

**QUANTIFICATION OF PHOSPHORUS IN
EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)
ASSOCIATED WITH THE ACTIVATED SLUDGE FLOCS**

by

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or part has been submitted at any university for a degree.

Signature: _____

A handwritten signature in black ink, appearing to be 'A. J. van der ...', written over a horizontal line.

Date: _____

04/08/2003

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Summary

Several mechanisms have been proposed to explain the enhanced uptake of phosphorus by microorganisms in waste water. It has been shown that for biological phosphorus removal to occur in waste water treatment plants, biomass first needs to pass through an oxygen and nitrogen free phase, before entering the phase where an electron acceptor is present. It has been indicated that not all phosphorus removed in activated sludge systems can be accounted for by polyphosphate accumulating bacteria (PAO), the extracellular polymeric substances (EPS) also play a role in phosphorus removal. EBPR in waste water treatment has been studied for about 20 years in many countries and there are many successful full scale applications in the treatment of municipal waste water. The aim of this study was to quantify the concentration of phosphorus in the EPS and determine the chemical composition of the EPS. Activated sludge samples were collected from five different municipal waste water treatment plants situated in South Africa (Gauteng province). The phosphorus concentration was determined using the scanning electron microscopy combined with energy dispersive spectrometry (SEM/EDS). The amount of

phosphorus in activated sludge flocs plus EPS was 50.5% of the total elemental analysis on average and the EPS alone contained 25% of phosphorus on average. The anaerobic activated sludge and EPS samples contained higher concentration of orthophosphate than in the aerobic activated sludge and the aerobic EPS samples. This was due to the fact that the orthophosphate in the aerobic zone was consumed. The method for quantifying mixed liquor suspended solids (MLSS) based on freeze drying was comparable to conventional method, but had an advantage of yielding high concentration of MLSS.

Four different extraction methods, i.e. formaldehyde-NaOH, regular centrifugation (RCF), formaldehyde and EDTA were employed to study their effectiveness in extracting the extracellular polymeric substances from aerobic and anaerobic activated sludge samples. The formaldehyde-NaOH extraction procedure yielded the highest concentration of EPS as compared to the other three methods. In general, all the methods extracted about 44% protein, 33% carbohydrates and 1-3% DNA. The EPS sample contained more proteins than carbohydrates, which might be due to the fact that more exo-enzymes are entrapped in the EPS matrix. The results indicated that possible mechanism of phosphorus removal by EPS is that, phosphorus binds to cations (Mg^{2+} , K^+ etc.) to make a insoluble compounds (Magnesium-phosphate compounds, Calcium phosphate compounds etc.) which precipitates out in the EPS associated with the activated sludge flocs. In conclusion it may be postulated that potassium, magnesium and calcium are important cations needed in phosphorus removal in terms of phosphate found in the EPS. In order to understand the mechanism of phosphorus removal by EPS further research is recommended.

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Opsoming

Verskeie meganismes is voorgestel om die vermeerderde opname van fosfor, deur mikroörganismes in afloopwater, te verklaar. Daar is bewys dat biologiese fosfor verwydering, in afloopwater behandelings aanlegte, slegs plaasvind wanneer die biomassa deur 'n suurstof/stikstof vrye fase beweeg, voordat dit die fase binnegaan waar 'n elektron akseptant teenwoordig is. Dit is duidelik dat die polifosfaat akkumuleer bakterieë (PAO), nie alleenlik verantwoordelik gehou kan word, vir die verwydering van fosfaat in geaktiveerde slyk sisteme nie. Die ekstrasellulêre polimeriese stowwe (EPS) speel ook 'n rol in fosfaat verwydering. EBPR in afloopwater behandeling, is vir die afgelope 20 jaar in baie lande gestudeer en daar bestaan baie suksesvolle, groot skaalse toepassings in die behandeling van munisipale afloopwater. Die doel van hiedie studie is om die konsentrasie van fosfor in die EPS te kwantifiseer, asook om die chemiese samestelling van die EPS te bepaal. Geaktiveerde slyk monsters is versamel vanaf vyf verskillende munisipale afloopwater behandeling aanlegte, geleë in Suid-Afrika (Gauteng provinsie). Die fosfor konsentrasie is bepaal deur gebruik te maak van die skandeer

elektron mikroskoop in kombinasie met energie dispergeer spektrometrie (SEM/EDS). Die hoeveelheid fosfor in geaktiveerde slyk vlokke, tesame met EPS was gemiddeld 50.5% van die totale elementale analiese. Die EPS alleenlik bevat gemiddeld 25% fosfor. Die anaerobiese geaktiveerde slyk en EPS monsters bevat hoër konsentrasies orthofosfaat as die aerobiese monsters. Dit is as gevolg van die feit dat die orthofosfaat in die aerobiese zone verteer is. Die metode, gebaseer die vries droging, vir die kwantifisering van mixed liquor suspended solids (MLSS) was vergelykbaar met die konvensionele metode. Hierdie vries droog metode is meer voordelig, aangesien hoë konsentrasies van MLSS opgebeur word.

Vier verskillende ekstraksie metodes nl. Formaldehid-NaOH, gewone sentrifugasie (RCF), formaldehid en EDTA. Hierdie metodes se effektiwiteit, in die ekstraksie van EPS vanaf aerobiese en anaerobiese monsters, is gestudeer. In vergelyking met die ander drie metodes het formaldehid-NaOH die hoogste konsentrasie EPS opgelewer. Oor die algemeen het al vier metodes 44% proteïne, 33% koolhidrate en 1-3% DNA opgelewer. Die EPS monster het meer proteïne as koolhidrate bevat, dit mag as gevolg van die feit wees dat meer ekso-ensieme vasgevang is in die EPS matriks. Die resultate dui op 'n moontlike meganisme vir fosfor verwydering deur EPS. Die fosfor bind aan die katione (Mg^{2+} , K^+ ens.) om onoplosbare verbindings (Magnesium-fosfaat verbindings, Kalium-fosfaat verbindings ens.) te vorm. Hierdie verbindings presipiteer uit die EPS geassosieer met die geaktiveerde slyk vlokke.

Ten slotte kan die volgende stelling gemaak word. Kalium (K^+), Magnesium (Mg^{2+}) en Kalsium (Ca^{2+}) is belangrike katione, nodig in die verwydering van fosfor in terme van die fosfaat gevind in die EPS. Verdere studie word aanbeveel, om die meganisme van fosfor verwydering deur EPS, te verstaan.

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CHAPTER 1

1. Introduction

In many countries including South Africa, water supplies are limited and droughts are a constant threat, making it essential to protect the aquatic environment from pollution. In many industrial countries phosphorus removal from waste water is required by the authorities, some also have set limits for nitrogen discharges. The requirement for nutrient removal usually involves the treatment of municipal wastewater (Pauli, 1994). Eutrophication is one of the processes which usually occurs from water pollution. Nutrient removal from waste water is thus important to prevent eutrophication (Cloete and Bosch, 1993).

Nutrient removal from waste water treatment is important to prevent eutrophication. Phosphorus removal is seen as one of the best method for eutrophication control. In many countries across the world large sums of money have been invested in the development of activated sludge plants for carbon, nitrogen and phosphorus removal from waste water. The orthophosphate concentration of effluents in South Africa has been limited to 1mg P/l by an amendment of the Water Act, Act no 54, 1956 (Slim, 1987). Phosphates can be removed from waste water by chemical precipitation or biological accumulation. Although the activated sludge systems are operating successfully, the mechanisms by which phosphate removal takes place are still not well understood, and due to this lack of knowledge, some biological waste water systems are unable to meet legislated standards. Currently FeCl_3 is being added to most of South Africa 's activated sludge systems to remove phosphorus to legislated levels. Biological phosphate removal, as an alternative to chemical removal, has gained support and activated sludge processes have been designed and operated for excess phosphate removal (Barnard, 1976).

Microorganisms have a vital role to play in both the aerobic and anaerobic treatment of waste water. They degrade the organic material present in the wastewater and convert the resultant products into microbial biomass, humic material, and waste gases such as

carbon dioxide, nitrogen compounds and methane (Houghton and Quarmby, 1999). Biopolymers (also referred to in the literature as extracellular polymeric substances (EPS), exocellular polysaccharides, exopolymeric substances, exopolymers, or microbial soluble products) are produced by many different types of bacteria. They are found either in a capsule like structure associated with the outer cell wall, or in a solution as loose slime polymers that are completely detached from the cell wall. Biopolymers (EPS) are of major importance in waste water treatment. Biopolymers act in conjunction with divalent metals ions, such as calcium, to aid the formation and settlement of sludge flocs in both aerobic and anaerobic treatment systems (Christensen, 1989). EPS is one of the main components of biofilms, which are utilized in treatment processes, such as membrane bioreactors, rotating biological contact units, and trickling filters. The structure of the EPS network determines many of the physical properties of the biofilm (Christensen, 1989).

Cloete and Oosthuizen (2001) indicated that EPS might play a role in biological phosphorus removal in activated sludge. However the contribution of EPS to the overall biological phosphorus removal process has not yet been quantified.

The aim of this study was to :

- quantify the concentration of phosphorus in the EPS associated with the activated sludge flocs
- understand the role played by EPS in phosphorus removal from waste water treatment systems
- use the freeze drying methods to quantify the concentration of EPS in activated sludge sample
- determine the chemical composition of the EPS

CHAPTER 2

Literature Review

1. Eutrophication

Eutrophication is a natural ageing process, which usually occurs in lakes or other quiescent bodies of water through introduction of plant nutrients like phosphorus and nitrogen to the impoundment. The process occurs naturally over hundreds of years, but the process is been greatly accelerated by human activities in sensitive areas, including the use of detergents and fertilizers. This enrichment of water in a impoundment results in some undesirable effects, the primary effect being the establishment of profuse algal blooms and excessive growth of nuisance aquatic plants.

The algal population which blooms as a result of the lake or pond becoming rich in nutrients which eventually crashes as a result of exhaustion of macronutrients or accumulation of toxic by products (Cloete and Oosthuisen, 2001). Therefore efficient methods have to be used to clean the water to make sure that potable water can be supplied.

1.1.1 End results caused by eutrophication

- Rapid oxygen uptake from the water causing lower layers of the water to be anaerobic
- Production of methane and sulphides from anaerobic digestion of dead plants which sink to the bottom of the water body
- These anaerobic acid conditions which occur cause the release of bound phosphates, iron and manganese from sediments
- Production of sulphides result in fish kills and a negative aesthetic appeal of the dam due to overturning of the water and the appearance that the water is black

- Sulphide also causes a strong rotten egg smell; the water can no longer be used for potable consumption purposes or for livestock or humans as certain species of the algae are toxic

The limitation of phosphorus to aquatic environments is thus seen as the best long-term solution to control eutrophication (Toerien *et al.*, 1975). Therefore efficient phosphate removal, from industrial and domestic waste water is essential. Phosphate removal from waste water has received considerable attention due to the effect on the eutrophication of natural waste water. There are chemical and biological methods of removing phosphate from the waste water. Nowadays biological phosphate removal is used all over the world as one of the best methods and has become the most commonly used method of liquid waste treatment (Striechen *et al.*, 1997).

There are many benefits for utilizing biological nutrient removal (BNR) for treatment of wastewater. They may be classified as environmental benefits, economical and operational benefits and the control of eutrophication in the effluent receiving water, which is an environmental benefit. Historically, treatment requirements were determined by the need to protect the oxygen resources of the receiving water and this was accomplished primarily through the removal of solids and dissolved organic matter from the waste water before discharge (Randall *et al.*, 1992).

2. The use of activated sludge as a process of biological phosphate removal

The activated sludge process has become the most commonly used method of liquid waste water treatment. The term "activated sludge" is generally used to denote an aerobic slurry of microorganisms which remove the organic matter from the wastewater and are then removed themselves usually by sedimentation and returned to the waste water stream (Grady and Lim, 1980). This process can be compared with a complex continuous culture with partial recycling of cells. Microbes are returned in to the system as activated sludge.

The activated sludge process consists mainly of two processes including aeration and sludge settlement in the clarifier. During microbial growth and respiration the organic matter of the effluent is converted into a new microbial biomass and inorganic compounds e.g.(CO₂). Organic matter and nutrients are removed from the system by taking out the biomass. The rest of the biomass is returned to the aeration tank (so called return sludge or recycled sludge) (Pauli, 1994).

There are, a number of activated sludge systems which have been developed and are now used, namely Bardenpho, UCT, VIP, Biotenpho, etc. All of these systems perform the same function in a similar way, the difference being the number of stages that are involved in the system. In some cases you may find that the system is just a modification of the previous in order to attain enhanced biological phosphate removal (EBPR). Generally an activated sludge system must include one or more unaerated zones and be a single sludge system in order to accomplish biological nutrient removal. Single sludge system means that each train contains only one secondary clarifier and settled biomass must be recycled through all the zones. Phosphorus removal can be maximized by placing the anaerobic zone first, so that the phosphorus-removing bacteria have the first opportunity to utilize the organic substrate. Herewith follows a brief description of the activated sludge processes.

2.1 The Pherodox activated sludge system (5-Stage Bardenpho activated sludge system)

The Pherodox system consists of an anaerobic zone followed by a primary anoxic zone and a primary aerobic zone, then a secondary anoxic and aerobic zone and lastly the clarifier. In this system, sludge is returned from the clarifier to the anaerobic zone. Mixed liquor is returned from the primary aerobic zone to the primary anoxic zone.

2.1.1. The primary anaerobic zone

The primary anaerobic zone is the zone where all the influent wastewater flows together with the return sludge. This zone is enriched by fermentative bacteria such as *Citrobacter*, *Proteus*, *Serratia*, *Enterobacter* etc (Buchan, 1984). The fermentative products formed in this zone such as lactic acid, succinic acid cannot be utilised and are to be used further on in the other zones (Murphy and Lotter, 1985). Organic materials are broken down into simpler substances or inorganic material such as phosphorus and nitrogen. Since microorganisms are only capable of accumulating polyphosphate under aerobic conditions, the anaerobic zone contains a high concentration of orthophosphate, due to the release of phosphate by microorganisms in this zone (Lotter, 1985).

2.1.2. The primary anoxic zone

The primary anoxic zone is where denitrification takes place and in this zone, microorganisms capable of denitrification, such as *Thiobacillus denitrificans*, *Pseudomonas spp* and others are found and play a major role.

2.1.3. The primary aerobic zone

The primary aerobic zone, is the zone where nitrification occurs and phosphate uptake takes place. Ammonium ions are oxidized to nitrite ions and then to nitrate ions and the microorganisms responsible for oxidation of ammonium ions to nitrite ions are, for example *Nitrosomonas*, *Nitrococcus* and microorganism responsible of oxidation to nitrate ions are amongst others *Nitrobacter*, *Nitrospira* (Cloete and Bosch, 1993). Many of the microorganisms found in this zone are capable of storing large quantities of polyphosphate, in the form of intracellular volutin granules. These microorganisms include *Pseudomonas*, *Aeromonas*, *Flavobacterium* and *Acinetobacter* (Cloete and Bosch, 1993). Some filamentous bacteria are also present in this zone and play an important role in floc formation, and this increases the sedimentation ability of the sludge.

2.1.4. The secondary anoxic zone

The secondary anoxic zone is where denitrification of any nitrates still present in the system occurs. From this zone the mixed liquor flows into the secondary aerobic zone where phosphate uptake can finally occur.

2.1.5. The clarifier

This is the last zone of the system where sedimentation of the flocs is allowed to take place so that the effluent (clean water) is easily removed from the system. Most of the sediment sludge is removed and it is mainly used as fertilizers and a small portion of the activated sludge is returned to the incoming waste water effluent which will serve as an inoculum.

3. Factors affecting phosphate removal in biological phosphate removal

3.1. pH of the mixed liquor

Not much work has been done specifically on the effect of pH on the functioning of *Acinetobacter* (Randall *et al.*, 1992). Due to the way in which the activated sludge system operates, more energy will be required to maintain the proton balance and thus to take up acetates against a higher H^+ concentration in the aerobic basin. Therefore overall phosphate removal may be reduced with a decrease in the pH value. This also suggests that the pH in the aerobic basin may be high. In many cases, optimum denitrification is required to restore the pH value.

3.2. Temperature

There are number of factors which affects the functioning of EBPR, but temperature seems to be the most uncontrollable, particularly in the field (Panswad *et al.*, 2003). Waste water treatment can plants can be subjected to wide temperature ranges giving a strong need to determine possible influences of temperature variations on the process. Most studies can be categorized studies can be categorized in those looking at the efficiency of the EBPR processes under varying temperatures, those which focus on the kinetics of the processes and also the effects of temperature on the stoichiometry of the EBPR process (Baetens, 2001). Looking at the EBPR efficiency at different temperatures, results from literature are revealing contrasting observations. Some studies indicated that phosphorus removal is achieved at low temperatures (5⁰C to 15⁰C) (Sell *et al.*, 1981; Erdal, 2002; Panswad *et al.*, 2003). The reason for better system performance being reduced competition for substrate in the non-oxic zones that results in an increased population of polyphosphate accumulating organisms (PAOs) relative to non-PAOs and, therefore resulting in greater EBPR efficiency. This is due to the fact that some of the PAOs are psychrophilic, i.e., they have alternate biochemical pathways that give them a competitive advantage over bacteria dependent upon glycogen metabolism. In the other hand higher EBPR efficiency are observed at elevated temperatures (20⁰C to 37⁰C) (McClintock *et al.*, 1993; Converti *et al.*, 1995; Jones and Stephenson, 1996).

3.3. Leaching from waste sludge

Since phosphates are released from the sludge under anaerobic conditions, it follows that anaerobic treatment will release phosphate to the liquid phase. Dewatering of sludge for disposal must be done under aerobic conditions to avoid too much leaching back. (Burdick *et al.*, 1982).

3.4. Maintaining the dissolved oxygen concentration

While some anaerobic and anoxic zones may be required for some reasons, a sufficient dissolved oxygen (DO) level is required in the aeration tank for phosphate uptake by Phosphate Accumulating Organisms (PAO).

3.5. Substrate

Certain substrates promote phosphate release while others promote phosphate uptake. The evidence given by Buchan. (1984) indicated that *Acinetobacter* growth in activated sludge decreases from butyrate, propionate, acetate, isobutyrate, ethanol. The substrate that produced the slowest growth, namely isobutyrate and ethanol also produced the largest polyphosphate inclusions. Cloete and Steyn (1988) found that mostly the smaller *Acinetobacter* cells contained polyphosphate granules, thus indicating that polyphosphate is mostly accumulated by slow growing cells.

4. Enhanced Biological Phosphate Removal in the Activated Sludge (EBPR)

Several mechanisms have been proposed to explain the enhanced uptake of phosphorus by microorganisms in waste water. It has been shown that for biological phosphorus removal to occur in waste water treatment plants, biomass first needs to pass through an oxygen and nitrogen free phase, i.e. an anaerobic phase, before entering the phase where an electron acceptor is present, i.e. anoxic phase where nitrate is present or an aerobic phase where oxygen is present. EBPR in waste water treatment has been studied for about 20 years in many countries and there are many successful full scale applications in the treatment of municipal waste water (Hysteen, 1990).

4.1. Polyphosphate (poly-P)

Polyphosphate is a polymer consisting of condensed inorganic phosphate with tetrahedral groups linked together by oxygen bridges. Mg, Ca, K, Mn and Zn are known to be

significant in polyphosphate metabolism (Buchan, 1981). In polyphosphate accumulating bacteria Mg is released to and taken up simultaneously with phosphate. Over the years there has been considerable debate as to whether the EBPR process was the consequence of biological metabolic events or whether it was mostly due to physiochemical mechanisms. Chemical precipitation of phosphate on cell surfaces or bound to extracellular polymer has been proposed to explain P removal in activated sludge (Caberry and Tenney, 1973). Precipitation of hydroxyapatite $[\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6]$ in the sludge may result in P removal at higher pH. However, solubility calculations suggest that phosphate concentrations are not sufficiently high for this mechanism in EBPR activated sludge, and a removal of Ca^{2+} ions concomitant with the phosphate removal has not been observed. Another precipitation process of possible importance in EBPR involves extracellular polymers forming a matrix for the cells in flocs, binding metals and trapping metal phosphate. In that case polymer forming characteristics of the biomass would be important in P removal, and not particular organisms accumulating phosphorus. However in the study of sludges with varying P contents, the sludges with the best EBPR characteristics had the least proportion of their P as extracellular precipitated metal phosphates (Seviour and Blackall, 1999). The major proportion was accumulated in the cells as polyP. Other studies have also indicated that sludges with increased P removal have a higher percentage of their P as intracellular polyP (Appeldorn *et al.*, 1992).

