2.13 The "G" bacteria

When assessing EBPR efficiency, a group of normal inhabitants found in activated sludge mixed liquor must be kept in mind: the "G" bacteria (Atkinson, 1999). There is a microbial community in activated sludge capable of organic substrate uptake and assimilation in the anaerobic zone with subsequent metabolism of these storage granules in the aerobic zone. This community is composed of two distinct groups of organisms with distinctly different modes of substrate uptake and synthesis of storage granules. The first group are the PAO, the metabolism of which has been previously described. The second group are the "G" or glycogen accumulating organisms (GAO). In conventional systems, GAO are involved in normal organic oxidation processes, but in selection systems like BPR, their presence and impact to system efficiency can become more prominent (Atkinson, 1999). These Gram negative cocci grow as tetrads and are able to out-compete PAO in anaerobic/aerobic systems by accumulating polysaccharide and not polyphosphate in the aerobic zone (Cech and Hartman, 1993; Cech *et al.*, 1994; Maszenan *et al.*, 1998). The GAO are thought to be able to effectively compete with fermentative organisms for RBCOD and PAO for VFA in the anaerobic zone as they are able to obtain the necessary reducing power and energy required for uptake through glycolysis (the Embden-Meyerhof pathway). Their proliferation in an EBPR system will eventually lead to a decline in phosphate removal (Atkinson, 1999). Cech and Hartman (1993) found that GAO were able to dominate in an anaerobic-oxic system when the influent consisted of an acetate and glucose mixture. Yet, when acetate alone was used as a substrate, the PAO were able to dominate their competitors. Influent P/COD ratios also affect the microbial community structure of EBPR activated sludge. Liu *et al.* (1997) found that a low P/COD ration enriches for GAO, while high P/COD ratios promote the growth of PAO, while suppressing the growth of GAO. Competing with the non-PAO (fermentative bacteria) for RBCOD in the anaerobic zone, reduces VFA production which ultimately influences P release by the PAO (Atkinson, 1999). Molecular phylogenetic classification of GAO isolates in the *Proteobacteria* phylum of the domain *Bacteria* shows their relatedness to many of the PAO, indicating that some relationship between the two competing bacteria does exist. This

implies that an identical or similar mode of metabolism between the two bacterial types must have existed at some stage of their evolution (Atkinson, 1999).

2.14 Failure of enhanced biological phosphate removal systems

The successful operation of nutrient removing activated sludge plants is dependent on good operation, coupled with good design and the maintenance of the correct biomass. Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986). EBPR fail either as a result of toxic shock due to the eccumulation of H_2S or the nitrate feedback into the anaerobic zone (Bitton, 1994). Operational optimization of the EBPR process is dependent on meeting the oxygen demand in the aerobic zone and providing the correct substrate in the anaerobic zone (for example SCFA). Other organisms can compete with poly-P bacteria in anaerobic-aerobic activated sludge systems, leading to system failure (Erasmus, 1997). The failure of EBPR process has attracted various methods to remedy this problem, for instance, augmenting of activated sludge by addition of readily biodegradable COD to promote the growth of phosphate removing bacteria has been applied by Osborn *et al.* (1986).

2.15 Bioremediation and bioaugmentation

The objective of wastewater treatment is to reduce or prevent the accumulation of pollutants in the environment, thus transforming them into harmless products such as biomass, carbon dioxide, methane and nitrate. This objective is achieved by the use of microbes which biodegrade these pollutants. From one perspective, activated sludge processes may be viewed as bioremedation of wastewater.

Atlas and Bartha (1993) define bioremedation as the use biological agents to reclaim soils and waters polluted by substances hazardous to human health, and/or the environment. It is an

extension of biological treatment processes that have traditionally been used to treat wastes in which microbes are typically used to biodegrade environmental pollutants. Bioremedation techniques are diverse and may involve the design of special reactors, as well as the use of specialized microbes, a process referred to as bioaugmentation.

Oellerman and Pearce (1995) explained that bioaugmentation involves the use of genetically manipulated or specially adapted microbes to enhance the biodegradation of specific toxic, hazardous and recalcitrant compounds which are difficult to remove from the environment by conventional treatment processes. Microbial strains for enhancing biodegradation of specific chemicals are generally isolated from environmental samples (wastewater, sludge, compost or soil) and selected by conventional enrichment techniques. They are grown in nutrient media that contain a specific organic chemical as the sole source of carbon and energy or as a sole source of nitrogen. Furthermore, strains that can handle relatively high concentrations of the target chemical are selected. Some of the microbial strains may be subsequently irradiated to obtain a desirable mutation (Bitton, 1994).

Exposure of wastewater microbial communities to toxic xenobiotics results in the selection of resistant microbes that have the appropriate enzymes to use the xenobiotic as the sole source of carbon and energy. This process is called acclimation or adaptation. Most microbes need acclimation prior to the onset of metabolism. Prior exposure to the xenobiotic helps reduce the acclimation period (Bitton, 1994).

In wider sense, augmentation also infers the stimulation of microbes indigenous to the contaminated environment and wastewater to enhance and improve the treatment performance. The aim is to attack preferential substrates which are marginally available or difficult to degrade as pollutants. Bioaugmentation technologies simply attempt to optimize the natural capacity of microbes to degrade and recycle such compounds by controlling pH, oxygen supply or anaerobicity and temperature, and supplying essential limiting nutrients to minimize the stress on such systems.

Bioaugmentation may involve the addition of selected bacteria to a bioreactor to maintain or enhance the biodegradation potential in the reactor. Although the technology has been known for decades, unfortunately, information on the formulation of the mixtures of the microbial cultures is scanty because of trade secrets (Bitton, 1994).

Biodegration techniques are versatile and can be utilized at various stages of treatment. Applications include removal of contaminants from raw materials prior to precessing, treatment of waste before discharge, treatment of effluent streams and decontamination of soils, sediments, surface water and groundwater (Oellermann and Pearce, 1995).

Enhanced biological phosphate removal (EBPR) is usually not optimized and routinely fails (Bond *et al.*, 1995). Under favourable conditions, phosphate-removing sludge has been observed to take several sludge ages to develop. This suggests that the phosphate-removing community might need to be established and may not occur merely as a result of conditioning of the existing population (Bond *et al.*, 1995). Therefore, it is of prime importance that a fixed biomass concentration be maintained if there is a need to obtain high reactor performance in every biological wastewater treatment process (Nicolella *et al.*, 1997). Another limitation of bioaugmentation is that there is usually a requirement by microbes used, for an acclimation period prior to the onset of the process. Short survival or lack of growth of microbial inocula in the seeded bioreactors is another well-known problem (Bitton, 1994). A sludge age ranging from 15 to 20 days is required for the removal of phosphate in activated sludge systems (Toerien *et al.*, 1990) and, thus, subsequent to toxic shock or the development of unfavorable conditions for phosphate uptake, effluents with high phosphate concentration may be discharged into waterways. The use of activated sludge biomass for augmentation may assist the wastewater plant operators to avoid discharging limiting nutrients into the rivers, lakes and ponds whilst the natural biomass of the plant is resuscitated.

Bioaugmentation could be used in this regard to rectify the depleted activated sludge microbial that fails to optimally remove phosphate.

2.16 Use of bioaugmentation in wastewater treatment

Some of the applications of bioaugmentation are the following (Grubbs, 1984; Rittmann *et al.*, 1990):

* increased BOD removal. Microbial strains may be used to increase BOD removal in wastewater treatment plants;

* reduction of sludge volume. Production of large amounts of sludge is a serious problem associated with aerobic waste treatment, and thus reduction of sludge volumes is highly desirable. The reduction is the result of increased organic removal following addition of a mixed culture of selected microbes. Reductions in 17 % to nearly 30 % have been documented;

* use of mixed cultures in sludge digestion. In aerobic digesters, the use of mixed cultures has led to significant savings in energy requirements. In anaerobic digesters, bioaugmentation has resulted in enhanced methane production;

* biotreatment/bioremedation of hazardous wastes. The use of added microbes for treating hazardous wastes (for example phenols, ethylene glycol, formaldehyde) has been attempted and has a promising future. Bioaugmentation with parachlorophenol-degrading bacteria achieved a 96% removal in 9 h, as compared with a control that exhibited 57 % removal after 58 h (Kennedy *et al.*, 1990). *Candida tropicalis* cells also have been used to remove high concentrations of phenol in wastewater (Kumaran and Shivaraman, 1988). *Desulfomonile tiedjei*, when added to a methanogenic upflow anaerobic granular-sludge blanket, increased the ability of the bioreactor to dechlorinate 3-chlorobenzoate (Arhing *et al.*, 1992). Oellermann and Pearce (1995) achieved significant results on phenol bioremedation;

Bioaugmentation by means of biomass could be used to rectify wastewater treatment plants in South Africa especially those plants which fail to remove phosphate.

2.17 General aspects of biomass

Atlas and Bartha (1993) define biomass as the dry weight, volume, or other quantitative estimation of organisms, thus, the total mass of living organisms in an ecosystem. The various types of organisms in activated sludge system thus constitute the biomass of that particular system. The biomass parameter measures the extent of growth and it enters into certain important derived parameters such as growth yields, metabolic quotients (Pirt, 1975), the quantity of energy being stored in a particular segment of the biological community, as well as the transfer of energy between trophic levels within an ecosystem (Atlas and Bartha, 1993). Biomass can be expressed in units of weight (g) that can be converted to units of energy (cal).

