Role of exopolymeric protein on the settleability of nitrifying sludges

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Abstract

The relationship of exopolymeric substances and the sludge volume indexes (SVI) of two nitrifying sludge were studied over a period of time (30 d) in two different types of reactors: (1) a stirred tank aerated nitrifying reactor (SANR) and (2) an air-lift nitrifying reactor (ALNR). Concentrations of lipids, carbohydrates and proteins of the EPS were determined in both reactors. The variation in lipids and carbohydrates was low (<10%), while the variation in the exopolymeric protein was higher. The SVI increased in accordance with the increase in the concentration of the exopolymeric protein. The number of bands of the exopolymeric protein was modified as a consequence of the change in the concentration of the exopolymeric protein. The molecular weights of the exopolymeric protein ranged from 31 to 97 in the stirred and 13 to 45 kDa in the air-lift reactor. The variations in the SVI were mainly due to the changes in the molecular weight of the exopolymeric proteins resulting in the modification of the sludge settleability characteristics.

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1. Introduction

Biological treatment is commonly used to remove pollutants from municipal and industrial wastewaters (Monroy et al., 2000). The microorganisms involved in this process aggregate to form flocs and/or granular sludge. The sludges are usually made up of two main fractions: (1) a heterogeneous microflora forming a consortium and (2) a complex mixture of exopolymeric substances (EPS) consisting mainly of carbohydrates, lipids and protein. The arrangement of the EPS in the complex structure of the sludge and its effect on sludge settleability are not well understood. It has been proposed that in activated sludge the concentration of exocellular polysaccharides is related to sludge settleability (Goodwin and Forster, 1985). Urbain et al. (1993) indicated that the EPS components of the flocs were linked to divalent cations like Ca2+ suggesting the possible participation of proteins.

The efficiency of wastewater treatment depends on the stability of the structure of the sludge. Changes in the stability or aggregation of the sludge can modify its settling characteristics resulting in microbial washout, thereby, reducing the efficiency of the reactor. There are diverse points of view about the causes leading to changes in the stability of sludge. It has been reported in the literature that stability of the activated sludge depends on various factors such as types of microorganisms, excessive microbial growth and changes in microbial activity due to chemical changes in the influent (Michael et al., 1985). Martínez et al. (1998) observed that settling of the sludge was variable in a steady state nitrifying reactor although the rate of feeding and consumption of ammonia remained constant. It has been suggested that the stability of the floccular sludge depends on the type of EPS and its concentration and that variation in EPS components can alter sludge settleability, mainly when the protein content changes (Urbain et al., 1993). These results reveal that variations in the concentration of the EPS alter the SVI which is usually considered as an indicator of stability or settleability of the sludge. Values of SVI less than 100 ml g⁻¹ normally indicate good stability of the sludge.
Lazarova and Manem (1995) reported that some components of EPS play an important role in substrate consumption and also in the formation and structuring of granules, flocs and biofilms. Similar points of view have also been expressed by others (Echeverría et al., 1992; Novák et al., 1993; Jahn and Nilsen, 1998; Bura et al., 1998; Jorand et al., 1998). Random sampling and one time chemical analyses of municipal sludge have shown that an increase in the concentrations of exopolymeric carbohydrates decreases the SVI value, but when the lipids increase in the EPS the SVI increases diminishing the sludge stability (Goodwin and Forster, 1985; Urbain et al., 1993).

The specific role of exopolymeric proteins with regard to stability and settleability of sludge has not been studied in detail. Jorand et al. (1998) working with activated sludge observed that hydrophobic and hydrophilic properties of different non-stabilized sludge were related to the presence of polysaccharides and proteins, which modified the SVI. Martínez et al. (1998) reported that sludge in steady state nitrification presented an oscillatory pattern in the exopolymeric protein concentration that correlated with changes in the SVI. However, they did not analyze the composition of proteins. Huber et al. (1998) observed that in a sequencing batch reactor, the type of protein produced in the sludge depended on the chemical composition of the influent. The authors concluded that the diversity of the proteins decreased when the culture medium was chemically more complex. Their results did not make any reference to the stability of the sludge or changes in the SVI. Periodical sampling to determine the chemical and physiological behavior of the sludges over a longer period has not been reported.