4.2. Localization and structure of polyphosphate.

Large polyP molecules may form complexes with other polymers such as proteins and nucleic acids. Early examination of volutin granules by extensive staining suggests that the granules contained polyP associated with RNA and lipoprotein held together with Mg^{2+} . Support that polyP is complexed with other macromolecules arises from the analysis of extracted granules which contained major constituents P, Mg^{2+} , Ca^{2+} , K^+ , RNA, PHA and protein (Heymann *et al.*, 1989). However there is a doubt as to whether all these are actually constituents of volutin granules. Considerable evidence exists suggesting that the polyP in activated sludge is stabilized by metal cations.

The release and uptake of phosphate in activated sludge and cultures of *Acinetobacter* spp. coincides with fluctuations in the extracellular concentrations of Mg^{2+} , K^+ and Ca^{2+} (Gerber *et al.*, 1987). Electron microscopy energy dispersive X-ray analysis (EDX) on electron dense granules in sludge cells and in *Acinetobacter* spp. indicates that the granules are composed of P, Mg and K, the likely composition being polyP (Buchan 1982). From the samples examined so far it appears that polyP is certainly associated with these cations and they probably play a role in stabilizing the polymer.

Localization of polyP in cells in activated sludge has been observed and suggested to be in :

- ❖ Volutin granules of the cytoplasm
- ❖ The cell membranes, complexed with cytoplasmic RNA
- ❖ The periplasm

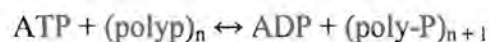
Numerous microscopic investigations have detected metachromatically staining intracellular granules in EBPR sludges (Seviour and Blackall, 1999).

4.3 Bacterial metabolism of polyphosphate

A number of methods that utilize or form poly-P have been identified and characterized from bacteria. Early interest was generated in poly-P metabolism partly due to the phenomena of 'overplus' and 'luxury' accumulation. Most bacteria do not normally accumulate poly-P unless induced by phosphate starvation or by limitation of nutrients. Overplus or luxury poly-P accumulation is equivalent to events that have been observed in *Escherichia coli* during phosphate starvation (Seviour and Blackall, 1999). Here phosphate starvation triggers a number of cellular events linked by the control of PHO regulon. Some studies have investigated the whole sludge and isolates for the presence of poly-P metabolising enzymes in attempts to better describe the EBPR metabolic processes.

4.3.1. Polyphosphate kinase

Poly-P is a highly-energy compound and it is assumed to form in conditions when energy is in excess. Its synthesis has been linked to the ATP-utilizing enzyme polyphosphate kinase (PPK) (Kulaev, 1979) whose activity has been observed in bacteria and eukarya to catalyse the reaction:



where n is the number of phosphate moieties. In activated sludge poly-P accumulation occurs in the aerobic zone, when the organism will have a high energy charge (ATP : ADP ratio), and these conditions will favour poly-P formation by PPK. PPK is associated with outer and inner membrane fusion of cell wall, which may have physiological significance as poly-P has been associated with cell membranes (Kornberg, 1995).

PPK may act to catalyse the above reaction towards the left. Thus, poly-P functions as an energy reserve polymer, and poly-P degradation produces ATP (Kulaev, 1979). Although there is a little direct evidence for the enzyme producing ATP in vivo, this activity could provide energy in the anaerobic zone of the EBPR, consistent with the biological EBPR model. PPK activity has been measured in activated sludge isolates and in activated sludge. Of several sludge isolates, *Acinetobacter* had the highest PPK activities, although only a few bacterial species were tested (T'Seyen *et al.*, 1985). In this same study PPK activity was relatively low in phosphate removing sludges. When levels of PPK activity and poly-P accumulating ability were investigated in a number of *Acinetobacter* activated sludge isolates, no correlation could be found between them (Visiliadis *et al.*, 1990).

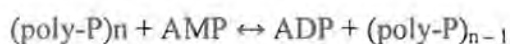
4.3.2. Polyphosphate glucokinase (and other sugar kinases)

Polyphosphate glucokinase catalyses the phosphorylation of glucose. Enzyme activity has been detected in bacteria from the mycobacteris and other actinomycetes (Kulaev and

Vagabov, 1983). High level of enzyme activity have been reported in some activated sludge isolates of unidentified coryneform bacteria (Bark *et al.*, 1993). These isolates were obtained from EBPR sludge and they could accumulate poly-P. The possible importance of this enzyme in EBPR has not been elucidated.

4.3.3. Polyphosphate: AMP phosphotransferase

Polyphosphate: AMP phosphotransferase (PPAT) catalyses the reversible reaction:



It is suggested that the degradation of poly-P may then be linked to the production of ATP in combination with the reaction catalyzed by the adenylate kinase (van Groenestijn and Dienema, 1987), described below:



One study into bacterial PPAT activity indicated that it required Mg^{2+} and was specific for AMP. Attempts have been made to obtain some correlation between this enzyme activity and poly-P accumulating ability in bacterial isolates. Good correlation between PPAT activity and poly-P accumulation was reported in certain *Acinetobacter* species (van Groenestijn and Dienema, 1987).

4.3.4. Polyphosphatase

Several enzymes can hydrolyse phosphoanhydride bonds in polyphosphates, without direct production of ATP or a source of phosphorylation. These enzymes include exo- and endopolyphosphatase (Wood, 1988). Bacterial exopolyphosphatase (PPX) is a major enzyme involved in poly-P degradation. The nature of PPX activity has been investigated

in *Kl. Aerogenes*. Mutants with no PPX activity synthesized poly-P, but poly-P degradation was inhibited (van Groenestijn *et al.*, 1987). In some bacteria the expression of the PPX gene has been associated with the PHO regulon. The activity of PPX has also been associated with cell membranes, indicating that its action may be on either intracellular or extracellular poly-P, or associated with the transport of phosphate (Akiyama *et al.*, 1993)

4.3.5. Possible functions of poly-P in activated sludge

The major biological functions which poly-P may participate have been discussed in a recent review (Kornberg, 1995). Possible functions in bacteria will include some of these;

- an energy reserve,
- a reserve of phosphate,
- a chelator metal ions,
- to buffer intracellular pH,
- a membrane channel for DNA,
- and a role in cellular stress and development responses (Seviour and Blackall, 1999).

In EBPR sludge it is not clear what the function of poly-P may be, yet accumulators must gain some selective advantage. Accumulators can compete with other bacteria, and in the anaerobic zone phosphate release coincides with carbon substrate uptake. So it appears that poly-P may be involved in an aspect of the anaerobic utilization of carbon substrate, and this is what is suggested in EBPR biological models (Seviour and Blackall, 1999).

4.4. Polyphosphate Accumulating bacteria (PAB)

According to the most commonly accepted theory, EBPR is based on the enrichment of activated sludge with bacteria capable of accumulating orthophosphate (PO_4^{3-}) in to the normal metabolic requirements of the cell. Many bacteria isolated from activated sludge,

for instance *Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Moraxella*, *Enterobacter*, some filamentous bacteria and others have been observed to accumulate polyphosphate in EBPR (Lotter 1985, Nakamura *et al.*, 1991, Okada *et al.*, 1992).

Excess phosphate uptake has been observed in various environments. In addition to waste water treatment the ability of microbes to accumulate phosphate has received attention in eutrophication and sediment studies (Sinke *et al.*, 1993). According to Cloete *et al.* (1985) and Lotter *et al.* (1986), EBPR would not be due to any major changes in the number of PAB, i.e. enrichments but instead, under appropriate conditions phosphate accumulation would be induced in the PAB already present in the activated sludge.

5. Microbial Ecology of activated sludge

The activated sludge processes like all other biological treatment processes, relies on a mixed culture of bacteria, with higher grazing microbes also present, forming a complete ecosystem which has various trophic levels. Microbes which play important role in activated sludge system include groups of bacteria, fungi, protozoa, rotifers and nematodes.

5.1. Bacteria

The bacteria constitute the major component of the activated sludge flocs and are the most active microorganisms in the system. The dominant bacteria found in the activated sludge flocs are *Pseudomonas*, *Acinetobacter*, *Corynebacteria*, *Commomonas*, *Bacillus* and others (Bitton, 1994). Both heterotrophic and chemolithotropic organisms are found in activated sludge. Activated sludge flocs harbor autotrophic bacteria such as nitrifiers (*Nitrosomonas*, *Nitrobacter*) which convert ammonium to nitrate and phototrophic bacteria such as the purple non-sulphur bacteria (*Rhodospirillaceae*), which are detected at concentrations of approximately 10 cells per ml of sludge. The purple and green sulfur bacteria are found at much lower levels (Bitton, 1994).

5.2. Fungi

Activated sludge does not usually favor the growth of fungi, although they may grow abundantly under specific conditions of low pH, toxicity and nitrogen deficient wastes. Some fungal filamentous microorganisms are occasionally observed in the activated sludge flocs. The predominant genera of fungi which are mostly found in the activated sludge flocs are *Geotrichum*, *Penicillium*, *Cephalosporium* and *Alternaria*.

5.3. Protozoa

The presence of protozoans is related to the effluent quality and plant performance. Protozoans play a secondary, but important role in the purification of aerobic waste water. Protozoans in the activated sludge treatment processes fall into four major classes: amoebae, flagellates ciliates and free-swimming ciliates. When amoebae are present in large numbers in the aeration basin, this usually indicates that there has been some sort of shock loading to the plant. Their presence also indicate that there is a low dissolved oxygen (D.O) environment in the aeration basin, because they can tolerate low concentrations of D.O. Protozoa are common components in activated sludge with population densities reaching up to 50 000 per ml. Three classes are important in activated sludge: the *Rhizopodia*, *Mastigophora*, and *Ciliophora*.

Most flagellates absorb dissolved nutrients. Flagellates and bacteria both feed on organic nutrients in the sewage as the nutrient level declines. If large numbers of flagellates are present in the later stages of the activated sludge development, this usually indicates that the waste water still contains a large amount of soluble organic nutrients (Gray, 1989).

Ciliates feed on bacteria and not on dissolved organics. While bacteria and flagellates compete for dissolved nutrients, ciliates compete with other ciliates and rotifers for bacteria. The presence of ciliates indicate a “good” sludge, because they dominate after the floc has been formed and after most of the organic nutrients have been removed.

6. Advantages of the activated sludge system for waste water treatment

- Flexibility
- Removes organics
- Oxidation and nitrification are achieved
- Biological nitrification is achieved without adding chemicals
- Biological nutrient removal (phosphate and nitrate)
- Solids/liquids separation
- Stabilization of the sludge is achieved
- Capable of removing approximately 97% of suspended solids.
- The most widely used waste water treatment processes

7. Disadvantages of the activated sludge system in waste water treatment

- Does not remove color from industrial wastes and may increase the color through formation of highly colored intermediates through oxidation.
- Does not remove all nutrients necessitating tertiary treatment
- Problem of getting well settled sludge
- Recycled biomass keeps high biomass concentrations in the aeration tanks

8. EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

8.1. What are Extracellular Polymeric Substances?

8.1.1. Definition

Microbial extracellular polymeric substances are high molecular weight mucous secretions of bacteria and microalgae. They range from tight capsules, which closely bind cells, to the loosely attached slime material. Biofilm EPS possesses many important functions in water and waste water treatment, including anchoring the microorganisms near food sources, protecting them from dehydration and toxic substances, and providing ion exchange due to negatively charged surface functional groups which allows them to

bind to cationic species such as heavy metals. EPS are mainly responsible for the structural and functional integrity of the biofilms and are considered as the key components that determine the physiochemical biological properties of the biofilm. Biofilms are an accumulation of microorganisms (prokaryotic and eukaryotic unicellular organism), extracellular polymeric substances (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds (Wingender *et al.*, 1999) EPS are biosynthesis polymers. EPS were defined as extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates (Geesey 1982).

EPS are also defined as organic macromolecules that are formed by polymerization of similar or identical building blocks of, which may be arranged as repeating units within the polymer molecule such as e.g. many polysaccharides. Activated sludge extracellular polymeric substances have been reported as a major sludge floc component (Urbain *et al.*, 1993; Liss *et al.*, 1996; Liao *et al.*, 2001; Frolund *et al.*, 1996; Bura *et al.*, 1998; Jorand *et al.*, 1998; Cloete and Oosthuisen, 2001) keeping the floc together in a three dimensional matrix due to bindings with bivalent cations and hydrophobic interactions (Frolund *et al.*, 1996). The EPS composition determines many important properties of biofilms such as density, porosity, diffusivity, strength, elasticity, frictional resistance, thermal conductivity and metabolic activity (Zhang *et al.*, 1999).

Individual (planktonic) bacterial cells have the ability to adhere to surfaces. Other planktonic bacteria can then attach to the adhered bacteria. This process of continuous adhesion eventually leads to multilayers of bacteria of the surface. A large amount of the extracellular polymeric substances (EPS) accompany the bacterial cells creating the matrix throughout the biofilms. A large part of floc structure in activated sludge is composed of EPS and attachment of bacterial cells to the surface is thought to be due to the presence of exopolymers (Cloete and Oosthuisen, 2001). The EPS of the contributing microbial flora provide a major part of the dry matter of biofilms, flocs and related structures. These polymers also play a role in determining the physical properties and structure of the microbial agglomerations. When biofilms or flocs are established, the

polysaccharide components of microbial origin may exhibit phenotypic differences from planktonic bacteria of the same species. However it is more likely that the microorganisms secrete exopolysaccharides identical in composition and probably also in physical properties with these formed the same bacteria when grown in planktonic cultures. (Wingender *et al.*, 1999)

8.1.2. Composition of the EPS

By definition, EPS are located at or outside the cell surface independent of their origin. EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as a repeating units within the polymer molecules (Wingender *et al.*, 1999). Polysaccharides have often been observed to be the most abundant component of the EPS in early biofilm research. Building blocks of the polysaccharides include: monosaccharides, uronic acids and amino sugars bound by glycosidic bonds, and which may have the organic substituents O-acetyl, N-acetyl, succinyl and puryl groups and the inorganic substituent groups sulphate and phosphate.

The chemical composition of the EPS is usually reported in literature to be very heterogeneous. Carbohydrate predominates and represents 65% of the extracellular polymeric substances, whilst other substances are also present, such as proteins, nucleic acids and lipids (Zhang *et al.*, 1999). The EPS component ratio varies, depending on the sample source. Activated sludge EPS has been found to have more carbohydrate than protein. Building blocks of proteins are amino acids linked by peptide bonds and may have the substituent oligosaccharides groups (in the case of glycoprotein) or fatty acids (in the case lipoproteins).

Nucleic acids are made up of nucleotides, while phospholipids consist of fatty acids, glycerol, phosphate, ethanolamine, serine, choline and sugars, linked by ester bonds. Humic substances are made up of phenolic compounds, simple sugars and amino acids, linked by ether bonds, C-C bonds and peptide bonds. Therefore, the EPS contains non

polymeric substituents of low molecular weight, which greatly alter their structure and physiochemical properties (Wingender *et al.*, 1999)

Most microbial polysaccharides are either homopolysaccharides composed of a single sugar unit or, heteropolysaccharides in which regular repeat units are formed from 2-8 monosaccharides. Bacterial EPS are mainly found to contain hexoses or methyl pentoses together with uronic acids of which D-glucuronic acid is most common bacterial EPS. The most widely known EPS is alginate from *Pseudomonas*. Bacterial alginates like their algal counterparts, are formed from irregular sequences of D-mannuronic acid and L-guluronic acid residues, but differ from the algal material in being heavily acetylated on many of the D-mannuronosyl residues (Sutherland, 2001).

The composition of EPS largely depends upon the method used for extraction. In general, no method has been developed which reliably yields complete extraction of EPS without contamination with the extracellular components. Nielsen and Jahn (1999) compared different methods of extracting EPS and the resulting quantities of EPS, where the highest concentration of EPS were polysaccharides, however the matrix also did contain other components such as proteins and nucleic acids (Flemming and Wingender, 2001) and also some lipids (Gehrke *et al.*, 1998).

8.2. Role and functions of the EPS

Biofilm may have both detrimental and beneficial effects. In either case their extracellular polymeric substances (EPS) play an important role, as this is the material which keeps microbial aggregates together and attaches biofilms to surfaces. The EPSs are prerequisite for existence of all microbial aggregates.

Table 1. Composition of EPS and range of component concentration (Flemming and Wingender, 2001)

Component	Content in the EPS
Polysaccharides	40-95%
Protein	< 1-60%
Nucleic Acids	< 1-10%
Lipids	< 1-40%

Although polysaccharides and proteins are the main EPS constituents in general, only the polysaccharide moiety has been investigated, while the role of proteins still remains largely unclear. They may be exoenzymes, but it is possible that some of the extracellular proteins have different functions.

The wealth of information about the bacterial EPS with respect to their genetics, biosynthesis, secretion, structure, function in natural, technical and pathogenic environments as well as their importance in industrial and medical application have been studied (Wingender *et al.*, 1999). The vast majority of microorganisms on earth live in aggregates such as films, flocs, and sludge. An everyday feature of such aggregates is addressed by the term slime, which refers to highly hydrated, thick biofilms. They are slippery and can have different effects (Flemming and Wingender, 2001). In general one of the most important functions of the extracellular polysaccharides is supposed to be their role as fundamental structural elements of the EPS matrix, determining the mechanical stability of the biofilm, mediated by non covalent interactions either directly or via multivalent cation bridges.

Among activated sludge extracellular polymers, proteins predominate and, on the basis of their relatively high content of negatively charged amino acids, are supposed to be more involved than sugars in the electrostatic bonds with multivalent cations, underlying their

key role in structure (Cloete and Oosthuisen, 2001). In addition, proteins have also been suggested to be involved in hydrophobic bonds with the EPS matrix.

8.3. Ecological functions of EPS

8.3.1. Adhesion and cohesion to the surface

A major ecological advantage of the biofilm is that consortia of various organisms can establish and maintain their position over a long period of time, compared to the planktonic forms. The EPS keeps the microorganisms together to form the biofilm. They are therefore responsible for the adhesion to a given surface, thereby providing the advantage to maintain their stable position for a longer period (Flemming and Wingender, 2001).

8.3.2. Protective barrier

Biofilms can tolerate a much higher concentration of biocides and antibiotics than their planktonic counterparts. Activated sludge flocs (biofilms) with EPS are resistant to certain biocides including detergents and disinfectants, and also, protection of cyanobacterial nitrogenase from harmful effects of oxygen. Wingender *et al.* (1999) indicated that oxidizing biocides can be consumed by reaction with EPS, as shown for chlorine, where they compared resistance in mucoid and non-mucoid strains of *Pseudomonas aeruginosa*.

8.3.3. Water Retention

Ophir and Gutnick (1994) have investigated the role of EPS in the protection of biofilm against desiccation. This is probably a very good ecological function of EPS because it maintains an environment in which microbial life is possible. As the EPS is highly hydrated it changes the surface properties, and biofilms develop hydrophobic surfaces. The water in the EPS will shield many of the potential binding sites which can bridge the

macromolecules by electrostatic interactions, hydrogen bonds and London dispersion forces (Flemming *et al.*, 2000).

When the biofilm starts drying out, more of these binding sites will interact, as the water will not separate them from each other. This leads to stronger cohesion which can be seen in many scanning electron micrographs where the EPS appears as the filamentous structures which is the result of the drying process required for SEM preparations and thus, this artefact. The adhesion forces to the substratum will also increase because they are based on the same mechanism as cohesion.

8.3.4. EPS and metal removal in waste water treatment

EPS are also used for metal removal from water. Liu *et al.* (2001) indicated the ability of bacterial EPS to remove Zn^{2+} , Ca^{2+} , Cr^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} and CrO_4^{2-} from waste water. In secondary waste water treatment plants, these metals are absorbed by biomass and removed from waste water along with the waste sludge.