The methods used to measure biomass are based on eight types of measurements: mass, volume or linear extent, mass of a biomass component, mass of substrate consumed or product formed, metabolic rates, light scattering, cell and organelle counts and staining methods (Pirt, 1975).

Choice of the method to be used for measuring biomass is a crucial decision to make and often limitations of the methods make the decision difficult. The factors which influence the choice are (Pirt, 1975):

* properties of the biomass;
* properties of the culture medium;
* accuracy required;
* sensitivity required; and
* required speed of measurement.

Properties of the biomass which affect the choice of the method to use are, whether it is filamentous or particulate, the ease at which separation from the medium can be obtained during quantification and the age of the biomass or its growth rate (Pirt, 1975).

The rate of phosphate removal in activated sludge process is dependent on the biomass concentration and cell sizes (Momba, 1995). Excess uptake of phosphate has been related to the increase in biomass (Bosch, 1992; Streichan *et al.*, 1990) and the rate of removal is dependent on concentration of the sludge (Srinath *et al.*, 1959). Momba and Cloete (1996a,b) demonstrated, using *Acinetobacter junii* that the concentration of phosphate uptake is related to the biomass concentration and nutrient gradients which have to be defined and, which in full-scale plants, are separated and governs P release and uptake.

Momba and Cloete (1996a,b) noted that:

* there was a rapid phosphate release when low initial cell biomass concentration were used in mixed liquor medium;
* the most favourable nett phosphate removal from mixed liquor medium was obtained by the use of a high initial biomass concentrations.

Biomass of 10^7 cells.ml^{-1} released phosphate during the logarithmic growth phase, whereas biomass of 10^8 cells.ml^{-1} (*Acinetobacter junii* and *Pseudomonas fluorescens*) removed phosphate during the lag phase, logarithmic growth phase and up to the stationary phase. Enhanced uptake of phosphate occurred when cells reached the stationary growth phase for *A. junii* and logarithmic growth phase for *Ps. fluorescens*.

Previous research has indicated that phosphate uptake was related to an increase in biomass concentration (Streichan *et al.*, 1990; Bosch, 1992). Momba and Cloete (1996a,b) reported that high initial cell concentrations of *Acinetobacter junii* (a PAO) removed more phosphate than low cell concentrations, and that phosphate uptake was therefore directly related to biomass concentration and high nutrient availability. Bosch (1992) stipulated that biomass was a more significant factor than the type of organism/s present with reference to biological

phoshorus removal. Sidat *et al.* (1999) also reported on findings suggesting a direct relationship between the biomass concentration and phosphate removal capacity. Optimal phosphate removal was achieved at a biomass concentration of 1900 $mg.l^{-1}$.

2.18 Limitations to maximum biomass determination

The maximum density of biomass of any kind that can be reached in a given medium is determined by using one of the following four conditions (Pirt, 1975):

* the amount of growth-limiting substrate supplied;
* maximum packing density of the biomass;
* the accumulation of an inhibitory producr;
* cell death.

Techniques for direct determination of biomass, such as filtration and dry weight or by centrifugation and packed cell volume, are rarely applicable to environmental samples. These techniques tend to measure mineral and detritus particles and fail to discriminate between trophic levels, that is, between producers and consumers. Consequently, determination of biomass by these methods is imprecise (Atlas and Bartha, 1993).

Current research in wastewater treatment has been directed towards mathematical modelling of basic design and operational procedures. One important parameter in such models is the amount of viable biomass. For this reason attempts have been made to find simple and reliable methods to determine the biomass in wastewater and activated sludge. The simplest and most often method is to measure SS or VSS. Such methods, however, do not distinguish between living cells and debris of either organic or inorganic origin. Using traditional plate techniques, the problem is normally an underestimation of the biomass due to selectivity of the media (Jørgensen *et al.*, 1992). Microbial activity has been used as a parameter to determine the microbial potential. Methods include respirometry, ATP content and different enzyme assays.

Very few attempts however have been done to relate activity to biomass. Roe and Bhagat (1982) estimated the ATP to SS ratio at maximum viability in activated sludge, and they found that viability varied significantly which mean cell residence time. Henze (1986) reported comparative studies of denitrification and oxygen utilization rates of raw wastewater and used conversion factors to relate activity to biomass. Kucknerowicz and Verstraete (1979) found a linear relationship between oxygen utilization rate (OUR) and ATP content suggesting that ATP reflected viable biomass. In a study to relate biomass activity with ATP content, oxygen respiration and fluorescein diacetate (FDA) hydrolysis, Jørgensen *et al.* (1992) reported that FDA hydrolysis fail to show any correlation.

Attempts at understanding microbial ecology are limited by the lack of suitable methods and it is thought that bacteria from aquatic and soil environments cannot be accurately enumerated. (Atlas and Bartha, 1987). The non-critical use of so-called standard methods may yield data of questionable accuracy as microbes live in micro-environments having physico-chemical properties, which may be distinctly different from those of the surrounding environment. Bacterial culture media used in laboratories are mostly designed for the cultivation of human pathogens or other fastidious bacteria and consists mainly of proteinaceous substrates such as peptones, meat and yeast extract and hence this may not necessarily satisfy the nutritional requirements of microbes isolated from the environment. Planktonic bacteria in water systems have different nutrient requirements to the fastidious bacteria (Brözel and Cloete, 1992).

The viable (cultivable) bacteria in activated sludge constitute only a small fraction (< 10 %) of the total number of cells present (Cloete and Steyn, 1988). Cloete (1984) has warned against the acceptance of organisms that can be cultivated as a true reflection of the entire population of the activated sludge. Cloete (1984) compared the total microscopic counts of two activated sludge samples that had been centrifuged onto density gradients with the number of *Acinetobacter* estimated with fluorescent antibodies and the numbers of volutin-containing bacteria revealed by metachromatic staining. *Acinetobacter* and volutin-containing bacteria occurred only in 3 out of 10 fractions and constituted only 2.4 -20.5 % of the total number of bacteria applied to the density gradients. The fluorescent antibody number of *Acinetobacter* corresponded closely to the colony forming units (cfu) of *Acinetobacter* of activated sludge

samples, thus giving reasonable justification that the bacteria which were non-cultivable were not *Acinetobacter*. The *Acinetobacter* (or cultivable bacteria) could therefore not represent the population structure of those bacterial groups that could not be cultivated. Bond *et al.* (1995) argued that culturing techniques have provided a misleading picture of bacterial community structure in activated sludge and, that in general, the role of *Acinetobacter* spp. in activated sludge processes has been overrated. For instance, non-culture-dependent methods such as quinone profiles and flourescent in situ hybridization (FISH) probes have indicated that *Acinetobacter* spp. are present in small proportions in activated sludge (*ca.* 3 to 6 %). A promising method for the quantification of uncultured microbes in the environment has been suggested by Polz and Cavanaugh (1997). This method is based on generating nucleic acid standards for the quantification of uncultured microbes. rRNA transcribed from cloned templates has been shown to afford a quantitative estimate of a species when added at different concentrations to an artificially assembled community. This replaces the need for nucleic acids standards obtained from pure cultures of the organisms to be quantified.

Although considerable research effort has been directed towards improving understanding of the BEPR phenomenon, designs of activated sludge systems to accomplish BEPR are still based on experience and semi-empirical methods. Clearly, the need exists for design procedures based on more fundamental behavioural patterns and kinetics (Wentzel *et al.*, 1990). Quantification, as well as kinetic models of biomass in activated sludge are routinely used in design of wastewater treatment plants, in spite of the limitations which are currently encountered in biomass determination. For instance, in the current steady state design and kinetic simulation models for activated sludge, the mixed liquor suspended solids is made up of a number of components. One key component is the heterotrophic active biomass, as this component mediates the biodegration processes of COD removal and denitrification. Thus, the rates for these processes are directly related to the heterotrophic active biomass present, and the specific rates should be expressed in terms of this parameter to allow a meaningful comparison of the rates measured in different systems. However, the heterotrophic active biomass parameter has been only hypothetical within the structure of these models; it has not been measured directly, primarily due to the lack of suitable simple measurement techniques. This deficiency has cast some measure of doubt on the entire framework within which the

steady state design and kinetic simulation models have been developed, in the past. However, Ubisi *et al.* (1997) reported close correlation between measured heterotrophic active biomass concentration with those calculated theoretically, thus promoting confidence in the application of the models for design, operation and control of activated sludge systems.

In the literature, principally microbiological techniques for biomass estimation has been proposed (Ubisi *et al.*, 1997), including pour plate or other culturing techniques (Gaudy and Gaudy, 1980), ATP analysis (Nelson and Lawrence, 1980), DNA analysis (Liebeskind and Dohmann, 1994), the use of fluorescent probes for rRNA (Wagner *et al.*, 1994c) and sequencing or ribosomal DNA (Blackall, 1994). However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory, while culturing techniques have been widely criticised for their unreliability (Cloete and Steyn, 1988). The RNA and the two DNA methods are still in their infancy. The last four named methods also require sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

2.19 Estimation of microbial numbers

A range of direct counting techniques has been developed to count dead and living cells separately. All these techniques are based on the indication of metabolic activity of the living cells (Roszak and Colwell, 1987).