Therefore, the purpose of this work was to evaluate sludge from two different steady state nitrification reactors during prolonged operation time to study: (a) fluctuations in the concentration of the exopolymeric proteins; (b) their molecular weights and (c) relate the changes in the composition of the EPS to changes in the SVI.

2. Methods

2.1. Reactor and culture conditions

Experiments were conducted using two different types of continuous reactors, (1) a stirred aerated nitrifying reactor (SANR) and (2) an air-lift nitrifying reactor (ALNR). The composition of the lithotrophic influent reactor (SANR) and (2) an air-lift nitrifying reactor of continuous reactors, (1) a stirred aerated nitrifying reactor were as follows (g l⁻¹): (NH₄)₂SO₄, 1.17; KH₂PO₄, 1.4; MgSO₄, 0.5; NaCl, 1; NH₄Cl, 0.96; FeSO₄, 0.15. The total NH₄⁺-N concentration in the input was of 0.5 g l⁻¹.

The working volume of the SANR was 2 l connected to a settler of 1 l. The operating conditions were: recycling rate 11 l·d⁻¹, stirring rate 300 rpm and temperature 28 °C. The pH was maintained at 7.6 ± 0.5 by adding a mixture of NaHCO₃ with CaCl₂·2H₂O (40 and 0.4 g l⁻¹, respectively). The flow rate of this mixture was maintained at 0.065 l·d⁻¹. In order to achieve a loading rate of 0.15 ± 0.01 g NH₄⁺-N l⁻¹ d⁻¹ the influent flow rate was of 0.6 l·d⁻¹. The hydraulic retention time (HRT) of the process was 3.3 d. The ALNR (2.3 l of working volume) operated under culture conditions similar to the SANR, but at a HRT of 0.115 d and a loading rate of 4 g NH₄⁺-N 1⁻¹ d⁻¹. Both reactors were fed in such a way as to keep the C:N ratio close to 1.25 as required in a dissimilative respiration process. The reactors were inoculated with a non-filamentous flocculent sludge obtained from a stirred steady state nitrifying reactor that had been operating continuously for three years. ALNR and SANR were operated continuously for 150 d.

2.2. Analysis

Soluble and microbial protein concentrations in the reactor were measured daily by a modified method described by Lowry et al. (1951) using three ml of sample volume and bovine serum albumin as the standard. The modification consisted in the addition of 0.1 ml of 10 M NaOH to 1 ml of sample and heating at 92 °C for 40 min in a water bath. Ammonium ion concentration was measured daily (50 ml of sample volume) using a specific ion electrode according to standard methods (APHA, 1985). Nitrite and nitrate were measured daily by capillary electrophoresis (Millipore Model 400) under the following conditions: a silica fused microcapillary column (60 cm long and 70 µm internal diameter) with 30 mA and 20 kV. A 10 mM Na₂SO₄ solution was used as the electrolyte. Each sample (0.5 ml of sample volume) application time was 4 s. Anion absorbance was measured with a 212 nm filter as previously described (Gómez et al., 1996) and a mixture of nitrite and nitrate (100 mg l⁻¹ each) was used as standard. For all methods, samples were analyzed in triplicate and the coefficient of variation was below 6%.

2.3. Extraction of exopolymeric substances from the sludge

Five milliliters sample drawn from the reactor was mixed with 5 ml of 0.1 M ethylene diamine tetracetic acid (EDTA), stirred for 10 min and then centrifuged at 3000g for 10 min. The supernatant was filtered through a 0.45 µm nylon membrane and the filtrate was divided into two parts. Six milliliters were used for measuring of the exopolymeric carbohydrates and lipids while 4 ml were dialyzed for 3 d at a constant temperature of 4 °C in distilled water in 10 KD cut off-bags (Spectrum) made
from regenerated cellulose (Martínez et al., 2000). Exopolymeric proteins were measured after dialysis according Lowry et al. (1951). The polysaccharides of the EPS were measured by quantitative determination of total sugars (Dubois et al., 1956) and lipids by the method reported by Frings and Dunn (1970). The SVI is defined as the volume occupied by 1 g of sludge after a period of 30 min in a graduated cylinder. The procedure we adapted here to determine the SVI was similar to that reported by Urbain et al. (1993) in which a sample of 100 ml was used in an Imhoff cone instead of a graduated cylinder. Tukey’s test was conducted to evaluate the difference in SVI values between the graduated cylinder and the Imhoff cone. The results showed (α = 0.05) that there was no significant difference (<10%) in the SVI values obtained from both. The total suspended solids content (TSS) of sludge was determined by standard methods. The volume of the settled sludge was related to the TSS concentration (Lee et al., 1983). In the case of the SANR, the exopolymeric substances analysis, the SVI and the TSS were carried out two times in a week, while in the ALNR these analyses were conducted three times in a week. For all methods, samples were analyzed in triplicate and the coefficient of variation was below 6%.