8.3.5. Sorption of exogenous organic compound and inorganic ions.

One of the major aims of waste water treatment is the removal of organic and inorganic pollutants. The distribution and fate of these pollutants is influenced by the sorption properties of the microbial biomass. The bacteria present in waste water may either take up the pollutant in the cell, or retain the pollutants externally, sorbed to either the cell wall or within the surrounding biopolymers (EPS) (Houghton and Quarmby, 1999). Both sorption processes are controlled by the rate of diffusion of the pollutant into the sludge floc or biofilm (EPS), the diffusion coefficient determining the overall reaction rate. The individual fates of pollutants in the waste water treatment systems are likely to vary depending on the pollutant type. Fractionation of the biomass from a sequencing batch biofilm reactor illustrated that organic and inorganic substances reacted differently, with >80% of the inorganic metals cadmium and zinc being found in the cellular fraction, and >60% of the organic compounds used (benzene, toluene, and *m*-xylene) localized in the

EPS fraction (Spath *et al.*, 1998). More detailed information about the accumulation and location of chlorinated organics in biofilm exopolymers was obtained using image analysis and dual channel imaging in conjunction with confocal scanning laser microscopy and lectin-specific fluorescent probes (Wolfaardt *et al.*, 1998). Dissolved substances can also be sorbed by biofilms and in particular by the EPS matrix (Flemming *et al.*, 1996). Some authors have pointed out that the carboxylic groups of polysaccharides bind to heavy metals (Ford and Ryan, 1995), such as Copper (Beech *et al.*, 1996), which accumulate at the cell surface in the sewage sludge. EPS also absorb ions thereby helping in the accumulation of toxic metal ion (detoxification), and promotion of polysaccharide gel formation and mineral formation (Flemming and Wingender, 2001).

8.3.6. EPS and phosphorus removal in waste water treatment.

Removal of phosphorus from domestic and industrial waste water is central in curbing the water pollution problem of eutrophication, a process resulting in excessive growth of algae and other photosynthesizing plants. Cloete and Oosthuizen (2001) indicated that not all the phosphorus in activated sludge system can be accounted for by poly-phosphate accumulating bacteria and indicated that the EPS associated with the activated sludge floc is also capable of removing phosphorus from the system. By means of using the X-ray microanalysis they indicated that EPS alone is capable of removing up to 30% of phosphorus from activated sludge.

8.3.7. Biopolymer content and the effect on sludge properties

One of the main areas of interest in biopolymer properties is the role that the biopolymer plays in the flocculation of particulate matter and bacterial cells during waste water treatment processes. Hydrophobic interactions of the secreted EPS (i.e. behavior of particles and molecules that are unable to interact with water, either via hydrogen bonds or electrostatically, to draw together in an aqueous environment) have been receiving attention of late. Increasing cell-surface hydrophobicities of bacterial cultures isolated

from waste water correlate well with the increased adhesion of cells to sludge flocs (Zita and Hermansson, 1997). Examination of the isolated exopolymeric substances (EPS) from activated sludge indicated that a significant proportion of this fraction is hydrophobic and consists of proteins but no carbohydrates (Jorand *et al.*, 1998).

The architecture of the activated sludge flocs is also dependent on the interaction between the microbially produced EPS and the cations present in the sludge. Small changes in ionic strength and ionic composition can alter the structural properties of activated sludge flocs (Keiding and Nielsen, 1997). Addition of the divalent cations, calcium and magnesium to the feed of laboratory grown activated sludge systems increased the bound protein content of the microorganisms present, which in turn improved the settling properties of the sludge. The presence of a single lectin-like protein in the biopolymer fraction allowed a new model of bioflocculation to be proposed. This model states that the biopolymer network is stabilized by lectin-like proteins binding polysaccharides that are cross-linked to adjacent proteins, and that the divalent cations provide further structural stability by bridging negatively charged sites on the biopolymers (Higgings and Novak, 1997).

8.4. Other functions of EPS

- Interaction of polysaccharides with enzymes for accumulation, retention and stabilization of the enzymes, (Wingender *et al.*, 1999).
- Enzymatic activities, including digestion of exogenous macromolecules for nutrient acquisition and release of biofilm cells by degradation of EPS structure, (Wingender *et al.*, 1999).
- In terms of biotechnology, some EPS are used for increasing the viscosity of technical materials and of food, (Wingender *et al.*, 1999).
- EPS are also used as biosurfactants in biotechnology industries (Wingender *et al.*, 1999).

9. Analysis of EPS

Apart from the high water content of up to 99%, biofilms consist mainly of EPS and cells. Several *in situ* techniques have been developed to investigate interfacial microbial communities. The major techniques include microelectrodes, gene probes, and new microscopic techniques. There are two main ways of analyzing EPS, including analysis by destructive and non-destructive methods (Wingender *et al.*, 1999).

9.1. Destructive analysis of EPS

Destructive analysis of EPS includes the extraction of EPS either by chemical or physical methods, the use of scanning electron microscopy and scanning probe techniques. The traditional approach to investigate polysaccharides is based on the isolation of the polysaccharides from a complex cell/EPS matrix. However using these approaches, the original structure of cells, aggregates, such as mats or biofilms matrix is disrupted. There are a number of reasons for studying the composition and properties of EPS in microbial aggregates, such as mats, biofilms, activated sludge, etc.

The approach to studying EPS from aggregates can be varied. It is often necessary to extract or separate the EPS from the cell to perform a more detailed study. Different extraction methods are used, either based on physical or chemical principles or a combination of both (Jahn and Nielsen, 1999). The selection of an appropriate method for a certain purpose must take place after careful evaluation of available methods.

10. Extraction of EPS

No universal extraction method exists for a quantitative extraction of bound EPS from microorganisms growing in suspension or in aggregates (Wingender *et al.*, 1999). A key point in determining the amount and composition of EPS in activated sludge is the extraction step (Frolund *et al.*, 1996).

A typical scheme of sampling, handling and analyzing of bound EPS in bioaggregates (Jahn and Nielsen, 1999) will now be discussed:

10.1. Sampling and pretreatment

Sampling of biological aggregates e.g. (activated sludge or biofilm in environmental samples or sampling from bioreactors). Transport of environmental samples to the laboratory and further storage should take place at 0-4⁰ C to prevent any changes occurring e.g. exoenzymatic activity. Biofilms and activated sludge can be stored for 1-2 days without significant changes in the extracted EPS composition. Pretreatment often includes a washing step and homogenization of the sample.

Different homogenizers are available, and a microscopic check of the particle size after homogenization is recommended. It is important to do homogenization at low temperature (Cloete and Oosthuizen, 2001). Washing of aggregates before extraction is often conducted to remove the soluble fraction. It is also important to select a washing buffer carrying an ionic strength and composition not too different from the sample, otherwise some bound EPS components might desorb and thus be washed away from the EPS matrix. Deionized water is mostly used to wash the activated sludge.

Matias *et al.* (2002) (lippel@peg.coppe.ufrj.br) have indicated the importance of ultrasonication in sludge as a step of homogenization in order to separate EPS from the activated sludge flocs without cell lysis. They have indicated that a high deflocculation was achieved as little as 30s after sonication in phosphate buffered saline (PBS), based on the total bacterial counts after each sonication, while exopolymer extraction increased with ultrasonication time. The results also indicated the use of Rutherin red (RR) to stain the samples in order to demonstrate the extracellular matrix of activated sludges, giving excellent staining of exopolymers for examination of ultrathin sections in Transmission electron microscopy. This shows the important and the need for sonication as a homogenizing step when extracting EPS from activated sludge. Indications were that ultrasonication for 30s in PBS and under specified conditions effectively deflocculated

the activated sludge, without greatly disrupting the cells. Samples can also be sonicated on Urea, EDTA, etc. (Matias *et al.*, 2002; lippel@peg.coppe.ufrj.br).

10.2. Extraction methods

A number of different methods have been applied in studies on pure culture or on undefined cultures, mainly related to activated sludge and biofilms. The extraction methods include various physical and chemical methods or combination of both. Zhang *et al.* (1999) compared different extraction methods in order to quantify extracellular polymeric substances in biofilms. He compared both physical and chemical methods where the physical methods (regular centrifugation) yielded a higher amount of EPS than others. Azeredo *et al.* (1999) also compared various methods of extracting the EPS, concentrating on both the physical and chemical methods. Frolund *et al.* (1996) also published a method of extraction, which considered to be yielding high amounts of EPS, called the cation exchange resin method.

There is no universal extraction method which exists for a quantitative extraction of bound EPS compounds from activated sludge, without contamination with intracellular macromolecules (Jahn and Nielsen, 1999). At present there are no standard procedures for EPS extraction, making it difficult to compare results from one study to another (Liu *et al.*, 2001).

Physical methods of extraction include centrifugation, mixing, shaking, sonication or heat treatment (Nielsen and Jahn, 1999). In general, methods employing only physical methods, give lower yields than those employing a combination of physical and chemical treatment. Heat treatment can cause significant lysis and disruption of macromolecules. Centrifugation is one of the most physical methods which is used and is most reliable. Centrifugation is often used to separate the soluble (slime) EPS fraction from cell biomass. Other physical methods used to extract the EPS are shaking, stirring or pumping (Nielsen and Jahn, 1999).

Chemical methods of extraction include addition of various chemicals into bacterial samples that can break different linkages in the EPS matrix and facilitate the release of EPS (Azeredo *et al.*, 1999, Frolund *et al.*, 1996 Nielsen and Jahn, 1999, Zhang *et al.*, 1999). Chemical extraction includes the addition of chemicals like NaOH. NaOH causes ionization of charged groups, like carboxylic groups in protein and polysaccharides, causing repulsion within the EPS matrix. Exchange of divalent cations (for example Ca^{2+} and Mg^{2+}) responsible for cross linking of charged compounds in the EPS matrix, with monovalent ions, can be removed by means of complexing agent like EDTA. Other chemical methods include cation exchange using a high concentrations of NaCl, or enzymatic digestion which is used to destabilize bioaggregates and enhance extraction. Enzymes are also used to deflocculate the cells before extraction (Nielsen and Jahn, 1999).

10.3. Combination of physical and chemical methods for EPS extraction.

Alkaline treatment has been combined with heat-treatment (70°C) to extract capsular EPS from *R. trifolii* (Bredveld *et al.*, 1990).

Ion exchange by Dowex extraction has been used in combination with shear (stirring) to extract EPS from activated sludge and biofilms (Frolund *et al.*, 1996, Jahn and Nielsen, 1999). As ion exchange is controlled by diffusion, it is very important to standardize the experimental conditions such as stirring time and temperature. NaCl, formaldehyde, and ultrasonic cation were also combined to extract EPS from anaerobic sludge (Nielsen and Jahn, 1999).

10.4. Contamination of EPS during extraction

In many extraction studies, the accumulation of protein and nucleic acids in the crude extract has been taken as an indication of cell lysis. The questions concerning the cell lysis has been described in detail for some of the extraction methods mentioned above. However, the EPS matrix usually contains large amounts of protein, nucleic acid and

probably also glycoprotein (Frolund *et al.*, 1996), so that the presence of the compounds in the extract is very difficult to use as an indicator of cell lysis. Boiling and alkaline treatment have been recorded to cause disruption of macromolecules (Karapanagiotis *et al.*, 1989). Deacylation of acylated alginates may take place, resulting in increased solubility and decreased hydrophobicity. High pH also breaks disulphide bonds in glycoproteins and uronic acids are degraded.

It is also important to realize that the enzymatic activities such as proteolytic/lipolytic/sugar cleaving activities can take place during extraction. These enzymes may be stable in the matrix and change the properties and composition of the macromolecules during extraction (Nielsen and Jahn, 1999). So whenever possible all procedures should be performed on ice.

10.5. Extraction efficiency

It is still uncertain which part of the exopolymers is extracted by the various methods. Many of the hydrophobic compounds together with some polysaccharides are not extracted by commonly used methods. This might explain why some tightly bound EPS associated with cell clusters are not extracted from activated sludge (Frolund *et al.*, 1996).

Extraction efficiency can be defined in one of two ways (Nielsen and Jahn, 1999):

- The total amount of EPS extracted from all the organic matter in a certain sample;
- The total amount of EPS extracted from the total EPS pool in a certain sample.

The second definition is the most correct definition, but since the total amount of EPS is usually unknown, this definition is rarely used. This definition does not reveal anything about the total concentration of EPS, but only how much of the EPS extracted with a certain method can be extracted from the total organic matter (Nielsen and Jahn, 1999). It is important to know how much EPS is extracted, because no direct quantification

methods for separation of cell biomass and EPS are available, and hence indirect methods must be used. In order to compare extraction yields, it is important to refer to a common measurement, for example the concentration of organic matter (volatile matter) (Nielsen and Jahn, 1999).

10.6. Purification and Analysis of EPS

In many cases when research is done on activated sludge, granular sludge and biofilms (Karapanagiotis *et al.*, 1987; Morgan *et al.*, 1990; Nielsen and Jahn, 1995; Frolund *et al.*, 1996) purification is done before analysis. They used heat extraction, precipitation of macromolecules in alcohol/acetone overnight, rinsing and dehydration in acetone/petroleum ether before analysis of the components.

10.7. Analysis of Extracted EPS

After extraction, chemical analysis of EPS includes sugar analysis, linkage analysis, sequencing, determination of anomeric configuration, and elemental composition (Wingender *et al.*, 1999). To better understand the role of each EPS component it requires one to perform a thorough characterization. The identification of different EPS components is not straightforward because of their complexity. Until now mainly spectrophotometric UV/VIS methods such as Dubois and Lowry methods, have been used to characterize the EPS, yielding only the total amount of polysaccharides and proteins. Polymer molecular weight is of particular importance as these biopolymers are thought to be the glue that holds sludge flocs together. The structure of biopolymer is unknown, especially the role of molecular weight. Previous studies indicated that high molecular weight polymers lead to round strong flocs while the enzymatic digestion of exocellular proteins initiates deflocculation. Low-pressure size exclusion chromatography (LPSEC) has been used to analyze and characterize activated sludge polymers. Recent high-pressure size exclusion chromatography (HPSEC) experiments of Frolund *et al.*, 1996 have revealed that EPS macromolecules can be separated according to chromatographic profiles composed of seven peaks. The method was found to be

successfully in showing differences and similarities between exopolymers from two different sludge treatment plants, showing the degradation of exopolymers compounds and impact of climatic conditions on chromatographic fingerprints (but without a closer knowledge about chemical nature of polymers). Recent work also used the same method of HPSEC together with FTIR to separate EPS molecules according to their molecular weight and analyze their peaks (Gorner *et al.*, 2002; email Tatiana.Gorner@ensic.inpl-nancy.fr.). They have indicated that proteins were present in all the peaks while polysaccharides were present in only three peaks. The results from an infrared spectrum indicated that the EPS contained one type of polysaccharides and two types of proteins.

10.8. The destructive analysis of EPS

The destructive analysis of EPS includes (Wingender *et al.*, 1999):

- ❖ Isolation by precipitation from the culture supernatant or extraction from the cell surface
- ❖ Purification by precipitation and size fraction by gel chromatography
- ❖ Release of single constituents by various types of methods
- ❖ Determination of charged compounds by high voltage electrophoresis
- ❖ Determination of elemental composition by scanning electron microscopy combined with Energy Dispersive X-ray spectroscopy or EDX) (Cloete and Oosthuisen, 2001)

10.9. Energy dispersive X-ray spectrometry as a method of destructive analysis

Electron microscopy combined with energy dispersive X-ray microanalysis is a method of elemental analysis within well-defined regions of a specimen (Buchan 1981). SEM can be an effective tool when used to investigate problems involving surface detail, both microstructural and microchemical. EDS is a spectrographic technique that identifies the elemental composition within single particles in a sample matrix, providing qualitative and semi-quantitative information.

Combining SEM with EDS produces a powerful and versatile tool able to obtain information, like:

- Identification of contaminants
- Verification of product integrity
- Mineral identification : allowing for the purity assessment of refined ore material
- Identification of inorganic inclusions or contaminants in polymers
- Analysis of metal and oxides : allowing for the evaluation of corrosion product
- Evaluation and identification of microstructures on sample surface

(http://www.clytongrp.com/semeds_art.htm)

10.9.1. The principle of SEM/EDS

In EDS high velocity incident electrons cause ionization of the atoms in the specimen. This ionization creates a vacancy in one of the energy levels of the atoms, which is almost immediately filled by an electron, which was at a higher energy level. As these electrons transfer to the lower energy levels, the excess energy is emitted in the form of X-ray photons. If the vacancy was created in the K-shell of the atom, it is called K X-ray. Since an electron from one of several higher energy levels could fill the vacancy, a photon corresponding to one of these transitions is emitted. In thin biological specimens, the amount of characteristic photons emitted by a particular element in a specimen is proportional to the number of atoms of that element present in the volume of material excited by the primary electron beam.

In the case of a biological specimen, the amount of bremsstrahlung is proportional to the mass of the specimen through which the electron passes. The ratio of the characteristic element X-rays to the total bremsstrahlung X-rays generated (or the peak to background ratio) is thus proportional to the ratio of the elemental mass to the total mass in the excited volume.

11. Other destructive analysis of EPS

11.1. Scanning probe techniques

These techniques include scanning tunneling electron microscopy, atomic force microscopy, scanning ion-conductance microscopy and scanning tunneling microscopy. In these techniques, a scanning probe provides a surface view of material, providing a resolution at the atomic level (Neu and Lawrence, 1999).

12. Non- Destructive analysis of EPS

Methods in this section include the infrared spectroscopy or FT-IR (with information given being a signal of chemical groups from condition films, coatings, EPS and biofilm events), nuclear magnetic resonance spectroscopy (NMR) (giving information about the biomass, flow of velocity, oxygen tension and cell distribution) and confocal laser scanning microscopy or CLSM (with a wide range of probes for polysaccharides, proteins and nucleic acids) (Neu and Lawrence, 1999).

13. Effects of enzymes on biofilms

As such a large proportion of the structure of biofilms is composed of polysaccharides secreted by constituents micro-organisms. The presence of enzymes (polysaccharases) acting on these polymers will inevitably have a very marked effect on the structure and on the integrity of the biofilm. It is also possible that glycosidases capable of cleaving exposed terminal monosaccharide residues may modify both polysaccharides and glycoproteins present in the biofilms. EPS may be degraded either by polysaccharides hydrolases or polysaccharide lyases. Polysaccharases may derive from three major sources i.e. endogenously from polysaccharide-synthesizing microorganisms, exogenously from a wide range of other microorganisms and, finally from bacteriophage particles or phage induced bacterial lysates (Sutherland, 1999).

The action of any polysaccharases or similar enzyme on a biofilm will depend very much on the nature of a biofilm. In particular if the polymer molecules present act synergistically to provide increased adhesion, destruction of a key component may have a significant effect. The effect of the phage enzyme attached to the viral base plate is to

carve a path through polysaccharide capsules. Thus if isolated cells within the outer reaches of a biofilm are infected, they will provide a focus for generation of further phage particles on lysis from within together with the release of soluble enzyme (Sutherland 1999). In a study of attached growth of alginate producing *P. aeruginosa*, Boyd and Chakrabarty (1994) observed that increased expression of alginate lyase caused alginate degradation and increased cell detachment. As the enzyme probably has a limited activity on the substrate, it probably caused cell detachment through reduced mass and viscosity. Exo-acting enzymes only cause a slow polymerization and a slow gradual release of oligosaccharides products.

14. Potential of EPS manipulation in waste water treatment

Biopolymers (EPS) produced by pathogenic bacteria and bacteria that are of industrial importance have been investigated in pure cultures. The structural genes involved in biopolymer production in bacterial species such as *Acetobacter xylinum*, *Xanthomonas campestris* and *Rhizobium spp.* along with inherent difficulties in obtaining this information, have been reviewed (Griffin *et al.*, 1996). This basic knowledge is necessary before attempts can be made to improve the biopolymer production of selected bacterial strains by genetic manipulation, thereby increasing the absorption properties or flocculation potential of the overall waste water treatment systems. The small number of successful bioaugmentation studies carried out so far have focused on the introduction of bacteria (*Pseudomonas spp.*) capable of enhancing pollution degradation. Results obtained highlight the need to use strains that are well adapted to the waste water treatment environment. Such techniques offer the potential to improve the efficiency of waste water treatment , but as with any application involving genetically modified organisms, they are likely to encounter legislative opposition to their implementation.

