

**MICROBIAL CHARACTERIZATION
OF ACTIVATED SLUDGE
MIXED LIQUOR SUSPENDED SOLIDS**

**Report to the
WATER RESEARCH COMMISSION**

By

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**WRC Project No 1191/1/03
ISBN No 1-86845-999-3**

March 2003

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EXECUTIVE SUMMARY

BACKGROUND:

In terms of these design the procedures and kinetic models, in the bioreactor of the non-nitrifying aerobic activated sludge system the mixed liquor organic suspended solids is made up of three components: heterotrophic active biomass; endogenous residue; and inert material. The heterotrophic active biomass arises from synthesis of living heterotrophic organisms on biodegradable organic substrates and is 'lost' via endogenous respiration/death processes; in the activated sludge system this mixed liquor component performs the biodegradation processes of COD removal and denitrification. Historically the mixed liquor suspended organic solids has been measured as a lumped parameter, via the VSS test or more recently, the COD test.

Currently, the heterotrophic active biomass exists only as a hypothetical parameter within the structure of the design procedures and kinetic models. The problem in measurement of this parameter has been the lack of suitable experimental techniques. In the literature, principally microbiological techniques have been proposed; for example, pour plate or other culturing techniques, ATP analysis and DNA analysis, using fluorescent probes. The active biomass is probably the most important process parameter and currently no reliable method exists to measure it. ATP is present in all microbes and can be measured with great sensitivity (Coetzee, 1999). Because ATP is rapidly lost following the death of cells, measuring ATP concentrations can be used to estimate living biomass (Holm-Hansen and Booth, 1966). The objective of this investigation was to use ATP as a method to determine the active biomass fraction in activated sludge.

SUMMARY OF MATERIALS AND METHODS:

Grab samples were taken from the aerobic zones of five activated sludge systems in and around Pretoria (i.e. Daspoort, Centurion, Baviaanspoort, Zeekoegat and Rooiwal). All samples were collected at the end of the aerobic zones. All samples were analysed within 8 h of sampling and all analyses were performed in triplicate.

ATP was measured on site as well as lab by means of the ATP Bioprobe (Hughes Whitlock) after 5 min homogenization. Total plate counts were done using the spread-plate technique on Nutrient Agar (NA) after 48h incubation at room temperature.

The following physicochemical analyses were conducted using standard procedures: MLSS, pH, NO_3^- , PO_4^{3+} , SO_4^{2+} and NH_4 . The OUR and ATP experiments were done at UCT using their laboratory reactors. The OUR was measured on-line while ATP was measured using ATP Bioprobe after sonication of the sample for 5 min.

DISCUSSION

Orthophosphate removal was consistently high with higher biomass concentrations as measured by TPC and ATP. This supports the notion that the viable biomass fraction of the MLSS is the key to orthophosphate removal by activated sludge. However, maintenance of large fractions of viable biomass in activated sludge will select for smaller flocs, causing poor settling (Roe and Bhagat, 1982). It is thus important to find a situation of equilibrium between viable biomass and settling performance to optimize the activated sludge process.

The method has been shown in the current study to be superior to the traditionally used methods of TPC, MLSS and MLVSS for biomass determination. MLSS and MLVSS did not resemble the viable population as measured by ATP or TPC. ATP was also found to be a better biomass estimator than TPC due to higher (at least one log unit) bacterial counts and smaller standard deviations. It is a cheap, simple and fast method, not requiring special training for laboratory personnel and with a small capital input for a portable luminometer, giving on-the-spot results.

The ATP results followed the same trend as the OUR results. The OUR of the organisms increased slightly with time, and then decreased after a few hours of the experimental period. The ATP values also increased with time, but in this case the value increases sharply after 2 hours of incubation before dropping down again. This is an indication that there is an increase in viable biomass numbers as the organisms utilizes the substrate. The gradual increase in the ATP value could be an indication that after some time the organisms are well adapted to the conditions in the bioreactor and that can optimally utilize the substrate, resulting in increases in their numbers, and hence an increase in the ATP and OUR values. This results indicate that ATP and OUR are good indicators of viable biomass numbers.

CONCLUSIONS:

- ATP proved to be a more reliable method for indicating the biomass concentration than TPC, due to the higher yield and a smaller standard deviation.
- Orthophosphate removal was consistently higher in the sludges with higher initial ATP and TPC values, indicating a relationship between viable biomass and orthophosphate removal.
- The MLVSS showed the same trend in orthophosphate removal as the MLSS, although always somewhat lower, due to it being the volatile fraction of the MLSS.
- Neither initial MLSS, initial MLVSS nor changes in the concentrations of these fractions could be directly linked to different orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS to indicate viable biomass and/or differences in the viable biomass fraction in activated sludge.

List of products

Degree:

MSc (Microbiology) Danie Oosthuizen

Presentations:

Posters

Cloete TE and Oosthuizen DJ: SEM-EDS for determining the phosphorus content in activated sludge EPS. International Water Association (IWA), Specialist Conference on Microbial population Dynamics, Rome, June 2001

Cloete TE and Oosthuizen DJ: Phosphorus removal capacity of MLSS and MLVSS fractions of activated sludge plants. IWA, Specialist Conference on Microbial Population Dynamics, Rome, June 2001

Oral

Cloete TE and Oosthuizen DJ: Evidence of phosphorus removal by extracellular polymeric substances in activated sludge using EDS analysis. IWA, Mülheim an der Ruhr, Germany, September 2000

Acknowledgements

The financial support of the Water Research Commission for the work and the support of the Steering Committee is acknowledged with gratitude. The members of the Steering Committee were:

Dr NP Mjoli	Water Research Commission (Chairperson)
Dr G. Offringa	Water Research Commission
Dr HC Kasan	Rand Water
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Prof GA Ekama	University of Cape Town
Assoc Prof MC Wentzel	University of Cape Town
Dr V Naidoo	University of Natal
Mrs CM Smit	Water Research Commission (Committee Services)

We would also like to thank the following wastewater treatment plants for their cooperation and for supplying samples for analysis:

Daspoort

Baviaanspoort

Zeekoegat

Rooiwal

LIST OF ABBREVIATIONS

ADP:	Adenosine diphosphate
ATP:	Adenosine triphosphate
BEPR:	Biological excess phosphate removal
BOD:	Biochemical Oxygen Demand
COD:	Chemical Oxygen Demand
DNA:	Deoxyribonucleic acid
DO:	Dissolved oxygen
MLSS:	Mixed liquor suspended solids
MLVSS:	Mixed liquor volatile suspended solids
OUR:	Oxygen uptake (utilization) rate
TBC:	Total bacterial counts
TPC:	Total plate counts
TSS:	Total suspended solids
VSS:	Volatile suspended solids
WTP:	Wastewater treatment plant

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CHAPTER 1

INTRODUCTION

To optimize the design and operation of activated sludge systems, over the past two decades a number of steady state design models have been developed, to progressively include aerobic COD removal and nitrification, anoxic denitrification and anaerobic/anoxic/aerobic BEPR (biological excess phosphate removal).