2.19.1 Direct count procedures

A means to overcome the quantitative and qualitative bias imposed by cultivation-dependent methods is the use of chemotaxonomic and molecular techniques for *in situ* analyses of communities. The term *in situ* indicates that all microbial cells in an environmental sample are not isolated prior to investigation, but are rather studied intact, within the original sample

(Atkinson, 1999). Techniques employed include application of biomarker approaches, immunofluorescence and sequence-based molecular methods (otherwise referred to as FISH). These methods have proven to be powerful tools in their ability to quantitatively enumerate specific species in diverse microbial communities and many perceptions (like *Acinetobacter* as the model poly-P organism have been shattered due to their accurate and reliable identification abilities (Atkinson, 1999).

2.19.1.1 Light microscopy

Microbes can be counted by direct microscopic observation. These procedures yield the highest estimations of numbers of microbes, although there are several major drawbacks to direct observational methods, including (Coetzee, 1999):

* dead and alive microbes are counted;
* different types of microbes, especially bacteria, cannot be distinguished on the basis of morphology;
* the sample cannot be used for further experimentation.

2.19.1.2 Epifluorescence microscopy

Epifluorescence microscopy, with stains such as acridine orange (AO), 4,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC), is widely used for direct counting of bacteria (Jacobs *et al.*, 1996). When AO is used as a stain, bacteria and other microbes fluoresce orange and green. DAPI, which stains the DNA of bacterial cells and produces an intense blue fluorescence, has been found to be superior to AO for visualizing small bacterial cells (Wolfaardt *et al.*, 1991).

Counts by direct epifluorescent microscopy are typically two orders of magnitude higher than counts obtained by cultural techniques (Coetzee, 1999). The value of the direct count epifluorescent microscopy approach to enumeration is that it is applicable to a variety of habitats without the bias inherent to viable count procedures (Wolfaaardt et al., 1991).

2.19.1.3 Scanning electron microscopy (SEM)

SEM is performed by scanning a focussed electron probe across the surface of the sample to be studied. Secondary electrons emitted from the sample are tyoically detected by a photomultiplier system, the uotput of which is used to modulate the brightness of a TV monitor that is rastered in synchronization with the electron beam scan. The more electrons a particular region emits, the brighter the image at that point. SEM images typically contain a good deal of topographical detail, in other words a very good depth of field. Electron microscopy (EM), instead of light microscopy, can be used for direct counting of microbes, although the technique is impractical for routine use (Caldwell *et al.*, 1997).

2.19.1.4 Confocal laser microscopy

This is a new, non-destructive technique with major advantages, bridging the gap between traditional light microscopy and SEM (Coetzee, 1999). It gives higher resolution and thinner non-invasive optical sections or planar views than those obtained by classical bright-field or dark-field microscopy and increased contrast is another advantage (Rochow and Tucker, 1994). Integrating a light microscope, a scanning laser and a computer, confocal microscopy allows the generation of three-dimensional images of biological cells and tissues, or successive profiles of multi-layer structures (Coetzee, 1999).

Confocal configuration of the microscope optics, along with the capability of the laser to scan the specimen in a point-by-point fashion to produce sharp, high contrast and high resolution images of very thin, well defined optical sections (Kuehn *et al.*, 1998).

In an image-processing system, a hundred or more very thin optical sections can be stored and combined into a composite three-dimensional image. These image views can resemble those from SEM, but the specimen need not be in a vacuum (Rochow and Tucker, 1994).

2.19.1.5 Fluorescent *in situ* hybridization (FISH)

Of the three categories of RNA in prokaryotes (mRNA, tRNA and rRNA), only rRNA can be used for probing purposes because of its central role in cell survival, maintenance and reproduction. In addition, rRNA and their genes are almost universally present in cellular life forms, are functionally constrained and therefore evolutionally conserved, which makes them valuable indicators of identity and relatedness (Atkinson, 1999). Perhaps their greatest quality as far as spatial distribution and identification in activated sludge or other environmental samples is concerned, is their natural amplification and high copy number per cell (usually > 10 000 per cell)(Atkinson, 1999). An intact rRNA molecule is comprised of three sub-unit molecules: 5S, 16S and 23S. An average bacterial 16S rRNA molecule is approximately 1 500 nucleotides long (Amann *et al.*, 1995). There is therefore sufficient information contained within this molecule to establish reliable phylogenetic analyses, even if they are not fully sequenced, although > 1000 nucleotides should be determined for accuracy and confidence (Amann *et al.*, 1995).

Each rRNA-targeted oligonucleotide is chemically linked to a fluorochrome which allows cells hybridized with the labelled oligonucleotide to be directly visualized using epifluorescence microscopy or scanning confocal laser microscopy (Amann *et al.*, 1995). The choice of fluorochrome for immunofluorescence or probe hybridization is dependent on availability in a stable, purified form, the ease with which it can be coupled to a carrier molecule (antibody or

oligonucleotide probe) without influencing biological activity, as well as the availability of optical apparatus that enable detection of the emitted fluorescent light (Bosch and Cloete, 1993).

The general procedure of bacterial cell quantification from an environmental sample using FISH, usually incorporates a total cell count using the DNA intercalating dye DAPI, followed by hybridization with an universal bacterial probe (Atkinson, 1999). AO can also be used to microscopically visualize DNA molecules, although there are a number of difficulties inherent to its application (Porter and Feig, 1980). DNA-DAPI complexes fluoresce bright blue when visulaized at wavelenghts of > 390 nm, while unbound DAPI and non-DNA-DAPI complexes fluoresce a weak yellow, which enhances the visibility of DAPI fluorescence (Porter and Feig, 1980). The eubacterial to DAPI ratio gives an indication as to the bacterial composition of the sample (Atkinson, 1999). Dual EUB/DAPI staining of activated sludge samples has revealed that approximately 80 % of the microbial cells in the samples were metabolically active bacteria, of which only 3-19 % could be cultivated on optimized media (Wagner and Amann, 1997). The specificity of oligoprobes is freely adjustable, dependent on the requirements of the user. Different phylogenetic levels ranging from kingdom, subclass, genus, species and subspecies can be probed (Wagner *et al.*, 1994a).

In situ identification of the organisms in a batch-type EBPR sludge has revealed that the four major bacterial groups present were the alpha and beta subclasses of the *Proteobacteria*, Gram positive bacteria with a high G+C content and bacteria belonging to the *Cytophaga-Flavobacterium* cluster of the *Cytophaga-Flavobacterium-Bacteroides* phylum (Kawaharasaki *et al.*, 1999). However, Wagner *et al.* (1994a), when characterizing the Proteobacterial microbial consortia in municipal mixed liquor, found that the beta subclass dominated over the alpha and gamma subclasses. Simultaneous plating on nutrient rich media showed dominance of the gamma subclass, emphasizing the bias introduced with cultivation techniques. FISH of mixed liquor samples at the family level of organization of the anaerobic and aerobic zones of the same plant indicated no markable population shift between zones in the EBPR process when characterized at the family level.

Direct *Acinetobacter* FISH counts of an enhanced pilot-scale culture based on the 3-stage Phoredox system by Atkinson (1999) revealed that the genus only constituted approximately 3 % of the total bacterial community. The alpha and gamma subclasses of the *Proteobacteria* appeared to dominate the system with approximate total DAPI counts of 23 and 21 %, respectively, followed by the beta subclass with approximately 16 %. The *Cytophaga-Flavobacterium* group and the high GC Gram positive bacteria only constituted 5 and 8 % of the bacterial community, respectively. Results indicate the definite involvement of the *Proteobacteria* in EBPR operations, although which specific genera still needs to be clarified. The author found that cell counts using plating techniques were generally 1 000-fold lower than counts obtained with membrane filtration and DAPI staining. The microbial population profile using FISH did not seem to alter throughout the system, with counts of the various classes of bacteria remaining fairly constant within the various reactor zones. These results compare well to those of a study done by Ehlers *et al.* (1998), who found little community variation by comparing protein profiles using SDS-PAGE of different plants and different zones in a particular plant. The results of the study by Atkinson (1999) seem to indicate that due to their diversity of metabolism, *Pseudomonas* spp. are able to achieve dominance in BNR activated sludge plants. Although they are not able to accumulate poly-P as efficiently as other PAO like *Acinetobacter*, their high cell counts in mixed liquors will enable them to remove high quantities of P from the system, which may account for the majority of the observed P removal.

Staining activated sludge samples with DAPI at elevated concentrations of those used for DNA staining, referred to as the polyphosphate probing concentration, results in the fluorescence of intracellular volutin and lipid inclusions (Nakamura *et al.*, 1998; Kawaharasaki *et al.*, 1999). Bacteria, which accumulate large quantities of poly-P, are easily distinguished by colour and intensity of fluorescence due to the following DAPI stain characteristics (Kawaharasaki *et al.*, 1999):

* DNA-DAPI fluorescence is blue-white;
* polyphosphate-DAPI fluorescence is bright yellow;
* lipid-DAPI fluorescence is weak yellow and fades rapidly (within seconds).

Dual staining of samples with DAPI at elevated concentrations and EUB will result in the determination of the PAO population in EBPR sludges by means of the PAO/EUB ratio. *In situ* identification of those bacteria exhibiting strong poly-P accumulation (identified through DAPI staining) can also be achieved through dual staining with class and subclass oligonucleotide probes which totally negates culture-dependent methods of isolation and/or Neisser (poly-P) staining procedures (Atkinson, 1999).

