sterilised.

(4) Settled sewage medium

Agar	15g
Settled sewage	1ℓ

Sterilised at 121°C for 15 min and pH adjusted to 7,0

(5) Fermented raw sludge medium

Raw sludge which had been left in a closed jar for 5 days at 20°C was centrifuged at 32 000g for 15 min. The pH of the supernatant was adjusted to 7,0, filter sterilised, aseptically added to molten concentrated agar and stirred with a magnetic stirrer at a temperature of 50°C until the agar was completely dissolved.

(6) Fermented mixed liquor medium

Mixed liquor from the anaerobic zone of the Northern works was diluted with an equal volume of settled sewage and left in a closed jar at 20°C for five days. Further preparation proceeded as for the fermented raw sludge medium.

3.3 REAGENTS FOR SAMPLE PREPARATION

3.3.1 Buffers

(1) Sodium cacodylate buffer 0,2M

Sodium cacodylate	42,806g
Double-distilled water	1ℓ

The pH was adjusted to 7,2 with concentrated HC1. The solution was filtered and stored at 4°C.

(2) Sodium cacodylate buffer 0,05M

Stock sodium cacodylate buffer	1 part
Double-distilled water	3 parts

The pH was adjusted to pH 7,2 when necessary.

(3) Stock tris buffer 0,2M

Tris (hydroxymethyl) methyl-amine	2,428g
Double-distilled water	1ℓ

The pH was adjusted to 7,2 with concentrated HC1 and the solution stored at 4°C.

(4) Tris buffer 0,05M

Stock tris buffer	1 part
Double-distilled water	3 parts

The pH was adjusted to 7,2 when necessary.

3.3.2 Fixatives

(1) Gluteraldehyde

Only fresh, high purity, EM grade gluteraldehyde, 25% biological grade, (Polysciences Inc) was used.

(i) 6% Gluteraldehyde in 0,05M sodium cacodylate buffer:

Gluteraldehyde 25%	25mℓ
Sodium cacodylate buffer 0,2M	24mℓ
Double-distilled water	51mℓ

When necessary the pH was adjusted to 7,2 with 1N NaOH

(ii) 6% Gluteraldehyde in 0,05M tris buffer:

Gluteraldehyde 25%	25mℓ
Tris buffer 0,2M	25mℓ
Double-distilled water	51mℓ

The pH was adjusted to 7,2 with NaOH

(2) Osmium tetroxide (OsO_4)

All operations with this substance were performed in a fume cupboard.

(i) 4% Osmium tetroxide:

OsO_4	1g
Double-distilled water	25mℓ

The OsO_4 and water were shaken in a glass-stoppered bottle till all the crystals were dissolved. The glass-stoppered reagent bottle was stored at 4°C within a padded screw-capped wide-mouth glass jar.

(ii) 2% Osmium tetroxide:

OsO_4 4%	2mℓ
Sodium cacodylate buffer 0,2M	1mℓ
Double distilled water	1mℓ

3.3.3 Dehydrating agents

(1) A graded concentration series of ethyl-alcohol was made up with double-distilled water (v/v). The series was 10, 20, 30, 50, 70, 80, 90, 100% alcohol.

(2) Absolute alcohol was stored over calcium chloride.

(3) After exposure to absolute alcohol the samples were infiltrated with 100% propylene oxide.

3.3.4 Embedding material

Spurr's resin (Spurr, 1969) was used for the embedding of the samples. The components of this resin are:

Vinylcyclohexane (VCD)
Diglycidyl ether (DER)
Nonenyl succinic anhydride (NSA)
Dimethylaminoethanol (SI)

(1) Spurr's resin 100%

VCD	10g
DER	6g
NSA	26g
SI	0,4g

The components were accurately massed into one container and mixed thoroughly by stirring with a glass rod. The density of the polymerised resin was 1,09g/cm^3

(2) Spurr's resin - 33% and 66%

Propylene oxide was used as a diluent (v/v).

3.3.5 Stains

(1) Light microscopy

The examination of sludges stained with methylene blue indicated that the amount of material which

stained metachromatically was related to the efficiency of a particular sludge in phosphorus uptake. Sludges which had released phosphorus into solution during anaerobiosis exhibited markedly less metachromacy. These observations indicated that phosphorus accumulation was related to polyanionic compounds in activated sludge. Methylene blue staining of specimens prepared for electron microscopy, was therefore adopted in initial attempts to locate phosphorus accumulations for analysis by energy dispersive analysis of X-rays.

Methylene blue stain:

Methylene blue	1g
Sodium borate	1g
Double-distilled water	100mℓ

The sodium borate was dissolved in a small quantity of double-distilled water, the methylene blue added and the volume made up to 100mℓ and the solution filtered.

When specimens exhibited inclusions with low electron density in the TEM, slides of the original cultures were stained for poly-β-hydroxybutyric acid with sudan black. (Burdon, K.L., 1946.)

(2) Electron microscopy

(i) Uranyl acetate:

Uranyl acetate	1g
Ethyl alcohol	1mℓ
Double-distilled water	50mℓ

Stored in foil-wrapped bottle at 4°C.

(ii) Lead nitrate (Reynolds, 1963)

Lead nitrate	1,33g
Sodium citrate	1,76g
NaOH 1N, freshly made	8mℓ
Double-distilled water, freshly boiled	50mℓ

The lead nitrate and sodium citrate were added to 30mℓ water and a gentle vacuum drawn until the bubbling stopped. The bottle was stoppered and shaken vigorously for 1min, then intermittently for 30min. The NaOH was added to the solution and the remaining 20mℓ of water added. The stains were stored at 4°C.

(3) Potassium permanganate 1% (Bray and Wagenaar, 1978)

$KMnO_4$	1g
Double-distilled water	100mℓ

The stain was stored at 4°C.

3.4 PREPARATION OF SAMPLES

3.4.1 Light microscopy

(1) Fresh activated sludge

Activated sludge was spread on microscope slides or on grated perspex discs (10mm diameter), the latter

being for eventual examination in the scanning electron microscope. After being allowed to dry at room temperature, the slides and perspex discs were flooded with methylene blue stain for two minutes, rinsed in tap water and again dried at room temperature.

(2) Sections

Slides: Sections of 2µm were picked off the glass knife of the microtome with a dissection needle and floated onto small drops of water on microscope slides. The slides were heated to evaporate the water and to flatten the sections by passage through the flame of a spirit burner to approximately 70°C. A drop of methylene blue was placed on the sections and the slides were again heated to approximately 70°C, whereafter the stain was gently rinsed in running tap water. The slides were then allowed to dry at room temperature.

Grids: Staining of 1µm sections on grids was accomplished by floating the grids face-downwards on a drop of methylene blue. After two minutes the grids were rinsed in three changes of double-distilled water, allowed to dry on filter paper, placed on microscope slides and examined.

3.4.2 Scanning electron microscopy

Two mℓ of mixed liquor was filtered by positive pressure through a 0,2µm millipore filter. The filter paper was placed face-upwards in a 10mℓ beaker, and all chemicals were then carefully added and drawn off with Pasteur pipettes. The following preparation schedules were then followed:

1) Preparation schedule for morphological examination

   (i) 6% Gluteraldehyde in 0,05M sodium cacodylate buffer. Left 60min.

   (ii) Sodium cacodylate buffer 0,05M, three changes, 10min each.

   (iii) 2% OsO_4 in 0,05M sodium cacodylate buffer. Left 60min.

   (iv) Sodium cacodylate buffer 0,05M, three changes, 10min each.

   (v) Dehydrated, in graded series of ethanol, 10, 20, 30, 50, 70, 80, 90, 100%. Left 10min at each concentration.

   (vi) Dehydrated, 3 changes, 10min each, absolute alcohol.

   (vii) Alcohol displaced with liquid CO_2 in Bomar SPC/900 EX critical point dryer.

(viii) Samples attached with 'scotch tape' double adhesive tape to SEM sample holders.

(ix) Samples coated with gold to a thickness of 30nm and stored in a dessicator till examined.

2) Preparation schedule for EDX analysis

The procedure was similar to the procedure described in 3.4.2(1). The sodium cacodylate buffer was replaced with 0,05M tris buffer. Steps (iii) and (iv) were omitted because sodium cacodylate interferes with the determination of calcium, and osmium with the determination of phosphorus. Because gold also interferes with the determination of phosphorus, the samples were carbon-coated.

3.4.3 Transmission electron microscopy

At the beginning of this study the problems associated with the possible effect of sample preparation, particularly on the mineral phase, were appreciated. It was, however, considered that if enhanced phosphorus removal is due to the accumulation of intracellular polyphosphate, as indicated by methylene blue staining, these water insoluble bodies might well be preserved during a selected preparation procedure. After due consideration of the problems

involved, a procedure to minimise the exposure of the samples to the various chemicals was followed, whereby the samples were fixated, dehydrated and transferred to 100% resin within 6 hours.

1) Activated sludge:

Five mℓ portions of mixed liquor were poured into centrifuge tubes and centrifuged at 3 000g for 10min. The supernatants were discarded and the precipitates prepared in the centrifuge tubes. After each treatment the precipitates were resuspended by stirring with a glass rod and by swirling for a few seconds on a vortex mixer. All centrifugation steps were performed at 3 000g for 5min and the supernatants were discarded. The following preparation schedules were followed with the precipitates:

Precipitate

(i) ↓ Resuspended in 5mℓ 6% gluteraldehyde in 0,05M sodium cacodylate buffer. Left for 60min. Swirled intermittantly. Centrifuged

Precipitate

(ii) ↓ Resuspended in 5mℓ 0,05M sodium cacodylate buffer.

Centrifuged

Precipitate

(iii) ↓ Repeated

Precipitate

(iv) ↓ Repeated

Precipitate

(v) ↓ Resuspended in 5mℓ 2% osmium tetroxide in 0,05M sodium cacodylate buffer. Left for 60min in fume cupboard.

Centrifuged

Precipitate

(vi) ↓ Resuspended in 5mℓ 0,05M sodium cacodylate buffer.

Centrifuged.

Precipitate

(vii) ↓ Repeated

Precipitate

(viii) ↓ Repeated

Precipitate

(ix) ↓ Resuspended in graded series of alcohol, 10, 20, 30, 50, 70, 80, 90, 100%, left 5min at each concentration. Centrifuged and resuspended after exposure to each concentration.

Precipitate

(x) ↓ Resuspended in absolute alcohol, three changes, 10min each.

Centrifuged and resuspended after each exposure.

Precipitate

(xi) ↓ Resuspended in 33% Spurr's in propylene oxide. Left 60min, with intermittent swirling. Centrifuged.

Precipitate

Resuspended in 100% Spurr's, left 12h under slight vacuum in dessicator at room temperature.

The suspensions in 100% Spurr's were transferred with Pasteur pipettes with broken-off tips (to increase the size of the apertures) into Beem capsules. The capsules were placed in a dessicator under slight vacuum for 10 min, removed, and placed in an oven at 70°C for 24h to polymerise the resin.

2) Cultures

Sterile razor blades were used to cut out colony sections of approximately 3 × 10mm with their supporting medium. The sections were placed in 10mℓ glass beakers and prepared by carefully pouring in and drawing off the required reagents with Pasteur pipettes.

The following preparation schedule was followed:

(i) Gluteraldehyde 6% in 0,05M sodium cacodylate buffer. Left for 60min.

(ii) Sodium cacodylate buffer 0,05M, three changes, 10min each.

(iii) Osmium tetroxide in 0,05M sodium cacodylate buffer. Left 60min.

(iv) Sodium cacodylate buffer 0,05M, three changes, 10min each.

(v) Dehydrated with graded series of alcohol, 10, 20, 30, 50, 70, 80, 90, 100%, 5min exposure to each concentration.

(vi) Dehydrated with absolute alcohol. Three changes, 10min each.

(vii) Infiltrated with 100% propylene oxide. Two changes of 10min each.

(viii) Infiltrated with propylene oxide 33%, resin 67%. Left 1h.

(ix) Infiltrated with propylene oxide 33%, resin 67%. Left 1h.

(x) Infiltrated with 100% resin. Left 12h under slight vacuum in dessicator.

(xi) Sections transferred to Beem capsules, capsules filled with 100% resin. Placed in dessicator under slight vacuum for 10min. Removed from dessicator and placed in oven at 70°C for 24h.

3.4.4 Scanning transmission electron microscopy - EDX analysis

1) Activated sludge

The same preparation schedule was followed as for 3.4.3(1) The sodium cacodylate buffer was replaced with 0,05M tris buffer, and steps (v), (vi), (vii) and (viii) were omitted.

2) Cultures

The same preparation schedule as for 3.4.3(2). Sodium cacodylate was replaced with 0,05M tris buffer and steps (iii) and (iv) were omitted.

3.5 SECTIONING OF EMBEDDED SAMPLES

After removal of the Beem capsule from the polymerised resin, about 4mm was cut off the bottom of the sample to remove most of the sedimented sand particles. The plane to be sectioned was then cut with a stout blade to leave a protruding section of 0,5 × 1mm, which was then smoothed off with a glass knife on the microtome. The sample was rotated in the chuck of the microtome and sections were prepared, to determine from which side final sectioning should proceed to cause the least amount of damage to the specimens (due to silica particles in the sample). Sections of 0,1 to 1µm were then floated onto water,

flattened with chloroform vapour and floated onto the required grids.

3.6 INSTRUMENTATION AND MODES OF OPERATION

1) Light microscopy

A Reichert Univar Research Microscope was used to examine methylene-blue-stained samples. The instrument was equipped with a Reichert Trimatic camera system, and photographs were recorded on 35mm Agfacolor 50L films or on Polaroid Type 58 colour films.

2) Scanning electron microscopy

The external morphology and the distribution of various elements of interest (X-ray area maps) of the specimens were examined in a Philips PSEM 500 scanning electron microscope (SEM). The instrument was equipped with an 'EDAX' energy dispersive X-ray detector. The accelerating voltage used was 20kV.

3) Transmission electron microscopy

Thin sections (0,1 to 1µm) were examined in a Philips 301 transmission electron microscope (TEM). The 'cold finger' of the specimen chamber was cooled with liquid nitrogen

to minimise contamination and volatilisation of the specimens. The intensity of the electron beam was always gradually increased over the areas to be examined. The instrument was operated at accelerating voltages of 60, 80 or 100kV, depending on section thickness. Selected area electron diffraction was carried out, using a 15µm diffraction aperture. Photomicrographs were recorded on X-ray sensitive photographic plates.

4) Scanning transmission electron microscopy - EDX analysis

These analyses were performed with a Jeol JSM-U3 scanning electron microscope, modified for scanning transmission (STEM) and equipped with an 'EDAX' energy dispersive X-ray detector with video display, and a 'Data General Corporation' data processor. The instrument was operated at 19, 20 and 40kV, and the 'cold finger' of the specimen chamber was cooled with liquid nitrogen to minimise contamination of the specimens by the residual oil vapour in the working chamber. Specimens were examined unstained on specially designed holders. Specimens were observed in the scanning transmission mode to identify from the light microscope and TEM photomicrographs the areas to be analysed. The probe diameter was 0,05µm. Polaroid photographs were recorded directly off the EDAX visual display.

3.7 DETERMINATION OF CALCIUM:PHOSPHORUS MASS RATIOS OF INTRACELLULAR PHOSPHORUS-RICH INCLUSIONS

The specimens were mounted on specially designed carbon supports in order to clearly identify the elements within the analysed volumes.

The electron-dense bodies were analysed with an accelerating voltage of 40kV, and mostly over 40sec counting intervals.

The Edax-Edit EP7 programme was used to subtract the background from each spectrum and to integrate the characteristic X-ray peak intensities. To convert the calcium:phosphorus X-ray intensity ratios to mass ratios, the P'values for the K_{α} emission lines for calcium and phosphorus at 40kV were utilised.

3.8 DETERMINATION OF ABSOLUTE MASS FRACTIONS OF CALCIUM AND PHOSPHORUS IN INTRACELLULAR PHOSPHORUS-RICH INCLUSIONS

1) Specimens

Sections of 1µm from Laboratory scale plant I and Brits were analysed. The sections were mounted on beryllium grids and analysed unstained.

2) Standard

The standard consisted of homogeneously dispersed calcium (0,5%m/m) in araldite resin, section thickness 0,1µm, and

mounted on an aluminium grid (Agar Aids, Essex). The standard was coated with a thin layer of carbon.

3) Specimen mounting

Quantitative analyses were initially performed with the specimens mounted as illustrated in Fig. 1(a). Spectra obtained from specimens in the chamber included:

(i) X-rays from the specimen

(ii) Contributing X-rays from the specimen grids and carbon supports, due to electrons scattered at the final aperture, i.e. I_i.

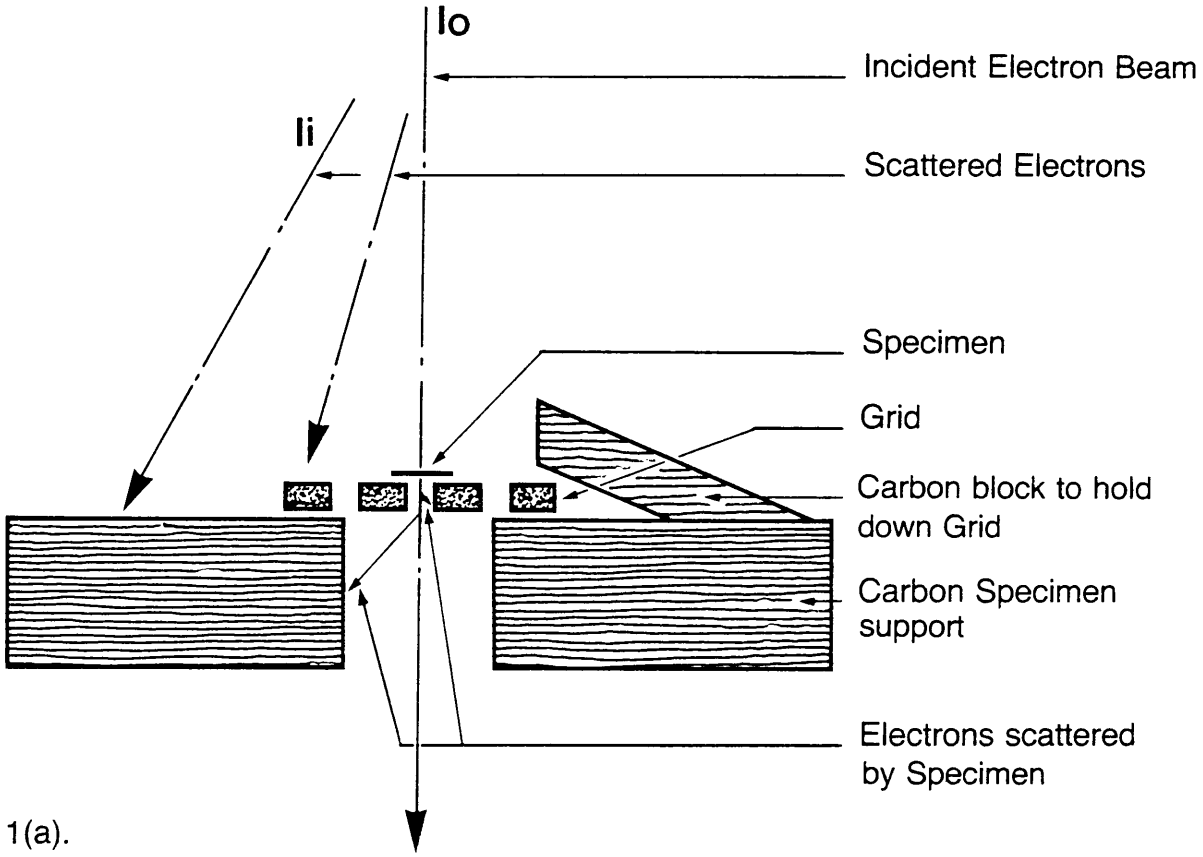
(iii) Contributing X-rays from the specimen grid and carbon supports, due to electrons which were scattered through wide angles during their passage through the specimen.

Because the contribution of the carbon mounting to the generated bremsstrahlung could not be evaluated, a mounting procedure as illustrated in Fig. 1(b) was utilised. Although the aluminium and iron must have made a larger contribution to the background in each spectrum than would have been the case if only carbon was used, these contributions could be accurately evaluated because they were identifiable by their characteristic peaks which appeared in the spectra.

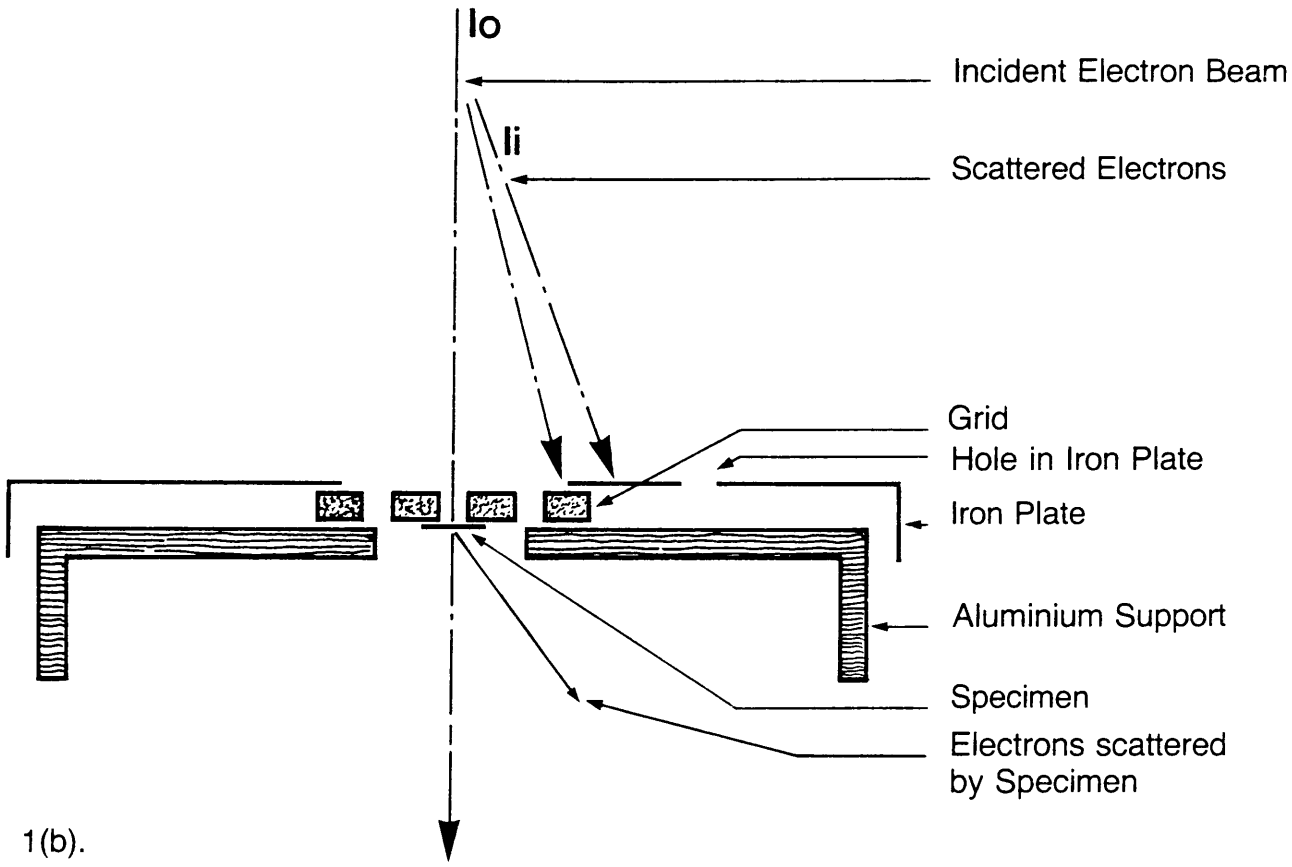
FIG. 1 In Fig. 1(a) the grid was mounted on a carbon support with the specimen film facing upwards.

In Fig. 1(b) the grid was mounted on an aluminium base with the specimen facing downwards. The grid was kept in position with a thin iron plate which was placed over it.

FIG. 1
Specimen supports for EDX-Analysis.



1(a).



1(b).

The bremsstrahlung (I_B) included X-rays from electrons scattered in the column striking the iron top cover of the sample holder and the aluminium specimen grid, plus that produced by electrons scattered in the sample which could only strike the aluminium. Both sources could be subtracted from the spectrum with reasonable precision, using pure element spectra of iron and aluminium to calculate the contribution of each source to the background.

4) Compensation for difference in thickness of the standard and the specimens

To compensate for the difference in the thickness of the standard (0,1µm) and that of the specimens (1µm), the probe voltage used was 19kV for the standard and 20kV for the specimens. This was done to allow for an estimated 2kV loss of beam energy in penetrating the specimens. This value of 2kV was estimated from the Thomson-Whiddington law as cited by Everhart and Thornley (1960).

