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Oscillations of Exopolymeric Composition and Sludge Volume Index in Nitrifying Flocs

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Abstract

Protein, carbohydrate, and lipid composition of the exopolymer fraction of a nitrifying sludge in steady-state culture was analyzed after dissociation with 50 m*M* EDTA and dialysis of the nonfilamentous flocs. Steady-state culture was established when the nitrification rate was constant. The nitrification efficiency at that regime was 93%, also constant. In steady state the concentration of exopolymer protein in the nitrifying sludge floc oscillated from 5 (lowest) to 45 (highest) mg/L with a consistent oscillating pattern having a duration period of 10 d each. Carbohydrate and lipid content in the flocs showed no significant variations (30 and 36 mg/L, respectively). Only 20% of the extracellular polysaccharides had molecular weights higher than 10 kDa, suggesting that the floc aggregation depended on smaller fractions of low-molecular-weight carbohydrates. The oscillations in the concentration of exopolymeric protein coincided with parallel variations in the sludge volumetric index (SVI) value (12.2 ± 2.1 mL/g). Analysis of the polymeric substances of the floc and suspended solids corroborated by statistical analysis indicated that the variations in the SVI of the nitrifying nonfilamentous flocs were mainly related to the changes in the exopolymeric protein content.

Index Entries: Nitrification; flocs; extracellular substances; sludge volume index.

Introduction

Nitrification is an aerobic biological process in which ammonium is converted to nitrite and nitrate by two groups of lithoautotrophic

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microorganisms: the ammonia-oxidizing and nitrite-oxidizing bacteria *(1,2)*. The nitrifying microorganisms are slime or exopolymer producers with a typical zoogloeae growth *(3)*. In activated sludge for wastewater treatment, the nitrifying bacteria form flocs associated with heterotrophic microorganisms enveloped in extracellular polymeric substances (EPS). The flocs can be constituted either by filamentous and nitrifying bacteria or by nonfilamentous nitrifying microorganisms. Carbohydrates, proteins, lipids, and nucleic acids are the major components of the EPS in the floc *(4–6)*. The EPS content of the floc depends on many factors, such as the degree of cellular lysis, type of microorganisms, metabolic activity of the microflora, and organic matter absorbed from wastewater. Thus, the degree of settleability of sludges may be related to the EPS content by affecting its sludge volumetric index ([SVI], milliliters/gram). When the SVI increases, the settleability decreases and the sludge could float in the reactor and be the cause of reactor failure. Therefore, the SVI value is normally used as an index to establish the state of the sludge. Sludges with a value <150 mL/g are considered to have good settleability properties, but others are unstable at that similar value.

Reports are contradictory about the relationship between the changes in SVI with respect to changes in EPS concentration. For example, Chaw and Keinath *(7)* did not observe any relationship between the changes in EPS and changes in SVI of the sludge. Additional work *(8)* showed that a decrease in the concentration of EPS induced increases in the SVI. Other evidence *(5)* indicates that the SVI decreased with an increase in the EPS concentration. These discrepancies in the relationship between SVI and EPS might be owing to different analytical methods used for extraction and measurement of EPS.

It is not yet clear which of the compounds present in EPS are responsible for the changes in SVI. It has been reported *(7)* that variations in the concentration of the carbohydrate fraction of the exopolymer changed the SVI and settling rate of the flocs. Others *(9)* have found that carbohydrates and proteins were responsible for the floc settleability and stability of the floc structure. Lipids in the floc may also increase the SVI value of the sludge. Lipids have also been related to the hydrophobicity of the floc and found to be an important factor in the settleability of the sludge *(10)*.

The major constraint in the study of the chemical composition of the exopolymer fraction of the floc is in the extraction process of EPS from the floc. Heat *(11)*, ultrasound *(5,8)* and alkali treatment *(12)* have been used to separate EPS from the cells in the sludge. Eriksson and Alm *(13)* used EDTA to chelate Ca^{2+}, Mg^{2+}, or Fe^{3+} ions bound to the exopolymers and also to dissociate the flocs. The ion-exchange method has also been used for extracting EPS from the microbial biomass *(14)*. All these methods have different modes of actions that may interfere with the process of extraction and measurement of the components of the EPS of the sludge flocs.