For all its promise and potential, there are still technical problems inherent to FISH when applying the technology to microbial systems such as activated sludge. Qualitatively, the protocol of hybridization and detection has been optimized at all levels of organization. However, due to the nature of activated sludge, quantitative results are often difficult and limited because of incomplete dispersion of flocs, a technical problem which limits the application (Atkinson, 1999). Other problems include DNA retrieval for sequence determination, PCR bias when amplifying the sequence of interest and an imposed selection of the retrieved or target sequences (Hiraishi *et al.*, 1998). One of the possible solutions directed towards these problems is the combination of molecular and biomarker methods (Atkinson, 1999).

2.19.1.6 Immunofluorescence

The immunofluorescence approach was introduced as an *in situ* identification technique prior to FISH in an attempt to avoid culture-dependent techniques and has been used to effectively identify *Acinetobacter* in activated sludge (Cloete *et al.*, 1985; Lötter and Murphy, 1985; Cloete and Steyn, 1988). Although the technique is highly specific for the bacterium in question, there are a number of limitations associated with it (Wagner and Amann, 1997). Firstly, the presence of extracellular polymeric substances in activated sludge flocs can inhibit the penetration of antibodies to the target cells. The method of raising antibodies in host animals requires initial culturing of the bacterium of interest. The cross-reaction of antibody with contaminants also does occur, resulting in high levels of background fluorescence. This

technique also permits aut-ecological studies (studies of individual microbes in their natural environment). This technique has been applied to studies on selected microbial species in their natural habitats, including ecologically important organisms (Cloete and Steyn, 1988).

2.19.1.7 Quinone profiles

Respiratory or isoprenoid quinones are a class of lipids, which are constituents of bacterial plasma membranes and play important roles in electron transport, oxidative phosphorylation and active transport across the plasmamembrane (Collins and Jones, 1981). The numerical analysis of lipoquinone profiles has offered an effective method for monitoring population shifts and for classifying bacterial communities in wastewater sludges (Hiraishi *et al.*, 1991). Quinones are usually extracted from an environmental sample using an organic solvent. After evaporation and re-extraction, the concentrated quinone is applied to column chromatography to separate menaquinone and ubiquinone fractions (Hiraishi *et al.*, 1998). Municipal sludges are usually characterized according to these fractions (Hiraishi *et al.*, 1989, Hiraishi *et al.*, 1998). Quinone components are then identified and quantified using spectrochromatography and mass spectrometry. Numerical analyses of quinone profiles can enhance the information regarding bacterial community dynamics in wastewater ecosystems (Atkinson, 1999). The strength of the technique lies not only in its ability to assess taxonomic structure of bacterial communities, but also that variations in bacterial population structure over space and time can be quantified (Hiraishi *et al.*, 1991).

2.19.1.8 Microautoradiography

Autoradiography has classically been used in the medical field, but has recently been introduced to environmental sample analysis to determine microbial community structures (Atkinson, 1999). Typically, a radiolabelled compound appears in the cell or biological structure of interest through either adsorption of a tracer or labelled substrate uptake. The

radiolabelled ample is then placed in contact with a radiosensitive emulsion and the emissions from the radioactive sample interact with silver bromide crystals in the emulsion. The emulsion is then developed using standard photographic procedures and the silver grains appear on top of the radioactive structure which can then be viewed microscopically (Nielsen *et al.*, 1998; Nielsen *et al*, 1999). Although autoradiography can be applied successfully to study the *in situ* physiology of various microbes, it is limited by its lack of proper identification of the organisms in question. However, Nielsen *et al.* (1998), through simultaneous use of autoradiography and FISH, were able to correlate function/activity with identification, which is a tremendous breakthrough for activated sludge identification/diversity/functional studies (Atkinson, 1999).

2.19.2 Viable count procedures

A revolution is occurring in the field of microbiology. Previously, microbiologists concerned with analysis of various microbial communities and population dynamics within a specific eco-system have relied heavily upon conventional plating techniques for isolation purposes (Atkinson, 1999). Although the determination of bacterial numbers in a given sample is a basic prerequisite for any microbiologist, attempts at understanding microbial diversity have been severely limited due to a lack of suitable isolation and identification techniques (Brözel and Cloete, 1992). The main concern amongst wastewater scientists is that many of the microbial constituents of the activated sludge community are viable but non-culturable (Wagner *et al.*, 1994a,b; Amann *et al.*, 1995), which inevitably results in biased assumptions regarding biodiversity. When plating on solid media, the number of cfu represents only a fraction (< 1 to 10 %) of the viable cell counts made by direct microscopic techniques (De Haas, 1998).

The activated sludge process is a habitat that consists of a complex mixture of generalist and specialist microbes. In an attempt to obtain a fundamental understanding and to optimize the biological component of key processes such as EBPR occurring within sludge, isolation and identification techniques have progressed to the molecular level of organization. The

conventional method of dilution and spread plating is the main reason why *Acinetobacter* has traditionally been implicated as the primary active agent in BPR operations (Atkinson, 1999).

A wide range of bacteria can be isolated from activated sludge mixed liquors using conventional microbiological techniques such as sample dilution and spread plate inoculation (Atkinson, 1999). A number of methods such as the Most Probable Number (MPN) technique, pour plate method, surface plate method and membrane filtration are available for estimating numbers of selected metabolic groups in sludge (Schade and Lemmer, 1994). However, these methods (either by the composition of the media or the incubation protocol) have been shown to be extremely selective as to which organisms are allowed to grow and form visible colonies (Atkinson, 1999). The two different types of cells which remain viable but non-culturable in environmental samples can either be (Amann *et al.*, 1995):

* known species for which the cultivation conditions are not suitable for growth or which have entered a non-culturable state;
* unknown species that have not yet been cultured for lack of suitable techniques.

Sufficient documentation of the true community structure is therefore not made available (Snaidr *et al.*, 1997). This becomes evident when one considers EBPR. The most significant effect of cultivation of activated sludge mixed liquor on nutrient rich solid media is the underestimation of bacteria belonging to the beta subclass of the *Proteobacteria* and a gross overestimation of members of the gamma subclass of the *Proteobacteria* (Wagner *et al.*, 1994b). Standard plate counts do not reveal the bacterial community structure of activated sludge, but rather reflect the selectivity of the growth media for certain bacteria (Wagner and Amann, 1997). In effect, typical isolation media and conditions give rise to typical bacteria, whilst the unculturable fraction of the population, which may also be functionally active, are overlooked (Atkinson, 1999).

As far as solid media are concerned, Casitone Glycerol Yeast Autolysate Agar (CGY) has been shown to yield the highest plate counts for activated sludge samples (Pike *et al.*, 1972; Bux *et al.*, 1994). However, due to its high nutrient content, the suitability of the medium to isolate

viable bacteria from activated sludge is questionable. Solid cultivation media should, as far as possible, imitate the environment from which the bacteria of interest originate. For example, bacteria from oligotrophic systems are best enumerated with low nutrient agars like R2A and R3A agars, while bacteria from eutrophic systems are best enumerated with high nutrient agars like CGY, trypticase soy broth, nutrient and plate count agar (Osborne *et al*, 1989; Brözel and Cloete, 1992; Kämpfer et al., 1996). Nutrient rich media, when applied to population community analyses of activated sludge, will render erroneous results as it will support the growth of faster growing bacteria, which may not actually play a major role in the system (Osborn *et al.*, 1989). When isolating bacteria from an EBPR activated sludge system, Kämpfer *et al.* (1996) found that recovery rates using R2A agar were usually approximately one order of magnitude higher than the nutrient rich trypticase soy agar. Osborn *et al.* (1989) found that diluted CGY agar yielded the highest recoveries from activated sludge when compared with dilute Fuhs and Chen media, balance tank effluent and settled sewage. Yet, not one of the media was able to isolate the total amount of bacteria present in the mixed liquor samples. Different media, however, result in different population compositions, so direct comparisons of the various culture-dependent methods available to evaluate isolation efficiency are not beneficial (Atkinson, 1999). Although no medium can be expected to recover all viable cells from wastewater samples, it seems appropriate to use either chemically defined or custom manufactured media designed to yield highest counts (Reasoner and Geldreich, 1985).

The agar plate count is based on the assumption that a viable bacterial cell is capable of multiplying to form two progeny, and so on, under "optimal" conditions for the cell concerned (Postgate, 1969). Continued growth under such conditions on agar will result in a visible colony (Hattori, 1988). The count therefore reflects the number of cells capable of dividing under the given conditions, and not necessarily the total viable number present in the original sample.

There are two basic approaches to viable count procedures: the plate count technique and the MPN technique (Postgate, 1969).

2.19.2.1 Plate count methods

Plate count procedures employ various media and incubation techniques. The number of colonies formed in the standard plate count is normally counted after 48 or 72 h (Gibbs and Hayes, 1988). Yet, water contains many slow-growing bacteria which take longer to form a visible colony (Hattori, 1988). Dilution of samples can be spread onto the surface of the agar (surface spread method), or the sample suspension can be mixed with the agar just before the plates are poured (pour plate method)(Coetzee, 1999). One must consider whether the microbes can survive the plating procedure. Some microbes are killed upon exposure to air in the spread plate method, while others cannot tolerate the temperatures (45°C) needed to maintain melted agar in the pour plate method (Postgate, 1969).