In terms of these design the procedures and kinetic models, in the bioreactor of the non-nitrifying aerobic activated sludge system the mixed liquor organic suspended solids is made up of three components: heterotrophic active biomass; endogenous residue; and inert material. In the nitrifying aerobic and anoxic/aerobic activated sludge systems, a fourth mixed liquor organic suspended solid component is included: autotrophic active biomass. The heterotrophic active biomass arises from synthesis of living heterotrophic organisms on biodegradable organic substrates and is "lost" via endogenous respiration/death processes; in the activated sludge system this mixed liquor component performs the biodegradation processes of COD removal and denitrification. The autotrophic active biomass arises from the synthesis of autotrophic organisms in the nitrification of ammonia to nitrate under aerobic conditions and is "lost" via endogenous respiration/ death processes. The endogenous residue is generated from the unbiodegradable portion of the heterotrophic and autotrophic active biomasses that are lost in the endogenous respiration/death processes. The inert material arises from the influent wastewater unbiodegradable particulate organics, which, on entry into the bioreactor are enmeshed in the mixed liquor suspended solids. All four mixed liquor organic suspended solids components settle out in the secondary settling tank and returned to the bioreactor to the bioreactor via the underflow recycle; these components leave the activated sludge system via the waste flow.

Historically the mixed liquor suspended organic solids has been measured as a lumped parameter, via the VSS test or more recently, the COD test. However, from the description above in the bioreactor of the aerobic and anoxic/aerobic activated sludge systems only a part of the mixed liquor organic suspended solids is heterotrophic active biomass, the active fraction, and only this part mediates the biological processes of COD removal and denitrification. Currently, the heterotrophic active biomass exists only as a hypothetical parameter within the structure of the design procedures and kinetic models. Although, indirect evidence provides support for this parameter (by consistency between observations and predictions over a wide range of conditions), it has not been directly measured experimentally and compared to theoretical values.

The problem in measurement of this parameter has been the lack of suitable experimental techniques. In the literature, principally microbiological techniques have been proposed; for example, pour plate or other culturing techniques, ATP analysis and DNA analysis, using fluorescent probes. However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory; the culturing techniques have been widely criticised for their unreliability and the molecular methods are still in their infancy.

The active biomass is probably the most important process parameter and currently no reliable method exists to measure it. ATP is present in all microbes and can be measured with great sensitivity (Coetzee, 1999). Because ATP is rapidly lost following the death of cells, measuring ATP concentrations can be used to estimate living biomass (Holm-Hansen and Booth, 1966). The objective of this investigation was to use ATP as a method to determine the active biomass fraction in activated sludge.

CHAPTER 2

LITERATURE REVIEW

2.1 Adenosine triphosphate (ATP)

Attempts have been made to find simple and reliable methods to determine the biomass in wastewater and activated sludge (Jørgensen *et al.*, 1992). The simplest and most often used method is to measure the Total suspended solids (TSS) or volatile suspended solids (VSS) concentration (Ali *et al.*, 1985). These methods, however, do not distinguish between living cells and debris of organic or inorganic origin. Using the traditional total plate count technique, an underestimation of the biomass is done due to the selectivity of the media employed (Jørgensen *et al.*, 1992).

All living things, including plants, animals and bacteria, require a continual supply of energy in order to function. This energy is used for all cellular processes which keep the organism alive. Some of these processes occur continually, such as the metabolism of food, the synthesis of large, biologically important molecules like proteins and DNA and the transport of molecules and ions throughout the organism. Other processes occur only at certain times, such as cellular movement. However, before the energy can be used, it must first be transformed into a form that the organism can easily handle. This special carrier of energy, is the ATP molecule (Brock, 1979).

The ATP molecule is composed of three components. At the center is a sugar molecule (ribose – the same molecule that forms the basis of DNA). Attached to one side of this sugar group is a base (a group consisting of linked rings of carbon and nitrogen atoms). In this case, the base is adenine. The other side of the sugar is attached to a string of phosphate groups, which are the key to the activity of ATP (Brock, 1979).

ATP is an endergonic molecule, requiring energy to be formed. Energy is stored in the covalent bonds between each phosphate group making up the tail of the molecule (Lee *et al.*, 1971). The last phosphate bond holds the most energy (approximately 7 kcal.mol⁻¹) and is called the pyrophosphate bond. In order to release its energy, ATP breaks down to form ADP (adenosine diphosphate) and an inorganic phosphate group, while releasing energy from the pyrophosphate bond. ADP is an exergonic molecule, yielding energy when formed. When ADP reacts and comes in contact with enough energy and an inorganic phosphate ion, it becomes ATP and stores energy yet again. ADP also needs the energy from the third phosphate group from respiration processes to become ATP (Lundin and Thore, 1976). More ATP is produced from aerobic respiration than from anaerobic respiration because there is more energy involved (Lundin and Thore, 1976).

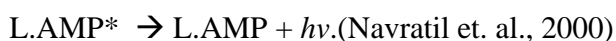
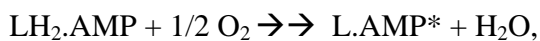
ATP \rightleftharpoons ADP + inorganic phosphate + energy

2.2. The luciferin–luciferase reaction

Luciferase is an enzyme, which reacts with a small molecule called luciferin in the presence of oxygen and ATP. The resulting high-energy compound releases its energy in the form of visible light in a fraction of a second. The emitted light is “cold” and has practically no waste heat (Lundin and Thore, 1976). Luciferins vary in chemical structure. For example, the luciferin in luminescent bacteria is completely different from that of fireflies. For each type of luciferin, there is a specific luciferase. One of the advantages of using luciferase as a reporter of biomass is the convenience and the speed of performing the assay (Stanley, 1989). Using luciferase assay reagents that support maximal luciferase activity is critical because the luminescent intensity of the luciferase-mediated reaction directly impacts on the detection sensitivity of the reporter assay (Stanley, 1989).

The firefly luciferase test for ATP in living cell is based on the reaction between the luciferase enzyme, luciferin (enzyme substrate), magnesium ions and ATP. Light is emitted during the reaction and can be measured quantitatively and correlated with the

quantity of ATP extracted from known numbers of bacteria. When all reactants are in excess, ATP is the limiting factor. Addition of ATP drives the reactions, producing a pulse of light that is proportional to the ATP concentration. The assay is completed in less than 1h. For monitoring microbial populations in water, the ATP assay is limited primarily by the need to concentrate bacteria from the sample to achieve the minimum ATP sensitivity level, which is 10^5 cells/ml. The luminometric method of determination of active biomass concentration is based on measuring the ATP content of cells. ATP serves as a carrier of chemical energy in cells, where available energy is stored in chemical bonds between two final phosphate groups. After cell death, the ATP concentration rapidly decreases. Because ATP is a good indicator of cell viability, its concentration is dependent on active biomass amount. The concentration of ATP can be conveniently determined by the bioluminescence method, where ATP is initially extracted from cells and then reacted with luciferin (LH_2)³ in a reaction catalysed by the enzyme luciferase, while bioluminescent radiation is emitted. The reaction, which takes place, is described as follows:



2.3 The application of ATP for monitoring microbial biomass in wastewater treatment plants

Operational control of biological waste treatment has long been dependent on estimates of *in situ* biomass in the waste stabilization process (Patterson *et al.*, 1970). A more appropriate and desirable parameter would evaluate the metabolic activity of those organisms responsible for the treatment (Patterson *et al.*, 1970). The standard parameter of biomass in activated sludge is mixed liquor suspended solids (MLSS), although it is recognized as an indirect and incomplete measure of the viable sludge floc (Fair and Geyer, 1954; Patterson and Brezonik, 1969, Patterson *et al.*, 1970). Other biomass parameters have been suggested, including particulate organic nitrogen and protein, but these are also unsatisfactory because of the variable concentrations of nonviable particulate organic material present in sewage (Patterson *et al.*, 1970). Furthermore, rapid changes in biological activity are only slowly reflected by changes in any of these parameters (Patterson *et al.*, 1970).