Although it is not clear how the floc is structured, it is presumed that the concentration and the type of the exopolymeric proteins and polysac-

charides play an important role in the structure of the floc and EPS linking. Polysaccharides increase or a decrease in the settleability of the sludge. The divalent ions could bind the ionic groups of the EPS linking carbohydrates or proteins and forming networks in the flocs *(15,16)*. A structural model of the floc was proposed *(17,18)*, however, the researchers did not mention which of the exopolymers could be linked to the divalent ion.

Low settleability of the sludge could also be owing to the formation of foam and to the phenomenon of bulking in the sludge resulting in poor sedimentation and compaction of the activated sludge together with an increase in SVI *(19)*. Factors such as excessive filamentous microbial growth, low oxygen concentration, and high EPS concentration in the floc may also induce bulking episodes in filamentous sludges *(20–22)*. However, in nonfilamentous sludges the reasons for bulking are not well understood, but it is assumed that bulking might be closely related to changes in the concentration of EPS.

The SVI value, normally conceived as *sui generis*, for the sludge is considered as constant in full-scale reactors. However, SVI determinations during the operation of wastewater treatment reactors are not done frequently and systematically. Similarly, studies on steady-state pilot reactors are scanty, and no specific experimental work has been done leading to establishing the nature of EPS and its relationship to the SVI.

The purpose of the present study was to investigate the main components of the exopolymers such as the polysaccharides, protein, and lipids in the flocs of a nonfilamentous nitrifying sludge. In addition, we wished to establish the possible relationship of each component with the SVI in steady-state nitrification in a reactor fed with a lithoautotrophic influent free of dissolved organic matter.

Materials and Methods

Reactor and Culture Conditions

A modified Husmann type of reactor *(23)* with a 2-L working volume was employed. Figure 1 presents a diagram of the reactor. The volume of the settler was 1 L. The nitrifying reactor operated at a high recycling rate (11 L/d) to avoid clogging by biomass accumulation at the tubes of connection of the reactor. The reactor was aerated at a saturation level of 65 ± 5%. Temperature was controlled at 28°C. The stirring of the culture was constant at 300 rpm. To hold the microbial protein concentration in the reactor at 0.27 ± 0.02 g/L, purges were made manually by drawing 27 mL/d of sludge from the settler. The pH was kept at 7.6 by adding a secondary medium (SM) made of $NaHCO_3$ (40 g/L) and $CaCl_2 \cdot 2H_2O$ (0.4 g/L), which was fed at a flow rate of 67 mL/d. The composition of the ammonium basal medium (BM) was 1.17 g/L of $(NH)_2SO_4$, 1.4 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of NaCl, 0.96 g/L of NH_4Cl, and 0.15 g/L of $FeSO_4$. Nitrification was always carried Out under lithoautotrophic conditions. The continuous flow rates of the SM and BM were adjusted to give a C:N