The plates are incubated under specific conditions for a period of time to allow the bacteria to multiply and form macroscopic colonies, after which these colonies are counted. It is assumed that each colony originated from a single bacterial cell. Plates with too many colonies (> 300) cannot be accurately counted because one overlapping colony may represent more than one original bacterium. Plates with too few colonies (< 30) also must be discarded from the counting procedure for statistical reasons (Postgate, 1969).

2.19.2.2 Most probable number

The MPN method is an alternative to the plate count method for determination of viable organisms and uses statistical analysis and successive dilutions of the sample to reach a point of extinction. Replicate dilutions, usually three to ten per dilution, are scored as positive or negative and the pattern of scores are used in connection with appropriate statistical tables to obtain the nost probable number of viable microbes. The MPN procedure gives a statistically based estimate of the number of microbes in the sample and when relatively few replicate tubes are used, the confidence intervals are quite large. The MPN method has the advantage of permitting the use of liquid culture, avoiding the need to add a solidifying agent such as agar

with its possible contaminants. However, the method is more laborious than the plate count (Postgate, 1969).

As in the plate count procedure, media and incubation conditions can be adjusted in the MPN procedure to select for particular groups of microbes or to differentiate microbes with desired characteristics. Each procedure must be carefully selected and tested to permit the correct interpretation of results (Postgate, 1969).

2.19.3 Biochemical assays for estimation of bacterial numbers

Common techniques for estimation of the biomass activity consist of biochemical tests to measure either certain specific enzymes or specific products of bacterial metabolism. The sensitivity, reproducibility and compatibility of a number of biochemical assays for bacterial activity estimations are discussed below.

2.19.3.1 Adenosine triphosphate (ATP)

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). ATP can be detected by the luciferin-luciferase assay in which reduced luciferin reacts with oxygen to form oxidized luciferin in the presence of the luciferase enzyme, magnesium ions and ATP. Light is emitted in this reaction, the amount of emitted light being directly proportional to the ATP concentration. There are some difficulties, however, in the accuracy of estimating microbial biomass based upon ATP measurements. Some microbes alter their ATP concentration radically when nutritional or physiological conditions change. Also, in some ecosystems such as soil, sediment and nearshore aquatic areas, ATP may be adsorbed on particles (Holm-Hansen and Booth, 1966).

2.19.3.2 Cell wall components

Almost all bacteria contain muramic acid in their cell walls and the specific relationship between murein and bacteria makes quantitation of this cell wall component useful for estimating bacterial biomass (Millar and Casida, 1970). There is a gradient of concentrations of muramic acid in Gram positive and Gram negative bacteria. To accurately use this method, it is necessary to estimate the proportion of Gram negative and Gram positive bacteria in the sample. Erroneous estimates of these proportions will yield inaccurate estimates of biomass (Millar and Casida, 1970).

2.19.3.3 Chlorophyll measurements

Chlorophyll a, the dominant photosynthetic pigment in algae and cyanobacteria, is a useful measure of the biomass of these photosynthetic microbes, even though there may not be a constant relationship between biomass and chlorophyll content (Bance, 1977). Estimation of the biomass of photosynthetic microbes based upon chlorophyll determinations has been found to correlate well with estimates such as those based upon ATP determination (Paerl *et al.*, 1976).

2.19.3.4 DNA concentration

Concentrations of DNA are maintained in relatively constant proportions within microbes and determination of DNA can be used as a measure of microbial biomass. Because DNA is synthesized in growing cells at a rate proportional to biomass, the rate of DNA synthesis reflects the growth rate of microbes. Microbial growth rates have been determined in environmental samples by incubating samples with tritiated thymidine, using autoradiography of samples to determine rates of nucleotide incorporation (Cloete and Flemming, 1997).

2.19.3.5 Photosynthesis

Both heterotrophic and autotrophic assimilation of carbon dioxide can be measured using radiolabelled bicarbonate by incubating a sample containing the indigenous microbial community with the radiolabelled substrate and then determining the amount of ^{14}C assimilated into the cellular organic matter by filtering the cells and counting the ^{14}C trapped on the filters. Washing the filters eliminates any unincorporated ^{14}C radiolabelled bicarbonate. The residual ^{14}C-containing organic compounds can be oxidized with acid dichromate and the released $^{14}CO_2$ trapped and quantitated (Cloete and Flemming, 1997).

2.19.3.6 Respiration

The release of $^{14}CO_2$ from labelled substrates can also be used to determine decomposition rates for specific substrates and, hence, microbial activity. The complete degradation of a compound to its mineral components, in which the organic carbon of the compound is converted to carbon dioxide by respiration, is called mineralization.

2.19.3.7 Specific enzyme assays

A variety of enzyme assays can be used for measuring the metabolic activities of microbes. Enzymes involved in the biogeochemical cycling of sulphur for example are of interest to microbial ecologists studying microbiologically induced corrosion. It is important that the microbial community not be altered during the assay procedure if the measurement of the enzymatic activity is to reflect *in situ* activities accurately. Caution must be taken in maintenance of *in situ* conditions, particularly with reference to temperature, moisture content and redox potential. Also, incubation periods must be short enough to preclude changes in numbers of microbes, which could alter the levels of enzymes present in the sample (Cloete and Flemming, 1997).

From the above it is clear that numerous techniques exist for the quantification of microbes, some of which represent greater ease and accuracy than others. However, direct microscopy and plate count procedures remain to be the most commonly used in industry (Coetzee, 1999).

2.19.4 Spectrophotometric measurements (turbidity and absorbance)

Light scattering is the most widely used, conveneient and least complex method for estimating total microbiological material in a liquid medium (Mallette, 1969). When light passes through matter, it is scattered apart from its original path by inhomogeneities present. If these inhomogeneities of interest are particles considerably larger than small molecules, scattering becomes relatively intense (Mallette, 1969). Light scattering is dependent upon the concentration, size and shape of the particles, the relative refractive indices of particle and medium and the wavelenght of the incident light (Mallette, 1969). Bacteria scatter light primarily in the forward direction and the amount of scattering is proportional to the mass of cells present (Kheshgi and Saunders, 1959). Scattering is measured by passing a beam of light through the culture in an instrument containing a photocell that registers the amount of light that is scattered at a 90°-angle (turbidity meter) or the amount of light that passes through without being scattered (photometer)(Sebata, 1998). The absorbancy measurement routinely employed in microbiology is more related to total bacterial mass than to bacterial numbers (Mallette, 1969). Most spectrophotometers have wavelenghts of between 350 and 800 nm and this flexibility is important as different substances absorb light at different wavelenghts (bacteria for example absorb most of the light at 540-550 nm)(Sebata, 1998). Spectrophotometers, even the simplest types, are convenient in this respect and have become the most widely used class of instruments in microbiological turbidimetry. However, several problems may arise, including light absorption, the need for calibration curves, collection of light in low scattering angles and the unwanted changes in the biological material (for example aggregation of *Pseudomonas aeruginosa* cells at high levels of phosphate) may introduce large uncertainties (Mallette, 1969).

2.20 Adenosine triphosphate (ATP)

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 and 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).

$$ATP \rightleftharpoons ADP + \text{inorganic phosphate} + \text{energy}$$

2.20.1 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).

2.20.2 The application of ATP for monitoring microbial biomass

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 SS or VSS (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).

Jørgensen *et al.* (1992) determined biomass of activated sludge growth cultures in terms of dry weight and compared the data with ATP content, the OUR and FDA hydrolysis data. ATP content showed the best correlation with biomass. A conversion factor of 3 mg ATP per g dry weight was calculated. With the same methods applied to 4 full-scale systems, ATP results indicated a relationship of 67 mg dry weight per gram SS.

Roe and Bhagat (1982) estimated the ATP to suspended solids ratio at maximum viability in activated sludge, and they found that viability varied significantly with mean cell residence time. Kucknerowicz and Verstraete (1979) found a linear relationship between OUR and ATP, suggesting that ATP reflected viable biomass. However, a requirement that must be met to obtain reliable biomass estimations, is that the activity/biomass ratio remains fairly constant during the measurement period (Jørgensen *et al.*, 1992). According to Jenkinson and Ladd (1981), however, the ATP concentration of a resting soil population did not differ much from ATP in pure cultures of actively growing microbes. This was in agreement with results by Jørgensen *et al.* (1992) which showed constant ATP content to biomass ratios, independent of growth phase. Van de Werf and Verstraete (1984) however, showed extensive variation in ATP to biomass ratios during different metabolic conditions.

It can be assumed that culture is at maximum activity when it is in the exponential phase of growth. In the study by Jørgensen *et al.* (1992), the amount of viable biomass was estimated on the basis of maximum activity measurements and the conversion factor of 3 mg per g dry weight found, was well in agreement with values reported by Patterson *et al.* (1970) and Nelson and Lawrence (1980). The percentage of viable biomass in the experiments by Jørgensen *et al.* (1992) also correlated well with values in literature (Patterson *et al.*, 1970). Roe and Bhagat (1982) measured ATP levels in activated sludge from a lab-scale sludge plant, and indicated that the fraction of SS made up by viable biomass depended on sludge age, so that the highest viable biomass was obtained at the lowest sludge age.

Few studies have dealt with the estimation of viable biomass in wastewater (Jørgensen *et al.* (1992). By means of OUR measurements, Henze (1986) found that viable biomass constitutes 6 to 78% of VSS, depending on treatment and the type of wastewater.

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.

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 (MLVSS), 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 µg per mg MLVSS.

