THE DETECTION OF *ACINETOBACTER* IN ACTIVATED SLUDGE AND ITS POSSIBLE ROLE IN BIOLOGICAL PHOSPHORUS REMOVAL

by

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

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SUMMARY

Biological phosphorus removal in activated sludge is not fully understood. *Acinetobacter* has been implicated as the principal phosphorus removing agent.

A fluorescent antibody and Acridine Orange staining technique combined with membrane filtration and epifluorescence microscopy was developed and applied for the *in situ* identification and enumeration of *Acinetobacter* and the total number of bacteria in activated sludge after chemical dispersion or sonication of activated sludge in a 0,5 % tripolyphosphate solution.

Whereas it has been generally assumed that all *Acinetobacter* cells were relatively large, the use of different membrane pore sizes combined with fluorescent antibody staining has shown that these cells can range from relatively very small to very large cells. This confirms the erroneous conclusions that can be made by considering morphological characteristics only for identification of bacteria using light microscopy.

The *Acinetobacter* numbers in the different activated sludge zones differed significantly in time. The primary aerobic and secondary anoxic *Acinetobacter* numbers differed significantly from the other zones when using membrane filters with a pore size of 0,45 μm suggesting that the size of these bacteria increased with higher metabolic activity in the primary aerobic zone resulting in carryover into the secondary anoxic zone.

Although statistical analysis showed no correlation between *Acinetobacter* numbers and phosphorus removal in activated sludge, density gradient centrifugation of activated sludge revealed that *Acinetobacter* was associated with phosphorus in activated sludge.

Using transmission electron microscopy the cell volume of volutin containing cells and the volutin volume were determined. This confirmed that only large *Acinetobacter* cells contained phosphorus. Electron dispersive micro-analysis of X-rays (EDAX) confirmed that the volutin contained phosphorus.

Using *Acinetobacter* numbers, volutin volumes,densities and phosphorus content it was determined that a maximum of 34% of the observed phosphorus removal in activated sludge could be removed by *Acinetobacter* as polyphosphate. Other organisms or mechanisms therefore also had to be involved in the observed phosphorus removal.

DIE WAARNEMING VAN *ACINETOBACTER* IN GEAKTIVEERDE SLYK EN DIE MOONTLIKE ROL VAN DIE ORGANISME TYDENS BIOLOGIESE FOSFORVERWYDERING

deur

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OPSOMMING

Biologiese fosforverwydering in geaktiveerde slyk is tot dusver nie ten volle begryp nie. *Acinetobacter* was beskou as die primêre fosforverwyderende agens.

'n Fluoresserende antiliggaam- en Akridien Oranje-kleuringstegniek, gekombineer met membraanfiltering en epifluoressensie-mikroskopie, is gebruik vir die *in situ* identifisering en telling van *Acinetobacter* en die totale aantal bakterieë in geaktiveerde slyk, na chemiese dispergering of sonikasie van geaktiveerde slyk in 0,5% tripolifosfaat-oplossing.

Voorheen was algemeen aanvaar dat alle *Acinetobacter*-selle relatief groot was. Die gebruik van membrane met verskillende poriegroottes tesame met fluoresserende antiliggaam-kleuring het egter getoon dat die selgroottes varieer van relatief baie klein tot baie groot. Dit bevestig dat verkeerde gevolgtrekkings gemaak kan word waar slegs die morfologie van die bakterieë in ag geneem word vir die identifikasie daarvan met behulp van ligmikroskopie.

Die *Acinetobacter*-getalle in die verskillende geaktiveerde slyksones het betekenisvol van mekaar verskil met tyd. Die *Acinetobacter*-getalle in die primêre aërobe en sekondêre anoksiese en die in ander sones het betekenisvol van mekaar verskil waar 0,45 μm filters gebruik was vir die telling. Dit dui op die moontlikheid dat die grootte van die selle toeneem in die twee sones, moontlik as gevolg van 'n toename in metaboliese aktiwiteit van die organismes in die primêre aërobe sone en die oordraging van die getalle na die sekondêre anoksiese sone.

Statistiese verwerkings het getoon dat daar geen korrelasie was tussen *Acinetobacter*-getalle en fosforverwydering nie. Nietemin het digtheidsgradiëntsentrifugering van geaktiveerde slyk getoon dat *Acinetobacter* met fosfor in geaktiveerde slyk geassosieer was.

Deurstraal-elektronmikroskopie was gebruik om die volume van volutinbevattende selle en die volume van die volutin te bepaal. Dit het bevestig dat slegs die groter *Acinetobacter*-selle fosfor bevat. EDAX ontledings het bevestig dat die volutin fosfor bevat.

Deur gebruik te maak van *Acinetobacter*- getalle, volutinvolumes, digthede- en fosfaatinhoud is bereken dat *Acinetobacter* vir 'n maksimum hoeveelheid van 34% van die waargenome fosforverwydering as polifosfaat verantwoordelik kon wees. Ander organismes of meganismes moet dus ook betrokke wees by fosforverwydering.

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CHAPTER 1

1. INTRODUCTION

The undesirability of phosphorus in sewage effluent is well documented and requires no further elaboration (Toerien, 1977). It has been known since 1959 (Srinath *et al.*, 1959) that phosphorus can be removed during the activated sludge wastewater treatment process. However, a critical appraisal of a recent manual (Wiechers *et al.*, 1984) indicates that the mechanism of phosphorus removal is not fully understood. This is exemplified that in 1985 most plants in South Africa are resorting to chemical precipitation of phosphorus to conform to Government legislation.

It was postulated that phosphorus removal can take place by chemical precipitation, biological accumulation and/or biologically mediated chemical precipitation. At least three research groups have provided data implicating *Acinetobacter* as the responsible agent. However, the Koch-Henle postulates adapted to microbial ecology have not yet been fulfilled, that is, a certain micro-organism must be associated with a certain phenomenon under all circumstances; the organism must be isolated and studied in pure culture; the same function performed in the natural habitat must be performed in pure culture, and finally, the organism must be present in sufficiently large numbers to account for the changes observed in the natural habitat. *Acinetobacter* was found to be predominant in activated sludge exhibiting enhanced phosphorus removal. This led to the association of *Acinetobacter* with the process of phosphorus removal in activated sludge.

Acinetobacter was isolated from activated sludge (Roïnestad, 1973; Fuhs and Chen, 1975; Buchan, 1980). Electron microscope studies revealed that pure cultures of *Acinetobacter* accumulated volutin on Acid Sludge Medium (Buchan, 1980). Energy dispersive micro-analysis of X-rays (EDAX) and chemical analysis indicated that the volutin contained *ca* 27% phosphorus (Friedberg and Avigad, 1968; Buchan, 1980). The bacterial cells containing volutin observed by metachromatic staining in activated sludge were not identified *in situ*. Therefore it was unknown whether these bacteria were *Acinetobacter*. The lack of quantitative data on *Acinetobacter* in activated sludge, left a void regarding the significance of *Acinetobacter* as a phosphorus removing agent (Barnard, 1976).

A problem in the study of *Acinetobacter* in activated sludge was the lack of suitable *in situ* identification and quantification techniques. To fulfil the Koch-Henle postulates *Acinetobacter* in activated sludge had to be enumerated. Accordingly, it was considered appropriate to study *Acinetobacter* in activated sludge, to obtain information required to test the Koch-Henle postulates with respect to the role of *Acinetobacter* in phosphorus removal.

The purpose of this study was therefore to develop a method for the *in situ* identification and enumeration of *Acinetobacter* in activated sludge and to apply this technique in order to :

(i) Enumerate the *Acinetobacter* in the different activated sludge zones; and

(ii) Determine whether the *Acinetobacter* numbers are sufficient to account for the observed phosphorus removal in the activated sludge system.

This thesis reports the experimental approach and findings of this study.

CHAPTER 2

2. LITERATURE REVIEW

Enhanced phosphorus removal in activated sludge has been studied intensively since Srinath *et al.* (1959) and Vacker *et al.* (1967), reported enhanced phosphorus removal in activated sludge. Since 1959 conflicting data have been put forward supporting either chemical precipitation of phosphorus, (Arvin and Bundgaard, 1982) biological accumulation (Buchan, 1980) and/or biologically mediated chemical precipitation of phosphorus (Arvin and Bundgaard, 1982). None of these mechanisms are clearly defined or fully understood (Barnard, 1976).

Experimental evidence has however been reported favouring the biological uptake of phosphorus, implicating *Acinetobacter* as the responsible agent (Roïnestad and Yall, 1970; Fuhs and Chen, 1975; Osborn and Nicholls, 1978; Buchan, 1980). No quantitative data have been put forward supporting this. This was mainly due to the lack of appropriate methodology to locate, identify and enumerate specific micro-organisms in their natural environment (Eren and Pramer, 1965; Prokasam and Dondero, 1967; Banks and Walker, 1976; Strayer and Tiedje, 1977).

This literature review discusses the basic principles of activated sludge and the methodology used to study phosphorus removing bacteria.

2.1 Basic principles of activated sludge

The following is a concise description of activated sludge as given by Ray (1983).

Activated sludge is formed by aerating a biological degradable waste for a period of time until a large mass of settleable solids forms. The settleable solids are active masses of micro-organisms and are designated as activated sludge. Sedimentation allows the activated sludge to flocculate and settle out, producing a clear effluent of low organic content.

When aeration is started, the nutrient to micro-organism ratio is large and nutrients are in abundance. Consequently the initial growth rate is exponential. During this growth phase the organic matter in the waste effluent is removed at its maximum rate with optimum conversion of organic wastes into new bacterial cells. At this stage the energy level is high enough to keep all the micro-organisms completely dispersed. It is therefore impossible to get activated sludge to form as long as the micro-organism remain in the exponential growth phase.

The nutrient to micro-organism ratio drops as the nutrients are consumed and become a limiting factor in further growth. Continued growth is hence directly proportional to the remaining nutrients. As the energy content of a system decreases, more and more bacteria lack the energy to overcome the forces of attraction between the cells once they have collided. Eventually when the endogenous phase is reached the system is inherently very stable and the nutrient to micro-organism ratio is constant. The demand for energy merely to stay alive, is very low compared to that required for growth. The nutrient concentration continues to decrease and the number of micro-organisms continues to increase, but at an everslowing rate until the bacteria are unable to obtain sufficient energy from the remaining nutrients in the system. As the energy level of the bacteria drops, the rate of floc formation increases very rapidly.

When the endogenous phase is reached, the tiny flocs which have formed are separated from the major fraction of the effluent by sedimentation. The sediment or concentrated floc is then fed to a fresh batch of organic matter and the cycle is repeated.

2.2 The Phoredox activated sludge system for nutrient removal

The following discussion is a resumé of a document published by the South African Water Research Commission which reviews the state of the art regarding biological phosphorus removal in South African activated sludge systems (Wiechers *et al.*, 1984).

The Phoredox activated sludge process is a five stage process designed to remove nitrogen and phosphorus by biological means (Figure 1).

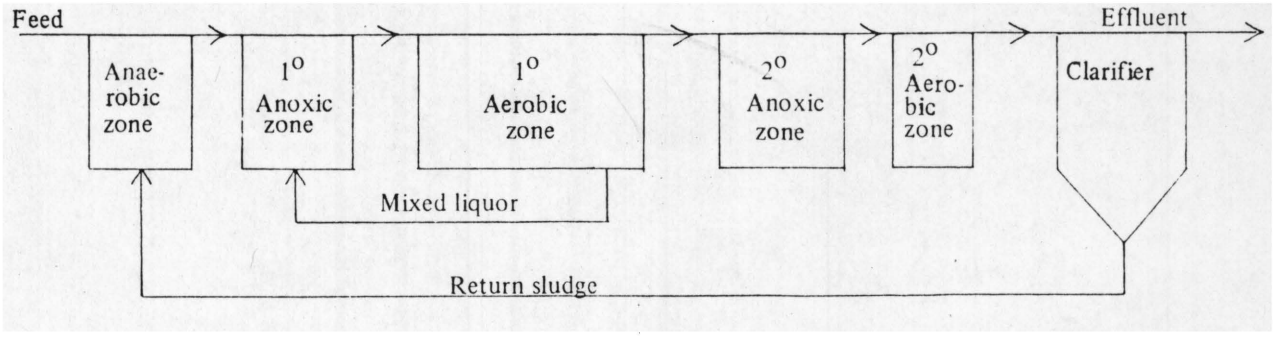


Figure 1: Phoredox (or modified Bardenpho) process for nitrification-denitrification-biological excess phosphorus removal

The Bardenpho activated sludge process is a four stage process designed primarily for removing nitrogen (Figure 2).

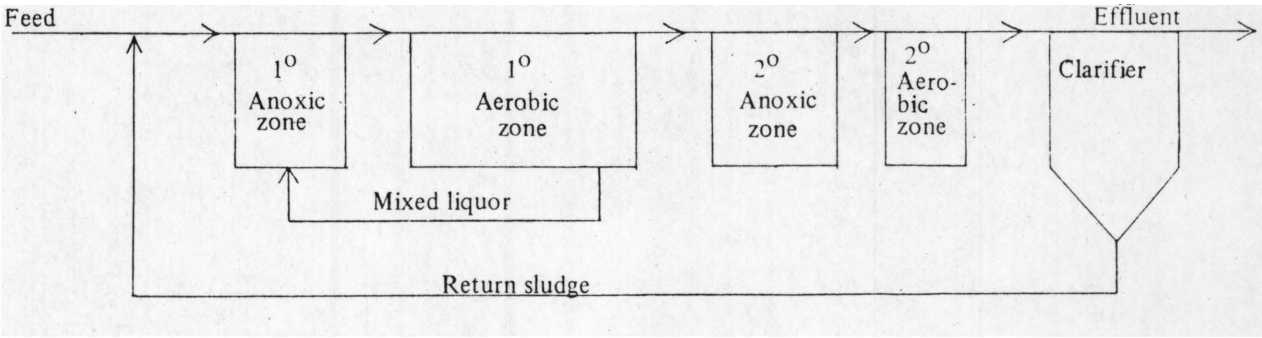


Figure 2: Bardenpho process for biological nitrogen removal – a combination of the modified Ludzack-Ettinger and Wuhrmann processes

This study was conducted on a Phoredox activated sludge system and therefore the different stages of this process will be discussed.

The first stage is the anaerobic zone. It is essential that the concentration of the dissolved oxygen be kept as low as possible in the sludge re-cycle (less than 1 $mgdm^{-3}$). This stage will only be anaerobic if no oxygen, nitrates or nitrites are present. Therefore the retention time in the anaerobic zone would depend on the incoming nitrate and oxygen concentrations. One of the few points of agreement amongst workers is that the release of phosphate is a basic requirement for successful phosphate uptake. This occurs primarily in the anaerobic zone and is the most important function of this stage. The mechanism of phosphate release is not clearly understood. Phosphate release in the anaerobic zone is probably symptomatic of some biochemical change in the system.

The second stage is the anoxic stage. The principal purpose of this stage is the denitrification of nitrates in the recycled mixed liquor from the primary aeration stage. Denitrification will commence only after the dissolved oxygen concentration has been reduced to such a level that denitrification is induced. The process will then continue until the available nitrate has been depleted or the mixed liquor leaves this stage and enters the primary aeration stage. The retention time in this stage is therefore influenced by the concentration of dissolved oxygen fed in from the primary aeration zone, since this must be reduced sufficiently to induce denitrification. If denitrification is completed in the primary anoxic stage, anaerobic conditions develop and phosphates will be released. This appears not to be detrimental to phosphorus removal.

The primary aeration zone is the third stage. This is the principal site for the oxidation of biodegradable nitrogen and carbonaceous material. The oxygen concentration should ideally be kept at 2 mg dm^{-3}, although concentrations as low as 1 mg dm^{-3} are sufficient to indicate that there is sufficient oxygen to support the oxidative process. Most of the phosphorus is removed in the primary aeration zone which is also the prime site for nitrification. The higher the rate of nitrification, the more nitrate will be available for denitrification in the primary anoxic zone.

The fourth stage is the secondary anoxic zone. The principle function of this stage being the removal of excess nitrates not removed in the primary anoxic zone. Due to the lower Chemical Oxygen Demand and hence lower energy available for denitrification, the retention time in the secondary anoxic stage is relatively long. Generally there is no change in the phosphorus concentration in this stage. Care should however be taken that anaerobic conditions do not develop, since this would lead to the release of accumulated phosphorus.

The fifth stage is the secondary aerobic zone. The primary function of this stage is to ensure that anaerobic conditions do not occur after the secondary anoxic stage. The secondary aeration stage also prevents anaerobic conditions from developing in the clarifier and hence prohibits phosphorus release, which would have resulted in higher phosphorus concentrations in the effluent. It appears as if the presence of the secondary anoxic zone was responsible for the introduction of the secondary aeration zone. When it is considered, that the primary function of the secondary anoxic zone is to remove excess nitrates and nitrites which may pass through the system and weigh this against the possibility of phosphate release due to the development of anaerobic conditions in the secondary aeration zone, the system would probably be better off without the secondary anoxic zone. This would minimize the risk of phosphate release into the effluent.

Based on the process dynamics the following has been put forward regarding the microbial population structure of a multi-stage activated sludge plant. The anaerobic stage will lead to the enrichment of bacteria capable of fermentation where organic compounds serve as electron donors and electron acceptors (Buchan, 1984). During fermentation, compounds such as lactic, succinic, propionic, butyric and acetic acid and ethanol are produced. Since these substrates cannot normally be used under anaerobic conditions, they are preferably utilized in the primary anoxic zone for denitrification and the excess will be passed onto the primary aerobic zone. Due to the slow changeover from a fermentative to a respiratory mode of metabolism, the facultative anaerobes stand a little chance of becoming the dominant organisms in activated sludge due to the short retention time and

competition of obligate aerobes in the system. However, the switchover from the respiratory mode of metabolism to the fermentative mode of metabolism is rapid. Fermentation can therefore commence immediately when the sludge enters the anaerobic stage.

In the presence of nitrate and nitrite and absence of oxygen, denitrifiers will be enriched in the *primary anoxic zone.*

In the *primary aerobic zone* nitrifying organisms will be enriched. Buchan (1984) stated that as *Acinetobacter* spp are metabolically very active the availability of their preferred and prestored intracellular substrates in the presence of oxygen could lead to their proliferation in the aerobic zone. Buchan (1984) refers to the intracellular accumulation of poly-β-hydroxybutyrate (PHB) which serves as an internal carbon and energy reserve in the anaerobic zone. As soon as these organisms enter the primary aerobic zone, these compounds can be utilized resulting in the proliferation of *Acinetobacter* in this zone.

Buchan (1980) observed the following whenever an activated sludge plant was removing phosphorus :

- The presence of clusters of cells containing volutin granules which proved to contain phosphorus using EDAX.
- Viable plate counts indicated that members of the genus *Acinetobacter* were the most numerous representatives of the Gram-negative bacteria in the aerobic zone.
- The clusters formed by activated sludge bacteria were highly resistant to dispersion and the viable plate count method was therefore considered to yield an underestimation of their true numbers.
- *Acinetobacter* in pure culture was able to accumulate phosphorus rich volutin granules when grown on Acid Sludge Medium.

Conclusive evidence that the clusters in activated sludge are members of the genus *Acinetobacter* has not yet been put forward. However, Buchan (1980) speculated (based on experimental data) on the quantity of phosphorus that could be removed by *Acinetobacter.* No conclusive evidence on the *in situ* numbers of *Acinetobacter* in activated sludge and the quantities of phosphorus removed has been produced.

Mainly three methods have been used for studying phosphorus removing bacteria in activated sludge :

- Agar plating techniques
- Microscopy techniques
- EDAX techniques

However, the following techniques showed promise for the study of phosphorus removing bacteria in activated sludge :

- Fluorescent techniques
- Immunofluorescent techniques
- Density gradient centrifugation

The abovementioned methods are discussed in the following part of this literature review.

2.3 Methodology used in the study of phosphorus removing bacteria in activated sludge

2.3.1 Agar plating techniques

(a) Plate count technique

In the past, agar plating methods were used to enumerate micro-organisms in activated sludge. However, no single culture medium has been developed that could support the growth of all nutritional types of heterotrophic bacteria present in activated sludge (Prokasam and Dondero, 1967). Culture media have several limitations (Hungate, 1962). Prokasam and Dondero (1967) stated that it was impossible to cultivate many of the bacteria in activated sludge on presently known culture media and seemingly impossible to formulate a media which would support the growth of all the heterotrophic bacteria. To set up an enrichment to detect primary niches in a complex habitat like activated sludge, the nutritional components in the food must be known (Hungate, 1962) However, the artificial niche does not simulate a niche in the natural environment and the results are susceptible to erroneous interpretation. However, it is possible to evaluate media and then reformulate media to yield greater numbers and greater heterogeneity of bacterial types.

The activity of a particular bacterium in its natural habitat is a function of many abiotic factors as well as biotic factors including the number of individual bacteria. Succession may occur in the natural habitat pre-determining the microbial population structure at a specific point in time. This phenomenon cannot be simulated in an enrichment culture, since additional niches are not formed in the culture media (Hungate, 1962).

The plate count gives a measure of viable bacteria and only those that can be resuscitated and capable of growing on the media used. The efficiency for enumerating total numbers of bacteria therefore depends on the extent to which the chemical and physical factors support growth of all viable bacteria.

It is seldom possible if not impossible to simulate all aspects of a habitat with just one medium. It is therefore also possible for bacteria occurring in very small numbers and of complete insignificance to utilize nutrients in the medium and outgrow the pre-dominant types. This difficulty can however, be overcome by dilution before plating out on solid media.

Further, many activated sludge bacteria form clusters or flocs. If not dispersed, this would lead to the underestimation of the total number of bacteria. Comparative studies by Banks and Walker (1976) indicated that a Glucose Casitone Yeast Agar (GCYA) compared most favourably with other existing nutrient media used for the recovery of activated sludge bacteria. They determined the total number of colony forming units (CFU) for six municipal treatment works using GCYA, Minimal Pyruvate Agar (MPA) and Tryptose Glucose Extract Agar (TGEA). The average number of CFU on GCYA was $4{,}2 \times 10^9$ CFU cm^{-3}, on TGEA $3{,}0 \times 10^9$ CFU cm^{-3} and on MPA $1{,}6 \times 10^9$ CFU cm^{-3}. To date, there has been no efforts to improve GCYA for the recovery of bacteria from activated sludge.

(b) The API-20E microtube technique for studying activated sludge bacteria

(1) Review of the API-20E microtube technique for studying the ecology of bacteria

The API-20E microtube technique was developed as a rapid method for the identification of the *Enterobacteriaceae* and other Gram-negative heterotrophic bacteria and has

been reviewed by Martin *et al.* (1971); Washington *et al.* (1971); Smith *et al.* (1972); Van Vuuren (1978) and Cloete (1981).

Although it is known that the API-20E technique has successfully been used to determine the population structure of aquatic systems, it has certain limitations for studying phosphorus-accumulating activated sludge. In the first place, this method is recommended for the identification of the *Enterobacteriaceae* and other Gram-negative heterotrophic bacteria. However phosphorus accumulation may take place in bacteria other than those that can be identified by using the API-20E technique.

The second disadvantage of the API-20E microtube technique, is the lack of suitable nutrient media which will support the growth of all activated sludge bacteria (Banks and Walker, 1976). Therefore some bacteria which may accumulate phosphorus in activated sludge might be excluded.

Thirdly, most bacteria form clumps in activated sludge, since it is a flocculated suspension. Some bacteria, like *Acinetobacter* form clusters in activated sludge. If the clusters are not dispersed the numbers of these bacteria will be underestimated in a population structure study.

The cost involved in using the API-20E microtube technique also becomes a limiting factor where the population structure of a multiple-stage process like activated sludge is studied. This problem is aggravated when, in order to get a representative picture of the bacteriology of phosphorus removing activated sludge, the process has to be studied over a period of time.

Nevertheless, the API-20E microtube technique has been used with some success to determine the population structure of activated sludge. (Buchan, 1980; Brodisch and Joyner, 1982).

(2) Bacterial population structure of activated sludge

After the initial work of Butterfield *et al.* (1935), it was generally believed that *Zoogloea ramigera* was the only important bacterium in sludge from the point of sewage purification. Since then, contradictory results have been reported.

According to Dias and Bhat (1964), McKinney and Horwood (1952) and McKinney and Weichlein (1953), focussed attention on other bacteria in activated sludge, but like the studies of Allen (1943), Jasewicz and Porges (1956) and Rogovskaya and Lazareva (1959), their investigations were far from comprehensive. Dias and Bhat (1964), indicated that Gram-negative bacteria of the genera *Zoogloea* and *Camamonas* predominated.

Anderson and McCoy (1963) found, however, that the population of activated sludge was dominated by *Pseudomonas* species. Buchan (1980), indicated that members of *Acinetobacter* were predominant in activated sludge. Brodisch and Joÿner (1982) found that *Aeromonas* and *Pseudomonas* were predominant in activated sludge. This correlated with the findings of Anderson and McCoy (1963).

Predominance of bacteria was always expressed as a percentage and not a real number. This limited quantitative analysis. It is therefore obvious that no suitable method presently exists for the enumeration of *Acinetobacter* in activated sludge. Hence, the role of this organism in biological phosphorus removal remains controversial.

Direct microscope studies of micro-organisms in activated sludge have been (Buchan, 1980) and could be even more informative if it were possible to identify the micro-organisms observed. This would also provide a method for the direct enumeration of the organisms involved. The fluorescent antibody (FA) technique makes possible the identification of specific micro-organisms in their natural habitats (Schmidt, 1974). The FA technique has therefore been extensively applied in the ecological study of micro-organisms in soil, aquatic systems and anaerobic sludge. (Eren and Pramer, 1965; Hill and Gray, 1967; Fliermans *et al.*, 1974; Schmidt, 1974; Strayer and Tiedje, 1977).

2.3.2 Microscope techniques

(a) Light microscopy

Light microscopy and metachromatic staining indicated that bacteria containing polyphosphate granules were present in activated sludge (Roïnestad, 1973; Osborn and Nicholls, 1978; Buchan, 1980). This method revealed numerous polyphosphate granules inside bacterial cells whenever a plant was exhibiting enhanced phosphorus removal (Osborn and Nicholls, 1978; Buchan, 1980), and was used to identify sludges with a high rate of phosphorus removal (Barnard, 1982). Therefore it was suggested that phosphorus

accumulation by bacteria might be significant during enhanced phosphorus removal by activated sludge (Barnard, 1976; McLaren, 1976). Although micro-organisms can be observed using light microscopy, they are rarely identified by this method (Schmidt, 1974). Therefore it was not proven that the relevant bacteria were present in sufficient numbers to be significant during the phenomenon of phosphorus removal in activated sludge.

(b) EM and EDAX techniques

Many different micro-organisms are capable of phosphorus uptake (Friedberg and Avigad, 1968; Terry and Hooper, 1970). Buchan (1980) using EM and EDAX techniques was able to confirm that some phosphorus in activated sludge was located inside bacterial cells. Due to the lack of identification techniques when using the EM technique, the phosphorus-accumulating bacteria in activated sludge were not identified.

As a result no quantitative data could be obtained regarding the significance of *Acinetobacter* phosphorus uptake in activated sludge. Indirect techniques were therefore used in efforts to explain the phosphorus removal ascribed to *Acinetobacter*. Density gradient centrifugation was used to quantify the constituent components of the volutin granules (Friedberg and Avigad, 1968) and the API-20E technique for the determination of the population structure study of activated sludge (Buchan, 1980; Brodisch and Joyner, 1982).

2.3.3 Fluorescent techniques

Due to the lack of suitable culture media for the recovery of all nutritional types of bacteria (paragraph 2.2) alternative methods have been used for enumerating bacteria in activated sludge. The direct count method using a fluorescent dye and epifluorescence microscopy has been used successfully to enumerate aquatic bacteria (Hobbie *et al.*, 1977). Staining of samples with fluorescent dye, Acridine Orange (AO), caused dead bacteria to fluoresce green and the viable red or yellow. This method enabled scientists to accurately and quickly enumerate all bacteria, dead and alive, in aquatic systems. When the AO concentration is kept low (0,01%), the dye appears to interact with the nuclear material of bacteria. Actively growing cells will fluoresce red-orange due to the pre-dominance of ribonucleic acid (RNA). Inactive bacteria have mostly deoxyribonucleic acid (DNA) and fluoresce green (Hobbie *et al.*, 1977). However, where a fixative is used to preserve the sample it can be expected that most of the bacteria will fluoresce green. Hobbie *et al.* (1977) stated that filtration onto Nuclepore filters and staining with a fluorescent dye, appeared to be the best way to make a direct count of bacteria.

This has been verified by a comparison of counts done with the scanning electron microscope, (Bowden, 1977) and with carbon replica and transmission electron microscope techniques (Watson *et al.*, 1977). The method developed by Hobbie *et al.* (1977) was therefore adopted for determining the total numbers of bacteria in activated sludge.

