Protein analysis as a measure of active biomass in activated sludge

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ABSTRACT

Conventional methods to determine the biomass in activated sludge are the measurement of total suspended solids (TSS) and volatile suspended solids (VSS). Such methods do not distinguish between active biomass and inactive organic material. In this study, biomass was determined with both conventional methods and also through measuring the protein content with the modified Lowry method. In order to investigate the relationship between activity and biomass concentration in terms of TSS, VSS and protein content, some starvation experiments were conducted. It was found that the protein fraction of VSS differs under different starvation conditions. The biological activity of the activated sludge was measured as oxygen uptake rate (OUR). The strongest correlation could be measured between protein and OUR under various conditions. The results show that protein is an appropriate parameter for the measurement of the biological activity.

Key words | activated sludge, active biomass, protein, starvation

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INTRODUCTION

The activated sludge process has been the most important treatment system for municipal wastewater. In the past only the reliability of the process was in focus. However, due to the increasing energy costs and CO_2 emissions, the price of the process has become as important as the reliability. The new motto is 'Less energy use and more reliability'. The improvement of treatment performance whilst decreasing the costs of operating requires information about the metabolic activity (Sherr *et al.* 2001) and the concentration of microorganisms (Hwang & Hansen 1998). Furthermore, mathematical modeling of basic design and operational procedures in wastewater treatment, which has been widely applied since starting off in the 1990s (Henze *et al.* 1987), requires a reliable measurement of the biologically active biomass. The activity and biomass concentration or biomass growth are not synonymous (Kirchman 2001). There are various methods to determine bacterial population. Robertson *et al.* (1998) reported that the accurate estimation of microbial mass is essential for the modeling of the biological processes. However current methods do not distinguish bacterial metabolic activity from biomass growth (Pollard *et al.* 1997). In addition, the methods to measure the biomass are not accurate enough, as they

do not separate active and inactive biomass, thus none of these methods are satisfactory.

The growth measurement parameters can be classified as physical, chemical and biological methods. Of these, the measurement of total suspended solids (TSS) and volatile suspended solids (VSS) are the most commonly used methods to determine the biomass concentration in wastewater treatment plants (Ali *et al.* 1985; Tchobanoglous *et al.* 2003). Operators have used these inaccurate and unreliable indicators because they are well established, simple and also the most economic way of quantifying biomass. The disadvantage of these methods is the fact that they cannot distinguish between the active biomass, and inactive debris of either organic or inorganic origin, which indeed do not play any role in the treatment of wastewater. Activated sludge extracellular polymeric substances (EPS) together with the living cells have been reported as the major and most important sludge floc component (Frølund *et al.* 1995). However, only a few parts of the whole floc are relevant for the biological activity. EPS typically amounts up to 15% of the suspended solids (SS) (Urbain *et al.* 1993; Frølund *et al.* 1995) and the cell mass does not exceed more than 10–15% of the total organic fraction (Frølund *et al.* 1995), so that the biggest part of the activated sludge

floc is not involved in the biological turnover of the waste-water pollutants. Nevertheless operators mostly use the measurement of SS to monitor the plant. According to Hwang & Hansen (1998), the most common methods of quantifying microorganisms in biological wastewater treatment are: (1) the TSS method, when the wastewater contains only soluble organic matter; and (2) the VSS method, when the wastewater contains a sizeable fraction of suspended inorganics. Nevertheless, both methods must be properly interpreted if the wastewater is coming from a source like agriculture, food processing industries, or from other sources, which comprise suspended organic materials (McComis & Litchfield 1989). In particular, anaerobically treated wastewater contains a lot of suspended organic matter. In such cases, it is not possible to differentiate between microbial cells and suspended organic material of wastewater. As Robertson *et al.* (1998) suggested, it is essential to avoid the overestimation of microbial biomass, in order to accomplish better results from the mathematical modeling of wastewater treatment processes.

There are various methods to determine the biomass concentrations rather than conventional dry weight measurements (Gaudy & Gaudy 1980; Atlas & Bartha 1987; Sutton 2002). The purpose of the analysis and the restrictions of the selected systems determine the most appropriate method. Such restrictions arise from the composition of wastewater and the extraction method used prior to the determination of biomass. A method should be simple, reliable, and fast. Furthermore, the cost of the method is also an important and an indispensible criterion for the choice of the method.