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, VSS 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 VSS 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).

Specific proportions of ATP in relation to total cellular carbon was found to be constant by Atlas (1982), with variations not succeeding 17 %.

2.21 Energy Dispersive Spectrometry (EDS)

EM combined with energy dispersive spectrometric X-ray microanalysis (EDS) is a method of elemental analysis within well-defined regions of a specimen (Buchan, 1980). However, there are many problems associated with this technique when applied to biological material, as the standard specimen preparation techniques for EM can displace, transform and dissolve many elements within biological samples, which are therefore not representative of the *in vivo* situation (Buchan, 1980). Morgan (1983) discusses the chemical artifacts that tend to be introduced by the exposure of biological material to aqueous sample preparation media. Artifacts discussed include:

* element loss;
* phase transformations in mineralized tissues;
* redistribution of elements within the specimen;
* introduction of extraneous elements.

The only preparation method known which would give a reasonable representation of the *in vivo* situation would be rapid freezing, freeze sectioning and X-ray analysis in the frozen state (Buchan, 1980; Zierold and Schäfer, 1988). However, this is a complex technique requiring special instrumentation (Buchan, 1980).

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 a K X-ray. Since an electron from one of several higher energy levels could fill the vacancy, a photon corresponding to one of these transitions is emitted. These are called $K\alpha_1$, $K\alpha_2$, $K\beta$ etc. X-ray photons.

Each element in the periodic table contains electrons in orbits with particular discrete energy levels and it is on the basis of the differences between these specific energies that each element can be identified (Buchan, 1980).

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 (Buchan, 1980).

In addition to the characteristic X-ray radiation emitted by each element in a specimen, a background radiation is also emitted over the whole energy spectrum up to the energy of the incident electrons. This radiation is known as background radiation or "bremsstrahlung" and arises from the deceleration of the electrons due to the interaction with the nuclei of all thew atoms in a specimen (Buchan, 1980).

In the case of thin biological specimens, the amount of bremsstrahlung is proportional to the mass of the specimen through which the electrons pass. The ratio of the characteristic elemental X-rays to the total bremsstrahlung X-rays generated (or the peak to background ratio) is thus proportional to the ratio of the elemental mass to the total mass in the excited volume. An example of an excitation volume can be seen in Figure 2.2. This relationship is the basis of quantitative analysis in biological specimens. The bremsstrahlung forms the background underlying the characteristic peaks as shown in an energy dispersive spectrum (Figure 2.3).

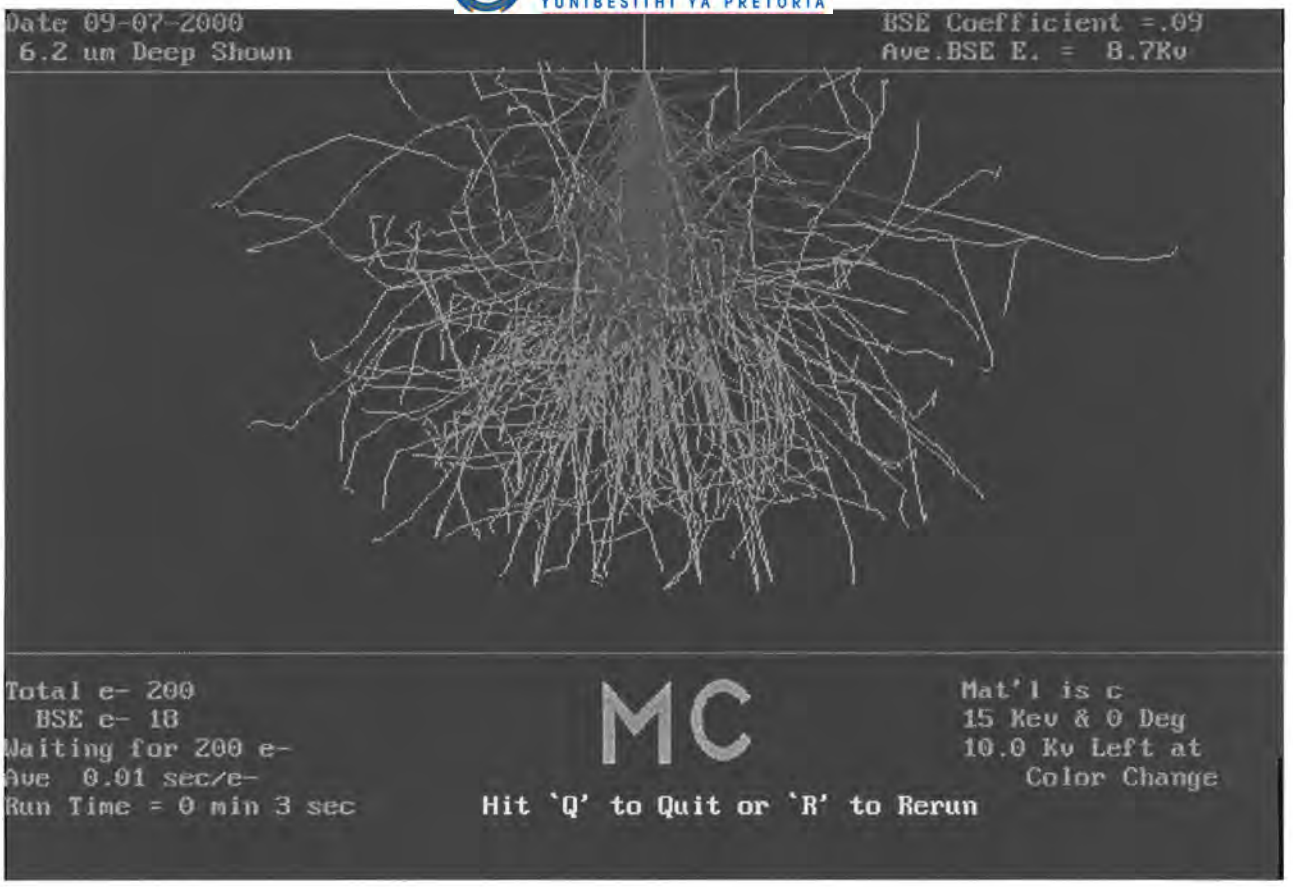

Figure 2.2: Example of the excitation volume created by EDS in a particular specimen (Monte Carlo electron flight simulator).

For quantitative analysis of biological samples, sections are considered to be thin if (Buchan, 1980):

* X-rays emitted by the elements in a specimen are not absorbed significantly by their passage through the specimen;
* the generation of additional X-rays by the process of fluorescence is also negligible.

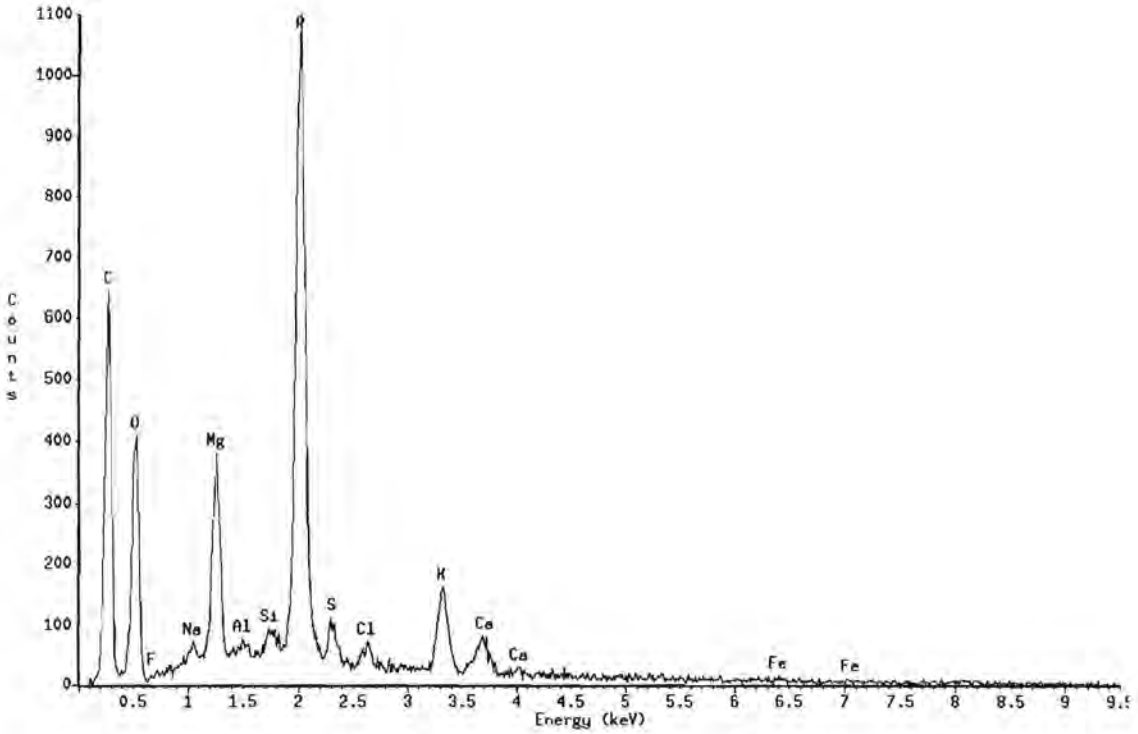

Figure 2.3: Typical EDS energy spectrum. Notice background bremsstrahlung between the Cl and K, as well as the Ca and Fe peaks.