Immunofluorescent staining was demonstrated for the first time by Coons (1942). According to Eren and Pramer (1965) this provided medical microbiologists with a reliable and quick diagnostic method. Since then the use of the FA technique has been applied for the *in situ* study of micro-organisms (Eren and Pramer, 1965; Hill and Gray, 1967; Fliermans *et al.*, 1974; Schmidt, 1974; Hobbie *et al.*, 1977; Strayer and Tiedje, 1977).

Immunofluorescent staining makes possible microscopic investigation of the antigen-antibody reaction. After coupling a specific antibody to a fluorochrome, it is permitted to react with its specific antigen and the reaction product is observed through a fluorescence microscope. Basically two methods, direct and indirect staining, can be employed to study the antigen antibody reaction. The advantage of the direct techniques used in this study lies in their simplicity.

2.3.4 Immunofluorescent techniques

(a) Factors influencing the effectivity of immunofluorescent techniques for the identification and enumeration of bacteria

(1) Fluorochromes

Compounds absorbing light of one wavelength and emitting the light of a longer wavelength are fluorescent. Many fluorescent dyes exist and are known as fluorochromes (Eren and Pramer, 1965). The ideal fluorochrome for immunofluorescent work must combine with the antibody to form a stable conjugate without unduly affecting its specificity or combining power and must emit light of suitable intensity and colour or irradiation (Pearce 1960). Due to their favourable characteristics, fluorescein-isothiocyanate (FITC), which fluoresces green, and rhodamine isothiocyanate (RITC) which fluoresces red, are the most commonly used fluorochromes for preparing FA. Other fluorochromes such as fluolite C and lissamineflavine FSS, which fluoresce blue, are unsuitable for many kinds of immunofluorescent work. Due to the low intensity of the fluorochrome 1 dimethylaminophthalene-5-sulphonic acid (DANS), it is hardly ever used. FITC is thus considered a very favourable fluorochrome for immunofluorescent studies.

(2) Light sources for microscopy

(i) ultra high pressure mercury lamps
(ii) high pressure xenon lamps
(iii) tungsten, halogen lamps and
(iv) lasers

The mercury burner and the tungsten halogen lamps have been the most common light sources in immunofluorescent studies (Thomason, 1976). Xenon lamps resulted in fluorescence 2,5 times as intense as obtained with the mercury lamp. Lasers have also been recommended as a light source for fluorescent antibody studies (Kaufman *et al.*, 1971). The advantage of this type of excitation appears to be that when it is linked to electronic fluorescence detection and recording devices, it eliminates human error and individual variability. The disadvantages of the latter method is that it is very expensive and can only produce reliable results where pure cultures are being examined. Therefore laser excitation will be of little value in an *in situ* study of activated sludge due to background fluorescence and non-specific adsorption of unreacted fluorescent dye and the variety of organisms found in activated sludge. Due to its favourable characteristics, FITC was chosen as the fluorochrome used in this study.

(3) Specificity of the antibody and non-specific absorption of unreacted dye

(i) Titre of the antisera

The effectivity of immunofluorescence depends on the selective combination of the fluorescent-labelled antibody to micro-organisms closely related to the antigen.

High or low titres can be used in FA studies. Usually antisera of a high antibody titre has been used in serological work. The latter however, often resulted in cross-reactions between the anti-serum and organisms related to the antigen (Carter and Leise, 1958). By producing antisera of low titre, antibodies to minor antigens are apparently not formed and this eliminates the forming of cross-reactions and renders the antisera suitable for identification purposes.

Low titres are therefore preferred for the identification of bacteria but has the disadvantage of small quantities of antisera that are recovered from the experimental animal. However high titre antisera are considered useful, because this can be diluted to give larger quantities of reactive serum. Cherry *et al.* (1960) suggested that it was better to use

isolated globulins of high specificity, prepared from high titre antisera than whole serum of low specificity. Isolation of globulins was however, only considered necessary if the high titre antisera were non-specific. Various methods exist to increase the specificity of antisera (Cherry *et al.*, 1960; Eren and Pramer, 1965).

(ii) Non-specific reactions

A problem regarding the effectivity of the FA technique is the occurrence of non-specific cross-reactions on the one hand (Moody and Jones, 1963), and non-specific adsorption of the unreacted dye on the other (Schmidt and Bankole, 1965).

Moody and Jones (1963) suggested that many non-specific reactions can be attributed to antibodies normally occurring in serum. Bergman *et al.* (1966) developed a method whereby pooled antiserum of uninfected animals is used to pre-stain the samples before staining with a specific fluorescein-labelled antibody, effectively blocking out non-specific staining.

Unreacted fluorescent dye is another major factor contributing to non-specific staining. It was therefore necessary to develop procedures to minimise the quantity of unreacted dye without impairing the specific activity of the conjugate.

Johnson and Holborrow (1978) found two methods to be very efficient for minimising the effect of unreacted dye. Unreacted dye may be removed by passing the conjugate through a G50 Sephadex column which acts as a "molecular sieve". This method is equivalent to dialysis but has the advantage of being much faster. A second effective method, was to determine the absolute fluorescein isothiocyanate concentration in relation to the protein concentration of the serum. For fluorescein isothiocyanate the optimum concentration was found to be 0,05 mg per mg protein. This rendered non-specific staining negligible (Johnson and Holborrow, 1978).

As mentioned before, non-specific adsorption of the fluorescent antibodies to tissue and other surfaces, caused problems when used to study the ecology of micro-organisms (Schmidt and Bankole, 1965). Bohlool and Schmidt (1968) therefore developed a counter-stain technique for controlling non-specific adsorption. They modified gelatin solutions by partial hydrolysis and conjugated this to the fluorochrome rhodamine isothiocyanate. The gelatin absorbed to the sites of non-specific staining and provided a counter-stain which fluoresced red. This provided a contrast with the green fluorescence of FITC. In some cases the red background fluorescence was too intense to obtain a good contrast (Bohlool

and Schmidt, 1968). Strayer and Tiedje (1977) found that prestaining of samples with 2% Bovine Serum Albumin (BSA) in the same way as gelatin-rhodamine isothiocyanate conjugate, was effective in blocking out non-specific adsorption.

The method of Strayer and Tiedje (1977) is considered to be the better of the two methods, since it eliminated the frequently encountered problem of obtaining a good contrast when using the gelatin-rhodamine isothiocyanate conjugate.

(4) pH of the mounting fluid

Pital and Janowitz (1963) found that maximum fluorescence intensity occurred at a pH of 9.

(5) Desorption of bacteria adhering to suspended colloids and dispersion of cluster-forming bacteria

Due to the tendency of the bacteria to adhere to soil particles, a method had to be developed to enumerate bacteria in soil (Schmidt, 1974). This involved the release of bacteria from soil in a dispersed suspension; flocculation of soil colloids out of suspension: concentration of the bacteria in the supernatant on a special membrane filter; staining with the specific fluorescent antibody and enumeration of reactive cells by epifluorescent microscopy.

Marshall (1971) emphasized the sorption of aquatic bacteria to particles. Sludges are rich in colloidal materials and bacteria and therefore resemble soil in suspension. Subsequently it was expected that similar problems with the enumeration of bacteria in soil and sludge might be encountered. This was substantiated by Strayer and Tiedje (1977) who adopted immunofluorescent techniques employed in the identification of soil organisms, to study the microbial ecology of lake sediments and anaerobic sludge. Nonspecific adsorption of the specific fluorescent antibody to the suspended colloids occurred. The colloids furthermore clogged the filter and masked the bacteria on the filter, causing inaccurate enumeration (Schmidt, 1974). Banks and Walker (1976) suggested sonication of activated sludge flocs for the recovery of bacteria on a solid nutrient medium. They indicated that the release of bacteria in activated sludge depended on the intensity and duration of sonication. Most viable bacteria were recovered after 80–100 s at a power output of 26 J s^{-1} (Banks and Walker, 1976). They also stated that the number of bacteria recovered after sonication depended very much so on the medium used for culturing the bacteria. In this respect GCYA yielded the highest numbers. They furthermore proved that after prolonged

sonication at the lowest intensity, no appreciable decrease in the number of bacteria recovered was observed. Due to the different release patterns in different sludges, it was concluded that until the characteristics of a sludge are assessed a range of sonication times between 1 and 2 minutes should be used (Banks and Walker, 1976).

The sorption phenomenon due to the interaction between bacteria and suspended colloids is attributed to high electrolyte concentrations (Marshall, 1971). Marshall (1971) illustrated that desorption of bacteria from colloids could be obtained by repeated washing of the bacterial-colloidal suspension with saline. However Schmidt (1974) found that the release of bacteria from colloids could effectively be accomplished by mechanical agitation of a diluted soil suspension using Tween 80 as surfactant and a specific antifoam to control foam build up during agitation.

In order to remove the colloids without removing the bacteria in the supernatant Harper (1942) recommended a mixture of calcium hydroxide and magnesium carbonate as a flocculant for soil colloids. This method was adopted in the studies of Schmidt (1974). Strayer and Tiedje (1977) found the abovementioned flocculant mixture to be very effective for the flocculation of colloids dispersed in sludge. Schmidt (1974) suggested that a certain percentage of micro-organisms were removed by flocculation. Strayer and Tiedje (1977) found that 15% of the total quantity of bacteria was removed by flocculation and therefore used 15% as a correction factor in their studies on methanogenic bacteria.

(6) Membrane filters

Hobbie *et al.* (1977) found that the type of membrane filter as well as the pore size were important considerations when using fluorescent techniques for enumerating bacteria and claimed that many more bacteria were visible on Nuclepore filters, than on cellulose filters. The lower numbers encountered when using cellulose filters were ascribed to the very rough surface in contrast with Nuclepore filters resulting in the embedding and masking of bacteria preventing their enumeration (Bowden, 1977). Nuclepore filters were therefore preferred when doing direct bacterial counts using a fluorescent dye and epifluorescence microscopy (Bowden, 1977; Hobbie *et al.*, 1977). Hobbie *et al.* (1977) furthermore found that many more bacteria were retained on filters with a pore size of 0,20 μm than on filters with a pore size of 0,45 μm.

Due to autofluorescence of white membrane filters they cannot be used in the identification or enumeration of bacteria using immunofluorescent techniques (Schmidt, 1974;

Hobbie *et al.*, 1977). To overcome this problem filters were dyed using Indian ink (Schmidt, 1974) or Irgalan black (Hobbie *et al.*, 1977). Currently, black membrane filters are commercially available, eliminating the necessity of staining filters with an appropriate dye. Filtration through black Nuclepore filters with a pore size of 0,20 μm therefore appeared the best method for the direct enumeration of bacteria in aquatic systems (Hobbie *et al.*, 1977). This was confirmed by Robarts and Sephton (1981). Neither the staining of white filters or the use of pre-stained black filters excluded the problem of non-specific adsorption of FA.

(7) Influence of numbers of organisms being filtered

Large numbers of organisms on a filter may depress the intensity of the fluorescence due to the excess of antigen over available antibody and may also result in multiple layers of organisms in a specific area on a filter, making it very difficult to identify and enumerate individual organisms. Due to large numbers of organisms occurring in activated sludge it was necessary to dilute samples to such an extent that only a sufficient number of organisms was filtered to form a single layer of bacteria on the filter.

(8) Intensity of fluorescence

Visual estimation of the degree of fluorescence of stained cells is often subjective and should therefore be conducted by an analyst trained in FA methodology (Thomason, 1976). The degree of fluorescence of cells was estimated on a scale of 0 to 4+ as follows (Thomason, 1976) :

4+	=	maximum fluorescence; brilliant yellow-green; distinct cell outline; sharply defined cell centre
3+	=	less brilliant yellow-green fluorescence; clear cut cell outline; sharply defined centre
2+	=	definite but dim fluorescence; cell outline less well-defined
1+	=	very subdued fluorescence; cell outline indistinguishable from cell centre
0	=	negligible or complete lack of fluorescence.

Although it may be argued, that a 2+ fluorescence together with morphological or other physical characteristics could be considered as a possible count, this was not recommended as it would increase the subjective nature of the technique and reproducibility of the results.

Apart from being very effective for the identification and enumeration of specific bacteria, immunological reactions have been used to identify areas of specific antigen in ultrastructural studies, using ferritin-labelled antibodies.

2.3.5 Ferritin-labelled antibodies (FLA)

Singer (1959) was the first to use FLA in ultrastructural studies. Ferritin is an iron containing protein. Under the electron microscope, this protein shows up as a electron-opaque spot enabling identification of specific antigen areas.

Regarding specificity, the same criteria apply to both FLA and FA (paragraph 2.3.4). The FLA can be used by pre-embedding staining, where the reaction takes place before preparing the specimen for EM. Post embedding staining of thin sections, with the FLA is an alternative method, but involves more technical difficulties.

The FLA technique has its own unique problems. Singer (1959) used M-xylylene di-isocyanate (XC) and later toluene-2,4-di-isocyanate (TC) as conjugating agents. With both the XC and TC the conjugation procedure is carried out in two stages. In the first stage, ferritin reacts with the coupling agent and in the second stage, the modified ferritin is conjugated with the antibody globulin (Vogt and Kopf, 1964). In most studies using ferritin-conjugated antibodies in electron microscopy, XC has been used as conjugating agent. However, the serological activity of the antibody-conjugate was very low (Vogt and Kopf, 1964). These results were in agreement with those obtained by Borek and Silverstein (1961). TC was used as the conjugation agent, but with little success (Borek and Silverstein, 1961; Vogt and Kopf, 1964).

The loss of antibody activity may be due to involvement of the determinants in the conjugation reaction or to steric hinderance by the large ferritin molecule. This could lead to the formation of functionally univalent antibody molecules carrying the ferritin label (Borek and Silverstein, 1961). This type of modified antibody would not be detected in the haemagglutination test, but would probably be able to combine with the antigen, depending on the specificity of the antibody and its binding mode to the antigen (avidity) (Vogt and Kopf, 1964).

The purity (ratio of ferritin to immunoglobulin) will also influence the antigen-antibody-conjugate reaction. Depending on how effectively the unconjugated antisera can be removed from the conjugated antisera, the antigen antibody-conjugate reaction would differ.

If too much unconjugated antibody is present, these would bind to the antigen, blocking the reaction with the ferritin-antibody conjugate (Johnson and Holborrow, 1978).

The success of the FLA technique therefore relies on striking the correct ratio of ferritin to antibody and no loss of the serological activity of the conjugate due to the conjugation procedure.

2.3.6 Density gradient centrifugation

Density gradient centrifugation is an established way of separating and purifying cells and subcellular particles. To isolate the volutin in bacterial cells, Friedberg and Avigad (1968) used gradient centrifugation to fractionate the cell debris of plasmolysed volutin containing *Micrococcus lysodeikticus*. Chemical analysis of the volutin indicated that 27% was phosphorus, 24% protein and 30% lipids. Density gradient centrifugation indicated that the specific gravity of the volutin was 1,23. This suggested that volutin containing cells could possibly have a higher buoyant density than other cells. This would facilitate the isolation of these cells from activated sludge if density gradient centrifugation were to be applied.

The limitations of this technique has been the physical characteristics of the gradient medium itself. However, Percoll possesses the ideal characteristics as a medium for gradient centrifugation (Pharmacia Chemicals).

2.4 Summary

The five stage Phoredox process was designed for biological nutrient and particularly phosphorus removal. Controversy exists whether phosphorus removal was a chemical, biological or chemically biologically mediated process.

Wherever phosphorus was removed :

- many clusters of bacteria containing volutin granules were observed and identified as *Acinetobacter*
- EM and EDAX analysis indicated that the volutin contained phosphorus
- indirect plating techniques indicated that *Acinetobacter* was the dominant bacteria whenever phosphorus was being removed.
- *Acinetobacter* could also accumulate phosphorus in the form of volutin when grown in pure culture on Acid Sludge Medium.

Based on these data, *Acinetobacter* has always been associated with phosphorus removal in activated sludge and it has been claimed to be mainly responsible for biological phosphorus removal.

However, the study of the role of *Acinetobacter* as a phosphorus removing agent in activated sludge has been restricted by :

- bacterial clusters being refractive to dispersion. Consequently *Acinetobacter* numbers could be underestimated using indirect enumeration techniques;
- the lack of suitable culture medium which would support the growth of all activated sludge bacteria. Consequently it gave a false impression of the bacterial population structure of activated sludge;
- the lack of *in situ* identification and enumeration techniques for *Acinetobacter* in activated sludge. Consequently no quantitative analysis of the role of *Acinetobacter* as a phosphorus removing agent in activated sludge was possible.

Therefore the following techniques showed great promise for studying the role of *Acinetobacter* as a phosphorus removing agent in activated sludge :

- dispersion of bacterial clusters by using sonication and a dispersant of some kind (e.g. 0,5% Tripolyphosphate).
- fluorescent techniques for determining the total number of bacteria in activated sludge.
- immunofluorescent techniques for the *in situ* identification and enumeration of *Acinetobacter* in activated sludge and determining its relation to the total number of bacteria.
- FLA technique for the *in situ* identification of *Acinetobacter* using EM.
- density gradient centrifugation to determine whether *Acinetobacter* is associated with phosphorus removal in activated sludge.
- using a combination of the abovementioned techniques to determine the possible role of *Acinetobacter* as a phosphorus removing agent in activated sludge.

This dissertation discusses the development and application of these techniques for studying the role of *Acinetobacter* as a phosphorus-removing agent in activated sludge.

CHAPTER 3

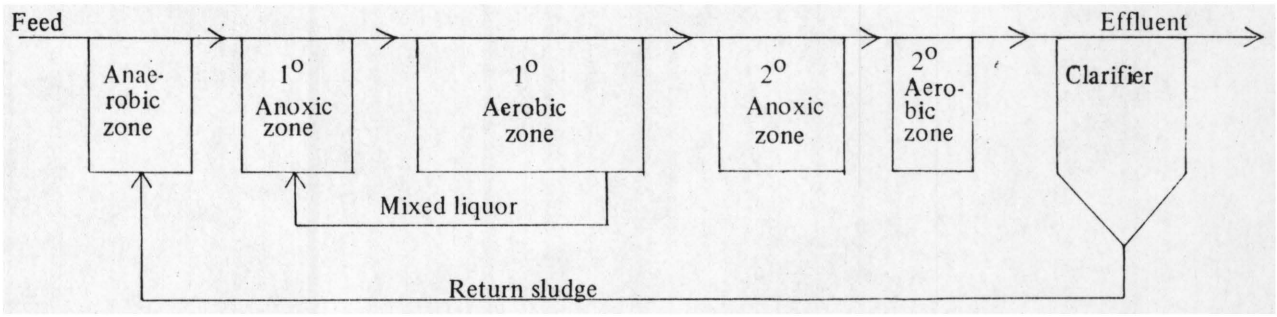
3. EXPERIMENTAL PROCEDURES

3.1 Materials

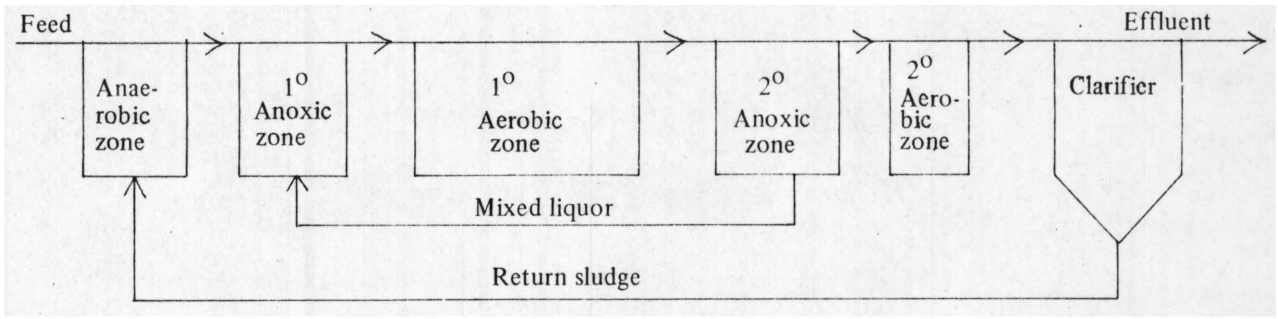
3.1.1 Experimental units

The following diagrams give a schematic representation of the experimental units studied.

(a) Northern Works- and Goudkoppies activated sludge plants



(b) Benoni activated sludge plant



3.1.2 Experimental animals

Adult Albino rabbits were used as experimental animals for the production of antibodies. These rabbits were specially bred for antibody production by the Onderstepoort Research Institute for Veterinary Science. During the period of antibody production the rabbits were kept under clean, but not sterile, conditions.

3.1.3 Bacterial cultures

The following bacterial cultures were used in this study:

(a) *Acinetobacter calcoaeceticus* var *lwoffii.* This culture was obtained from Buchan (1980) and identified as *A. calcoaceticus* var *lwoffii* in this study and confirmed by the Deutsche Sammlung von Microorganismen (DSM).

(b) *Acinetobacter calcoaceticus.* Two authentic cultures were obtained from the American Type culture collection (ATCC) number E23055.

(c) *Acinetobacter lwoffii.* Two authentic cultures were obtained from the ATCC number 17925.

(d) *Acinetobacter phosphodevorans* was obtained from the DSM.

Stock cultures were maintained by subculturing onto Glycerol Casitone Yeast Agar every second week.

3.1.4 Culture media

(a) General culture media

(1) Glycerol - Casitone - Yeast Agar (GCYA) (Banks and Walker, 1976)

Casitone	1 g
Yeast Extract	1 g
Agar	13 g
Distilled Water	1 dm^3
pH	7,2

Sterilised at 121°C for 15 min.

(b) Acinetobacter culture media

(1) *Acinetobacter*- Agar. (Fuhs and Chen, 1975)

Sodium acetate	5 g
$(NH_4)_2SO_4$	2 g
$MgSO_4.7H_2O$	0,5 g
KH_2PO_4	0,25 g
$CaCl_2.2H_2O$	0,2 g
Tap Water	1 dm^3

Sterilised at 121°C for 15 min.

(2) Acid Sludge Medium (Buchan, 1980)

The same procedure used by Buchan (1980) for preparing Fermented Raw Sludge Medium was followed to prepare Acid Sludge Medium.

3.1.5 Reagents

(a) Buffers

(1) Phosphate buffered saline, 0,01 M, pH 7.2 (PBS)

NaCl		8,5 g
Solution A:	Na_2HPO_4	1,4 g
	Distilled water	100 cm^3
Solution B:	NaH_2PO_4	1,4 g
	Distilled water	100 cm^3

The 8,5 g of NaCl was added to 500 cm^3 of distilled water. Of solution A, 84,1 cm^3 was added to 15,9 cm^3 of solution B. This mixture was added to the 500 cm^3 of distilled water containing 8,5 g of NaCl and made up to 1 dm^3 with distilled water, and sterilised at 121°C for 15 min.

(2) Carbonate buffer, 0,5 M, pH 9.0

Solution A:	Na_2CO_3	5,3 g
	Distilled water	100 cm^3
Solution B:	$NaHCO_3$	4,2 g
	Distilled water	100 cm^3

Of solution A, 4,4 cm^3 was mixed with 100 cm^3 of solution B. The pH was checked after mixing, and when necessary adjusted to 9,0 by adding more of solution A.

(3) Carbonate buffered glycerol (Mounting fluid)

One part of the carbonate buffer was added to 9 parts of glycerol.

(4) Borate buffer (Garvey *et al.*,1977)

Boric acid	6,184 g
Borax	9,536 g
Distilled water	1 dm^3

(b) Preservative

(1) Sodium azide 0,1%

Sodium azide	0,1 g
Distilled water	100 cm^3

(c) Fixatives

(1) Formalin

Formaldehyde solution (37%–40%)

(2) Phosphate buffered formal solution

Formaldehyde solution (37%–40%)	10 cm^3
0,01 M Phosphate buffered saline	100 cm^3

(3) Acetone

Acetone was purchased from N.T. Laboratories (Pty) Ltd.

(4) Osmic acid 1%

Osmic acid	1 g
Distilled water	100 cm^3

(d) Dehydrating agents

A graded concentration series of ethyl alcohol was made up with double distilled water (v/v). The series was 50, 60, 70, 80, 90 and 100% alcohol.

(e) Embedding material

L.R. White Resin (Wirsam Scientific and Precision Equipment (Pty) Ltd.)

(f) Stains

(1) Methylene blue stain.

Methylene blue	1 g
Sodium borate	1 g
Double distilled water	100 cm^3

(2) Fluorescent stain

Fluorescein Isothiocynate (FITC) (Merck Chemicals) was used as described in paragraph 3.2.4(a).

(3) Ferritin stains

Ferritin containing trace Cd (Merck Chemicals) was used as described in paragraph 3.2.9.

(4) Bovine serum albumin fraction 5.

Bovine serum albumin (2%)	2 g
Distilled water	100 cm^3

(5) Irgalan black stain (Hobbie *et al.*, 1977)

Irgalan black	2,0 g
Acetic acid	20,0 cm^3
Water	1,0 dm^3

(6) Acridine orange (AO) stain (Hobbie *et al.*, 1977)

Acridine orange	0.1 g
Water	100 cm^3

(g) Dispersants

(1) Tween 80 was obtained from Merck Chemicals.

(2) Ansan 6 was obtained from Chemserve Systems (Pty) Ltd.

(3)

Sodium Tripolyphosphate	(0,5% m/v)
Sodium Tripolyphosphate	0,5 g
Distilled Water	100 cm^3

(h) Antifoam

Silicone silcolapse 5001 (Merck Chemicals).

(i) Flocculant

$Ca(OH)_2$	2 g
$Mg\ CO_3$	5 g

The 2 g of $Ca(OH)_2$ was mixed with 5 g of $MgCO_3$ and dried overnight at 90°C (Strayer and Tiedje, 1977). Of this mixture, 0,7 g was added, to 100 cm^3 of mixed liquor for flocculation.

3.1.6 McFarland scale

The McFarland scale was used to standardize bacterial suspensions (Table 1).

Table 1 Standardisation of bacterial suspensions by nephelometry (McFarland, 1970)

McFarland Scale	1% $BaCl_2$ (m/v) cm^3	1% H_2SO_4 (v/v) cm^3	Number of bacteria (value listed x 10^6 cm^{-3})
1	0,1	9,9	300
2	0,2	9,8	600
3	0,3	9,7	900
4	0,4	9,6	1 200
5	0,5	9,5	1 500
6	0,6	9,4	1 800
7	0,7	9,3	2 100
8	0,8	9,2	2 400
9	0,9	9,1	2 700
10	1,0	9,0	3 000

3.1.7 Sephadex column

(a) A Sephadex column was used to purify the FITC labelled antisera. The column was prepared as follows :-

(1) Thirty gram Sephadex G – 25 fine was added to 250 cm^3 PBS and well shaken.

(2) The suspension in (1) was allowed to stand for 5 to 10 min. The supernatant was carefully decanted. Fresh PBS (250 cm^3) was then added and again the suspension was shaken. This procedure was repeated three times.

(3) The suspension was boiled for 1 h, allowed to cool and the supernatant decanted. Fresh PBS (250 cm^3) was added, the suspension shaken and allowed to stand for 5 to 10 min. The latter was repeated twice.

(4) The slurry was added to the column and allowed to stabilise overnight (Garvey *et al.*,1977).

3.1.8 API-20E microtubes

API-20E microtubes were obtained from Ayerst Laboratories Inc.

3.1.9 Membrane filters

Black Nuclepore filters (0,45 μm and 0,20 μm pore size, 47 mm diameter) were used for all filtration work (Atomic Export Import Company).

3.1.10 Light microscopy apparatus

A Reichert Univar research microscope equipped with epifluorescence facilities was used to study methylene blue – and fluorescent antibody stained samples. The microscope was equipped with a trimatic camera system and photographs were recorded on 35 mm Agfachrom 50 c film.

3.1.11 Electron microscopy apparatus

(a) Microtome

A Reichert Jung microtome fitted with a diamond blade was used to make ultra-thin sections.

(b) TEM

An Hitachi H600 transmission electron microscope was used to study all ultra-thin sections

3.1.12 EDAX

A Jeol JSM–U3 scanning electron microscope, modified for scanning transmission and equipped with an EDAX detector with video display, and a 'Data general Corporation' data processor was used.

3.1.13 Apparatus and reagents for gradient centrifugation

(a) Apparatus

(1) Centrifuge

A Beckman L5–50 ultracentrifuge was used for centrifugation.

(b) Reagents

(1) Saline

NaCl	8,5 g
Distilled Water	1 dm^3

Sterilised at 121^{o}C for 15 min.

(2) Percoll was obtained from Pharmacia Chemicals (Pty) Ltd.