2.4. Polyacrylamide gel electrophoresis

The number of bands of exopolymeric protein was assessed by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) by denaturing the proteins (Laemmli, 1970) with a resolving polyacrylamide gel electrophoresis (SDS-PAGE) by denaturing the assessed by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis was carried out at 150 V for 60 min. Silver nitrate staining was done as follows (V/V): the gel was fixed with 40% methanol plus 0.5 ml of 37% formaldehyde for 10 min and then poured into a 0.2 g l⁻¹ Na₂S₂O₃ solution for 1 min and then in 0.1% AgNO₃ for 10 min. The gel was developed with a solution of 3% Na₂CO₃, 0.0004% Na₂S₂O₃ and 0.5 ml l⁻¹ of 37% formaldehyde (W/V). The reaction was stopped by mixing the reactants with 5 ml of 2.3 M citric acid.

Molecular weights of the exopolymeric proteins from SANR were determined two times in a week using a kit (Sigma) containing a total concentration of 2 mg ml⁻¹ of macroglobulin, galactosidase, fructose 6 phosphate quinase, pyruvate quinase, fumarase, lactate dehydrogenase and 1-triose phosphate isomerase. Molecular weights of the reference proteins ranged from 43 to 91 kDa. Molecular weights of exopolymeric proteins extracted from the ALNR were determined three times in a week using a kit (Dalton Mark VI, Sigma) containing a total protein concentration of 2 mg ml⁻¹ of bovine albumin, egg albumin, lactoglobulin, lysozyme, trypsinogen and pepsin. The molecular weights of these proteins ranged from 14 to 66 kDa. The range covered by the two kits allowed a broader determination of the protein components. In both reactors the electrophoretic protein analysis was conducted as duplicates.

3. Results and discussion

The production rate of nitrate (g NO₃⁻N l⁻¹ d⁻¹) in the SANR was 0.14 ± 0.01 and 3.75 ± 0.24 in the ALNR. The output of NH₄⁺-N was low in both reactors indicating that the consumption efficiency of ammonia was high (95% ± 3). The low variation in the nitrate production rate coupled with high efficiency of NH₄⁺-N consumption noted in both reactors were indicators of steady state nitrification. Thus, the respiratory processes in both reactors were similar. During steady state nitrification, the mean concentrations of total suspended solids were of 7773 ± 610 and 7637 ± 650 mg l⁻¹ for the SANR and ALNR, respectively, of which around 40% were non-volatile suspended solids. Under these conditions, nitrifying sludge samples drawn from the steady state reactors were dissociated with EDTA and electrophoretic analyses of the extracellular proteins were performed. The interference of EDTA in the measurement of protein was successfully eliminated by dialysis without affecting the cell structure or producing cell lysis, as reported earlier (Eriksson and Alm, 1991; Martínez et al., 2000).

The concentrations of exopolymeric proteins, carbohydrates and lipids in the SANR and the ALNR are shown in Table 1. Carbohydrates and lipids showed a low variation, close to 10% during the entire experimental period. However, exopolymeric proteins showed significant changes (Fig. 1) while nitrification rate remained nearly constant in steady state. Dignac et al. (1998) had earlier reported that in activate sludge the main exopolymers were proteins, but considering that the protein content was not analyzed over time, they failed to observe any variation in its concentration. The profiles for exopolymeric proteins and SVI in the SANR are shown in Fig. 1A. The fluctuations in SVI and protein followed very similar patterns during the entire experimental period. Namely, when the protein increased the SVI also increased and vice versa. Thus, the SVI fluctuations were closely linked to variations in the exopolymeric protein concentration of the nitrifying sludge. These results indicate that changes in settleabil-

<table>
<thead>
<tr>
<th>Reactor</th>
<th>SVI (ml g⁻¹)</th>
<th>Carbohydrates (mg l⁻¹)</th>
<th>Proteins (mg l⁻¹)</th>
<th>Lipids (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SANR</td>
<td>6 ± 0.7</td>
<td>33 ± 3</td>
<td>51 ± 9</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>ALNR</td>
<td>22 ± 8</td>
<td>18 ± 1</td>
<td>56 ± 25</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

Values are averages over all samples taken.
ity were due to the changes in the concentration of protein. Similar results were also found by Urbain et al. (1993).