A suitable parameter must fulfill certain criteria to be a useful and appropriate estimate of biomass. For example, the measured quantity should be proportional to some cellular entity (Patterson *et al.*, 1970), such as total organic carbon or dry weight. Also, the substance should have a short survival time after cell death, otherwise it would not be specific for viable biomass. There should also be a sensitive and accurate analytical procedure available to measure the parameter. The authors investigated the occurrence of ATP in activated sludge for the purpose of utilizing this parameter as a measure of metabolic activity and/or biomass. The ATP pool measured, approximated 2 g per mg mixed liquor volatile suspended solids (MLVSS).

Patterson *et al.* (1970) developed the method for ATP measurement, using the reaction between luciferin, luciferase and ATP. The finalized procedure was highly sensitive and reliable. The authors reported relative standard deviations of less than 2 % for activated sludge replicates and nearly 100 % recovery of added ATP from activated sludge. Also, the authors claimed ATP levels in activated sludge to be relatively constant under endogenous conditions, indicating the potential of ATP as an estimate of viable biomass.

To relate ATP concentration to microbial biomass, it is necessary to know the approximate ATP concentration per cell of the microbial species present (Patterson *et al.*, 1970). If ATP is also related to metabolic activity, the physiological state of the culture must be determined (Patterson *et al.*, 1970). Since it is impossible to make a taxonomic analysis of the microbiota present in activated sludge, the accuracy of biomass estimations would depend upon the constancy of the ATP pool among species (Patterson *et al.*, 1970). D'Eustachio and Levin (1967) reported a constant pool of ATP for *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*, which was also constant during all growth phases. In a later study, D'Eustachio and Johnson (1968) investigated the endogenous ATP pool of 13 species of Gram positive and Gram negative aerobic bacteria and found a mean ATP pool of 2.1 μg per mg dry cell material. Also, a linear correlation existed between the endogenous ATP pool and standard plate count for the species involved.

It was uncertain, in the study of Patterson *et al.* (1970), as to the response of the ATP pool to changes in metabolic activity. If there was no change, or only erratic variation, then ATP could not be used as an activity parameter in studies on activated sludge. Thus, an experiment was designed to this extent. Results indicated that the ATP pool is affected by the metabolic activity of an activated sludge culture and may be expected to respond rapidly and decisively to an increase in substrate loading, while only being gradually reduced as the organisms enter an endogenous phase.

Results by Patterson *et al.* (1970) indicated that a significant portion of the MLVSS is non-viable organic material not associated with the oxidative degradation of the substrate. Assuming a mean endogenous ATP pool of 2 μg per mg, dry cell material would result in an estimate that only 40% of the laboratory unit MLVSS was actually viable cell material. In a separate experiment carried out on a contact stabilization plant indicated that only 15 to 20 % of the MLVSS may be active biomass under actual operating conditions.

Upadhyaya and Eckenfelder (1975) found, in a laboratory-scale activated sludge setup, that in general, the viable fraction, as measured by ATP analysis, was found to be higher in experiments with low MLVSS because of less accumulation of non-active mass at lower MLVSS levels. Also, ATP per mass of MLVSS decreased with increases in the cell detention period. The authors also found that the ATP per plate count colony was fairly stable, substantiating the claim that ATP is a measure of viable biomass.

Levin *et al.* (1975) conducted tests at two full-scale municipal treatment plants where ATP was used to control the return sludge flow rate. BOD decreased, MLVSS remained constant and ATP increased for progression through a plug-flow aeration basin. The result seemed to indicate that ATP will measure increased biomass formation by oxidation and incorporation of the BOD, but MLVSS will not. However, as with the investigation of Upadhyaya and Eckenfelder (1975), the ATP content of the return sludge fluctuated substantially, possibly by environmental stress in the form of low dissolved oxygen levels (Roe and Bhagat, 1982).

Jørgensen *et al.* (1992) determined biomass of activated sludge growth cultures in terms of dry weight and compared the data with ATP content and the oxygen uptake rate (OUR). ATP content showed the best correlation with biomass. A conversion factor of 3 mg ATP per g dry weight was calculated. Specific proportions of ATP in relation to total cellular carbon was found to be constant by Atlas (1982), with variations not more than 17 %.

2.4 OTHER APPLICATIONS OF ATP.

2.4.1 ATP as an indicator of cell physiological status: Oxygen transfer applications

When oxygen supply to aerobic populations is reduced, their cellular ATP content is rapidly reduced. Providing the air supply is returned before serious changes occur to the organism, their ATP rapidly rises again after aeration is returned. In contrast to

measuring dissolved oxygen (DO), the measurement of ATP is a direct measurement of cells. Furthermore, it is possible that ATP may more quickly reflect cellular response to oxygen changes. Populations, which are strictly anaerobic, are killed by aeration. Therefore aeration would cause a large reduction in cellular ATP.

2.4.2 Detection of response to toxic substances

This is another of the common uses of the ATP assay in industrial water treatment. When cells are killed, their ATP content is reduced. Observing this reduction of ATP has been used to monitor the effectiveness of a variety of biocides. It could be used to assess the toxicity of various waste streams to a biological waste treatment process (<http://user.fundy.net/pjwhalen/adenosinetriphosphate.html>).

2.4.3 Early detection of poor settling conditions

Poor settling is probably the most common problem in activated sludge plants. Often, long correlation studies are required to solve these problems. The use of turbidity to monitor these problems may be too insensitive. ATP is a much more sensitive and specific detector of microorganisms than turbidity. It has potential to reveal on-coming settling problems much sooner and establish correlation of problems much quicker (<http://user.fundy.net/pjwhalen/adenosinetriphosphate.html>).

Research has indicated the relationship between biomass and phosphorus removal in activated sludge. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) are often used as indicators of biomass, and used as such in the mathematical modelling of biological phosphorus removal. A good biomass assay must measure something relatively constant in concentration and common to all bacteria, but which is absent from all nonliving material, even recently dead cells. Enough is known about microbial intermediary metabolism today to be certain that ATP meets these criteria very well; probably better than any biomolecule (Archibald et al, 2001).

The objective of this study was therefore to use the standard ATP method to measure the viable or active biomass fraction of mixed liquor suspended solids.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

Grab samples were taken from the aerobic zones of five activated sludge systems in and around Pretoria (i.e. Daspoort, Centurion, Bavianspoort, Zeekoegat and Rooiwal). All samples were collected at the end of the aerobic zones. Samples were taken in sterile Schott bottles, transported on ice and initial analysis performed immediately upon return to the laboratory. All samples were analysed within 8 h of sampling and all analyses were performed in triplicate.

3.1.1 Sample preparation (homogenization)

The sample was homogenised for different time intervals (5min, 10 min and 15 min) using an ultrasonic homogenization (Cole-Parmer) at 50% output.

3.2 Microbiological analyses:

3.2.1 ATP

ATP was measured on site as well as lab by means of the ATP Bioprobe (Hughes Whitlock) after 5 min homogenization as in paragraph 3.1.1.

3.2.2 Total plate counts

Total plate counts were done using the spread-plate technique on Nutrient Agar (NA) after 48h incubation at room temperature.

3.3 Physico-chemical analyses:

3.3.1 MLSS

MLSS was determined by filtering 100 ml of sample through a glass fibre filter (Whatman), after which the filter paper was dried for one hour at 105°C (Standard Methods, 1995) and the dry weight was determined.

3.3.2 pH

pH was measured with a Beckman Φ 6 pH meter and a relevant probe.