15. Conclusion

Biopolymers (also referred to in the literature as extracellular polymeric substances (EPS), exocellular polysaccharides, exopolymeric substances, exopolymers, or microbial

soluble products) are produced by many different types of bacteria. They are found either in a capsule like structure associated with the outer cell wall or in a solution as loose slime polymers that are completely detached from the cell wall. Our knowledge of the role played by microorganisms and their associated biopolymers in waste water treatment is slowly being elucidated. Biopolymers (EPS) are of major importance in waste water treatment. Biopolymers act in conjunction with divalent metals ions, such as calcium, to aid the formation and settlement of sludge flocs in both aerobic and anaerobic treatment systems. EPS can be considered as one of the important structures of cell clusters (biofilms, activated sludge flocs etc.). In terms of waste water treatment, if the role of EPS in phosphorus removal can be understood, then this will enhance phosphorus removal in waste water treatment. The approach to studying EPS from the aggregates can be varied. Therefore it is often necessary to extract or separate the EPS from the cells to perform the detailed study. Comparing the results of EPS extraction from one study to another is still difficult since there are no universal standard methods for extraction of EPS. Therefore for a detailed analysis of EPS, an extraction method which yields a high concentration of EPS should be given a priority.

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CHAPTER 3

Quantification of phosphorus in extracellular polymeric substances (EPS) from the activated sludge using SEM and EDS

Abstract

Scanning Electron Microscopy combined with Energy Dispersive spectrometry (SEM/EDS) was used in order to quantify the amount of phosphorus in the EPS of the activated sludge flocs. Analysis was performed on activated sludge and extracellular polymeric substance (EPS) samples from five different wastewater treatment plants situated in South Africa (Gauteng Province). The quantity of EPS and its composition was investigated. The mixed liquor suspended solids (MLSS) was quantified using standard methods and the freeze drying method. Both methods gave similar results in each of the waste water treatment plants. The EPS contained more carbohydrates than proteins. The EPS in the aerobic zone was $\pm 0.05\text{mg/ml}$ higher than in the anaerobic zone. The phosphorus content in cell cluster and EPS was related to the concentration bivalent cations present in activated sludge and EPS, e.g. magnesium, potassium and calcium. The amount of phosphorus in activated sludge flocs plus EPS was 50.5% of the total elemental analysis on average and the EPS alone contained 25% of phosphorus on average for Daspoort, Bavianspoort and Zeekoegat. The amount of phosphorus in activated sludge cell clusters and EPS of Waterval and Vlakplaats plants was low (25% in cell clusters with EPS and 5% in EPS) which might be due to the fact that these EBPR plants function under potassium, magnesium and calcium limitation. The possible mechanism of phosphorus removal by EPS is that, phosphorus binds to cations (Mg^{2+} , K^{+} etc.) to make an insoluble compounds (Magnesium-phosphate compounds, Calcium phosphate compounds etc.) which precipitates out in the EPS associated with the activated sludge flocs. In conclusion it may be postulated that potassium, magnesium and calcium are important in phosphorus removal in terms of phosphate found in the EPS. The anaerobic activated sludge and EPS samples contained higher concentration of

orthophosphate in the aerobic activated sludge and the aerobic EPS samples. The role of EPS in the biological removal of phosphorus in activated sludge is not well understood. More studies still need to be done to understand the possible mechanism by which EPS removes phosphorus from activated sludge.

1. Introduction

The production of extracellular polymeric substances (EPS) is a general property of microorganisms in natural environments and occurs in both prokaryotic (archae and bacteria) and eukaryotic (algae, fungi) microorganisms. EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeating units within polymer molecules such as many polysaccharides. Activated sludge EPS has been reported as the major floc component (Urbain *et al.*, 1993; Liss *et al.*, 1996; Frolund *et al.*, 1996; Bura *et al.*, 1998; Jorand *et al.*, 1998) keeping the floc together in a three dimensional matrix (Wingender *et al.*, 1999) due to the bonding with bivalent cations and hydrophobic interactions. EPS is mainly composed of polysaccharides, although other compounds can be found in smaller concentrations, such as proteins, DNA and other humic substances (Jahn and Nielsen, 1995; Frolund *et al.*, 1996).

The EPS chemical composition determines many important properties of biofilms or activated sludge such as density, porosity, diffusivity, elasticity, strength, frictional resistance, thermal conductivity and metabolic activity (Zhang *et al.*, 1999). More information about the composition will contribute to a better understanding of the physiological and physical behavior of biofilms in environmental systems. Cells and EPS distribution do not always correspond to each other (Zhang *et al.*, 1999). EPS has been defined as any polysaccharides or peptidoglycan structure of bacterial origin lying outside the outer membrane of cells, although it is now recognized that proteins and other macromolecules represent significant quantities in EPS in many environments (Wingender *et al.*, 1999).

The chemical compositions of the EPS are usually reported in literature to be very heterogeneous. Carbohydrate predominates and represents 65% of the extracellular polymeric substances, together with proteins, nucleic acids and lipids (Zhang *et al.*, 1999). The EPS chemical component ratio varies, depending on the sample source. Activated sludge EPS has been found to have more carbohydrate than protein (Azeredo *et al.*, 1999).

The approach to studying EPS from aggregates can be varied. It is often necessary to extract or separate the EPS from the cell to perform a more detailed study. Different extraction methods are used, either based on physical or chemical principles or a combination of both (Nielsen and Jahn, 1999). The selection of an appropriate method for a certain purpose must take place after careful evaluation of available methods. No universal extraction method exists for a quantitative extraction of bound EPS from microorganisms growing in suspension or in aggregates (Wingender *et al.*, 1999). A key point in determining the amount and composition of EPS in activated sludge is the extraction step (Frolund *et al.*, 1996).

Scanning Electron Microscopy (SEM) can be an effective tool when used to investigate problems involving surface detail, both microstructure and microchemical. EDS is a spectrographic technique that identifies elemental composition within single particles in a sample matrix, providing qualitative and semi-qualitative information. SEM/EDS technology produces a powerful versatile tool to obtain a wide range of information, like identification of inorganic inclusions or contaminants in polymers.

The aim of this work was to:

- quantify the amount of EPS in activated sludge
- characterize the chemical composition of EPS in activated sludge
- quantify the amount of phosphorus in EPS associated with activated sludge flocs using SEM/EDS.
- To quantify the mixed liquor suspended solids (MLSS) using the freeze drying method.

2. Materials and Methods

2.1 Sampling

Activated sludge samples were collected from five enhanced biological phosphate removal (EBPR) waste water treatment plants (WTP) i.e. Waterval, Vlakplaats and Daspoort which function exclusively biological without addition of any chemicals and Baviaansport and Zeekoegat which function both biologically and chemically. All these waste water treatment plants are situated in the Gauteng province, South Africa. Activated sludge samples were collected at the end of the aerobic and anaerobic zone in all the plants and transferred into 500ml Schott bottles. The samples were stored on ice and transported to the laboratory for analysis.

2.2 Sample preparation for SEM/EDS analysis

Ten milliliter of activated sludge sample was transferred to a centrifuge tube. The sample was removed from liquid material by centrifugation at 5000rpm for 2 min using a Sorvall RC-5B Refrigerated superspeed centrifuge. The pellet was washed three times with 10ml of sterile double distilled water by centrifugation at 10 000rpm for 5min. The samples were diluted 1:10 in sterile double distilled water and freeze dried for 18h. Samples were then gold coated for 10 s for SEM/EDS analysis and 30s (3times) for three dimensional imaging of the both EPS and activated sludge samples. Samples were autocoated using a SEM autocoating unit E5200.

2.3. Orthophosphate analysis

Orthophosphate analyses were done on both the aerobic and anaerobic activated sludge samples and EPS. Five analyses were done on each sample. These analyses were done immediately after sampling from the Daspoort waste water treatment plant. The orthophosphate analyses were done using a SQ118 spectrophotometer (Merck) and the relevant test kit. For analyses of orthophosphate in the EPS samples, the analyses were

done before and after filtration of EPS sample through a 0.22 μ m Millipore filter. For analyses of orthophosphate in the activated sludge samples, analyses were done after filtering 10ml of activated sludge sample through Whatman no. 1 filter paper.

2.4 Mixed Liquor Suspended Solids (MLSS)

MLSS was determined using the standard method (Greenberg *et al.*, 1992) and a freeze drying method. Ten ml of each sample was filtered through a whatman no.1 filter paper and the MLSS was analysed by the standard method (APHA *et al.*, 1989). In the freeze drying method for MLSS analysis, 10ml of each sample was transferred into a centrifuge tube. The samples were freeze dried using liquid nitrogen and then freeze dried for 24h. After freeze drying the solid sample then weighed using a Mettler AE 163 balance.

2.5 Extraction of EPS from activated sludge samples

EPS material was removed from the samples using a regular centrifugation method (Zhang *et al.*, 1999) with modifications. For the washing step, 10ml of each activated sample was added into a centrifuge tube. Samples were centrifuged for 5min at 3500rpm at room temperature. The supernatant was removed and stored for further use. 10ml of sterile double distilled water was added to the pellet and the samples were homogenized 3 times for 30s on ice using a Cole-Parmer homogenizer with an output of 50%. After homogenization, the previously removed supernatants were added to the homogenized samples and centrifuged for 30min at 12000rpm at 4⁰C using a Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant containing the EPS was gently collected using a 20ml syringe needle without disturbing the pellet. The supernatant was filtered through a 0.22 μ m Millipore filter. The filtrate was freeze dried using nitrogen liquid and freeze dried for 24h. The EPS was weighed using a Mettler AE 163 balance. After weighing of the samples, EPS was checked for purity using scanning electron microscopy.

2.6 Chemical composition analysis of the EPS

Extracellular polymeric substances (EPS) for chemical analysis was extracted as mentioned in 2.4, the difference was that the filtrate was not freeze dried. A phenol sulfuric method (Dubois method) was used to quantify carbohydrates using glucose as the standard. The absorbance was read at 490nm. Proteins were quantified using the Lowry method, with a Hitachi U –2000 double beam spectrophotometer. The concentration of protein (mg/ml) was calculated using the following formula: $\text{Protein (mg/ml)} = 1.55A_{280} - 0.76A_{260}$ wherein A_{280} was the absorbance of proteins and A_{260} the absorbance of nucleic acids if they were present.

2.7 SEM/EDS analysis on EPS and activated sludge.

Samples were analyzed using a Jeol model JSM 5800 LV, using a backscatter detector for better compositional contrast and depth resolution than secondary electrodetectors. Samples were analyzed by a pre-standardized Noran Voyager system at 15keV for a lifetime of 300s. Digital images were captured by means of an Orion frame grabber.

3 Results and Discussion

3.1 Quantification of MLSS and EPS

The concentration of both the MLSS and EPS was given in mg/ml of activated sludge samples from both aerobic and anaerobic samples of three waste water treatment plants which function exclusively biological. Two methods were used for MLSS analysis, the standard method and a freeze drying method. Mixed liquor suspended solids is one of the physical characteristics of activated sludge samples which varies from one plant to the other depending on the amount and type of the sludge and characteristics of the plant. Usually the activated sludge of plants which function chemically contain sludge quantities which are higher than that of the biologically functioning plants, due to chemical precipitation (Gray, 1989; Wanner, 1994).

Table 1a. MLSS of activated sludge samples from the anaerobic and the aerobic zone of three different wastewater treatment plants.

Method	Waste water treatment Plant	Mass of MLSS in mg/ml (aerobic zone) mean	Mass of MLSS in mg/ml (anaerobic zone) mean
Standard	Waterval	3.82	6.34
Freeze Drying	Waterval	3.95	6.50
Standard	Vlakplaats	0.88	0.61
Freeze Drying	Vlakplaats	0.95	0.65
Standard	Daspoort	2.45	3.77
Freeze Drying	Daspoort	2.66	3.95

The Waterval waste water treatment plant contained the highest concentration of MLSS (6.42mg/ml in the anaerobic and 3.88mg/ml in the aerobic zone) followed by Daspoort (3.82 mg/ml in the anaerobic and 2.55 in the aerobic zone) and Vlakplaats with concentrations of 0.63mg/ml in the anaerobic and 0.95mg/ml in the aerobic zone. The concentration of MLSS suspended was more or less the same (with the difference of ± 0.14 mg/ml in the aerobic samples and 0.12mg/ml in the anaerobic samples in all the plants). Higher concentrations (Table 1a) of MLSS were observed when determined by the freeze drying method than the standard method. This might be due to the fact that in the standard method there is an oven drying step at high temperature (105°C), which might cause some of molecules in the sample to evaporate which then lowers the concentration of the MLSS. In freeze drying there is no temperature step involved which might vaporize any components in the sample.

The concentration of the MLSS in the anaerobic zone (Table 1a) of the Waterval and Daspoort plants were higher than that of the aerobic zone. In the Vlakplaats works the concentration of MLSS in the anaerobic zone was lower than in the to the aerobic zone (Table 1a). The sludge age is a property which determines the MLSS. A longer sludge

age results in high sludge and MLSS (<http://lemnatechnologies.com/wtclass.html>). The freeze drying method was a reliable, fast and efficient in determining MLSS concentration.

Table 1.b. Concentration of the extracted EPS in the anaerobic and aerobic samples of three waste water treatment plants

Plant	Aerobic EPS mg/ml (mean)	Anaerobic EPS mg/ml (mean)
Waterval	0.18	0.27
Vlakplaats	0.15	0.12
Daspoort	0.16	0.20

EPS concentration in activated sludge was measured in mg/ml. The total amount of extracted EPS from both the aerobic and anaerobic samples of three waste water treatment plants, that function exclusively biological were determined (Table 1b). Waterval and Daspoort plants contained a higher concentration of EPS than the Vlakplaats plant. The concentration of the EPS in the anaerobic zone of both Waterval and Daspoort was higher than in the aerobic zone, while the Vlakplaats waste water treatment plant contained a higher concentration of EPS in the aerobic zone than in the anaerobic zone. The total concentration of extracted EPS was related to the concentration of MLSS of the sludge and represented 12-16% of the MLSS.

3.2 Chemical composition of the EPS

The chemical composition of EPS was determined on samples from the Daspoort waste water treatment plant.

3.2.1 Protein concentration

EPS is described as heterogeneous because it contains different compounds in different concentrations i.e. proteins, carbohydrates, nucleic acids, uronic acids etc (Zhang *et al.*, 1999).

Table 2.1 Protein concentration in Daspoort aerobic and anaerobic wet EPS samples.

Zone	Concentration (mg/ml)	Concentration (mg/ml)
	Range	Mean
Aerobic	0.128-0.463	0.265
Anaerobic	0.056-0.281	0.136

The protein concentration in the aerobic zone was two times higher (0.265mg/ml) than in the anaerobic zone (0.136mg/ml) (Table 2.1). For years, carbohydrates were considered the main constituents of EPS in pure cultures (Sutherland, 1997; Sutherland and Kennedy, 1996). Recent studies of mixed cultures in waste water treatment systems indicated that protein was also an important constituent of EPS (Dignac, 1998). One of the possible reasons for the large quantities of protein in EPS might be due to the fact that large quantities of exoenzymes are entrapped in the EPS.

3.2.2 Carbohydrate concentration

Table 2.2. Carbohydrate concentrations in Daspoort aerobic and anaerobic wet EPS

Zone	Concentration (mg/ml)	Concentration (mg/ml)
	Range	mean
Aerobic	0.41-0.91	0.82
Anaerobic	0.35-0.53	0.38

Carbohydrate concentrations were also two times higher in the aerobic zone than in the anaerobic zone (Table 2.2). Large quantities of carbohydrates might be contributed to the washing step (recovery of loosely slime material from the biofilm). Studies have shown

that many of the carbohydrates are collected from the washing step, rather than carbohydrates which can be removed from the stripping step (recovery of the capsule bound material from the biofilm) (Zhang *et al.*, 1999). In general the EPS contained more carbohydrates than protein. The concentration of nucleic acid was very low (0.01% w/v of the total EPS) which indicated, that, cell lysis did occur to a lesser extent (Azeredo *et al.*, 1999; Liu and Fang, 2002; Zhang *et al.*, 1999). Regular centrifugation does not cause a high a degree of cell lysis during extraction as compared to other chemical extraction methods (Zhang *et al.*, 1999). Liu and Fang (2002) indicated that EDTA extraction caused cell lysis due to the removal of cations by EDTA from the cell-membrane, causing cell lysis and the release of intracellular DNA. Therefore it is important to do nucleic acid tests on the pellet in order to compare the intracellular and extracellular concentration of the nucleic acid when determining the composition of EPS. In this study the method for quantifying EPS and MLSS from activated sludge was discovered which is based on freeze drying technique.

3.3 SEM/EDS

3.3.1 Scanning electron micrographs of the activated sludge and EPS samples

In order to optimize the EDS analysis and imaging of the activated sludge flocs, the samples were freeze dried.

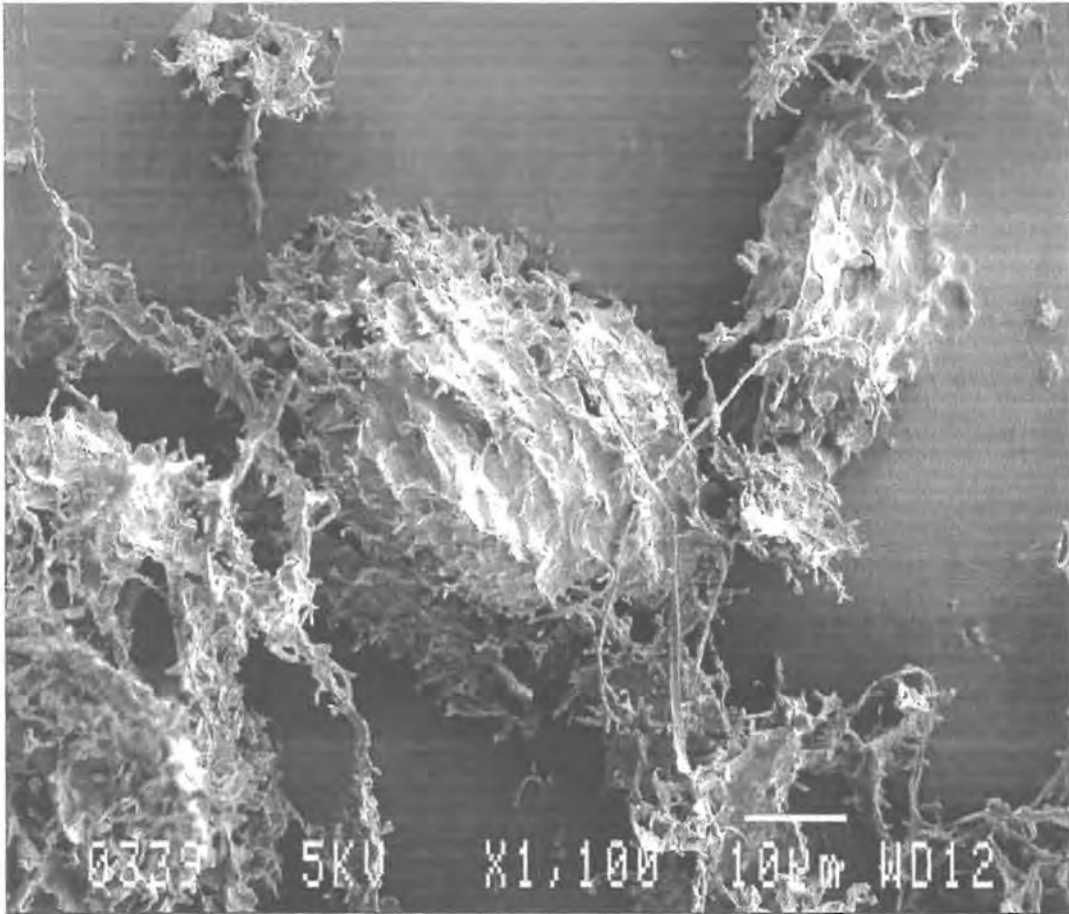


Fig. 1a. SEM of Daspoort Sludge flocs. (1100X).