Russ (1978) concluded that regardless of the details of the procedure, the general accuracy of the method is in the order of 10 %, and that in the analysis of biological thin sections, the greatest, and therefore, limiting errors arise in counting statistics, specimen preparation variables and in the difficulty in determining the density and thickness of specimens.

Buchan (1981) used electron microscopy in combination with EDS to examine the nature of accumulated P in seven activated sludge plants exhibiting EBPR. Large P accumulations were located in identical structures in the sludges examined. The P was located in large electron-dense bodies within large bacterial cells, characteristically grouped in clusters and later identified as *Acinetobacter* spp. (Buchan, 1983). The calcium to P ratio of these bodies precluded them from being any form of calcium phosphate precipitate. Quantitative analysis

indicated that the electron-dense bodies contained in excess of 30 % P. Extracellular P containing precipitates were not located.

2.22 Extracellular polymeric substances (EPS)

2.22.1 Definition of EPS

The production of extracellular polymeric substances (EPS) is a general property of microbes in natural environments and has been shown to occur both in prokaryotic and eukaryotic microbes. Microbial EPS are biosynthetic polymers (biopolymers). EPS were defined by Geesey (1982) as extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates. The abbreviation EPS has been used for extracellular polysaccharides, exopolysaccharides, exopolymers and extracellular polymeric substances (Wingender *et al.*, 1999). Polysaccharides have often been assumed to be the most abundant components of EPS in early biofilm research (Costerton et al., 1981). However, proteins and nucleic acids (Frølund *et al.*, 1996; Nielsen *et al.*, 1997), as well as amphiphilic compounds including phospholipids (Neu, 1996; Takeda *et al.*, 1998) have been shown to appear in significant amounts or even predominate in EPS preparations from activated sludges, biofilms and pure cultures of bacteria. In addition, some researchers described humic substances as components of EPS matrices (Nielsen *et al.*, 1997, Jahn and Nielsen, 1998).

2.22.2 Composition, secretion and spatial arrangement of EPS

EPS are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeating units within the polymer molecules (Wingender *et al.*, 1999). Building blocks of polysaccharides include: monosaccharides, uronic acids and amino sugars bound by glycocidic bonds, and which may have the organic substituents O-acetyl, N-acetyl, succinyl and pyruvyl groups and the inorganic substituent

groups, sulphate and phosphate. Building blocks of proteins are amino acids linked by peptide bonds and my have the substituent groups oligosaccharides (in the case of glycoproteins) or fatty acids (for 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. As described above, EPS may contain nonpolymeric substituents of low molecular weight, which greatly alter their structure and physicochemical properties (Wingender *et al.*, 1999).

By definition, EPS are located at or outside the cell surface, independent of their origin (Wingender *et al.*, 1999). The extracellular localization of EPS and their composition may be the result of different processes such as active secretion, shedding of cell surface material, cell lysis and adsorption from the enviroment. In all cases, EPS form the outermost surface layers of bacteria as boundary structures mediating contact and exchange processes with their biotic and abiotic environments (Wingender *et al.*, 1999).

Although EPS are not essential structures of bacteria in laboratory cultures (as loss of EPS does not impair growth and viability of cells), under natural conditions, EPS production seems to be an important survival feature, with most bacteria occur in microbial aggregates such as flocs, whose structural and functional integrity is based upon the presence of an EPS matrix (Wingender *et al.*, 1999).

2.22.3 Functions of EPS

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 electrostatic bonds with multivalent cations, underlining their key role in the floc structure (Wingender *et al.*, 1999). In addition, proteins have also been suggested to be involved in hydrophobic bonds within the EPS matrix (Dignac *et al.*, 1998).

General functions of EPS (some of which are relevant in activated sludge) include (Wingender *et al.*, 1999):

* adhesion to surfaces;
* aggregation of bacterial cells and formation of flocs and biofilms;
* cell-cell recognition, including symbiotic relationships with plants and animals and initiation of pathogenic processes;
* structural elements of biofilms including mechanical stability and shape of EPS structures;
* protective barrier, including resistance to host defenses and resistance to biocides, disinfectants and antibiotics;
* retention of water, preventing desiccation;
* sorption of exogenous organic compounds, including scavenging and accumulation of nutrients from the environment and sorption of xenobiotics;
* sorption of inorganic ions, including accumulation of toxic metal ions (detoxification), promotion of polysaccharide gel formation and mineral formation;
* enzymatic activities, including digestion of exogenous macromolecules for nutrient acquisition and release of biofilm cells by degradation of EPS structure;
* interaction of polysaccharides with enzymes for accumulation/retention and stabilization of secreted enzymes.

2.22.4 Analysis of EPS

Historically, microbial polysaccharides were studied for three reasons (Neu and Lawrence, 1999):

* they represent a structural feature of the microbial cell, therefore investigated for pure and basic research interests;

* polysaccharides were recognized as antigen determinants of the microbial cell surface, the knowledge of their structure being of great importance in medical microbiology;
* they were recognized as a source of polymers with unique properties.

These applied aspects of polysaccharides were a reason to study their structure, properties and production on the pilot- and industrial-scale. Generally due to the pure culture philosophy in microbiology, the production, isolation and preparation of EPS were done with pure cultures only. Nowadays, more microbiologists are starting to work on defined mixed or even fully complex cultures derived from natural or technical habitats (Neu and Lawrence, 1999). This is mainly because of the fact that a study of a natural system does not involve a pure culture, but a very complex microbial community (Caldwell *et al.*, 1996), and as these complex communities produce complex mixtures of EPS, we are confronted with the challenge of analyzing these complex EPS matrices (Neu and Lawrence, 1999).

2.22.4.1 Destructive analysis of EPS

2.22.4.1.1 Physical and chemical techniques

Apart from a high water content of up to 99 %, biofilms consist mainly of EPS and cells (Neu and Lawrence, 1999). The traditional approach to investigate polysaccharides is based on the isolation of the polysaccharides from the complex cell/EPS matrix. However, these techniques disrupt the original structure of the cells, aggregates or biofilm matrix (Neu and Lawrence, 1999). Several methods have been suggested to isolate polysaccharides from biofilm systems and most are concerned with the extraction of polysaccharides from activated sludge (Nielsen and Jahn, 1999). In early publications, a variety of methods were used for extraction, although recently, employment of cation exchange resins seems to be the method of choice (Neu and Lawrence, 1999).

In typical scheme of sampling, handling and analysis of bound EPS in bioaggregates involves (Nielsen and Jahn, 1999):

* sampling and pretreatment (including washing steps, homogenization or storage);
* extraction by a suitable method;
* purification before analysis;
* analysis for macromolecular composition or other characteristics.

For optimal extraction, samples should be well homogenized without cell disruption. Microscopic checks of particle size during and after homogenization is recommended.

Washing before extraction is often conducted to remove soluble EPS (Nielsen and Jahn, 1999). It is important to select a washing buffer carrying an ionic strength and composition not too different from the sample preventing some bound EPS from desorption and being washed away from the EPS matrix (Nielsen and Jahn, 1999). The mixing of a sample with deionized water can, for example, be regarded as a simple form of extraction (Gaudy and Wolfe, 1962).

Physical methods of extraction include centrifugation, mixing or shaking, sonication or heat treatment (Nielsen and Jahn, 1999). In general, methods employing only physical methods, give lower yields than those employing both physical and chemical techniques. Heat treatment may cause significant lysis and disruption of macromolecules.

Chemical methods of extraction include addition of various chemicals to the bacterial sample that can break different linkages in the EPS matrix and facilitating a release of EPS into the water. Treatments include (Nielsen and Jahn, 1999):

* alkaline treatment by addition of NAOH, causing ionization of charged groups, like carboxylic groups, in proteins 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; the divalent ions can be removed by means of a complexing agent like EDTA;
* cation exchange by a high concentration of NaCl;
* enzymatic digestion.

The more hydrophobic part of the EPS is usually not extracted by the above methods, but may be extracted from pure cultures by the use of detergents (Nielsen and Jahn, 1999).

In many extraction studies, the accumulation of protein and nucleic acids in the crude extract has been taken as an indication of cell lysis (Nielsen and Jahn, 1999). However, it has been found that the EPS matrix usually contains large amounts of protein, nucleic acids and probably also glycoprotein (Frølund *et al.*, 1996), so that the presence of these compounds in the extract is very difficult to use as an indicator for cell lysis. Instead, other methods must be applied, for example substances that are truly intracellular and do not accumulate in the EPS matrix (Nielsen and Jahn, 1999). ATP has been used in addition to DNA as intracellular marker, but difficulty is experienced in the accuracy of the ATP measurements (Grotenhuis *et al.*, 1991). Another promising method is the appearance of intracellular enzymes like glucose-6-phosphate dehydrogenase (G6PDH) in the extract, indicating the extent of lysis (Platt *et al.*, 1985). Cell counts can only be used to see whether cells are destroyed, but not to assess whether leakage of intracellular material occurs (Frølund *et al.*, 1996).

Despite problems with the analysis of cell lysis, boiling and addition of NaOH seem to cause severe cell lysis in activated sludge (Karapanagiotis *et al.*, 1989). The cation exchange method with Dowex resin did not show strong lysis if short extraction times (less than 2 h) were conducted (Frølund *et al.*, 1996). It can be argued that some lysis does not make much of a difference on EPS composition during extraction (Frølund *et al.*, 1996), since in activated sludge for example, the cell biomass represents only 10-20 % of the total organic matter (Wanner, 1994; Frølund *et al.*, 1996; Jahn and Nielsen, 1998; Münch and Pollard, 1997).