3.3.3 Chemical analyses

Chemical analyses (NO_3^- , PO_4^{3-} , SO_4^{2-} and NH_4^+ etc) were done on the filtrate from the MLSS determination by means of the Spectroquant (SQ118) spectrophotometer (Merck) and the relevant test kits.

3.4 Batch experiment using ATP, TBC and MLSS to determine the relationship between these parameters and phosphate removal

3.4.1 Batch Experimental design

40g of wet sludge pellets from five activated sludge plants were used to evaluate orthophosphate uptake from sterile mixed liquor growth medium containing 219 mg.l^{-1} orthophosphate. Experiments were done in triplicate and the experimental period was 8h. Average orthophosphate removal was expressed as mg P removed per g wet sludge, as well as mg P removed per g of initial MLSS.

3.4.2 Preparation of sterile mixed liquor growth medium

Mixed liquor from Daspoort was used as a nutrient media for the experiments (Table 1). Grab samples from the anaerobic zone (already containing orthophosphate after anaerobic release from polyphosphate and influent wastewater) were taken. After settling (1 h), the top clear mixed liquor was filtered through Whatman No. 1 filter papers using a vacuum pump (Edwards E.B.3). The mixed liquor obtained was sterilized by autoclaving in 5 l Schott bottles for 60 min. After cooling, the pH of the liquor was determined and adjusted to 6.89

for both experiments with concentrated sulphuric acid. The phosphate concentration was measured and adjusted to 219 mg.l⁻¹ for experiment 1 and 28 mg.l⁻¹ for experiment 2 with sterile 2M KH₂PO₄ (made up in mixed liquor and autoclaved).

Table 1: Characteristics of sterile mixed liquor from Daspoort used in the experiments. Standard Deviations are shown in brackets.

Analysis	Experiment 1
ATP cells.ml ⁻¹	2.15x10 ³ (1.77x10 ³)
MLSS mg.l ⁻¹	400.00 (140.00)
MLVSS mg.l ⁻¹	ND
COD mg.l ⁻¹	87.50 (7.78)
TPC cfu.ml ⁻¹	<10 (0)
pH (original)	8.65 (0.00)
pH (adjusted)	6.89 (0.00)
PO₄³⁻ (original) mg.l ⁻¹	13.60 (0.00)
PO₄³⁻ (adjusted) mg.l ⁻¹	219.50 (0.00)
NO₃⁻ mg.l ⁻¹	<5.00 (0.00)
SO₄²⁻ mg.l ⁻¹	165.50 (4.95)
NH₄⁺ mg.l ⁻¹	16.59 (0.14)

* ND = Not determined

3.4.3 OUR and ATP experiments

Batch experiments were performed using raw sewage as feed. Concentrate particles in the feed were precipitated using aluminium sulphate. Supernatant was filtered using 100 nm membrane, and then warmed up to room temperature before being added to the two reactors. The total volume in the reactors was 3 l (one reactor with 10 day sludge age activated sludge and the other with 20 day sludge age). The first sample was taken

immediately after addition of feed into the reactors. OUR was measured by an on-line OUR meter. The next samples for ATP measurement were taken after every hour from both reactors, for 4 h. The samples were sonicated for 5 min before taking the ATP measurement. ATP was measured using the ATP Bioprobe (Hughes Whitlock).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characteristics of the activated sludge systems studied

Table 2: Characteristics of the activated sludge plants used in this study

	Daspoort	Baviaanspoort	Zeekoegat	Centurion	Rooiwal
Sludge age	12 days	13 days	13 days	12 days	12 days
Plant configuration	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho	3-stage Bardenpho
Mean daily flow	45 megalitres	35-40 megalitres	35 megalitres	36 megalitres	120 megalitres
Inflow characteristics	Domestic and industrial	Domestic and industrial (85:15)	Domestic and industrial (60:40)	Domestic and industrial (80:20)	Domestic and industrial (70:30)
Type of treatment process	Biological	Biological and chemical*	Biological and chemical*	Biological and chemical**	Biological

* chemical treatment with ferric chloride

** chemical treatment with aluminium oxide

Three of the 5 systems studied use biological and chemical means to remove phosphorus (Table 2).

Table 3: Characteristics of the activated sludge collected from five different full scale activated sludge plants used in this study. Standard deviations are shown in brackets.

Analysis	Daspoort	Centurion	Zeekoegat	Rooiwal	Baviaanspoort
ATP (cells.ml ⁻¹) (on site)	4.17x10 ⁷ (9.02x10 ⁶)	3.95x10 ⁷ (6.35x10 ⁶)	4.30x10 ⁷ (7.21x10 ⁶)	5.13x10 ⁷ (1.05x10 ⁷)	3.27x10 ⁷ (4.04x10 ⁶)
(laboratory)	5.90x10 ⁷ (2.18x10 ⁷)	5.36x10 ⁷ (2.34x10 ⁷)	7.23x10 ⁷ (3.06x10 ⁶)	5.13x10 ⁷ (9.19x10 ⁶)	5.33x10 ⁷ (4.16x10 ⁶)
MLSS (mg.l ⁻¹)	4700 (420)	4600 (0)	4740 (20)	4600 (170)	2800 (120)
COD (mg.l ⁻¹)	63 (29.70)	54 (4.24)	76 (12.02)	60 (30.41)	6 (0.00)
TPC (cfu.ml ⁻¹)	4.37x10 ⁶ (1.58x10 ⁶)	7.32x10 ⁶ (3.69x10 ⁶)	3.30x10 ⁶ (3.96x10 ⁶)	3.46x10 ⁶ (9.09x10 ⁵)	2.42x10 ⁶ (8.24x10 ⁵)
pH	6.92 (-)	7.10 (-)	6.95 (-)	7.00 (-)	7.23 (-)
PO₄³⁻ (mg.l ⁻¹)	11.00 (0.00)	8.00 (0.00)	7.33 (0.58)	2.00 (0.00)	6.67 (0.58)
NO₃⁻ (mg.l ⁻¹)	5.50 (3.54)	9.00 (5.66)	6.03 (1.21)	11.40 (2.71)	34.40 (1.25)
SO₄²⁻ (mg.l ⁻¹)	45.67 (3.21)	104.33 (4.62)	50.00 (1.73)	54.00 (3.61)	41.00 (3.46)
NH₄⁺ (mg.l ⁻¹)	10.32 (0.36)	7.53 (0.04)	5.07 (0.15)	0.08 (0.02)	9.95 (1.00)

* ATP analysis was done on samples from the aerobic zone.

No significant difference was observed in the bacterial cell numbers in the different full scale activated sludge plants studied (Table 3).

4.2 ATP analysis, Total bacterial counts and MLSS during an 8h batch experiment in sterile mixed liquor inoculated with aerobic sludge.