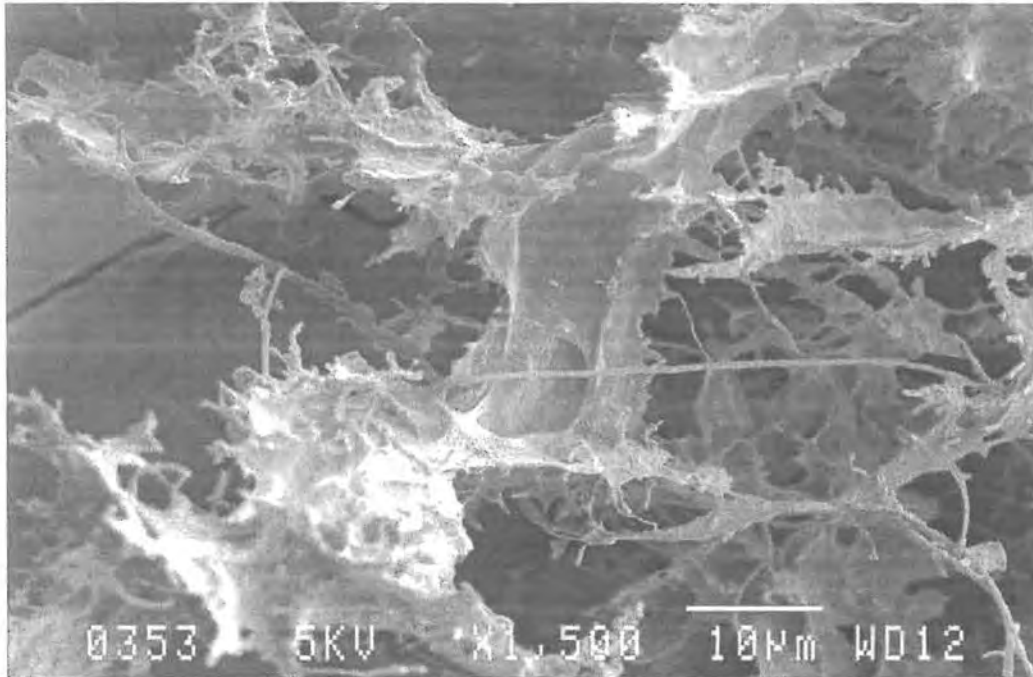


Fig 1b. SEM of Bavianspoort sludge floc (1500X).

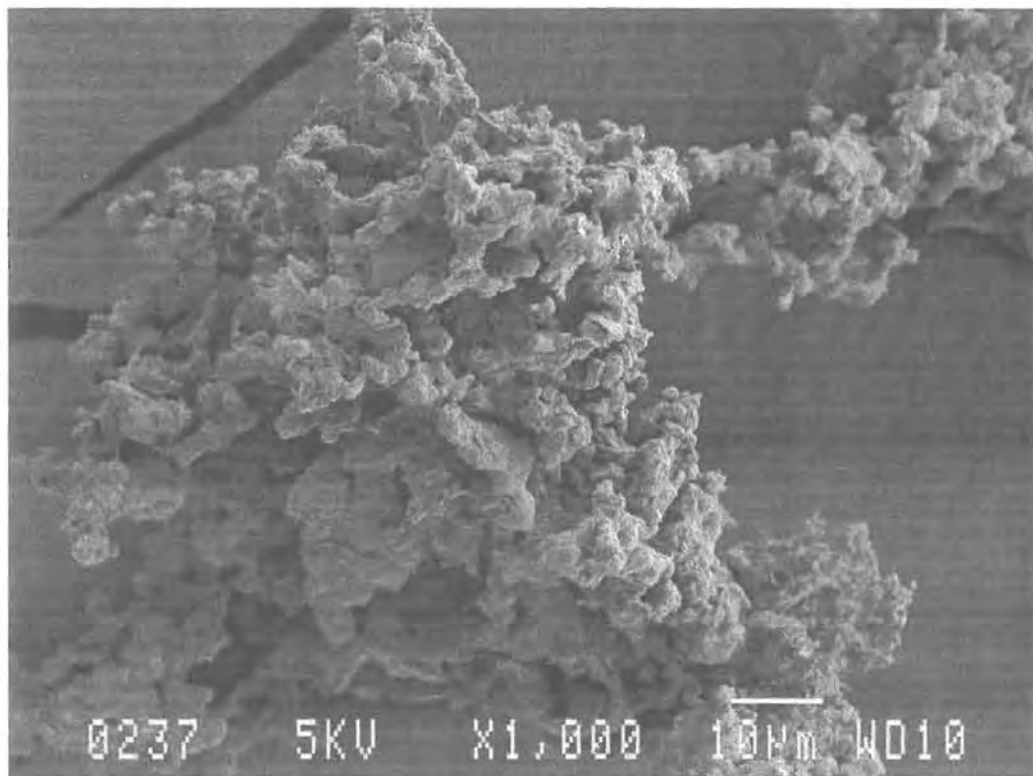


Fig 1c. EPS structure of Bavianspoort (1000X).

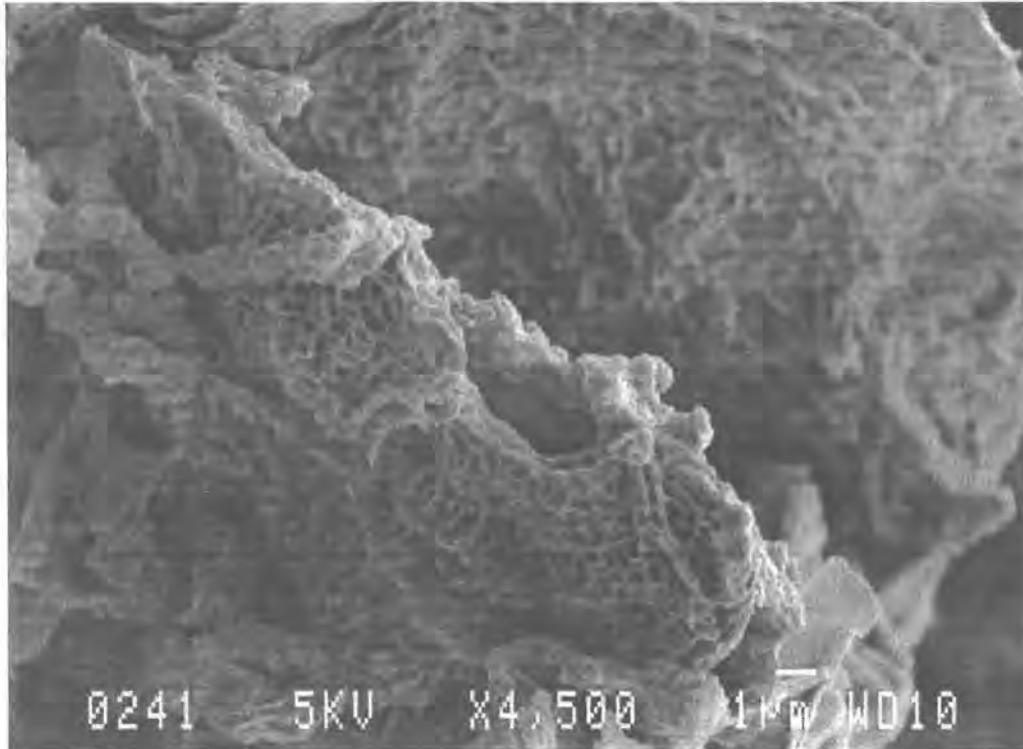


Fig 1d. EPS structure of Baviaanspoort
(4500X).

Freeze drying method was used in order to avoid displacement and/or migration of ions in the samples (Cloete and Oosthuisen, 2001). Freeze drying did not have any effect on the elemental analysis and the structure of the flocs (Fig. 1a-b). The floc structures and EPS structures were different from one plant to the other (Fig. 1a-d). Figure (1a-d) indicate the three dimensional image of the activated sludge flocs and EPS at different magnification from different plants. The activated sludge floc contained both EPS and cells. Filamentous microorganisms also occurred in the flocs (Fig. 1a-b). Both EPS and filamentous microorganisms are involved in holding the sludge together, resulting in bioflocculation and settling of the sludge. The floc structures differed from one plant to the other. EPS in some of the floc samples was loosely associated (Fig. 1b) to the floc, which made the EDS analysis easy, because it was not difficult to distinguish the bacterial cells from the EPS. Extracted EPS (Fig. 1c-d) was cell free. This also indicated EPS purity after extraction.

3.3.2 EDS analysis of activated sludge flocs and EPS.

The elemental composition of both the EPS associated with activated sludge flocs (cell clusters) and the EPS alone was determined using SEM/EDS. Buchan (1982) used EDS to determine the location of phosphorus volutin granules in activated sludge, although little attention was paid to phosphorus located in the EPS of the cell cluster. SEM/EDS technology produces a powerful versatile tool to obtain a wide range of information, like the identification of inorganic inclusions e.g. phosphorus, magnesium etc or contaminants in polymers (<http://clytongrp.com/sem-eds-art.htm>).

Previous studies by Cloete and Oosthuisen (2001) indicated that not all phosphorus which is removed by in EBPR is accounted for by polyphosphate accumulating bacteria (PAB), but that the EPS was also capable of removing a certain amount of phosphorus. They indicated that EPS alone could remove as much as 30% of phosphorus based on the total elemental analysis. Schonborn *et al.* 2001 used X-ray spectrum analysis and found phosphorus in the polyphosphate granules when Mg, K and Ca were present in higher concentrations (80-158mg/l Ca and 24-31mg/l Mg). They indicated that during the addition of 10-20mg/l Mg, the mean phosphorus removal efficiency amounted to 97% compared to 85% without metal dosage and besides phosphorus, the cations Mg^{2+} , K^{+} and Ca^{2+} were the main components of polyphosphate granules (Schornborn *et al.*, 2001). The phosphorus content from anaerobic and aerobic samples from each waste water treatment plant was not different in both the cell clusters and EPS. In this study the EDS indicated that in all the samples, the elements which were dominating were phosphorus, magnesium, potassium, calcium, silicon and aluminum. The presence of these cations suggests the possible mechanism whereby phosphorus is bound to these cations to form insoluble compounds which precipitate in the EPS.

Table 3. Summary of EPS and activated sludge floc phosphorus content in the aerobic and anaerobic zones of five different waste water treatment systems.

Plant and zone	Range (%)	Average (%)
Daspoort cell cluster (anaerobic)	53-59	57
cell cluster (aerobic)	53-57	55
EPS (anaerobic)	19-26	26
EPS (aerobic)	18-24	23
Baviaanspoort cell cluster (anaerobic)	45-56	53
Cell cluster (aerobic)	44-51	50
EPS (anaerobic)	18-23	20
EPS (aerobic)	19-21	17
Zeekoegat cell cluster (anaerobic)	38-45	45
Cell cluster (aerobic)	37-44	42
EPS (anaerobic)	16-22	18
EPS (anaerobic)	15-19	16
Waterval cell cluster (anaerobic)	22-28	25
Cell cluster (aerobic)	25-27	26
EPS (anaerobic)	6-8	7.1
EPS (aerobic)	3-7	4.8
Vlakplaats cell cluster (anaerobic)	20-25	22
Cell cluster (aerobic)	31-35	33
EPS (anaerobic)	1.61-3	2.1
EPS (aerobic)	4-6	4.4

Table 4.1 A typical data set of the elemental analysis of the cell clusters and EPS from the Daspoort anaerobic zone.

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.34	Na-K	12.02
Mg-K	17.30	Mg-K	14.37
Al-K	0.00	Al-K	0.30
Si-K	1.05	Si-K	8.59
P-K	60.29	P-K	24.72
S-K	1.83	S-K	5.50
Ca-K	1.56	Ca-K	6.02
Fe-K	0.76	Fe-K	0.04
Cl-K	0.15	Cl-K	12.82
K-K	16.12	K-K	15.62
Ti-K	0.10	Ti-K	0.00

Table 4.2 A typical data set of the elemental analysis of the cell clusters and EPS from the Daspoort aerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.25	Na-K	8.84
Mg-K	13.36	Mg-K	11.18
Al-K	0.00	Al-K	0.26
Si-K	0.94	Si-K	9.22
P-K	59.31	P-K	24.50
S-K	1.87	S-K	5.64
Ca-K	1.98	Ca-K	7.71
Fe-K	1.35	Fe-K	0.07
Cl-K	0.17	Cl-K	14.54
K-K	20.63	K-K	19.55
Ti-K	0.14	Ti-K	0.00

Table 5.1 A typical data set of the elemental analysis of the cell clusters and EPS of the Baviaanspoort anaerobic zone

Cell cluster	weight(%)	EPS	Weight(%)
Na-K	0.45	Na-K	2.83
Mg-K	18.21	Mg-K	7.14
Al-K	0.77	Al-K	3.93
Si-K	2.14	Si-K	7.49
P-K	56.56	P-K	23.39
S-K	0.62	S-K	15.23
Ca-K	2.69	Ca-K	17.40
Fe-K	1.11	Fe-K	14.96
Cl-K	0.30	Cl-K	3.03
K-K	16.85	K-K	4.59
Ti-K	0.30	Ti-K	0.00

Table 5.2 A typical data set of the elemental analysis of the cell clusters and EPS of the Baviaanspoort aerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.33	Na-K	4.15
Mg-K	14.01	Mg-K	9.91
Al-K	0.66	Al-K	4.92
Si-K	1.90	Si-K	8.99
P-K	55.45	P-K	25.48
S-K	0.63	S-K	16.02
Ca-K	3.41	Ca-K	14.65
Fe-K	1.97	Fe-K	9.04
Cl-K	0.34	Cl-K	2.88
K-K	20.85	K-K	3.96
Ti-K	0.46	Ti-K	0.00

Table 6.1 A typical data set of the elemental analysis of the cell clusters and EPS of the Zeekoegat aerobic zone

Cell cluster	weight(%)	EPS	Weight(%)
Na-K	1.01	Na-K	16.61
Mg-K	11.75	Mg-K	7.72
Al-K	2.98	Al-K	0.00
Si-K	5.11	Si-K	1.66
P-K	45.45	P-K	22.79
S-K	9.10	S-K	11.67
Ca-K	7.14	Ca-K	6.07
Fe-K	3.06	Fe-K	0.04
Cl-K	0.44	Cl-K	14.93
K-K	13.30	K-K	18.50
Ti-K	0.36	Ti-K	0.00

Table 6.2 A typical data set of the elemental analysis of the cell clusters and EPS of the Zeekoegat anaerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.72	Na-K	22.26
Mg-K	8.78	Mg-K	9.79
Al-K	2.47	Al-K	0.00
Si-k	4.41	Si-K	1.82
P-K	43.26	P-K	22.67
S-K	8.96	S-K	11.21
Ca-K	8.79	Ca-K	4.67
Fe-K	5.26	Fe-K	0.02
Cl-K	0.48	Cl-K	12.77
K-K	16.34	K-K	14.58
Ti-K	0.53	Ti-K	0.00

Table 7.1 A typical data set of the elemental analysis of the cell clusters and EPS from the Waterval anerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.97	Na-K	26.64
Mg-K	6.71	Mg-K	6.80
Al-K	10.63	Al-K	0.01
Si-K	13.82	Si-K	3.96
P-K	23.58	P-K	7.32
S-K	21.51	S-K	9.24
Ca-K	11.21	Ca-K	8.75
Fe-K	4.05	Fe-K	0.10
Cl-K	0.06	Cl-K	24.85
K-K	6.64	K-K	12.26
Ti-K	0.27	Ti-K	0.00

Table 7.2 A typical data set of the elemental analysis of the cell clusters and EPS from the Waterval aerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	3.90	Na-K	13.69
Mg-K	6.16	Mg-K	3.93
Al-K	10.42	Al-K	0.00
Si-K	12.21	Si-K	2.47
P-K	25.78	P-K	3.26
S-K	17.78	S-K	4.66
Ca-K	12.95	Ca-K	14.85
Fe-K	5.01	Fe-K	0.25
Cl-K	0.60	Cl-K	51.65
K-K	5.14	K-K	5.19
Ti-K	0.06	Ti-K	0.07

Table 8.1 A typical data set of the elemental analysis of the cell clusters and EPS from the Vlakplaats aerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.95	Na-K	25.02
Mg-K	5.96	Mg-K	5.01
Al-K	8.61	Al-K	0.04
Si-K	13.49	Si-K	4.36
P-K	33.31	P-K	4.52
S-K	11.10	S-K	16.01
Ca-K	8.47	Ca-K	5.03
Fe-K	5.89	Fe-K	0.45
Cl-K	0.71	Cl-K	31.55
K-K	10.44	K-K	8.16
Ti-K	1.07	Ti-K	0.12

Table 8.2 A typical data set of the elemental analysis of the cell clusters and EPS from the Vlakplaats anaerobic zone

Cell cluster	Weight (%)	EPS	Weight (%)
Na-K	0.48	Na-K	19.78
Mg-K	3.04	Mg-K	6.47
Al-K	10.93	Al-K	0.11
Si-K	16.99	Si-K	11.40
P-K	21.27	P-K	1.60
S-K	15.90	S-K	11.22
Ca-K	11.61	Ca-K	8.77
Fe-K	9.23	Fe-K	0.09
Cl-K	0.18	Cl-K	25.54
K-K	8.78	K-K	9.01
Ti-K	1.59	Ti-K	0.02

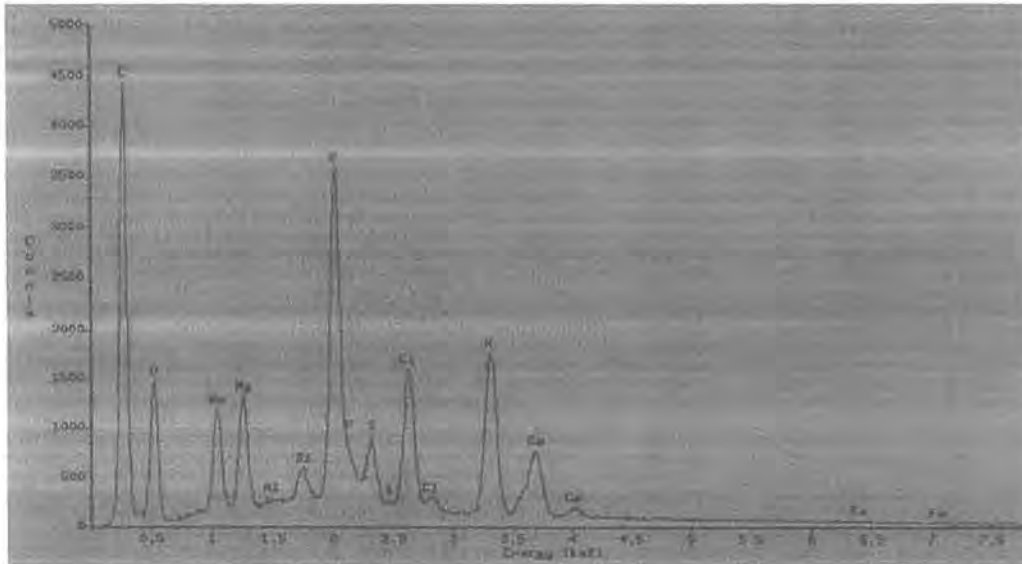


Fig 2. A typical X-ray spectrum of Zeekoegat anaerobic sludge cell clusters.

Schonborn *et al.* (2001) indicated that phosphorus could bind to these cations to form magnesium-phosphate compounds (MAP) and calcium-phosphate compounds. The phosphorus content in both cell clusters with EPS and EPS alone was high when the concentration of magnesium and calcium were high (Fig 2, 3 & 4, Table 4.1& 4.2, 5.1 & 5.2, 6.1 & 6.2). This situation was different to the other two plants i.e. Waterval and Vlakplaats (Fig 5-6 and Table 7.1 & 7.2, 8.1 & 8.2). The EDS analyses of activated sludge samples and EPS in the Waterval and Vlakplaats wastewater treatment plants indicated a low concentration of phosphorus (average of 25%P in cell clusters) and 5%P in the EPS alone.