3.2 Methodology

3.2.1 Preparation of antisera

(a) Preparation of somatic O antigens

Acinetobacter isolates (paragraph 3.1.3(a), (b) only ATCC E23055 and (d)) were used as antigens for the stimulation of antibody production. The antisera using above-mentioned antigens were produced separately for each antigen as follows:

(1) The isolates, maintained on GCY medium, were transferred to 400 cm^3 *Acinetobacter* medium and incubated at 37°C for 72 h.

(2) The bacteria were collected by centrifugation at 8 000 rpm for 10 min. The supernatant was discarded and the bacteria resuspended in saline. This procedure was repeated three times.

(3) The bacterial suspension was then placed in a boiling water bath for 1 h.

(4) The bacteria were again collected by centrifugation (8 000 rpm for 10 min) and diluted with saline to a final concentration of *ca* 1,2 x 10^9 bacteria cm^{-3} using the McFarland scale (Table 1), for injection into the experimental animals.

(b) Injection into animals

Intravenous injections were made as described by Garvey *et al.* (1977). In order to prevent the animals from becoming hypersensitive and susceptible to anaphylactic shock the inside of the sterile syringe was rinsed with adrenalin before filling with the right volume of antigen.

The following programme was used :

Day 1	0,5 cm^3 antigen
Day 4	1,0 cm^3 antigen
Day 7	1,5 cm^3 antigen
Day 10	2,0 cm^3 antigen
Day 13; 16; 19 and 22	2,0 cm^3 antigen
Day 29	The titre of the antiserum was determined (paragraph 3.2.1(c)). No booster injection was necessary because of a satisfactory titre (Table 2) indicating a good antibody response.

(c) Agglutination test

The highest dilution of serum causing agglutination of the antigen is defined as the titre (Garvey *et al.*,1977).

To determine the titre it was necessary to draw 5 cm^3 of blood from the ear of each experimental animal, according to a method described by Garvey *et al.* (1977). The agglutination test was then used to determine the titre of each antiserum from each experimental animal.

(d) Separation and preservation of antisera

(1) Blood (*ca* 50 cm^3) was sampled from each experimental animal using the cardiac puncture technique (Garvey *et al.*,1977).

(2) The freshly drawn blood was allowed to stand for 2 to 3 h at room temperature for clot formation. The clot was separated from the wall of the tube by using a sterile applicator stick. The tube was then stored in a refrigerator for 12 to 24 h to permit clot contraction.

(3) The serum was decanted into clean sterile centrifuge tubes (autoclaved at 121°C for 15 min) and centrifuged at 1 000 rpm for 20 min. The supernatant serum was again decanted into clean sterile centrifuge tubes and again centrifuged at 1 000 rpm for 20 min.

(4) The serum was preserved by adding 0,1% sodium azide and storing in a refrigerator (Garvey *et al.*,1977).

3.2.2 Precipitation of the immunoglobulins

All the work was carried out at 4°C.

The serum volume was determined using a measuring cylinder. Polyethyleneglycol 6000 (12% v/v) was added to the antiserum prepared in paragraph 3.2.1. The suspension was centrifuged at 2 000 rpm for 20 min and the supernatant decanted and the precipitate was dissolved in 0,01 M phosphate buffer (pH 7,2). This was repeated twice. The precipitate was then dissolved in PBS a third of the original serum volume.

3.2.3 Preparation of the immunoglobulins for conjugation

During this experimental procedure, fluorescein isothiocyanate (FITC) was used as a fluorochrome for labelling the antibodies. In order to calculate the quantity of FITC to be added to the immunoglobulin for conjugation it was necessary to determine the protein concentration of the immunoglobulin (Paragraph 3.2.3), the total protein and the volume of conjugate to have a protein concentration of 10 mg cm^{-3} (Paragraph 3.2.3).

(a) Determination of the immunoglobulin protein concentration and total protein using spectrophotometry (Porter, 1955 as cited by Walker *et al.*, 1971)

(1) The absorbancy of the immunoglobulin (paragraph 3.2.2), was determined at 280 nm and 260 nm.

(2) Protein concentration (mg cm^{-3}) = (1,45 x absorbancy at 280 nm – 0,74 x absorbancy at 260 mm) x dilution.

(3) Total protein = protein concentration (mg cm^{-3}) x volume of the immunoglobulin.

(b) Determination of the total volume to have a protein concentration of 10 mg cm^{-3}

(1) Total volume = mg total protein 10 mg cm^{-3}.

(2) A carbonate buffer plus FITC (paragraph 3.2.3) was added to the immunoglobulin to obtain a 10 mg cm^{-3} protein concentration. (The volume carbonate buffer added = total volume for a protein concentration of 10 mg cm^{-3}/10).

(3) The final mixture therefore consisted of :
The original serum volume + the volume of carbonate buffer + PBS. The volume of the PBS = total volume necessary for a protein concentration of 10 mg cm^{-3} – (serum volume + carbonate buffer).

3.2.4 Conjugation of immunoglobulins with FITC

(a) Labelling antisera with FITC

The quantity of FITC to be added to the immunoglobulin = total protein (paragraph 3.2.3(a) 3 x 0,05):

(1) The FITC was dissolved in the carbonate buffer (sterile)

(2) The FITC-carbonate solution was carefully added to the immunoglobulin using a sterile Pasteur pipette.

(3) The volume of PBS as calculated in paragraph 3.2.3 was then added to (2) and stirred overnight at 4°C.

(b) Purification of the FITC labelled antisera

(1) Dialysis

Initially, dialysis against PBS was used to purify the fluorescent antibodies (FA) produced using *A. calcoaceticus* (ATCC E23055) and *A. phosphodevorans* (DSM) as antigens. The process of dialysis was not effective for purifying the FA, since cross reactions occurred with *Enterobacter aerogenes* and *E. agglomerans* when tested against these bacteria. Further purification was considered necessary and was done using column chromatography. This eliminated the cross reactions of the FA with *E. aerogenes* and *E. agglomerans.*

(2) Column chromatography

A Sephadex column (paragraph 3.1.7) was used to purify the FA. The FA was individually added to the column and the eluate was collected and used to stain the antigen.

3.2.5 Staining procedure

(a) FA staining

(1) Air dried smears of the bacteria were fixed by gentle heat (60°C) and by submerging the slides into acetone for 10 min.

(2) The smears were then gently washed, using PBS and again allowed to air dry.

(3) The FA was added to the slide, using a sterile inoculation loop. The slides were then placed under an inverted Petri dish cover (to avoid the air-drying of the slides) and incubated at room temperature for 30 min.

(4) The slides were drained free of the FA and gently washed with PBS. Slides were mounted in carbonate buffered glycerol for optimum fluorescence (Pital and Janowitz, 1963).

3.2.6 FA specificity tests

To test for the specificity of the FA it was necessary to obtain bacteria both related and unrelated to the original antigen.

(a) Related bacteria

Related bacteria were, for the purpose of this study, considered as bacterial isolates accumulating phosphorus during the activated sludge process and belonging to the genus *Acinetobacter*. In order to obtain the related bacteria the following procedure was followed :-

One hundred pure cultures were isolated from the aerobic zone of the Northern Works activated sludge plant exhibiting enhanced phosphorus removal. These isolates were obtained by making serial dilutions of water samples collected from the activated sludge plant at the Northern Works. One cm^3 of each dilution was pipetted aseptically into sterile Petri-dishes and *ca* 15 cm^3 GCYA was added. The Petri-dishes were incubated at 20°C for 76 h. All colonies were picked off the Petri-dishes containing between 30 and 300 colonies, which were used for determining the viable plate count in activated sludge.

The colonies were repeatedly streaked out onto GCYA until pure bacterial cultures were obtained.

All the phosphorus-accumulating bacteria in the abovementioned pure cultures were collected. Phosphorus-accumulating bacteria were isolated and identified by transferring all of the abovementioned isolates onto Acid sludge agar, and incubated at 30°C for 48 h ± 2 h. Microscope slides were prepared of the isolates and were then metachromatically stained. Microscopic investigation of the stained samples was used to identify phosphorus-accumulating bacteria.

By using this method, isolates shown to accumulate phosphorus were selected for further study and were identified using the API-20E microtube technique. The experimental procedure was carried out strictly as prescribed by the manufacturers. The Analytical Profile Index was used for the identification of the isolates. (Analytical Profile Index, 1983).

In addition the *Acinetobacter* cultures (paragraph 3.1.3) were used to test the specificity of the FA against related bacteria.

(b) Unrelated bacteria

For the purpose of this study, unrelated bacteria were considered as all the bacterial isolates not belonging to the genus *Acinetobacter*. In order to obtain bacteria unrelated to *Acinetobacter* the following procedure was followed :-

The step described in paragraph 3.2.6(a) was repeated, except that the activated sludge sample was obtained from the primary aerobic zone of the Goudkoppies plant which was at the time exhibiting enhanced phosphorus removal.

All the isolates obtained from Goudkoppies were phenotypically characterised using the API-20E microtube technique (paragraph 3.2.6). From then on, the phenotypic characterisation was used to identify all isolates using the API-20E system for the *Enterobacteriaceae* and other Gram-negative bacteria.

3.2.7 Extension of the FA technique for the identification of all the phosphorus-accumulating Acinetobacter isolated from activated sludge

If the individual FA did not cross react with all the related bacteria it would be necessary to pool the individual FA by mixing the individual FA in a 1 : 1 : 1 ratio.

3.2.8 Method for the enumeration of bacteria in activated sludge

In this study a modification of the procedure described by Schmidt (1974) was used to obtain direct counts of AO and FA reacting bacteria in activated sludge.

(a) Dispersion and declustering of the bacteria in activated sludge

(1) The method of Schmidt (1974)

Ten cm^3 of a fresh activated sludge sample was added to 90 cm^3 sterile distilled water. To this, 0,1 cm^3 of Tween 80 (*ca* 3 drops) and 0,075 cm^3 of an Antifoam (Silicone silcolapse 5001 emulsion) (*ca* 2 drops) were added. The mixture was blended for 5 min using an Ultra-Turrax blender. Microscopic investigation revealed that this procedure dispersed the sludge but did not break up the clusters formed by bacteria.

(2) Declustering using Ansan 6

One cm^3 of activated sludge was suspended in 9 cm^3 of sterile water. Three drops of Ansan 6 was added, and the suspension was shaken vigorously by hand for 2 min.

(3) Dispersion and declustering using a 0,5% Tripolyphosphate solution and sonication.

Five cm^3 of activated sludge were added to 95 cm^3 of a sterile 0,5% Tripolyphosphate solution. This was sonicated for at least 15 minutes at 150 Watt, 20°C using a Bransonic 32. It was sometimes necessary to sonicate for longer periods to obtain complete dispersion and declustering of flocs and activated sludge bacteria. Ten cm^3 of this solution was furthermore diluted to 90 cm^3 of sterile water before staining and filtration.

(b) Flocculation

The dispersed activated sludge was transferred to a sterile 500 cm^3 bottle containing 0,07 g of flocculant mixture (paragraph 3.1.5). The suspension was shaken vigorously by hand for 2 min and allowed to stand for 30 min to permit complete flocculation. This method was not used, due to the loss of bacteria during flocculation.

(c) Filtration

(1) Filters

Black Nuclepore membrafil filters with a pore size of 0,45 μm and 0,20 μm were used. Where pre-stained black Nuclepore filters were not available, white Nuclepore were stained using Irgalan black (Hobbie *et al.*, 1977).

(2) Filtering procedure

Method 1

This method was initially used to determine *Acinetobacter* numbers in activated sludge using black Nuclepore filters with a pore size of 0,45 μm. The Black Nuclepore filter was placed in the filter assembly. One cm^3 of activated sludge treated as described in paragraph 3.2.8(a) 2, was placed in 20 cm^3 sterile water prior to filtration. This ensured an even distribution of the bacteria on the filter surface. The filter was removed from the filter assembly and stained (paragraph 3.2.8(d)1), before microscopic examination (paragraph 3.2.8(e)).

Method 2

This method was used to compare the recovery of bacteria using Nuclepore filters with a pore size of 0,45 μm and 0,20 μm. This method was also used for the enumeration of the total number of bacteria and *Acinetobacter* numbers in activated sludge using Nuclepore filters with a pore size of 0,2 μm.

Where *Acinetobacter* numbers were to be determined, 2 cm^3 of the 5 cm^3 in 95 cm^3 0,5% Tripolyphosphate solution (paragraph 3.2.8(a)3)) were filtered prior to staining (paragraph 3.2.8(d)1)) and microscopic investigation (paragraph 3.2.8(e)).

Where the total number of bacteria were to be determined, 2 cm^3 of the final solution in paragraph 3.2.8(a)3) was stained with AO (paragraph 3.2.8(d)2)) before filtration. The Nuclepore filter was placed in the filter assembly. Two cm^3 of the AO stained sample was filtered (Hobbie *et al.*, 1977). Two cm^3 of a 70% alcohol solution was added to fix the bacteria. A few drops of water were added hereafter to keep the filter wet during microscopic investigation. When the filter is not kept wet, it is difficult to mount the filter on the microscope slide and Hobbie *et al.* (1977) furthermore indicated that the AO stained bacteria should be examined while still moist. The filter was removed from the filter assembly and prepared for microscopic investigation (paragraph 3.2.8(e)).

(d) Staining

(1) FA - staining

The filter was carefully removed and transferred to a Petri dish. The filters were covered with 2% Bovine Serum Albumin (BSA) fraction 5, to control nonspecific adsorption of FA on the filters (Strayer and Tiedje, 1977). The filter was then brought to near dryness at 50 – 60^{o}C.

An area *ca* 10 mm in diameter was marked on the filter. To this area, 0,5 cm^3 of a 1:1 diluted specific pooled FA was added. The filter was incubated under an inverted Petri-dish cover for 30 min and returned to the filter assembly. Excess FA was washed through the filter unit using 100 – 150 cm^3 sterile distilled water.

(2) AO - staining

Two cm^3 of the final activated sludge solution (paragraph 3.2.8(a)3)) was stained with a 0,1% AO solution to give a final AO concentration of 0,01%. It was con-

venient to place 2,0 cm^3 subsample in a small test tube and add 0,2 cm^3 of a 0,1% AO as suggested by Hobbie *et al.* (1977). The AO stained sample was incubated for 1 to 2 minutes before filtering.

(e) Microscopic examination

The filter was removed from the filter assembly, the marked area was cut from the filter using a pair of scissors. Each section was placed on a microscope slide and a drop of mounting fluid (paragraph 3.1.5) was added and covered with a coverslip. Only those cells rating a 3+ and 4+ according to the degree of fluorescence of cells as described by Thomason (1976) were counted.

The method was strictly adhered to since errors in preparation of the sample smears, conjugate and other reagents could lead to unreliable results. Microscopic observation of stained filtered samples were performed with critically aligned and properly functioning equipment.

A Reichert Univar Research Microscope equipped with epifluorescence facilities, was used to study the FA stained samples. The instrument was also equipped with a trimatic camera system and photographs were recorded on 35 mm Agfachrome 50.L film.

The actual number of bacteria cm^{-3} was calculated as described in paragraph 3.3.1.

(f) Samples

Grab samples of *ca* 2 dm^3 each were collected from the different sampling points in each zone from activated sludge plants, and shaken vigorously before sub-sampling.

3.2.9 Preparation of FLA

(a) Preparation of immunoglobulins

Unconjugated immunoglobulin produced in paragraphs 3.2.1 – 3.2.4 was used. The concentration was adjusted to 1,5% (w/v) in Borate buffer. (Johnson and Holborrow, 1978).

(b) Ferritin preparation, conjugation and purification of the conjugate

The methods described by Walker *et al.* (1971) were used to: (1) prepare cadmium free ferritin, (2) conjugate the ferritin and immunoglobulin using toluene 2,4 - di-isocyanate and, (3) purify the conjugate.

(c) Staining for electron microscopy

Activated sludge samples were fixed for 18 h in 0,01 M phosphate (pH 7,5) containing 10% (v/v) formalin. The preparation of mixed liquor precipitates was done using the method of Buchan (1980). The precipitates were washed with saline, and concentrated by centrifugation. Of the concentrate, 0,2 cm^3 was shaken with 0,1 cm^3 of the FLA at 37^{o} for 30 min, centrifuged and washed with PBS. The deposit was fixed for 1 h in 1% (w/v) osmic acid. Further preparation of the samples was done as described in paragraph 3.2.10.

3.2.10 Electron Microscopy

(a) Activated sludge: All samples were fixed using 2% formalin concentration. Mixed liquor precipitates were prepared according to the method of Buchan (1980).

The following preparation schedules were then followed using the precipitates :

(b) The formalin-fixed precipitate was resuspended in a graded series of alcohol (50; 70; 80 and 90%) and left for 5 min in each concentration, centrifuged and resuspended after every exposure to the different concentrations.

(c) Thereafter, the precipitate was resuspended in absolute alcohol, three changes of 10 min each, centrifuging and resuspending the bacteria after each treatment.

(d) The precipitate was then resuspended in a 30% solution of LR White resin in alcohol for 1 h with intermittent swirling and again centrifuged.

(e) The precipitate was again resuspended, this time in a 70% solution of LR White resin in alcohol for 1 h with intermittent swirling and centrifuged.

(f) At this stage the precipitate was exposed to absolute resin for 18 h. This process was repeated and after transferring the samples to Beem capsules, curing or polymerisation of the resin took place in an oven at $55^{o}C$ for 18 h.

(g) Ultra-thin sections (0,5 μm) of the prepared samples were made using a Reichert Jung microtome fitted with a diamond blade after removal of the Beem capsules.

(h) Thin sections were studied using an Hitachi H600 Electron microscope.

3.2.11 EM combined with the EDAX

(a) Thin sections were prepared as described in paragraph 3.2.10.

(b) The EDAX technique described by Buchan (1980) was used.

3.2.12 Determination of the enterobacterial and other Gram-negative bacterial population structure of activated sludge

(a) Cell treatment for declustering

The following method for declumping bacteria in activated sludge was followed (Lötter and Murphy, 1983).

(1) Forty cm^3 of activated sludge was centrifuged at 20 000 rpm for 10 min.

(2) The clear supernatant was decanted and the pellet resuspended in 40 cm^3 of an 0,5 percent Tripolyphosphate solution and sonicated at 20°C for 15 min at 150 Watt.

(b) Isolation of, phenotypic characterisation and identification of bacteria

The same procedure as described in paragraph 3.2.6(a) was followed, except that the activated sludge samples were obtained from the anaerobic, primary anoxic and primary aerobic zone of the Northern Works activated sludge plant and dispersed using the technique in paragraph 3.2.12(a).

3.2.13 Isolation of phosphate rich Acinetobacter using density gradient centrifugation, metachromatic staining and the FA technique

(a) Preparation of samples

(1) Control

A pure culture of *Acinetobacter calcoaceticus* var *lwoffii* was used as a control The *A. calcoaceticus* var *lwoffii* was cultured in GCYA at 37°C for 48 h. The cell suspension was washed in saline twice before density gradient centrifugation.

(2) Activated sludge

Mixed liquor samples from the aerobic zone of a phosphorus-removing activated sludge plant was used for the purpose of this study. The bacteria were dispersed as described in 3.2.8(a)3 and washed in saline before density gradient centrifugation.

(b) Density gradient centrifugation

Initially a Percoll gradient (Pharmacia Fine Chemicals) ranging in buoyant density from 1,017 g cm^{-3} to 1,143 g cm^{-3} was used. Initially 5 cm^3 of activated sludge and 5 cm^3 of Perçoll was used. However after gradient centrifugation of five activated sludge samples, it became apparent that all the bacteria accumulated in one gradient fraction. The range of the gradient was therefore extended by using 3 cm^3 Percoll and 7 cm^3 activated sludge. This resulted in the improved fractionation of the activated sludge. The Percoll gradient was prepared and calibrated strictly according to the manufacturers' instructions. (Pharmacia Fine Chemicals).

Density gradient centrifugation was done using the calibrated Percoll, the control in Percoll and the activated sludge samples in Percoll at 20 000 g for 15 min.

The gradient fractions of the control and activated sludge was carefully removed from the centrifuge tubes using a sterile 10 cm^3 syringe. One cm^3 fractions were collected starting at the top of the test tube.

(c) Enumeration of volutin containing bacteria in the different density gradient fractions

Activated sludge fractions as prepared in paragraph 3.2.13(b) were dispersed as described in paragraph 3.2.8(a)3), but using 1 cm^3 of sample in 9 cm^3 of 0,5% Tripolyphosphate. Of this dispersed sample 0,005 cm^3 was spread out over a surface area of 3,8 cm^2, pre-marked on a microscope slide using a circular coverslip with a diameter of 22 mm and a water fast marker pen. The smear was allowed to air dry before gentle heat fixation. Hereafter the smear was metachromatically stained (paragraph 3.1.5(f)(1), mounted and all the bacteria containing volutin enumerated. The number of fields to be counted as well as the calculation of the bacterial number was done as described in paragraph 3.3.1 except that the surface area over which the sample was spread was used instead of the effective filtering area.

(d) Enumeration of the total number of bacteria and Acinetobacter numbers in the different density gradient fractions

Activated sludge fractions (paragraph 3.2.13(b)) were dispersed (paragraph 3.2.8(a)3) using 1 cm^3 of sample in 9 cm^3 of 0,5% Tripolyphosphate. The total number of bacteria was hence determined using the procedure described in paragraph 3.2.8(d)(2) and (e). The *Acinetobacter* number was determined as described in paragraph 3.2.8(d)(1) and (e).

3.2.14 Comparative analysis of the total viable plate count, total AO bacterial and FA Acinetobacter count using 0,45 µm and 0,20 µm membrane filters

(a) Introduction

Four grab samples from the same sampling point were collected. All four samples were dispersed using the technique described in paragraph 3.2.8(a)3). These dispersed samples were used for further study.

(b) Total viable plate count (TPC)

The TPC was determined by making serial dilutions of the dispersed samples in paragraph 3.2.14(a). One cm^3 of each dilution was added to Petri dishes to which *ca* 15 cm^3 of GCYA was added. The plates were incubated at 20°C for 5 days. All plates containing between 30 and 300 colonies were counted to determine the total viable plate count.

(c) AO and FA enumeration techniques

The same techniques described in paragraph 3.2.8 were used for determining the total AO bacterial count and FA *Acinetobacter* count.

3.2.15 Phosphorus analysis

Standard procedures as described in the Standard Methods for the Examination of Water and Wastewater were used (Orland, 1965).

3.3 Calculations

3.3.1 Calculation of the bacterial numbers using the FA and AO technique

The equation of Schmidt (1974) was used to calculate the number of bacteria :

Number of bacteria cm^{-3} = (Nf x A x D)/aV

Nf = average number of bacteria per microscope field

A = effective filtering area (15,89 cm^2)

D = dilution factor

a = area of microscope field (1,54 x 10^{-4} cm^2)

V = volume of sample passed through filter (cm^3)

The number of microscope fields to be counted was determined as follows :-

x_1 = number of bacteria as observed for 1 microscope field

x_2 = average number of bacteria calculated for 2 microscope fields

x_3 = average number of bacteria calculated for 3 microscope fields

x_n = average number of bacteria calculated for a number (n) of microscope fields (n = the stage when x_1, x_2 and x_3 etc. is within a deviation of 1 bacterium per microscope field.)

3.3.2 Analysis of variance (Steel and Torrie, 1960)

Step 1: The data were arranged as in Table 5. The treatment totals (ΣXi), block totals ($\Sigma X.j$) and grand total ΣX were determined. The ΣX^2 for each treatment and block ($\sum_j X^2$ ij for each i and ΣX^2 ij for each j) was calculated. (Table 5).

Step 2: The adjusted sums of squares were determined as follows :

Correction term (C) = $\frac{X^2}{rt}$ ..... (i)

Total Sum of Squares (SS) = $\sum_{i,j} X^2 ij - C$ ..... (ii)

Block SS $\frac{\sum_j X^2 j}{t} - C$ ..... (iii)

Treatment SS = $\frac{\sum_i X^2 i}{r} - C$ ..... (iv)

Error SS = total SS – block SS – treatment SS ..... (v)

The analysis of variance was calculated using the values obtained in Step 2 and is illustrated in Table 6.

3.3.3 Duncan's new multiple range test for determining the least significant difference

Step 1: The following was determined :

$S_x = \sqrt{(\text{error mean square})/r}$

Step 2: The means were ranked starting at the lowest mean (ie. secondary aeration).

Step 3: The differences amongst the zones were tested by subtraction in the following order. (Steel and Torrie, 1960) : largest minus smallest, largest minus second smallest, ..., largest minus second largest, then second largest minus smallest, second largest minus second smallest etc., to second smallest minus smallest. Each difference exceeding the corresponding least significant range (LSR) was considered to be significant at the 5% level.

3.3.4 Common statistical calculations

(a) Sample mean determination :

$\bar{x} = \frac{\text{sum total of all treatments}}{\text{number of treatments}}$

(b) Standard deviation

The standard deviation was determined using a Sharp EL–506H desk top calculator pre-programmed for standard deviation analysis.

(c) Coefficient of variation

$$\text{Coefficient of variation} = \frac{\text{standard deviation}}{\bar{x}} \times 100$$

(d) Correlation coefficient

The correlation coefficient was calculated using a Hewlett Packard 12C desk top calculator, pre-programmed for calculating correlation coefficient.

3.3.5 Calculation of the cell volume of Acinetobacter

Measurements were made of the *Acinetobacter* cells, directly from electron-micrographs. It was reported, that *Acinetobacter* are round or rod shaped.

(a) Volume for round Acinetobacter cells

For round cells the *Acinetobacter* volume =

$$\frac{4}{3} \pi r^3 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad \text{(i)}$$

r = radius

(b) Volume for elongated Acinetobacter cells

For elongated cells of *Acinetobacter,* the volume was calculated as follows : See Figure 3.

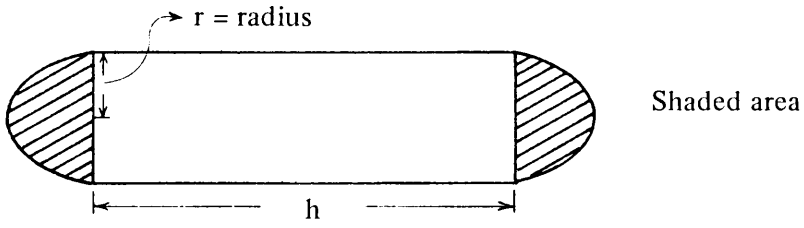


Figure 3: Schematic representation of an elongated Acinetobacter cell

For Figure 3, the volume of the shaded area was calculated using the following equation :

$$\frac{4}{3}\ \pi\ r^3 \quad \text{.....} \quad \text{(ii)}$$

For Figure 3, the volume of the unshaded (cylinder) area, the volume was calculated using the following equation :

$$\pi\ r^2\ h \quad \text{.....} \quad \text{(iii)}$$

$$\therefore \text{The total cell volume} = \frac{4}{3}\ \pi\ r^3 + \pi\ r^2\ h \quad \text{.....} \quad \text{(iv)}$$

3.3.6 Calculation of the volutin volume inside Acinetobacter

The volutin granule inside the *Acinetobacter* was usually spherical and calculated using equation (i) paragraph 3.3.6. Equation (iv) paragraph 3.3.6 was used wherever a rod-shaped volutin granule was encountered.

3.3.7 Calculation of the total cell volume of Acinetobacter in activated sludge

The total *Acinetobacter* cell volume = ($\bar{x}$ total *Acinetobacter* number) X ($\bar{x}$ cell volume).

3.3.8 Calculation of the total volutin volume in activated sludge

The total volutin volume = ($\bar{x}$ total *Acinetobacter* number) X ($\bar{x}$ volutin volume).

3.3.9 Calculation of the total volutin mass

Total volutin mass = ($\bar{x}$ total volutin volume) X (specific gravity of the volutin) where the specific gravity of the volutin = 1,23 g cm^{-3} (Friedberg and Avigad, 1968).

3.3.10 Calculation of the total phosphorus mass

Total phosphorus mass = (Total volutin mass) X (estimated phosphorus content of the volutin) where the estimated phosphorus content of the volutin = 27% (Friedberg and Avigad, 1968; Buchan, 1980).