4.2.1 ATP analysis

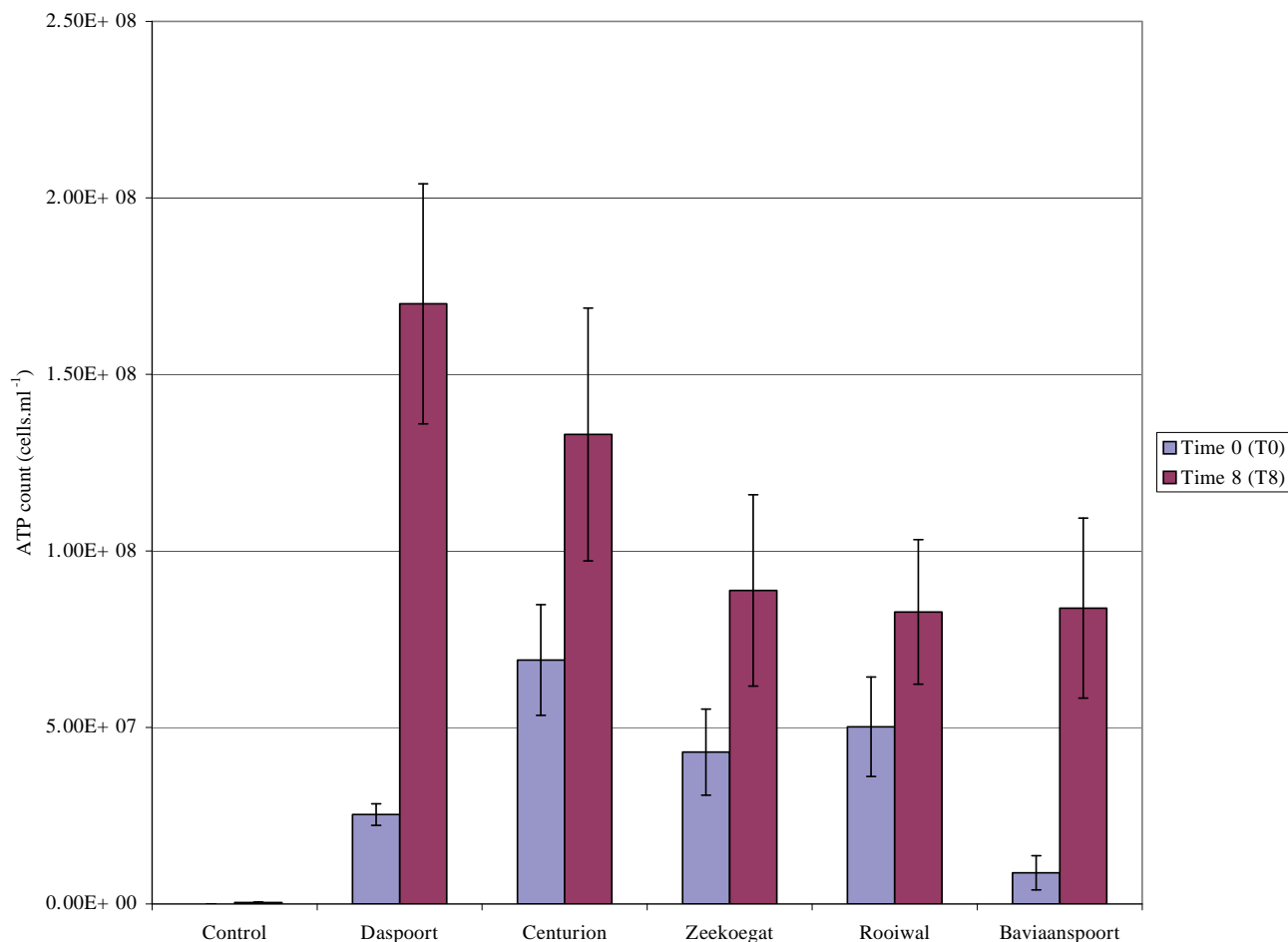


Figure 1: **Average ATP results at Time 0 and Time 8.**

The ATP values indicated that the initial active biomass fraction in the MLSS from the different systems varied. Centurion had the highest ATP concentration, followed by Rooiwal, Zeekoegat, Daspoort and Baviaanspoort (Figure 1). After 8 h, Daspoort had the highest ATP concentration, followed by Centurion, Zeekoegat, Baviaanspoort and Rooiwal. Daspoort showed the largest increase in ATP concentration, followed by Baviaanspoort, Centurion, Zeekoegat and Rooiwal. The increase in MLSS values during the experimental period was attributed to the increase in bacterial numbers as indicated by the ATP

concentrations (Figure 1). The increase in ATP concentrations indicated that bacterial growth took place during the experimental period.

4.2.2 Total bacteria count

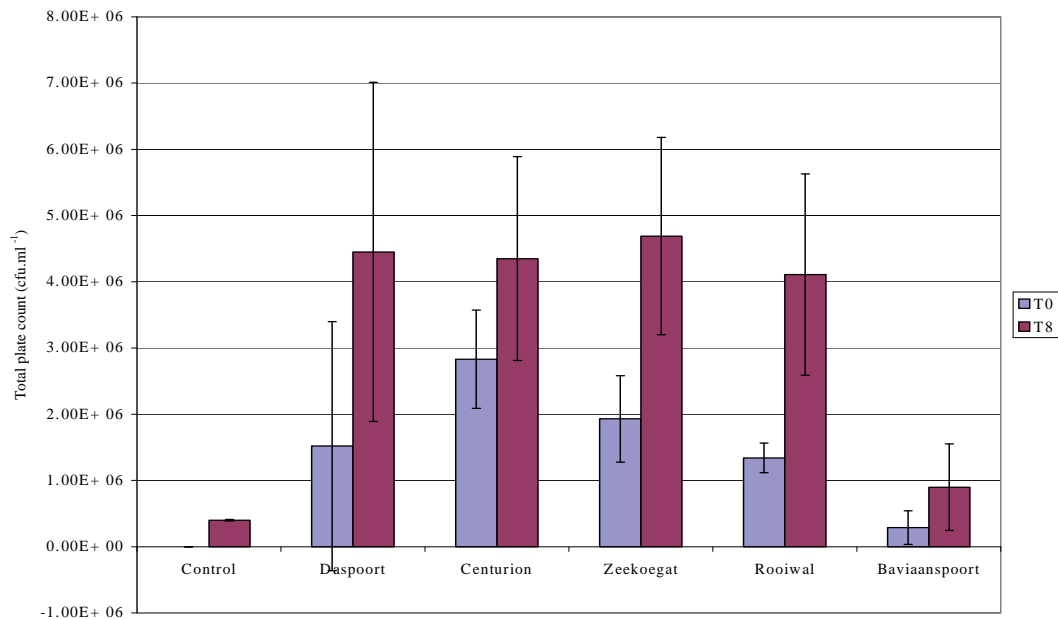


Figure 2: **Average total plate count (TPC) results at Time 0 and Time 8.**

On average, the TPC was the highest for the Centurion sludge, followed by Zeekoegat, Daspoort, Rooiwal and Baviaanspoort (Figure 2). The TPC indicated an increase in cell numbers during the experimental period. This was in agreement with the ATP concentrations (Figure 1). The standard deviation for the TPC was larger than the standard deviations for ATP analysis (Figure 1). The larger variation in the TPC data was ascribed to the method, which relies on colony formation. The colony forming unit in activated sludge would be the floc, which may contain any number of individual bacteria. Since the floc size and distribution in a sample will vary, one would expect a greater variance in the result, as was observed in this study (Figures 1 and 2). On the other hand, ATP analysis relies on an extraction method, which is not reliant on floc size or distribution, hence the smaller variation in the results. This is furthermore

substantiated by the higher ATP cell number values compared to the TPC (on average a one log difference). This also confirms previous data indicating that less than 10% of the viable organisms in activated sludge are culturable (Cloete and Steyn, 1988). These results indicated that ATP was the better method for determining the biomass concentration in activated sludge. This is in agreement with results in previous studies (Jørgensen *et al.*, 1992; Roe and Bhagat, 1982).