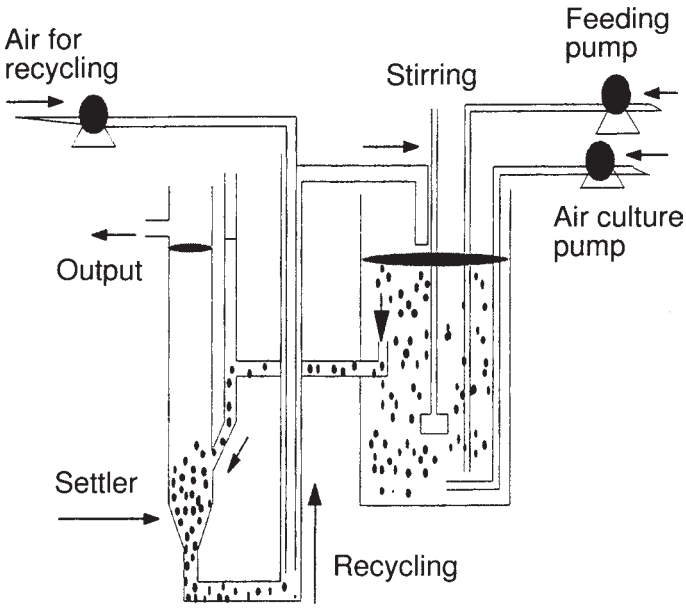

Fig. 1. Nitrifying aerated reactor. The reaction vessel was connected to the settler for biomass recycling.

ratio of 1.5 with a hydraulic retention time (HRT) of 3 d. The input concentration of nitrogen as ammonium salts (NH_4^+-N) was 0.5 g/L and the flow rate was 0.6 L/d.

Methods of Analysis

Determination of total protein in the nitrifying sludge was done by digesting 1 mL of sample with 0.1 mL of 10 *N* NaOH at 90°C for 30 min. Absorbance was measured at 750 nm using bovine serum albumin (Sigma, St. Louis, MO) with 16% nitrogen content as standard *(24)*. This ratio was used as a factor to convert protein to nitrogen. The determination of total carbohydrate of the sludge or exopolymer was done by a colorimetric method *(25)* by drawing 1 mL of sample culture. Floccular lipids of the EPS were analyzed using 1 mL of sludge according to the method described by Frings and Dunn *(26)*. Ammonium was measured by using an ammonia-specific electrode (Phoenix) by the following procedure: twenty milliliters of nitrifying sludge was mixed with 0.5 mL of 10 *N* NaOH, and then the mixture was made up to 50 mL in a volumetric flask with distilled water. NH_4Cl dissolution was used as the standard for measuring NH_4^+-N *(27)*. Nitrite and nitrate were measured using a capillary electrophoresis instrument (model 4000; Millipore) with a microcapillary silica-fused column *(28)*. An electrolyte of 10 m*M* Na_2SO_4 was used as reference. A mixture of nitrite and nitrate (100 mg/L each) was used as the standard. The total suspended solids (TSS), volatile suspended solids (VSS), and fixed suspended solids (FSS) expressed in grams/liters and SVI (milliliters of cul-

ture/gram of VSS) were measured following methods described by the American Public Health Association *(27)*.

Floc Dissociation and Analysis

The sludge flocs were dissociated by mixing 5 mL of sample from the nitrifying reactor with 5 mL of 0.1 *M* EDTA (final concentration of 50 m*M*), homogenizing them for 10 min, and centrifuging them for 10 min at 3000*g*. One milliliter of the supernatant was used to measure the total carbohydrate concentration of the exopolymer. Lipids in the flocs were also measured following a similar procedure *(26)* by using 5 mL of the supernatant dialyzed for 3 d in distilled water in 10-kDa cutoff bags.

Results and Discussion

Figure 2 shows the performance of the nitrifying reactor at 3 d of HRT. The microbial production rate (Q_{out}) for prot-N was kept constant at 0.013 g/(L·d). The loading rate (Q_{in}) for NH_4^+-N and the production rate (Q_{out}) for NO_3^--N were constant at 0.15 and 0.135 g/(L·d), respectively. The output loading rate (Q_{out}) for the NH_4^+-N was constant and low in contrast to the high-rate production (Q_{out}) for NO_3^--N. Therefore, it can be established that the reactor was in steady-state nitrification, at constant respiration rate. Under this regime, the ammonium consumption (close to 100%) and nitrification efficiency (93%) of the sludge were high, and precisely at these culture conditions the analysis of the chemical components of the EPS and SVI of the nitrifying sludge were evaluated.