3.3.11 Calculation of the maximum and minimum Acinetobacter numbers, cell volume, volutin volume, total cell volume, total volutin volume, total volutin mass and total phosphorus mass

(a) Calculation of the maximum and minimum numbers of Acinetobacter

The maximum number of *Acinetobacter* = maximum number of *Acinetobacter* observed ..... (i)

The minimum number of *Acinetobacter* = minimum number of *Acinetobacter* observed ..... (ii)

(b) Calculation of the maximum and minimum total cell volume

Maximum total cell volume = (i) X ($\bar{x}$ cell volume)..... (iii)

Minimum total cell volume = (ii) X ($\bar{x}$ cell volume)..... (iv)

(c) Calculation of the maximum and minimum total volutin volume

Maximum total volutin volume = (i) X ($\bar{x}$ volutin volume)... (v)

Minimum total volutin volume = (ii) X($\bar{x}$ volutin volume)..... (vi)

(d) Calculation of the maximum and minimum total volutin mass

Maximum total volutin mass = (i) x 3.3.9 ..... (vii)

Minimum total volutin mass = (ii) x 3.3.9 ..... (viii)

(e) Calculation of the maximum and minimum phosphorus mass

Maximum phosphorus mass = (vii) x percentage of volutin containing phosphorus (ie 27%) ..... (ix)

Minimum phosphorus mass = (viii) x percentage of volutin containing phosphorus (ie 27%) ..... (x)

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Experiments to develop immunofluorescent techniques for the identification and enumeration of *Acinetobacter* in activated sludge

In order to identify and enumerate *Acinetobacter* in activated sludge, immunofluorescent techniques were used.

Factors influencing the application of the FA technique for the quantification of *Acinetobacter* were :

- Specificity of the antisera
- suspended colloids in mixed liquor samples of activated sludge and numbers of organisms being filtered
- membrane filter pore size
- intensity of fluorescence
- cluster-forming bacteria
- number of fields to be counted

4.1.1 Specificity of the antisera

The results in Table 2 list the tube agglutination titres of the antisera as prepared in paragraph 3.2.1.

Table 2: Tube agglutination titres of antisera and fluorescence with homologous antigens

Antigen	Agglutination titre	Immunofluorescence*
A.calcoaceticus var *lwoffii*	320	4+
A.calcoaceticus (ATCC E23055)	660	4+
A.phosphodevorans (DSM)	660	4+

*According to the intensity of fluorescence as measured by Thomason (1976)

The unconjugated antisera gave high agglutination titres. The results obtained with each conjugated serum furthermore demonstrated excellent immunofluorescent reactions with its homologous antigen (Table 2).

It was indicated that the titre of the antibody might influence the specificity of the FA-antigen reaction (Carter and Leise, 1958; Hill and Gray, 1967). Immunofluorescent staining must be selective if it is to be used to detect a particular organism in activated sludge.

Possible cross-reactions with other bacteria occurring in activated sludge, due to high antisera titres, suggested that it would be essential to test the specificity of the antisera to related and unrelated bacteria. This would lead to the elimination of cross-reactions and the possible misinterpretation of the significance of *Acinetobacter* in phosphorus removal by activated sludge.

All *Acinetobacter* isolates from the Northern Works and Goudkoppies activated sludge plants and five authentic *Acinetobacter* isolates (four from ATCC and one from DSM) were regarded as related bacteria (34 isolates in total).

To determine the specificity, the reaction of the FA with related isolates were tested and these results are presented in Table 3.

The *A. calcoaceticus* var *lwoffii* isolated by Buchan cross-reacted with isolates No's 25; 28; 36; 47; 56; 72; 73; 87; 88 and 92 (Table 3). *A. calcoaceticus* (ATCC E23055) cross-reacted with all the isolates excepting isolate No's 28 and 92. *A. phosphodevorans* (DSM) cross-reacted with all the isolates except Nos 28; 29; 56; 73 and 87. From these results it was calculated that *A. calcoaceticus* var *lwoffii* cross-reacted with only 29% of the related bacteria, *A. calcoaceticus* (ATCC E23055) with 94% of all the related isolates and *A. phosphodevorans* with 85% of the isolates. These results indicated that the low titre antisera (that produced from *A. calcoaceticus* var *lwoffii*) was more specific than the high titre antisera produced using the ATCC and DSM *Acinetobacter* isolates. This supported the findings of Carter and Leise (1958) who indicated that low titre antibody was more specific than high titre antibody.

None of the antisera produced could be used individually to enumerate all *Acinetobacter* in activated sludge because none of the antisera cross reacted with all the *Acinetobacter* isolates (Table 3). However, by pooling the antisera, cross-reactions could be produced to all the *Acinetobacter* listed in Table 3. By using the pooled antisera in this study, it was ensured that all the phosphorus-accumulating bacteria in activated sludge belonging to the *Acinetobacter* group of bacteria were identified.

Table 3: Immunofluorescent specificity tests of antisera with related bacterial isolates

ISOLATE NUMBER	ISOLATE IDENTIFICATION	SOURCE	Immunofluorescent Reactions			
			A. calcoaceticus var *lwoffii* (Buchan)	*A. calcoaceticus* (ATCC E23055)	*A. phosphodevorans* (DSM)	Pooled Antisera
2, 11, 21, 89	*A. calcoaceticus* var *lwoffii*	Northern Wks A/S	–	+	+	+
25, 36, 47, 72, 88	" " " "	" " "	+	+	+	+
27, 89	" " " "	" " "	–	+	+	+
28	" " " "	" " "	+	–	–	+
29	" " " "	" " "	–	+	–	+
32	" " " "	" " "	–	+	+	+
49	" " " "	" " "	–	+	+	+
56	" " " "	" " "	+	+	–	+
64, 68	" " " "	" " "	–	+	+	+
73	" " " "	" " "	+	+	–	+
87	" " " "	" " "	+	+	–	+
92	" " " "	" " "	+	–	+	+
10, 11, 24, 31 36, 62, 63, 65,	*A. calcoaceticus* var *lwoffii*	Goudkoppies A/S	–	+	+	+
17, 19, 21, 28, 47, 52, 53, 32,	" " " "	" "	–	+	+	+
	A. calcoaceticus var *lwoffii* (2 isolates)	ATCC 17925	–	+	+	+
	A. calcoaceticus (2 isolates)	ATCC 23055	–	+	+	+
	A. phosphodevorans	DSM	–	+	+	+

A/S = Activated sludge – = Negative cross-reaction + = Postive cross-reaction

Due to the high titre of the antisera produced by using the *A. calcoaceticus* (ATCC) and *A. phosphodevorans* (DSM), the possibility existed that the antisera might cross-react with organisms in activated sludge other than *Acinetobacter*. This would lead to the misinterpretation of the results, since bacteria other than *Acinetobacter* would also be enumerated. Therefore, it was essential to test all the antisera produced in this study for cross-reactions with unrelated bacteria isolated from activated sludge.

Initially dialysis was used to purify the FA produced using *A. calcoaceticus* (ATCC E23055) and *A. phosphodevorans* (DSM) as antigens. On testing the immunofluorescent reactions of this FA with unrelated bacteria, it was found that the FA cross-reacted with *Enterobacter aerogenes* and *E. agglomerans*. In an attempt to eliminate these cross-reactions, the FA was purified using column chromatography. This resulted in the elimination of the cross-reactions and it was hence concluded that column chromatography was more effective for purifying conjugated antisera. The reactions of the FA used in this study with unrelated bacteria are presented in Table 4.

None of the antisera cross-reacted with any of the unrelated bacterial isolates. The cross-reactions between *A. calcoaceticus* (ATCC E23055) and *A. phosphodevorans*(DSM) and *Citrobacter freundii, Shigella dysenteriae,* CDC Group VEI and *Klebsiella ozaenae* were not separately tested. Cross-reactions with the pooled antisera and abovementioned bacteria were however tested, without any positive cross-reactions occurring.

The reactions of the individual and pooled antisera were thus considered to be specific enough for the purpose of this study. It was therefore not necessary to isolate specific globulins to make the antisera more specific.

Pooled antisera used in this study ensured that all the *Acinetobacter* isolates obtained from activated sludge were detected. Initially only reactions of the antisera with pure bacterial cultures, either related or unrelated, were considered. However, activated sludge preparations treated with FA should retain the labelled antisera at sites corresponding only to the distribution of the antigen used to prepare the FA. This would enable the enumeration of these antigens in activated sludge.

Table 4: Immunofluorescent specificity tests of three individual and pooled antisera with unrelated bacterial isolates

ISOLATE NUMBER	IDENTIFICATION	SOURCE	Immunofluorescent Reaction			
			A. calcoaceticus var *lwoffii* (Buchan)	*A. calcoaceticus* (ATCC E23055)	*A. phosphodevorans* (DSM)	Pooled Antisra
1, 4, 5, 33, 46, 48, 55, 67, 69, 70, 72, 74, 75, 78, 81, 85, 88, 94	*Aeromonas hydrophila*	Activated sludge	–	–	–	–
3	*Enterobacter aerogenes*	Activated sludge	–	–	–	–
30, 43	*Enterobacter agglomerans*	Activated sludge	–	–	–	–
34, 45, 77, 87, 96	*Enterobacter cloacae*	Activated sludge	–	–	–	–
6	*Pseudomonas maltophila*	Activated sludge	–	–	–	–
37, 49, 91	*Pseudomonas* sp	Activated sludge	–	–	–	–
57	*Pseudomonas stutzeri*	Activated sludge	–	–	–	–
7	*Escherichia coli*	Activated sludge	–	–	–	–
8, 22, 29 90	*Flavobacterium*	Activated sludge	–	–	–	–
18	*Citrobacter freundii*	Activated sludge	–	ND	ND	–
25	*Shigella dysenteriae*	Activated sludge	–	ND	ND	–
71, 99	*Pasteurella* sp	Activated sludge	–	–	–	–
83	Presumptive *Brucella*	Activated sludge	–	–	–	–
97	CDC Group VEI	Activated sludge	–	ND	ND	–
98	*Klebsiella ozaenae*	Activated sludge	–	ND	ND	–

ND = Not determined

4.1.2 Dispersion of bacteria adsorbed to colloids

The activated sludge bacteria adsorbed to suspended colloids made it difficult to identify the *Acinetobacter* to be enumerated using the pooled FA (Plate 1). This problem was also experienced by Eren and Pramer (1965). Adsorption of bacteria to suspended colloids also made it difficult to enumerate the bacteria stained with AO.

Three methods were investigated to disperse the sludge and break the clusters formed by certain bacteria. The method of Schmidt (1974) was found to be useful for dispersing the sludge, but did not break up the clusters formed by bacteria (Plate 2). The second method, using Ansan 6 as dispersant, resulted in the effective dispersion of the sludge as well as the disperson of clusters formed by *Acinetobacter* (Plate 3).

However, the Ansan 6 method of dispersion (paragraph 3.2.8(a)2) only proved to be useful when small volumes and low dilutions of activated sludge were used. (1,0 cm^3 activated sludge in 9 cm^3 of sterile water). The use of Ansan 6 in the larger volume (5 cm^3 activated sludge in 95 cm^3) sterile water did not prove to be successful, for unkown reasons.

In order to obtain a more representative sample of the activated sludge, 5 cm^3 were used in 95 cm^3 of sterile 0,5% Tripolyphosphate. Effective dispersion of the latter could only be obtained after sonification for at least 15 minutes at 150 watt and 20^{o}C.

A problem in enumerating fluorescent-stained bacteria was that the microscope field viewed was an extremely small area (1,54 x 10^{-4} cm^2). Consequently, only a limited number of colloids could be tolerated in each field due to the interference of these colloids with the fluorescent staining technique. Furthermore, microbial populations had to be high in number in order to encounter a reasonable number of cells per microscope field. Initially during this study a volume of 1 cm^3 of a 10x dilution of activated sludge in sterile distilled water was filtered. By using this volume a minimum of 1,03 x 10^6 bacteria cm^{-3} had to be present to detect one bacteria per microscope field. Where soil micro-organisms were studied (Schmidt, 1974), it was necessary to filter 0,1 cm^3 of a 10 times dilution of soil in water, in order to overcome the colloid problem. By using this volume, ten times more bacteria (1,03 x 10^7 bacteria cm^{-3}) had to be encountered, than in the case with activated sludge, in order to detect one bacteria per microscope field.

However, more than 100 organisms per microscope field made counting difficult (Plate 3). Hobbie *et al.* (1977) found that 2 cm^3 was a sufficient volume to obtain an even distribution of bacteria on the filter surface upon filtration. At a later stage during this study 2 cm^3 of a 20 x (in the case of *Acinetobacter* counts) and 200 x (in the case of total counts) dilution of activated sludge was used. Consequently an even distribution of between 40 and 100 organisms per microscope field was obtained.

The ratio of bacterial cells to colloids was found to be large and it was therefore not necessary to remove colloids out of suspension after separating them from the bacteria (Plate 3). The choice of filters was also important in developing a successful enumeration technique.

4.1.3 The role of membrane pore size in the enumeration technique

A comparison was carried out on activated sludge samples, dispersing the bacteria using sonication and a 0,5% Tripolyphosphate solution and filtering through Nuclepore filters with pore sizes 0,45 μm and 0,2 μm respectively. The results are represented in Table 5.

Table 5: Total counts of bacteria retained on Nuclepore filters with two different pore sizes[1]

Sample Number	Nuclepore filter pore size	
	0,45 μm	0,20 μm
1	1,7 x 10^8 bacteria cm^{-3}	8,0 x 10^8 bacteria cm^{-3}
2	1,6 x 10^8 bacteria cm^{-3}	8,2 x 10^8 bacteria cm^{-3}
3	2,2 x 10^8 bacteria cm^{-3}	6,5 x 10^8 bacteria cm^{-3}
4	1,6 x 10^8 bacteria cm^{-3}	6,7 x 10^8 bacteria cm^{-3}
5	1,7 x 10^8 bacteria cm^{-3}	7,6 x 10^8 bacteria cm^{-3}
$\bar{x}$	1,7 x 10^8 bacteria cm^{-3}	7,4 x 10^8 bacteria cm^{-3}

1) The AO technique combined with sonication and dispersion in a 0,5 Tripolyphosphate solution was used

An average 22,9% of the bacteria retained by the 0,20 μm filter were retained by the 0,45 μm filter (Table 5). This substantiates the findings of Hobbie *et al.* (1977) who found that the size of bacteria varies from water mass to water mass. Hobbie *et al.* (1977) found

that there was no fixed relationship between very small and very large bacteria and that higher numbers (99%) of the bacteria studied in a lake were retained on filters with a pore size of 0,20 μm, than on filters with a pore size of 0,40 μm which retained only 56% of the bacteria from the same system.

Buchan (1980), using electron micrographs concluded that the volutin-containing bacteria in activated sludge were much larger than the other bacteria. This study concentrated on the role of *Acinetobacter* in phosphorus removal and due to the larger size of the volutin containing bacteria (presumed to be *Acinetobacter*) filters with a pore size of 0,45 μm were initially used for the enumeration of *Acinetobacter*. However, at a later stage 0,20 μm filters were also used to enumerate *Acinetobacter* in activated sludge.

4.1.4 The effect of fluorescence intensity on the enumeration technique

It was essential to have a good contrast between the FA-stained bacteria and the Nuclepore filter background. Although mounting fluid with a pH of 9 was used to enhance fluorescence, the intensity of fluorescence varied. In order to obtain reproducible results, it was decided to count only bacteria exhibiting a 4+ and 3+ intensity (Plate 3). A 2+ fluorescence (Plate 2) together with physical or morphological characteristics were not used in this study as this would have resulted in inaccurate results due to its subjective nature brought about by individual interpretation of morphological characteristics.

A 1:1 dilution of the pooled antisera resulted in less FA available to adsorb to suspended colloids. This made it easier to distinguish the fluorescing cells. Non-specific adsorption of the FA was effectively controlled by pre-staining with 2% BSA as was found by Strayer and Tiedje (1977).

4.1.5 Declustering of cluster-forming bacteria

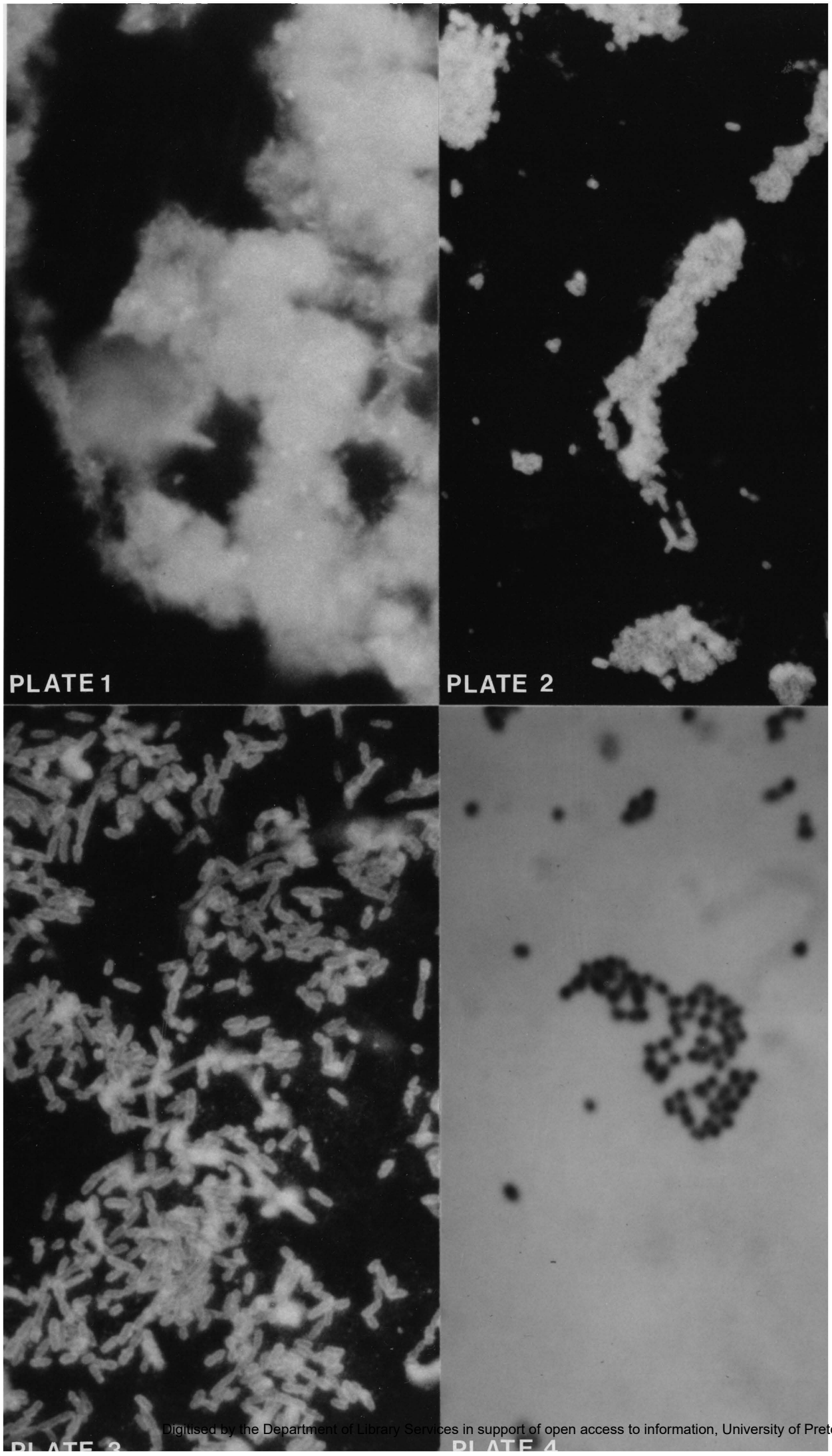
Acinetobacter formed clusters in activated sludge (Plate 2 also see Appendix VI) (also observed by Buchan, 1980). This rendered the direct enumeration of these bacteria impossible. The cluster-formation of *Acinetobacter* was ascribed to the extracellular production of an emulsifying agent (anionic heteropolysaccharide containing fatty acid side chains) (Kaplan and Rosenberg, 1982). The use of Ansan 6 resulted in the breaking up of *Acinetobacter* clusters and the dispersion of bacteria adhering to colloids in suspension (Plate 3). Individual bacteria could hence be identified and enumerated. The use of sonication of activated sludge diluted in a 0,5% Tripolyphosphate solution also resulted in the dispersion of bacteria adhering to colloids and dispersion of bacterial clusters (Appendix VI).

PLATE 1 Non-specific adsorption of FA to colloids in activated sludge and masking of specific FA stained antigens. X2000

PLATE 2 *Acinetobacter* clusters, stained with FA and separated from colloids in activated sludge by dispersion with Tween 80. X2200 (Arrow indicates 2+ fluorescense).

PLATE 3 FA stained *Acinetobacter* clusters, after dispersion with Ansan 6. X2500

PLATE 4 Metachromatic stain of the fraction containing numerous *Acinetobacter* after gradient centrifugation of activated sludge. X3500



This agreed with the findings of Banks and Walker (1977). An input energy of 150 Watts was used in this study. Using this energy the activated sludge had to be sonicated for at least 15 min to obtain complete dispersion. The Bransonic 32 sonicator used in this study unfortunately only had one setting and could therefore not be standardized to the energy output used by Banks and Walker (1977) and therefore a direct comparison of the two methods could not be made.

4.1.6 Number of microscope fields to be counted

During this study the number of fields to be counted was calculated as described in paragraph 3.3.1. This ensured that an adequate number of microscope fields were counted to provide reliable data. By overcoming the problems initially encountered, an FA enumeration technique was developed successfully for the enumeration of *Acinetobacter* in activated sludge. Not less than 10 fields were ever counted.

4.2 Experiments to determine the *Acinetobacter* numbers in activated sludge

During this study, three methods were used to determine the *Acinetobacter* numbers in activated sludge.

4.2.1 The FA technique

Two different methods were used. In method one Ansan 6 and shaking by hand was used to decluster the bacteria before filtration through a black Nuclepore filter with pore size 0,45 μm. The results obtained using this technique are presented in Table 6.

In method 2 black Nuclepore filters with a pore size of 0,20 μm were used. A 0,5% Tripolyphosphate solution and sonication was used for declustering the activated sludge bacteria. The results are presented in Table 7.

(a) Analysis of variance

The analysis of variance of results for method 1 contained in Table 8 indicated that the calculated F for the blocks exceeded 1% of Tabular F at the 0,05 level. This indicated a significant difference amongst the block means at the 0,05 level. However the calculated F for the blocks was less than the Tabular F at the 0,01 level, indicating no significant difference at this level (Table 8).

Table 6: The *Acinetobacter* numbers in different activated sludge zones over a period of time, corresponding phosphorus removal at the time of sampling and representation of statistical data[1]

Source of sample	Date (Blocks)[3]	Number of *Acinetobacter* $cm^{-3} \times 10^6$ (Treatment)[2]						Total phosphorus removal mg P dm^{-3}	ΣX.i.	$\Sigma X^2 i.j$ j
		Anaerobic zone	Primary anoxic zone	Primary aerobic zone	Secondary anoxic zone	Secondary aerobic zone	Effluent			
Northern Works	21 March 1983	2,10	1,00	7,50	6,80	2,20	1,00	11,30	20,60	$113,74 \times 10^{12}$
Northern Works	25 March 1983	2,30	4,50	5,00	6,60	2,00	1,00	9,40	21,40	$99,10 \times 10^{12}$
Northern Works	21 April 1983	3,00	1,00	6,40	5,70	1,10	1,00	3,50	18,20	$85,66 \times 10^{12}$
Northern Works	17 May 1983	1,00	2,10	4,30	4,30	1,00	1,00	6,50	13,70	$44,39 \times 10^{12}$
Northern Works	19 May 1983	2,70	2,70	6,40	1,00	1,60	1,60	11,30	16,00	$61,66 \times 10^{12}$
Northern Works	24 May 1983	2,10	3,70	7,50	8,40	2,10	3,80	6,80	27,60	$163,76 \times 10^{12}$
Northern Works	26 May 1983	2,70	4,30	7,00	6,90	1,00	1,00	7,00	22,90	$124,39 \times 10^{12}$
Northern Works	24 June 1983	2,10	3,20	7,60	7,60	2,10	6,90	7,90	29,50	$182,19 \times 10^{12}$
Benoni Works	29 September 1983	4,70	1,20	1,40	2,60	3,30	ND	15,70		
ΣX.j.		18,00	22,50	51,70	47,30	13,10	17,30		169,90	
$\bar{x}$		2,25	2,81	6,46	5,91	1,64	2,16	7,96		
s		0,60	1,36	1,22	2,33	0,53	2,14			
Coefficient of variation		26,66	48,39	18,88	39,42	32,31	99,07			
$\Sigma X^2 ij$ i		$43,10 \times 10^{12}$	$76,37 \times 10^{12}$	$344,67 \times 10^{12}$	$317,71 \times 10^{12}$	$23,43 \times 10^{12}$	$69,61 \times 10^{12}$			$874,89 \times 10^{12}$

ND = Not determined

NOTE: The *Acinetobacter* numbers as determined for the Benoni works were not used in the statistical calculations

1) Ansan 6 and shaking by hand was used to decluster the activated sludge bacteria before filtration through a black Nuclepore filter with pore size 0,45 μm
2) Treatments = Different activated sludge zones
3) Blocks = Different sampling dates

Table 7: The *Acinetobacter* numbers in different activated sludge zones over a period of time, corresponding phosphorus removal at the time of sampling and representation of statistical data[1]

Source of sample	Date (Blocks)[3]	Number of *Acinetobacter* cm^{-3} x 10^6 (Treatment)[2]						Total phosphorus removal mg P dm^{-3}	ΣX.i.	$\Sigma X^2 i.j.$ j
		Anaerobic zone	Primary anoxic zone	Primary aerobic zone	Secondary anoxic zone	Secondary aerobic zone	Effluent			
Northern Works	10 January 1985	5,88	42,92	5,88	6,70	10,31	11,34	14,0	83,03	$2,19 \times 10^{15}$
Northern Works	11 January 1985	39,62	16,92	20,84	44,36	41,27	15,80	14,5	178,80	$6,21 \times 10^{15}$
Northern Works	14 January 1975	54,10	52,72	54,58	34,66	40,65	22,86	14 3	259,57	$12,06 \times 10^{15}$
Northern Works	15 January 1985	7,53	3,61	16,81	11,45	9,18	5,70	16,0	54,28	$0,60 \times 10^{15}$
Northern Works	16 January 1985	45,18	36,62	37,40	44,98	44,51	13,30	13,2	221,99	$8,96 \times 10^{15}$
ΣX.j.		152,31	152,79	135,51	142,15	145,92	69,00	14,4	797,68	
$\bar{x}$		30,46	30,55	27,10	28,43	29,18	13,80			
s		22,30	19,95	19,08	18,21	17,81	6,28			
Coefficient of variation		73,21	65,32	70,41	64,06	61,03	45,54			
$\Sigma X^2 ij$ i		$6,63 \times 10^{15}$	$6,26 \times 10^{15}$	$5,13 \times 10^{15}$	$5,37 \times 10^{15}$	$5,53 \times 10^{15}$	$1,11 \times 10^{15}$			$30,03 \times 10^{15}$

1) Sonication of activated sludge in a 0,5% Tripolyphosphate solution was used to decluster the bacteria before filtering through a 0,20 μm filter

2) Treatments = Different activated sludge zones

3) Blocks = Different sampling dates

It can therefore be concluded that at the 5% level a significant difference existed amongst *Acinetobacter* numbers at different times, but no significant difference existed at the 1% level. The significant difference at the 5% level is probably due to the fluctuation in the organic feed which varies with time. This would have a direct influence on the microbial population since the activated sludge system is a biological process.

The calculated F for the treatments exceeded 1% of the Tabular F at the 0,05 and 0,01 level, indicating significant differences amongst treatments (Table 8). These results did however not indicate which of the treatments differed significantly. The least significant difference (LSD) using Duncan's new multiple range test (Steel and Torrie, 1960) was used to determine the LSD of the treatments (Table 9).

Table 8: Analysis of variance for data in Table 6

Source of variation	df	SS	MS	Calculated F
Treatment (zones)	5	$1,75 \times 10^{14}$	$3,49 \times 10^{13}$	18,96
Blocks (dates)	7	$0,34 \times 10^{14}$	$4,85 \times 10^{12}$	2,66
Error	35	$0,64 \times 10^{14}$	$1,82 \times 10^{12}$	
Total	47			

Tabular F0,05 and F0,01 for 7 and 35 degrees of freedom = 2,29 and 3,21 (For blocks)
Tabular F0,05 and F0,01 for 5 and 35 degrees of freedom = 2,49 and 3,60 (For treatments)

Table 9: Multiple range values in order to determine the difference amongst the activated sludge zones of the Northern works system at the 5% level of confidence[1]

Zone	Zone compared with	Mean range value
Primary aerobic	primary anoxic	$3,65 \times 10^6$ *
	anaerobic	$4,21 \times 10^6$ *
	secondary aerobic	$4,82 \times 10^6$ *
	effluent	$4,30 \times 10^6$ *
	secondary anoxic	$0,55 \times 10^6$ *
Secondary anoxic	secondary aerobic	$4,27 \times 10^6$ *
	effluent	$3,75 \times 10^6$ *
	primary anoxic	$3,07 \times 10^6$ *
	anaerobic	$3,66 \times 10^6$ *
Primary anoxic	secondary aerobic	$1,17 \times 10^6$
	effluent	$0,65 \times 10^6$
	anaerobic	$0,56 \times 10^6$
Anaerobic	secondary aerobic	$0,62 \times 10^6$
	effluent	$0,09 \times 10^6$
Effluent	secondary aerobic	$0,52 \times 10^6$

1) The values in Table 6 were used for the calculations

* Indicates significant differences

Least significant range (LSR) = $1,36 \times 10^6 - 1,54 \times 10^6$. If the mean range value exceeds the LSR the differences are significant. If the mean range value is smaller than the LSR the differences are not significant.