Among various culture based, genetic and biochemical methods, the most popular approach to determine the actual biomass concentration is the determination of protein, which is a biochemical method. This wide usage depends on the ease of measurement and the fact that the protein is the most abundant biochemical compound of cell material. Proteins are essential parts of living cells and participate in every biological process. They are readily degraded when cell lysis occurs and easy to extract, purify and analyze (Denecke 2006). Ehlers & Cloete (1999) used the total protein content from different activated sludge samples in order to make fingerprints to describe the biological diversity of the sludge biocenosis. Schmitz *et al.* (2000) used the protein analysis as a simple method for the quantitative assessment of sewage sludge disintegration. Wilmes & Bond (2004) separated protein extracts from a laboratory WWTP with 2D-PAGE and found protein spots which originated from a polyphosphate-accumulation organism (*Rhodocyclus*-type). None of the authors investigated the protein content of activated sludge systems under various operation conditions e.g. absence of C, N, P or K (starvation experiments).

In this investigation the activated sludge protein from labscale sequencing batch reactors (SBR) operated under different starvation conditions was determined. The activity measured as oxygen uptake rate (OUR) was correlated with the amount of protein.

MATERIAL AND METHODS

Experimental set-up

Two aerated and stirred SBR, (8 l) with monitoring of temperature, pH and oxygen (IKS Aquastar) were used at 20 °C. The cycle time was 8 h including 4 min filling, 48 min settling, 116 min denitrification (anaerobic), 300 min nitrification (aerated) and 12 min discharging. The synthetic wastewater used as influent in the reference reactor consisted of 1,060 mg/l $C_2H_3NaO_2$; 95 mg/l NH_4Cl; 9 mg/l H_2KPO_4; 23 mg/l $HNaPO_4 * 12 H_2O$; 5 mg/l $MgSO_4$; 6 mg/l $CaCl_2$ and 5 mg/l $FeCl_3 * 6 H_2O$. The influents for the starvation experiments were similar but the carbon-starvation influent was made without $C_2H_3NaO_2$, the nitrogen-starvation influent without NH_4Cl, the phosphorous-starvation influent lacked H_2KPO_4 and $HNaPO_4$ and the potassium-starvation influent lacked H_2KPO_4. The sludge retention time was 20 d, the hydraulic retention time was 0.6 d. The operation time per starvation experiment was 30 d. The average of three samples before and after the starvation experiment was taken. The experiment was performed according to Wang *et al.* (2006). Seed sludge was taken from the wastewater treatment plant of Duisburg-Kasslerfeld, Germany.

Sampling and sample preparation

Sludge samples of 10 ml were taken daily and cooled at 4 °C. The samples were centrifuged at 2,075 g for 15 min. The pellet was resuspended in 10 ml extraction buffer (200 mM NaCl, 200 mM TRIS, 2 mM Na Citrate, 10 mM $CaCl_2$, 50 mM EDTA, pH 8.0) and then sonicated (Branson Sonic B30, micro tip, output control 7, 50% duty cycle). Two milliliters of the sonified sample were centrifuged at 11,180 g for 10 min. The supernatant was tested for protein.

Protein analysis

Two parallel assays were conducted for each sample to determine the color formations with and without $CuSO_4$ in the reagent. Total absorbance indicates the color formation of both proteins and humic compounds with the reagent including $CuSO_4$ and blind absorbance indicates the formation of color due to humic compounds and 20% of proteins (Frølund *et al.* 1995). Solving the following equations provides both actual protein absorbance and humic acid absorbance as well.

$$A_{Total} = A_{Protein} + A_{Humicacid}$$

$$A_{Blind} = 0.2 A_{Protein} + A_{Humicacid}$$

$$A_{Protein} = 1.25(A_{Total} - A_{Blind})$$

$$A_{Humicacid} = A_{Total} + A_{Protein}$$

where A_{total} is the total absorbance with $CuSO_4$, A_{blind} is the total absorbance without $CuSO_4$, $A_{Humicacid}$ is the absorbance due to humic compounds, and $A_{Protein}$ is the absorbance due to proteins.

The measurement was done at 750 nm with a Shimadzu UV-1602 spectrophotometer.

The chemicals used for the preparation of reagents are summarized below.