Disruption of macromolecules during extraction may take place. It is critical if the chemical structure or some macromolecular properties are to be investigated, but not if only the total amount of EPS is determined (Nielsen and Jahn, 1999). Boiling and alkaline treatments have been reported 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 bindings in glycoproteins and uronic acids are degraded (Nielsen and Jahn, 1999). Possible disruption has not been investigated in detail for extraction by heating or sonication (Nielsen and Jahn, 1999), although no disruption is caused by the Dowex cation exchange method (Karapanagiotis *et al.*, 1989; Frølund *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 (Nielsen and Jahn, 1999). The second definition does not reveal anything about the total amount of EPS, but only how much EPS a certain method can extract of the total organic matter. Comparative extraction studies using different extraction and analysis methods reflect large variation. In order to compare extraction yields, it is important to refer to a common measurement, for example the amount of organic matter (volatile matter)(Nielsen and Jahn, 1999).

It is still uncertain which part of the exopolymers is extracted by the various methods (Nielsen and Jahn, 1999). Many of the hydrophobic compounds, together with some polysaccharides, are not extracted by commonly used methods. This might explain why some tightly bound exopolymers associated with cell clusters are not extracted from activated sludge (Frølund *et al.*, 1996). It is important to realize that use of even well-standardized extraction procedures is still qualitative in nature, and perhaps only a minor part of the EPS is extracted (Nielsen and Jahn, 1999).

In many studies, it is important to know how much of the EPS is extracted (Nielsen and Jahn, 1999). Because no direct quantitative methods for separation of cell biomass and EPS are available, indirect methods must be used. A possibility is to measure the total biovolume by measuring the number and sizes of bacteria present and using conversion factors from biovolume to total organic carbon (TOC), or protein, to calculate the total contribution rom the cellular biomass fraction (Nielsen and Jahn, 1999). Cellular molecules like DNA or polysaccharide cannot be used, since the cellular content varies. Considering the usually very low extraction yields from the total organic matter recorded in many studies (3-27 %), the extraction yields in respect of the total EPS amount are in general very small (Nielsen and Jahn, 1999).

In many studies of EPS, no further purification of the crude extract is conducted before analysis of the EPS composition and amount (Nielsen and Jahn, 1999). This is common in investigations of activated sludge, granular sludge and biofilms in technical systems (Karapanagiotis *et al.*, 1989; Frølund *et al.*, 1996). In some studies, however, purification before any further analysis takes place. Methods include heat extraction, macromolecular precipitation in alcohol/acetone overnight and rinsing and dehydration in acetone/petroleum ether before analysis of components (Nielsen and Jahn, 1999). This purification step will remove various macromolecules from the EPS matrix. Further purification steps include precipitation of polysaccharides in cold alcohols, removal of protein by protease treatment or phenol extraction, or the use of gel filtration chromatography (Domenico *et al.*, 1989).

After extraction, chemical analyses include sugar analyses, linkage analysis, sequencing and determination of the anomeric configuration. The destructive analysis of polysaccharides includes (Neu and Lawrence, 1999):

* isolation by precipitation from the culture supernatant or extraction from the cell surface;
* purification by precipitation and size fractionation by gel chromatography;
* release of single constituents by various types of hydrolyses;
* determination of charged compounds by high voltage electrophoresis;

* analysis of polysaccharide constituents (sugar and non-sugar) by high performance liquid chromatography (HPLC) or gas chromatography (GC).

Additional investigation may include methylation analysis to determine the linkage pattern of the carbohydrates, analysis of the products by gas chromatography-mass spectroscopy (GC-MS), specific degradation and isolation of di- or oligosaccharides to confirm the structure (Lindberg *et al.*, 1975). ^{1}H NMR and ^{13}C NMR spectroscopy may also be used to follow derivatization procedures of the polysaccharide molecule (Perlin and Casu, 1982). Finally the anomeric configuration (D or L form) of the carbohydrates has to be determined and if the polysaccharide is available in a pure form, the three-dimensional structure may be investigated by X-ray crystallography (Rees *et al.*, 1982).

2.22.4.1.2 Electron microscopy

Electron microscopy is another destructive analysis method for EPS and the presence and significance of EPS in microbial adhesion and biofilm development has been demonstrated by different electron microscope techniques (Neu and Lawrence, 1999). SEM includes fixation and dehydration techniques and results in thin dried strands between organisms, representing the remains of the original EPS (Costerton *et al.*, 1986; Richards and Turner, 1984). Newer SEM techniques, using a cryo-transfer chamber and cold stage, were more gentle, but revealed similar strands of polymers (Richards and Turner, 1984). A technique claiming to allow examination of fully hydrated environmental samples, is the environmental SEM (ESEM)(Danilatos, 1991). However, in reality the process starts with fully hydrated samples, moves to partially hydrated samples and ends with dehydrated samples. Highest resolution is obtained in the latter stage, the result being similar to cryo-SEM techniques.

Transmission electron microscopy (TEM) also requires fixation and dehydration, as well as embedding and sectioning of the specimen. Thereby, high-resolution images of the dried EPS fibrils became visible (Costerton *et al.*, 1986).

The major disadvantage of all electron microscopic techniques lies in the creation and interpretation of artifacts caused by the various preparation steps (Neu and Lawrence, 1999).

2.22.4.1.3 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 the material, providing resolution at the atomic level (Neu and Lawrence, 1999).

2.22.4.2 Non-destructive analysis of EPS

Methods in this section include infrared spectroscopy or FT-IR (with information given being a signal of chemical groups from conditioning films, coatings, EPS and early biofilm events), nuclear magnetic resonance spectroscopy (NMR) (giving information about biomass, flow 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).

2.23 Summary

It is evident from accomplishments to date that engineers have succeeded, to a certain degree, in modelling wastewater treatment systems, especially if one considers the tremendous successes achieved with biological P removal and nitrification denitrification processes at full-scale (Atkinson, 1999). However, there are limitations to this empirical approach and EBPR processes occasionally deteriorate in P removal efficiency (Atkinson, 1999), or even routinely fails (Bond *et al.*, 1995).

The successful operation of nutrient removing activated sludge plants is dependent on good operation, coupled with good design and the maintenance of correct biomass levels. Biological phosphate removal plants in South Africa have not always given reliable and satisfactory performance (Osborn *et al.*, 1986).

Although considerable research effort has been directed towards improving understanding of the EBPR phenomenon, designs of activated sludge systems to accomplish EBPR are still based on experience and semi-empirical methods. Clearly, the need exists for design procedures based on more fundamental behavioural patterns and kinetics (Wentzel *et al.*, 1990). Quantification, as well as kinetic models of biomass in activated sludge are routinely used in design of wastewater treatment plants, in spite of the limitations which are currently encountered in biomass determination. For instance, in the current steady state design and kinetic simulation models for activated sludge, the mixed liquor suspended solids is made up of a number of components. One key component is the heterotrophic active biomass, as this component mediates the biodegration processes of COD removal and denitrification. However, the heterotrophic active biomass parameter has been only hypothetical within the structure of these models; it has not been measured directly, primarily due to the lack of suitable simple measurement techniques.

In the literature, principally microbiological techniques for biomass estimation has been proposed (Ubisi *et al.*, 1997), including pour plate or other culturing techniques (Gaudy and Gaudy, 1980), ATP analysis (Nelson and Lawrence, 1980), DNA analysis (Liebeskind and Dohmann, 1994), the use of fluorescent probes for rRNA (Wagner *et al.*, 1994c) and sequencing or ribosomal DNA (Blackall, 1994). However, these techniques have not yet been adequately integrated with the design and kinetic modelling theory, while culturing techniques have been widely criticised for their unreliability (Cloete and Steyn, 1988). The RNA and the two DNA methods are still in their infancy. The last four named methods also require sophisticated equipment and experimental techniques that are not widely available (Ubisi *et al.*, 1997).

Although the macro-environmental conditions of activated sludge have been well described,

very little is known about the micro-environment in activated sludge flocs, including diffusion gradients and the role of EPS in EBPR. Also, data on the P content of EPS has not been included in modelling of activated sludge systems and/or processes. In previous studies, Buchan (1980) used EDS to determine the location of phosphorus volutin granules in activated sludge, without attention to the phosphorus content of EPS.

The traditional approach to investigate polysaccharides is based on the isolation of the polysaccharides from the complex cell/EPS matrix. However, these techniques disrupt the original structure of the cells, aggregates or biofilm matrix (Neu and Lawrence, 1999). Several methods have been suggested to isolate polysaccharides from biofilm systems and most are concerned with the extraction of polysaccharides from activated sludge (Nielsen and Jahn, 1999).

Physical methods of extraction include centrifugation, mixing or shaking, sonication or heat treatment (Nielsen and Jahn, 1999). In general, methods employing only physical methods, give lower yields than those employing both physical and chemical techniques. Heat treatment may cause significant lysis and disruption of macromolecules.

In many extraction studies, the accumulation of protein and nucleic acids in the crude extract has been taken as an indication of cell lysis (Nielsen and Jahn, 1999).

The need exists to study the role of EPS in P removal *in situ*, generating data that can be directly incorporated into activated sludge models.

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