The *Acinetobacter* numbers in the primary aerobic and secondary anoxic zones did not differ significantly, but were significantly higher than the *Acinetobacter* numbers in the other zones (Table 9). This suggested that the physical size of *Acinetobacter* cells in these two zones (primary aerobic and secondary anoxic zones) were either larger than those in other zones (hence more cells were retained by the 0,45 μm filter) or that more *Acinetobacter* cells were present in these two zones due to the increased activity of *Acinetobacter* under aerobic conditions resulting in the carry-over of these numbers to the secondary anoxic zone and hence the higher numbers in this zone.

The analysis of variance for the data in Table 7 (Table 10) indicated that the calculated F for the treatments (different activated sludge zones) was less than 1% of the tabular F at the 0,05 and 0,01 level.

Table 10: Analysis of variance for data in Table 9

Source of variation	df	SS	MS	Calculated F
Treatments (zones)	5	$1,0 \times 10^5$	$2,0 \times 10^{14}$	1,90
Blocks (date)	4	$5,2 \times 10^5$	$1,3 \times 10^{15}$	12,38
Error	20	$2,1 \times 10^5$	$1,0 \times 10^{14}$	
Total	29			

Tabular F0,05 and F0,01 for 4 and 20 degrees of freedom = 2,87 and 4,43 (F or blocks)
Tabular F0,05 and F0,01 for 5 and 20 degrees of freedom = 2,71 and 4,10 (F or treatments)

These results indicate that there was no significant difference amongst the different activated sludge zones. This was ascribed to the large coefficient of variation (Table 7) which was probably due to the smaller cells which might have passed through the 0,45 μm filter, but were retained on the 0,2 μm filter. Since activated sludge should ideally be operated in the endogenous phase (Wiechers *et al.*, 1984) it can be expected that the relative number of very small bacteria may be high and may fluctuate to a large extent resulting in the large coefficient of variation (Table 7) and no significant difference amongst the zones.

The calculated F for the blocks exceeded the Tabular F at the 0,05 and 0,01 level (Table 10). This indicated that the *Acinetobacter* numbers differed significantly in time. Again this is not surprising since the organic load varies daily consequently affecting the microbial activity and numbers.

4.2.2 Determination of the Acinetobacter numbers in activated sludge using the API-20E system

The total viable plate count was determined in triplicate for the anaerobic, primary anoxic and primary aerobic zone. All colonies from plates poured from a 10^5 dilution were counted, being between 30 and 300 colonies per plate. All colonies from the plate with a viable count closest to the average of the three plates were picked up and purified (paragraph 3.2.6). Biological phosphorus removal in activated sludge has always been associated with Gram-negative bacteria (Roïnestad, 1973; Fuhs and Chen, 1975; Buchan, 1980; Brodisch and Joyner, 1982). Therefore only the Gram-negative isolates were identified using the API-20E system.

The percentage representation of each isolate was calculated by using only those isolates giving an excellent or good identification. These results are represented in Table 11 and were used to enumerate *Acinetobacter* in activated sludge for comparative purposes with the FA technique as previously discussed (Table 12).

Table 11: Distribution of aerobic and facultative anaerobic *Enterobacteriaceae* and other Gram-negative bacteria in different zones of the Northern Works activated sludge system

Bacteria	Activated sludge zone					
	Anaerobic		Primary anoxic		Primary aerobic	
	Number cm^{-3}	%	Number cm^{-3}	%	Number cm^{-3}	%
Acinetobacter calcoaceticus var *lwoffii*	$2,6 \times 10^6$	66,6	$2,9 \times 10^6$	67,4	$5,5 \times 10^6$	76,3
Pseudomonas	$4,0 \times 10^5$	10,1	$4,0 \times 10^5$	9,3	$4,0 \times 10^5$	5,5
Aeromonas hydrophila	$3,0 \times 10^5$	7,6	$6,0 \times 10^5$	13,9	$4,0 \times 10^5$	5,5
Citrobacter freundii	$2,0 \times 10^5$	5,1	–	0	$1,0 \times 10^5$	1,3
CDC – Group 11 K-2	$1,0 \times 10^5$	2,5	–	0	–	0
CDC – Group 11 F	$1,0 \times 10^5$	2,5	–	0	–	0
Shigella	$1,0 \times 10^5$	2,5	–	0	–	0
Klebsiella oxytoca	$1,0 \times 10^5$	2,5	–	0	–	0
Escherichia coli	–	0	$1,0 \times 10^5$	2,3	–	0
Serratia	–	0	$3,0 \times 10^5$	6,9	–	0
Pasteurella	–	0	–	0	$5,0 \times 10^5$	6,9
CDC – Group 1 V	–	0	–	0	$1,0 \times 10^5$	1,3
Flavobacterium	–	0	–	0	$1,0 \times 10^5$	1,3
Yersinia	–	0	–	0	$1,0 \times 10^5$	1,3
Total	$3,9 \times 10^6$		$4,3 \times 10^6$		$7,2 \times 10^6$	

Note: Percentages are expressed as the percentage of the total number of bacteria that could be identified and *not* the total number of bacteria

The viable counts of identifiable Gram negative bacteria were 3,9 x 10^6, 4,3 x 10^6 and 7,2 x 10^6 for the anaerobic -, primary anoxic - and primary aerobic zones respectively. In all three these zones *A. calcoaceticus* var *lwoffii* was found to be dominant (66,6%, 67,4% and 76,3% respectively for the three different zones). *Acinetobacter* was followed by *Pseudomonas* and *Aeromonas* in the anaerobic zone (10,1% and 7,6% respectively) and were equally represented in the primary aerobic zone (5,5% each), whilst *Aeromonas* was more important than *Pseudomonas* in the primary anoxic zone (13,9% *vs.*9,3%). Members of the other genera or groups constituted the less important members of the identifiable Gram-negative isolates. In all three zones, the obligate aerobes were found to be pre-dominant.

Brodisch and Joyner (1982) determined the bacterial population structure of a pilot plant and laboratory activated sludge unit. They found that *Aeromonas, Pseudomonas* and *Alcaligenes* (not always in this order) were the dominant genera in the anaerobic, primary anoxic and primary aerobic zone. This differed from the results obtained in this study where *Acinetobacter* was found to be the dominant organism in these zones (Table 11). However, a common factor was that in both studies relatively high numbers of *Pseudomonas* and *Aeromonas* were encountered.

Buchan (1980) found *Acinetobacter* (with an average representation of 57%) to be the dominant organism in the primary aerobic zone of five different activated sludge plants. In principal, these results correspond with the results obtained in this study. However, the percentage of *Acinetobacter* encountered by Buchan (1980) was lower than in this study (57% *vs.*76%).

The results from the abovementioned studies substantiate the statement of Buchan (1984) that organisms such as *Pseudomonas* and *Acinetobacter* which have a low growth factor requirement would proliferate in activated sludge.

The pre-dominance of *Acinetobacter* in the different activated sludge zones studied in this study was ascribed to :

(i) the low growth factor requirement

(ii) storage of poly-β-hydroxybutyrate as an internal carbon and energy reserve enabling the organism to survive anaerobic and primary anoxic conditions, and

(iii) it has been shown at the Contra Costa Phostrip plant in California that up to 10 hours of anaerobiosis has no effect on respiratory rates when aerobic organisms are exposed to oxygen (Buchan, 1984). Buchan (1984) therefore concluded that anaerobic conditions probably do not adversely affect the aerobic populations established in the primary aerobic zone of activated sludge. The presence of oxygen could thus lead to the proliferation of *Acinetobacter*, which is a strictly aerobic organism, in the primary aerobic zone of activated sludge.

Many explanations may be offered for the differences in results obtained during the different studies. Some possible explanations could be the number of isolates obtained and identified, operational procedures of the various plants, dispersion of bacterial clusters before analysis and the fact that a pilot plant or laboratory scale activated sludge plant was compared with a full-scale plant.

Due to the lack of information in the publication of Brodisch and Joyner (1982), the number of isolates identified could not be calculated and consequently this could not be discussed as a possible explanation for the differences in the results.

Different operational procedures did exist amongst the various systems studied (Brodisch and Joyner, 1982). However, this was also the case in the studies of Buchan (1980). During these studies the different operational procedures did not influence the dominance of *Acinetobacter* (Buchan, 1980). Therefore different operational procedures were not considered a major factor contributing to the differences in the results of Brodisch and Joyner (1982) on the one hand and Buchan (1980) on the other hand and this study.

Due to the numerous variables in activated sludge, this process will be extremely difficult to simulate under laboratory and even pilot plant conditions. The different results obtained when laboratory scale units and pilot plant systems were compared with full scale plants were therefore not surprising.

Another possible contributing factor is the observation that *Acinetobacter* formed large clusters in activated sludge (Roïnestad, 1973; Fuhs and Chen, 1975; and Buchan, 1980) a phenomenon also encountered in this study. Dispersion of *Acinetobacter* and other cluster forming bacteria in activated sludge will result in different viable counts and possibly the percentage representation of these and other bacteria, when the bacterial population is determined. Brodisch and Joyner (1982) and Buchan (1980) did not disperse

any of the activated sludge samples effectively, prior to analysis. In this study, the activated sludge samples were dispersed effectively resulting in the declumping of cluster forming organisms like *Acinetobacter* (Plate 3; Appendix VI).

This resulted in an increased number of colony forming units as was also experienced by Banks and Walker (1977). Instead of having a single colony developing from a cluster of organisms, many colonies would develop. This led to a more accurate estimate of the numbers of bacteria of which the numbers would have been under-estimated if not dispersed as was possibly the case in the study of Brodisch and Joyner (1982).

Clumping of bacterial cells was therefore considered the most important single contributing factor for the different results obtained in the various studies as discussed.

The interpretation of the API-20E microtube results is another important factor which will influence the outcome of the results. During this study, only those Gram-negative isolates giving a good or excellent identification were used for calculations. Often the outcome of an API-20E microtube identification is expressed as a probability e.g. 1/500; 1/1000 etc. If all isolates (also those with a low probability) were to be included in the calculations, the results may differ significantly. Unfortunately Buchan (1980) and Brodisch and Joyner (1982) did not state which isolates were used in their calculations. The interpretation of the API-20E microtube results will not be discussed any further as a possible explanation for the different results.

The dilution from which the colonies are selected for identification and the number of colonies selected will definitely influence the outcome of the API-20E microtube results. As the dilution increases, many of the bacteria occurring in low numbers are diluted out, hence the percentage of the dominant bacteria will increase with increase in dilution. During this study all colonies growing on the 10^5 dilution were selected for determining the bacterial population structure.

4.3 Comparison of counts obtained by using the API-20E technique and the two methods of *Acinetobacter* enumeration using FA

For comparative purposes, the results obtained using the different techniques are listed in Table 12.

Table 12: Comparison of the average *Acinetobacter* numbers as determined using the API-20E technique and the two methods employing the FA technique

Technique	*Acinetobacter* numbers cm^{-3}		
	Anaerobic zone	Primary anoxic zone	Primary aerobic zone
API-20E	$2,60 \times 10^6$	$2,90 \times 10^6$	$5,50 \times 10^6$
Method 1 and FA	$2,20 \times 10^6$	$2,80 \times 10^6$	$6,40 \times 10^6$
Method 2 and FA	$3,00 \times 10^7$	$3,00 \times 10^7$	$2,70 \times 10^7$
S for method 1	$0,60 \times 10^6$	$1,36 \times 10^6$	$1,22 \times 10^6$
S for method 2	$2,23 \times 10^7$	$1,99 \times 10^7$	$1,90 \times 10^7$

S = Standard deviation

Method 1: 0,45 μm Nuclepore filters and Ansan 6 dispersion

Method 2: 0,20 μm Nuclepore filters and 0,5% Tripolyphosphate and sonication dispersion

In the anaerobic, primary anoxic and primary aerobic zone, the *Acinetobacter* numbers determined by using the API-20E method was within the standard deviation of the *Acinetobacter* numbers determined by using the 0,45 μm Nuclepore filters and FA technique (method 1) (Table 12).

However, the *Acinetobacter* numbers as determined using the API-20E technique were lower and not within the standard deviation of the *Acinetobacter* numbers as determined using the 0,20 μm filters and FA (method 2) in the abovementioned zones (Table 12).

The API-20E determination of *Acinetobacter* numbers relies on the viability of the organisms and their capability of growing on GCYA. Since *Acinetobacter* grew on GCYA, viability was the determining factor when determining the *Acinetobacter* numbers using the API-20E technique. The results in Table 12 indicate, that the *Acinetobacter* numbers determined using the API-20E method, were closely related to the determination of the *Acinetobacter* numbers using the 0,45 μm filter and FA technique (method 1) but were not within the standard deviation of the method using 0,20 μm filters and FA (Table 12). This was ascribed to *Acinetobacter* cells being larger when viable and actively growing.

The higher numbers of *Acinetobacter* obtained using a 0,2 μm filter however indicated that many of the *Acinetobacter* cells were small enough to pass through the 0,45 μm filters.

Buchan (1980) stated that the bacteria containing volutin in activated sludge were larger than other bacteria in activated sludge. However, the size of these bacteria were never accurately determined. Since the size of the bacteria could influence the quantity of phosphorus that could be accumulated, it was considered necessary to determine the actual size of the volutin containing bacteria using TEM. The results are reported on page 73 and 74.

4.4 Experiments to determine the total number of bacteria in activated sludge

Due to the lack of suitable nutrient media which would support the growth of all viable nutritional types of bacteria in activated sludge (Banks and Walker, 1977),the AO-staining technique was used in this study for determining the total number of bacteria in activated sludge (paragraph 3.2.8). The results are presented in Table 13. A comparative analysis was also done using the AO technique and the viable plate count technique (Table 17).

Table 13: The total number of bacteria in the Northern Works activated sludge plant as determined by the AO-staining technique

Date	Number of bacteria x 10^8 cm^{-3} in the different activated sludge zones					
	anaerobic zone	primary anoxic zone	primary aerobic zone	secondary anoxic zone	secondary aerobic zone	effluent
10/1/1985	1,92	1,27	1,69	0,95	0,47	2,68
11/1/1985	1,59	6,55	3,35	2,63	2,45	0,41
14/1/1985	4,70	4,73	4,66	5,17	6,42	3,72
15/1/1985	4,47	4,59	5,73	2,53	1,56	2,90
16/1/1985	4,82	7,32	6,64	6,54	7,83	3,92
$\bar{x}$	3,50	4,89	4,41	3,56	3,74	2,72
S	1,60	2,33	1,95	2,24	3,20	1,39
Coefficient of variation	45,70	47,60	44,21	62,92	85,50	51,10

NOTE: The filter pore size was 0,2 μm

When the average *Acinetobacter* numbers determined on the same sample as the total count and using the same technique, except that FA was used instead of AO, it was found that *Acinetobacter* constituted 8,5%, 6,2%, 6,1%, 7,8%, 7,7% and 5,0% of the total count in the respective zones (anaerobic, primary anoxic, primary aerobic, secondary anoxic and secondary aerobic zone) and effluent. (Comparison of the average *Acinetobacter* numbers in Table 7 and the results in Table 13). These percentages were lower than the percentage representation as determined using the API-20E technique (Table 11). This was ascribed to the fact that the API-20E technique relies on the viability of the activated sludge organisms whereas by using the FA technique dead and live cells are counted and in Table 11 the comparison was not done with the total count, but with the count of identifiable bacteria.

It was probable that the GCYA favoured the growth of *Acinetobacter* giving it an unfair advantage over other bacteria and hence the high percentage representation when using the API-20E technique.

Due to the lack of agar plating techniques for the direct enumeration of *Acinetobacter* in activated sludge, it was not possible to compare the results obtained by the FA technique with the viable count of *Acinetobacter*. The numbers of *Acinetobacter* obtained by using the FA technique were nevertheless compared with the total viable count in activated sludge.

4.4.1 Comparative analysis using the AO, FA and viable plate count technique

Prokasam and Dondero (1967) obtained a maximum number of viable bacteria ($6,10 \times 10^8$ bacteria cm^{-3}) when using Sewage Extract Agar. The average total viable bacterial counts encountered by Prokasam and Dondero (1967) in all activated sludge samples cultured on different media was $1,4 \times 10^8$ bacteria cm^{-3}. Banks and Walker (1977) on average encountered a viable bacterial count of $4,28 \times 10^8$ bacteria cm^{-3} when using GCYA as culture medium.

The average viable plate count obtained in this study was $4,32 \times 10^6$ bacteria cm^{-3} (Table 14). This was lower than the average viable plate counts encountered by Prokasam and Dondero (1967) and Banks and Walker (1977). The higher counts obtained by Prokasam and Dondero (1967) than in this study and that of Banks and Walker (1977) was due to the high rate activated sludge plant which they examined supporting the growth of more viable cells.

Table 14: Comparative analysis of bacterial numbers in activated sludge using the viable plate count, total AO and FA count using a 0,20 μm filter and total AO and FA count using a 0,45 μm filter

	Number of Bacteria cm^{-3}				$\bar{x}$	s	Coefficient of Variance %
	1	2	3	4			
Viable plate count	$5,10 \times 10^6$	$3,90 \times 10^6$	$3,50 \times 10^6$	$4,80 \times 10^6$	$4,32 \times 10^6$	$0,75 \times 10^6$	17,36
Total AO count using a 0,20 μm filter	$1,29 \times 10^9$	$2,0 \times 10^9$	$1,50 \times 10^9$	$1,36 \times 10^9$	$1,53 \times 10^9$	$0,32 \times 10^9$	20,91
Total AO count using a 0,45 μm filter	$6,65 \times 10^7$	$8,28 \times 10^7$	$5,80 \times 10^7$	$7,96 \times 10^7$	$7,17 \times 10^7$	$1,15 \times 10^7$	16,03
Total FA *Acinetobacter* count using a 0,20 μm filter	$2,68 \times 10^6$	$2,27 \times 10^6$	$2,43 \times 10^6$	$2,46 \times 10^6$	$2,46 \times 10^6$	$0,16 \times 10^6$	6,5
Total FA *Acinetobacter* count using a 0,45 μm filter	$2,18 \times 10^6$	$2,06 \times 10^6$	$1,96 \times 10^6$	$2,16 \times 10^6$	$2,09 \times 10^6$	$0,10 \times 10^6$	4,7

Naturally, the viable count obtained in this study was also lower than the viable count obtained by Banks and Walker (1977) although the same procedure was followed.

The total bacterial count using the AO technique and 0,20 μm filter yielded the highest number of bacteria (1,53 x 10^9 bacteria cm^{-3}) followed by the AO technique using 0,45 μm filters (7,17 x 10^7 bacteria cm^{-3}) followed by the viable plate count (4,32 x 10^6 bacteria cm^{-3}) Table 14.

Higher bacterial numbers were always encountered when using the AO technique and 0,20 μm filters, than in any of the viable plate counts including those of Prokasam and Dondero (1967) and Banks and Walker (1977). This was probably due to the lack of suitable nutrient medium for the growth of all viable bacteria in activated sludge. Furthermore, by using the AO technique, dead and live cells are counted. This would result in higher numbers when compared to viable counts of bacteria on GCYA.

Higher bacterial numbers were also encountered when using the AO technique and 0,20 μm filters than was the case using the AO technique and 0,45 μm filters (Table 14). This was ascribed to smaller bacteria not being retained by the 0,45 μm filter, but retained by the 0,20 μm filter, a phenomenon also encountered by Hobbie *et al.* (1977).

The average *Acinetobacter* count using a 0,20 μm filter constituted 56% of the total viable plate count, 0,16% of the total AO count using a 0,20 μm filter and 3,4% of the total AO count using a 0,45 μm filter (Table 14). The coefficient of variation was low at 6,5% (Table 14). The average *Acinetobacter* count using a 0,45 μm filter constituted 48% of the total viable plate count, 0,13% of the total AO count using a 0,20 μm filter and 2,9% of the total AO count using a 0,45 μm filter (Table 14). The coefficient of variation was low at 4,7% (Table 14). Eighty four percent of the *Acinetobacter* numbers retained on the 0,20 μm filter was also retained on the 0,45 μm filter (Table 14). However only 4,6% of the total number of bacteria, as determined using the AO technique, were retained on the 0,45 μm filter (Table 14). These results indicate that there was a marginal difference in the size of *Acinetobacter* in the primary aerobic zone, but that the size of the total number of bacteria varied to a large extent. The small variation in the size of *Acinetobacter* in the primary aerobic zone is ascribed to viability of these organisms in this zone. The 56% and 48% representation of the total viable count supports the statement that *Acinetobacter* is viable in the primary aerobic zone.

Buchan (1980) stated that the volutin containing cells in activated sludge were larger than other cells. He did however, not identify these cells *in situ* and did not determine their actual size. If these cells were identified as *Acinetobacter,* this would have tied in with the small difference in the *Acinetobacter* cell size in the primary aerobic zone (Table 14) as discussed and their large representation of the total viable count (56% and 48%) which suggested that the *Acinetobacter* cells were larger than other cells in activated sludge.

Therefore the determination of the actual size of the volutin containing cells as well as the identification of these cells was essential. TEM was used to determine the actual cell volume.

4.5 TEM studies to determine the volume of volutin containing bacterial cells in activated sludge

Sections (*ca* 1 μm thick) for TEM were prepared from samples collected from the primary aerobic zone of the Northern Works activated sludge plant at a time of enhanced phosphorus removal. Numerous bacterial cells containing volutin were encountered (Plate 5). This was also observed by Buchan (1980). The volumes of 60 cells containing volutin were calculated (paragraph 3.3.5). The volumes of the volutin granules inside the bacterial cells were also calculated (paragraph 3.3.6). The average bacterial cell volume was 1,0 μm^3 (with a standard deviation of 0,8) and the average volutin volume 0,28 μm^3 (with a standard deviation of 0,26).

A frequency table was compiled for the size distribution of the volutin containing cells (Table 15).

These results are illustrated in Figure 4. The largest percentage (20%) of the volutin containing cells had a volume of between 0,50 and 0,59 μm^3 (Table 15). The smallest cells measured had a volume of 0,12 μm^3 and hence an effective spherical diameter of 0,612 μm, indicating that even these cells would be retained on a filter with a 0,45 μm pore size. These cells would therefore have been retained on the 0,45 μm Nuclepore filter.

The small difference in the average *Acinetobacter* counts using 0,45 μm and 0,20 μm Nuclepore filters (Table 14) indicated that some of the *Acinetobacter* cells were small enough to filter through the 0,45 μm filter. At this stage of the study, it was assumed that the volutin-containing bacteria were *Acinetobacter* due to suggestions that this was the case by other researchers (Roïnestad, 1973; Fuhs and Chen, 1975, Buchan, 1980).

The smallest volutin containing cell measured would be retained by the 0,45 μm Nuclepore filter. This suggested that the smaller *Acinetobacter* cells passing through the 0,45 μm filter and retained on the 0,20 μm filter did not contain volutin. This also suggested that the volutin-containing *Acinetobacter* were larger than the other bacteria in activated sludge as was suggested by Buchan (1980). Furthermore, this suggested that the bacteria small enough to go through the 0,45 μm filter did not contain volutin.

Table 15: Frequency table for the size of volutin containing bacterial cells

Cell volume μm^3	Number of cells	% of Total
0,10 – 0,19	4	6,60
0,20 – 0,29	6	10,00
0,30 – 0,39	3	5,00
0,40 – 0,49	1	1,70
0,50 – 0,59	12	20,00
0,60 – 0,69	2	3,33
0,70 – 0,79	1	1,67
0,80 – 0,89	2	3,33
0,90 – 0,99	5	8,33
1,00 – 1,09	2	3,33
1,10 – 1,19	2	3,33
1,20 – 1,29	0	0
1,30 – 1,39	0	0
1,40 – 1,49	0	0
1,50 – 1,59	2	3,33
1,60 – 1,69	2	3,33
1,70 – 1,79	3	5,00
1,80 – 1,89	1	1,67
1,90 – 1,99	1	1,67
2,00 – 2,09	1	1,67
2,10 – 2,19	1	1,67
2,10 – 2,29	1	1,67
3,30 – 3,39	1	1,67
3,70 – 3,79	1	1,67
4,10 – 4,19	1	1,67

4.6 Experiments to determine the role of *Acinetobacter* in phosphorus removal by activated sludge

Speculation and controversy exist whether only *Acinetobacter* in the primary aerobic zone is responsible for phosphorus removal in activated sludge. In order to investi-

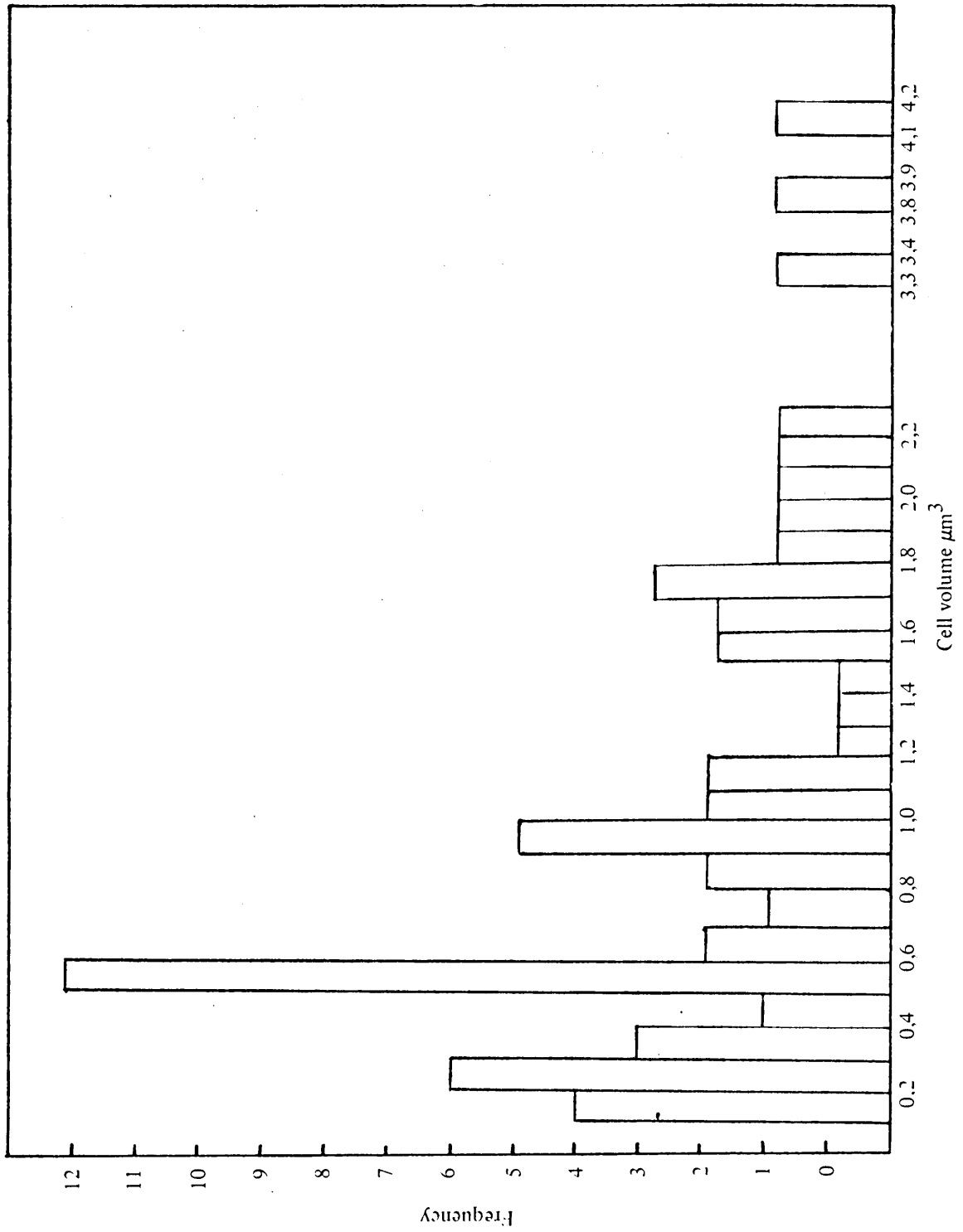


Figure 4. *Histogram and frequency polygon for data in Table 15*

gate this, it was necessary to enumerate *Acinetobacter* in activated sludge. This in itself, did not supply sufficient evidence to indicate whether *Acinetobacter* was solely responsible for phosphorus removal. Additional data were therefore obtained which were used in conjunction with the quantitative data of *Acinetobacter* numbers. The first factor that was determined, was the correlation between the *Acinetobacter* numbers in the different Northern Works activated sludge system zones and total phosphorus removal.