4.2.3 Mixed liquor suspended solids (MLSS)

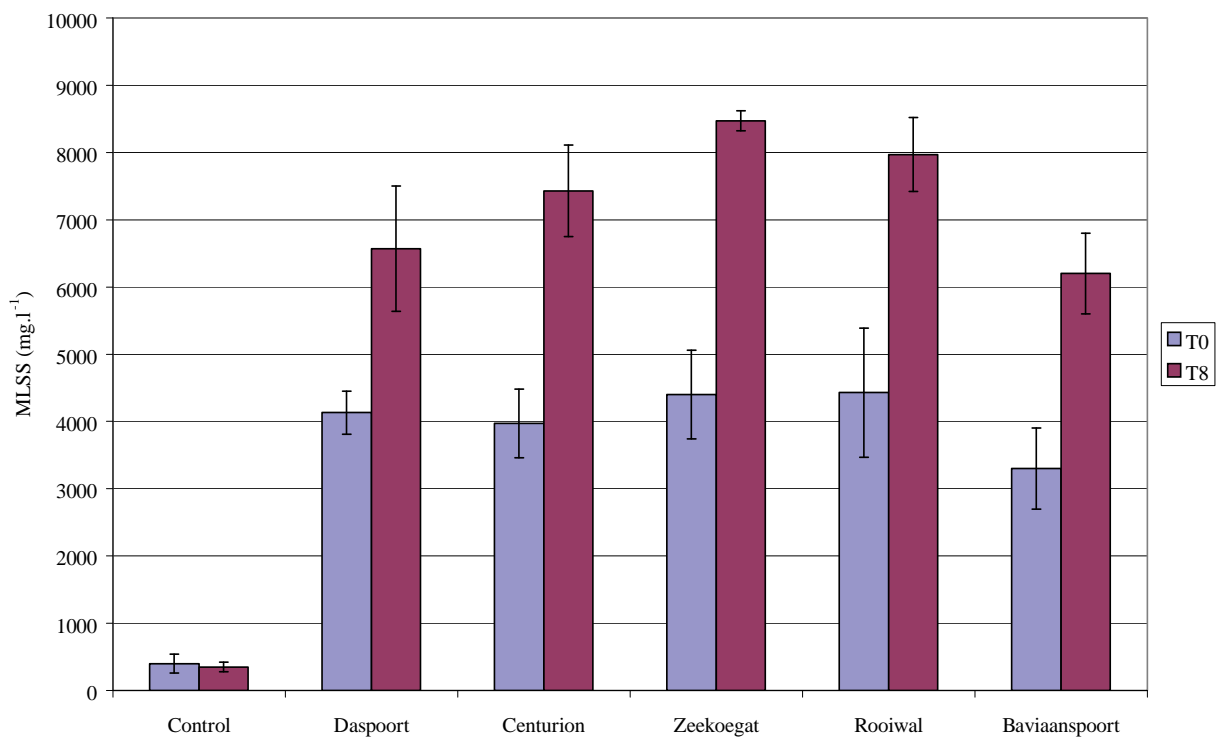


Figure 3: **Average MLSS values at Time 0 and Time 8 during experiment 1.**

MLSS values at time 0 h were similar for all the systems. Values increased for all the systems during the 8 h experiment. MLSS values increased by 4070, 3540, 3370, 2900 and 2440 mg.l⁻¹ for the Zeekoegat, Rooiwal, Centurion, Baviaanspoort and Daspoort WTPs, respectively. The increase in MLSS values was attributed to an increase in viable bacterial cell numbers as indicated by total plate counts and ATP analyses (Figures 1 and

2). The good orthophosphate removal by the Centurion system can, however, not only be attributed to the increase in MLSS, as it did not show the greatest increase in MLSS. It is therefore clear that the MLSS composition, especially in terms of viable cells might play an important role in different orthophosphate uptake abilities of different sludges, as observed in better orthophosphate removal in sludges containing higher initial TPC and ATP counts (Figures 1 and 2). No pattern could be observed for the rest of the WTPs, although the Daspoort WTP showed the

4.2.4 Orthophosphate removal during the batch experiment

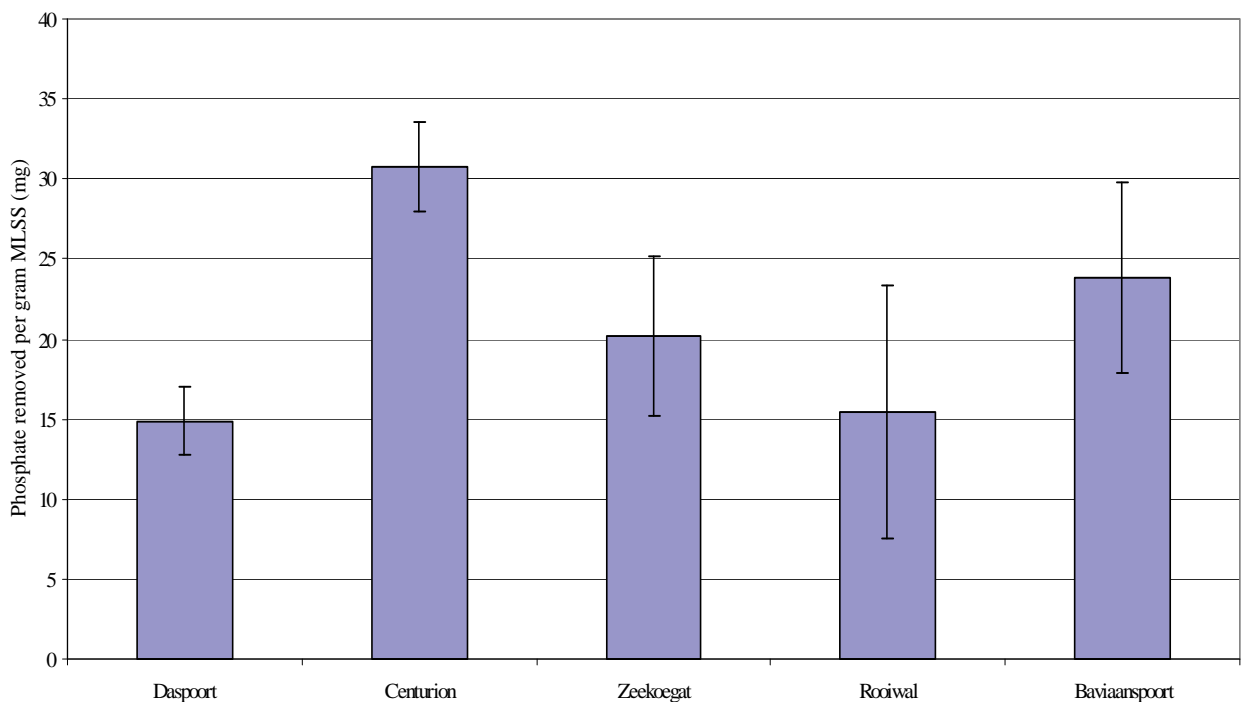


Figure 4: **Average orthophosphate uptake (mg) per gram of initial MLSS.**

smallest increase in MLSS (2440 mg.l^{-1}), while removing the least amount of orthophosphate (62 mg P.l^{-1})(Figure 4.4). The control flasks showed an initial MLSS value of 400 mg.l^{-1} at time 0 h that can be attributed to residue not filtered out in the preparation of the sterile growth medium. With the orthophosphate removed calculated in terms of initial MLSS, Centurion performed the best ($30.79 \text{ mg P.g}^{-1}$ removal)

followed by Baviaanspoort, Zeekoegat, Rooiwal and Daspoort with values of 23.78, 20.17, 15.40 and 14.88 mg P.g⁻¹ MLSS, respectively (Figure 3). Although the initial MLSS values were similar for all the systems, the quantities of orthophosphate removed over the experimental period were different. Judging from the standard deviations, it was concluded that these differences were, however, not significant for Baviaanspoort, Daspoort, Zeekoegat and Rooiwal. However, the orthophosphate removal was significantly higher in the Centurion WTP (Figure 4).