5) Fluorescence and absorption

That absorption could be ignored was confirmed by calculating the X-ray absorption, using published absorption coefficients and an estimated density for the specimen (Appendix II).

6) Beam current and counting intervals

In the application of the method any beam currents and counting intervals could be used, as they were normalized by the use of the P/B ratio.

7) Equation for calculating mass fractions

$$C_i = \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot (C_i)_{st} \cdot \frac{(\sum_j C_j \cdot Z_j^2/A_j)_{sp}}{(\sum_j C_j \cdot Z_j^2/A_j)_{st}} \quad \text{----------(3)}$$

The derivation of this equation is included in the appendix (Appendix I).

C_i = mass fraction of calcium in specimen.

$(I_P/I_B)_{sp}$ = corrected peak to background ratio for the characteristic calcium peak in the specimen.

$(I_P/I_B)_{st}$ = corrected peak to background ratio for the characteristic calcium peak in the standard.

$(C_i)_{st}$ = Mass fraction of calcium in the standard (0,005).

$(\sum_j C_j \cdot Z_j^2/A_j)sp$ and $(\sum_j C_j \cdot Z_j^2/A_j)st$: the sums are over all the constituent atoms.

C, Z and A are the mass fractions, the atomic numbers and the atomic weights respectively, of all the elements in the matrixes of the specimen and the standard.

After calculation of the concentrations of calcium in the specimens, these values were used to obtain the concentration of phosphorus by use of the equation:

$$C_P = C_{Ca} \cdot \frac{I_P}{I_{Ca}} \cdot \frac{P'_{Ca}}{P'_P} \qquad \text{----------(4)}$$

The P´values for the K_α emission lines for calcium and phosphorus at 19kV were utilised.

8) Analysis of the specimens and the standard

The Edax-Edit EP7 programme was utilised to obtain the integrated counts for the characteristic peaks of each element. The counts in the background windows were obtained from a teletype printout.

(i) Background windows utilised for normalizing the background counts of the specimens and standard. Window no. 2, set at 2940eV, 13 channels width with 20eV per channel, was used to determine the background counts in all analyses. The window was set in this region of the energy spectrum because neither the specimens nor the

standard exhibited characteristic peaks in that energy range.

(ii) Counting statistics. For the purpose of calculating the standard deviations of the X-ray counts for the specimens, the backgrounds under the characteristic peaks were taken to be similar to the backgrounds recorded in window no. 2. See Fig. 5 for example.

Standard deviation = $\sqrt{N_o+N_1+N_1}$ (Russ, 1979)

N_o = X-ray counts of peak after background subtraction.

N_1 = X-ray counts of window no. 2.

(iii) Determination of bremsstrahlung generated from sources extraneous to the specimens and the standard.
Iron: The electron beam was focussed on the iron top cover and a spectrum recorded. The ratio of counts recorded in background window no. 2 to the count in the K_α peak of iron, was used as the factor to correct for the contribution of the iron to the bremsstrahlung in background window no. 2.

Aluminium: The electron beam was focussed on the aluminium exposed in the hole in the iron top cover,

and a spectrum recorded. The ratio of counts recorded in background window no. 2 to the count in the K_α peak of aluminium, was used to obtain the factor to correct for the contribution of the aluminium to the bremsstrahlung in background window no. 2.

(iv) Evaluation of the effects of contamination and volatilisation of the specimens by the electron beam.

The contamination of a specimen in an electron microscope by hydrocarbons, particularly oil vapour from the vacuum pumps, is a well known phenomenon. This can be reduced, but not eliminated, by the use of techniques such as cold condensing surfaces adjacent to the specimen.

Contamination increases the effective mass of material analysed, and would decrease the P/B ratio progressively during repeated analysis. Similarly if the compound being analysed was decomposing or the elements were migrating out of the irradiated volume, a progressive decrease in the P/B ratio or change in the ratio of elements being analysed would be observed.

An electron-dense body in a specimen of Brits sludge was analysed on the same spot for five consecutive intervals of 1min. The spectra were recorded for each analysis and these counts were examined to evaluate the effects of volatilisation and contamination on the P/B ratios.

(v) Determination of the corrected peak to background ratio for the 0,5% calcium standard, i.e. $(I_P/I_B)_{st}$.

Several spectra were recorded at different positions on the standard, the off-focus beam being scanned over a small area. The areas were scanned at 19kV with different probe currents and for different counting intervals, to verify that a consistent value of the peak to background was obtained.

The integrated counts for the calcium, aluminium and iron peaks and for the calcium background window and background window no. 2, were recorded for each analysis.

The corrected background counts were calculated by subtracting the contribution of iron and aluminium from the counts obtained in background window no. 2.

(vi) Determination of the corrected peak to background ratios for the specimens, i.e. $(I_P/I_B)_{sp}$.

The conditions of analysis were: accelerating voltage 20kV; probe current 1×10^{-9} ampere; beam diameter 0,05µm. The diameters of the electron-dense bodies analysed were mostly approximately 0,5µm (as measured off the video screen of the STEM). The electron beam was focussed in the centre of the dense bodies. It could thus be approximated that the volume of the dense bodies comprised 50% of the total analysed volume. The remainder of the analysed volume was composed of biological material and Spurr's resin.

The corrected peak to background ratios for calcium and phosphorus were obtained after subtraction of the contribution of the iron and aluminium counts to the counts obtained in background window no. 2.

9) Graphical illustration of quantitative analysis

The technique used to obtain the corrected peak to background counts for the standard and specimens is graphically illustrated in Figs. 2 to 7.

FIG. 2 Uncorrected energy dispersive X-ray spectrum of the 0,5% calcium standard.

FIG. 3 Energy dispersive X-ray spectrum of 0,5% calcium standard after subtraction of the contribution of the iron and aluminium peaks to the spectrum. The uncorrected spectrum is superimposed with the dotted outline.

FIG. 4 The same spectrum as Fig. 3 with the superimposed uncorrected spectrum removed. This represents the corrected spectrum of the 0,5% calcium standard.

FIG. 2

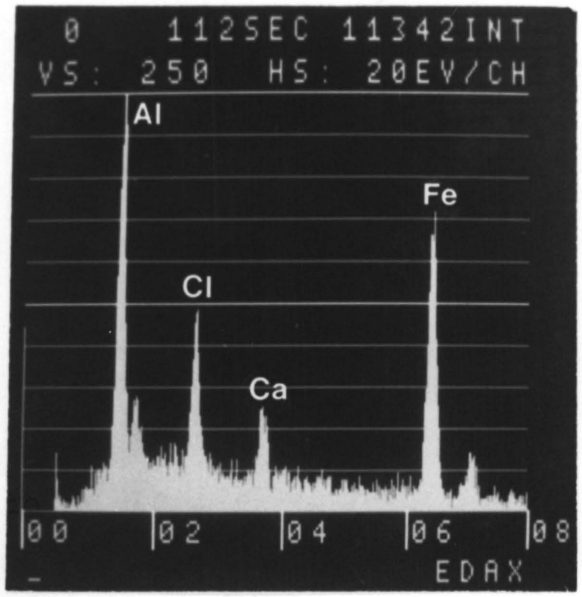


FIG. 3

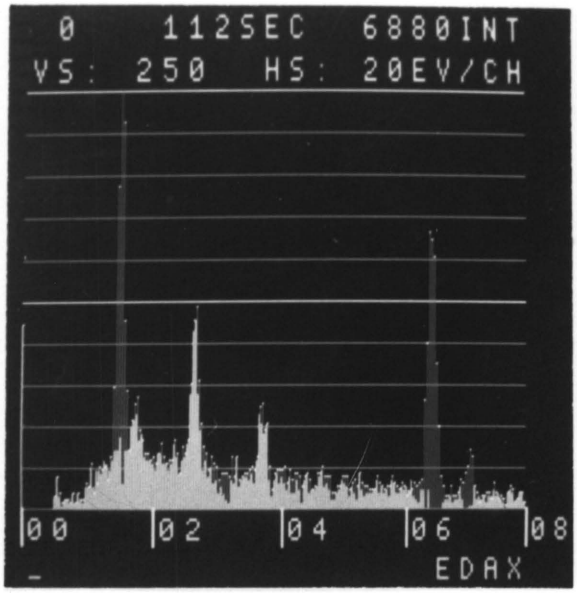


FIG. 4

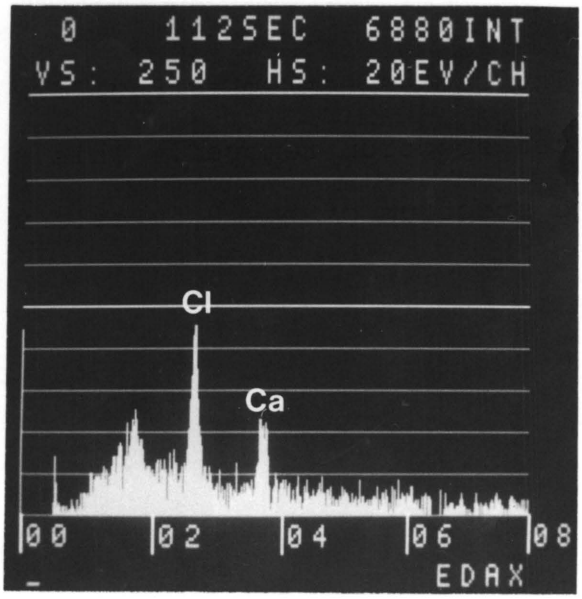


FIG. 5 The corrected spectrum of the 0,5% calcium standard. The lower, brighter area indicates the background due to bremsstrahlung. The darker peaks are the elemental peaks, which were integrated to obtain the peak counts.

FIG. 6 The corrected spectrum of the 0,5% calcium standard. The bright area indicates background window no. 2.

FIG. 7 The uncorrected energy dispersive X-ray spectrum generated by an electron-dense body from Laboratory scale plant I. This figure serves to illustrate the calcium and phosphorus peaks generated relative to about the same background count in background window no. 2, as was generated in this window in Fig. 6. The vertical scale is larger by a factor of four in this figure.

FIG. 5

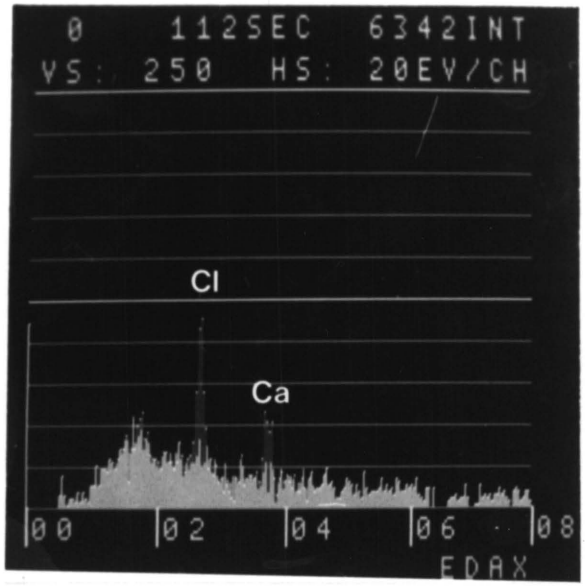


FIG. 6

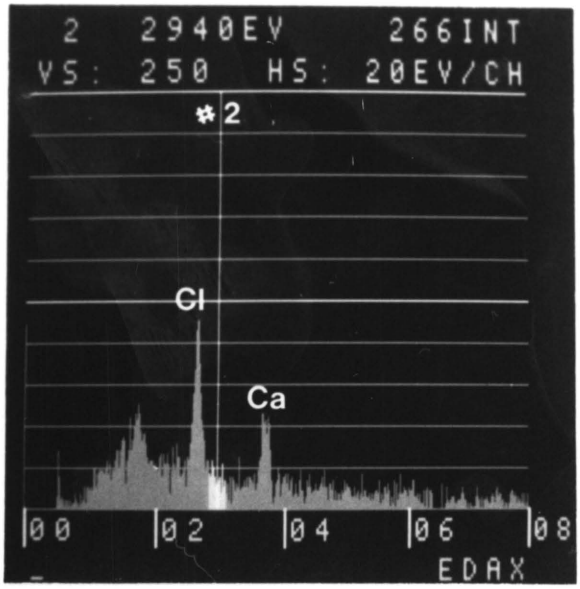
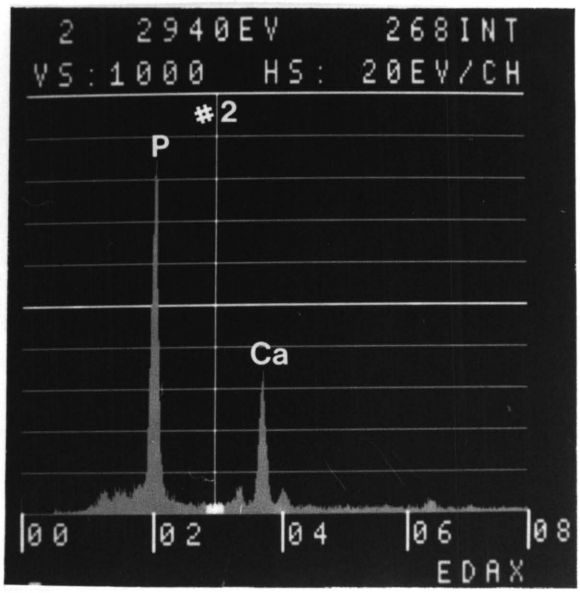


FIG. 7



3.9 ULTRAMICROTOMY

Glass knives were made with a LKB knife maker, with glass strips supplied by LKB. The knives were cut at an angle of 45° and had a cutting edge width of 6,35mm. The knives were mounted in a Reichert OMU - 3 ultramicrotome at an angle of 6°.

3.10 CHARACTERIZATION OF THE GRAM NEGATIVE, HETEROTROPHIC BACTERIAL POPULATIONS IN ACTIVATED SLUDGE

1. Enumeration of relative bacterial population structures

Mixed liquor was homogenized by swirling on a vortex mixer for two minutes. The homogenized mixed liquor was diluted in sterilised tap water in order to obtain 100± colonies after spreading 0,1mℓ of the homogenate on GCY agar, and after incubation at 20°C for five days. The dilution normally adopted was 10^{-6} to 10^{-7}. The colonies were picked off after five days of incubation and streaked onto GCY agar. After 48h incubation at 20°C the cultures were Gram stained. The Gram positive cultures were enumerated and discarded. The remaining Gram negative cultures were identified using the API 20E system (1976 Analytab Products Inc., U.S.A.), in conjunction with the API Profile Recognition System.

2. Characterization of metachromatic cell clusters

Methylene blue stain (0,5mℓ) was added to 25mℓ of Laboratory Scale plant I and Brits mixed liquor, and the suspensions swirled for 1min, 25mℓ molten agar (50°C) was added to each suspension and the suspensions again swirled for 1min. One mℓ of each suspension was spread on separate microscope slides and allowed to set. Six metachromatic cell clusters were then extracted with a micromanipulator from each slide. After two subcultures of 48h at 20°C on GCY-agar the cultures were identified with the API 20E system, in conjunction with the API Profile Recognition System.

CHAPTER 4

RESULTS AND DISCUSSION

The technique to locate and analyse the intracellular phosphorus-rich inclusions in activated sludge was developed with sludge drawn from the Umhlatuzana works. After succeeding in locating and analysing the phosphorus-rich inclusions in the Umhlatuzana sludge, sludges from the other plants were examined and analysed.

Sludges which had been subjected to anaerobiosis were then examined to locate the origin of the orthophosphate released into the supernatant.

Having obtained a clear picture of the appearance and nature of the intracellular phosphorus-rich inclusions and of the morphology of the bacterial cell types involved, the bacterial population characteristics of activated sludge from four plants were examined.

Finally, the possible application of the developed techniques in the elucidation of factors affecting enhanced phosphorus uptake was examined. Although these were not considered to be definitive experiments, the results were considered of such significance that they are included in this report. It was evident that the results supported reports in published literature (Fuhs and Chen, 1975; Yall *et.al.*, 1974).

To simplify the presentation the results and discussion are grouped into six major sections viz:

1) The location and nature of accumulated phosphorus in sludge from the Umhlatuzana works.
2) Comparison of the intracellular phosphorus-rich inclusions in the seven sludges examined.
3) Quantitative analysis of electron-dense bodies.
4) The fate of the intracellular phosphorus-rich inclusions in sludges subjected to anaerobiosis.
5) The population characteristics of the Gram negative, heterotrophic bacteria in activated sludge from four plants.
6) The induction of the accumulation of intracellular phosphorus-rich inclusions in a pure culture and in activated sludge.

4.1 THE LOCATION AND NATURE OF ACCUMULATED PHOSPHORUS IN ACTIVATED SLUDGE FROM UMHLATUZANA

1) Light microscopy

Methylene-blue-stained smears of the aerated sludge exhibited a great number of metachromatic cell clusters (Figs. 8 and 9). When the sludge was left anaerobic for 24h, very few metachromatic clusters were observed.

To determine the effect of sample preparation for electron microscopy on the metachromatic properties of the cell clusters, methylene-blue-stained smears were prepared at the final step before infiltration with resin, i.e. after infiltration with propylene oxide. It was apparent that although much debris had been removed by the treatment, the metachromatic cell clusters were still present (Fig. 10).

Examination of methylene-blue-stained sections revealed that metachromatic structures were clearly visible down to section thicknesses of 0,5µm (Figs. 11 and 12).

Photomicrographs were recorded and used to locate the metachromatic areas in the various electron microscopes (Fig. 13).

FIG. 8 Metachromatic cell clusters in aerated activated sludge from Umhlatuzana ×970.

FIG. 9 A metachromatic cell cluster in aerated activated sludge from Umhlatuzana ×2 400.

FIG. 10 The appearance of a metachromatic cell cluster in aerated Umhlatuzana sludge after exposure of the sludge to the various reagents used for sample preparation ×970.

FIGS. 11 and 12 Metachromatic cells in a 2µm section of aerated Umhlatuzana sludge in Spurr's resin. Note the purple tinge of the cells and the large polyphosphate granules within the cells. As these were representative specimens, the large number of polyphosphate-accumulating cells in the sludge is clearly evident.
Fig. 11 ×970; Fig. 12 ×2 400.

FIG. 13 Metachromatic cells in a 1µm section of aerated Umhlatuzana sludge in Spurr's resin and mounted on a copper grid ×970.

FIG. 8

FIG. 9

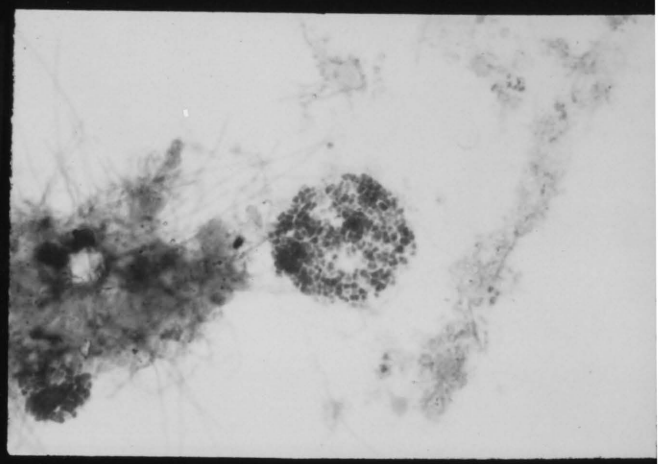


FIG. 10

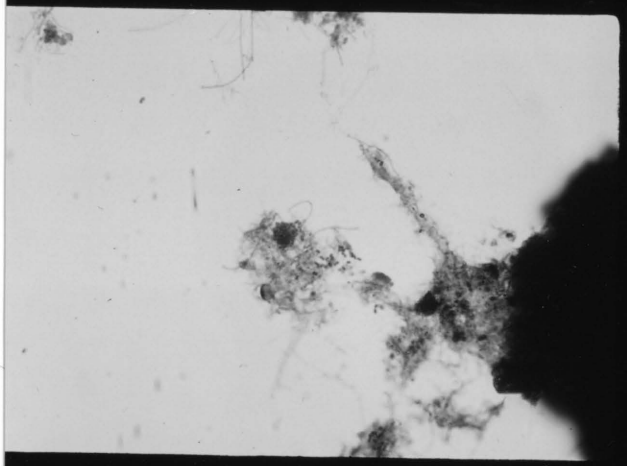


FIG. 11

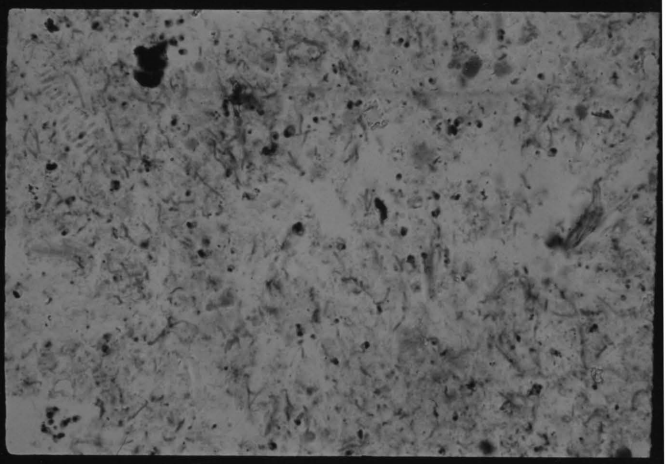


FIG. 12

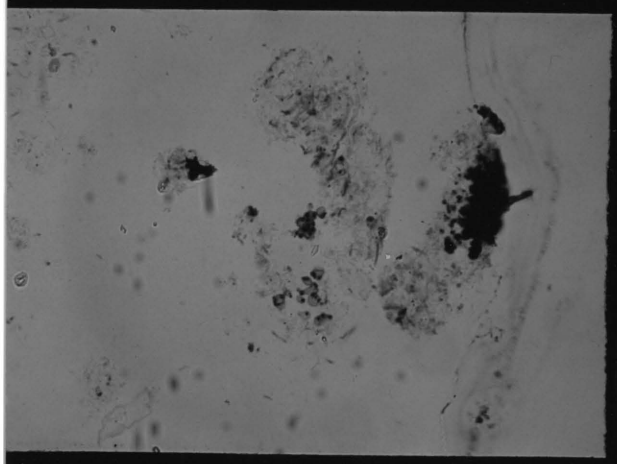
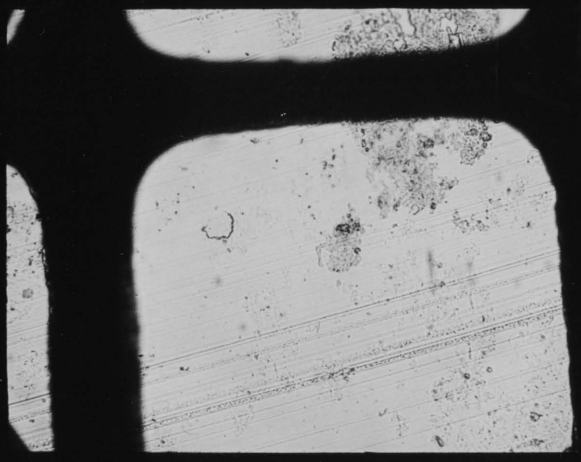


FIG. 13



2) Scanning electron microscopy

After the metachromatic areas in the air-dried samples on the perspex discs were recorded on photomicrographs, the discs were coated with carbon and examined in the SEM. The metachromatic areas were located by referring to their position on the grid pattern of the perspex discs.

Examination of the metachromatic areas revealed that they were clusters of large cells (Fig. 14a). X-ray area maps for various elements were obtained by scanning the field under observation ten times for each element (Fig. 14b, c,d,e,f). From these observations it was evident that a high concentration of phosphorus was associated with the cell clusters. It also appeared as if there was no association of the other elements with the phosphorus.

To obtain more morphological detail of the cell clusters, a sample prepared as described in section 3.4.2(2) was scanned to locate cell clusters. Figure 15 illustrates the morphological appearance and X-ray area maps of calcium and phosphorus obtained for a typical cell cluster. A marked association of phosphorus with the cell cluster (framed area) was clearly visible. Calcium was also associated with the cell cluster, but to a lesser extent.

To obtain increased morphological detail of the cell clusters, a sample was prepared as described in section 3.4.2(1). Figure 16 illustrates the appearance of a cell cluster and its environment as obtained with this method. X-ray area maps were not obtained because of the interference of the various reagents used in the sample preparation with calcium and phosphorus.

FIG. 14(a) Aerated sludge from Umhlatuzana as observed in the SEM. The sludge was air dried, stained with methylene blue and carbon-coated. The cell clusters are indicated in the framed areas ×1 250.

FIG. 14(b,c,d,e,f) X-ray area maps for the same area as Fig. 14(a).

(b) phosphorus (c) calcium (d) magnesium (e) potassium (f) sulphur.