The flocs of the sludge are dissociated owing to the chelating properties of EDTA for divalent cations, which interfere with protein determination by Lowry's method. Strong acids such as HCl produce efficient floc dissociation, but they also promote cell disruption, resulting in the release of intracellular proteins, which hampers the determination of the true proteins of the EPS, thereby, masking the real values. To eliminate the interference of EDTA in the measurement of protein, the samples were dialyzed. Likewise, to optimize the dissociation of the floc without cell disruption, different EDTA concentrations were tested for the extraction of exopolymeric protein. With an EDTA concentration of 1 m*M*, the protein extracted was 2 mg/L, but with 10, 50, and 100 m*M* EDTA, the protein extracted from the floc increased and remained constant (21 ± 0.5 mg/L). The total protein in the reactor was controlled at 0.27 g/L and, hence, the protein concentration extracted from the sludge remained constant. Therefore, there was no evidence of cell disruption by the addition of EDTA, but it effectively extracted protein from the floc.

Following a similar procedure, a test was done to dissociate the sludge using different HCl concentrations (25, 50, and 500 m*M*, and 1 *M*). These acid treatments produced a high level of sludge dissociation and the concentration of protein extracted was in the range of 30–72 mg/L. There was an increase in the disruption of microorganisms of the sludge as the concen-

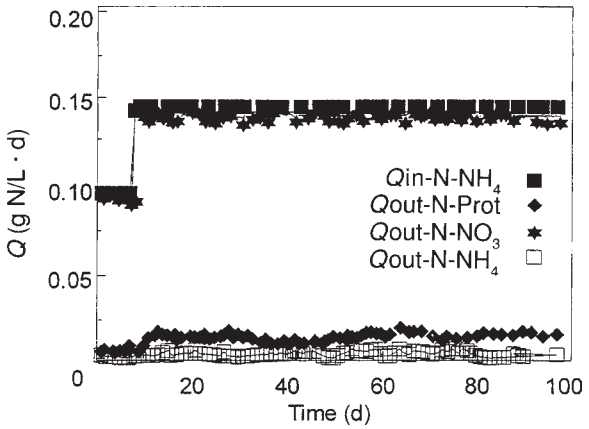

Fig. 2. Volumetric rates of input (Q_{in}) and output (Q_{out}) in the nitrifying reactor in steady state. The N-NH_4 concentration in the input was 0.5 g/L. Culture conditions were pH 7.8 ± 0.3, 28°C, and 3 d of HRT.

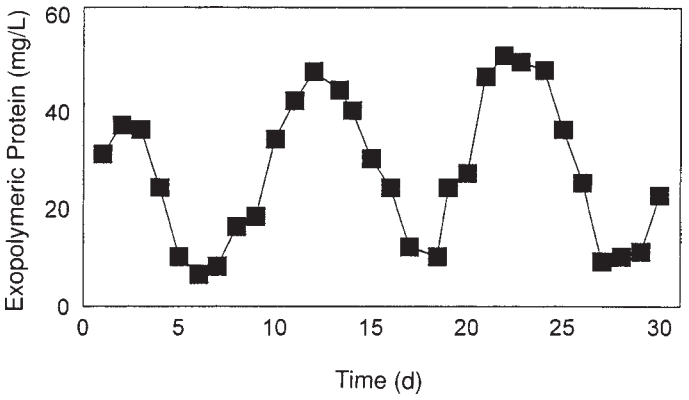

Fig. 3. Exopolymeric protein profiles of the nitrifying floc in steady-state culture. EDTA (0.05 *M*) was used to dissociate the nitrifying sludge floc.

tration of HCl was increased. Therefore, a concentrations of 50 m*M* EDTA was considered for floc dissociation and was employed throughout in the study. After floc dissociation, the samples were dialyzed for 5 d. However, it was seen that after 3 d of dialysis no interference by EDTA was detected in the measurement of protein.