Table 4: Chemical analysis of the inflow and outflow of the Zeekoegat wastewater treatment plant

Analysis	Inflow	Outflow
COD (mg/l)	532	36
pH	7.64	7.20
Orthophosphate (mg/l)	6.01	0.95
Total phosphate (mg/l)	7.67	1.65
Nitrate (mg/l)	0.36	7.48
Sulphate (mg/l)	94	64
NH₄ (mg/l)	32.95	2.70
MLSS (mg/l)	2044	10.2
Settled solids (mg/l)	12.0	ND
Alkalinity (mg/l)	238	108
ATP*	9,16 x 10 ⁴	
Number of bacteria	8,75 x 10 ⁴	
	9,73 x 10 ⁴	
x	9,21 x 10 ⁴	
s	4,91 x 10 ⁴	

*Samples taken from the aerobic zone - ND: not determined

The ATP cell count in Zeekoegat was 9,21 x 10⁴ bacteria/ml (Table 4). This is an extremely low value for an activated sludge system. Ferric chloride is used in the system to achieve phosphorus removal (Table 2). The latter is probably necessitated by the low biomass component of the MLSS.

Table 5: Chemical analysis of the inflow and outflow of the Rooiwal wastewater treatment plant

Analysis	Inflow	Outflow
COD (mg/l)	170	121
pH	7.83	7.49
Orthophosphate (mg/l)	6.28	7.44
Total phosphate (mg/l)	10.66	8.80
Nitrate (mg/l)	0.01	5.40
Sulphate (mg/l)	78	ND
NH₄ (mg/l)	23.82	23.62
MLSS (mg/l)	486	27
Settled solids (mg/l)	15	0.01
Alkalinity (mg/l)	275	238
ATP*	2,46 x 10 ⁴	
Number of bacteria	2,14 x 10 ⁴	
	1,54 x 10 ⁴	
x	2,04 x 10 ⁴	
s	4,64 x 10 ⁴	

*Samples taken from the aerobic zone

ND: not determined

The inflow COD concentration was 170 mg l⁻¹ and the outflow COD concentration 121 mg l⁻¹ (Table 5). This indicates that very little COD was being removed in the system, at the time of sampling. The MLSS concentration was also very low (486 mg l⁻¹) (Table 5). This could explain the low bacterial cell numbers (2,04 x 10⁴ bacteria/ml) observed in this system (Table 5).

Table 6: Chemical analysis of inflow and outflow for Daspoort wastewater treatment plant.

Analysis	Inflow	Outflow
COD (mg/l)	412	28
pH	7.62	7.60
Orthophosphate (mg/l)	4.60	0.36
Total phosphate (mg/l)	7.02	1.06
Nitrate (mg/l)	0.23	3.97
Sulphate (mg/l)	ND	39
NH₄ (mg/l)	22.40	1.49
MLSS (mg/l)	3045	6.0
Settled solids (mg/l)	10.00	0.01
Alkalinity (mg/l)	2	140
ATP*	2,59 x 10 ⁶	
Number of bacteria	1,26 x 10 ⁶	
	1,96 x 10 ⁶	
x	1,93 x 10 ⁶	
s	6,6 x 10 ⁵	

*Samples taken from the aerobic zone

ND: not determined

The bacterial cell number was 1,93 x 10⁶bacteria/ml in the Daspoort system (Table 6).

This system is a fully biological system, achieving good phosphorus removal (Table 6).

Table 7: Chemical analysis for inflow and outflow of Baviaanspoort wastewater treatment plant

Analysis	Inflow	Outflow
COD (mg/l)	636	ND
pH	7.39	7.23
Orthophosphate (mg/l)	3.84	0.82
Nitrate (mg/l)	1.52	2.94
Sulphate (mg/l)	66	ND
NH₄ (mg/l)	17.77	0.59
MLSS (mg/l)	3500-4500	ND
Settled solids (mg/l)	ND	ND
Alkalinity (mg/l)	ND	ND
ATP* Number of bacteria	3,28 x 10 ⁴ 2,91 x 10 ⁴ 2,76 x 10 ⁴	
x s	2,99 x 10 ⁴ 2,68 x 10 ³	

*Samples taken from the aerobic zone

ND: not determined

The Baviaanspoort system also makes use of chemical methods to remove phosphorus (Table 2). Again, as was the case with Zeekoegat, chemical treatment was necessitated by the low biomass concentration (Table 7).

Table 8: ATP analysis on the activated sludge sample from the Daspoort wastewater treatment plant, after different times of sonication. Readings were taken in duplicates.

Sample	ATP (number of bacteria)	Average (x)
Mixed liquor (not sonicated)	270590 283778	2,7 x 10 ⁵
Mixed liquor (sonicated for 30s)	2198190 1969178	2,08 x 10 ⁶
Mixed liquor (sonicated for 5min)	3398984 2993432	3,19 x 10 ⁶

The number of bacteria increased, with the increase in sonication time (Table 8). Sonication therefore did not “kill” the organisms over the 5 min period, but improved the release of ATP (Table 8). The number of bacteria in the sonicated samples was significantly higher than in the original non-sonicated sludge (Table 8). The scanning electron micrographs (Appendix I) indicate that 5 min of sonication dispersed the sludge flocs effectively and hence the higher ATP results.

4.3 Correlation between ATP and oxygen utilization rates (OUR) in measuring viable biomass numbers.

The ATP amount and the OUR for the two bioreactors, the one reactor at 10 day sludge age and the other at 20 day sludge age, were measured. The batch experiment was done over four hours. The reactors were fed with raw sewage whose particulates were concentrated using aluminium sulphate, as the substrate.

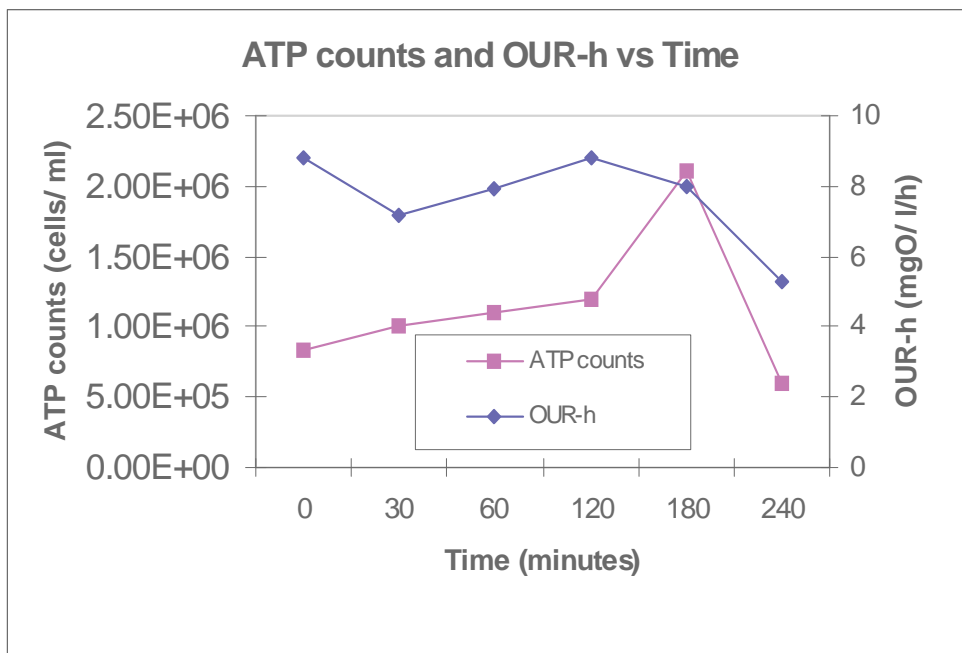


Figure 5. ATP and OUR profiles for the 10 day sludge age bioreactor.