FIG. 14a

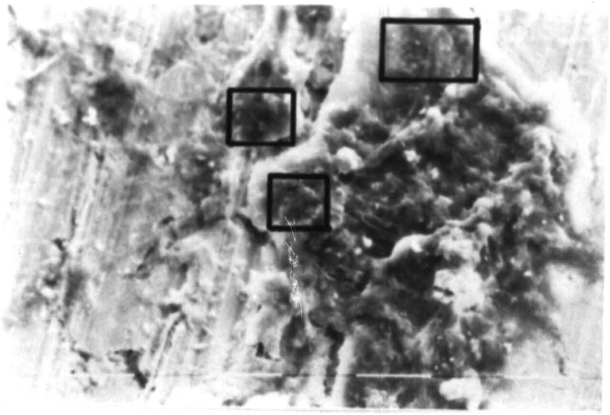


FIG. 14b

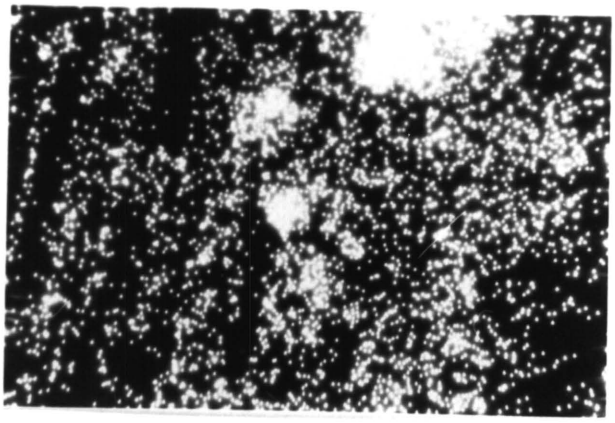


FIG. 14c

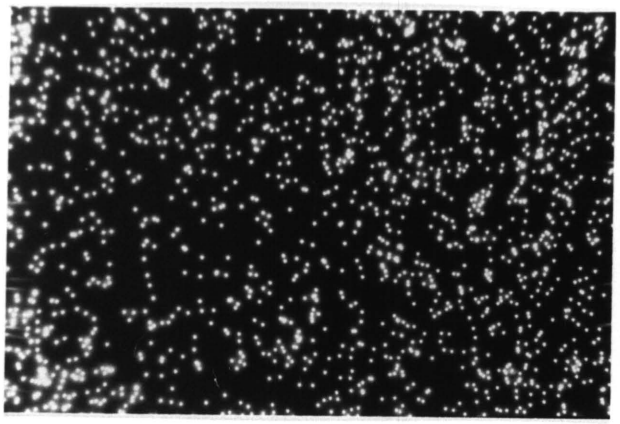


FIG. 14d

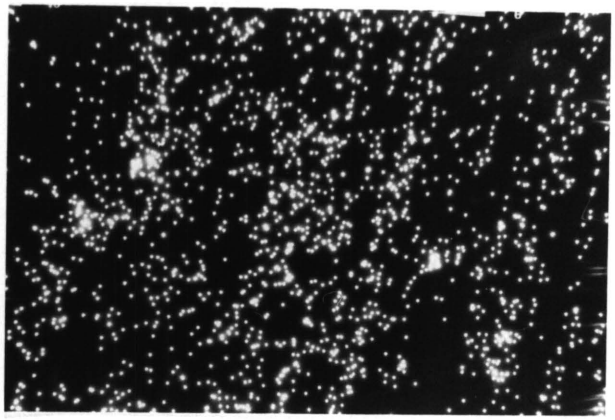


FIG. 14e

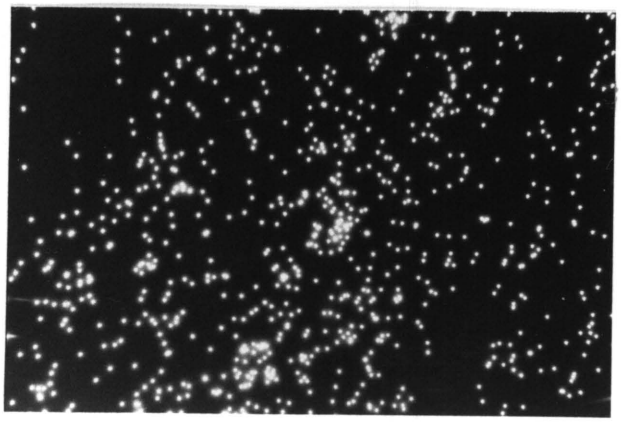


FIG. 14f

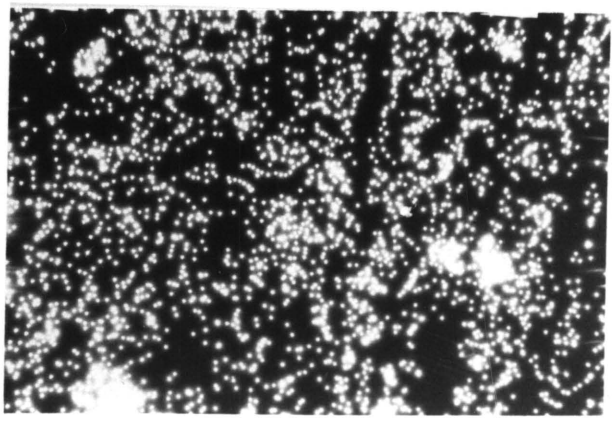


FIG. 15(a) The appearance of a sample of aerated Umhlatuzana sludge in the SEM. The sample was fixed in gluteraldehyde, dehydrated, critical point dried, and coated with carbon ×1 250.

FIG. 15(b) and (c) X-ray area maps for the same area as 15(a)
(b) Phosphorus (c) Calcium.

FIG. 15a

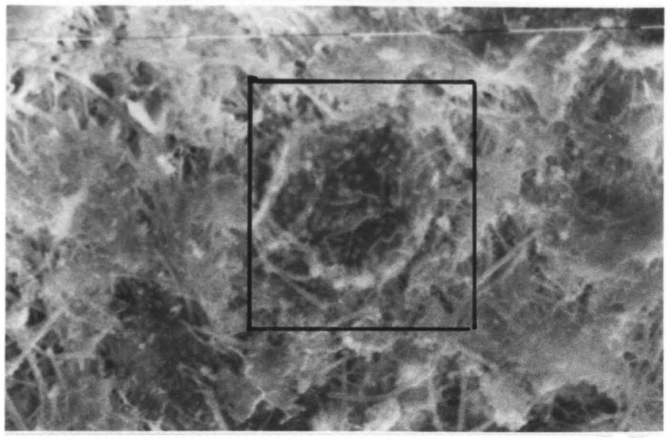


FIG. 15b

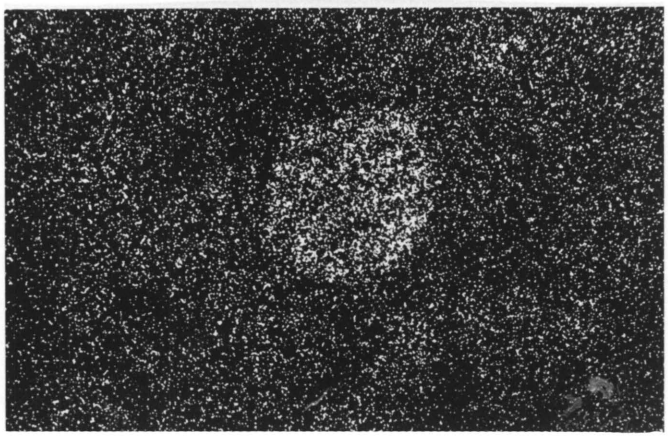


FIG. 15c

FIG. 16 The appearance of a sample of aerated Umhlatuzana sludge in the SEM. The sample was fixed in gluteraldehyde and osmium tetroxide, dehydrated, critical point dried, and coated with gold.

16(a) ×160 16(b) ×640 16(c) ×1 250

16(d) ×2 500 16(e) ×5 000 16(f) ×20 000

FIG. 16a

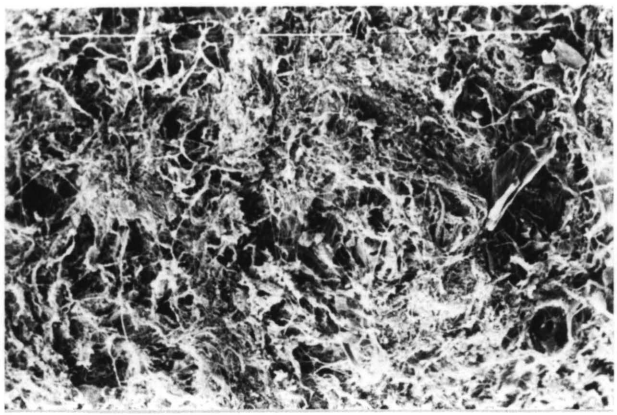


FIG. 16b

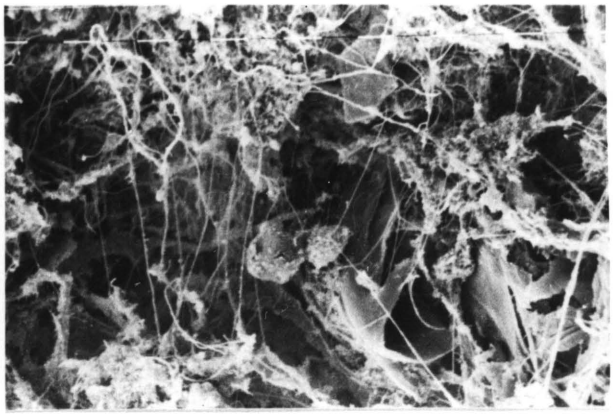


FIG. 16c

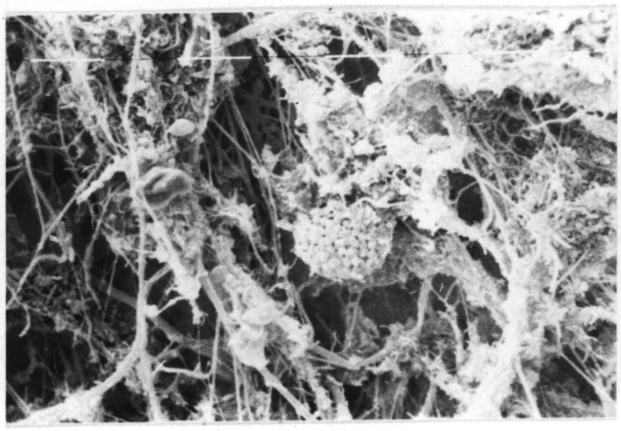


FIG. 16d

FIG. 16e

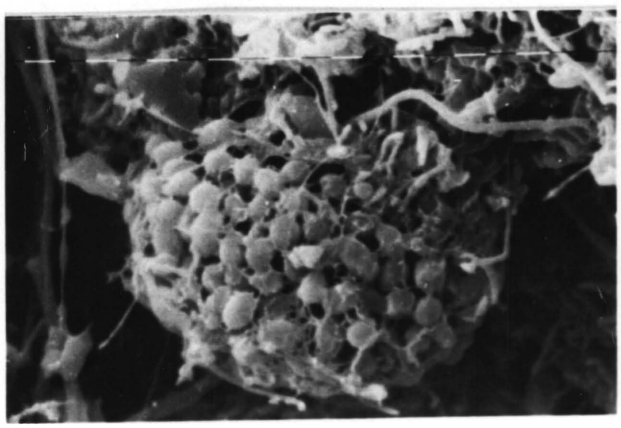
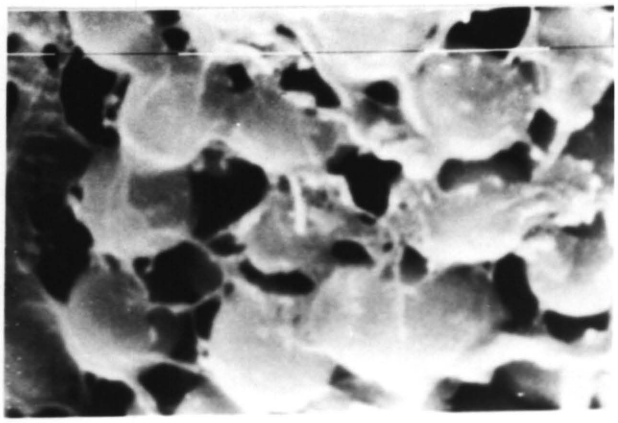


FIG. 16f



3) Transmission electron microscopy

After obtaining Polaroid photomicrographs of methylene-blue-stained 1µm sections on copper grids, the sections were stained with uranyl acetate and lead citrate. Examination in the TEM revealed that the metachromatic areas were tight clusters of large cells, characterized by the presence of large intracellular electron-dense bodies and by areas within the cells where their contents had been torn out during sectioning (Fig. 17).

After being assured of the consistent association of the intracellular electron-dense bodies with the metachromatic areas observed in the light microscope, an effort was made to examine the fine structure of these cells in 0,1µm sections, using the electron-dense bodies as markers to locate the cells. These efforts failed initially because no intracellular electron-dense bodies could be located in sections of less than 0,4µm. It eventually became apparent that due to the size of these bodies and their suspected physical density, they were all sheared out of the resin in sections of less than 0,4µm.

Sections of 1µm on beryllium grids which were prepared for STEM-EDX analysis were not stained with uranyl acetate and lead citrate. This procedure led to a significant observation which greatly obviated further investigations. When the electron beam was focussed on the electron-dense bodies

in unstained sections, they seemed to 'boil' and eventually lose their electron density in the central area, finally leaving an electron-dense perimeter (Fig. 18).

It was suspected that some form of degradation occurred during this process. Electron diffraction patterns were then obtained of several of these electron-dense bodies before and after the suspected degradation. Before exposure to the intensified electron beam, amorphous diffraction patterns were always obtained which could not be distinguished from the diffraction patterns obtained on adjacent Spurr's resin. After the bodies had reacted in the electron beam, a definite diffraction pattern was occasionally observed (Fig. 19). Dark field images of such bodies indicated the presence of scattered crystals (Fig. 20). From these observations it was concluded that a molecular rearrangement occurred during this process.

The degradation also occurred when sections were stained with potassium permanganate. Multivalent cations but not monovalent cations have been shown to stabilize polyphosphates (Friedberg and Avigad, 1968). It is also known that uranyl ions possess a strong affinity towards polyanions such as DNA and RNA (Zobel and Beer, 1961). The observation that potassium ions did not prevent the electron-dense bodies from undergoing degradation, coupled with the fact that these bodies exhibited amorphous electron

diffraction patterns, indicated that these bodies were not calcium phosphate precipitates, but probably polyphosphates. The volatilisation of polyphosphates with high electron beam intensity is also a known characteristic of these structures (Harold, 1966).

Careful observation of the process of degradation indicated that the electron-dense material was arranged around a central spherical structure of low electron density (Fig. 21). The polyphosphate granules in *Micrococcus lysodeikticus* have been shown to be arranged in a similar fashion (Friedberg and Avigad, 1968).

At this stage of the investigation it was evident that the intracellular electron-dense bodies and the holes in the resin where many of these bodies had been sheared out, were so conspicuous in the TEM that localization with the aid of methylene-blue-stained sections in the light microscope was no longer necessary. It was also evident that the metal content of the sludge samples was sufficient to obtain relatively sharp images in the TEM. Metal staining of sections was thus not considered necessary.

Figures 22(a) and (b) illustrate clusters of cells as observed in unstained 1µm sections.

During the morphological examination with the TEM it became clear that the electron-dense bodies were

characteristically found in a single morphological type of organism. These organisms were also numerically predominant. The second most abundant were clusters of smaller cells which were presumed to be nitrifying organisms, as was indicated by their lamellar structure (Figs. 23 and 24). These organisms were never found to contain the characteristic electron-dense bodies.

4) Scanning transmission electron microscopy and EDX analysis

Phase contrast photomicrographs at X100 magnification were obtained in the light microscope of 1µm sections on beryllium grids to assist in the eventual orientation of the grids in the STEM. Thereafter photomicrographs of cell clusters were obtained in the TEM at low magnification and using low electron beam intensity to prevent degradation of the electron-dense bodies.

Although the image obtained in the STEM was poor, the holes and electron-dense bodies in the specimens were easily located.

The energy dispersive X-ray spectra generated by the electron-dense bodies indicated that they were composed mainly of calcium and phosphorus, with suggestions of magnesium, pottasium and chlorine occasionally occurring (Fig. 25). Random microanalysis over the surface of the electron-dense bodies generated identical spectra, indica-

ting a uniform composition.

The energy dispersive spectra, generated over 25 electron-dense bodies, were examined. Similar spectra were generated for all the bodies although the intensity ratios for the individual bodies varied. The number of counts under the elemental peaks for nine of the electron-dense bodies were integrated over 40sec counting intervals, and the intensity ratios were converted to mass ratios for calcium and phosphorus by using the equation:

$$\frac{C_{Ca}}{C_P} = \frac{I_{Ca}}{I_P} \cdot \frac{P'_p}{P'_{Ca}}$$

The results are recorded in Table 3. Reference to the Ca:P molar ratios of various calcium phosphate species (Landis and Glimcher, 1978) indicated that the mass ratios obtained, totally excluded these bodies from being any form of calcium phosphate precipitate.

X-ray microanalyses were also performed on an electron-dense body after inducing degradation in the TEM. The analyses were carried out in a progressive sequence across the diameter of the body (Table 4). These results indicated that after degradation certain regions exhibited Ca:P mass ratios which could be related to some form of calcium phosphate precipitate. Such precipitates could

possibly have formed with the disintegration of the poly-phosphate chains. The lower counts obtained also indicated that a considerable degree of volatilisation had occurred.

FIG. 17 A cluster of large cells in a 1µm section of aerated Umhlatuzana sludge, stained with potassium permanganate.

The outline of one of the cells is indicated with the dotted line.
Bar represents 1µm.

FIG. 18 An electron-dense body which had lost its electron density after degradation was induced by the electron beam of the TEM.

Bar represents 1µm

FIG. 19 The electron diffraction pattern obtained after degradation of an electron-dense body was induced by the electron beam of the TEM.

FIG. 20 A dark field image of an electron-dense body after degradation, showing the presence of scattered crystals.

Bar represents 1µm.

FIG. 17

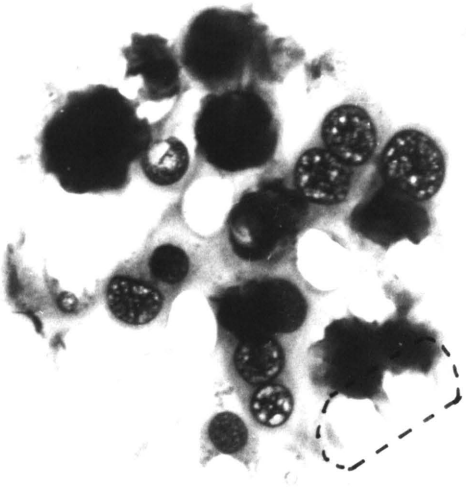


FIG. 18

FIG. 19

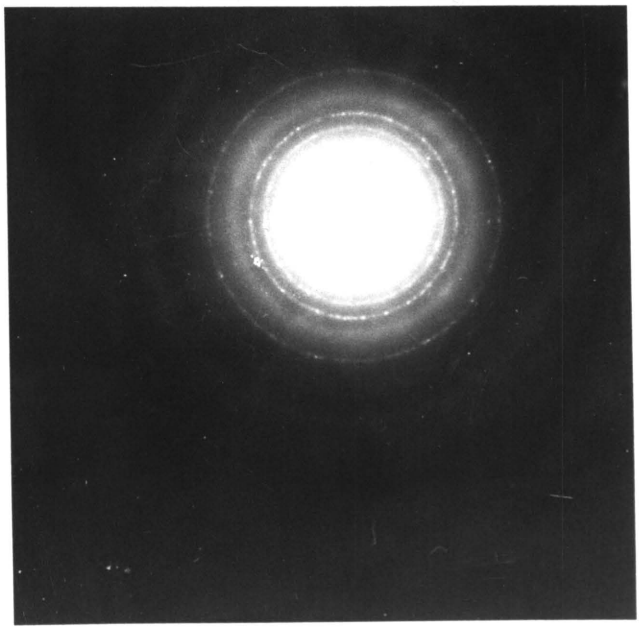


FIG. 20

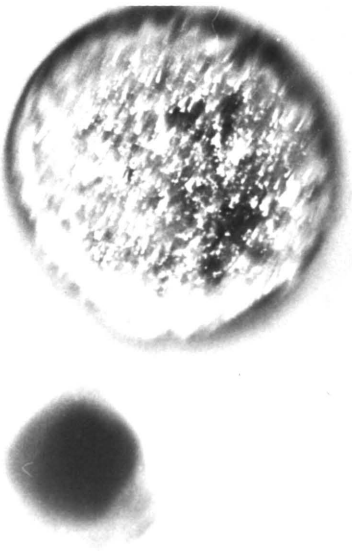


FIG. 21 An electron-dense body illustrating the central spherical structure (framed area). The arrow indicates the central structure.

Bar represents 1µm.

FIG. 22(a) and (b) Phosphorus-rich cell clusters in 1µm unstained sections of aerated Umhlatuzana sludge.

In Fig. 22(b) one of the cells are marked with the dotted line.

Bars represent 1µm.

FIG. 21

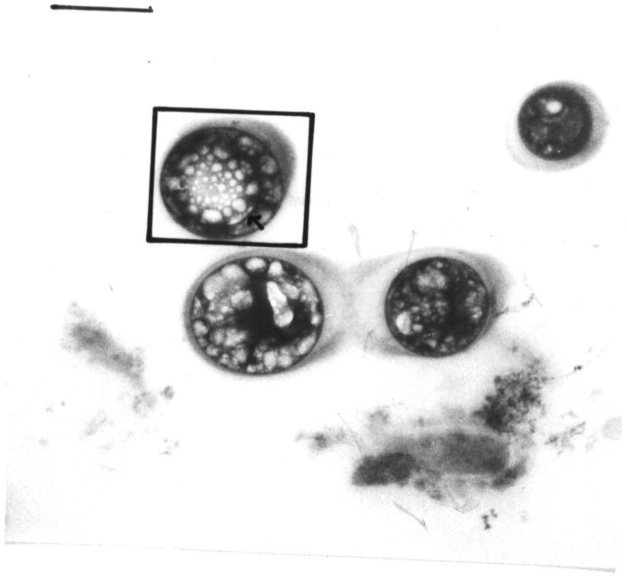


FIG. 22a

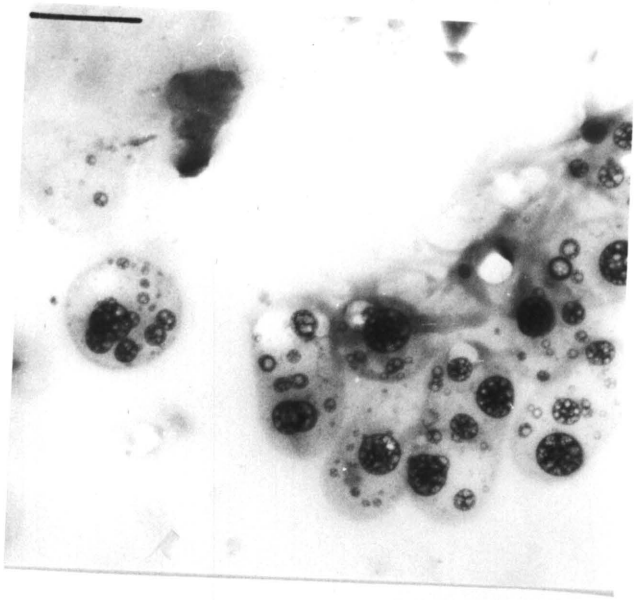


FIG. 22b

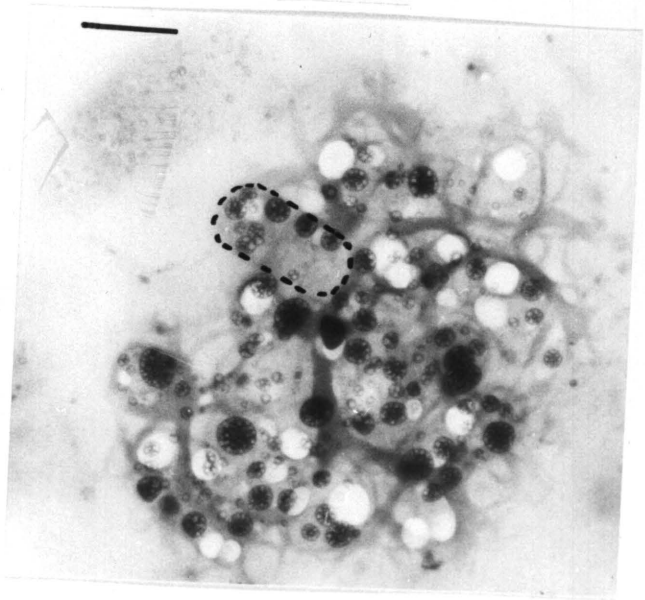


FIG. 23 Clusters of cells which were presumed to be nitrifying organisms, in a 1µm section of Umhlatuzana sludge.

Bar represents 1µm.