Thereafter, the exopolymeric protein of the EPS was analyzed following dissociation of sludge flocs with EDTA and its subsequent dialysis. The exopolymer protein content in the sludge floc ranged from 5 to 45 mg/L, a variation about 90% following a consistent oscillatory pattern with a period of 10 d, as can be seen in Fig. 3. These significant changes in the concentration of exopolymeric protein were observed in spite of the fact that the reactor was operating in steady-state nitrification at an efficiency of 93%. During the oscillatory period, grams of EPS-protein/grams of total

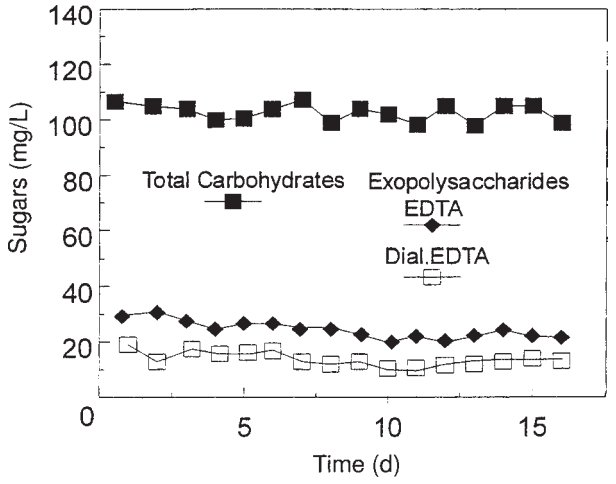

Fig. 4. (■) Total sugar from biomass and floc, (◆) exopolymeric carbohydrates after dissociating with 0.05 *M* EDTA, and (□) carbohydrates after 3 d of dialysis.

protein ratio ranged from 1.85×10^{-2} to 1.67×10^{-1}. Some investigators *(6,17)* report that the exopolymeric protein was the main fraction in the sludge from municipal wastewaters. However, they do not mention any variations or oscillations in the concentration of protein in the sludge flocs. We are reporting, for the first time, a consistent oscillatory pattern in the behavior of the exopolymeric protein oscillation in a nitrifying sludge.

Results from the published data indicate that the EPS concentration is dependent on the type of microorganisms or microbial concentration. The culture conditions in our study were established so that the total sludge protein concentration (or microbial biomass) in the nitrifying reactor would be constant, presuming, therefore, that the EPS content would also be constant. But, on the contrary, we observed that the protein showed a consistent oscillating behavior in steady-state nitrification. At present, we have no hypothesis to explain this behavior, and more work is needed to elucidate the significance of the oscillatory patterns observed in the exopolymeric proteins.

Figure 4 shows total carbohydrates and the exopolymeric carbohydrates extracted from the sludge flocs after dissociation with EDTA. Both fractions showed low variations in concentration: 110 ± 10 mg/L for total carbohydrates and 30 ± 4 mg/L for exopolymeric carbohydrates. Thus, about 80% of the carbohydrates in the sludge corresponded to microbial biomass. Note that the total carbohydrates were measured in a nitrifying sludge sample whereas the exopolymers were estimated after dissociation with EDTA without dialysis. The exopolymer carbohydrates were determined after 3 d of dialysis (open squares in Fig. 4). The carbohydrate concentration in the dialysate showed a 40% reduction, suggesting that the exopolymeric carbohydrates contained a high proportion of low molecular weight carbohydrates (<10 kDa).

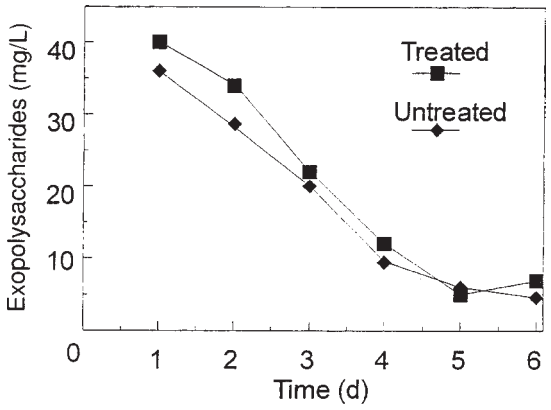

Fig. 5. Floccular carbohydrates after dissociation in 0.05 *M* EDTA. (■) Series thermally treated at 121°C for 15 min; (◆) control with no thermal treatment. Both series were dialyzed for 6 d.