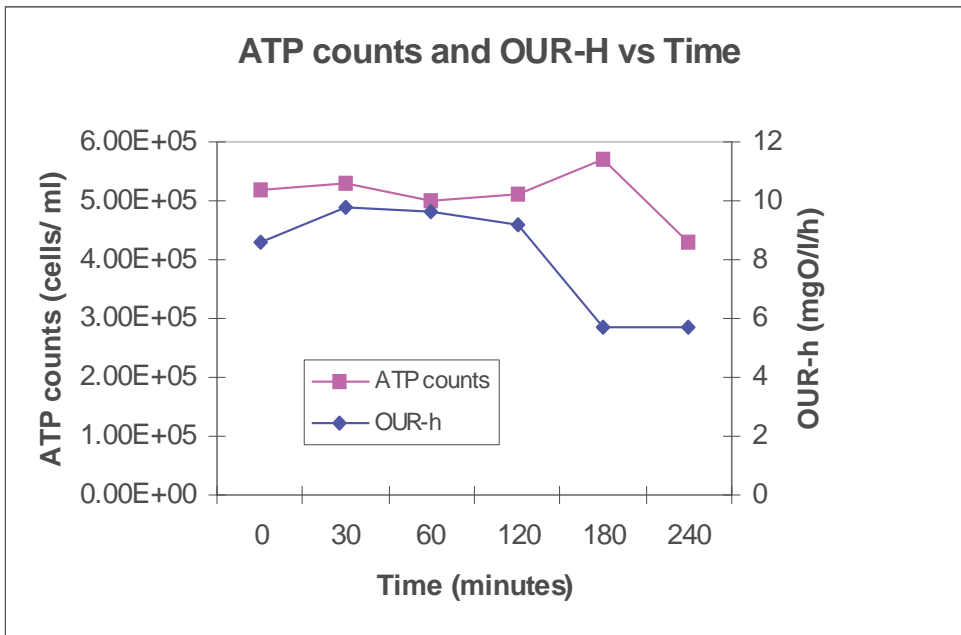


Figure 6. ATP and OUR profiles for the 20 day sludge age bioreactor

Both the ATP and OUR follow the same trend over the experimental period for both reactors (**Fig.5 and 6**). The values start by increasing gradually with time and then decreases sharply after 2 hours. The ATP value increases exponentially after 2 hours before dropping again to a lower value after about an hour. The initial readings taken from both reactors at time 0 (before the start of the experiment) were different for both reactors. The 10-day sludge age bioreactor had high number of live organisms than the 20-day sludge age bioreactor.

CHAPTER 5

GENERAL DISCUSSION

Historically, the mixed liquor organic suspended solids have been measured as a lumped parameter via the VSS test, or more recently, the COD test. However, as stated above, only a part of the mixed liquor organic suspended solids is heterotrophic active biomass, the active part of activated sludge, and only this part mediates the biological processes of COD removal and denitrification. Currently, the heterotrophic active biomass exists only as a hypothetical parameter within the structure of the design procedures and kinetic models. Although indirect evidence provides support for this parameter (by consistency between observations and predictions over a wide range of conditions) it has not been directly measured experimentally and compared to theoretical values.

The problem in measurement of this parameter has been the lack of simple, suitable experimental techniques. Current techniques have not yet been adequately integrated with the design and kinetic modelling theory. The culturing techniques have been widely criticized for their unreliability, while molecular methods are still in their infancy, requiring sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

In the current study, orthophosphate removal was consistently high with higher biomass concentrations as measured by TPC and ATP. This supports the notion that the viable biomass fraction of the MLSS is the key to orthophosphate removal by activated sludge. However, maintenance of large fractions of viable biomass in activated sludge will select for smaller flocs, causing poor settling (Roe and Bhagat, 1982). It is thus important to find a situation of equilibrium between viable biomass and settling performance to optimize the activated sludge process.

Although most authors claim ATP measurement to be an accurate measure of viable biomass (Weddle and Jenkins, 1970; Roe and Bhagat, 1982), there is still concern as to its constancy under different metabolic conditions. In the experiments by Weddle and Jenkins (1970) and Upadhyaya and Eckenfelder (1975) the ATP per plate count colony was fairly stable, substantiating the claim that ATP is a measure of viable biomass. However in the current study, at time 0 h (before the start of experiment 1) the ATP cell count ranged between 17 and 37 times the TPC cell count, while at the end of the experiment (time 8 h) with controlled aeration and conditions, this range differed even more, ATP counts being between 19 and 93 times the TPC cell count. ATP measurements have shown variation in response to environmental stresses like anoxic conditions in return sludge (Roe and Bhagat, 1982). However, the method has been shown in the current study to be superior to the traditionally used methods of TPC, MLSS and MLVSS for biomass determination. MLSS and MLVSS did not resemble the viable population as measured by ATP or TPC. ATP was also found to be a better biomass estimator than TPC due to higher (at least one log unit) bacterial counts and smaller standard deviations. It is a cheap, simple and fast method, not requiring special training for laboratory personnel and with a small capital input for a portable luminometer, giving on-the-spot results.

The ATP results followed the same trend as the OUR results. The OUR of the organisms increased slightly with time, and then decreased after a few hours of the experimental period. The ATP values also increased with time, but in this case the value increases sharply after 2 hours of incubation before dropping down again. This is an indication that there is an increase in viable biomass numbers as the organisms utilize the substrate. The gradual increase in the ATP value could be an indication that after some time the organisms are well adapted to the conditions in the bioreactor and that can optimally utilize the substrate, resulting in increases in their numbers, and hence an increase in the ATP and OUR values. This results indicate that ATP and OUR are good indicators of viable biomass numbers. OUR value remain increasing by nearly the same rate which can indicate that the OUR is independent of the metabolic activity of the organisms while the drastic increase in ATP indicates that when the cells are metabolically active the ATP amount present also increases. When the substrate is depleted the number of organisms

decrease, which in this experiment is indicated by the decrease in the ATP and OUR values. ATP counts for the 10 day sludge age bioreactor are higher than those for the 20 day sludge age. These correlates with the literature that the longer the sludge age the less the number of viable organisms as the amount of dissolved or suspended particles increases. The OUR value for the two bioreactors are significantly the same, which indicated the OUR is not influenced by the environmental conditions.

CONCLUSIONS:

- ATP proved to be a more reliable method for indicating the biomass concentration than TPC, due to the higher yield and a smaller standard deviation.
- Orthophosphate removal was consistently higher in the sludges with higher initial ATP and TPC values, indicating a relationship between viable biomass and orthophosphate removal.
- The MLVSS showed the same trend in orthophosphate removal as the MLSS, although always somewhat lower, due to it being the volatile fraction of the MLSS.
- Neither initial MLSS, initial MLVSS nor changes in the concentrations of these fractions could be directly linked to different orthophosphate uptake abilities of different sludges, indicating the unsuitability of MLSS and MLVSS to indicate viable biomass and/or differences in the viable biomass fraction in activated sludge.

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