4.6.1 Correlation between total Acinetobacter numbers in activated sludge and total phosphorus removal

The correlation coefficient results are contained in Table 16 and 17.

Table 16: Correlation coefficient between total *Acinetobacter* numbers in the different zones of the Northern Works activated sludge system and phosphorus removal calculated for the data in Table 6

Activated sludge zone	Correlation Coefficient	Error df	Significant values for r and R	
			P 0,05	P 0,01
Anaerobic	- 0,06	7	0,666	0,798
Primary anoxic	0,105	7	0,666	0,798
Primary aerobic	0,120	7	0,666	0,798
Secondary anoxic	0,304	7	0,666	0,798
Secondary aerobic	0,580	7	0,666	0,798
Effluent	- 0,041	7	0,666	0,798

NOTE: When r < P 0,05 and P 0,01, the correlation coefficient is not significant
When r > P 0,05 and P 0,01, the correlation coefficient is significant

Table 17: Correlation coefficient between the total *Acinetobacter* numbers in the different zones of the Northern Works activated sludge system and phosphorus removal calculated for the data in Table 9

Activated sludge zone	Correlation Coefficient (r)	Error df	Significant values for r and R	
			P 0,05	P 0,01
Anaerobic	- 0,5041	4	0,811	0,917
Primary anoxic	- 0,7220	4	0,811	0,917
Primary aerobic	- 0,3039	4	0,811	0,917
Secondary anoxic	- 0,5017	4	0,811	0,917
Secondary aerobic	- 0,5875	4	0,811	0,917
Effluent	- 0,4701	4	0,811	0,917

NOTE: When r < P 0,05 and P 0,01, the correlation coefficient is not significant
When r > P 0,05 and P 0,01, the correlation coefficient is significant

Total *Acinetobacter* numbers were for the first time expressed as real numbers in this study (Table 6 & 7). This provided a more accurate determination of the correlation coefficient between *Acinetobacter* numbers in the primary aerobic zone and phosphorus removal. Workers previously have determined the microbial population structure of the primary aeration zone and, due to the pre-dominance of *Acinetobacter,* speculated that there was an association between *Acinetobacter* numbers in this zone and phosphorus removal.

No significant correlation existed between total *Acinetobacter* numbers in the different zones (Table 16 and 17). This however did not imply that *Acinetobacter* was not responsible for all the phosphorus accumulation and removal in activated sludge. It was therefore necessary to determine the actual quantity of phosphorus that could be associated with intracellular accumulation by *Acinetobacter* in activated sludge.

Uncertainty existed whether all the cells containing volutin in activated sludge belonged to *Acinetobacter.* Before any further calculations were made, it was necessary to determine whether the cells containing phosphorus all belonged to *Acinetobacter.* This was attempted by using ferritin labelled antibodies, density gradient centrifugation, metachromatic and FA staining.

4.6.2 The use of FLA, density gradient centrifugation, metachromatic- and FA staining

(a) FLA

Activated sludge samples were collected from the primary aerobic zone at a time when enhanced phosphorus removal occurred. Activated sludge samples for TEM were stained with FLA prior to preparation. This was done to identify *Acinetobacter* using TEM and determining whether volutin containing cells in activated sludge were *Acinetobacter*. The FLA technique gave unsatisfactory results. Very few *Acinetobacter* in activated sludge and in pure culture were detected by this method, and those detected did not contain any volutin granules (Plate 6). This implied that *Acinetobacter* in activated sludge did not contain volutin. *Acinetobacter*, isolated from activated sludge, and cultured on Acid Sludge Media however exhibited phosphorus uptake (Buchan, 1980).

However, these results were not conclusive due to the many problems that may be encountered using the FLA technique (Borek and Silverstein, 1961; Vogt and Kopf, 1964). Unconugated sera however reacted with the antigen indicating a high serological activity of the antisera. Haemagglutination tests using the FLA and antigen indicated that no reaction took place between the FLA and antigen. This indicated a loss of serological activity of the FLA. This was also found by Borek and Silverstein (1961) and Vogt and Kopf (1964) when using FLA. The cause of the loss of serological activity of the FLA in this study is not known. It is possible that the antibody became univalent after conjugation with the ferritin resulting in the lack of binding between the FLA and antigen as was experienced by Borek and Silverstein (1961) and Vogt and Kopf (1964).

(b) Density gradient centrifugation of activated sludge

Activated sludge samples from the primary aerobic zone were collected at a time of enhanced phosphorus removal in the Northern Works activated sludge plant. These samples were prepared for density gradient centrifugation (paragraph 3.2.13). The density gradient fraction corresponding to the control density gradient fraction (pure culture of *Acinetobacter* containing volutin) was collected. All the other fractions were also collected for further study. FA staining of all fractions revealed that only the fraction corresponding to the control, contained bacterial cells.

This procedure was repeated five times yielding the same results. The observation that the bacterial cells in activated sludge accumulated in one fraction suggested that the range of the density gradient used was possibly too narrow. Hence, the range of the density gradient was

extended in order to fractionate the activated sludge samples (paragraph 3.2.13). This yielded at least six distinguishable fractions (Appendix VI). These fractions were very close to each other which made it difficult to extract the individual fractions for further study. For further analysis one cm^3 fractions were collected starting at the top of the tube containing the different fractions. The total number of bacteria, *Acinetobacter* number as well as a count of the bacteria containing volutin upon metachromatic staining and total phosphorus concentration were determined on each fraction (Table 18).

The percentage of *Acinetobacter* expressed as a percentage of the total count was calculated for each fraction (Table 18). The highest percentages of *Acinetobacter* in sample 1 were recorded in fraction 3, 4 and 5 (84,87%; 89,94% and 79,94%) (Table 18). This corresponded with the highest phosphorus concentrations which were also encountered in fraction 3, 4 and 5 (122, 126, 124 mg dm^{-3}) (Table 20).

In sample 2, the highest percentage of *Acinetobacter* was in fraction 4 (81,20%). Again this corresponded with the highest phosphorus concentration which was recorded in the same zone (54,0 mg dm^{-3}). The percentage of *Acinetobacter* was very low in all the other fractions in sample 2 (Table 18).

The percentage of *Acinetobacter* which was made up by bacteria containing metachromatic granules (Plate 4) was calculated (Table 18). In sample 1, the number of metachromatic stained bacteria constituted 91,0%, 95,8% and 93,2% of the *Acinetobacter* number in fractions 3, 4 and 5 respectively (Table 18). In sample 2, the number of bacteria which stained metachromatically constituted 41,1%, 97,8% and 47,8% of the *Acinetobacter* numbers in fraction 3, 4 and 5 respectively (Table 18).

No *Acinetobacter* nor any bacteria containing volutin were detected in any of the other gradient fractions (Table 18). *Acinetobacter* as well as the bacteria containing volutin upon metachromatic staining were detected in the gradients containing the highest concentration of phosphorus (Table 18). This again confirmed the association between *Acinetobacter*, volutin containing cells and phosphorus.

By calculating the percentage of volutin containing bacteria as a percentage of the total number of bacteria, the following results were obtained, in sample 1: 77,3%, 86,1% and 74,5% of the total number of bacteria in fraction 3, 4 and 5 respectively were volutin-containing bacteria (Table 18).

In sample 2: 30,2%, 75,4% and 7,7% of the total number of bacteria were volutin containing bacteria in fraction 3, 4 and 5 (Table 18). However a very high percentage of the total count also consisted of *Acinetobacter* (Table 18). This indicated, that the majority of the volutin-containing bacteria, if not all, belonged to *Acinetobacter*. This is supported by the fact that the number of volutin-containing bacteria decreased whenever the number of *Acinetobacter* decreased as illustrated in Table 13, sample 2, fraction 3 and 5.

The observation that *Acinetobacter* and volutin-containing bacteria were detected in three of the gradient fractions can be explained by the method of fraction collection. As was stated previously, because of the proximity of different fractions 1 cm^3 fractions were collected. This lead to the overlapping of certain gradient fractions e.g. fractions 3, 4 and 5 (Figure 5).

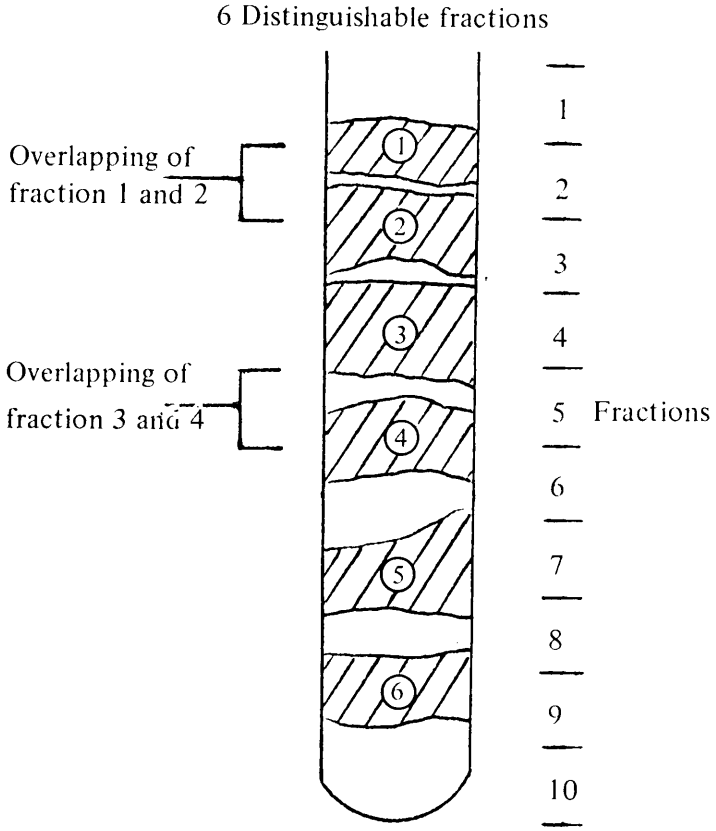


Figure 5: Typical example of how the fraction collection procedure resulted in the overlapping of distinguishable fractions (Appendix vi)

If it were possible to collect distinct fractions, which was not the case, it would probably have been possible to collect one gradient fraction consisting of fractions 3, 4 and 5. This would however not necessarily have resulted in more accurate analysis,

Table 18: Total number of bacteria, *Acinetobacter* number, metachromatic number and total phosphorus concentration in the different activated sludge density gradient fractions

Sample No.	Fraction No.	Total number of bacteria cm^{-3}	*Acinetobacter* number cm^{-3}	Metachromatic (M) number cm^{-3}	M as a % of the total number of bacteria	M as a % of the *Acinetobacter*	*Acinetobacter* as a % of the total number	Total phosphorus concentration mg dm^{-3}
1	1	$1,84 \times 10^8$	0	0	0	0	0	42,0
	2	$9,27 \times 10^6$	0	0	0	0	0	73,0
	3	$7,67 \times 10^7$	$6,51 \times 10^7$	$5,93 \times 10^7$	77,3	91,0	84,87	122,0
	4	$8,75 \times 10^6$	$7,87 \times 10^6$	$7,54 \times 10^6$	86,1	95,8	89,94	126,0
	5	$7,98 \times 10^6$	$6,38 \times 10^6$	$5,95 \times 10^6$	74,5	93,2	79,94	124,0
	6	$1,00 \times 10^7$	0	0	0	0	0	92,0
	7	$4,53 \times 10^7$	0	0	0	0	0	46,0
	8	$1,87 \times 10^7$	0	0	0	0	0	46,0
	9	$2,42 \times 10^7$	0	0	0	0	0	60,0
	10	$2,96 \times 10^6$	0	0	0	0	0	46,0
2	1	$3,15 \times 10^7$	0	0	0	0	0	32,0
	2	$1,08 \times 10^8$	0	0	0	0	0	32,0
	3	$4,89 \times 10^7$	$3,60 \times 10^6$	$1,48 \times 10^6$	30,2	41,1	7,30	36,0
	4	$1,00 \times 10^7$	$8,12 \times 10^6$	$7,54 \times 10^6$	75,4	92,8	81,20	54,0
	5	$5,35 \times 10^7$	$3,09 \times 10^6$	$1,48 \times 10^6$	2,7	47,8	5,70	22,0
	6	$6,78 \times 10^7$	0	0	0	0	0	16,0
	7	$6,78 \times 10^7$	0	0	0	0	0	26,0
	8	$1,73 \times 10^7$	0	0	0	0	0	17,0
	9	$2,36 \times 10^7$	0	0	0	0	0	21,0
	10	$1,28 \times 10^6$	0	0	0	0	0	20,0

since it was impossible to know beforehand where the fractions that had to be combined because of similar characteristics, would be. By collecting the fractions using the method in this study, more information could be obtained than would have been the case by collecting composite gradient fractions. These results indicated that *Acinetobacter* has a buoyant density ranging between 1,070 g cm^3 and 1,120 g cm^3 (Figure 6). The same was true for the volutin-containing cells.

The lower number of *Acinetobacter* in fractions 3 and 5 of sample 1 and 2 was ascribed to the dilution effect of fractions 2 and 6 which did not contain any *Acinetobacter* and overlapped with fractions 3 and 5 (Table 18) due to the fraction collection procedure.

These data obtained by *in situ* identification and enumeration techniques substantiated observations and speculations that *Acinetobacter* in activated sludge could accumulate volutin (Roïnestad, 1973; Fuhs and Chen, 1975; Buchan, 1980).

The quantity of phosphorus which *Acinetobacter* could accumulate still had to be determined. This was done using EM and EDAX techniques combined with the FA identification and enumeration of *Acinetobacter* in activated sludge.

4.6.3 EM and EDAX techniques for the quantification of phosphorus accumulated by Acinetobacter

(a) EDAX analysis of the volutin in Acinetobacter

EDAX analysis was carried out on numerous *Acinetobacter* cells containing volutin granules (Plate 5). This indicated that the volutin contained phosphorus (Plate 7). This was also found by Buchan (1980) who used the EDAX analysis to determine the phosphorus content of the volutin as being *ca* 27,0% P. This corresponded with the values obtained by Friedberg and Avigad (1968). By using these data the values in Table 19 and 20 were calculated.

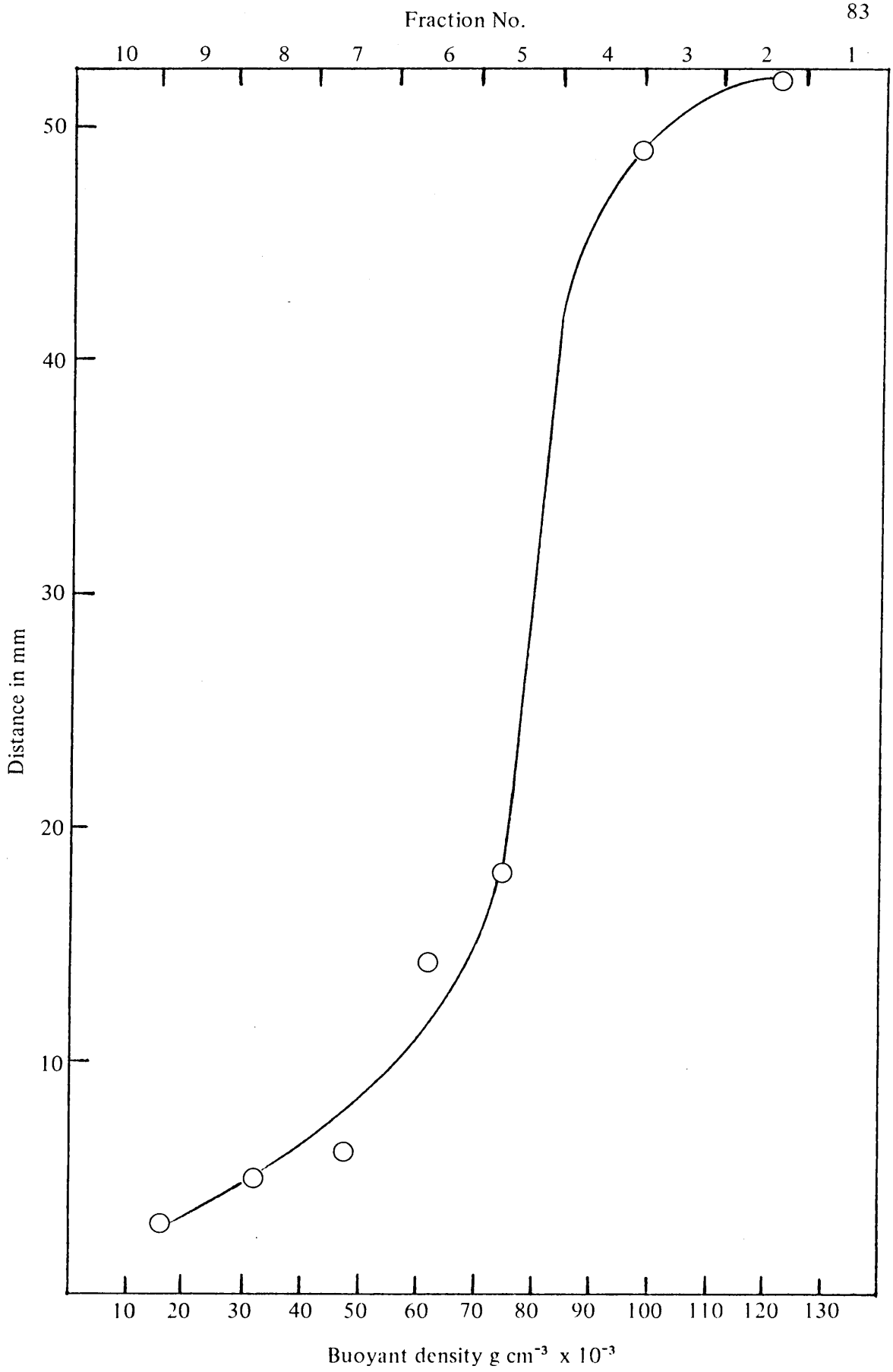


Figure 6: *Standard curve for Percoll gradient and fraction buoyant density distribution*

Table 19: *Acinetobacter* numbers in the primary aerobic zone of the Northern Works activated sludge system and corresponding total cell volume, total volutin volume, total volutin mass and total phosphorus mass (calculated using the values in Table 6)

		Average	Maximum	Minimum
1.	*Acinetobacter* cm^{-3}	$6,46 \times 10^6$	$7,60 \times 10^6$ *	$4,30 \times 10^6$ **
2.	*Acinetobacter* cell volume (μm^3)	1,00	3,79	0,12
3.	Volutin volume (μm^3/cell)	0,28	1,14	0,02
4.	Total cell volume (μm^3 cm^{-3})	$6,46 \times 10^6$	$7,60 \times 10^6$	$4,30 \times 10^6$
5.	Total volutin volume (μm^3 cm^{-3})	$1,80 \times 10^6$	$2,12 \times 10^6$	$1,20 \times 10^6$
6.	Total volutin mass (mg dm^{-3})	2,21	2,60	1,47
7.	Total phosphorus mass (mg dm^{-3})	0,59	0,70	0,39

* Maximum number of *Acinetobacter* counted

** Minimum number of *Acinetobacter* counted

Table 20: *Acinetobacter* numbers in the primary aerobic zone of the Northern Works activated sludge system and corresponding total cell volume, total volutin volume, total volutin mass and total phosphorus mass (calculated using the values in Table 7)

		Average	Maximum	Minimum
1.	*Acinetobacter* cm^{-3}	$2,71 \times 10^7$	$5,45 \times 10^7$ *	$5,88 \times 10^6$ **
2.	*Acinetobacter* cell volume (μm^3)	1,00	3,79	0,12
3.	Volutin volume (μm^3/cell)	0,28	1,14	0,02
4.	Total cell volume (μm^3 cm^{-3})	$2,71 \times 10^7$	$5,45 \times 10^7$	$5,88 \times 10^6$
5.	Total volutin volume (μm^3 cm^{-3})	$7,58 \times 10^6$	$1,52 \times 10^7$	1.64×10^6
6.	Total volutin mass (mg dm^{-3})	9,32	18,60	2,01
7.	Total phosphorus mass (mg dm^{-3})	2,51	5,02	0,54

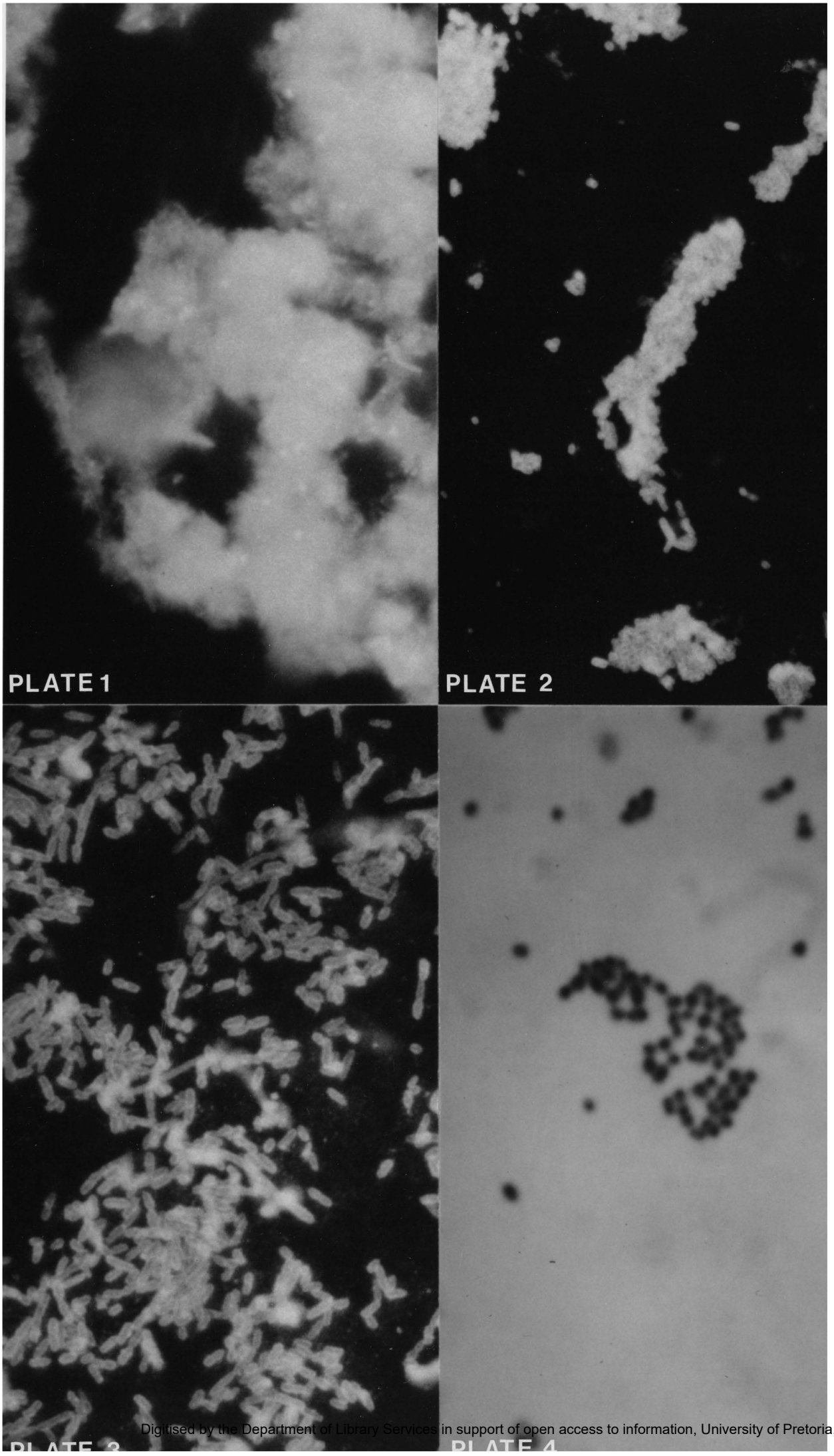
* Maximum number of *Acinetobacter* counted

** Minimum number of *Acinetobacter* counted

PLATE 5 Electron micrograph of volutin-containing bacterial cells in the primary aeration zone of the Northern Works activated sludge system. (Arrows indicate volutin) X25000

PLATE 6 Electron micrograph of an *Acinetobacter* cell stained with ferritin labelled antibody. (Arrows indicate FLA) X30000

PLATE 7 The uncorrected energy dispersive X-ray spectrum generated by an electron-dense body (volutin) within bacterial cells from the primary aeration zone of activated sludge. This figure serves to illustrate the phosphorus and calcium peaks generated.



The following assumptions were made for calculating the values in Table 19 and 20.

(i) All the *Acinetobacter* cells contained volutin,

(ii) The specific gravity of the volutin was 1,23 g cm^3 (Friedberg and Avigad, 1968), and

(iii) That the volutin contained 27% phosphorus (Friedberg and Avigad, 1968; Buchan, 1980).

Based on these assumptions and subsequent calculations (Table 19) it was calculated that *Acinetobacter* could on average accumulate 7,00% of the average, a maximum of 8,00% and a minimum of 4,00% of the average phosphorus removed (7,9 mg P dm^3) during the study period. These results indicated, that *Acinetobacter* could not account for all the phosphorus being removed.

The results in Table 20 indicate, that *Acinetobacter* could account for an average of 17,4%, a maximum of 34,86% and a minimum of 3,00% of the average phosphorus removed (14,4 mg P dm^3) at the time of the analysis.

EM studies revealed that the smallest volutin containing cell with a volume of 0,12 μm^3 had a diameter of 0,61 μm and would have been retained by the 0,45 μm filter. Therefore the phosphorus removal as calculated for *Acinetobacter* numbers determined using 0,20 μm filters (Table 20) is probably an overestimation of the actual quantity of phosphorus that could be removed by *Acinetobacter*.

Buchan (1980) usually prepared *ca* one hundred pure cultures for identification, by plating out 0,1 cm^3 from plates containing the 10^6 or 10^7 dilution on GCYA. If these bacterial isolates were prepared from the 10^6 dilution, the real number of bacteria encountered by Buchan (1980) would have been *ca* 10^8 cells cm^{-3}. If the isolates were prepared from the 10^7 dilution, the real number of bacteria encountered by Buchan (1980) would have been *ca* 10^9 cells cm^{-3}. Buchan (1980) indicated that 35 of the 98 isolates obtained from the Northern Works activated sludge system were *Acinetobacter*. Unfortunately it was not stated from which dilution (10^6 or 10^7) these isolates were prepared. The real number of *Acinetobacter* was nevertheless calculated as for both dilutions. (For the 10^6 dilution the real *Acinetobacter* number was calculated as 3,5 x 10^8 cells cm^{-3} and for 10^7 dilution 3,5 x 10^9 cells cm^{-3}). The total mass of phosphorus that these cells could contain was calculated using the average total phosphorus mass (Table 19). The values obtained were 31,9 mg P dm^{-3} (for 3,5 x 10^8 *Acinetobacter* cm^{-3})

and 319,0 mg P dm^{-3} (for 3,5 x 10^9 cm^{-3}). When the quantity of phosphorus removed in this study is considered (Table 6 and 7) it is clear, that if the *Acinetobacter* cells encountered by Buchan (1980) contained the same quantity as the cells in this study, adequate numbers of *Acinetobacter* were encountered to account for all the phosphorus removed by intracellular accumulation. A possible explanation for the lower numbers of *Acinetobacter* encountered in this study as compared to those obtained by Buchan (1980), was the fact that *Acinetobacter* numbers in activated sludge were determined during the winter months. This could result in less microbial activity and hence lower numbers of *Acinetobacter*.

This study indicates that other organisms or mechanisms had to be involved to account for all the observed phosphorus removed during the activated sludge process.

In this regard Suresh *et al.* (1984) indicated that *Pseudomonas vesicularis* could accumulate phosphorus in activated sludge. Gersberg and Allen (1984) furthermore indicated that *Klebsiella pneumoniae* could remove phosphorus from activated sludge by intracellular accumulation. These results indicate that at least two additional types of bacteria apart from *A. calcoaceticus* and *A. lwoffii* could contribute to phosphorus removal in activated sludge. The quantities of phosphorus removed by *P. vesicularis* and *K. pneumoniae* have not yet been quantified. The techniques developed in this study could be used to determine the quantity of phosphorus removed by the latter two bacteria. This would indicate what contribution these organisms would make to phosphorus removal in activated sludge.

Secondly, normal metabolic phosphorus requirements of all organisms in activated sludge could contribute to phosphorus removal. (Jenkins, *et al.*, 1971).

Furthermore, Spatzierer *et al.* (1984) indicated that biological phosphorus removal and simultaneous precipitation of phosphorus by adding a chemical precipitant were possible. They also found that the quantity of precipitant added during biological phosphorus removal was significantly lower than when there was no biological phosphorus removal.