The decrease in carbohydrates noted was thought to be owing to enzymatic hydrolysis. To reject this possibility, samples of the nitrifying sludge were dissociated with EDTA and dialyzed. One set of samples was subjected to a temperature of 121°C for 15 min in order to inactivate the enzymes. The other set of a similar number of samples without any thermal treatment was used as control. Exopolymeric carbohydrates were measured in both sets of samples. The results revealed no difference between control and heat-treated samples (Fig. 5), corroborating that the decline in exopolymeric carbohydrate concentration after dialysis was not owing to enzymatic activity, but was owing to the presence of low molecular weight carbohydrates. After 5 d of dialysis, the exopolymeric carbohydrate fraction with a molecular size >10 kDa represented only 20% of the total exopolymer carbohydrates. These results are in contrast with previous reports, which suggested that the carbohydrates present in the EPS were composed of abundant quantities of high molecular weight carbohydrates forming the backbone of the flocular structure *(11,12,19)*. After dissociation of the sludge flocs during the same period of time, the lipids showed only minor variations (36 ± 4 mg/L). Therefore, in the steady-state nitrification the variations in content of lipids and exopolymeric carbohydrates in the EPS of the floc sludge were not significant.

To eliminate the possibility that oscillations in the concentration of the exopolymer protein were owing to the activity of proteolytic enzymes, a sample was dialyzed for 6 d at room temperature and tested for exopolymeric proteins. It was observed that the protein remained constant at 45 ± 2 mg/L, indicating that oscillations in the concentration of protein in the floc were independent of any enzyme activity and were not affected by dialysis.

The analysis of the nitrifying sludge under steady-state conditions showed that TSS, VSS, and FSS were 9.24 ± 0.6, 6.24 ± 0.4, and 3.2 ± 0.2 g/L,

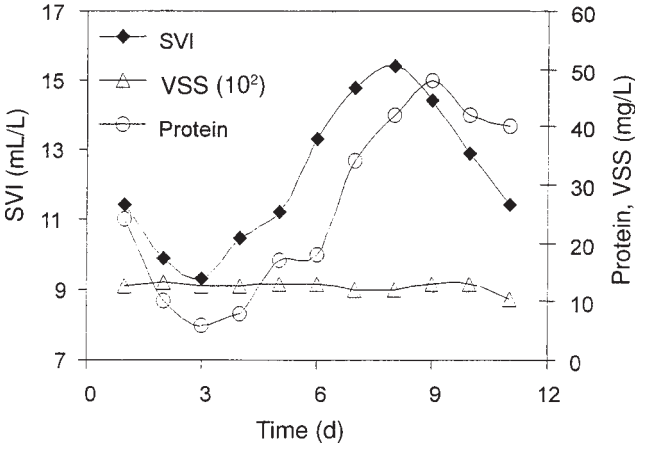

Fig. 6. Evolution of the SVI, VSS, and exopolymeric protein of the sludge during a period of oscillation of the exopolymeric protein in steady-state nitrification. The real value of VSS is obtained by multiplying by 10^2.

respectively. Although the variation in exopolymeric protein was significantly high, the variation in VSS in the sludge was not significant in steady-state culture. The exopolymer protein concentration represented only a very small fraction of the VSS. Therefore, the variations in the EPS:VSS ratio must mainly be a function of the exopolymeric protein fraction.