- *Lowry Copper Reagent (6 ml)*: 400 μl of 20 g/l $CuSO_4$ for A_{Total} or 400 μl for deionized water for A_{Blind}; 400 μl of 175.2 g/l Na-Tartrate ($C_4H_4Na_2O_6$); 5.2 ml of 76.9 g/l Na_2CO_3.
- *Lowry Reagent (10 ml)*: 6 ml of Lowry copper reagent; 2 ml of 1% sodium dodecysulfate (SDS); 2 ml of 1 M NaOH.
- *Folin-Ciocolteu reagent*: 1:10 dilution of 2 N Folin reagent (Sodium 3,4-dioxo-3,4-dihydronaphthalene-1-sulfonate).

The Lowry assay was conducted according to the following procedure:

1. 10 ml of two different copper reagents were prepared, one for A_{Total} and the other for A_{Blind}.
2. 400 μl of $CuSO_4$ solution was added into the first centrifuge tube, which was labelled as A_{Total}.
3. Same amount of deionized water as $CuSO_4$ was added to the second centrifuge tube labelled as A_{Blind}.
4. 400 μl of Na tartrate solution was added to both of the tubes. Note that Na tartrate was used instead of NaK tartrate, as the potassium salt makes SDS insoluble.
5. 5.2 ml of Na_2CO_3 was given to each tube. It is important to add Na_2CO_3 after mixing $CuSO_4$ and Na tartrate. Otherwise precipitation may occur as the chemicals are mixed.
6. 2 ml of 1% SDS (w/v) was added to each tube.
7. 2 ml of 1 M NaOH was added to each tube.
8. 800 μl of samples which were diluted 40 times were pipetted into Eppendorf tubes.
9. For blank 800 μl of deionized water was used.
10. 800 μl of copper reagents were pipetted into each Eppendorf tube.
11. After an incubation time of 10 min at room temperature, 400 μl of 0.2 N Folin reagent was added to each tube. Tubes were vortexed immediately after addition.
12. The absorbance values of samples were read at a wavelength of 750 nm after half an hour.
13. The blank of A_{Blind} should be first read and be adjusted to zero. This sample is taken as absolute blank, as it doesn't contain any chemicals which develop color.
14. The blank of A_{Total} should not be adjusted to zero, as the $CuSO_4$ solution itself develops minimum amount of color and should be considered in each test. The color formation by $CuSO_4$ depends on the composition of the reagent and may vary when the reagents are new.

Analytical methods

Dr. Lange test kits were used to analyze COD (LCI400, LCK614 and LCK314), ammonium nitrogen (LCK 341&342), total nitrogen (LCK 238&338), nitrate nitrogen (LCK 339&340) and phosphorous levels in each test. TS, VS and SVI were determined by German standard methods (DIN EN 12789, DIN EN 12880 and DIN 38 412). OUR was measured using Standard Method 2710B (*Standard Methods for the Examination of Water and Wastewater* 1998). Carbohydrates were analyzed using the Dreywood's Anthrone Reagent (Beck & Bibby 1961).

RESULTS AND DISCUSSION

Effect of carbon and nutrient starved waste water on the biomass growth

Wang *et al.* (2006) investigated the effects of carbon and nutrient starvation on aerobic granules. They compared

the net biomass growth of aerobic granules before and after starvation and found that under carbon and potassium starvation conditions, the growth of aerobic granules is seriously suppressed due to the fact that carbon and potassium are essential elements which actively participate in the energy metabolism of the cells. On the other hand the results of Wang *et al.* (2006) indicate an increase in biomass growth by means of weight under nitrogen and phosphorus starvation conditions.