Kerdachi and Roberts (1980) suggested that *Acinetobacter* served as a biological phosphorus pump, accumulating phosphorus in the aerobic zone and releasing phosphorus under anaerobic conditions. Firstly, the phosphorus is concentrated inside

the cell and secondly released into the medium (ie. anaerobic zone). This results in an increase of the phosphorus concentration to such an extent that nucleation of phosphorus occurs resulting in precipitation.

To date it therefore seems that at least the following mechanisms are involved and may be used for phosphorus removal in activated sludge :

i) Biological excess phosphorus removal by *A. calcoaceticus, A. lwoffii, P. vesicularis* and *K. pneumoniae,*

ii) Chemical precipitation of phosphorus combined with biological phosphorus removal, and

iii) Normal metabolic phosphorus requirements of organisms in activated sludge,

iv) Biologically mediated chemical precipitation.

CHAPTER 5

5. CONCLUSIONS

5.1 Fluorescent antibodies were successfully produced using three *Acinetobacter* isolates as antigens. It was however necessary to pool the antisera to obtain cross-reactions with all the *Acinetobacter* isolates obtained from activated sludge.

5.2 The FA technique was successfully applied for the recognition and enumeration of *Acinetobacter* in all the different zones of the activated sludge process.

5.3 Bacterial clusters and bacteria adsorbed to colloids were effectively dispersed using Ansan 6 or sonication of activated sludge in a 0,5% Tripolyphosphate solution.

5.4 The *Acinetobacter* numbers differed significantly in the various zones over a period of time.

5.5 When using 0,45 μm filters the *Acinetobacter* numbers in the primary aerobic and secondary anoxic zones differed significantly from the other zones.

5.6 When using 0,20 μm filters no significant difference existed amongst the activated sludge zones.

5.7 The API-20E techniques employed in this study indicated that *Acinetobacter* was the dominant Gram-negative organism in the anaerobic-, primary anoxic- and primary aerobic zone of the Northern Works activated sludge plant.

5.8 A comparative analysis indicated that the AO technique (using 0,20 μm filters) yielded the highest number of bacteria followed by numbers obtained by using the AO technique using 0,45 μm filters, followed by the viable plate count.

5.9 A comparative analysis furthermore indicated that the *Acinetobacter* numbers determined using the FA technique and 0,20 μm filters and 0,45 μm filters respectively constituted 56% and 48% of the total viable plate count in the primary aerobic zone. Furthermore, 84% of the *Acinetobacter* retained on the 0,20 μm were also retained on the 0,45 μm filter indicating a small difference in the physical size of these bacteria in the primary aerobic zone.

5.10 The average volume of volutin-containing bacterial cells was determined as 1,0 μm^3 using TEM, and the average volutin volume as 0,28 μm^3. All volutin containing cells (even the smallest cells measured with a volume of 0,12 μm^3) would be retained by a filter with pore size 0,45 μm.

5.11 No correlation was found between the *Acinetobacter* numbers obtained in this study and phosphorus removal.

5.12 The FLA technique and TEM could not be used successfully for the identification of *Acinetobacter* as a result of the loss of serological activity of the antisera upon conjugation with the ferritin.

5.13 Density gradient centrifugation of activated sludge samples revealed an association amongst *Acinetobacter* numbers, metachromatic-stained cells and phosphorus. These results for the first time proved that *Acinetobacter* could accumulate volutin *in situ*. The buoyant density of *Acinetobacter* in activated sludge was furthermore determined to be between 1,07 and 1,12 g cm^{-3}.

5.14 Quantitative analysis of the quantity of phosphorus which could be accumulated by *Acinetobacter* in activated sludge revealed that *Acinetobacter* could at the utmost remove 34,86% of the quantity of phosphorus removed during the activated sludge process. However, at times quantities as low as only 4,0% could be removed. On average (as calculated for the *Acinetobacter* numbers determined by the FA technique using 0,45 μm filters), 8,0% of the phosphorus could be removed by *Acinetobacter*. When using 0,20 μm filters and the FA technique, an average percentage of 17,40% of the phosphorus removal was attributed to accumulation by *Acinetobacter*. It was therefore concluded that *Acinetobacter* was not solely responsible for phosphorus removal in activated sludge.

The null-hypothesis was that *Acinetobacter* was not present in the activated sludge process in numbers sufficiently large to account for the removal of phosphorus by intracellular accumulation, this null-hypothesis could not be rejected on the ground of results obtaining this study. The Koch-Henle postulates were therefore not fulfilled and other organisms or mechanisms have to be involved in phosphorus removal during the activated sludge process.

BIBLIOGRAPHY

ALLEN, L.A., 1943. The bacteriology of activated sludge. *J. Hyg.* 43, 424–431.

ANALYTICAL PROFILE INDEX (1983). Anylab Products Division of Ayerst Laboratories of American Homeproducts Corporation, U.S.A.

ANDERSON, R. and McCOY, E., 1963. Floc-forming bacteria from activated sludge. *Bacteriol. Proc.* 162.

ARVIN, E. and BUNDGAARD, E., 1982. Full scale experience with phosphorus removal in an alternating system. In : *Proceedings of a post conference seminar on phosphate removal in biological treatment processes,* Council for Scientific and Industrial Research, Pretoria, South Africa.

BANKS, C.J. and WALKER, I., 1976. Sonication of activated sludge flocs and the recovery of their bacteria on solid media. *J. gen. Microbiol.* 98, 363–368.

BARNARD, J.L., 1976. A review of biological phosphorus removal from the activated sludge process. *Water S.A.* 2, 136–144.

BARNARD, J.L., 1982. Background to biological phosphorus removal. In : *Proceedings of a post conference seminar on phosphate removal in biological treatment processes.* Council for Scientific and Industrial Research, Pretoria, South Africa.

BERGMAN, S., FORSGREN, A. and SWAHN, B., 1966. Effect of normally occurring rabbit antibodies on fluorescent-antibody reactions. *J. Bacteriol.* 91, 1664–1665.

BOHLOOL, B.B. and SCHMIDT, E.L., 1968. Nonspecific staining : its control in immunofluorescence examination of soil. *Science* 162, 1012–1014.

BOREK, F. and SILVERSTEIN, A.M., 1961. Characterization and purification of ferritin antibody globulin conjugate. *J. Immunol.* 87, 555.

BOWDEN, W.B., 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. *Appl. environ. Microbiol.* 33, 1229–1232.

BRODISCH, K.E.U. and JOYNER, S.J., 1982. The role of organisms other than *Acinetobacter* in biological phosphate removal in activated sludge processes. In : *Proceedings of a post conference seminar on phosphate removal in biological treatment processes.* Council for Scientific and Industrial Research, Pretoria, South Africa.

BUCHAN, L. 1980. The location and nature of accumulated phosphorus in activated sludge, D.Sc. thesis, University of Pretoria, Pretoria, South Africa.

BUCHAN, L. 1984. Microbiological aspects. In : *Theory, design and operation of nutrient removal activated sludge processes.* (eds.) Wiechers, H.N.S., Ekama, G.A., Gerber, A., Keay, G.F.P., Malan, W., Marais, G.V.R., Osborn, D.W., Pitman, A.R., Potgieter, D.J.J., Pretorius, W.A. Water Research Commission, Pretoria, South Africa.

BUTTERFIELD, C.T., RUCHHOFT, C. and McNAMEE, P.A., 1935. Studies of sewage purification. VI. Biochemical oxidation by sludges developed by pure cultures of bacteria isolated from activated sludge. *Publ. Health Dept.* 52, 387–412.

CARTER, C.H. and LEISE, J.M., 1958. Specific staining of various bacteria with a single fluorescent antiglobulin. *J. Bacteriol.* 76, 152–154.

CHERRY, W.B., GOLOMAN, M. and CARSKI., 1960. Fluorescent antibody techniques in the diagnosis of communicable diseases. Public Health Service. Publication. No. 729. (Full details not available).

CLOETE, T.E., 1981. Heterotrophic aspects and algal-bacterial interactions in a pond treatment system for cattle feedlot effluent. M.Sc. thesis, University of the Orange Free State, Bloemfontein, South Africa.

COONS, A.H., 1942. The demonstration of pneumococcal antigens in tissues by the use of fluorescent antibody. *J. Immunol.* 45, 157–170.

DIAS, F.F. and BHAT, J.V., 1964. Microbial ecology of activated sludge. I. Dominant bacteria. *Appl. Microbiol.* 12, 412–417.

EREN, E. and PRAMER, D., 1965. Application of immunofluorescent staining to studies of the ecology of soil micro-organisms. *Soil Sci.* 101, 39–45.

FLIERMANS, C.B., BOHLOOL, B.B. and SCHMIDT, E.L., 1974. Aut-ecological study of the chemo-autotroph *Nitrobacter* by immunofluorescence. *Appl. Microbiol.* 27, 124–129.

FRIEDBERG, I. and AVIGAD, G., 1968. Structures containing polyphosphate in *Micrococcus lysodeikticus*. *J. Bacteriol.* 96, 544–553.

FUHS, G.W. and CHEN, M., 1975. Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. *Microbial Ecol.* 2, 119–138.

GARVEY, J.S., CREMER, N.E. and SUSSDORF, D.H., 1977. *Methods in Immunology : A laboratory text for instruction and research*, 3rd ed. W.A. Benjamin Inc., London.

GERSBERG, R.M. and ALLEN, D.W., 1984. Phosphorus uptake by *Klebsiella pneumonia* and *Acinetobacter calcoaceticus*. In : *Proceedings of a post conference seminar on enhanced biological phosphorus removal from wastewater.* International Association on Water Pollution Research and Control, Paris, France.

HARPER, H.J., 1942. The accurate determination of nitrates in soils. *Ind. Eng. Chem.* 16, 180–185.

HILL, I.R. and GRAY, T.R.G., 1967. Application of the fluorescent antibody technique to an ecological study of bacteria in soil. *J. Bacteriol.* 93, 1888–1896.

HOBBIE, J.E., DALEY, R.J. and JASPER, S., 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. environ. Microbiol.* 33, 1225–1228.

HUNGATE, R.E., 1962. In: *The Bacteria.* Gunsalus, I.C., and Stanier, R.Y. (eds.). Academic Press, New York.

JASEWICZ, L. and PORGES, N., 1956. Biochemical oxidation of dairy wastes. VI. Isolation and study of sludge micro-organisms. *Sewage Ind. Wastes.* 28, 1130–1136.

JENKINS, D., MENAR, A.B. and FERGUSON, J.F., 1971. Chemical processes for phosphate removal. *Water Res.*, 5, 369–389.

JOHNSON, G.D. and HOLBORROW, E.J., 1978. Immunofluorescence and immunoenzyme techniques. In : *Handbook of experimental immunology, vol. I. Immunochemistry,* (ed.) D.M. Weir. 3rd ed. Blackwell Scientific Publications, London.

KAPLAN, N. and ROSENBERG, E., 1982. Exopolysaccharide distribution of an bioemulsifier production by *Acinetobacter calcoaceticus* BO4 and BO413. *Appl. environ. Microbiol.* 44, 231–246.

KAUFMAN, G.I., NESTER, J.F. and WASSERMAN, D.E., 1971. An experimental study of lasers as excitation sources for automated fluorescent antibody instrumentation. *J. Histochem. Cytochem.* 19, 469–476.

KERDACHI, D.A. and ROBERTS, M.R. 1980. The ability of the extended aeration activated sludge process to remove phosphorus consistently to less than 0,1 mg P/litre in a simple surface aerated reactor. Presented to the S.A. Branch of the Institute of Water Pollution Control Conference, Pretoria.

LÖTTER, L. and MURPHY, M., 1983. Unpublished method for the declustering of cluster forming bacteria in activated sludge. Johannesburg City Council Research Laboratories, Johannesburg.

MARSHALL, K.G., 1971. *Sorptive interaction between soil particles and microorganisms,* Vol. II (eds.) A.D. McLaren and J.J. Skuijns. Marcel and Decker Inc, New York.

MARTIN, W.J., PAULINE, K.W.Y.U. and WASHINGTON, J.A., 1971. Evaluation of the enterotube system for identification of members of the family Enterobacteriaceae. *Appl. Microbiol.* 22, 96–99.

McFARLAND, J., 1970. The nephelometer : An instrument for estimating the number of bacteria in suspensions used for calculating the opsonix index and for vaccines. *J. Amer. Med. Assoc.* 49, 1176.

McKINNEY, R.E. and HORWOOD, M., 1952. Fundamental approach to the activated sludge process. I. Floc-producting bacteria. *Sewage Ind. Wastes.* 24, 117–123.

McKINNEY, R.E. and WEICHLEIN, R., 1953. Isolation of floc-producing bacteria from activated sludge. *Appl. Microbiol.* 1, 259–261.

McLAREN, A.L. 1976. Effective phosphorus removal from sewage by biological means. *Water S.A.* 2, 47.

MOODY, M.D. and JONES, W.L. 1963. Identification of *Corynebacterium, Diphtheriae* with fluorescent antibacterial reagents. *J. Bacteriol.* 86, 285.

ORLAND, H.P., 1965. (ed.) Standard methods for the examination of water and wastewater. APHA, New York.

OSBORN, D.W. and NICHOLLS, H.A.,1978. Bacterial stress : A prerequisite for the biological removal of phosphorus. *Wat. Pollut. Control Fed.* 51, 557–569.

PEARCE, A.G.E., 1960. *Histochemistry, Theoretical and Applied.* Churchill, London.

PITAL, A. and JANOWITZ, S.L., 1963. Enhancement of staining intensity in the fluorescent-antibody reaction. *J. Bacteriol.* 86, 888–889.

PROKASAM, T.B. and DONDERO, N.C., 1967. Aerobic heterotrophic bacterial populations of sewage and activated sludge. I. Enumeration. *Appl. Microbiol.* 15, 461–467.

RAY, M.S., 1983. A review of activated sludge treatment – Part 1. *Effluent and Water Treatment Journal.* 23, 225–232.

ROBARTS, R.D. and SEPHTON, L.M., 1981. The enumeration of aquatic bacteria using DAPI. *J. Limnol. Soc. Sth. Afr.* 7, 72–74.

ROGOVSKAYA, Z.I. and LAZAREVA, M.F., 1959. Intensification of biochemical purification of industrial sewage. I. A microbiological specification of activated sludges purifying various industrial sewages. *Mikrobiologiya.* 28, 565–573.

ROÏNESTAD, F.A., 1973. Volutin accumulation by activated sludge micro-organisms. University Microfilms International. 300N ZEEB Road, Ann Arbor, MI 48106, 18 Bedford Row, London, WCIR 4EJ.

ROÏNESTAD, F.A. and YALL, I., 1970. Volutin granules in *Zoogloea ramigera. Appl. Microbiol.* 19, 973–979.

SCHMIDT, E.L., 1974. Quantitative aut-ecological study of micro-organisms in soil by immunofluorescence. *Soil Sci.* 118, 141–149.

SCHMIDT, E.L. and BANKOLE, R.O., 1965. Specificity of immunofluorescent staining for study of *Aspergillus flavus* in soil. *Appl. Microbiol.* 13, 673–679.

SINGER, S.J., 1959. Preparation of an electron dense antibody conjugate. *Nature,* 183, 1523.

SMITH, P.B., TOMFOHRDE, K.M., RHODEN, L.D. and BALOWS, A., 1972. API system : a multitube micromethod for identification of Enterobacteriaeceae. *Appl. Microbiol.* 24, 449–452.

SPATZIERER, G., LUDWIG, C. and MATSCHÉ, N., 1984. Biological phosphorus removal in combination with simultaneous precipitation. In : *Proceedings of a post conference seminar on enhanced biological phosphorus removal from wastewater.* International Association on Water Pollution Research and Control, Paris, France.

SRINATH, E.G., SASTRY, G.A. and PILLAI, S.C., 1959. Rapid removal of phosphorus from sewage by activated sludge. *Experientia.* 15, 339–340.

STEEL, R.G.D. and TORRIE, J.H., 1960. *Principles and Procedures of Statistics.* McGraw-Hill Book Company, Inc New York.

STRAYER, R.F. and TIEDJE, J.M., 1977. Application of the fluorescent antibody technique to the study of methanogenic bacterium in lake sediments. *Appl. environ. Microbiol.* 35, 192–198.

SURESH, N., WARBURG, R., TIMMERMAN, M., WELLS, J., COCCIA, M., ROBERTS, M.F. and HALVORSON, H.O., 1984. New strategies for the isolation of micro-organisms responsible for phosphate accumulation. In : *Proceedings of a post conference seminar on enhanced biological phosphorus removal from wastewater.* International Association on Water Pollution Research and Control, Paris, France.

TERRY, K.R. and HOOPER, A.B., 1970. Polyphosphate and orthophosphate content of *Nitrosomonas europaea* as a function of growth. *J. Bacteriol.* 103, 199–206.

THOMASON, B.M., 1976. Fluorescent antibody detection of *Salmonellae.* In : *Compendium of Methods for the Microbiological Examination of Foods.* (ed.) M.C. Speck. American Public Health Association, 1015–18th Street, N.W. Washington D.C. 20036.

TOERIEN, D.F., 1977. *A review of eutrofication and guidelines for its control in South Africa.* Special Report WAT48, Council for Scientific and Industrial Research, Pretoria.

VACKER, P., CORNELL, C.H. and WELLS, W.N., 1967. Phosphate removal through municipal wastewater treatment at San Antionio Texas. *J. Wat. Pollut. Control Fed.* 39, 750–771.

VAN VUUREN, H.J.J., 1978. Identification and physiology of Enterobacteriaeceae isolated from South African larger breweries, Ph.D. thesis, University of the Orange Free State, Bloemfontein, South Africa.

VOGT, A. and KOPF, R., 1964. Loss of specific agglutinating activity of purified ferritin-conjugated antibodies. *Nature.* 27, 1350–1351.

WALKER, P.D., BATTY, L. and THOMSON, R.O., 1971. The localization of bacterial antigens. In : *Methods in Microbiology* (eds.) J.R. Norris and D.W. Ribbons. Academic Press, New York.

WASHINGTON, J.A., YU, P.K.W. and MARTIN, W.J., 1971. Evaluation of accuracy of multitest micro-method system for identification of Enterobacteriaeceae. *Appl. Microbiol.* 22, 267–296.

WATSON, S.W., NOVITSKY, T.J., QUINBY, H.C. and VALOIS, F.W., 1977. Determination of bacterial number and biomass in the marine environment. *Appl. environ. Microbiol.* 33, 940–954.

WIECHERS, H.N.S., EKAMA, G.A., GERBER, A., KEAY, G.F.P., MALAN, W., MARAIS, G.V.R., OSBORN, D.W., PITMAN, A.R., POTGIETER, D.J.J. and PRETORIUS, W.A., 1984. (eds.) *Theory, design and operation of nutrient removal activated sludge processes.* Water Research Commission. Pretoria. South Africa.

APPENDIX I

1. API-20E microtube reactions and identification of isolates from activated sludge using the Analytical Profile Index, 1983.

Note: All *Acinetobacter calcoaceticus* var *lwoffii* will be reported as *A lwoffii*

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
Goudkoppies primary aeration zone																						–
1	+	+	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
2	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	Unacceptable
3	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–	+	–	*Enterobacter aerogenes*
4	+	+	–	–	–	–	–	–	+	–	–	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
5	+	–	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
6	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*Pseudomonas maltophila*
7	+	–	+	–	–	–	–	–	+	–	–	++		–	+	+	+	+	–	+	–	*Escherichia coli*
8	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	*Flavobacterium*
9																						No growth
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
12																						No growth
13																						No growth
14	–	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	Unacceptable
15	+	–	+	+	–	–	–	–	–	+	+	+	+	–	+	–	+	+	+	–	–	Unacceptable
16	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	+	Unacceptable
17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
18	+	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	–	–	+	–	*Citrobacter freundii*
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
20																						No growth
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
22	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	*Flavobacterium*
23	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	No growth
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
25	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	*Shigella dysenteriae*
26																						No growth
27	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
28	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
29	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	*Flavobacterium*
30	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–	+	–	*Enterobacter agglomerans*
31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
32	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
33	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
34	+	+	–	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	–	*Enterobacter cloacae*
35																						No growth
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
37	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas* sp
38																						No growth
39																						No growth
40																						No growth

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
41																						No growth
42																						No growth
43	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	+	–	*Enterobacter agglomerans*
44																						No growth
45	+	+	–	+	+	–	–	–	–	+	–	+	+	+	+	+	+	+	+	+	–	*Enterobacter cloacae*
46	+	+	–	–	–	–	–	–	+	+	–	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
48	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	–	+	+	*Aeromonas hydrophila*
49	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas* sp
50																						No growth
51																						No growth
52	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
54																						No growth
55	+	+	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	–	+	+	*Aeromonas hydrophila*
56	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella* sp (NO_2+)
57	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*P. stutzeri* (N_2+)
58																						No growth
59																						No growth
60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Moraxella* sp
61																						No growth
62	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
63	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
64	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
65	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
66	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	+	–	Unacceptable
67	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
68																						No growth
69	+	+	–	–	–	–	–	–	+	–	–	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
70	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
71	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	*Pasteurella* sp
72	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
73																						No growth
74	+	+	–	–	–	–	–	–	+	–	–	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
75	+	+	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
76																						No growth
77	+	+	–	+	+	–	–	–	–	+	–	+	+	+	+	+	+	+	+	+	–	*Enterobacter cloacae*
78	+	+	–	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–	+	+	*Aeromonas hydrophila*
79																						No growth
80																						No growth
81	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	–	+	+	*Aeromonas hydrophila*
82																						No growth
83	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Brucella* (NO_2+)
84																						No growth
85	+	+	–	–	+	–	–	–	+	+	+	+	+	–	–	–	+	–	–	–	+	*Aeromonas hydrophila*
86																						No growth
87	+	+	–	+	+	–	–	–	–	+	–	+	+	–	+	–	+	–	+	+	–	*Enterobacter cloacae*
88	+	+	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
89																						No growth

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
90	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	*Flavobacterium*
91	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas* sp
92																						No growth
93																						No growth
94	+	+	–	–	–	–	–	–	+	–	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
95	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	Unacceptable
96	+	+	+	+	+	–	–	–	–	+	–	+	+	–	+	+	+	+	+	+	–	*Enterobacter cloacae*
97	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–	CDC Group VE I
98	+	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	+	–	–	*Klebsiella ozaenae*
99	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella* sp (NO_2+)
100																						
Northern Works																						
2	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
25	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
32	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
56	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
64	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
68	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
72	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
73	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
87	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
88	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
89	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
Northern Works anaerobic zone																						
1																						No growth
2	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
3	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
4	–	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	Unacceptable
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
8	+	+	–	–	–	–	–	+	+	–	+	+	–	–	–	+	–	–	+	+	+	*Aeromonas hydrophila*
9																						Unacceptable
10	+	+	–	–	–	–	–	+	+	–	+	+	–	–	–	+	–	–	+	+	+	*Aeromonas hydrophila*
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
13	+	–	+	+	–	–	–	–	–	+	+	+	+	–	+	–	+	+	+	–	–	Unacceptable
14	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	+	+	Unacceptable
15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
17	+	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	–	–	+	–	*Citrobacter freundii*
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
19	+	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	–	–	+	–	*Citrobacter freundii*
20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
23	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	*Shigella*
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
25																						No growth
26	–	–	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	+	–	–	CDC group II K-2
27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
28	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	–	–	+	+	*Aeromonas hydrophila*
29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
32																						No growth
33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
36	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	+	+	Unacceptable
37	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	CDC group II F
38																						No growth
39	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
41	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
42	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
45	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
48	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+	+	–	*Klebsiella oxytoca*
49																						*Pseudomonas*
50	–	–	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	+	+	Unacceptable
Northern Works primary anoxic zone																						
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
2	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
3	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
7	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
11	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
13	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
17	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	*Escherichia coli*
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
21	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+	+	–	*Serratia*
22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
25	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
32	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+	+	–	*Serratia*
33	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	*Pseudomonas*
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
36	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
38	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+	+	–	*Serratia*
39	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
41	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
42	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
43	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2+)
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
45	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
48	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
Northern Works primary aeration zone																						
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	*Pseudomonas*
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella*
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella*
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	*Pseudomonas*
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella*
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
9																						CDC Group IV*
10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
13	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	*Flavobacterium*
14																						*Yersinia**
15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
20	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
21	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
22	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–		*Citrobacter freundii*
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella*
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
27	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*Pseudomonas*
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	*Pasteurella*
29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
31	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
35	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
39	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
40																						Unacceptable*
41																						Unacceptable*
42																						Unacceptable*
43																						Unacceptable*
44	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
45	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*

APPENDIX I – (Continued)

Source of sample and isolate number	Biochemical reaction 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Isolate
46	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
47	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	Unacceptable
48	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
51	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
52	–	–	–	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	+	+	Unacceptable
53																						Unacceptable
54	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	*Pseudomonas*
55	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
56	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
57	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
58	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
59	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
60	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
61	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
62	–	–	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	+	Unacceptable
63	–	–	–	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	+	+	Unacceptable
64	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	Unacceptable
65																						Unacceptable*
66																						Unacceptable*
67	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
68	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
69	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
70	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
71	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
72	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	No growth
73	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
74																						No growth
75																						No growth
76																						No growth
77																						No growth
78																						No growth
79	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
80	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
81	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
82	+	+	–	–	–	–	–	–	+	+	+	+	+	–	–	–	+	–	+	+	+	*Aeromonas hydrophila*
83	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii* (NO_2 +)
84	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
85-90																						Unacceptable*
91																						No growth
92																						No growth
93																						No growth
94																						No growth
95	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
96	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
97	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
98	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
99	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*
100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*A. lwoffii*

*Not available

APPENDIX I – (Continued)

Biochemical reaction number	Biochemical reaction
1	ONPG
2	ADH
3	CDC
4	ODC
5	CIT
6	H_2S
7	URE
8	TDA
9	IND
10	VP
11	GEL
12	GLU
13	MAN
14	INO
15	SOR
16	RHA
17	SAC
18	MEL
19	AMY
20	ARA
21	OX

APPENDIX II

1. Calculation of the *Acinetobacter* numbers in activated sludge using 0,45 μm Nuclepore filters, the Ansan 6 dispersion technique combined with FA (paragraph 3.2.8c(2)). The equation in paragraph 3.3.1 was used for all calculations :

Date	Sampling point	Average number of *Acinetobacter* per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
	Northern Works		
21 March 1983	Anaerobic zone	2,03	2,10
	Primary anoxic zone	0,97	1,00
	Primary aerobic zone	7,78	7,50
	Secondary anoxic zone	6,60	6,80
	Secondary aerobic zone	2,13	2,20
	Effluent	0,97	1,00
25 March 1983	Anaerobic zone	2,23	2,30
	Primary anoxic zone	4,36	4,50
	Primary aerobic zone	4,85	5,00
	Secondary anoxic zone	6,40	6,60
	Secondary aerobic zone	1,94	2,00
	Effluent	0,97	1,00
21 April 1983	Anaerobic zone	2,91	3,00
	Primary anoxic zone	0,97	1,00
	Primary aerobic zone	6,21	6,40
	Secondary anoxic zone	5,53	5,70
	Secondary aerobic zone	1,06	1,10
	Effluent	0,97	1,00
7 May 1983	Anaerobic zone	0,97	1,00
	Primary anoxic zone	2,03	2,10
	Primary aerobic zone	4,17	4,30
	Secondary anoxic zone	4,17	4,30
	Secondary aerobic zone	0,97	1,00
	Effluent	0,97	1,00
19 May 1983	Anaerobic zone	2,62	2,70
	Primary anoxic zone	2,62	2,70
	Primary aerobic zone	6,21	6,40
	Secondary anoxic zone	0,97	1,00
	Secondary aerobic zone	1,55	1,60
	Effluent	1,55	1,60
24 May 1983	Anaerobic zone	2,03	2,10
	Primary anoxic zone	3,59	3,70
	Primary aerobic zone	7,28	7,50
	Secondary anoxic zone	7,96	8,20
	Secondary aerobic zone	2,03	2,10
	Effluent	3,68	3,80
26 May 1983	Anaerobic zone	2,62	2,70
	Primary anoxic zone	4,17	4,30
	Primary aerobic zone	6,79	7,00
	Secondary anoxic zone	6,69	6,90
	Secondary aerobic zone	0,97	1,00
	Effluent	0,97	1,00

Appendix II – (Continued)

Date	Sampling point	Average number of *Acinetobacter* per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
24 June 1983	Anaerobic zone	2,03	2,10
	Primary anoxic zone	3,10	3,20
	Primary aerobic zone	7,37	7,60
	Secondary anoxic zone	7,37	7,60
	Secondary aerobic zone	2,03	2,10
	Effluent	6,69	6,90
	Benoni		
29 September 1983	Anaerobic zone	4,56	4,70
	Primary anoxic zone	1,16	1,20
	Primary aerobic zone	1,35	1,40
	Secondary anoxic zone	2,52	2,60
	Secondary aerobic zone	3,20	3,30

2. Calculation of the *Acinetobacter* numbers in activated sludge using 0,20 μm Nuclepore filters and the tripolyphosphate, sonification dispersion technique and FA (paragraph 3.2.8c(2).