The cations detected in these two plants by EDS analysis were also low (5%Mg in cell clusters and 4%Mg in EPS on average for both plants and 10%K in cell clusters and 7%K in EPS on average for both plants) (Table 7.1 & 7.2, 8.1 & 8.2 and Fig 5-6).

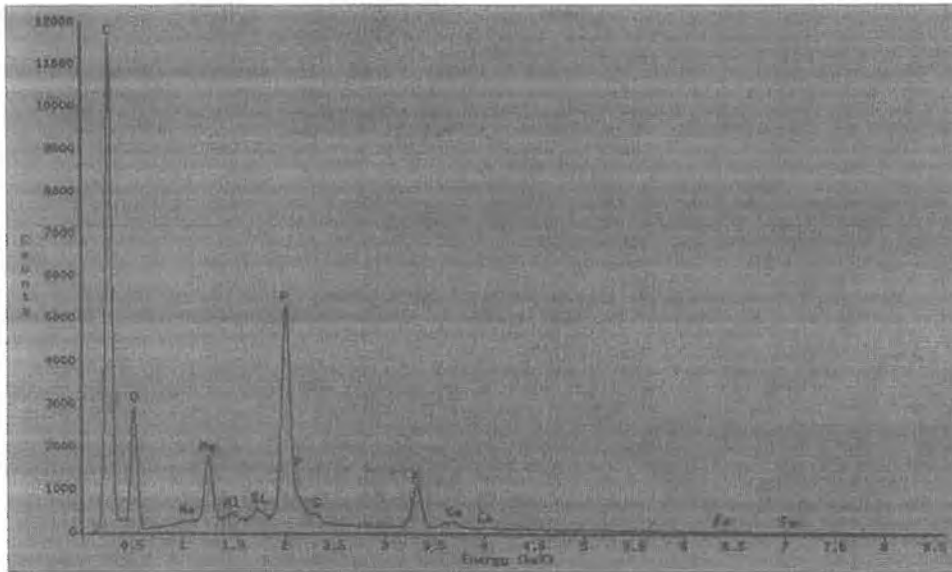


Fig 3. A typical X-ray spectrum of the Bavianspoort aerobic activated sludge EPS.

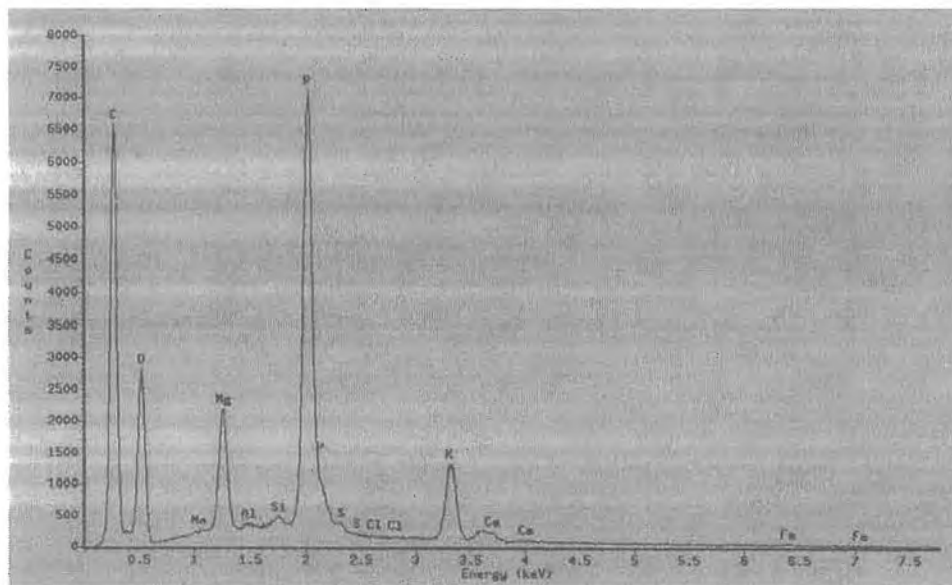


Figure 4. A typical X-ray spectrum of Daspoort anaerobic sludge cell clusters.

The Daspoort wastewater treatment plant which works exclusively biological, contained a higher amount (57%) of phosphorus compared to other plants (Fig. 4 and Table3 & 4.1-4.2). The elemental analysis indicated that the phosphorus content was related to magnesium and potassium (Table 4.1 – 8.2). The phosphorus content in the Zeekoegat

waste water treatment plant was 15% (phosphorus) lower compared to that of the Daspoort waste water treatment plant and 10% (phosphorus) lower as compared to Baviaanspoort waste water treatment plant (Table 3). Daspoort waste water treatment plant is an EBPR process which does not seem to be magnesium and potassium limited. Plants which function under magnesium and potassium limitation tend to be inefficient in terms of phosphorus removal (Pattarkine and Randall, 1999).

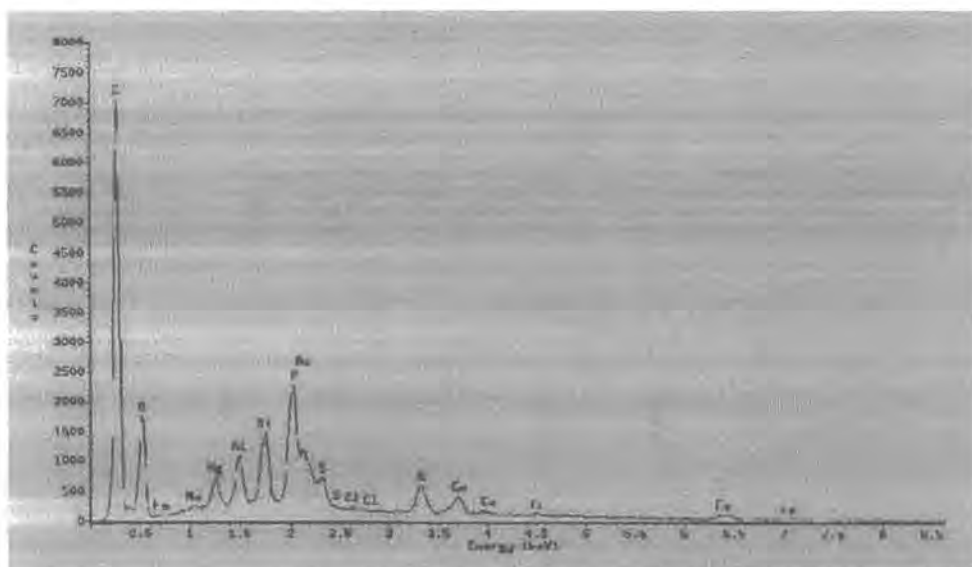


Figure 5. A typical X-ray spectrum of Waterval anaerobic EPS sample.

EDS analyses (Fig.5 & 6) of Waterval and Vlakplaats waste water treatment indicated that they are EBPR processes which functions under magnesium and potassium limitation. Metal cations tend to be important in terms of phosphorus removal in EBPR systems. One possible mechanism of explaining phosphorus removal by the EPS is that, phosphorus binds to Mg^{2+} , K^+ and Ca^{2+} ions to form insoluble compounds which then precipitate on the EPS. This then indicates that phosphorus which is bound to EPS and cell cluster is mostly in the form of magnesium-phosphate compounds and potassium-phosphate compounds and some of phosphorus is also bound to calcium to form calcium-phosphate compounds. Bonting *et al.*, (1993) investigated the influence of the Ca-, Mg- and K- concentration the culture medium on the elemental composition of polyphosphate

granules in *Acinetobacter johnsonii*. They found that under conditions of a low Ca-concentration (Ca=16.4mg/l, Mg=59mg/l and K=126mg/l), the polyphosphate granules contained besides phosphorus, only magnesium and potassium with no calcium and at a low concentration of magnesium and high Ca-concentration (Mg=0.73mg/l; Ca=164mg/l) only Ca- rich granules were found. If the concentrations of all the cations were high (Ca=164mg/l; Mg=59.3mg/l; K=126mg/l) all three cations were detected as the components of the polyphosphate granules. Investigations of P-release and P-uptake behavior have shown that magnesium and potassium are released and taken up simultaneously with phosphorus (Pattarkine and Randall, 1999) and therefore possibly bind to the EPS. This fact is regarded as an evidence that Mg^{2+} and K^{+} are bound as counter-ions in polyphosphate chains (Schonborn *et al.*, 2001).

Chemical precipitation of phosphate on cell surfaces or bound to extracellular polymers has been proposed to explain P removal in activated sludge (Seviour and Blackall, 1999). Precipitation of hydroxyapatite in the sludge may result in P removal at high pH, however, solubility calculations suggest that phosphate concentrations are not sufficiently high for this mechanism in EBPR activated sludge, and a removal of Mg^{2+} , K^{+} and Ca^{2+} ions concomitant with the phosphate removal has been observed (Carberry and Tenney 1973). Considerable evidence exists suggesting that poly P in activated sludge is stabilized by cations. In conclusion it may be postulated that potassium, magnesium and calcium are important in phosphorus removal in terms of phosphate found in the EPS.

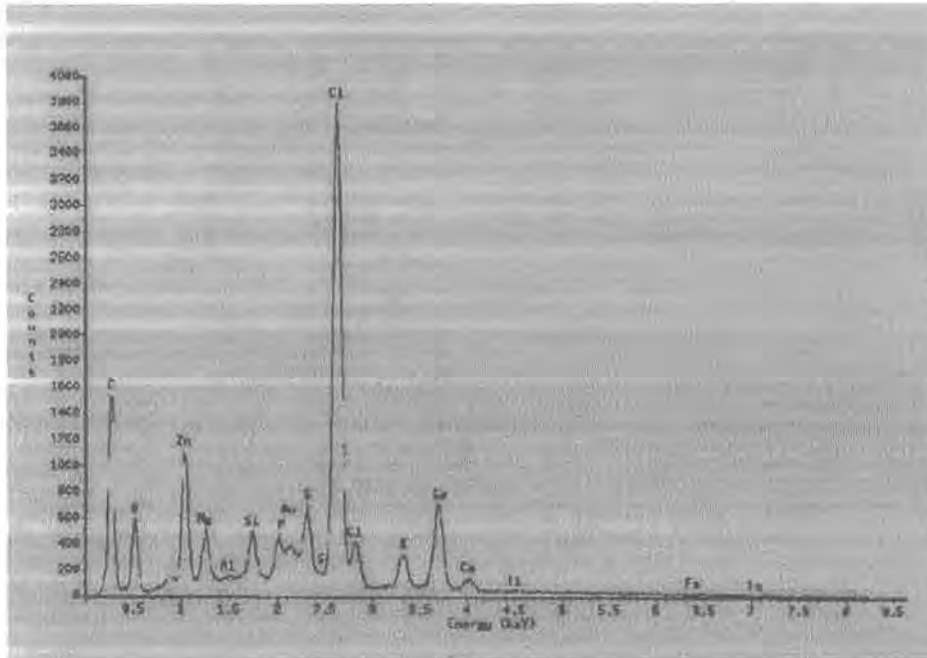


Figure 6. A typical X-ray spectrum of Vlakteplaats aerobic sludge cell clusters.

The role of EPS in the biological removal of phosphorus in activated sludge is not well understood. More studies still need to be done to understand the possible mechanism by which EPS removes phosphorus from activated sludge.

3.4 Orthophosphate analysis

Orthophosphate analyses were performed on the aerobic and anaerobic activated sludge samples and also in both the anaerobic and aerobic extracted EPS samples. Analyses on EPS were performed before EPS can be filtered through a 0.22µm Millipore filter, and after the EPS was filtered through a 0.22µm Millipore filter paper. Anaerobic samples (sludge and EPS) contained the highest concentration of the PO_4^{3-} (Fig. 7.). The anaerobic activated sludge sample contained a maximum of 11.63mg/l PO_4^{3-} , while the anaerobic EPS before filtration and after filtration contained respectively 10.05mg/l and 7.96mg/l PO_4^{3-} (Fig 7). The orthophosphate concentration of the anaerobic sample as compared to

that of unfiltered EPS where slightly equivalent (Fig. 7). This might have been caused by the fact that the unfiltered EPS sample did contained some bacterial cells which were in the supernatant after centrifugation. This then caused an increase in orthophosphate concentration in the anaerobic EPS sample due to the fact that the orthophosphate concentration in the cells was also included. The filtered aerobic EPS contained the lowest orthophosphate concentration (7.96mg/l) since the EPS was not contaminated with bacterial cells.

The orthophosphate concentration of the aerobic activated sludge, unfiltered EPS and filtered EPS were all less than 1mg/l (Fig. 8). The aerobic activated sludge sample contained the highest orthophosphate concentration (0.87mg/l). The orthophosphate concentration of the unfiltered aerobic EPS sample was two and half times higher than the aerobic filtered EPS samples (Fig. 8). This was due to the fact that the unfiltered EPS sample was not free from cells. Orthophosphate concentration was higher in the anaerobic zone EPS samples than the aerobic zone EPS samples. This indicated that high a concentration of phosphorus was removed by anaerobic EPS as compared to the aerobic EPS samples. Anaerobic conditions favoured the production of EPS and phosphorus adsorption (precipitation). Aeration in the aerobic zone could have been responsible for the decrease in EPS production. Due to carbon limitation in the aerobic zone, aerobic conditions would favour phosphorus metabolism. This suggests that phosphorus removal under anaerobic conditions involves cation induced precipitation in the EPS and under aerobic conditions phosphorus metabolism is involved.

The orthophosphate concentration of both the samples from the anaerobic zone and the aerobic zone were compared (Fig. 9). The orthophosphate concentration in the anaerobic samples (sludge, unfiltered and filtered EPS) was 10 times higher than orthophosphate concentration in the aerobic samples (sludge, unfiltered and filtered EPS) (Fig. 9). Several mechanisms have been proposed to explain the enhanced uptake of phosphorus by microorganisms in waste water (Cloete and Bosch, 1993; Pauli, 1993, Baetens, 2001).

It has been shown that for biological phosphorus removal to occur in waste water treatment plants, biomass first needs to pass through an oxygen free and nitrate free phase, i.e. an anaerobic, before entering a phase where an electron acceptor is present, i.e. an anoxic phase where nitrate is present or an aerobic phase where oxygen is present (Cloete and Bosch, 1993).

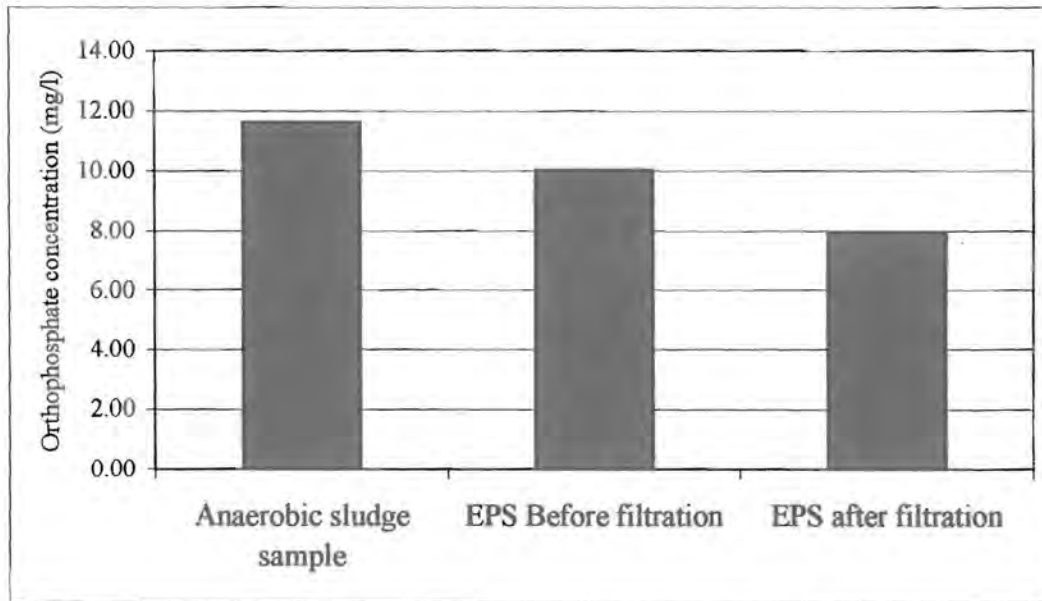


Figure 7. The orthophosphate (PO_4^{3-}) of anaerobic activated sludge, Unfiltered EPS and filtered EPS.

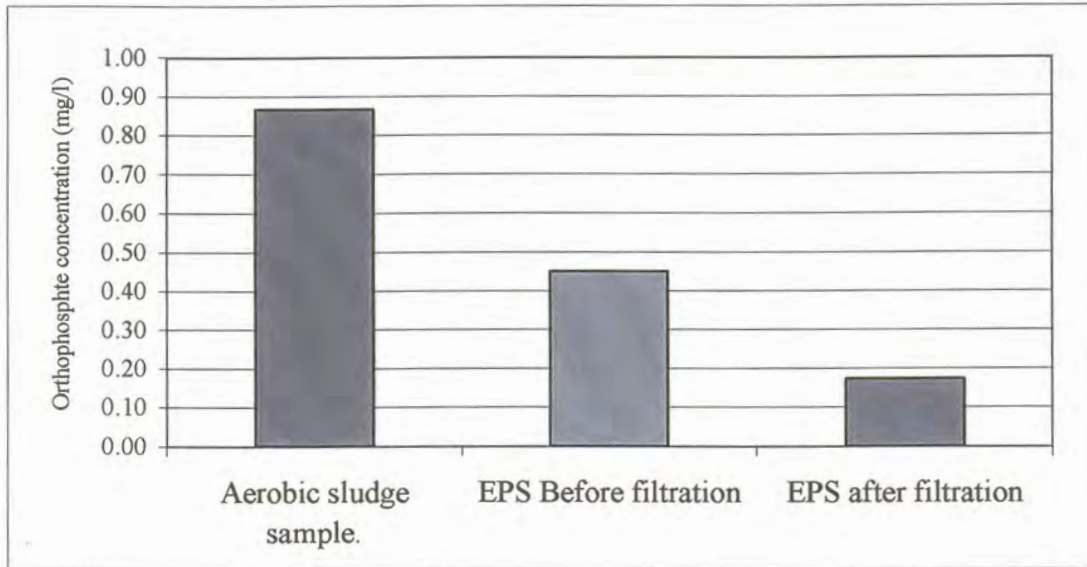


Figure 8. The orthophosphate (PO_4^{3-}) of aerobic activated sludge, Unfiltered EPS and filtered EPS.

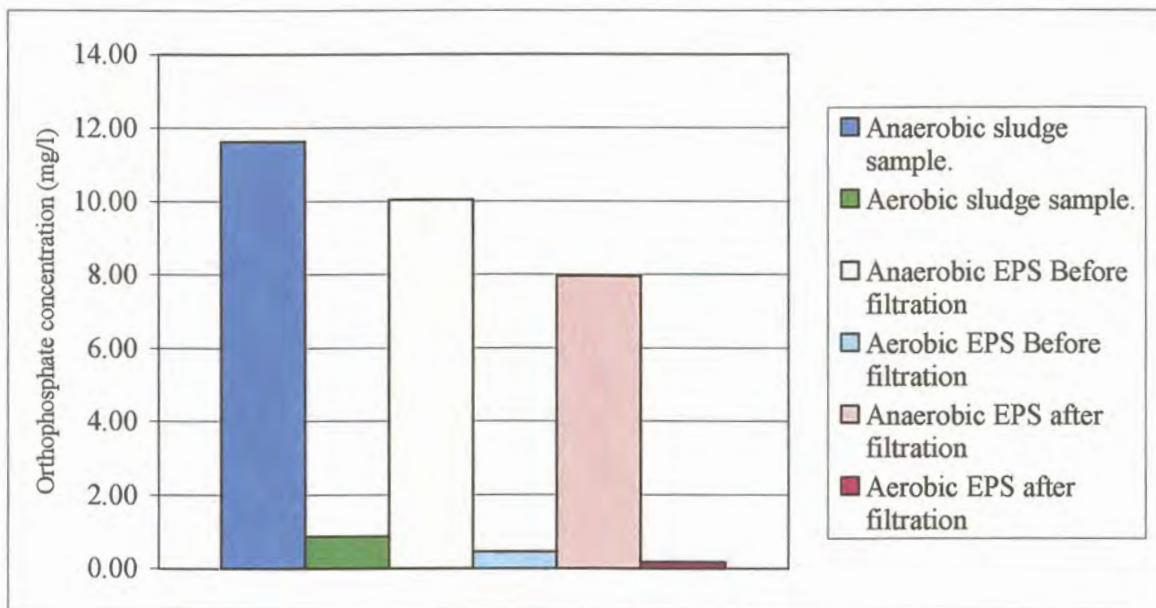


Figure 9. Comparison of the orthophosphate concentration of the anaerobic and aerobic sludge and EPS samples.