In the studies of Wang *et al.* (2006) the biomass growth was measured in terms of volatile solids (g VS l^{-1}). In the scope of this study, the biomass growth was measured in terms of total solids, volatile solids and protein concentration. Figures 1(a–d) show the comparison of biomass
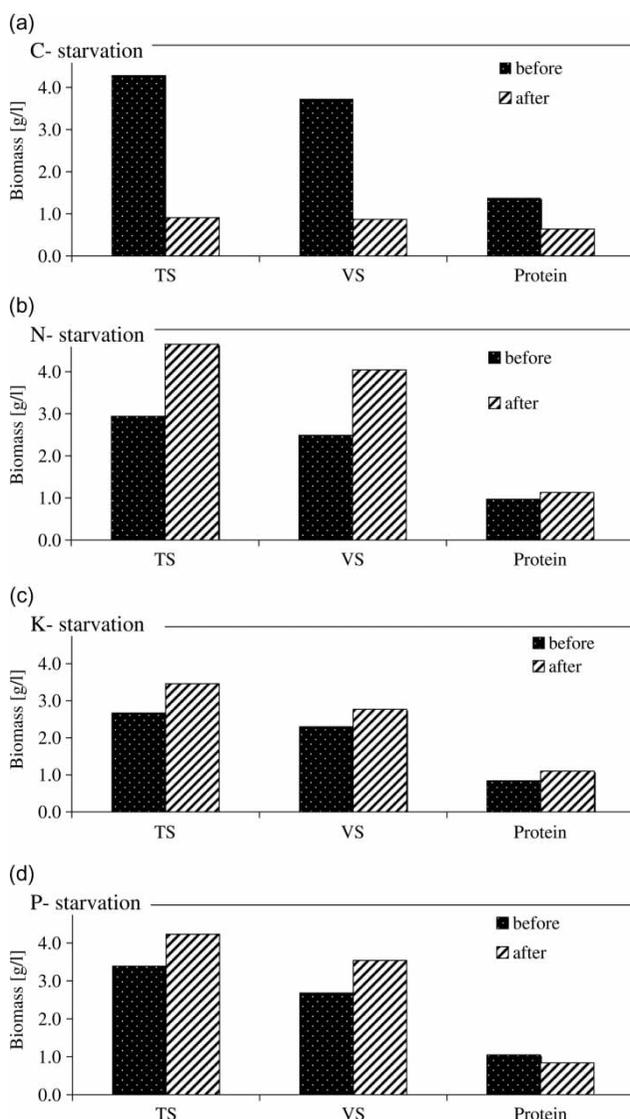
(a)

(b)

(c)

(d)

Figure 1 | (a–d) Changes in biomass measurement parameters before and after the C-, N-, K- and P-starvation.

growth parameters under carbon and nutrient starvation conditions.

In accordance with the results described by Wang *et al.* (2006), all biomass growth measurement parameters decrease after carbon starvation. The decrease in total solids and volatile solids are higher than the decrease in protein content. The decreases of total solids and volatile solids are 79 and 77% respectively. In contrast, the decrease of protein content under carbon starvation is about 50% (Figure 1(a)). This extensive difference is due to the fact that microorganisms use firstly non biomass carbon as an energy source and then after depleting non-biomass carbon, biomass decay starts leading to a decrease in all biomass measurement parameters including protein content. Thus, considering TS and VS as a biomass measurement parameter would result in overestimation of biomass loss (about 80%). However actual loss in biomass is only 50% according to protein measurements. The increase of activated sludge biomass under K-starvation was lower compared to N-starvation (Figures 1(c) and 1(b)). Since potassium is also required for the energy metabolism of the cells, absence of potassium leads to a suppression of the biomass growth.

Nitrogen and phosphorous are essential nutrients for the biomass growth, as they are required for the synthesis of proteins and so for new cell production. As described by Droste (1997) the required ratio of COD : N : P for biosynthesis of biomass is 100 : 5 : 1. In the absence of those nutrients, biomass growth is not possible and the excess carbon in wastewater which can not be converted to new cells, is stored by microorganisms both intracellularly and extracellularly. As a result of accumulation of storage compounds, the cell mass increases. It is important to note that the increase in biomass is only an increase in cell weight, whilst no cell division occurs. According to Robarts & Zohary (1993), bacterial growth is marked by cell division, and thus an increase in cell numbers rather than cell weight. They indicate that cell division occurs only when new cellular compounds such as proteins, DNA, RNA and membranes are synthesized. Durmaz & Sanin (2001) found that an increase in C/N ratio results in a decrease in protein concentration. Figures 1(b) and 1(d) show the changes in biomass measurement parameters due to the nitrogen – and phosphorous starvations, respectively. In both cases, conventional biomass measurement parameters, which are based on the dry weight of the sludge, increase. In contrast, the protein concentration is almost constant under both nitrogen and phosphorous starvation conditions.
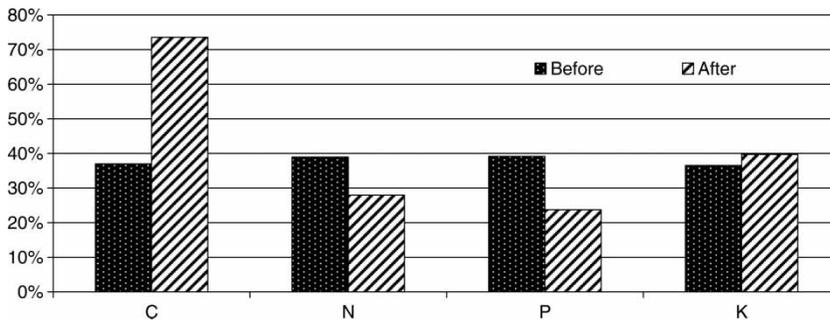
Figure 2 │ Changes in protein fraction of VS before and after C-, N-, P- and K-starvation.