FIG. 24 Clusters of cells which were presumed to be nitrifying organisms in a 1µm section of Umhlatuzana sludge.

Note the lamellar structure of the cells.

Bar represents 1µm.

FIG. 25 X-ray energy dispersive spectrum generated by an electron-dense body in a 1µm section of Umhlatuzana sludge. The lines observed were Magnesium 1,25keV, Phosphorus 2,01keV, Chlorine 2,62keV, Potassium 3,3keV, Calcium 3,69keV.

FIG. 23

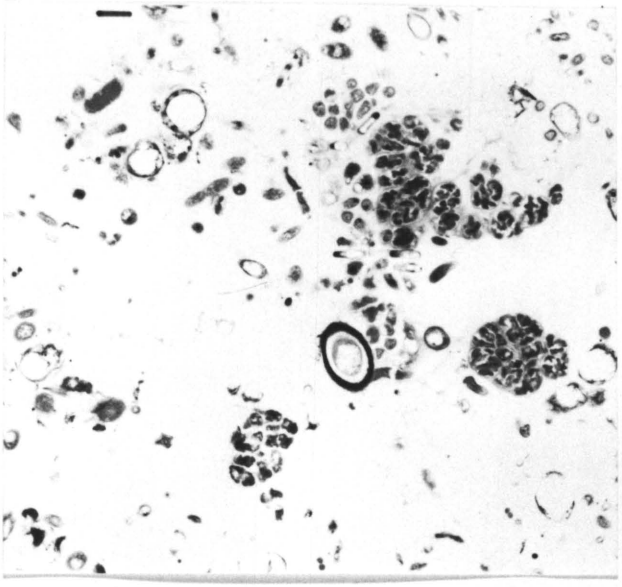


FIG. 24

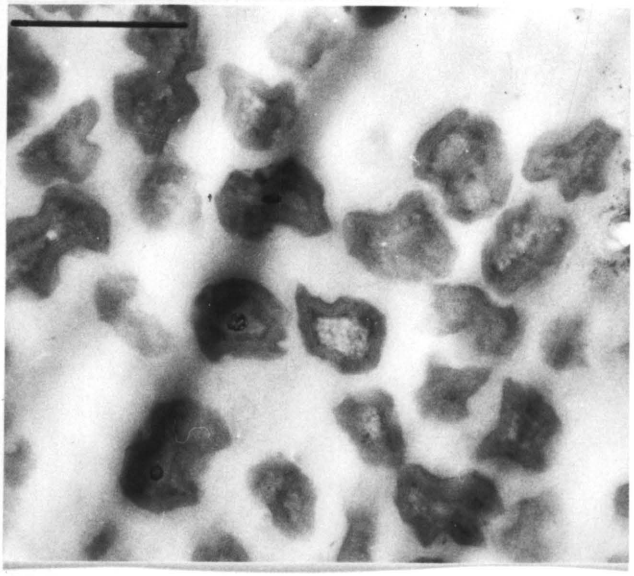


FIG. 25

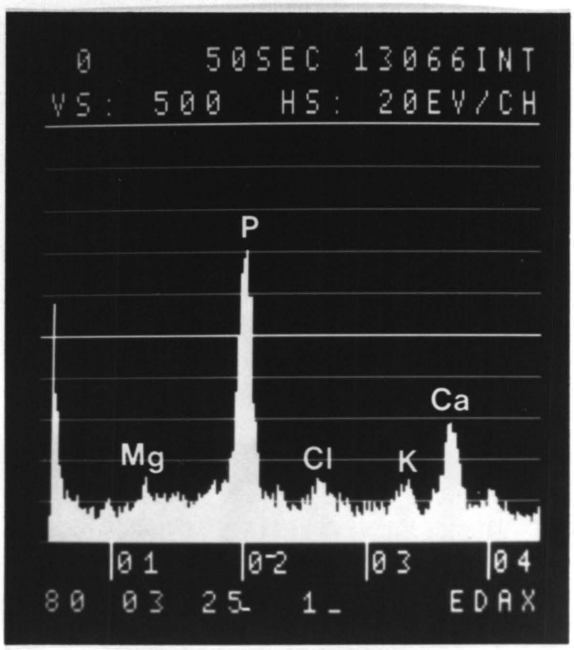


TABLE 2. Master curves for P´values at 40kV and 19kV

```
RUN
DIRECT ELEMENT WEIGHT FRACTION RATIO IN THIN SECTIONS
CALCULATE CONC. 1 : CONC. 2=INT.1/P.1 : INT.2/P.2 ETC.
ACCELLERATING VOLTAGE (KV.)=? 40
WINDOW CONSTANT =? 3.68
                                RELATIVE P VALUE ->
Z  P(K)   P(L)   0                          1
```

Z	P(K)	P(L)
11	.08	0
12	.3	0
13	.61	0
14	.93	0
15	1.2	0
16	1.38	0
17	1.5	0
18	1.56	0
19	1.59	0
20	1.58	0
21	1.55	0
22	1.5	0
23	1.44	0
24	1.37	0
25	1.3	0
26	1.22	0
27	1.14	0
28	1.06	.01
29	.98	.02
30	.9	.05
31	.83	.1
32	.76	.16
33	.69	.25
34	.62	.34
35	.56	.43
36	.51	.53
37	.46	.61
38	.41	.69
39	.36	.76
40	.32	.82
41	.28	.86
42	.24	.9
43	.21	.93
44	.18	.96
45	.15	.97

P'_P == 1,2

P'_{Ca} = 1,58

```
*READY
RUN
DIRECT ELEMENT WEIGHT FRACTION RATIO IN THIN SECTIONS
CALCULATE CONC. 1 : CONC. 2=INT.1/P.1 : INT.2/P.2 ETC.
ACCELLERATING VOLTAGE (KV.)=? 19
WINDOW CONSTANT =? 3.68
                                RELATIVE P VALUE ->
Z  P(K)   P(L)   0                          1                          2
```

Z	P(K)	P(L)
11	.08	0
12	.29	0
13	.59	0
14	.89	0
15	1.12	0
16	1.27	0
17	1.35	0
18	1.38	0
19	1.37	0
20	1.34	0
21	1.28	0
22	1.21	0
23	1.13	0
24	1.05	0
25	.96	0
26	.87	0
27	.78	0
28	.7	.01
29	.62	.02
30	.54	.05
31	.46	.1
32	.4	.16
33	.33	.24
34	.28	.33
35	.22	.42
36	.17	.5
37	.13	.58
38	.09	.65
39	.06	.71
40	.03	.76
41	0	.8
42	0	.83
43	0	.85
44	0	.86
45	0	.87

P'_P = 1,12

P'_{Ca} = 1,34

TABLE 3. EDX-analysis of nine electron-dense bodies in Umhlatuzana sludge

Integrated X-ray counts for elements					Calcium: Phosphorus X-ray intensity ratio	Calcium: Phosphorus mass ratio
Ca	P	Mg	S	C1		
329	1009				0,33	0,25
383	958			3	0,40	0,30
1707	3014				0,57	0,43
326	1840		758		0,18	0,14
784	2495	137			0,31	0,24
716	2309	157			0,31	0,24
856	2886	89			0,30	0,23
1289	3917	173			0,33	0,25
331	1244				0,27	0,21

Mean 0,254

s = 0,078

TABLE 4. EDX-analysis of an electron-dense body after degradation

Integrated X-ray counts for elements(i)			Calcium: Phosphorus intensity ratio	Calcium: Phosphorus mass ratio
Ca	P	Mg		
124	446	0	0,28	0,21
626	419	0	1,50	1,14
898	690	188	1,30	0,99
858	780	155	1,10	0,84
641	886	168	0,72	0,55
324	743	0	0,44	0,33

(i) The counts were obtained in a progressive sequence across the diameter of the body.

4.2 COMPARISON OF THE INTRACELLULAR PHOSPHORUS-RICH INCLUSIONS IN ACTIVATED SLUDGE FROM SEVEN PLANTS

1) Light microscopy

Examination of methylene-blue-stained smears revealed that all the sludges contained metachromatic cell clusters. Very few cell clusters were observed in Northern Works specimens whereas the specimens from the other plants indicated that the metachromatic clusters constituted the bulk of the biomass present. The cells in the clusters were in the form of large plump rods.

2) Electron microscopy

Triplicate samples of each sludge were prepared according to schedule 3.4.4(1). Ten sections were examined for each replicate, i.e. 30 sections for each sludge.

3) Transmission electron microscopy

The morphology of the samples was compared, using 0,4µm sections on copper grids. The specimens were mostly examined unstained.

To obtain information of the fine structure of the phosphorus accumulating cells, sections of 0,1µm were prepared and stained with potassium permanganate.

In all observations, electron-dense particles were only considered to contain phosphorus if they underwent degradation when the electron beam was focussed on them. Various electron-dense particles which did not undergo degradation were analysed in the STEM-EDX-system. Not one of these particles was found to contain phosphorus.

The fine structure of a group of phosphorus accumulating cells in a 0,1µm section from Laboratory scale plant 1, is illustrated in Fig. 26.

A small number of cells in short chains which contained the typical electron-dense inclusions, as illustrated in Fig. 26, were repeatedly observed in the vicinity of the cell clusters in all the sludges examined. During the examination of the sludges it became evident that there was some relationship between the cell chains and the clusters of phosphorus-accumulating cells.

Figures 27 to 31 illustrate typical cell clusters observed in the comparative examination.

The sludges from Umhlatuzana and from Laboratory scale plants I and II contained large clusters of large cells. The intracellular phosphorus-rich inclusions were so large that most had been sheared from the sections.

The only other cells, which were observed only occasionally, to contain the polyphosphate inclusions, were clusters of smaller cells which were always closely associated with the clusters of large cells (Fig. 27). This observation indicated that these small cells were possibly undeveloped forms of the large cells.

The phosphorus-accumulating cell clusters in the Brits and Goudkoppie sludges seemed to be smaller and there were fewer large cells. The large cells also contained large intra - cellular phosphorus - rich inclusions.

Northern works sludge which had the poorest phosphorus uptake capacity of all the sludges, contained a sparse distribution of the large phosphorus - rich cells.

Observation of the sludges from Brits, Goudkoppie I and II, and Northern works created the impression of phosphorus-accumulating cells being present in various stages of development, i.e. there were cells of varying sizes with small phosphorus - rich inclusions and in the same clusters there were also large cells with large polyphosphate inclusions (Figs. 29 and 31).

From the observations made in the comparative study it appeared as if the sludges from Goudkoppie I and II and Northern works possessed the necessary bacterial types to improve their phosphorus-uptake capacities. The organisms probably needed to be enriched and induced to increase their cell size and apparently associated phosphorus-accumulating properties.

FIG. 26 The fine structure of phosphorus-accumulating cells in a 0,1µm section of sludge from Laboratory scale plant I. The specimen was stained with potassium permanganate. The arrows indicate where large electron-dense bodies were sheared out of the section, and the framed areas indicate some of the remaining electron-dense bodies (confirmed by induction of slight degradation).
Bar represents 1µm.

FIG. 27 A large cluster of phosphorus-accumulating cells in an unstained 0,4µm section of Umhlatuzana sludge. Most of the electron dense bodies had been sheared from the large cells. Adjacent to the cluster of large cells were smaller cells which also contained phosphorus-rich inclusions. These cells were suspected to be undeveloped forms of the larger cells.
Bar represents 1µm.

FIG. 28(a) A cluster of phosphorus-accumulating cells in a 0,4µm unstained section of sludge from Laboratory scale plant I. Note that most of the electron-dense bodies were sheared from the section.
Bar represents 1µm.

FIG. 28(b) A cluster of phosphorus-accumulating cells in a 0,4µm unstained section of sludge from Laboratory scale plant II. Note that most of the electron-dense bodies were sheared from the section.
Bar represents 1µm.

FIG. 26

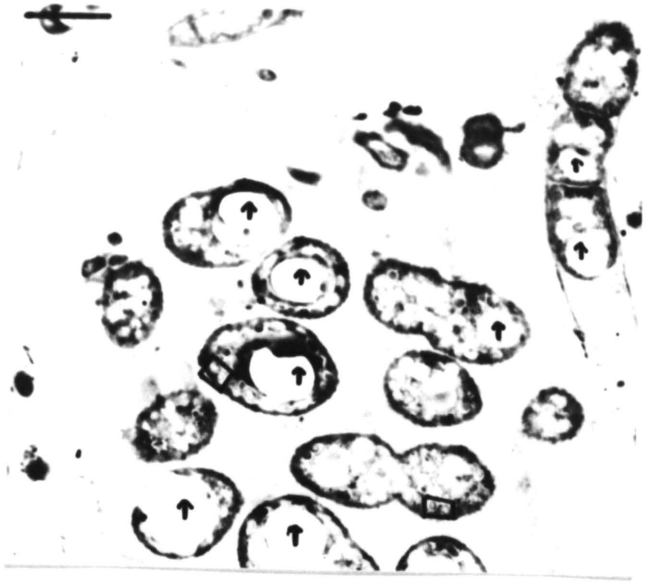


FIG. 27

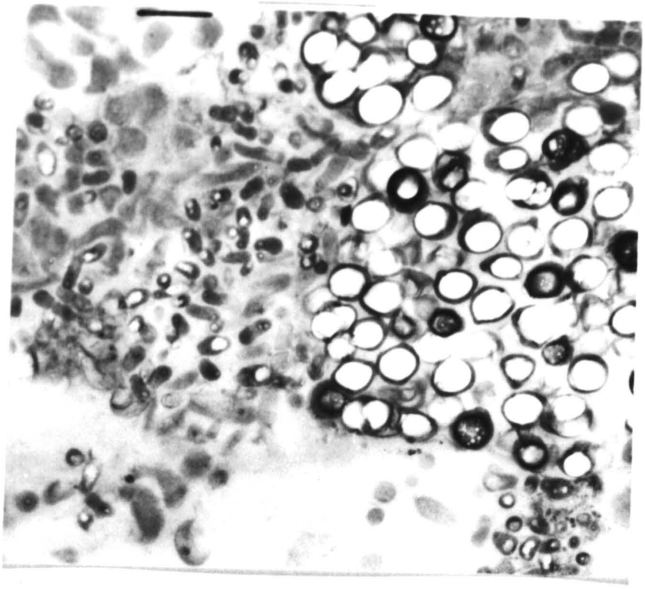


FIG. 28a

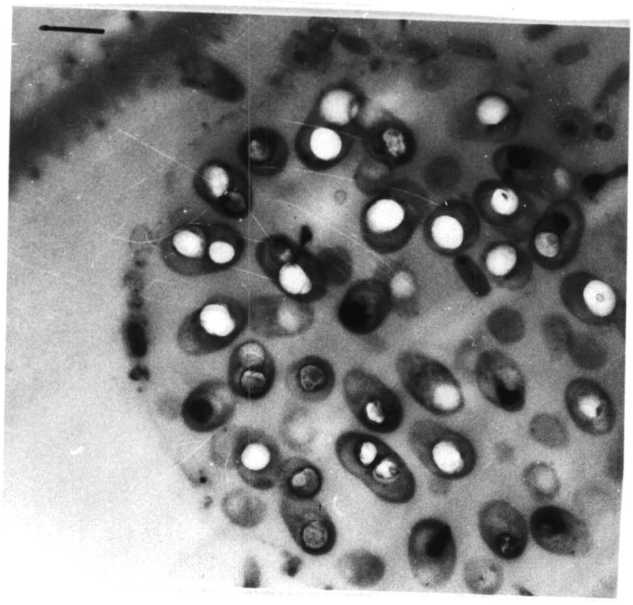


FIG. 28b

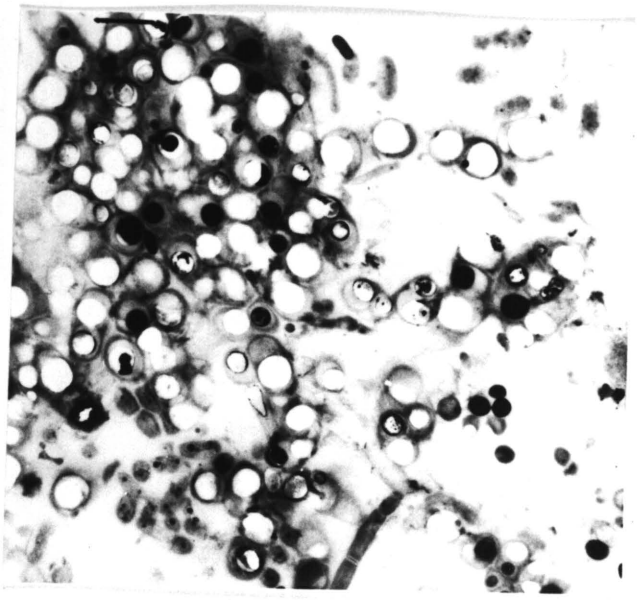


FIG. 29 A cluster of phosphorus-accumulating cells in a 0,4µm unstained section of Brits sludge. Note the small electron-dense inclusions in the smaller cells of the cluster.
Bar represents 1µm.

FIG. 30(a) and (b) Clusters of phosphorus-accumulating cells in 0,4µm unstained sections of sludge from Goudkoppie 1(a) and Goudkoppie 11(b). Note the chain forms in the framed area of (b).
Bars represent 1µm.

FIG. 31 A cluster of phosphorus-accumulating cells in a 0,4µm unstained section of Northern works sludge. Note the smaller phosphorus-rich inclusions in the smaller cells of the cluster (arrows).
Bar represents 1µm.

FIG. 29

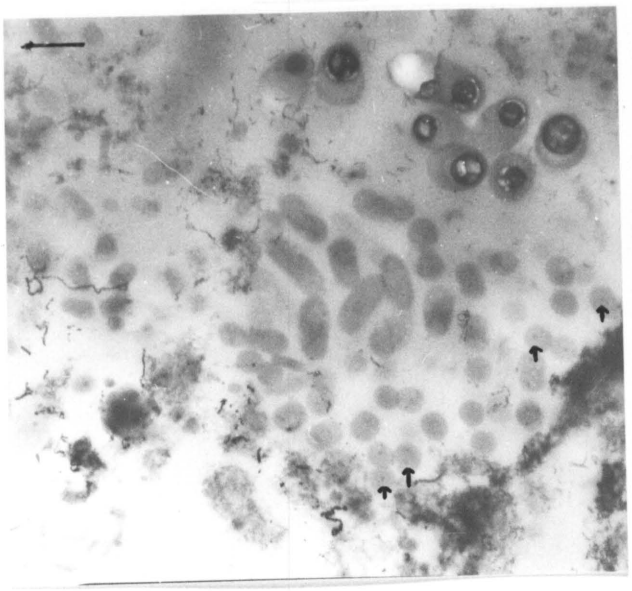


FIG. 30a

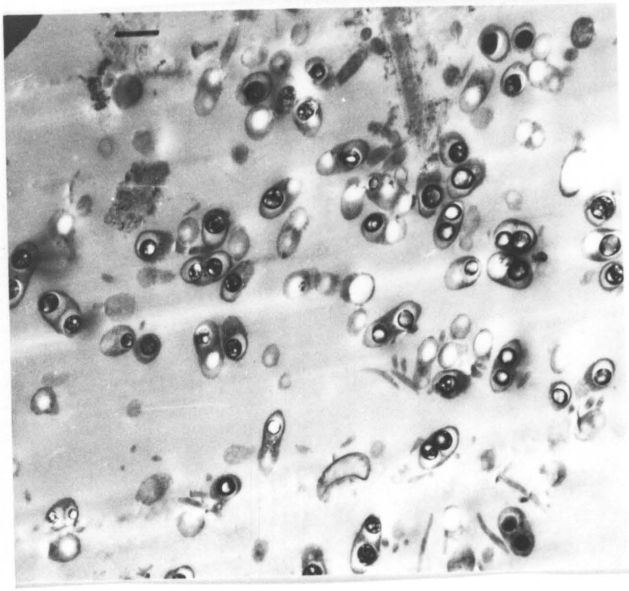


FIG. 30b

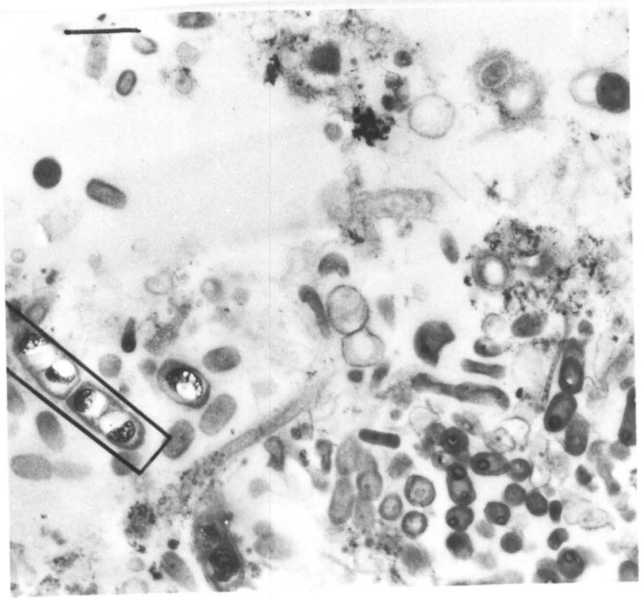
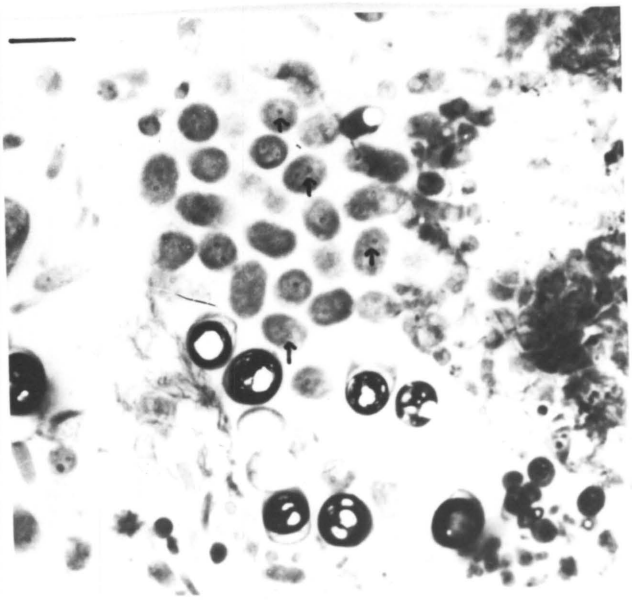


FIG. 31



4.3 STEM-EDX ANALYSIS OF THE ELECTRON-DENSE BODIES IN THE SLUDGES

4.3.1 Determination of the mass ratios of calcium and phosphorus in the electron-dense bodies

Specimens of 1µm on beryllium grids from Goudkoppie I, Laboratory scale plant 1 and Brits were analysed with the STEM-EDX-system. The electron-dense bodies were located, as previously described, and analysed to determine the elemental X-ray intensity ratios of calcium and phosphorus. Typical spectra generated by the different specimens are illustrated in Fig. 32.

The elemental peaks for calcium and phosphorus were integrated, using the Edax Edit EP7 programme, and the intensity ratios were converted to mass ratios (Table 5). From these results it was evident that the electron-dense bodies within the different specimens were similar in composition, with small variations in the mass ratios.

FIG. 32(a), (b) and (c) Energy dispersive X-ray spectra generated by electron-dense bodies in 1µm sections of sludge from Brits (a), Goudkoppie 1 (b), and Laboratory scale plant I (c).