High orthophosphate concentrations (Fig. 9) in the anaerobic sludge and the EPS samples and low orthophosphate concentration in the aerobic sludge and the EPS samples could be explained as follows; when the waste water enters the anaerobic zone (phase), specialised microorganisms, called the polyphosphate accumulating bacteria (PAOs) accumulate carbon sources as an internal polymer called PolyHydroxyAlkanoates (PHAs) (Dienema *et al.*, 1983). The energy to store this polymer is obtained from breakdown of glycogen and hydrolysis of energy rich internal phosphorus chain called poly- phosphate (poly-P). Since poly-P is broken down to orthophosphate for energy supply, the phosphate concentration in the anaerobic zone increases (Barnard, 1976; Buchan, 1983; Lotter, 1985; Rybicki, 1997; Mino *et al.*, 1998; Baetens, 2001). In the aerobic zone the stored PHB is consumed, generating energy for growth, for uptake of orthophosphate from the liquid and generating energy and carbon for replenishment of the glycogen and poly-P pools. Under these conditions the orthophosphates concentration thus decreases (Yeoman, 1998). In these study the EPS also indicated that is capable of removing some of phosphorus from the EBPR as the orthophosphate. The reason for low concentration of orthophosphate detected in the aerobic samples might be that, high concentration of orthophosphate was consumed in aerobic zone (phase).

4. Conclusion

The following conclusions were made from this study:

- ❖ The freeze drying method proved to be a reliable method for quantifying the amount of EPS and MLSS from activated sludge samples.
- ❖ The EPS contained more carbohydrates than protein.
- ❖ The concentration of EPS and carbohydrates were higher in the aerobic than in the anaerobic zone
- ❖ The concentration of EPS extracted from activated sludge was different from one plant to plant.
- ❖ The EPS yield depended on the extraction methods.

- ❖ EPS constituted about 12% of the suspended solids in activated sludge
- ❖ The phosphorus content in cell clusters and EPS was related to the amount of cations present in the activated sludge and EPS. The amount of phosphorus in activated sludge flocs plus EPS was 50.5% of the total elemental analysis on average and the EPS alone contained 25% of phosphorus on average for Daspoort, Baviaanspoort and Zeekoigat. The amount of phosphorus in activated sludge cell clusters and EPS of Waterval and Vlakplats plants was low (25% in cell clusters with EPS and 5% in EPS) which might be due to the fact that this EBPR plants functions under potassium, magnesium and calcium limitation. The anaerobic activated sludge and EPS samples contained higher concentration of orthophosphate than the orthophosphate concentration in the aerobic activated sludge and the EPS samples
- ❖ The possible mechanism of phosphorus removal by EPS is that, phosphorus binds to cations (Mg^{2+} , K^+ etc.) to make an insoluble compound (Magnesium-phosphate compounds, Calcium phosphate compounds etc.) which precipitate in the EPS associated with the activated sludge flocs.

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Website: <http://clytongrp.com/semeds-art.htm>

CHAPTER 4

Chemical analysis in extracellular polymeric substances extracted from EBPR activated sludge

Abstract

Four different extraction methods, i.e. formaldehyde-NaOH, regular centrifugation (RCF), formaldehyde and EDTA were employed to study their effectiveness in extracting the extracellular polymeric substances from aerobic and anaerobic activated sludge samples. The results indicated that, the formaldehyde-NaOH yielded the highest (468mg EPS/l anaerobic activated sludge and 320mgEPS/l aerobic activated sludge) concentration of the EPS. The formaldehyde and the EDTA method yielded respectively (327mgEPS/l; 330mgEPS/l of anaerobic sludge and 261mgEPS/l; 266mgEPS/l of aerobic activated sludge) concentration of EPS. The EDTA and formaldehyde methods lead to 20-30% more EPS extracted than with the regular centrifugation method. The regular centrifugation method yielded the lowest concentration of extracted EPS. The formaldehyde extraction procedure extracted equivalent concentrations of carbohydrates compared with the EDTA method and about 8% more carbohydrates than the regular centrifugation method using and anaerobic EPS sample. In general, all the methods extracted about 44% protein, 33% carbohydrates and 1-3% DNA.

1. Introduction

Phosphorus removal from wastewater can be achieved through chemical precipitation and coagulation, through biological treatment or a combination of both (Pauli, 1994). Enhanced biological phosphorus removal (EBPR) in wastewater treatment has long been studied (Toerien *et al.*, 1990). According to the most commonly accepted theory, EBPR is based on the enrichment of activated sludge with bacteria capable of accumulating orthophosphate (PO_4^{3-}) in excess to the normal metabolic requirements of the cell (Pauli, 1994). Phosphorus removal in sludge may also be the result of natural simultaneous precipitation, that is, if cations such as Ca^{2+} , Fe^{2+} , Mg^{2+} , K^+ , Al^+ and Zn^+ are present (Pauli, 1994; Baetens, 2001). It has been demonstrated in some full-scale wastewater treatment plants, that the EBPR process is able to reduce phosphorus concentrations to very low levels (Oldham, 1985; Ketchum *et al.*, 1987). Cloete and Oosthuizen (2001) indicated that extracellular polymeric substances (EPS) are also capable of removing phosphorus in EBPR.

Microbial extracellular polymeric substances (EPS) are high molecular weight mucous secretions of bacteria and microalgae (Zhang *et al.*, 1999). EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeating units within polymer molecules such as many polysaccharides (Wingender *et al.*, 1999). The abbreviation “EPS” is used as a more general and comprehensive term for different classes of macromolecules such as polysaccharides, proteins (including enzymes), nucleic acids, (phospho) lipids, and other polymeric compounds which have been found to occur in the intracellular space of microbial aggregates (Wingender *et al.*, 1999). The EPS are responsible for cohesive forces which keep these aggregates together, i.e. biofilms, flocs and sludge (Urbain *et al.*, 1993; Frolund *et al.*, 1996; Liss *et al.*, 1996; Bura *et al.*, 1998; Jorand *et al.*, 1998; Liao *et al.*, 2001; Liu *et al.*, 2001)) due to the bonding with bivalent cations and hydrophobic interactions. The presence of multivalent cations is essential for the formation of ionic bridges and maintenance of ordered structures. The polymer could also act as a reservoir of ions to supplement those which might be deficient under oligotrophic growth conditions. The ionic complement of any biofilm polysaccharides, which is usually polyionic in nature, could be expected to change with the nature of the fluid perfusing the biofilm (Sutherland, 1999).

Some exopolysaccharides may resemble algal alginates in highly specific binding of cations such as Ca^{2+} and Sr^{2+} , but more commonly binding will be less specific (Sutherland, 1983). Other exopolysaccharides bind cations and alter their ordered structure, others are precipitated by a range of metallic ions. Multivalent cations including Sn^{2+} , Al^{3+} , Fe^{3+} and Th^{4+} may all, under certain conditions, precipitate polymers (Corzo *et al.*, 1994). Similar results were observed with various polysaccharides produced marine bacteria and originating from biofilm on rock surfaces (Sutherland, 1999). Ion concentration and pH play an important role in these reaction which may have secondary effects on biofilm structure and function (Sutherland, 1999).

The composition of EPS determines many important properties of biofilm such as density, porosity, diffusivity, strength, elasticity, frictional resistance, metabolic activity and thermal resistance (Zhang *et al.*, 1999). The EPS are considered important for physico-chemical properties of activated sludge flocs and have been implicated in determining the floc structure and in addition keeping the floc in a three-dimensional matrix (Eriksson and Hardin, 1984). The precise role of the EPS in determining the sludge physico-chemical properties is however not completely understood and sometimes the reported role of EPS is contradictory (Urbain *et al.*, 1993).

The chemical composition of the EPS matrix is reported to be very heterogeneous (Frolund *et al.*, 1996). Carbohydrate was identified as the predominant constituent in the EPS of many pure cultures (Cescutti *et al.*, 1999), whereas protein was found in substantial quantities in the sludge of many wastewater treatment reactors (Fang and Jia, 1996; Viegas *et al.*, 1997). Humic substances (18-23% of VS), uronic acids (about 6%) and DNA (about 1- 1.2%) were also detected in EPS (Frolund *et al.*, 1996; Liu and Fang, 2002). The EPS normally contains small quantities of DNA, which are released from the dead cells after lysis. Variations in the EPS composition can be attributed to several factors. Firstly, activated sludge from plants with different process design can give different EPS extracts (Urbain *et al.*, 1993). Secondly, different extraction procedures affects the EPS yield (Rudd *et al.*, 1983) and finally, different analytical tools are used for analyzing the chemical composition of the extracted EPS, which can cause further variability in the results.

Quantification of EPS is dependent upon the extraction method used (Wingender *et al.*, 1999). Many methods have been used to extract the EPS from different bacterial cultures and activated sludge. The physical extraction methods include centrifugation, ultrasonication and heating, whereas common chemical extractions include the use of alkaline, EDTA, cation exchange resin, NaOH and formaldehyde. The extraction procedure must be selected for each case considering the specific needs and constraints. In some studies, a certain fraction, e.g., the polysaccharides are extracted for a more detailed chemical or structural analysis. In this case lysis of the bacteria may not be a problem, given that impurities are removed before further analysis. In other cases a quantitative extraction of all EPS is desired, and here it is of critical importance that no lysis (breakage of the cell membrane, and the release of cell material) takes place (Wingender *et al.*, 1999). A number of different methods have also been applied in studies related to activated sludge and biofilms.

Only a few of the methods have been thoroughly evaluated, to obtain an optimal extraction procedure with a high extraction efficiency, without unwanted cell lysis and disruption of macromolecules. The best extraction method will depend on the type of interactions that keep the EPS components together in the matrix. The main forces involved in the binding of the polymers in the EPS matrix is the van der Waal forces electrostatic interactions, hydrogen bonds and hydrophobic interactions (Wingender *et al.*, 1999), and in some cases, covalent bonds and disulfide bonds in glycoproteins (Wingender *et al.*, 1999). The dominating forces may be different from one EPS matrix to another and hence various methods must often be evaluated. Many chemical extraction methods rely on breakage of the electrostatic interactions, thereby promoting the extraction of water soluble compounds. Less focus has been on the hydrophobic components, probably because it is difficult not to destroy the cells with the procedure (Wingender *et al.*, 1999).

With the physical methods, a shear is applied to extract the EPS by centrifugation, mixing, shaking sonication or heat treatment. In general, the extraction yield is lower when using a physical treatment than when using combined chemical and physical treatments (Nielsen and Jahn, 1999). Centrifugation is often used to separate the soluble (slime) EPS from cell biomass. The shear applied by centrifugation may only

to a very limited extent extract bound EPS. High speed centrifugation was proposed as the most effective method for EPS extraction (Wingender *et al.*, 1999). However, centrifugation does not provide any significant extraction of bound EPS from microbial aggregates from natural systems, as the components are usually strongly bound (Wingender *et al.*, 1999). Centrifugation is, however, almost always used after any extraction procedure to separate the extracted EPS from cells and other particles.

The chemical extraction methods include addition of various chemicals to the bacterial sample that can break different linkages in the EPS matrix, facilitating a release of EPS (Nielsen and Jahn, 1999). The repulsion among the components in the EPS matrix and the water solubility can also be increased by an exchange of divalent cations with monovalent ions. Chemical and physical methods are sometimes combined in order to increase EPS quantity. Ion exchange by Dowex extraction has been used in combination with shear forces (stirring) to extract EPS from activated sludge and biofilms (Jahn and Nielsen, 1995).

However, there is no standard extraction procedure, making it very difficult to compare and interpret published results. For example, Horan and Eccles (1986) and Morgan *et al.* (1990) found more carbohydrate than proteins in activated sludges with a ratio of 0.16 – 0.70. Foster and Clarke, (1983) found more proteins than carbohydrates in digested sludge with a ratio of 1.1 – 5.1.

The aim of this work was to:

- compare different extraction methods in extracting the EPS from activated sludge
- characterize the chemical composition of EPS in activated sludge

2. Materials and Methods

2.1. Sampling

Activated sludge samples were collected from the Daspoort municipal wastewater treatment plant situated in Pretoria, South Africa. This is an enhanced biological

phosphorus removal (EBPR) wastewater treatment plant which functions exclusively biological without addition of any chemicals. Activated sludge samples were collected at the end of aerobic and anaerobic zone and transferred into 500ml schott bottles. The samples were stored on ice and transported into laboratory for analysis.

2.2. Extraction of EPS

The EPS was extracted using four different methods i.e. Regular centrifugation (RCF), EDTA, Formaldehyde (Zhang *et al.*, 1999) and formaldehyde plus NaOH (Liu and Fang, 2002). The samples were washed by centrifuging 10ml of each activated sludge samples at 3500rpm for 5 min. The supernatant was removed and stored for further use. 10ml of sterile double distilled water was added to the pellet and the samples were homogenised 3 times for 30s on ice using a Cole-Parmer homogenizer with an output of 50%. After homogenisation the previously removed supernatants were added to the homogenised samples. For RCF the EPS was removed for 30min at 12000rpm at 4⁰C using a Sorvall RC-⁰B refrigerated superspeed centrifuge. For EDTA extraction method, 10ml of 2% EDTA was added to the samples and extraction took place at 4⁰C for 3h. For formaldehyde extraction, 0.06ml of 36.5 % formaldehyde was added to the sample and extraction took place at 4⁰C for 1h. For the formaldehyde plus NaOH extraction method, the same procedure as for the formaldehyde extraction method was followed, thereafter 4ml of 1N NaOH was added to the sample and extraction took place at 4⁰C for 3h. For all later methods, the EPS was collected by centrifugation at 15000rpm at 4⁰C for 20min. In order to remove the microbial cells from the EPS, the supernatant was collected and filtered through a 0.22 μ m Millipore filter. For the chemical and orthophosphate analysis, the filtrate was not freeze dried. The filtrate was freeze dried (18h) for EPS quantification. The EPS was weighed using Mettler AE 163 balance.

2.3. Chemical composition analysis of the EPS

Extracellular polymeric substances (EPS) for chemical analysis was extracted as mentioned in 2.2, the difference was that the filtrate was not freeze dried. A phenol sulfuric method (Dubois *et al.*, 1956) was used to quantify carbohydrates using

glucose as the standard. The absorbance was read at 490nm. Proteins were quantified using the Lowrey method (Lowrey *et al.*, 1951), with a Hitachi U –2000 double beam spectrophotometer. The concentration of protein (mg/ml) was calculated using the following formula: Protein (mg/ml) = $1.55A_{280} - 0.76A_{260}$ wherein A_{280} was the absorbance of proteins and A_{260} the absorbance of nucleic acids if they were present.

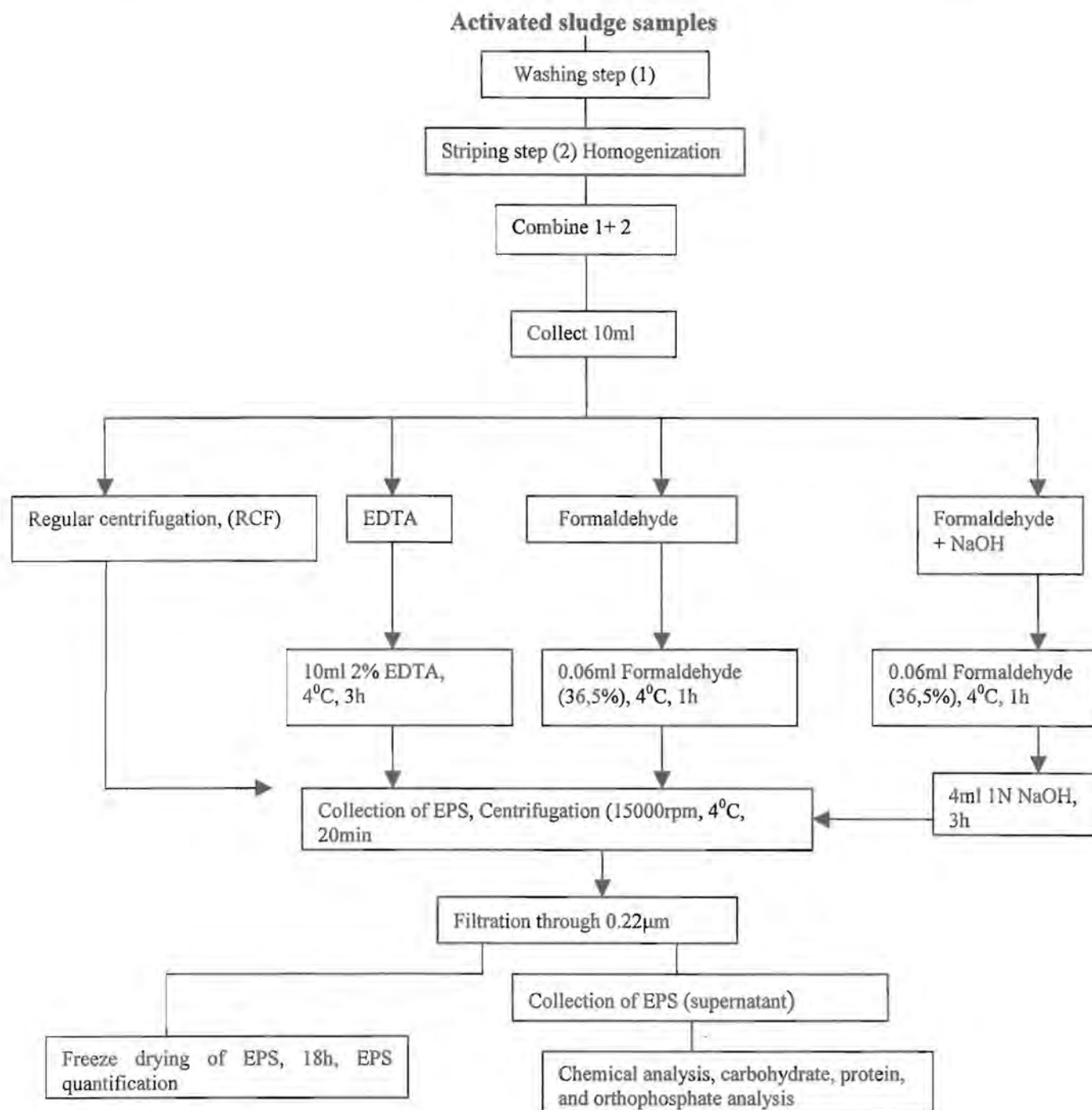


Fig 1. Procedure for the EPS extraction

3. Results and discussion

3.1. Extraction of the EPS

Four different extraction methods Regular centrifugation (RCF), (Zhang *et al.*, 1999) with some modification; EDTA, Zhang *et al.*, 1999 with some modification, formaldehyde; formaldehyde plus NaOH, (Liu and Fang, 2002) were used in this study and compared (Figure 1).

The methods were compared for two analyses:

- The efficiency of the method for extracting the EPS from the activated sludge samples with respect to the quantity of EPS extracted
- Comparison of the methods with respect to the chemical composition of the EPS extracted, mainly carbohydrates and the proteins.

3.1.1. The quantity of extracted EPS from the activated sludge samples

The concentration (quantity) of the extracellular polymeric substances extracted by four different processes (one physical and three chemical extraction procedures) are summarized in Table 1. In this study, the effect of time on EPS extraction was not examined.