Effect of carbon and nutrient starved wastewater on the protein fraction of volatile solids

Conventional biomass measurement parameters are only valid if the process is steady and if the wastewater does not contain highly organic matter. In such a case, the protein fraction of volatile solids is constant. Thus, measuring VSS or protein content delivers the same biomass concentration. However when the microorganisms are under stress due to nutrient or dissolved oxygen deficiency, presence of inert and toxic substances or pH and temperature changes, the active biomass concentration cannot be measured by conventional biomass measurement methods. Figure 2 presents how the protein fraction of biomass changes due to carbon and nutrient starvation conditions.

Under carbon starvation condition the protein fraction of volatile solids increases, since the non-biomass organic matter is depleted. Almost 75% of the volatile solids are made up of proteins. On the other hand, the protein fraction of volatile solids decreases under nitrogen and phosphorous starvation conditions. The reason for such an increase is the accumulation of storage products. Fractional changes in protein content of volatile solids enable a comparison of two different biomass measurement methods: (1) conventional, volatile solids measurement method; and (2) protein analysis method.

Comparison of biomass measurement methods under carbon and nutrient starvation conditions

Figures 3(a–d) demonstrate the result of the correlation analysis of activity, which is given in terms of OUR and the biomass concentrations in terms of TS, VS and protein. Under N-, K- and P-starvation conditions, the conventional biomass measurement parameters do not indicate any significant correlations between activity and biomass
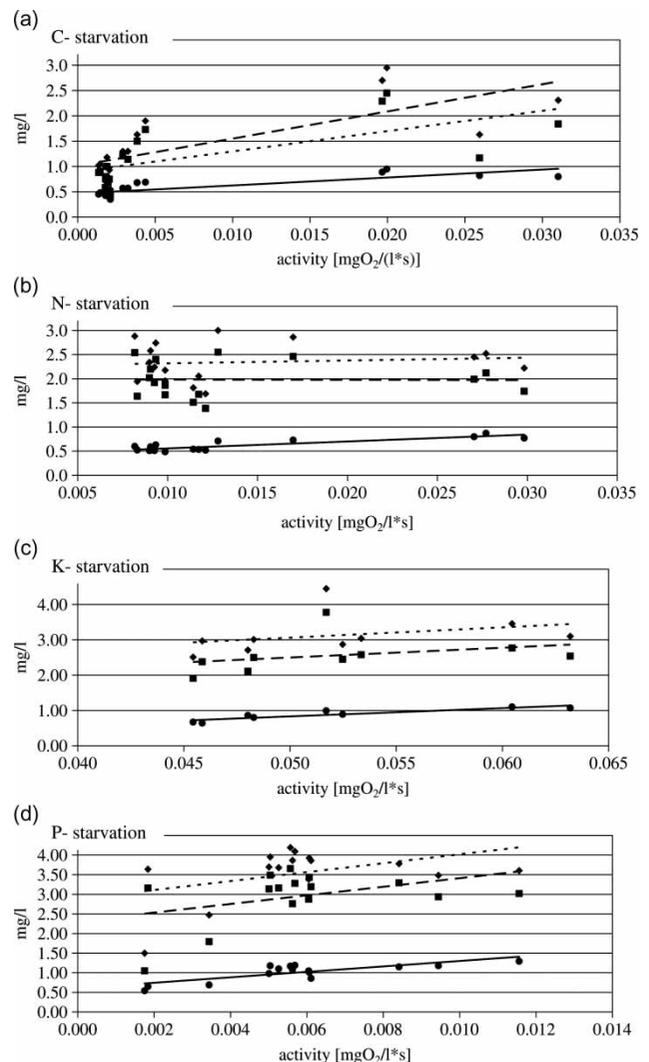