FIG. 32a

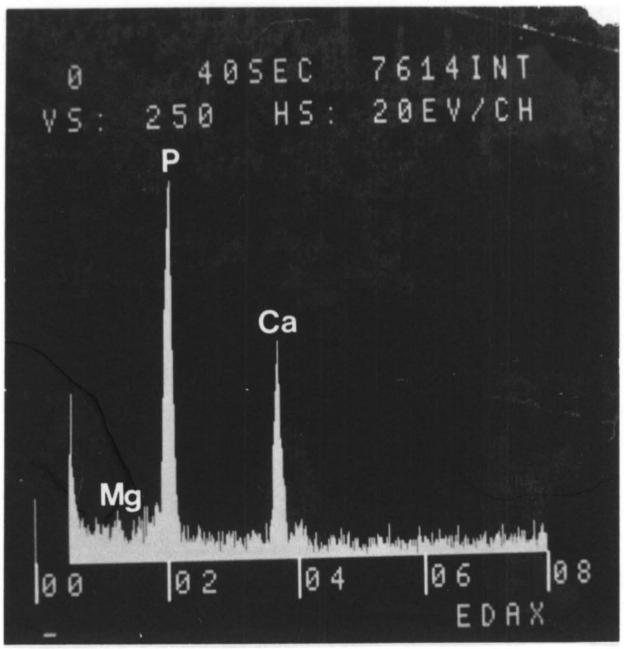


FIG. 32b

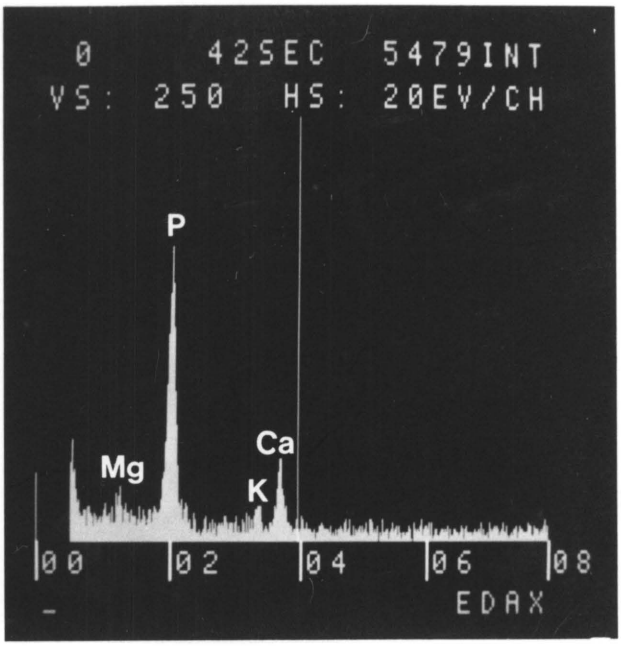


FIG. 32c

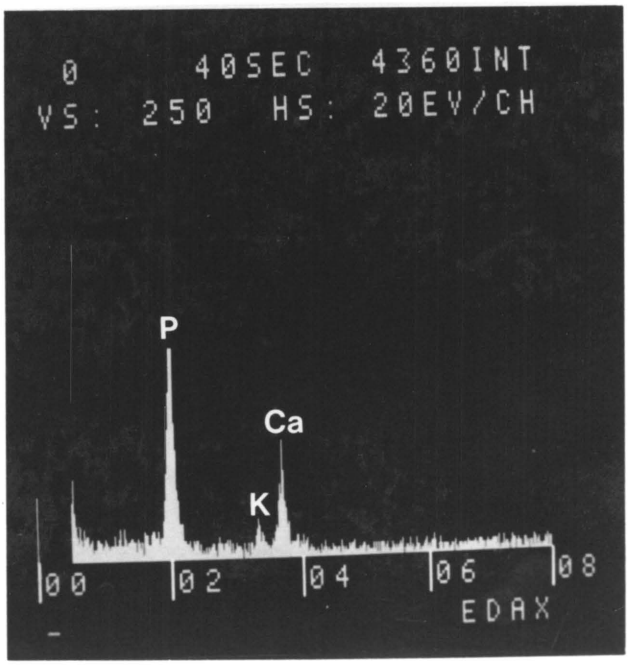


TABLE 5. EDX analysis of electron-dense bodies in three activated sludge specimens*

Plant	Integrated X-ray counts for elements		Calcium : Phosphorus X-ray intensity ratio	Calcium : Phosphorus mass ratio
Brits	Calcium	Phosphorus		
	273	557	0,49	0,37
	235	443	0,53	0,40
	235	436	0,54	0,41
	175	466	0,38	0,29
	266	602	0,44	0,33
			Mean:	0,36
			s =	0,05
Laboratory scale plant I	228	573	0,40	0,30
	321	835	0,38	0,29
	415	1251	0,33	0,25
	195	487	0,40	0,30
	233	672	0,35	0,27
	255	501	0,51	0,39
			Mean:	0,30
			s =	0,048
Goudkoppie I	72	471	0,15	0,11
	238	1296	0,18	0,14
	153	518	0,30	0,23
	219	1387	0,16	0,12
	177	785	0,23	0,17
			Mean:	0,15
			s =	0,048

*Accelerating voltage 40kV.

4.3.2 Quantitative analysis of electron-dense bodies

The data obtained with the EDX analyses are recorded in Table 6.

4.3.2.1 The effect of volatilisation and contamination by the electron beam on the P/B ratio

The uncorrected P/B ratios for calcium and phosphorus, after five consecutive analyses on the same spot in a specimen from the Brits works, are plotted in Fig. 33.

From this data it was evident that, although a small decline of the P/B ratio with time occurred it did not seriously affect the results, as short counting intervals (1-3min) were used.

4.3.2.2 Calculation of mass fractions

1) Correction factors for the contribution of stray radiation from iron and aluminium to the background counts (cf. p. 49).

Iron; $\frac{6578}{163203}$ = 0,040 error 1,2%

Aluminium; $\frac{4060}{297992}$ = 0,0136 error 1,6%

2) Correction of background counts.

The method which was used for the background correction is illustrated with reference to standard spectrum 1,

(Table 6).

Aluminium; The X-ray count generated for the aluminium peak was 1379.

The contribution to the count obtained in window no. 2 of the standard from scattered electrons striking the aluminium, therefore was:

$$1379 \times 0,0136 = 19 \text{ counts}$$

Iron; X-ray counts generated by iron = 1309.

The contribution of iron to window no. 2:

$$1309 \times 0,04 = 52 \text{ counts}$$

Corrected background count in window no. 2 for standard spectrum 1:

$$353-19-52 = 282 \text{ counts.}$$

The corrected background counts for window no. 2 are recorded in Table 7.

3) Corrected peak to background ratios.
The method to determine these ratios is also illustrated with reference to standard spectrum 1.

X-ray counts in characteristic calcium peak = 262.

Corrected background count for calcium = 282.

Corrected P/B ratio:

$$\frac{262}{282} = 0,93$$

4) Counting statistics.

The counting statistics of the X-ray counts and the corrected peak to background ratios for the standard and the specimens, are recorded in Tables 8 to 11.

Spectrum	Approximate diameters of electron-dense bodies (µm)	Accelerating voltage (kV)	Probe current (ampere)	Counting interval (min)	Characteristic elemental X-ray counts generated								X-ray counts generated in background windows (a) 2 4(d) (b) 13 9 (c) 2940 3700		Phosphorus: Calcium X-ray intensity ratio
					Al	Si	Cl	Ca	Fe	Mg	P	K			
Beam on Iron	-	19		Not recorded					163-203				6578		
Beam on Aluminium	-	19		Not recorded	297-992								4060		
Standard 1	-	19	1.10^{-9}	10	1379	126	504	262	1309				353	229	
" 2	-	19	2.10^{-9}	3	1203	270	439	175	1449				349	225	
" 3	-	19	3.10^{-9}	4	1328	339	628	263	2155				490	310	
" 4	-	19	1.10^{-9}	20	712	125	251	78	724				195	139	
" 5	-	19	1.10^{-9}	10	1602	321	503	194	1141				344	207	
Brits 1.1	0,5	20	1.10^{-9}	1	21	61	193	522	139	97	3056	98	208		
" 1.2	0,5	20	1.10^{-9}	1	0	85	113	1228	152	113	3030	105	209		
" 1.3	0,5	20	1.10^{-9}	1	0	149	137	1348	89	145	3152	143	209		
" 1.4	0,5	20	1.10^{-9}	1	0	81	138	1237	155	112	3128	109	243		
" 1.5	0,5	20	1.10^{-9}	1	32	163	190	1290	167	165	3056	91	218		
" 2	0,7	20	1.10^{-9}	Not recorded	186	1178	439	8716	656	717	18014	704	1164		2,06
" 3	0,5	20	1.10^{-9}	Not recorded	178	770	421	4170	624	511	9879	283	769		2,37
" 4	0,5	20	1.10^{-9}	Not recorded	179	1024	560	5403	508	507	11394	542	859		2,10
" 5	0,5	20	1.10^{-9}	Not recorded	111	636	399	3519	386	316	8088	186	631		2,3
Laboratory scale plant I															
" " " 1	0,8	20	1.10^{-9}	3	146	219	0	3136	309	607	9627	445	512		3,07
" " " 2	0,5	20	1.10^{-9}	3	191	333	0	3891	457	498	9615	636	656		2,47
" " " 3	0,5	20	1.10^{-9}	3	199	154	0	3187	381	409	7809	437	575		2,45
" " " 4	0,7	20	1.10^{-9}	3	325	317	59	5325	402	430	12205	822	720		2,29
Beam on Spurr's		20	1.10^{-9}	3	134	74	0	0	352	0	0	0	293		
Beam in specimen grid hole		20	1.10^{-9}	3	0	0	0	0	274	0	0	0	9		

(a) Background window number

(b) Number of channels in window

(c) Energy (eV) at centre of window

(d) Background window for calcium

FIG 33

The variation of the P/B Ratio due to Contamination and Volatilisation.

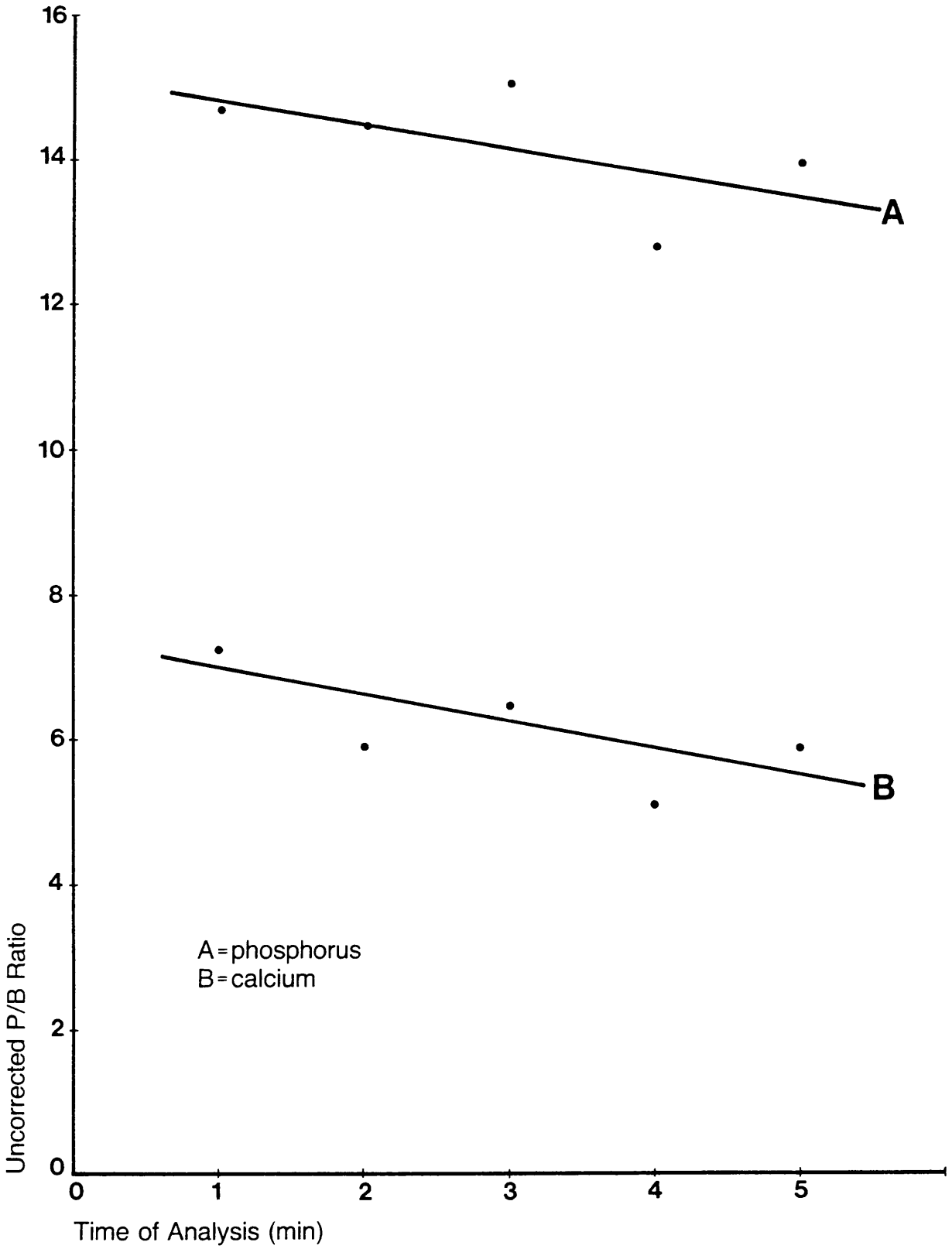


TABLE 7: Corrected background counts for standard and specimens

A		B	C	D	E	F
Spectrum		X-ray counts generated in background window no. 2	X-ray counts contributed to background window no. 2 by stray radiation	X-ray counts obtained in background window after correction for stray radiation	Standard deviation (a)	Coefficient of variation (b)
Standard	1	353	71	282	21	0,075
	2	349	74	275	21	0,076
	3	490	104	386	24	0,062
	4	195	39	156	15	0,096
	5	344	68	276	20	0,072
Brits	2	1164	29	1135	34	0,030
	3	769	27	742	28	0,038
	4	859	22	837	30	0,036
	5	631	17	614	25	0,041
Laboratory scale plant I	1	512	14	498	23	0,046
	2	656	21	635	26	0,041
	3	575	18	557	24	0,044
	4	720	20	700	27	0,039

(a) Standard deviation $= \sqrt{D+C+C}$

(b) Coefficient of variation $= \frac{E}{D}$

TABLE 8: Counting statistics of the integrated peak counts for the 0,5% calcium standard

A	B	C	D	E
Spectrum	X-ray counts generated by calcium	X-ray counts generated in calcium background window	Standard deviation(á)	Coefficient of variation(b)
1	262	229	27	0,10
2	175	225	25	0,14
3	263	310	30	0,11
4	78	139	19	0,24
5	194	207	25	0,13

(a) Standard deviation = $\sqrt{B+C+C}$

(b) Coefficient of variation = D/B

TABLE 9: Counting statistics of the integrated peak counts for calcium and phosphorus in the specimens

A		B		C	D		E	
Spectrum		Characteristic X-ray counts generated		X-ray counts generated in window no. 2	Standard deviation (a)		Coefficient of variation (b)	
		Ca	P		Ca	P	Ca	P
Brits	2	8716	18014	1164	105	142	0,012	0,008
	3	4170	9879	769	75	107	0,018	0,011
	4	5403	11394	859	84	115	0,016	0,010
	5	3519	8088	631	69	97	0,012	0,012
Laboratory scale plant I								
	1	3136	9627	512	65	103	0,021	0,011
	2	3891	9615	656	72	104	0,019	0,011
	3	3187	7809	575	66	95	0,021	0,012
	4	5325	12205	720	82	117	0,015	0,010

(a) Standard deviation = $\sqrt{B+C+C}$

(b) Coefficient of variation = D/B

TABLE 10: Corrected peak to background ratios for the 0,5% calcium standard

A	B	C	D	E
Spectrum	X-ray counts generated by calcium	Corrected X-ray count for window no. 2	Corrected P/B ratio $\{(I_P/I_B)_{st}\}$	Coefficient of variation for ratio[a]
1	262	282	0,93	0,13
2	175	275	0,64	0,16
3	263	386	0,68	0,13
4	78	156	0,50	0,26
5	194	276	0,70	0,15
			Mean 0,69 s_{n-1} = 0,16[b]	

(a) Coefficient of variation = $\sqrt{(\text{coefficient of variation of B})^2 + (\text{coefficient of variation C})^2}$

(b) The coefficient of variation of the P/B ratio derived from counting statistics could not explain the variation observed in the five values for the P/B ratio. Experimental variation must have influenced the ratio, i.e. changes in analytical conditions or non-uniformity of the standard.

The standard deviation of the mean P/B ratio was therefore used as a measure of total variation (22%).

TABLE 11: Corrected peak to background ratios for specimens

A		B		C	D		E	
Spectrum		Characteristic X-ray counts generated		Corrected X-ray counts for window no. 2	Corrected P/B ratio $\{(I_P/I_B)_{sp}\}$		Coefficient of variation for ratio[a]	
		Ca	P		Ca	P	Ca	P
Brits	2	8716	18014	1135	7,7	15,9	0,03	0,03
	3	4170	9879	742	5,6	13,3	0,04	0,04
	4	5403	11394	837	6,5	13,6	0,04	0,04
	5	3519	8088	614	5,7	13,2	0,04	0,04
Laboratory scale plant I								
	1	3136	9627	498	6,3	19,3	0,05	0,05
	2	3891	9615	635	6,1	15,1	0,05	0,04
	3	3187	7809	557	5,7	14,0	0,05	0,04
	4	5325	12205	700	7,6	17,4	0,04	0,04

(a) Coefficient of variation $= \sqrt{(\text{coefficient of variation of B})^2 + (\text{coefficient of variation of C})^2}$

5) $\Sigma CZ^2/A$ for standard

Element	C	Z^2	A	CZ^2/A
H	0,048	1	1	0,048
C	0,735	36	12	2,205
O	0,190	64	16	0,760
Ca	0,005	400	40	0,050
C1	0,022	289	35	0,182

$CZ^2/A = 3,245$

6) $\Sigma CZ^2/A$ for specimens

By iterative calculation the following approximate compositions for the analysed volumes were obtained:

Laboratory scale plant I

Spectra 1,2,3,4.

Element	C	Z^2	A	CZ^2/A
H	0,030	1	1	0,03
C	0,122	36	12	0,37
N	0,080	49	14	0,28
O	0,480	64	16	1,92
Mg	0,030	144	24	0,18
P	0,190	225	31	1,38
K	0,008	361	39	0,07
Ca	0,060	400	40	0,60

$\Sigma CZ^2/A = 4,83$

Brits

Spectrum 2

Element	C	Z^2	A	CZ^2/A
H	0,030	1	1	0,03
C	0,128	36	12	0,38
N	0,040	49	14	0,14
O	0,500	64	16	2,00
Mg	0,042	144	24	0,25
P	0,180	225	31	1,30
K	0,010	361	39	0,09
Ca	0,070	400	40	0,70

$\Sigma CZ^2/A = 4,89$

Spectra 3,4,5

Element	C	Z^2	A	CZ^2/A
H	0,030	1	1	0,03
C	0,122	36	12	0,37
N	0,080	49	14	0,28
O	0,480	64	16	1,92
Mg	0,030	144	24	0,18
P	0,190	225	31	1,38
K	0,008	361	39	0,07
Ca	0,060	400	40	0,60

$\Sigma CZ^2/A = 4,83$

7) Mass fractions obtained for calcium after substitution of $(I_P/I_B)_{sp}$, $(I_P/I_B)_{st}$, $(C_i)_{st}$, $(\sum_j C_j Z_j^2/A_j)_{st}$ and $(\sum_j C_j Z_j^2/A_j)_{sp}$ into equation (3)

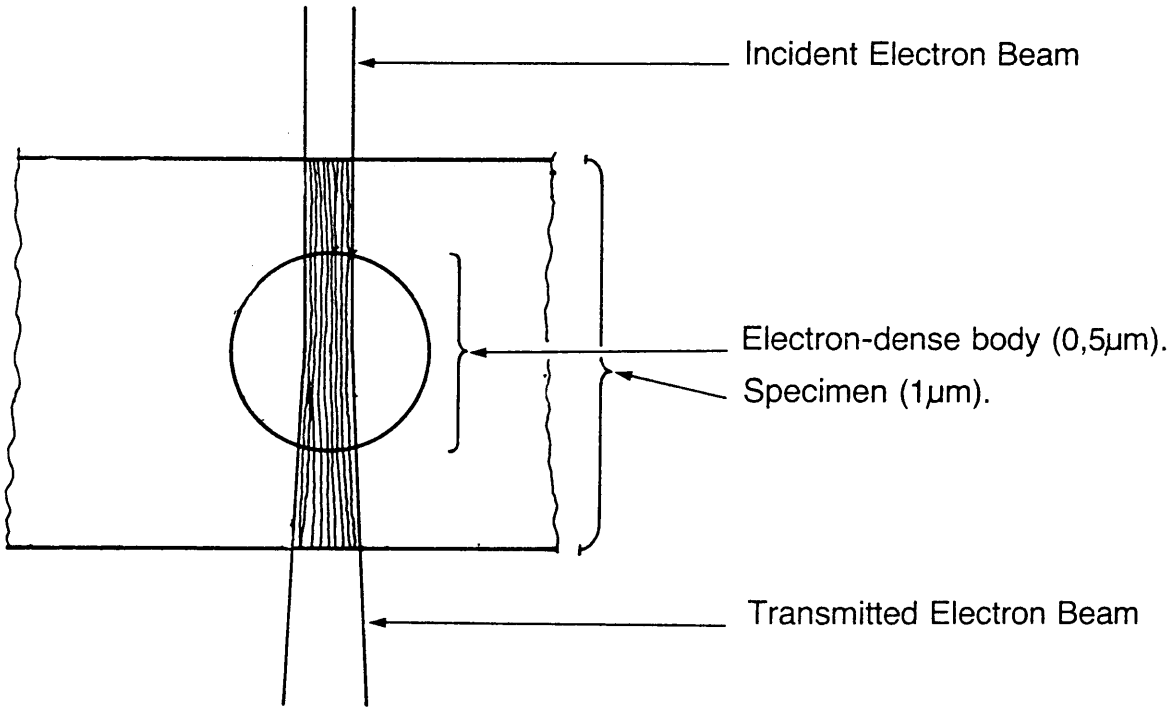
Spectrum		% Ca
Laboratory scale plant I		
	1	6,8
	2	6,5
	3	6,1
	4	8,2
Brits		
	2	8,3
	3	6,0
	4	7,0
	5	6,1

8) Mass fractions obtained for phosphorus after substitution of the calcium mass fractions into equation (4)

Spectrum		%P
Laboratory scale plant I		
	1	25
	2	19
	3	18
	4	23
Brits		
	2	21
	3	18
	4	19
	5	18

9) Concentration of phosphorus and calcium in the electron-dense bodies

The figures obtained represent the composition of the analysed volumes. The electron-dense bodies analysed were mostly approximately 0,5µm in diameter and the section thicknesses were 1µm. The following illustration serves to illustrate this concept.



The shaded area represents the analysed volume. This volume included the Spurr's resin, the electron-dense

body and other biological material.

Because of uncertainty as to the density of the electron-dense bodies and the exact thickness of the sections, it was difficult to estimate accurately the concentrations of phosphorus and calcium in the electron-dense bodies. It can, however, be accepted that the concentrations are larger than those indicated in 4.3.2(7) and (8).

10) Precision of analysis

Because of counting statistics and errors in background subtraction when the background is high compared to net peak intensity, the principal limitation was due to the low peak intensity of the calcium standard (P/B ratio, s_{n-1} = 22%).

11) Accuracy of analyses

The accuracy was primarily dependent on:

(i) The quality of the standard (which was obtained from a reputable manufacturer).

(ii) The accuracy of the composition of the analysed volumes as determined by iterative calculation:

From Fig. 33 it is evident that elemental migration, volatilisation and specimen contamination did not seriously affect the results. The error, which could have been introduced by these phenomena, would have led to an underestimation of the concentrations obtained.

The bremsstrahlung from the beryllium grid was not considered. This was a systematic error which was considered to be very small. If the correction was made, it would also have increased the concentrations obtained.

In the calculations to determine whether fluorescence and absorption played a significant role in the X-ray counts obtained (appendix II), relatively high concentrations were ascribed to the major elements in the matrix. A relatively high density for the electron-dense bodies was also assumed. From the results obtained with these calculations it appeared as if fluorescence and absorption effects were neglible.

The method is based on the model of Kramers (1923), which is cited as being 90% accurate.

4.4 THE FATE OF THE INTRACELLULAR PHOSPHORUS-RICH INCLUSIONS IN SLUDGES SUBJECTED TO ANAEROBIOSIS

1) Sludges examined

(i) Umhlatuzana sludge left anaerobic for 24h. This led to the release of 40% of the total phosphorus in the sludge into the supernatant as orthophosphate.

(ii) Sludge drawn from the anaerobic zone of Laboratory scale plant I.

2) Sample preparation and examination

The sludges were prepared according to schedule 3.4.4(1) and examined unstained. The presence of phosphorus-rich inclusions was confirmed by inducing degradation with the electron beam of the TEM.

Electron microscopic examination revealed that a large number of the phosphorus-accumulating cells in the sludge which had been left anaerobic for 24h contained virtually no intracellular electron-dense inclusions. It also appeared as if the cell clusters had been dispersed (Fig. 34).

With the specimen from Laboratory scale plant I it was apparent that the electron-dense bodies in many of the phosphorus-accumulating cells had virtually disappeared. It was also evident that the electron-dense bodies which were still present were dispersing into smaller electron-dense particles (Fig. 35).

FIG. 34 Phosphorus-accumulating cells in a 0,4µm unstained section of 24h anaerobic Umhlatuzana sludge. Note that the cells had lost most of their electron-dense inclusions, with only a few small inclusions remaining.
Bar represents 1µm.

FIG. 35(a) and (b) Phosphorus-accumulating cells in 0,4µm unstained sections of sludge drawn from the anaerobic zone of Laboratory scale plant I.
(a) Cell cluster (i) had lost practically all its electron-dense inclusions, and in cluster (ii) the electron-dense bodies had dispersed into smaller particles.
(b) Note that the large electron-dense body (i) had lost some of its density, which probably occurred due to the smaller particles being released from the central spherical body.
Bars represent 1µm.