Table 1. The concentration of EPS extracted from both anaerobic and aerobic activated sludge samples by four different procedures

Extraction procedure	Anaerobic (mg/l sludge)	Aerobic (mg/l sludge)
Regular centrifugation	230 ± 1.15	175 ± 2.64
EDTA	330 ± 1.52	266 ± 3.00
Formaldehyde	327 ± 1.22	261 ± 2.00
Formaldehyde-NaOH	468 ± 2.00	320 ± 4.35

Mean value (n=3) ± SD

The concentration of EPS extracted from the activated sludge sample was dependent upon the extraction method (Table 1). Since there is no standard method for the extraction of the EPS, it remains difficult to compare the results from one study to another. Amongst all those methods used, the formaldehyde-NaOH yielded the highest concentration of the EPS (Table 1). The formaldehyde method and the EDTA method yielded respectively 327mgEPS/l and 330mg/l in the anaerobic sludge and 261mgEPS/l and 266mgEPS/l in the aerobic activated sludge (Table 1). The formaldehyde-NaOH extracted two times more than the other three extraction methods from both the anaerobic and the aerobic activated sludge samples, while the EDTA and formaldehyde extracted 20-30% more than the regular centrifugation method. The regular centrifugation method yielded the lowest concentration of EPS in both the anaerobic and the aerobic activated sludge samples (Table 1).

The EPS has been reported to contribute up to 15% of the sludge total solids (TS) content (Urbain *et al.*, 1993). The study by Frolund *et al.* (1996) indicated an EPS content of 20-25% of activated sludge TS (or 33-42% of volatile solids (VS)), while the work by Liu and Fang, (2002) indicated an EPS content of around 10-18% of VS. Different extraction procedures have been used and are still used to extract the EPS from either the activated sludge samples, pure culture samples or biofilm samples which give varying results from one study to the other. Only few of the methods have been thoroughly evaluated to obtain an optimal extraction procedure with high extraction efficiency. It is of most important to note that almost all methods rely on a certain water solubility of the components extracted, where the more hydrophobic compounds cannot be expected to be extracted.

In this study the formaldehyde-NaOH procedure yielded the highest concentration of EPS from both the anaerobic and the aerobic activated sludge samples (Table 1). Formaldehyde could fix the cell, and thus prevent the lysis, by reacting with amino, hydroxyl, carboxyl, and sulfhydryl groups of proteins and nucleic acid of the cell membrane (Alcamo, 1997). Alkaline treatment by addition of NaOH causes many charged groups, such as carboxylic groups in proteins and polysaccharides to be ionised because their isoelectric points are generally below pH 4-6 (Wingender *et al.*, 1999). NaOH increases the pH, resulting in the dissociation of acidic groups in EPS

and the repulsion between the negatively charged EPS. This also increases the EPS solubility in water and therefore allows more EPS to be extracted.

The EDTA also yielded higher a concentration of EPS than the regular centrifugation procedure. The EDTA is a complexing agent which can remove the divalent cations, mainly Ca^{2+} and Mg^{2+} which are very important for the cross linking of charged compounds in the EPS matrix (Wingender *et al.*, 1999). Therefore, by removing these divalent cations the EPS matrix tends to fall apart. The regular centrifugation method yielded the smallest concentration of EPS as compared to other three methods. This might have been caused by the shear applied by the centrifugation leading to cell disruption. In general, the extraction yield was lower when using a physical treatment (Wingender *et al.*, 1999).

The EPS concentrations were higher in the anaerobic zone than the aerobic zone (Table 1). The results for this might be due to the different in sludge solids content. Liu and Fang, (2002) found higher (179mg/g VS) concentration of EPS from anaerobic sample than in the aerobic sample (165mg/g VS).

3.1.2. The composition of EPS extracted from activated sludge samples.

The EPS samples contained both the carbohydrates and proteins with a small quantity of DNA. The results in Table 2 and 3 indicate that the formaldehyde-NaOH was the most effective method for extracting the carbohydrates and proteins.

Table 2. Carbohydrate and protein concentrations in the anaerobic wet EPS samples

Extraction procedure	Protein (mg/l sludge)	Carbohydrates (mg/l sludge)
Regular centrifugation	98 ± 4.84	85 ± 3.60
EDTA	104 ± 1.73	90 ± 3.60
Formaldehyde	117 ± 1.73	93 ± 1.15
Formaldehyde-NaOH	213 ± 5.03	185 ± 3.61

Mean value (n=3) ± SD

Table 3. Carbohydrate and protein concentrations in the aerobic wet EPS samples

Extraction procedure	Protein (mg/l sludge)	Carbohydrates (mg/l sludge)
Regular centrifugation	71± 3.61	67± 4.36
EDTA	81 ± 3.22	74 ± 2.33
Formaldehyde	88 ± 2.88	61 ± 1.52
Formaldehyde-NaOH	104 ± 1.86	83 ± 2.14

Mean value (n=3) ± SD

The formaldehyde-NaOH method extracted the highest concentration (213mg/l anaerobic sample; 104mg/l aerobic sample) of proteins, with carbohydrate concentrations of (185mg/l anaerobic sample; 83mg/l aerobic sample) (Table 2-3). In general, the formaldehyde-NaOH extracted twice as much proteins and carbohydrates than the other three methods from both anaerobic and aerobic EPS samples. Formaldehyde, EDTA and regular centrifugation, in descending order, followed the formaldehyde-NaOH method (Table 2- 3). The formaldehyde extraction procedure extracted about 9-12% more proteins than the EDTA method and about 18-20% more proteins than the regular centrifugation method, from the anaerobic EPS samples and aerobic EPS samples.

The formaldehyde extraction procedure extracted equivalent concentrations of carbohydrates compared to the EDTA method and about 8% more carbohydrates than the regular centrifugation method from the anaerobic EPS sample. In the aerobic EPS sample, the situation was different, the formaldehyde extraction procedure extracted less concentration (61mg/l) compared to regular centrifugation method which extracted 67mg/l of carbohydrates. The EDTA procedure extracted about 8% more carbohydrates than the regular centrifugation method and about 13% more carbohydrates than the formaldehyde method.

For years carbohydrates have been considered to be the main constituents of EPS in pure cultures (Sutherland and Kennedy 1996; Sutherland, 1997). Recent studies of

mixed cultures in wastewater treatment systems indicated that protein was also an important constituent in the EPS (Dignac *et al.*, 1998). In this study the EPS content was about 44% of proteins and 33% of carbohydrates. The higher protein concentration could be due to the presence of large quantities of exoenzymes from bacterial excretions, such as lysis products (Frolund *et al.* 1995; Dignac *et al.* (1998). Sponza, (2002) indicated that the reason for the high protein concentration in activated sludge flocs, may be due to the limited utilization of extracellular enzymes excreted from the cells, since the readily biodegradable organic substances are degraded easily by microorganisms and this probably caused the accumulation of proteins. In other words limited utilization of extracellular material released from the bacteria may cause accumulation of enzymes in the cell wall in the presence of readily biodegradable organics in waste water (Sponza, 2002).

Although localization of extracellular enzymes has not been clearly established in activated sludge flocs, a few studies have indicated that exoenzymes are associated with the EPS (Cadoret *et al.*, 2002). However, the distribution of the extracellular enzymes between the cell surface and the EPS is still unknown. These extracellular enzymes are involved in the biodegradation of high molecular weight compounds by sewage biofilms and activated sludge flocs. Complex wastewater organic matter is biodegraded by various enzymes expressed by the mixed-species microbial aggregates (Cadoret *et al.*, 2002). Availability of the extracellular enzymes in the EPS might have caused the increase in protein quantity. In general, all the methods extracted about 44% protein, 33% carbohydrates. This indicated that extraction of carbohydrates (polysaccharides) need more severe extraction procedures. Frolund *et al.* (1996) indicated that many intact micro colonies were embedded in the EPS, probably the polysaccharides, after examining the flocs after extraction. Frolund *et al.* (1996) also indicated that a significant fraction was not extractable, such as organic fibres and intracellular storage products (e.g. glycogen) in the bacteria. This had an effect on the low concentrations of carbohydrates in this study. Microscopic examination of the flocs after extraction is recommended in order to check the availability on non extracted carbohydrates.

All extraction procedures resulted in 1-2% of DNA in the EPS, except the EDTA extraction procedure which yielded about 2-3% DNA. The low levels of DNA can be

explained by low mortalities and limited cell lysis (Wingender *et al.*, 1999; Sponza, 2002) as a result of high cell numbers of living microorganisms in the activated sludge system (Sponza *et al.*, 2002). The 2-3% DNA yielded using the EDTA extraction method might be due to the fact that, EDTA can remove divalent cations such as Mg^{2+} and Ca^{2+} from the cell wall causing cell lysis, thus releasing components such as lipopolysaccharides (LPS) and also possible contamination with intracellular macromolecules such as DNA (Wingender *et al.*, 1999).

Variations in the EPS composition can be attributed to several factors i.e.

- Activated sludge from different plants with different process design can give different EPS extracts (Frolund *et al.*, 1996)
- Different extraction procedures may be used which affect the yield (Frolund *et al.*, 1996)
- Different analytical tools are used for analysing chemical composition of the extracted EPS, which can cause further variability in the results (Frolund *et al.*, 1996).
- Different cultures are used, for example, pure cultures, mixed cultures, biofilms, activated sludge flocs from different activated sludge systems or different laboratory reactors, which also affect the results.

4. Conclusion

Microbial extracellular polymeric substances were extracted from anaerobic and aerobic activated sludge samples using four different methods. Extraction of EPS from activated sludge includes sampling, washing, extraction and analysis. No universal extraction method exists due to a variation in the properties of EPS components in various bioaggregates, but also due to different aims for performing an extraction. Before an extraction procedure is selected, it is important to consider that the methods are not quantitative and that often only a minor part of the total quantity of EPS is extracted. The efficiency of the extraction methods was evaluated. The orthophosphate concentration in the anaerobic and aerobic sludge samples and EPS samples was investigated.

The following conclusions were made from this study:

- The formaldehyde-NaOH extraction procedure yielded the highest concentration of EPS as compared to the other three methods.
- In general a higher concentration of EPS was extracted from the anaerobic samples than from the aerobic samples.
- In general, all the methods extracted about 44% protein, 33% carbohydrates and 1-3% DNA.

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CHAPTER 5

Determination of mixed liquor suspended solids in enhanced biological phosphorus removal activated sludge using the freeze drying method.

Abstract

The freeze drying method and the standard method (APHA *et al.*, 1989) were used in order to determine the concentration of mixed liquor suspended solids in aerobic and anaerobic activated sludge samples. Analysis were performed on activated sludge samples from three different waste water treatment plants situated in South Africa (Gauteng province). The Waterval waste water treatment plant contained the highest concentration of MLSS (6.42mg/ml in the anaerobic and 3.88mg/ml in the aerobic zone) followed by Daspoort (3.82 mg/ml in the anaerobic and 2.55mg/ml in the aerobic zone) and Vlakplaats with concentrations of 0.63mg/ml in the anaerobic and 0.95mg/ml in the aerobic zone. The concentration of MLSS was more or less the same (with the difference of ± 0.14 mg/ml in the aerobic samples and 0.12mg/ml in the anaerobic samples in all the plants). Higher concentrations of MLSS (± 0.05 mg/ml) were observed when determined by the freeze drying method as compared to the standard method, due to the fact that in freeze drying, samples are not exposed to high temperatures (105°C). Both freeze drying method and standard method gave similar results in each of waste water treatment plants.

1. Introduction

The enhanced biological phosphorus removal (EBPR) process is an economical and environmental friendly method for removing phosphate (P) from waste water. In the process an anaerobic stage is typically placed at the head of the aerobic stage. Enhanced P removal is attributed to the activities of the polyphosphate accumulating bacteria (PAB). In the anaerobic stage the PAB degrade intracellular polyphosphate to liberate

energy for the uptake of organic substrate. The substrate is oxidized in the aerobic stage to yield energy for the regeneration of polyphosphate.

There are different types of solids in the activated sludge processes e.g. Total suspended solids (TSS), Suspended solids (SS), Volatile suspended solids (VSS), Mixed liquor suspended solids (MLSS) etc (APHA *et al.*, 1989). Mixed liquor is a mixture of raw or settled wastewater and activated sludge contained in an aeration basin in the activated sludge process. Mixed liquor suspended solids (MLSS) is the concentration of suspended solids in mixed liquor that are combustible at 105⁰C, usually expressed in milligrams per liter (mg/l) (APHA *et al.*, 1989). Within the aeration basin, dissolved oxygen (DO), pH, active biological mass, mixing, rate of oxygen utilization, temperature, MLSS concentration and retention time are critical factors that must be closely monitored.

Effluent from a primary clarifier and return activated sludge from a secondary clarifier are mixed together and pumped to an aeration basin. In the basin, aerobic microbes decompose organic matter in the mixed liquor. The MLSS concentration is typically 2500 mg/l with a calibrated range of 0-5000 mg/l (APHA *et al.*, 1989) (http://www.pmlpprocess.com/Industry_Application/ZA_applicMLSS).

Depending on the temperature, type of sludge, size of tank and other variables, the retention time of mixed liquor in an aeration tank ranges from 10 to 20 days. The process operates on either a batch or a continuous basis. In some operations the aeration system is shut down for one to two hours to allow the sludge to settle and a clear supernatant (surface water) to form. The supernatant is then drawn off to allow the addition of more sludge. Due to settling and supernatant pump-out, MLSS concentration in the effluent (activated sludge) is typically double that of the influent MLSS concentration. Part of the mixed liquor is returned to the secondary clarifier. The rest is wasted, that is, diverted from the return loop and pumped to the digesting stage for further processing. Removing the waste activated sludge maintains the MLSS loading level in the aeration basin. (http://www.pmlpprocess.com/Industry_Application/ZA_applicMLSS)

The suspended solid test that is run on the aeration tank mixed liquor is called the MLSS test. It is used as a control test to help find out whether to increase or decrease the rate of sludge return and when to waste sludge. An estimate of the quantity of MLSS to be wasted from the aeration tank of an extended aeration plant may be determined by the rate of settling and centrifuge test on the sludge solids. (http://www.pmlpprocess.com/Industry_Application/ZA_applicMLSS)

The aim of this study was to quantify the concentration of MLSS using the freeze drying method and compare it with the standard method.

2. Materials and methods.

2.1 Sampling

Activated sludge samples were collected from three enhanced biological phosphate removal (EBPR) waste water treatment plants (WTP) i.e. Waterval, Vlakplaats and Daspoort which function exclusively biological without addition of any chemicals. All these waste water treatment plants are situated in the South Africa, Gauteng province. Activated sludge samples were collected at the end of the aerobic and anaerobic zone in all the plants and transferred into 500ml Schott bottles. The samples were stored on ice and transported to the laboratory for analysis.

2.2 Determination of Mixed Liquor Suspended Solids (MLSS)

MLSS was determined using the standard method (Greenberg *et al.*, 1992) and a freeze drying method. Ten ml of each sample was filtered through a whatman no.1 filter paper, after which the filter paper was dried at 105⁰C and the dry weight was determined (APHA *et al.*, 1989). In the freeze drying method for MLSS analysis, 10ml of each sample was transferred into a centrifuge tube. The samples were freeze dried using liquid nitrogen and then freeze dried for 24h. After freeze drying the solid sample then weighed using a Mettler AE 163 balance.

3. Results and Discussion

3.1 Quantification of MLSS

The concentration of the MLSS was given in mg/ml of activated sludge samples from both aerobic and anaerobic samples of three waste water treatment plants which function exclusively biological. Two methods were used for MLSS analysis, the standard method (APHA *et al.*, 1989) and a freeze drying method.

Table 1. MLSS of activated sludge samples from the anaerobic and the aerobic zone of three different wastewater treatment plants.

Method	Waste water treatment Plant	Mass of MLSS in mg/ml (aerobic zone) mean	Mass of MLSS in mg/ml (anaerobic zone) mean
Standard method	Waterval	3.82	6.34
Freeze Drying	Waterval	3.95	6.50
Standard method	Vlakplaats	0.88	0.61
Freeze Drying	Vlakplaats	0.95	0.65
Standard method	Daspoort	2.45	3.77
Freeze Drying	Daspoort	2.66	3.95

Mixed liquor suspended solids is one of the physical characteristics of activated sludge samples which varies from one plant to the other depending on the amount and type of the sludge and characteristics of the plant. Usually the activated sludge of plants which function chemically contain sludge quantities which are higher than that of the biologically functioning plants, due to chemical precipitation (Gray, 1989; Wanner, 1994). The Waterval waste water treatment plant contained the highest concentration of MLSS (6.42mg/ml in the anaerobic and 3.88mg/ml in the aerobic zone) followed by Daspoort (3.82 mg/ml in the anaerobic and 2.55 in the aerobic zone) and Vlakplaats with concentrations of 0.63mg/ml in the anaerobic and 0.95mg/ml in the aerobic zone. The

concentration of MLSS was similar with the difference of $\pm 0.14\text{mg/ml}$ in the aerobic samples and 0.12mg/ml in the anaerobic samples in all the plants. Higher concentrations of MLSS were observed when determined by the freeze drying method than the standard method (Table 1). This might be due to the fact that in the standard method there is an oven drying step at high temperature (105°C), which might cause some of molecules in the sample to evaporate which then lowers the concentration of the MLSS. In freeze drying there is no temperature step involved which might vaporize any components in the sample.

The concentration of the MLSS in the anaerobic zone (Table 1) of the Waterval and Daspoort plants were higher than that of the aerobic zone. In the Vlakplaats works the concentration of MLSS in the anaerobic zone was lower than in the to the aerobic zone (Table 1). The sludge age is a property which determines the MLSS. A longer sludge age results in more wasted sludge (i.e. sludge removed from the system) and MLSS (<http://lemnatechnologies.com/wtclass.html>). In conclusion the freeze drying method was a reliable and efficient in determining MLSS concentration. Future research in determining the concentration of MLSS and suspended solids using the freeze drying method is proposed.

4. References

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CHAPTER 6

General conclusion

The following conclusions were made from this study:

- The concentration of EPS extracted from activated sludge was different from one plant to plant. The EPS yield depended on the extraction methods.
- The formaldehyde-NaOH extraction procedure yielded the highest concentration of EPS as compared to the other three methods.
- All the methods extracted about 44% protein, 33% carbohydrates and 1-3% DNA from the EPS sample.
- The phosphorus content in cell clusters and EPS was related to the amount of cations present in the activated sludge and EPS. The amount of phosphorus in activated sludge flocs plus EPS was 50.5% of the total elemental analysis on average and the EPS alone contained 25% of phosphorus on average for Daspoort, Baviaanspoort and Zeekoigat. The amount of phosphorus in activated sludge cell clusters and EPS of Waterval and Vlakplats plants was low (25% in cell clusters with EPS and 5% in EPS) which might be due to the fact that this EBPR plants functions under potassium, magnesium and calcium limitation.
- The possible mechanism of phosphorus removal by EPS is that, phosphorus binds to cations (Mg^{2+} , K^+ etc.) to make an insoluble compound (Magnesium-phosphate compounds, Calcium phosphate compounds etc.) which precipitate in the EPS associated with the activated sludge flocs.
- The method for quantifying mixed liquor suspended solids (MLSS) based on freeze drying was comparable to conventional method, but had an advantage of yielding high concentration of MLSS.

- The anaerobic activated sludge and EPS samples contained higher concentration of orthophosphate than the orthophosphate concentration in the aerobic activated sludge and the EPS samples

From this study it was postulated that removal of phosphorus by EPS from waste water treatment is enhanced by the presence of cations present in the waste water system. The more the cations in the waste water system the more the phosphorus is precipitated in the EPS. Introduction of compounds into the waste water system which contains cations (Mg^{2+} , K^+ , Ca^{2+}) is recommended to ensure enhanced biological phosphorus removal by EPS. Orthophosphate concentration was higher in the anaerobic zone EPS samples than the aerobic zone EPS samples. This indicated that high a concentration of phosphorus was removed by anaerobic EPS as compared to the aerobic EPS samples. Anaerobic conditions favoured the production of EPS and phosphorus adsorption (precipitation). Aeration in the aerobic zone could have been responsible for the decrease in EPS production. Due to carbon limitation in the aerobic zone, aerobic conditions would favour phosphorus metabolism. This suggests that phosphorus removal under anaerobic conditions involves cation induced precipitation in the EPS and under aerobic conditions phosphorus metabolism is involved.