Figure 3 │ (a–d) Correlation analysis under C-, N-, K- and P-starvation (activity vs. concentrations of (♦) TS, (■) VS and (●) Protein).

concentration, whilst protein content indicates under all starvation conditions significant correlations with activity (see Table 1).

Table 1 | Summary of correlations between biomass measurement parameters and activity under all given starvation conditions ($r*$ = critical correlation coefficient for an error rate of 0.01, r = correlation coefficient)

	Parameter	C-starvation $r*$ = 0.623	N-starvation $r*$ = 0.606	K-starvation $r*$ = 0.798	P-starvation $r*$ = 0.641
Correlation coefficient r	Total solids	0.76	0.14	0.32	0.42
	Volatile solids	0.67	0.00	0.33	0.42
	Protein	0.83	0.81	0.91	0.79

Under carbon starvation conditions not only the correlation between protein content and activity but also the correlations between TS and VS concentrations and the activity were significant. The reason why TS and VS indicate significant correlation under a C-starvation condition, unlike under N-, K-, and P-starvation conditions, is the fact that under C-starvation the amount of protein fraction in VS is very high. The fraction of protein content in VS increased from 37 to 74% during C-starvation period (see Figure 2).

As a result of these correlation analyses, it is concluded that the protein content is the only reliable parameter under all circumstances to reflect the actual biomass concentration.

CONCLUSION

In this study, a new approach for the determination of actual biomass concentration is introduced. As proteins are the most abundant compounds in living cells, and they are subjected to rapid degradation when cell lysis occurs, the actual biomass concentration can be determined via measuring the protein content of the biomass. This study showed that measuring protein content of the biomass supplies rapid and correct information about the state of the activated-sludge biomass.

Carbon starvation results in a decrease of all biomass measurement parameters, including protein content. The decrease in TS and VS are greater than the decrease in protein content. The correlation analysis between biomass measurement parameters and activity concluded a significant correlation with all parameters. The reason why TS and VS show a significant correlation under carbon starvation is the fact that the protein fraction in VS was up to 75%.

Nitrogen starvation leads to an accumulation of excess carbon and an increase in cell mass. As without nitrogen proteins cannot be synthesized, this increase is only an increase in cell weight rather than cell number. Thus, as the conventional biomass parameters increase, the protein content remains constant. As the protein fraction in VS decreases, the correlation of conventional biomass measurement parameters with activity differs from the correlation of protein content with the activity. The correlation analysis showed that only protein content has a significant correlation with the activity. Due to the high amount of non-biomass organic matter in TS and VS, the correlations of those parameters with activity are not significant.

The same effect as in nitrogen starving conditions was observed with phosphorus starvation as well. While TS and VS concentrations increase under phosphorus starvation, protein content decreases, resulting in a decrease of protein fraction in VS. Correlation analyses indicate that only protein content is significantly correlated with the biological activity.

Under potassium starvation, an increase in all biomass measurement parameters was recorded. However, the biomass growth with respect to protein content is limited due to the limited energy source. The energy available is used firstly for the purpose of maintenance and then only the remaining energy is used for the growth of biomass. The correlation analyses show a significant correlation between protein content and activity, whereas conventional biomass measurement parameters do not significantly correlate with activity.

The results show that protein is a more appropriate parameter for the measurement of the active biomass than TS or VSS. The protein method has particular relevance in times of trouble with the operation of a plant. In this study the protein changes were monitored before and after a starvation period in a laboratory-scale plant. The method has to be evaluated in full-scale plants to get a protein pattern especially in times of malfunctions.

REFERENCES

Ali, I., Khararjian, H. & Ahmed, M. 1985 Viability of microbial mass in compartmentalised single activated sludge process. *Water Research* **19** (7), 927–932.