FIG. 34

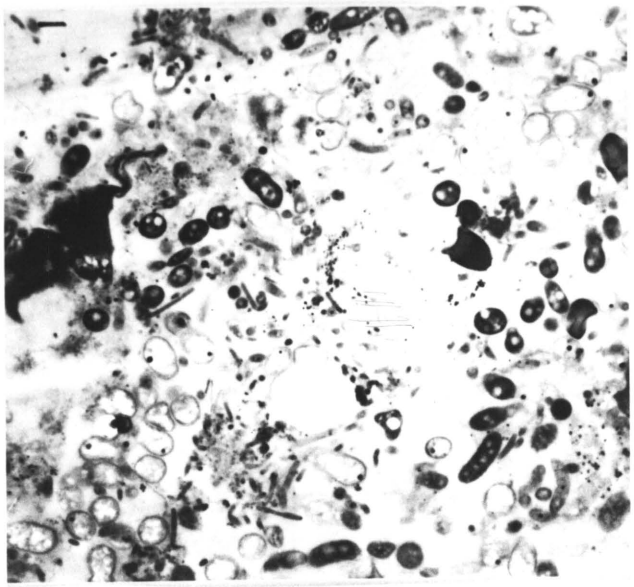


FIG. 35a

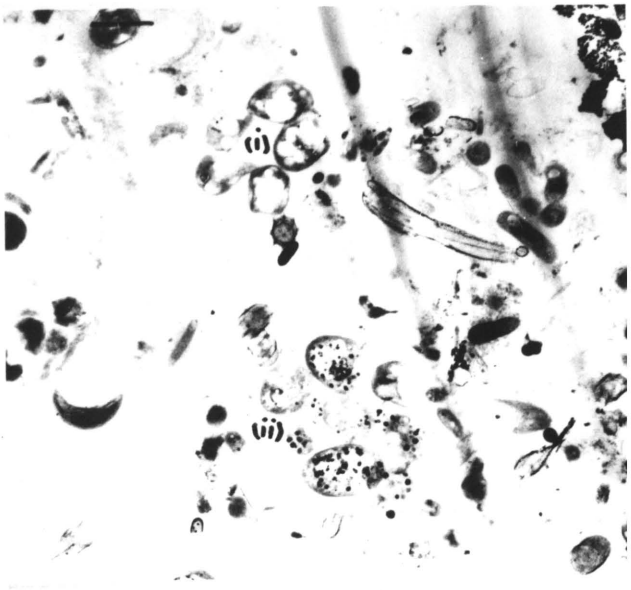
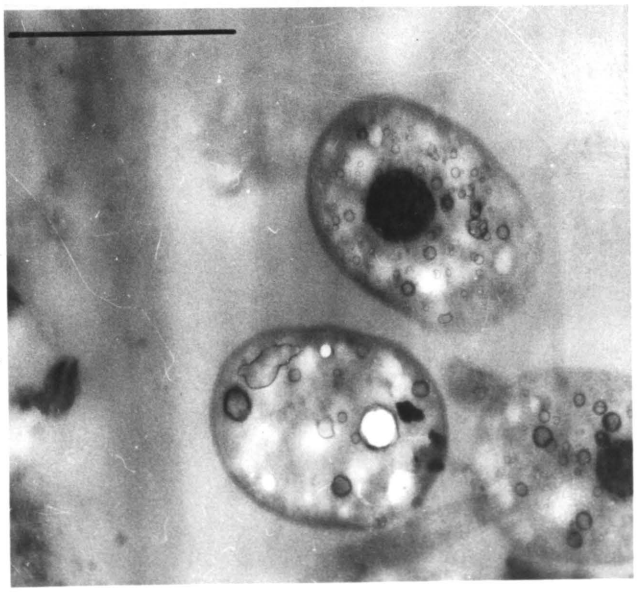


FIG. 35b



4.5 POPULATION CHARACTERISTICS OF THE GRAM NEGATIVE, HETEROTROPHIC BACTERIA IN ACTIVATED SLUDGE FROM FOUR PLANTS

Having ascertained that a particular morphological type of organism, which was numerically predominant, was responsible for phosphorus accumulation in all the sludges examined, a survey which was being made of the bacterial population structure of the Goudkoppie works was extended to some of the other plants (Table 12).

Although the homogenization procedure adopted in this survey would not have affected efficient dispersal of the organisms, the predominance of *Acinetobacter* spp was clearly indicated. *Acinetobacter* spp have been implicated as being responsible for enhanced phosphorus removal (Fuhs and Chen, 1975).

The previous examination of the sludges in the TEM showed that even after the vigorous sample preparation procedure the phosphorus-accumulating cells were present in large clusters. If it were to be accepted that the cell clusters were *Acinetobacter* spp, it is evident that the values obtained for *Acinetobacter* spp in this survey would have been considerably higher had the cells been dispersed during homogenization. The colonies isolated after the initial spreading on G.C.Y. agar had obviously been initiated by cell clusters and not by single cells.

The exceptionally large clusters observed in the TEM in the case of the Umhlatuzana sludge indicated that the count for *Acinetobacter* spp would have been greatly increased had the cell clusters been dispersed.

Because of the large error introduced into the percentage population distributions by the clumping of the cells, the value of these surveys were only in the supportive evidence they supplied as to the role of *Acinetobacter* spp in the process.

The 12 cell clusters extracted by micromanipulation from Laboratory scale plant I and Brits mixed liquor, were all identified as *Acinetobacter* spp.

Acinetobacter posesses a relatively characteristic cellular morphology, i.e. short plump rods 1,0-1,5µm by 1,5-2,5µm in logarithmic phase, approaching coccus shape in stationary phase and predominantly in pairs and short chains (Buchanan and Gibbons, 1971; Juni, 1978). The morphology of the phosphorus-accumulating organisms (Fig. 26) corresponded with this description. Juni (1978) made use of the characteristic cellular morphology, lack of motility, gram-negative reaction and oxidase-negative reaction to isolate acinetobacters from soil, water and sewage. It was subsequently shown with transformation assay that 105 out of 114 strains isolated on the basis of the aforementioned characters were acinetobacters.

Source of activated sludge		Brits	Goud Koppie I	Goud Koppie II	Northern works	Umhlatuzana
Date of sampling		80.07.23	80.01.18	80.02.14	80.08.13	79.10.04
Colonies subcultured onto GCY-agar		100	100	105	100	96
Number of colonies which did not grow		6	0	10	2	0
Number of Gram positive organisms		1	14	11	0	15
Number of colonies put onto API 20E		93	86	84	98	28 (i)
% Obligate aerobic organisms		94	44	76	63	42
% Facultative anaerobic organisms		4	37	12	37	54
% Denitrifiers		7	25	13	7	
Organisms identified	Mode of respiration	(ii)	(ii)	(ii)	(ii)	(iii)
Acinetobacter spp	Aerobic	64	35	39	35	16
Aeromonas hydrophila	Facultative Anaerobic	2	9	11	6	
Alcaligenes spp	Aerobic	24		12	6	5
Bordetella bronchi-septica	Aerobic	2	1	4	2	
Brucella spp	Aerobic		1	1		
CDC Group 11F	Facultative Anaerobic				26	
Citrobacter spp	Aerobic	2				
Enterobacter agglom erans	Facultative Anaerobic		2			
Enterobacter freundii	Facultative Anaerobic		6			
Escherichia coli	Facultative Anaerobic			1		
Flavobacterium spp	Facultative Anaerobic			6	4	
Moraxella spp	Aerobic	1	6	14	14	4
Pasteurella spp	Facultative Anaerobic		26	1		
Pseudomonas spp	Aerobic	4	8	10	7	3
Shigella spp	Facultative Anaerobic					
Unidentifiable		2	6	1		1

(i) Only obligate aerobes were put onto API.

(ii) Figures are for the percentage of organisms put onto API.

(iii) Figures are for the percentage of organisms subcultured onto GCY.

4.6 THE ROLE OF OXYGEN DEPRIVATION AND SUBSTRATE QUALITY ON THE ACCUMULATION OF PHOSPHORUS

The possible application of the developed techniques in the elucidation of the role of oxygen deprivation and substrate quality was investigated. These investigations were conducted with the pure culture of *A. calcoaceticus* and with activated sludge from the Northern works. The Northern works sludge was utilised because of its poor phosphorus-uptake capacity.

1) *A. calcoaceticus* grown on acetate as sole carbon source

Acinetobacter which was maintained on acinetobacter-agar prepared as described in section 3.2(2), was streaked onto acinetobacter-agar and incubated aerobically at 20°C for 48h. A section of the culture was then prepared for electron microscopy as described in section 3.4.4(2). Sections of 1µm were then examined in the TEM. The sections were scanned to find the characteristic electron-dense bodies. Degradation of the electron-dense bodies by the intensified electron beam was used as confirmation for the presence of polyphosphate inclusions.

The examination revealed that electron-dense bodies were present in a few isolated large cells (Fig. 36), or in small clusters of large cells (Figs. 37 and 38). The appearance of the electron-dense bodies ranged from scattered small particles to relatively large bodies (Figs. 38, 39, 40, 41 and 42).

The polyphosphate-containing cells were conspicuous because they were much larger and possessed a far greater electron density than the other cells. The high electron density was probably due to the presence of scattered small polyphosphate granules. A few filamentous forms which contained some polyphosphate granules were also observed (Fig. 43).

It was puzzling that only a small percentage of the total number of cells contained the polyphosphate inclusions.

FIG. 36 *A. calcoaceticus* grown on acetate as sole carbon source. The cells containing polyphosphate inclusions were considerably larger and more electron-dense than the other cells (framed areas).
Bar represents 1µm.

FIGS. 37 and 38 *A. calcoaceticus* grown on acetate as sole carbon source. The cells containing polyphosphate inclusions were grouped in clusters and were larger and more electron-dense than the other cells.
Bars represent 1µm.

FIG. 39 *A. calcoaceticus* grown on acetate as sole carbon source. The framed areas and arrows indicate the various sizes of some of the polyphosphate inclusions.
Bar represent 1µm.

FIG. 36

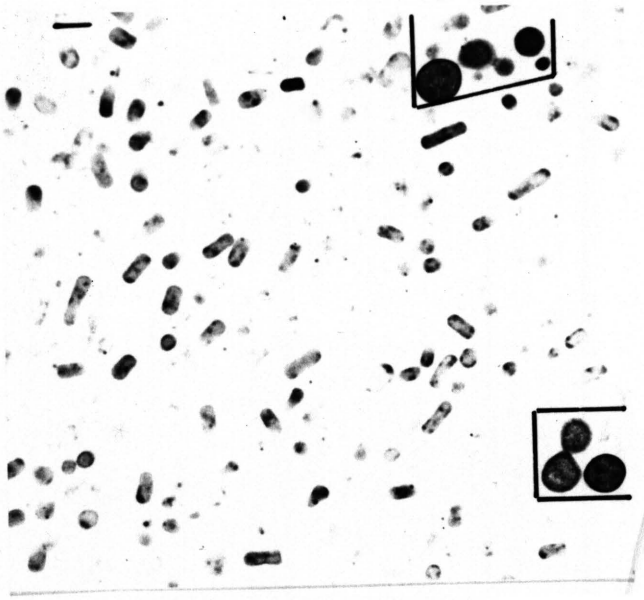


FIG. 37

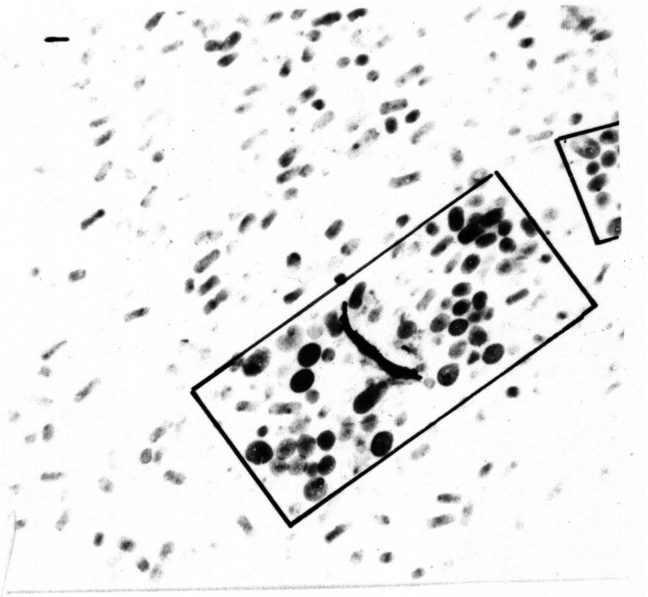


FIG. 38

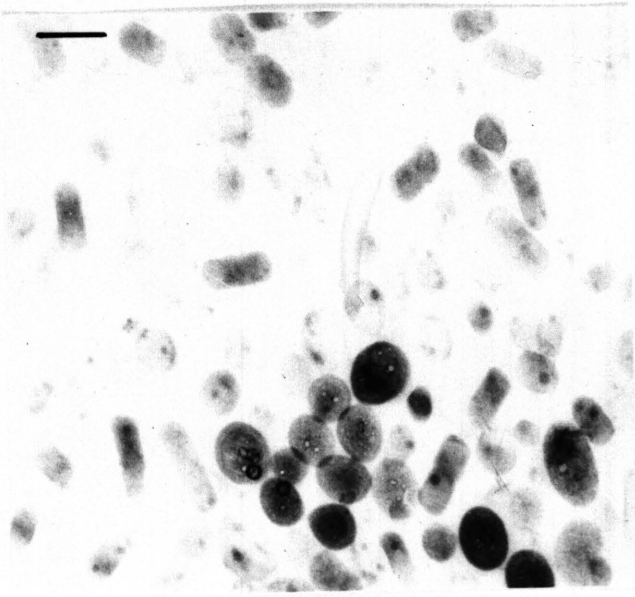
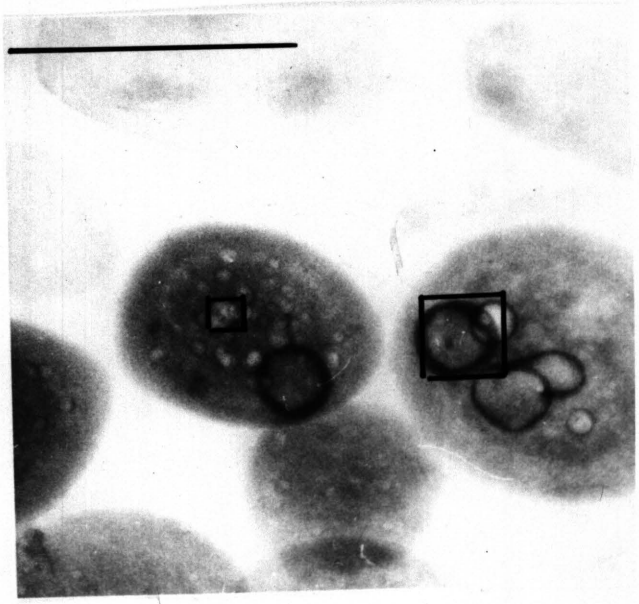


FIG. 39



FIGS. 40 and 41 A. *calcoaceticus* grown on acetate as sole carbon source. The framed areas and arrows indicate the various sizes of some of the polyphosphate inclusions.
Bars represent 1µm.

FIG. 42 A. *calcoaceticus* grown on acetate as sole carbon source. The large dark cells contained polyphosphate inclusions.
Bar represents 1µm.

FIG. 43 A. *calcoaceticus* grown on acetate as sole carbon source. The filamentous form of the organism contained polyphosphate inclusions.
Bar represents 1µm.

FIG. 40

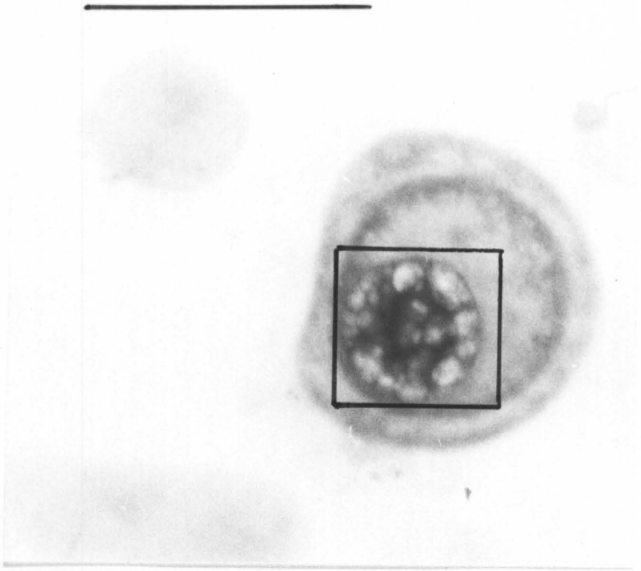


FIG. 41

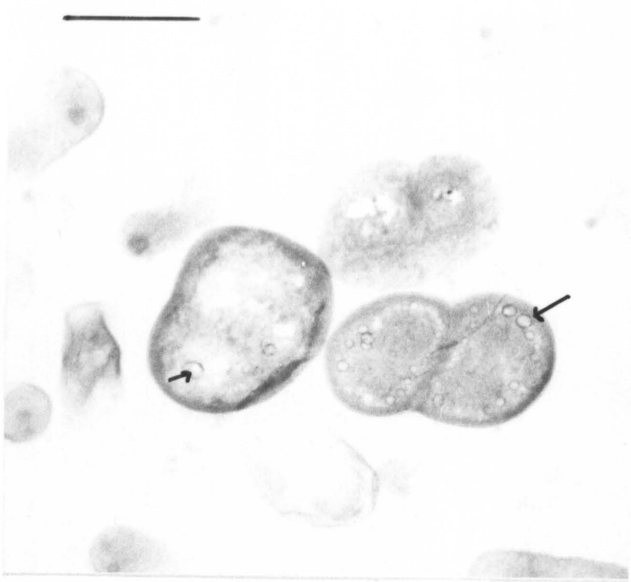


FIG. 42

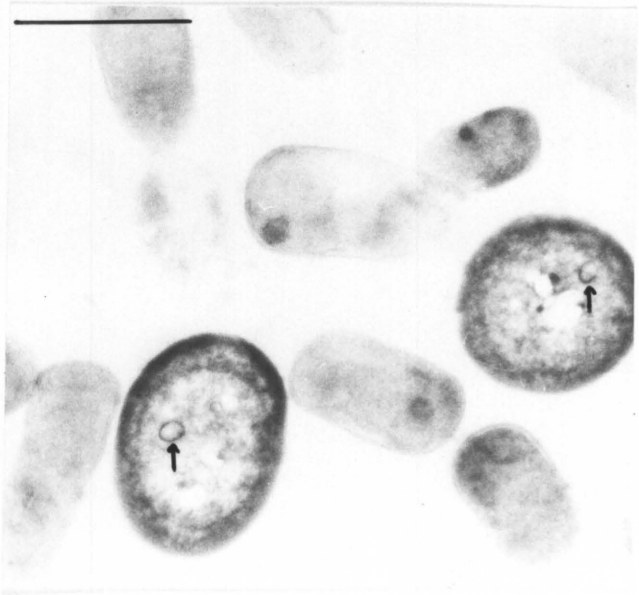
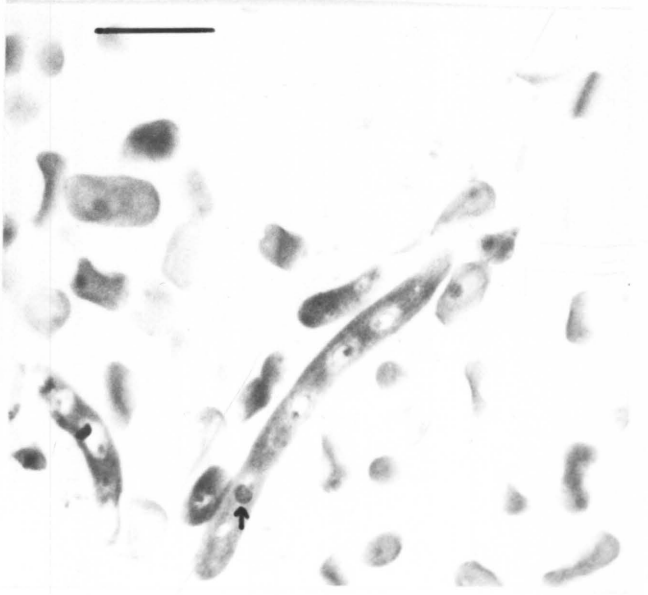


FIG. 43



2) A. *calcoaceticus* grown on ethanol, sodium propionate, sodium butyrate and sodium iso-butyrate as sole carbon source

The *Acinetobacter* culture maintained on acinetobacter-agar, was streaked onto the modified acinetobacter-agar containing the various carbon sources. The cultures were incubated aerobically at 20°C for 48h and then prepared for electron microscopy as described in section 3.4.4(2).

The cells grown on ethanol showed very scant growth, but the cells all contained relatively large polyphosphate inclusions when viewed in the TEM. The cells grown on sodium propionate showed substantial growth, but there were no polyphosphate inclusions when viewed in the TEM. The butyrate-grown cells showed the best growth of all the substrates, including acetate, and the cells all contained many small polyphosphate inclusions when viewed in the TEM (Fig. 44). Cells grown on iso-butyrate showed very poor growth, but the cells all contained relatively large polyphosphate inclusions when viewed in the TEM (Fig. 45).

3) A. *calcoaceticus* grown on acinetobacter-agar, with aerobic, anaerobic, and aerobic exposure consecutively

The culture was streaked onto acinetobacter-agar, incubated aerobically for 48h at 20°C and a section then prepared for examination in the TEM. The culture was then placed

in an anaerobic jar and the air replaced with nitrogen gas. After 24h at 20°C a section was again prepared for examination in the TEM. The culture was then incubated aerobically at 20°C for 24h and a section again prepared for examination in the TEM. Electron microscopic examination revealed that very few of the cells contained polyphosphate inclusions, and that the morphological appearance and polyphosphate content of the cells did not change as a result of the three treatments.

4) Activated sludge from Northern works inoculated onto acetate and butyrate as sole carbon source

Drops of activated sludge drawn from the anaerobic zone and from the end of the aeration basin of the Northern works, were placed onto acetate and butyrate media. The inoculated media were incubated aerobically at 20°C for 48h and prepared for electron microscopy according to schedule 3.4.4(2). The cultures were examined for the presence of PHB with sudan black.

Electron microscopic examination of 1µm unstained sections revealed that the appearance of the anaerobic and of the aerobic samples were very similar. With both media it was apparent that a single morphological type of organism predominated. The cells either contained poly-β-hydroxy-butyrate or polyphosphate inclusions and many cells contained both storage products (Figs. 46, 47, 48 and 49).

5) Activated sludge from Northern works inoculated onto settled sewage medium, fermented mixed liquor medium and fermented raw sludge medium

Drops of aerobic and anaerobic sludges were placed onto the three substrates prepared as described in section 3.2(4), (5) and (6). The inoculated media were incubated aerobically at 20°C for 48h and prepared for electron microscopy according to schedule 3.4.4(2). The cultures were examined for the presence of PHB with sudan black.

Electron microscopic examination of 1µm unstained sections revealed that there was no difference between the aerobic and anaerobic samples.

The appearance of the cells in the sludge inoculated onto the settled sewage medium, appeared to be very similar to the Northern works specimen examined in section 4.2.3.

The sludge inoculated onto the fermented mixed liquor medium contained a greater number of polyphosphate-accumulating cells than the sludge inoculated onto the settled sewage medium. With the sludge inoculated onto the fermented raw sludge medium, a single morphological type of organism predominated. These organisms contained large accumulations of polyphosphates (Figs. 50 and 51).

FIG. 44 A. *calcoaceticus* grown on butyrate as sole carbon source. The cells all contained innumerable small polyphosphate inclusions.
Bar represents 1µm.

FIG. 45 A. *calcoaceticus* grown on iso-butyrate as sole carbon source. The cells all contained relatively large polyphosphate inclusions.
Bar represents 1µm.

FIG. 46 Activated sludge from Northern works grown on acetate as sole carbon source. The electron-dense bodies were polyphosphate inclusions and the globules with low electron density were probably poly-β-hydroxybutyrate inclusions.
Bar represents 1µm.