The equation in paragraph 3.3.1 was used for all calculations :

Date	Sampling point	Average number of *Acinetobacter* per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
	Northern Works		
10 January 1985	Anaerobic zone	11,39	5,88
	Primary anoxic zone	83,19	42,92
	Primary aerobic zone	11,39	5,88
	Secondary anoxic zone	12,98	6,70
	Secondary aerobic zone	19,98	10,31
	Effluent	21,98	11,34
11 January 1985	Anaerobic zone	76,79	39,62
	Primary anoxic zone	32,79	16,92
	Primary aerobic zone	40,39	20,84
	Secondary anoxic zone	85,98	44,36
	Secondary aerobic zone	79,99	41,27
	Effluent	30,62	15,80
14 January 1985	Anaerobic zone	104,10	54,10
	Primary anoxic zone	102,18	52,72
	Primary aerobic zone	105,79	54,58
	Secondary anoxic zone	67,18	34,66
	Secondary aerobic zone	78,79	40,65
	Effluent	44,31	22,86

Appendix II – (Continued)

Date	Sampling point	Average number of *Acinetobacter* per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
15 January 1985	Anaerobic zone	14,59	7,53
	Primary anoxic zone	6,99	3,61
	Primary aerobic zone	32,58	16,81
	Secondary anoxic zone	22,19	11,45
	Secondary aerobic zone	17,79	9,18
	Effluent	11,04	5,70
16 January 1985	Anaerobic zone	87,57	45,18
	Primary anoxic zone	70,98	36,62
	Primary aerobic zone	72,49	37,40
	Secondary anoxic zone	87,18	44,98
	Secondary aerobic zone	86,27	44,15
	Effluent	25,77	13,30

3. Calculation of the total number of bacteria in activated sludge using the AO technique (paragraph 3.2.8c(2)).

The equation in paragraph 3.3.1 was used for all calculations :

Date	Sampling point	Average number of bacteria per micro-scope field	Average number of bacteria $cm^{-3} \times 10^8$
	Northern Works		
10 January 1985	Anaerobic zone	18,60	1,92
	Primary anoxic zone	12,30	1,27
	Primary aerobic zone	16,37	1,69
	Secondary anoxic zone	9,20	0,95
	Secondary aerobic zone	4,55	0,47
	Effluent	25,97	2,68
11 January 1985	Anaerobic zone	15,40	1,59
	Primary anoxic zone	63,48	6,55
	Primary aerobic zone	32,46	3,35
	Secondary anoxic zone	25.48	2,63
	Secondary aerobic zone	23,74	2,45
	Effluent	3,97	0,41
14 January 1985	Anaerobic zone	45,55	4,70
	Primary anoxic zone	45,84	4,73
	Primary aerobic zone	45,16	4,66
	Secondary anoxic zone	50,10	5,17
	Secondary aerobic zone	62,22	6,42
	Effluent	36,05	3,72

Appendix II – (Continued)

Date	Sampling point	Average number of bacteria per microscope field	Average number of bacteria $cm^{-3} \times 10^8$
15 January 1985	Anaerobic zone	43,32	4,47
	Primary anoxic zone	44,48	4,59
	Primary aerobic zone	55,53	5,73
	Secondary anoxic zone	24,51	2,53
	Secondary aerobic zone	15,11	1,56
	Effluent	28,10	2,90
16 January 1986	Anaerobic zone	46,71	4,82
	Primary anoxic zone	70,94	7,32
	Primary aerobic zone	64,35	6,64
	Secondary anoxic zone	63,38	6,54
	Secondary aerobic zone	75,88	7,83
	Effluent	37,99	3,92

4. Calculation of the total number of bacteria in activated sludge density gradient fractions using the AO technique (paragraph 3.2.8c(2)).
The equation in paragraph 3.3.1 was used for all calculations

Sample number	Fraction	Average number of bacteria per microscope field	Average number of bacteria $cm^{-3} \times 10^7$
1	1	17,83	18,400
	2	0,89	0,927
	3	7,43	7,670
	4	0,84	0,875
	5	0,77	0,798
	6	0,96	1,000
	7	4,39	4,530
	8	1,81	1,870
	9	2,34	2,420
	10	0,28	0,296
2	1	3,05	3,1500
	2	10,46	10,800
	3	4,73	4,890
	4	0,96	1,000
	5	5,18	5,350
	6	6,57	6,780
	7	6,57	6,780
	8	1,67	1,730
	9	2,28	2,36
	10	0,12	1,28

5. Calculation of the *Acinetobacter* and metachromatic stained bacteria numbers in activated sludge gradient fractions using the FA technique (paragraph 3.2.8c(2)). The equation in paragraph 3.3.1 was used for all calculations

5.1 *Acinetobacter* numbers

Sample number	Fraction	Average *Acinetobacter* number per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
1	3	126,18	65,10
	4	15,23	7,86
	5	12,36	6,38
2	3	6,97	3,60
	4	15,73	8,12
	5	5,98	3,09

5.2 Metachromatic stained bacteria number

Sample number	Fraction	Average metachromatic stained bacteria number per microscope field	Average number of metachromatic stained bacteria $cm^{-3} \times 10^6$
1	3	114,94	59,30
	4	14,61	7,54
	5	11,53	5,95
2	3	2,86	1,48
	4	14,61	7,54
	5	2,86	1,48

$= 0,34 \times 10^{-4}$

5. Calculation of the *Acinetobacter* and metachromatic stained bacteria numbers in activated sludge gradient fractions using the FA technique (paragraph 3.2.8c(2)). The equation in paragraph 3.3.1 was used for all calculations

5.1 *Acinetobacter* numbers

Sample number	Fraction	Average *Acinetobacter* number per microscope field	Average number of *Acinetobacter* $cm^{-3} \times 10^6$
1	3	126,18	65,10
	4	15,23	7,86
	5	12,36	6,38
2	3	6,97	3,60
	4	15,73	8,12
	5	5,98	3,09

5.2 Metachromatic stained bacteria number

Sample number	Fraction	Average metachromatic stained bacteria number per microscope field	Average number of metachromatic stained bacteria $cm^{-3} \times 10^6$
1	3	114,94	59,30
	4	14,61	7,54
	5	11,53	5,95
2	3	2,86	1,48
	4	14,61	7,54
	5	2,86	1,48

$= 0,34 \times 10^{-4}$

1.1.5 Error SS = total SS – replicate SS – treatment SS

= $2,73 \times 10^{14} - 0,34 \times 10^{14} - 1,75 \times 10^{14}$

= $0,64 \times 10^{14}$

1.2 Analysis of variance using the abovementioned data. (The results are represented in Table 6).

1.2.1 Degrees of freedom (df)

Degrees of freedom for treatments = r–1 = 6–1 = 5

Degrees of freedom for blocks = t–1 = 8–1 = 7

Error degrees of freedom = (r–1) (t–1) = 35

Total degrees of freedom = (rt–1) = 47

1.2.2 Adjusted sums of squares (SS)

Treatment SS = $1,75 \times 10^{14}$

Block SS = $0,34 \times 10^{14}$

Error SS = $0,64 \times 10^{14}$

Total SS = Block SS + Treatment SS + Error SS

= $2,73 \times 10^{14}$

1.2.3 Mean sum of squares (MS)

Treatment MS = Block SS/Block df

= $1,75 \times 10^{14}/5$

= $3,49 \times 10^{13}$

Block MS = Treatment SS/Treatment df

= $0,34 \times 10^{14}/7$

= $4,85 \times 10^{12}$

Error MS = Error SS/Error df

= $0,64 \times 10^{14}/35$

= $1,82 \times 10^{12}$

1.2.4 Calculated F values

F block = Treatment MS/Error MS
= $4,85 \times 10^{12} / 1,82 \times 10^{12}$
= 2,66

F treatment = Block MS/Error MS
= $3,49 \times 10^{13} / 1,82 \times 10^{12}$
= 18,96

1.2.5 Tabular F values

Tabular F0,05 for the blocks = Tabular value for 7 and 35 degrees of freedom = 2,29

Tabular F0,01 for the blocks = Tabular value for 7 and 35 degrees of freedom = 3,21

Tabular F0,05 for the treatments = Tabular value for 5 and 35 degrees of freedom = 2,49

Tabular F0,01 for the treatments = Tabular value for 5 and 35 degrees of freedom = 3,60

1.3 The adjusted sums of squares for the values in Table 9 were determined as follows: (These values are not listed in Table 5).

1.3.1 Correction term (C) $= \frac{X^2}{rt}$

$= \frac{(797,68 \times 10^6)^2}{30}$

$= 2,12 \times 10^{16}$

1.3.2 Total sum of squares (SS)

$= \sum_{i,j} X^2 i.j. - C$

$= 2,19 \times 10^{15} + ... + 8,96 \times 10^{15} - 2,12 \times 10^{16}$

$$= 6{,}63 \times 10^{15} \mp \ldots\ldots \mp 1{,}11 \times 10^{15} - 2{,}12 \times 10^{16}$$

$$= 8{,}3 \times 10^{15}$$

1.3.3 Treatment SS $= \dfrac{\sum_j X^2 .j - C}{t}$

$$= \frac{(152{,}31 \times 10^6)^2 \mp \ldots\ldots \mp (69{,}0 \times 10^6)^2}{5} - 2{,}12 \times 10^{16}$$

$$= 1{,}0 \times 10^{15}$$

1.3.4 Block SS $= \dfrac{\sum_i X^2 i - C}{r}$

$$= \frac{(83{,}03 \times 10^6)^2 + \ldots\ldots + (221{,}99 \times 10^6)^2}{6} - 2{,}12 \times 10^{16}$$

$$= 5{,}2 \times 10^{15}$$

1.3.5 Error SS = total SS – replicate SS – treatment SS

$$= 8{,}3 \times 10^{15} - 5{,}2 \times 10^{15} - 1{,}0 \times 10^{15}$$

$$= 2{,}10 \times 10^{15}$$

1.4 Analysis of variance using the abovementioned data. (The results are represented in Table 6).

1.4.1 Degrees of freedom (df)

Degrees of freedom for treatments = r–1 = 6–1 = 5
Degrees of freedom for blocks = t–1 = 5–1 = 4
Error degrees of freedom = (r–1) (t–1) = 20

Total degrees of freedom = (rt–1) = 29

1.4.2 Adjusted sums of squares (SS)

Treatment SS	=	$1{,}00 \times 10^{15}$
Block SS	=	$5{,}20 \times 10^{15}$
Error SS	=	$2{,}10 \times 10^{15}$
Total SS	=	Block SS + Treatment SS + Error SS
	=	$8{,}30 \times 10^{15}$

1.4.3 Mean sum of squares (MS)

Treatment MS	=	Block SS/Block df
	=	$1{,}00 \times 10^{15}/5$
	=	$2{,}00 \times 10^{14}$
Block MS	=	Treatment SS/Treatment df
	=	$5{,}20 \times 10^{15}/4$
	=	$1{,}30 \times 10^{15}$
Error MS	=	Error SS/Error df
	=	$2{,}10 \times 10^{15}/20$
	=	$1{,}05 \times 10^{14}$

1.4 .4 Calculated F values

F block	=	.Treatment MS/Error MS
	=	$1{,}30 \times 10^{5}/1{,}05 \times 10^{14}$
	=	12,38
F treatment	=	Block MS/Error MS
	=	$2{,}00 \times 10^{14}/1{,}05 \times 10^{14}$
	=	1,90

1.4.5 Tabular F values

Tabular F0,05 for the blocks	=	Tabular value for 4 and 20 degrees of freedom = 2,87
Tabular F0,01 for the blocks	=	Tabular value for 7 and 20 degrees of freedom = 4,43
Tabular F0,05 for the treatments	=	Tabular value for 5 and 20 degrees of freedom = 2,71
Tabular F0,01 for the treatments	=	Tabular value for 5 and 20 degrees of freedom = 4,10

APPENDIX IV

1. Duncan's new multiple range test for determining the least significant difference.

1.1 Determination of Sx

$$Sx = \sqrt{(\text{error mean square})/r}$$

$$= \sqrt{(1{,}82 \times 10^{12})/8}$$

$$= 4{,}76 \times 10^{5}$$

1.2 Determination of the Significant studentized ranges (SSR) at the 5% level and the least significant ranges (LSR)

Error degrees of freedom = 35

p = 2 to 6

Value of p	2	3	4	5	6
SSR	2,870	3,020	3,110	3,185	3,235
LSR	$1{,}36 \times 10^{6}$	$1{,}43 \times 10^{6}$	$1{,}48 \times 10^{6}$	$1{,}51 \times 10^{6}$	$1{,}54 \times 10^{6}$

LSR = (SR) x (Sx)

APPENDIX V

1.1 Calculation of the *Acinetobacter* cell and volutin volume.

The equations in paragraph 3.3.7 were used to colculate the *Acinetobacter* cell and volutin volume.

Cell No.	*Acinetobacter* cell volume μm^3	Volutin volume μm^3
1	0,29	0,038
2	0,12	0,060
3	3,79	1,140
4	4,18	0,960
5	0,59	0,220
6	1,40	0,380
7	1,43	0,690
8	3,33	0,520
9	1,99	1,000
10	1,88	0,500
11	1,79	0,500
12	1,79	0,500
13	1,76	0,380
14	0,69	0,260
15	0,35	0,060
16	0,86	0,120
17	2,00	0,120
18	0,56	0.310
19	0,20	0,090
20	0,70	0,020
21	0,51	0,220
22	0,52	0,110
23	0,52	0,220
24	0,52	0,220
25	0,26	0,030
26	0,52	0,220
27	0,52	0,110
28	0,52	0,110
29	0,80	0,200
30	0,90	0,700
31	0,90	0,600
32	2,29	0,600
33	0,69	0,110

Appendix V – (Continued)

Cell No.	*Acinetobacter* cell volume μm^3	Volutin volume μm^3
34	1,14	0,140
35	0,30	0,110
36	0,38	0,110
37	0,38	0,110
38	1,13	0,120
39	0,52	0,170
40	1,52	0,120
41	1,68	0,220
42	2,14	0,100
43	0,90	0,380
44	0,90	0,520
45	0,57	0,030
46	0,17	0,130
47	0,17	0,060
48	0,98	0,060
49	0,50	0,030
50	0,52	0,170
51	0,49	0,146
52	0,26	0,179
53	1,08	–
54	1,67	0,900
55	1,00	0,195
56	1,99	0,232
57	1,52	0,905
58	0,26	0,040
59	0,29	0,030
60	0,12	0,060
x	1,0	0,28
s	0,8	0,27

1.2 Calculations based on the results in Table 6.

1.2.1 Calculation of the total cell volume of *Acinetobacter* in activated sludge.

The total *Acinetobacter* cell volume = ($\bar{x}$ total *Acinetobacter* number in the primary aeration zone) x ($\bar{x}$ *Acinetobacter* cell volume) = 6,46 x 10^6 x 1,0 = 6,46 x 10^6 μm^3.

1.2.2 Calculation of the total volutin volume in the primary aeration zone of activated sludge.

The total volutin volume = ($\bar{x}$ total *Acinetobacter* number in the primary aeration zone) x ($\bar{x}$ volutin volume) = 6,46 x 10^6 x 0,28 = 1,80 x 10^6 μm^3.

1.2.3 Calculation of the total volutin mass.

Total volutin mass = ($\bar{x}$ total volutin volume) x (the specific gravity of the volutin)
= 1,80 x 10^6 μm^3 x 1,23 g dm^{-3} x 10^3
= 2,21 mg dm^{-3}

1.2.4 Calculation of the total phosphorus mass.

Total phosphorus mass
= (total volutin mass) x (estimated phosphorus content of the volutin)
= 2,21 mg dm^{-3} x 27%
= 0,59 mg dm^{-3}

1.2.5 Calculation of the maximum and minimum number of *Acinetobacter* in the primary aeration basin.

(a) The maximum number of *Acinetobacter*

= maximum *Acinetobacter* number in the primary aeration zone
= 7,60 x 10^6 cells cm^{-3}

(b) The minimum number of *Acinetobacter*

= minimum *Acinetobacter* number in the primary aeration zone
= 4,30 x 10^6 cells cm^{-3}

1.2.6 Calculation of the maximum and minimum total cell volume.

(a) Maximum total cell volume

= (maximum number of *Acinetobacter*)
x ($\bar{x}$ cell volume)
= (7,60 x 10^6) x 1,00 μm^3
= 7,60 x 10^6 μm^3

(b) Minimum total cell volume

= (minimum number of *Acinetobacter*)
x ($\bar{x}$ cell volume)
= (4,30 x 10^6) x 1,00 μm^3
= 4,30 x 10^6 μm^3

1.2.7 Calculation of the maximum and minimum volutin volume.

(a) Maximum volutin volume

= (Maximum number of *Acinetobacter*)
x ($\bar{x}$ volutin volume)
= (7,60 x 10^6) x 0,28 μm^3
= 2,12 x 10^6 μm^3

(b) Minimum volutin volume

= (minimum number of *Acinetobacter*)
x ($\bar{x}$ volutin volume)
= (4,30 x 10^6) x 0,28 μm^3
= 1,20 x 10^6 μm^3

1.2.8 Calculation of the maximum and minimum volutin mass.

(a) Maximum volutin mass

= (Maximum volutin volume)
x (specific gravity of the volutin)
= (2,12 x 10^6 μm^3) x 1,23 g dm^{-3}
= 2,60 mg dm^{-3}

(b) Minimum volutin mass

= (minimum volutin volume)
x (specific gravity of the volutin)
= 1,20 x 10^6 μm^3 x 1,23 g dm^{-3}
= 1,47 mg dm^{-3}

1.2.9 Calculation of the maximum and minimum phosphorus mass.

(a) Maximum phosphorus mass

= (Maximum volutin mass)
x (percentage of volutin containing phosphorus)
= 2,60 mg dm^3 x 27%
= 0,70 mg dm^{-3}

(b) Minimum phosphorus mass

= (Minimum volutin mass)
x (percentage of volutin containing phosphorus)
= 1,47 mg dm^{-3} x 27%
= 0,39 mg dm^{-3}

1.3 Calculations based on the results in Table 7.

1.3.1 Calculation of the total cell volume of *Acinetobacter* in activated sludge.

The total *Acinetobacter* cell volume = ($\bar{x}$ total *Acinetobacter* number in the primary aeration zone) x ($\bar{x}$ *Acinetobacter* cell volume) = 2,71 x 10^7 x 1,0 = 2,71 x 10^7 μm^3.

1.3.2 Calculation of the total volutin volume in the primary aeration zone of activated sludge.

The total volutin volume = ($\bar{x}$ total *Acinetobacter* number in the primary aeration zone) x ($\bar{x}$ volutin volume) = 2,71 x 10^7 x 0,28 = 7,58 x 10^6 μm^3.

1.3.3 Calculation of the total volutin mass dm^{-3}

Total volutin mass = (x total volutin volume)
x (the specific gravity of the volutin x 10^3
= 7,58 x 10^6 μm^3 x 1,23 g cm^{-3} x 10^3
= 9,32 mg dm^{-3}

1.3.4 Calculation of the total phosphorus mass dm^{-3}

Total phosphorus mass
= (total volutin mass)
x (estimated phosphorus content of the volutin)
= 9,32 mg dm^{-3} x 27%
= 2,51 mg dm^{-3}

1.3.5 Calculation of the maximum and minimum number of *Acinetobacter* in the primary aeration basin.

(a) The maximum number of *Acinetobacter*
= maximum *Acinetobacter* number counted in the primary aeration zone
= 5,45 x 10^7 cells cm^{-3}

(b) The minimum number of *Acinetobacter*
= minimum *Acinetobacter* number counted in the primary aeration zone
= 5,88 x 10^6 cells cm^{-3}

1.3.6 Calculation of the maximum and minimum total cell volume.

(a) Maximum total cell volume
= (maximum number of *Acinetobacter*)
x (x cell volume)
= (5,45 x 10^7) x 1 μm^3
= 5,45 x 10^7 μm^3 cm^{-3}

(b) Minimum total cell volume

= (minimum number of *Acinetobacter*)
x ($\bar{x}$ cell volume)
= $(5,88 \times 10^6) \times 1\ \mu m^3$
= $5,88 \times 10^6\ cm^{-3}$

1.3.7 Calculation of the maximum and minimum volutin volume.

(a) Maximum volutin volume

= (maximum number of *Acinetobacter*)
x ($\bar{x}$ volutin volume)
= $(5,45 \times 10^7) \times 0,28\ \mu m^3$
= $1,52 \times 10^7\ \mu m^3\ cm^{-3}$

(b) Minimum volutin volume

= (minimum number of *Acinetobacter*)
x ($\bar{x}$ volutin volume)
= $(5,88 \times 10^6) \times 0,28\ \mu m^3$
= $1,64 \times 10^6\ \mu m^3\ cm^{-3}$

1.3.8 Calculation of the maximum and minimum volutin mass.

(a) Maximum volutin mass

= (maximum volutin volume)
x (specific gravity of the volutin)
= $(1,52 \times 10^7\ \mu m^3) \times 1,23\ cm^{-3}$
= $18,69\ mg\ dm^{-3}$

(b) Minimum volutin mass

= (minimum volutin volume)
x (specific gravity of the volutin)
= $(1,64 \times 10^6) \times 1,23\ g\ cm^{-3}$
= $2,01\ mg\ dm^{-3}$

1.3.9 Calculation of the maximum and minimum phosphorus mass.

(a) Maximum phosphorus mass

= (maximum volutin mass)
x (percentage of volutin containing phosphorus)
= 18,69 mg dm^3 x 27%
= 5,02 mg dm^{-3}

(b) Minimum phosphorus mass

= (minimum volutin mass)
x (percentage of volutin containing phosphorus)
= 2,01 mg dm^{-3} x 27%
= 0,54 mg dm^{-3}

APPENDIX VI

PLATE 8 Clusters of bacteria in activated sludge. AO stain.

PLATE 9 Dispersed bacteria in activated sludge using 0,5% Tripolyphosphate and sonication. AO stain.

PLATE 10 FA stained activated sludge after dispersion, dilution and filtration.

PLATE 11 FA stained activated sludge after dispersion, dilution and filtration.

PLATE 12A Density gradient centrifuged activated sludge.

B Standard used for drawing standard curve.

PLATE 13 Density gradient centrifuged activated sludge samples.

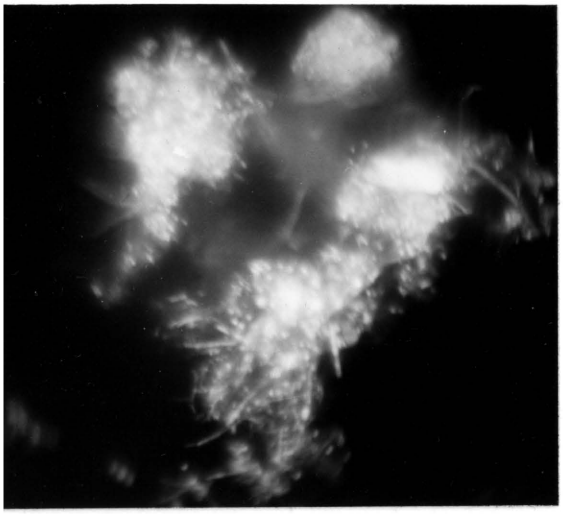


Plate 8

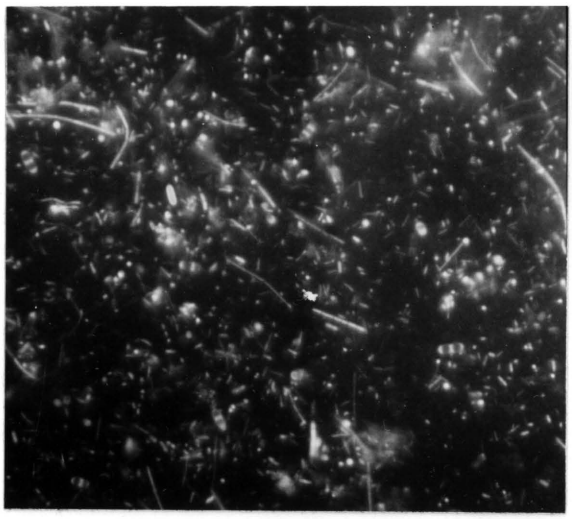


Plate 9

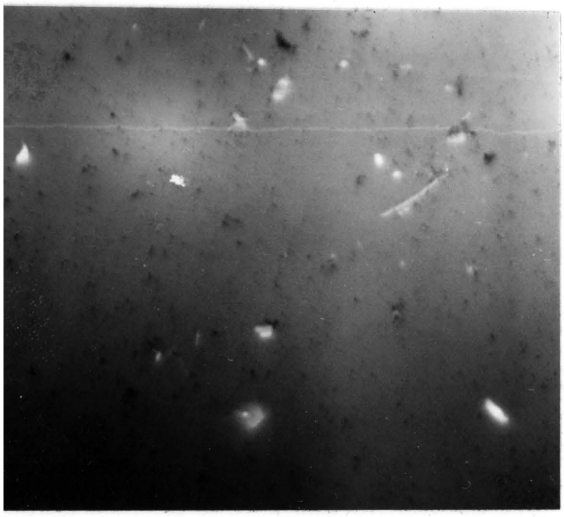


Plate 10

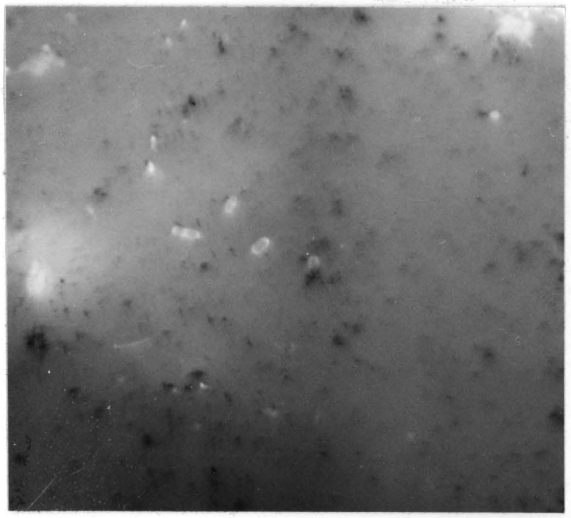


Plate 11

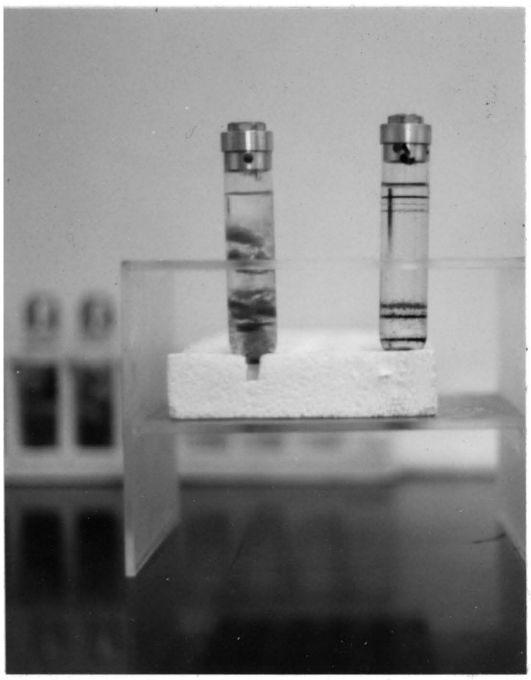


Plate 12

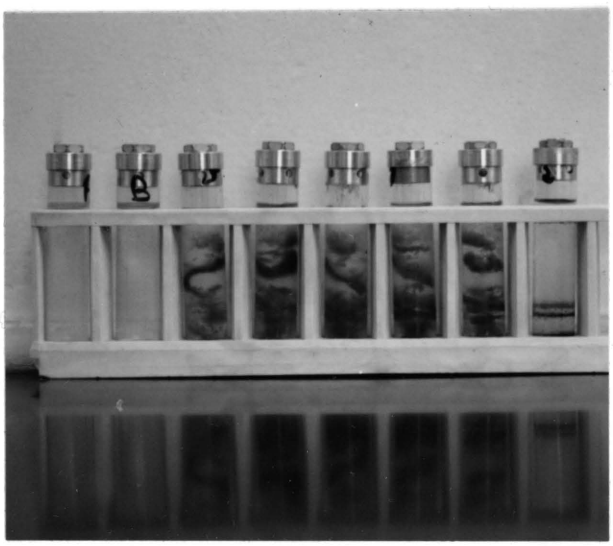


Plate 13