During the period of exopolymeric protein oscillation the SVI value was 12.2 ± 2.1, which represented an important variation considering that the nitrification was constant. These changes in the SVI of the nitrifying sludge suggest that the variations could be related to the variations in the concentration of the EPS, specifically to the exopolymeric protein. To support this point of view, a study of the relationship of the SVI with the VSS and the exopolymeric protein during the period of oscillation was made (Fig. 6). The SVI of the sludge changed only when the protein showed variations in its concentration. However, the results shown in Fig. 7 indicated that no relationship existed between the variations in SVI and the concentration of the exopolysaccharides and lipids of the EPS of the sludge. To corroborate these observations, the dependence among the different variables in the sludge statistical analysis of linear correlations was determined. The analysis included the VSS, EPS (protein, polysaccharides, and lipids), and SVI (Table 1). The SVI had the higher positive correlation with the exopolymeric protein changes (r = 0.783).

It has been mentioned that SVI variations in municipal sludges can be associated with changes in the exopolymers concentration, but no mention has been made about the role of the exopolymeric protein in the sludge settleability. There are reports *(5,29)* indicating that the changes in the settleability of the SVI are dependent on the lipid concentration in the EPS of the sludge, but in the present study the lipids of the EPS did not correlated with the variations in the SVI.

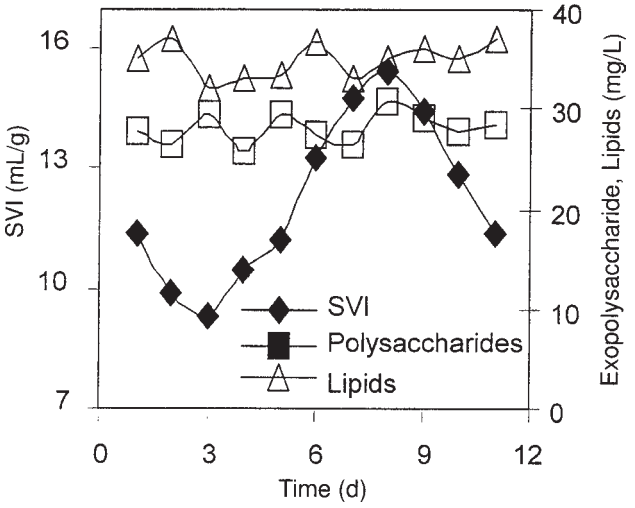

Fig. 7. Profiles of the exopolymeric polysaccharides and lipids of the dissociated sludge floc and SVI in a nitrifying reactor in a steady-state culture.

Table 1
Linear Coefficients of Correlations (*r*) of Statistical Analysis of Nitrifying Sludge[a]

	Polysaccharides	Lipids	Protein	VSS	SVI
Polysaccharides	1.000	—	—	—	—
Lipids	–0.039	1.000	—	—	—
Protein	0.349	0.381	1.000	—	—
VSS	–0.157	–0.376	–0.439[b]	1.000	—
SVI	0.255	0.177	0.783[c]	–0.176[b]	1.000

[a]The coefficients *r* (Pearson coefficient) were estimated by Excel 97 for Windows 95.
[b]$\propto$ = 1%.
[c]$\propto$ = 5%.

It is well known that an increase in the SVI value would alter the degree of compaction of the sludge. The results presented herein show that the changes in the compaction of the nitrifying sludge were owing to the changes in the exopolymeric protein concentration in the floc. It is possible that the increase in the SVI value by the increase in exopolymeric protein was owing to the biosurfactant properties of some proteins produced by anaerobic microorganisms *(30)*. Thus, an increase in the concentration of exopolymeric protein could provoke a decrease in the surface tension in the nitrifying sludge resulting in an increase in the SVI of the sludge.

Conclusion

The efficient dissociation of the nitrifying sludge and the extraction of the EPS from the sludge was achieved by using EDTA and subsequent dialysis. This procedure eliminated interferences during the exopolymeric

and total protein assays of the nitrifying sludge. In steady-state, nitrifying sludge floc showed a significant variation in exopolymer protein and also showed a consistent oscillating profile with a period of 10 d. Analysis of the exopolymeric polysaccharides showed that 80% were of molecular weight <10 kDa. Under the same nitrifying conditions, the exopolymer polysaccharide, lipids of the floc, as well as VSS concentration of the sludge did not show any significant variation. It was also observed that the SVI of the nitrifying sludge during the oscillating period showed variations associated with changes in the concentration of the exopolymeric protein. The statistical analysis of correlation corroborated that the SVI variations were dependent on the exopolymeric protein changes.