The exopolymeric proteins were resolved by SDS-PAGE. For the SANR, the analysis of the maximum and minimum proteins concentration points (arrows in Fig. 1A) are shown in Table 2. As can be seen, molecular weights assessed for the exopolymeric proteins ranged from 31 to 97 kDa. At the maximum concentration (days 7 and 10) there were 18 different proteins while at the minimum (days 16 and 18) only 9. Therefore, the number of bands of exopolymeric proteins varied with protein concentration. In spite of the protein concentration at maximum days were approximately the same, it was observed that some protein bands had disappeared. This behavior might be due to the turnover of the exopolymeric substances, as it has been observed that while carbohydrates are relatively stable exopolymeric component, proteins undergo a more rapid turnover (Flemming and Wingender, 2001; Wingender et al., 2001). However, further investigation focused in protein turnover is required. The results of this work show that the EP is constituted by a higher number of protein bands than have been previously reported in other works (Higgins and Novak, 1997). Flemming and Wingender (2001) observed in activated sludge that the molecular weight ranged from 5 to 150 kDa, but no mention was made on the number of protein bands.

In our work the variations in the concentrations of the exopolymeric proteins in the SANR were close to 20%, despite the fact that the feed was a chemically simple lithoautrophic solution and the nitrification process was at steady state. These results are in contrast to those of Huber et al. (1998) who reported that feeding

Table 2
Molecular weights of exopolymeric proteins assessed in the stirred aerated nitrifying reactor and air-lift nitrifying reactor during days of maximum and minimum concentrations

<table>
<thead>
<tr>
<th>Protein</th>
<th>Day</th>
<th>Maximum concentration (kDa)</th>
<th>Minimum concentration (kDa)</th>
<th>Day</th>
<th>Minimum concentration (kDa)</th>
<th>Maximum concentration (kDa)</th>
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<tr>
<td></td>
<td>7</td>
<td>97,450</td>
<td>97,450</td>
<td>17</td>
<td>44,462</td>
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<tr>
<td></td>
<td>10</td>
<td>93,480</td>
<td>93,480</td>
<td>19</td>
<td>42,032</td>
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<tr>
<td></td>
<td>16</td>
<td>89,673</td>
<td>42,032</td>
<td>21</td>
<td>37,563</td>
<td>42,032</td>
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<tr>
<td></td>
<td>18</td>
<td>86,020</td>
<td>42,032</td>
<td>23</td>
<td>37,563</td>
<td>37,563</td>
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<td>Stirred aerated nitrifying reactor (SANR)</td>
<td>V</td>
<td>82,680</td>
<td>35,510</td>
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<td>31,734</td>
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<tr>
<td></td>
<td>VI</td>
<td>76,082</td>
<td>33,569</td>
<td></td>
<td>30,000</td>
<td>30,000</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>72,983</td>
<td>30,000</td>
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<td>28,360</td>
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<tr>
<td></td>
<td>VIII</td>
<td>70,010</td>
<td>25,344</td>
<td></td>
<td>23,959</td>
<td>23,959</td>
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<tr>
<td></td>
<td>IX</td>
<td>67,158</td>
<td>22,650</td>
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<tr>
<td></td>
<td>X</td>
<td>61,921</td>
<td>21,412</td>
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<tr>
<td></td>
<td>XI</td>
<td>59,399</td>
<td>19,135</td>
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<tr>
<td></td>
<td>XII</td>
<td>52,432</td>
<td>17,100</td>
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<td></td>
<td>XIII</td>
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<tr>
<td></td>
<td>XIV</td>
<td>44,486</td>
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<td></td>
<td>XV</td>
<td>42,674</td>
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<td>XVI</td>
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<tr>
<td></td>
<td>XVII</td>
<td>34,731</td>
<td>13,657</td>
<td></td>
<td>13,657</td>
<td>13,657</td>
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<tr>
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<td>XVIII</td>
<td>31,960</td>
<td>13,657</td>
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<td>Air-lift nitrifying reactor (ALRN)</td>
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Fig. 1. Behavior of exopolymeric protein and SVI in nitrifying reactors. (-•-) SVI, (− - ) exopolymeric protein. Arrows show the maximum and minimum of exopolymeric protein concentration. (A) Stirred aerated nitrifying reactor, maximum concentration (7 and 10 d, respectively), minimum concentration (16 and 18 d, respectively). (B) Air-lift nitrifying reactor, minimum concentration (17 and 19 d, respectively), maximum concentration (21 and 23 d, respectively).
with a complex organic medium significantly influenced the number of protein bands of the sludge in a sequencing batch reactor. Although the changes in exopolymeric protein concentrations were due to changes in microbial activity, the differences in our results compared to those reported by Huber et al. (1998) were probably influenced by the type of reactor used. The operating mode of each type of reactor can promote growth of different microorganisms which may alter not only the respiratory behavior of the microorganisms, but also the kind of proteins produced by the sludge (Lazarova and Manem, 1995).