Atlas, R. & Bartha, R. 1987 *Microbial Ecology: Fundamentals and Applications*, 2nd edition. The Benjamin Cummings Publishing Co Inc., Menlo Park.

Beck, D. J. & Bibby, B. G. 1961 A modified anthrone colorimetric technique for use in investigations related to the cariogenicity of foodstuffs. *Journal of Dental Research* **40**, 161.

Denecke, M. 2006 Protein extraction from activated sludge. *Water Science and Technology* **54** (1), 175–181.

DIN EN 12789 Characterization of sludges – Determination of the loss on ignition of dry mass.

DIN EN 12880 Characterization of sludges – Determination of dry residue and water content.

DIN NORM 38412 German standard methods for the examination of water, waste water and sludge Bio assays.

Droste, R. 1997 *Theory and Practice of Water and Wastewater Treatment*. John Wiley and Sons, New York.

Durmaz, B. & Sanin, F. 2001 Effect of carbon to nitrogen ratio on the composition of microbial extracellular polymers in activated sludge. *Water Science and Technology* **10** (22), 1–9.

Ehlers, M. M. & Cloete, T. E. 1999 Direct extraction of proteins to monitor an activated sludge system on a weekly basis for 34 weeks using SDS-page. *Water SA* **25** (1), 57–62.

Frølund, B., Palmgren, R., Keiding, K. & Nielsen, P. H. 1995 Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Research* **30** (8), 1749–1758.

Gaudy Jr, A. & Gaudy, E. 1980 *Microbiology for Environmental Scientists and Engineers*. McGraw Hill, New York.

Henze, M., Grady, C., Gujer, W., Marais, G. & Matsuo, T. 1987 A general model for single-sludge wastewater treatment systems. *Water Research* **21** (5), 505–515.

Hwang, S. & Hansen, C. 1998 Evaluating a correlation between volatile suspended solid and adenosine 5′-triphosphate levels in anaerobic treatment of high organic suspended solids wastewater. *Bioresource Technology* **63** (3), 243–250.

Kirchman, D. 2001 Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments. *Methods in Microbiology. Marine Microbiology* **30**, 227–237.

McComis, W. & Litchfield, J. 1989 Meat, fish, and poultry processing wastes. *Journal of the WPCF* **61** (6), 855–865.

Pollard, P., Münch, E., Lant, P. & Greenfield, P. 1997 The impact of microbiological tools on mathematical modelling of biological wastewater treatment. *Water Science and Technology* **36** (10), 97–108.

Robarts, R. & Zohary, T. 1993 Fact or fiction – Bacterial growth rates and production as determined by [methyl-H]-thymidine? In: *Advances in Microbial Ecology* (G. Jones, ed.). Plenum Press, New York, pp. 371–425.

Robertson, B., Button, D. & Koch, A. 1998 Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. *Applied Environmental Microbiology* **64** (10), 3900–3909.

Schmitz, U., Berger, C. R. & Orth, H. 2000 Protein analysis as a simple method for the quantitative assessment of sewage sludge disintegration. *Water Research* **34** (14), 3682–3685.

Sherr, B., Sherr, E. & del Giorgio, P. 2001 Enumeration of total and highly active bacteria. *Methods in Microbiology. Marine Microbiology* **30**, 129–159.

Standard Methods for the Examination of Water and Wastewater, 1998 A. E. Greenberg, L. S. E. Clesceri & A. D. Eaton (eds). American Public Health Association, Washington D. C., USA.

Sutton, S. 2002 Quantification of microbial biomass. In: *Encyclopedia of Environmental Microbiology* (G. Bitton, ed.). John Wiley & Sons, New York, USA, pp. 2652–2660.

Tchobanoglous, G., Burton, F. L. & Stensel, H. D. 2003 *Wastewater Engineering Treatment and Reuse*. McGraw-Hill, California.

Urbain, V., Block, J. C. & Manem, J. 1993 Bioflocculation in activated sludge: analytical approach. *Water Research* **27** (5), 829–838.

Wang, Z.-W., Li, Y., Zhou, J.-Q. & Liu, Y. 2006 The influence of short-term starvation on aerobic granules. *Process Biochemistry* **41** (12), 2373–2378.

Wilmes, O. & Bond, P. L. 2004 The application of two dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. *Environmental Microbiology* **6** (9), 911–920.