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References

1. Prosser, J. I. (1989), *Adv. Microb. Physiol.* **30,** 125–181.
2. Böck, E., Koops, H. P., Harms, H., and Ahlers, B. (1991), in *Variations in Autotrophic Life*, Böck, E., ed., Academic, London, pp. 171–200.
3. Watson, S. W., Böck, E., Harms, H., Koops, H. P., and Hooper, A. B. (1989), in *Bergy's Manual of Systematic Bacteriology*, vol. 3, Stanly, J. T., Brynt, M., Pfennig, N., and Holt, J. G., eds., Williams & Wilkins, Baltimore, pp. 1807–1834.
4. Michael, G., Shimizu, G. P., and Jenkins, D. (1985), *J. Water Pollut. Control Fed.* **57,** 1152–1162.
5. Urbain, V., Block, J. C., and Manem, J. (1993), *Water Res.* **27,** 829–838.
6. Jahn, A. and Nielsen, P. H. (1998), *Water Sci. Technol.* **37,** 17–24.
7. Chaw, A. C. and Keinath, T. M. (1979), *Water Res.* **13,** 1213–1223.
8. Magnusson, K. E. (1980), *Scand. J. Infect. Dis.* **24,** 131–134.
9. Goodwin, J. A. S. and Forster, C. F. (1985), *Water Res.* **19,** 527–533.
10. Forster, C. F. (1985), *Water Res.* **19,** 1259–1264.
11. Sato, T. and Ose, Y. (1980), *Water Res.* **14,** 333–338.
12. Horan, N. J. and Eccles, C. R. (1986), *Water Res.* **20,** 1427–1432.
13. Eriksson, L. and Alm, B. (1991), *Water Sci. Technol.* **24,** 21–28.
14. Jahn, A. and Nielsen, P. H. (1995), *Water Res.* **32(8),** 157–164.
15. Sutherland, I. W. (1977), *Am. Chem. Soc. Symp. Ser.* **45,** 40–56.
16. Forster, C. F. and Dallas-Newton, J. (1980), *J. Water Pollut. Control Fed.* **79,** 338–351.
17. Jorand, F., Zartarian, F., Thomas, F., Block, J. C., Bottero, J. Y., Villemin, G., Urbain, V., and Manem, J. (1995), *Water Res.* **29,** 1639–1647.
18. Nielsen, P. H., Jahn, A., and Palmgren, R. (1997), *Water Sci. Technol.* **36,** 11–19.
19. Novak, L., Larrea, L., Wanner, J., and Garcia-Heras, J. L. (1993), *Water Res.* **27,** 1339–1346.
20. van Leeuwen, J. (1988), *Water S.A.* **15,** 127–132.
21. Jenkins, D. (1992), *Water Sci. Technol.* **26,** 215–230.
22. Foot, R. J., Robinson, M. S., and Forster, C. F. (1994), *Water Sci. Technol.* **29,** 213–220.
23. Rudling, L. and Solyom, P. (1974), *Water Res.* **8,** 115–119.
24. Lowry, O. H., Rosenbrouth, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
25. Dubois, M., Gilles, K. A., Hamilton, J. K., Roberts, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–352.

26. Frings, C. S. and Dunn, R. T. (1970), *J. Clin. Pathol.* **53,** 89–91.
27. APHA. (1985), *Standard Methods for Examination of Water and Wastewater*, 16th ed. American Public Health Association, Washington, DC.
28. Gomez, J., Mendez, R., and Lema, J. M. (1966), *J. Appl. Biochem. Biotechnol.* **57/58,** 869–876.
29. Khan, A. R. and Forster, C. F. (1990), *Enzyme Microb. Technol.* **12,** 788–793.
30. Nue, T. R. (1996), *Microbiol. Rev.* **60,** 151–166.