Periodic chemical determinations of EPS in the biofilm from the ALNR are shown in Fig. 1B. The exopolymeric protein concentration in the sludge from the ALNR was higher by approximately 10% compared to the SANR (Fig. 1A). This difference in exopolymeric content was quite significant for sludge stability. Similarly, fluctuations observed in the concentration of exopolymeric proteins over the experimental time were also higher. These fluctuations in the concentrations of exopolymeric proteins and the SVI followed a pattern similar to that of the SANR. Neither the exopolymeric protein concentration nor the SVI were constant in spite of the nitrifying process being at steady state. When the exopolymeric protein changed, SVI changed in a similar manner. Thus, the behavior of EPS and the SVI was similar both for the ALNR and the SANR. However, the analysis of variance for the SVI values for both reactors showed ($z = 0.0005$) that there was a significant difference.

Results from electrophoresis of the exopolymeric protein from the ALNR are shown in Table 2. The number of different exopolymeric protein bands was 15 at the maximum concentration (days 21 and 23), three less than for the SANR. In the case of SANR, the higher number of protein bands were also noted at the maximum concentration of exopolymers. The difference in number of protein bands between the maximum concentration points can be explained in the same terms as those in the SANR reactor. The molecular weights of the exopolymeric proteins ranged from 13 to 44 kDa differing by a factor of 2. The SVI for the ALNR was higher by approximately 10% compared to the SANR. Results from electrophoresis showed major differences in the exopolymeric proteins from both sludge although the feed had the same chemical composition. Molecular weights of the exopolymeric proteins from the SANR ranged from 31 to 97 while those from the ALNR ranged from 13 to 44 kDa differing by a factor of 2. The SVI for the ALNR ($22 \pm 8 \text{ ml g}^{-1}$) was about 4 times greater than for SANR ($6 \pm 0.7$). These results indicated that variations in the SVI in both reactors were related to exopolymeric protein concentrations and to the differences in their molecular weights. Exopolymeric proteins appear to affect the settleability and the stability of nitrifying sludge. It is, however, necessary to establish conditions to control exopolymeric protein production in the nitrifying sludge to improve the efficiency of reactor operation.

4. Conclusions

Results obtained from this work with two different types of reactors (SANR and ALNR) at steady state for nitrification of lithoautotrophic culture medium indicated a close relationship between exopolymeric proteins and settleability of the sludge. During the experiment the concentrations of lipids and carbohydrates of the EPS showed little variation, while the exopolymeric protein concentration varied from 20% to 45% for SANR and ALNR, respectively. Exopolymeric protein concentrations were lower in the SANR than in the ALNR by about 10%. Results from electrophoresis showed major differences in the exopolymeric proteins from both sludge although the feed had the same chemical composition. Molecular weights of the exopolymeric proteins from the SANR ranged from 31 to 97 while those from the ALNR ranged from 13 to 44 kDa differing by a factor of 2. The SVI for the ALNR ($22 \pm 8 \text{ ml g}^{-1}$) was about 4 times greater than for SANR ($6 \pm 0.7$). These results indicated that variations in the SVI in both reactors were related to exopolymeric protein concentrations and to the differences in their molecular weights. Exopolymeric proteins appear to affect the settleability and the stability of nitrifying sludge. It is, however, necessary to establish conditions to control exopolymeric protein production in the nitrifying sludge to improve the efficiency of reactor operation.

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