FIG. 44

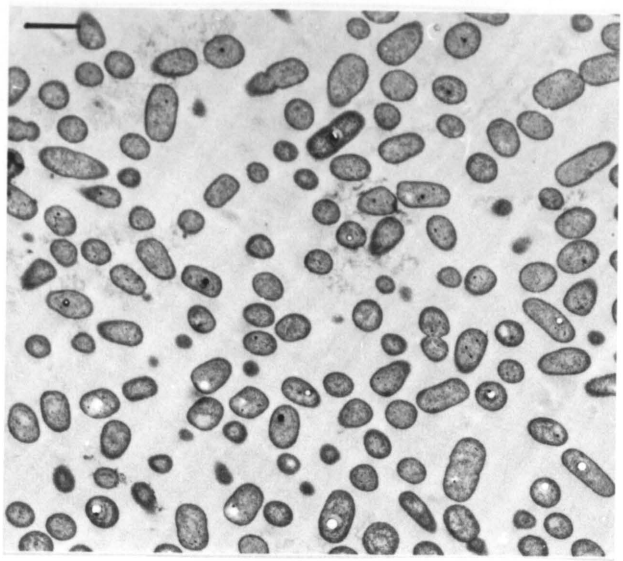


FIG. 45

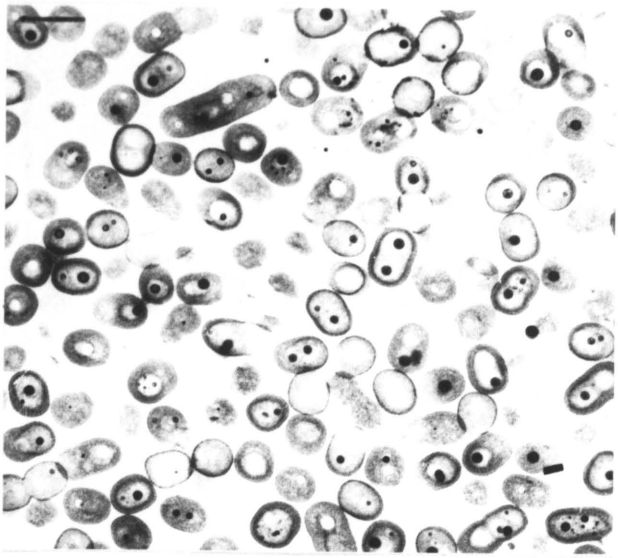


FIG. 46

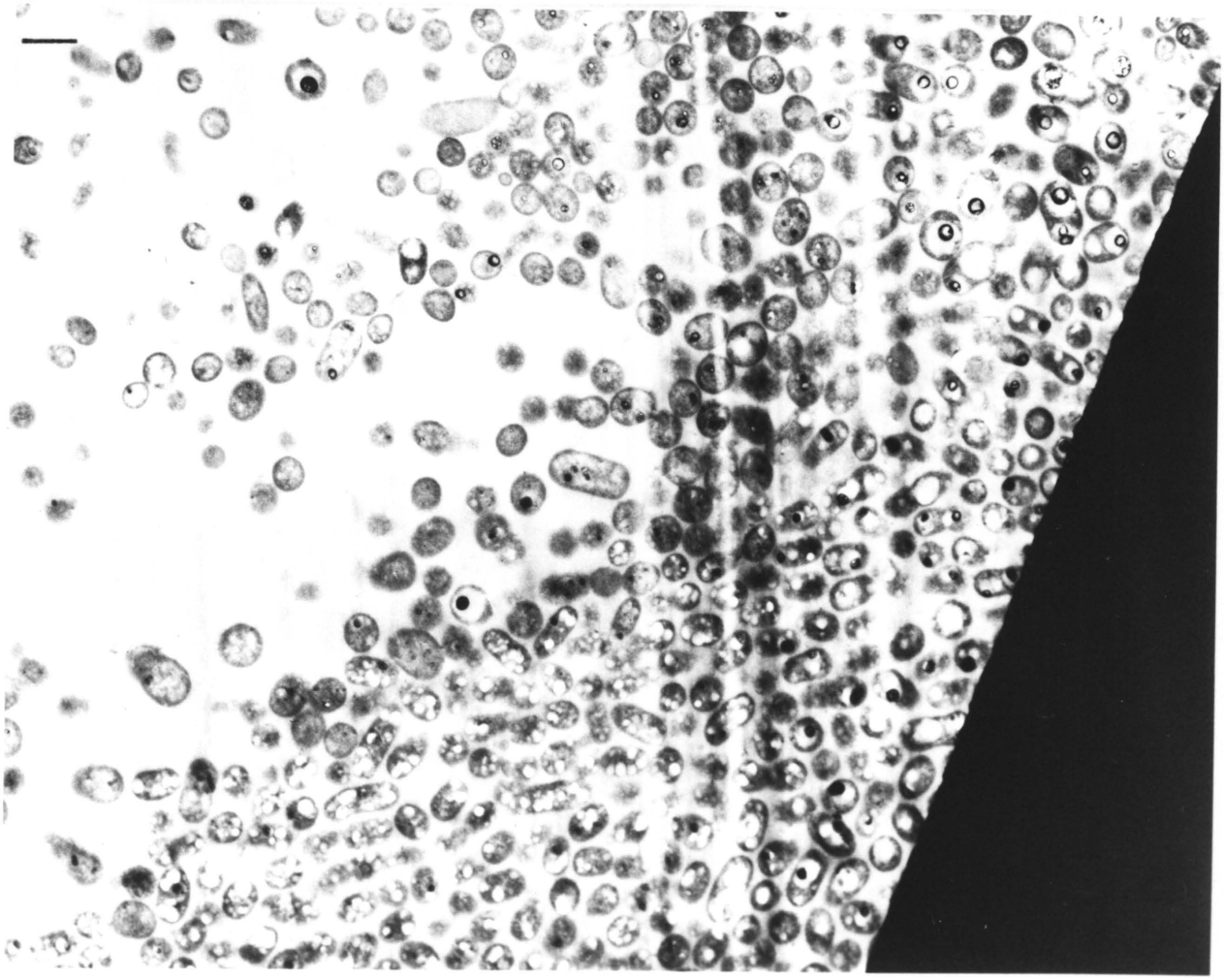


FIG. 47 Activated sludge from Northern works grown on acetate as sole carbon source. The electron-dense bodies were polyphosphate inclusions and the globules with low electron density were probably poly-β-hydroxybutyrate inclusions.
Bar represents 1µm.

FIG. 48 Activated sludge from Northern works grown on butyrate as sole carbon source. The electron-dense bodies were polyphosphate inclusions and the globules with low electron density were probably poly-β-hydroxybutyrate inclusions.
Bar represents 1µm.

FIG. 47

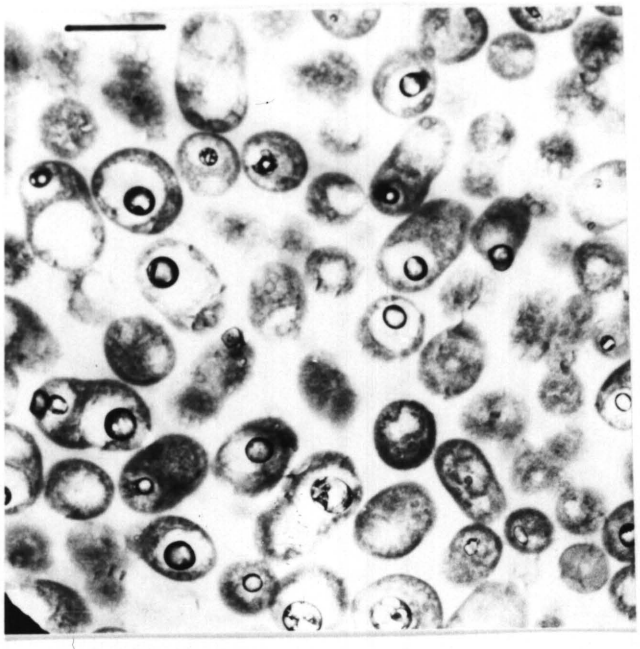


FIG. 48

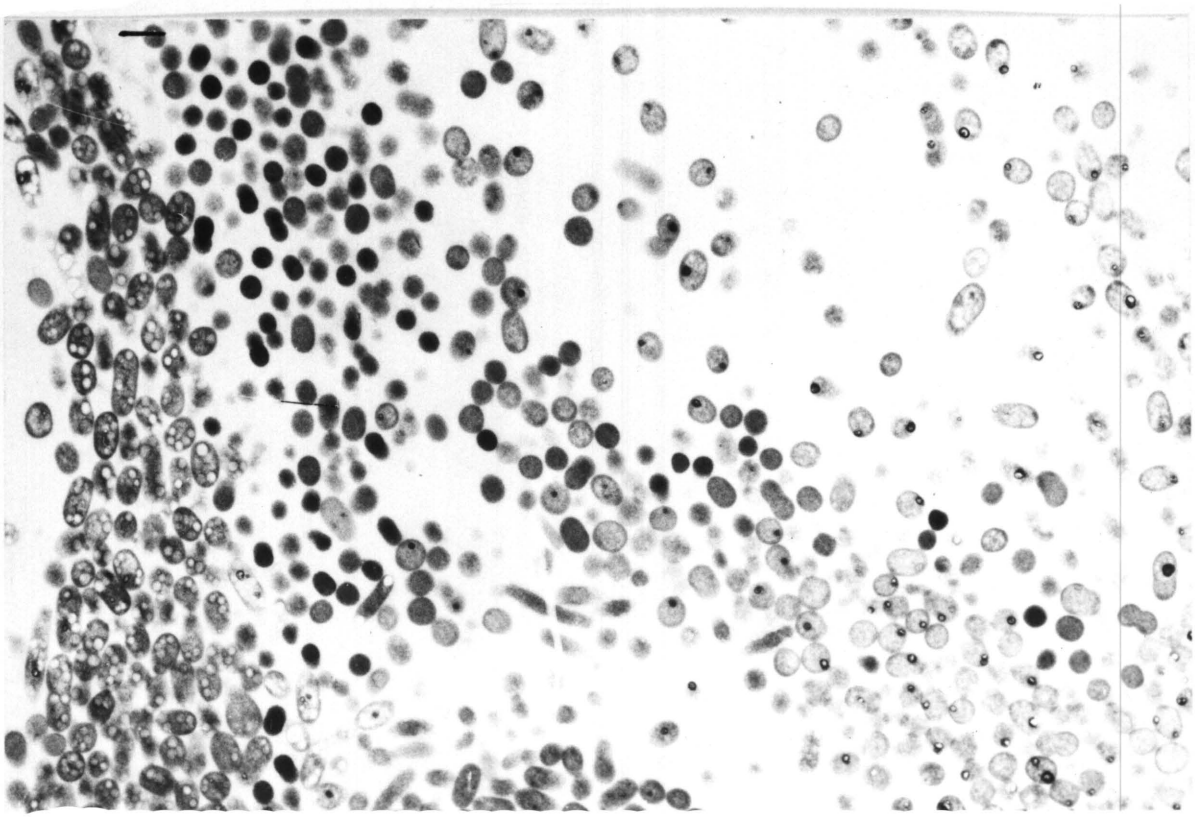


FIG. 49 Activated sludge from Northern works grown on butyrate as sole carbon source. The electron-dense bodies were polyphosphate inclusions and the globules with low electron density were probably poly-β-hydroxybutyrate inclusions.
Bar represents 1µm.

FIGS. 50 and 51 Activated sludge from Northern works grown on fermented raw sludge medium. Note the predominance of the cells with abundant polyphosphate inclusions. The polyphosphate-accumulating cells proliferated and had virtually overgrown all the other cell types.
Bars represent 1µm.

FIG. 49

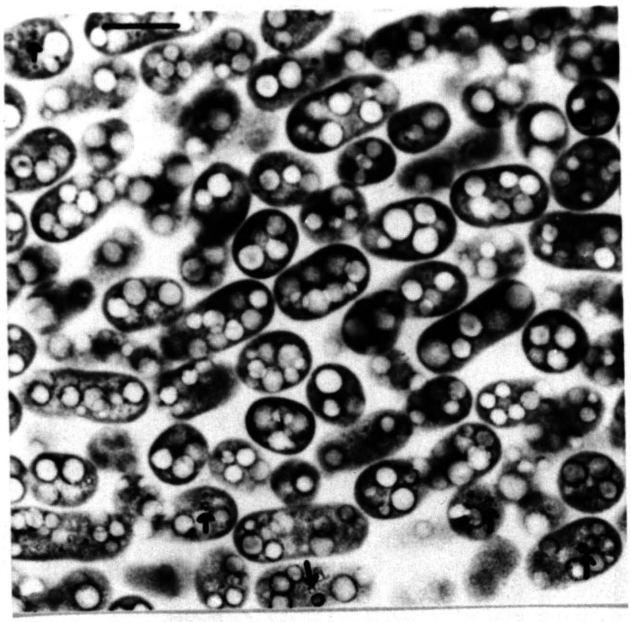


FIG. 50

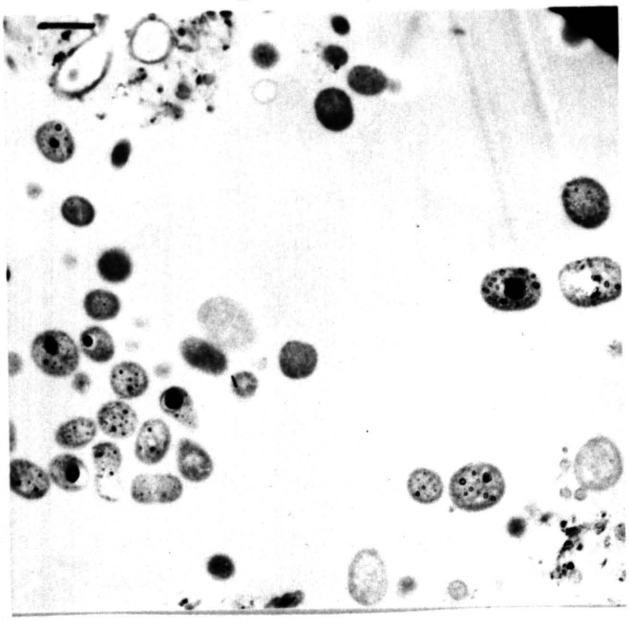
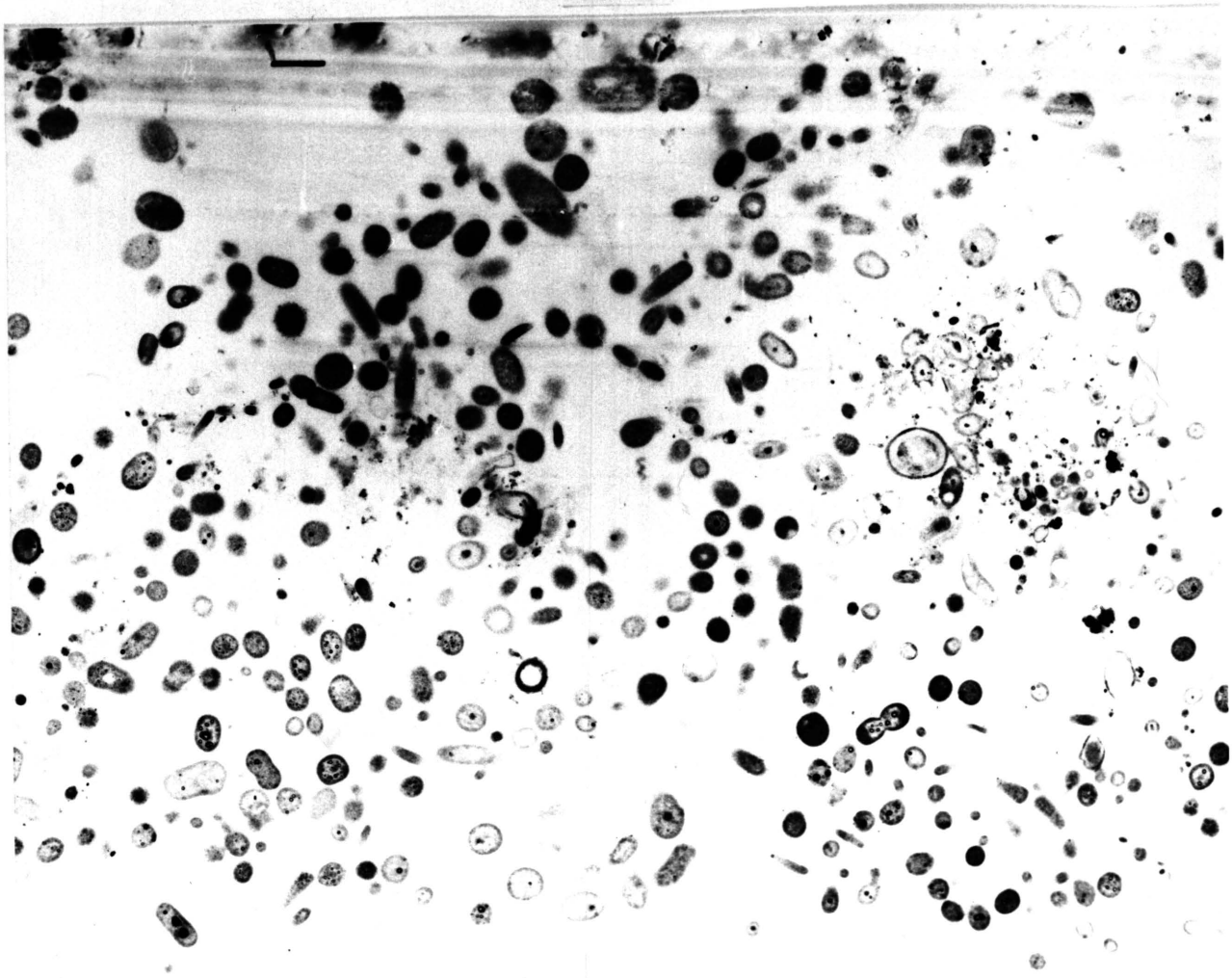


FIG. 51



6) Light microscopic observation of *A. calcoaceticus* grown on the media utilised in 5.5(5) and 4.6.(1) and (2)

The culture was streaked onto the various media and incubated at 20°C for 48h. The culture showed no growth on the settled sewage medium, very scant growth on the fermented mixed liquor medium and substantial growth on the fermented raw sludge medium. Examination in the light microscope of methylene-blue-stained slides of the culture grown on the acetate medium revealed a few small metachromatic cell clusters. The cultures grown on ethanol and isobutyrate contained no cell clusters but the cells all contained small metachromatic granules. The butyrate grown culture did not contain cell clusters but the cells all exhibited a faint purple tinge. The cells grown on fermented mixed liquor were very similar to the butyrate grown cells. The culture grown on the fermented raw sludge medium revealed a marked metachromatic reaction of the cells with large polyphosphate granules clearly visible within the cells. Clusters of cells were also evident and the polyphosphate granules within these clustered cells were larger than those within the other cells.

CHAPTER 5

GENERAL DISCUSSION AND PROPOSALS

The morphology of the phosphorus-accumulating cells as observed in the TEM, combined with the bacterial population analyses support previous observations (Fuhs and Chen, 1975; Yall *et.al.*, 1974) that the organisms involved are *Acinetobacter* spp.

Comparison of sludges with different uptake capabilities suggested that intracellular phosphorus accumulation is related to the tendency of the *Acinetobacter* cells to increase in size and to aggregate. This conclusion is also supported by the observations in the TEM when a pure culture of *Acinetobacter* was grown with acetate as sole carbon source.

In the sludges with the highest uptake capabilities the phosphorus-accumulating cells were grouped into very large clusters and the cells all contained polyphosphate inclusions which occupied almost the entire cell.

In the other sludges the clusters were smaller and the cells within the clusters were not all uniform in size. The smaller

cells contained small polyphosphate inclusions, whereas the larger cells contained inclusions which occupied almost the entire cell volume.

In all the sludges examined a few short chains of large cells with polyphosphate inclusions were frequently associated with the cell clusters. In a study of an encapsulated strain of *A. calcoaceticus*, Juni and Janik (1969) found that the cells clumped into large masses when the capsules were removed from the cells. Their investigations indicated that the tendency to form chains was a manifestation of the clumping ability of the cells. They also found that these cell clumps could not be dispersed by the action of proteolytic enzymes, lipase, deoxyribonuclease, ribonuclease, temperature or pH treatments.

In this study it was also apparent that the *Acinetobacter* cell clusters were not easily dispersed as they were still intact after the vigorous preparation procedures for electron microscopy. The organisms which were presumed to be nitrifiers were also found to be still grouped in clusters after sample preparation (Fig. 23). Because of the stability of the cell clusters and the various degrees of clumping observed in the different sludges, it is evident that meaningful quantitative enumeration of the microbial populations in activated sludge is virtually

impossible. It is probably for this reason that Davelaar *et al* (1978) and Toerien *et al* (1979) could not relate the phosphorus removal capabilities of activated sludge to the selection of certain population groups.

The normal cellular calcium content of bacterial cells is in the order of 0,5% m/m (Luria, 1960). The concentration of calcium which was found to be associated with the polyphosphates in this study thus indicated that an exceptionally high concentration of calcium is associated with these structures. Magnesium and potassium have been repeatedly reported to be important cations for the neutralisation of charges of the polyphosphate chains. It has now been clearly determined that calcium plays a predominating role in the stabilization of these polyanions in activated sludge. It is interesting that in *M. lysodeikticus*, which accumulates polyphosphates on complete growth media, calcium was found to be an important stabilizing cation.

Reported biochemical analyses of the phosphorus distribution in activated sludge has failed to supply evidence of the important role of polyphosphates in the process. This work has clearly shown that the organisms can accumulate a remarkable amount of intracellular polyphosphate. Considering Fig. 21 it would be conservative to estimate the volume of the polyphosphate inclusions to be 60% of the total cell volume. If these inclusions contained 30% phosphorus it would imply an intracellular phosphorus content (in the form of polyphosphate) of 18% (m/v). Because these bodies possess a high density, the actual percentage on mass to mass basis could be higher. Thus, if the dry mass of a sludge is 3000mg/ℓ and it contains 5%P on a dry mass

basis, it would contain 150mg/ℓP. If 80% of the dry mass is volatile and 50% of this volatile fraction is viable, then 150mgP would have to be stored in 1200mg viable biomass. The normal metabolic phosphorus content of the activated sludge biomass has been estimated at 3%, i.e. 36mg in the example cited. The balance of 114mgP would therefore have to be stored in the form of polyphosphates, i.e. equivalent to 9,5% of the viable biomass. From the value of 18%P in the form of polyphosphates for the *Acinetobacter* cells it is evident that 50% of the viable biomass as *Acinetobacter* could account for the 114mgP. The phosphorus associated with volatile suspended solids, other than the active mass, is not considered in this calculation.

Potgieter (personal communication) observed improved settling properties in enhanced phosphorus-removing sludges. This was probably due to the clumping properties of the polyphosphate-accumulating cells and to the density of the inclusions. Friedberg and Avigad (1968) determined the density of the inclusions in *M. lysodeikticus* as 1,23g/cm³. The settling properties of these cell clusters would also lead to their retention in the system.

Anaerobiosis of enhanced phosphorus removing activated sludge normally results in the release of orthophosphate into the supernatant (Barnard, 1976). In the sludges examined it was evident that the metachromatic properties of the sludges was greatly reduced as a result of anaerobiosis. Furthermore TEM examination of anaerobic sludges revealed that anaerobiosis led to the disintegration or total disappearance of the polyphosphate granules. Therefore the results obtained indicated that the phosphorus

released into the supernatant during anaerobiosis is at least partially derived from the intracellular polyphosphate inclusions.

Dawes and Senior (1973) suggested that reserve polymers appear to enhance survival only if they are degraded at comparatively slow rates. If faster metabolism occurs the energy runs to waste and the bacteria die more rapidly.

The rapidity at which the polyphosphate is solubilised during the anaerobiosis of activated sludge suggests an uncontrolled degradation of these compounds. TEM examination of sludge from the anaerobic zone of Laboratory scale plant I revealed that the release was preceded by the dispersal of the large granules into smaller particles.

A number of low molecular weight compounds derived from bacterial metabolism, particularly low molecular weight aliphatic acids, have been shown to be responsible for the solubilisation of phosphates in soils. It has also been shown that 2-ketogluconic acid, produced by many soil bacteria, chelates calcium from a number of calcium salts and minerals (Mortenson, 1963).

It may be possible that during the anaerobiosis of activated sludge, chelating compounds are formed due to fermentation by facultative anaerobes and possibly due to the intracellular accumulation of compounds resulting from

the blocking of metabolic pathways of aerobic organisms. These chelating compounds could then possibly bind with the calcium ions of the polyphosphates, thereby destabilizing the latter and releasing the smaller granules into the cytoplasm, where they are degraded by polyphosphatase enzymes, with the resultant release of orthophosphate. It is thus conceivable that the release of orthophosphate during anaerobiosis is not a necessary prerequisite for the subsequent uptake during aeration, but is indicative of events occurring in the anaerobic zone, i.e. the production of short-chain fatty acids which in turn serve as substrates for enrichment of *Acinetobacter* spp during aeration.

The investigations conducted with the pure culture of *Acinetobacter* indicated that cells which had not been exposed to anaerobiosis and which were cultured under aerobic conditions on complete growth media, accumulated intracellular polyphosphates when certain carbon/energy sources were incorporated into the growth media. The growth on the various substrates decreased in the order butyrate>propionate>acetate>isobutyrate>ethanol. The butyrate-grown organisms contained a considerable amount of small polyphosphate granules; the propionate grown organisms contained none; the acetate grown cells contained relatively large inclusions but only in a small proportion of the cells, and the isobutyrate and ethanol-grown cells all contained relatively large inclusions. (It is interesting to note that many workers seem to associate the name

Acinetobacter with acetate. The name is in actual fact derived from the Greek word 'akinetos', meaning, 'unable to move' (Buchanan and Gibbons, 1974).)

Subjection of an acetate grown culture of *Acinetobacter*, to anaerobiosis and subsequently to aerobiosis failed to induce the organisms to increase their polyphosphate accumulating abilities. This observation indicated that oxygen deprivation *per se* did not induce the uptake of phosphorus. That oxygen deprivation is not a prerequisite for polyphosphate accumulation was also indicated when activated sludge from the end of the aeration basin of Northern works was inoculated onto acetate and butyrate media. In both instances organisms which were presumed to be *Acinetobacter* proliferated. The organisms exhibited marked storage properties and contained massive deposits of what was presumed to be poly-β-hydroxybutyrate and polyphosphate.

Because these organisms are normally found in environments such as soil and water which are normally subject to considerable variation in composition and temperature, they must regulate their metabolism to adjust to these changes (Juni, 1978). It is possible that given sufficient quantities of easily metabolizable substrates, these organisms have developed mechanisms to accumulate these massive reserves.

The poly-β-hydroxybutyrate reserves which the organisms are capable of accumulating could explain the observations of Yall *et al.* (1972), that activated sludge was capable of removing phosphorus from distilled water.

The results obtained with Northern works sludge inoculated onto fermented raw sludge, pointed to the importance of this material as a substrate for inducing phosphorus uptake under practical conditions. This substrate led to the enrichment of large *Acinetobacter* cells with large intracellular polyphosphate inclusions.

The importance of substrate was confirmed by streaking the pure culture onto the settled sewage medium, the fermented mixed liquor medium and the fermented raw sludge medium. Light microscopic examination of methylene-blue-stained slides revealed a dramatic metachromatic reaction with large polyphosphate granules clearly visible, in the case of the cells grown on the fermented raw sludge medium.

In the reviews of Harold (1966), Dawes and Senior (1973) and Kulaev (1975) on polyphosphates, the accumulation of polyphosphates by *Acinetobacter* spp is never mentioned. The accumulation of polyphosphates by *M. lysodeikticus* under normal conditions of growth is designated as atypical by Dawes and Senior (1973). It appears from the observations in this investigation that polyphosphate accumulation under normal growth conditions may not be atypical.

The cells in the pure culture never accumulated nearly as much phosphorus as the cells in activated sludge. It is evident that only certain species of *Acinetobacter* are capable of accumulating the massive polyphosphate inclusions. It is possible that the cells with the massive polyphosphate inclusions observed in this investigation were related to the organism *Acinetobacter phosphadevorus*, described by Yall *et al.* (1974). It was reported by these authors that this organism is capable of taking up large quantities of phosphorus in complete growth media, during exponential growth, and under continual aeration.

From the work of Fuhs and Chen (1975), Yall *et al* (1974) and from the results of this report, it appears as if enhanced phosphorus removal can be achieved in an activated sludge system if the process is manipulated in such a way that the necessary *Acinetobacter* population is enriched.

The results obtained with the various substrates indicated that the ability of the organisms to accumulate polyphosphates varied considerably on the different substrates. When grown on fermented raw sludge, the amount of polyphosphate granules accumulated by the cells in the pure culture and in Northern works sludge, greatly exceeded the amount accumulated on any of the individual substrates. These results indicated that enhanced phosphorus removal in activated sludge could possibly be induced by bleeding fermented raw sludge or acid digestor supernatant liquor into the system,

as proposed by Fuhs and Chen (1975).

Juni and Heym (1963) working with an encapsulated strain of *Acinetobacter* found that the capsules constituted 41% of the dry mass of the cells. In this strain most of the triose phosphate formed from glucose or gluconate was converted to capsular polysaccharide. In strains which cannot utilise cabohydrates, hexoses are formed by reversal of the Embden Meyerhoff metabolic path (Juni, 1978).

Juni and Janik (1969) also observed that unencapsulated mutants increased in size due to the accumulation of phosphorylated intermediates which could not be utilised for capsular synthesis. As polyphosphate accumulation in activated sludge is associated with clumping of the cells, and as the clumped cells are possibly devoid of capsules, it could be that the energy saved by not synthesising capsules is diverted to polyphosphate accumulation.

Potgieter (1978) reviewed 'well established' findings as regards enhanced phosphorus removal in terms of known fundamental biological and biochemical principles, and concluded *inter alia*:

(i) The ability to accumulate polyphosphate is most probably shared by many different microbial groups among the facultative anaerobes as well as among strict aerobes.

(ii) Polyphosphate accumulation requires preconditioning of the sludge under specified conditions in the anaerobic zone.

(iii) Polyphosphate accumulation in the aerobic zone is initiated by a deficiency of one or more nutrients. If the latter is supplied in abundance luxury uptake will be impaired.

The results of this study do not support these three conclusions. Firstly, polyphosphate accumulation in the sludges examined was observed to be due to the action of one morphological type of organism, i.e. *Acinetobacter*. Secondly, it appears as if preconditioning of the sludge is not a necessary prerequisite for polyphosphate accumulation, but that the supply of suitable substrates to the aerobic zone will suffice. Thirdly, it appears as if the accumulation of polyphosphate in activated sludge is not dependent on the deficiency of certain nutrients but rather on the sufficiency of certain nutrients.

It is generally accepted that the basin configuration necessary for inducing phosphorus removal in activated sludge systems, is that of a sequence of anaerobic/anoxic/aerobic/anoxic/aerobic[(i)] zones. This configuration, with

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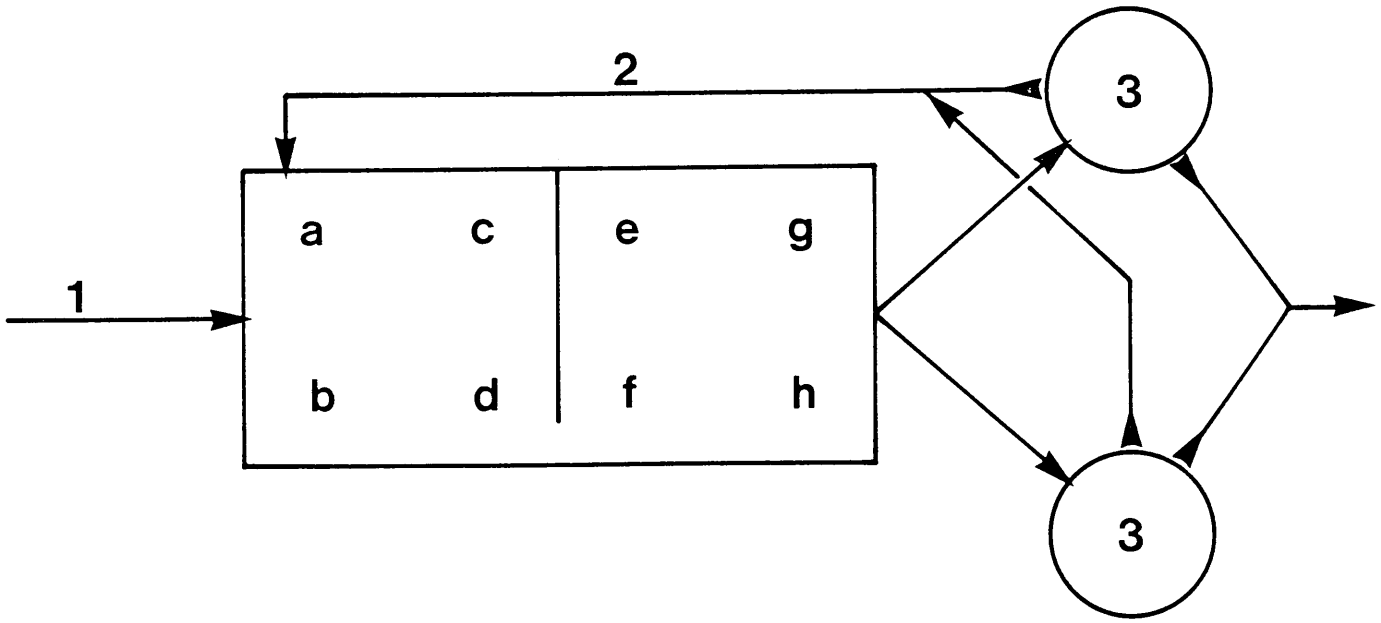
(i) Anoxic refers to the condition where nitrates replace oxygen as electron acceptor. Anaerobic refers to the absence of both nitrates and dissolved oxygen.

minor modifications, was the mode of operation of Goud-koppie I and II, Northern works and Laboratory scale plants I and II at the time of sampling for this study.

The Brits and Umhlatuzana plants were operated on totally different principles. It was thus considered necessary to seek explanations for the exceptionally good phosphorus removal of these plants.

The Brits works consists of a single aeration basin with eight surface aerators (Fig. 52).

FIG. 52
Brits Town Council: Activated sludge plant



1) Influent sewage 2) Return sludge 3) Clarifiers
a,b,c,d,e,f,g,h surface aerators

Only two aerators were operated at a time: a and h for three hours alternating with b and g for three hours.

Based on values obtained from the town chemist, the influent COD was 285mg/ℓ; the influent ammonia was 5mg/ℓ; the nitrate concentrations in the aeration basin and effluent were 3mg/ℓ; the effluent COD and total dissolved solids were 30mg/ℓ and 510mg/ℓ respectively. (Despite the predominance of *Acinetobacter* spp this plant produced a high quality effluent.)

The dissolved oxygen levels throughout the plant were 1,0 to 1,2mg/ℓ. That the plant was in fact aerobic, despite the low level of aeration, was clearly evident from the results of the population analysis, i.e. 93% of the organisms were obligate aerobes. The plant is pulse fed from various sumps in the town. These sumps take up to eight hours before they fill up, and, when full, the sewage is automatically pumped to the sewage works. Because Brits is located in a semi-tropical area, it can be expected that substantial fermentation occurs in these sumps. Toerien and Hattingh (1969) reviewed the literature on anaerobic digestion of sewage, and concluded that the main end-products formed by the non-methanogenic bacteria were acetic, propionic and butyric acids. The likely presence of these acids in the influent sewage was probably the reason why *Acinetobacter* spp were the predominant organisms in the system.

As the plant consistently maintains effluent phosphorus levels of less than 1mg/ℓ, this casts doubt on the necessity of equalizing the load with balancing tanks. It appears that, when the organisms are supplied with the necessary fatty acids, they can accumulate vast quantities of poly-β-hydroxybutyrate, which could probably tide them through limited periods of energy deficiency.

At one stage a welding plant factory was dumping ferrous chloride into the Brits sewer. The iron content of the sludge reached a level of 17% on a dry mass basis. The dynamic nature of the biological uptake mechanism was clearly illustrated during this period, as the cells still contained vast quantities of intracellular polyphosphates. EDX-analysis indicated that these inclusions did not contain any traces of iron.

Four months after the dumping of the ferrous chloride into the sewer was prohibited, the effluent phosphorus was still maintained at near zero values.

The configuration of the Umhlatuzana works is similar to that of the Brits works. The plant consists of a 4800m³ single tank reactor with six surface aerators. An extraordinarily high rate of sludge recycling and the maintenance of low oxygen levels in the aeration reactor were characteristics of the mode of operation of this plant.

Based on values obtained from the town chemist, the unsettled influent sewage had a COD of 780mg/ℓ, COD:TKN of 15:1 and a phosphorus load of 9mg/ℓ. The effluent phosphorus, ammonia and nitrate nitrogen values were all close to zero.

As complete nitrification and denitrification occurred in the aerated reactor, it is obvious that, probably due to the high influent COD and low dissolved oxygen levels, zones of oxygen deficiency must have existed to attain such low nitrate values in the effluent. Extending this argument, it is reasonable to suggest that fermentation also occurred in the sludge environment.

Reference to the population structure obtained with activated sludge from this plant did indicate a large facultative anaerobic population. The extremely large clusters of *Acinetobacter* observed in the sludge specimen of this plant, probably led to a substantial overestimation of the facultative anaerobic population. However, supportive evidence for the existence of an active facultative anaerobic population in the sludge was obtained from the observation that the sludge, when left anaerobic overnight, was malodorous the next day. Of all the sludges examined this particular sludge seemed to be the most unstable.

The mechanism proposed for this plant is, that due to the high COD and low dissolved oxygen levels, aerobic respira-

tion, denitrification and fermentation occur within the same reactor. The *Acinetobacter* spp were therefore able to compete directly with the denitrifying organisms for the products released by the fermentative organisms.

It is suggested that the problem with the sequential anaerobic/anoxic/aerobic mode of operation is that any fermentation products produced in the anaerobic zone are utilised in the anoxic zone by the denitrifying organisms, which are known to have a high affinity for substrates such as short-chain fatty acids. Consequently only limited amounts of these compounds pass through to the aerobic zone for enrichment of, and utilisation by, the *Acinetobacter* spp.

The procedures normally adopted for the preparation of biological material are lengthy, usually lasting several days. In this investigation a procedure was adopted whereby the time for processing the samples from the initial step of fixation to immersion in 100% Spurr's resin was performed within six hours. That the procedure gave satisfactory results, could be seen in 0,1µm sections where the cell wall components of Gram negative organisms and certain other structures e.g. mesosomes, were clearly defined.

The fact that unstained sections of 0,4 to 1µm were sufficient to clearly identify the state of phosphorus accumulation, greatly simplified sample preparation as

these sections were easy to prepare.

The degradation of the polyphosphate inclusions, which could be induced by the electron beam, appeared to be a reliable indicator of the phosphorus-rich inclusions.

These rapid and easily performed preparation procedures can be of great value as accessory tools in the elucidation of the mechanism of enhanced phosphorus removal. The technique of inoculating activated sludge onto defined agar media in petri dishes, and subsequent examination in the TEM or STEM-EDX-system, has great potential in determining the effect of various physical and chemical factors on the phosphorus-uptake of the organisms under exactly defined and replicable conditions. It is possible that the morphological appearance of the organisms as observed in the TEM could be used as a measure of the degree of phosphorus accumulation. Many variables could thus be examined without the necessity of chemical and biochemical monitoring. The problems associated with the operation and availability of chemostats and pilot plants could also be obviated in such initial investigations.

The field could thus be narrowed, whereafter the identified relevant factors could either be applied on a larger scale, e.g. pilot plants, chemostats, and full scale plants, or as guidelines in the elucidation of the basic biological and biochemical principles involved.

CHAPTER 6

CONCLUSIONS

By the application of the technique of microprobe analysis, hitherto largely unexploited in microbiology, it was possible to provide greater clarity as to the phenomenon of enhanced phosphorus removal by activated sludge.

The results of this study strongly support the pioneering work and concepts proposed by Fuhs and Chen (1975).

It has been shown that enhanced phosphorus uptake by different sludges is due to the action of one type of organism, i.e. *Acinetobacter*. These organisms are capable of storing vast quantities of polyphosphates when grown on complete growth media which contain certain carbon/energy sources.

Although the polyphosphate storage capabilities of these organisms are not cited in the extensive reviews on these compounds, it has become apparent that these organisms can store quantities in excess of any other organism reported to date.

The analyses of the elemental content of the polyphosphate inclusions indicated that a high concentration of calcium is always associated with these structures, with lesser quantities of magnesium and potassium also occurring.

The polyphosphate-containing cells are characteristically grouped into clusters, which are not easily dispersed. The tendency of the cells to aggregate is visibly more pronounced in sludges with high phosphorus-uptake capabilities. The possibility that the phenomenon of cell clumping and the accumulation of polyphosphates are associated responses, warrants investigation.

The assumed necessity of subjecting *Acinetobacter* to anaerobiosis to induce polyphosphate accumulation is questioned. A pure culture of this organism which had been maintained under aerobic conditions for two years, could be induced to accumulate polyphosphates by the inclusion of certain carbon/energy sources into its growth medium. The cells in an aerobic sludge which had poor phosphorus-uptake capabilities, could be enriched and induced to accumulate large quantities of polyphosphate, when grown on certain carbon/energy sources.

The ability of the *Acinetobacter* cells in activated sludge to accumulate vast quantities of poly-β-hydroxybutyrate under aerobic conditions and given the necessary substrate, was also clearly shown.

The orthophosphate released into the supernatant during anaerobiosis has been shown to be derived from the intracellular polyphosphate inclusions.

It is suggested that phosphate release is not a necessary prerequisite for uptake, but that this release is an indication that fermentation is occurring in the anaerobic zone.

The dependence of these organisms on fermented substrates was strongly suggested by the observation that neither the pure culture of *Acinetobacter* nor Northern works sludge accumulated polyphosphates on settled sewage medium whereas, they accumulated substantial quantities on fermented raw sludge medium.

The suggestion by Fuhs and Chen (1975) that fouled primary effluent or acid digestor supernatant liquor could be fed into the aerobic zone to induce phosphorus-uptake is supported by the findings in this study. It seems as if a high proportion of the biomass as *Acinetobacter* will not be detrimental to the performance of an activated sludge system.

Note added in proof

Ten litres of Northern Works mixed liquor, which was inactive in phosphorus uptake, was drawn from the end of the aeration basin. (The effluent orthophosphate value at the time of sampling was 12mg/ℓ.) Ten mℓ acid sludge (COD = 40 000mg/ℓ) was added to the 10ℓ mixed liquor, and the mixed liquor aerated for 4h. During this period the supernatant orthophosphate dropped from 16mg/ℓ to 5mg/ℓ.

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APPENDIX I

THE DERIVATION OF EQUATION (3)

Basic assumption (Kramers, 1923)

$$\frac{\left(\frac{I_P}{I_B}\right)_{sp}}{\left(\frac{I_P}{I_B}\right)_{st}} = \frac{\left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{sp}}{\left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{st}} \qquad \text{----------(1)}$$

N: number of atoms in analysed volume.

$\left(\frac{I_P}{I_B}\right)$: P/B ratio for element i.

Other formulae concerning element i (there are j elements present).

$$\text{Atomic fraction } n_i = \frac{N_i}{\sum_j N_j} \qquad \text{----------(2)}$$

$$\sum_j n_j = 1.$$

$$\text{Mass fraction } C_i = \frac{N_i A_i}{\sum_j N_j A_j} \qquad \text{----------(3)}$$

$$= \frac{\frac{N_i}{\sum_j N_j} A_i}{\sum_j \left(\frac{N_i}{\sum_j N_j} . A_j\right)}$$

$$= \frac{n_i . A_i}{\sum_j n_j A_j} \qquad \text{----------(3a)}$$

From equations (1) and (3); C_i for specimen:

$$C_i = \frac{A_i}{(\sum_j N_j A_j)_{sp}} \cdot \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot \left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{st} \cdot (\sum_j N_j Z_j^2)_{sp}$$

which can be written:

$$C_i = A_i \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot \left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{st} \cdot \left(\frac{\sum_j N_j Z_j^2}{\sum_j N_j A_j}\right)_{sp}$$

$$= A_i \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot \left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{st} \cdot \left(\sum_j \left\{\frac{N_j A_j}{\sum_j N_j A_j} \cdot \frac{Z_j^2}{A_j}\right\}\right)_{sp} \text{---(4)}$$

substituting eq. (3) into (4);

$$C_i = A_i \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot \left(\frac{N_i}{\sum_j N_j Z_j^2}\right)_{st} \cdot \left(\sum_j C_j \frac{Z_j^2}{A_j}\right)_{sp} \qquad \text{--------(5)}$$

which is the equation given by Hall *et al* (1973), p. 178 equation (3).

If the numbers of atoms are converted to atomic fractions for the standard (eq.3(a)) then eq. (5) can be written as;

$$C_i = A_i \frac{(I_p/I_B)_{sp}}{(I_p/I_B)_{st}} \cdot \left(\frac{\bar{n}_i}{\sum_j n_j Z_j^2}\right)_{st} \cdot \left(\sum_j \frac{C_j Z_j^2}{A_j}\right)_{sp} \qquad \text{----------(6)}$$

To express the factors in the standard as mass fractions, rewrite eq. (6) as:

$$C_i = \frac{\left(\frac{I_p}{I_B}\right)_{sp}}{\left(\frac{I_p}{I_B}\right)_{st}} \cdot \left(\frac{n_i A_i}{\sum_j n_j Z_j^2}\right)_{st} \cdot \left(\sum_j \frac{C_j Z_j^2}{A_j}\right)_{sp} \qquad \text{----------(7)}$$

substitution of (3a) into (7)

$$= \frac{(I_p/I_B)_{sp}}{(I_p/I_B)_{st}} \cdot \left(\frac{C_i \cdot \sum_j n_j A_j}{\sum_j \left| n_j A_j \frac{Z_j^2}{A_j} \right|}\right)_{st} \cdot \left(\sum_j \frac{C_j Z_j^2}{A_j}\right)_{sp}$$

$$= \frac{\left(\frac{I_p}{I_B}\right)_{sp}}{\left(\frac{I_p}{I_B}\right)_{st}} \cdot \left(\frac{C_i}{\sum_j \left| \frac{n_j A_j}{\sum n_j A_j} \cdot \frac{Z_j^2}{A_j} \right|}\right)_{st} \cdot \left(\sum_j \frac{C_j Z_j^2}{A_j}\right)_{sp}$$

$$= \frac{\left(\frac{I_P}{I_B}\right)_{sp}}{\left(\frac{I_P}{I_B}\right)_{st}} \cdot \left(\frac{C_i}{\sum_j C_j \cdot \frac{Z_j^2}{A_j}}\right)_{st} \cdot \left(\sum_j \frac{C_j Z_j^2}{A_j}\right)_{sp}$$

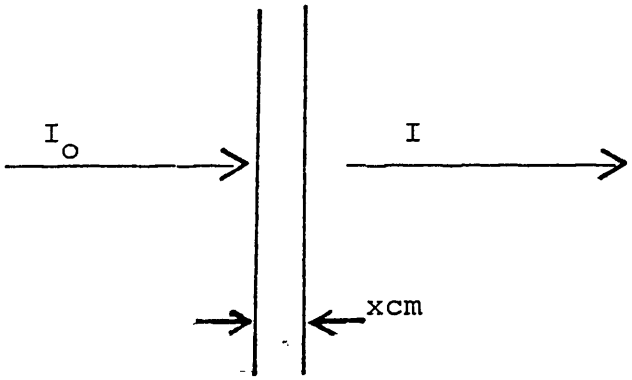
$$\therefore \quad C_i = \frac{(I_P/I_B)_{sp}}{(I_P/I_B)_{st}} \cdot (C_i)_{st} \cdot \frac{\left(\sum_j C_j \cdot \frac{Z_j^2}{A_j}\right)_{sp}}{\left(\sum_j C_j \cdot \frac{Z_j^2}{A_j}\right)_{st}}$$

APPENDIX II

1. ESTIMATION OF THE ABSORPTION OF X-RAYS WITHIN A SPHERE, BY THE MATRIX OF THAT SPHERE (Russ, 1979)

Basic equation for absorption through a thin barrier

$$I = I_o e^{-\mu\rho x}$$



ρ = density (g/cm^3)

With a compound barrier:

$$\bar{\mu} = \sum_j C_j \mu_j$$

μ is the matrix absorption coefficient which must be estimated from an assumed composition, using C_j for each major element and their individual mass absorption coefficients, μ

Calculation: Assumed composition,

36%P, 14%Ca, 8%Mg, 2%K, 40%0

Assumed density of electron-dense bodies, 1,5g/cm³

Radius of electron-dense bodies, 0,25µm

$\therefore$ $x = 0,25 \times 10^{-4}$cm.

Energy of X-rays (keV)	Mass absorption coefficients of absorbers P	Ca	Mg	K	0	$\bar{\mu}$	µρx	I/I_o
2,012(P)	280	728	1877	615	639	620	0,025	0,975
2,940(Ar)	1193	255	643	643	216	607	0,023	0,977
3,690(Ca)	645	139	346	346	116	349	0,013	0,987

Conclusions: These figures indicate that although a small degree of absorption of the radiation occurred within the bodies, it was effectively uniform over the range of energies of interest and therefore did not affect the P/B ratio.

2. ESTIMATE OF THE FLUORESCENCE EFFECT ON THE PHOSPHORUS PEAK INTENSITY

(a) The Kα X-rays of phosphorus are fluoresced by calcium.

(b) None of the elements of interest in this study fluoresce the Kα X-rays of calcium.

(c) The bremsstrahlung will slightly fluoresce the Kα X-rays of calcium and phosphorus. This correction is generally regarded as neglible and is not included in most published data processing programmes, such as FRAME. (Yakowitz, Mykleburst and Heinrich, 1973.)

By the use of the 'FRAME' programme, the increase in the phosphorus peak intensity due to fluorescence by calcium was estimated at 0,3%.

This degree of fluorescence did not effect the P/B ratio significantly.

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