Effects of colloidal organic matter on nitrification and composition of extracellular polymeric substances in biofilms

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Abstract: The effects of colloidal organic matter on nitrification and the composition of extracellular polymeric substances (EPS) in biofilms were studied in two parallel bench-scale biological aerated filters (BAFs) fed with different organic carbon sources. The study filter used starch and glucose to model colloidal and soluble organic carbon, respectively, while the control filter used glucose as the sole organic carbon source. Studies with different COD/NH₄⁺ (C/N) ratios (0.5–6.0) showed that the increase in organic matter in the influent resulted in the displacement of nitrification from the bottom to the upper part of filter bed. It was observed that the greater reduction in nitrification was caused by colloidal organic matter rather than by soluble organics at the same COD concentration. The starch hydrolysis took place in the bottom 40 cm of the filter bed, which implied that hydrolysis of colloidal organic matter into smaller molecules could not be the limiting step of its oxidation in the biofilm. In addition, biofilm surface morphology and EPS composition were subject to different substrate conditions. Polysaccharide was the primary and protein the minor component of the EPS extract. In the study filter, the protein content of EPS diminished from the bottom to the upper layer of the filter bed, which was strongly correlated to the hydrolysis of colloidal organic matter.

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Keywords: biofilm; nitrification; colloidal organic matter; extracellular polymeric substance

1 INTRODUCTION

Biological nitrification is the most widely used process for removal of nitrogen from wastewater, required by many regulatory agencies, based on the issues of toxicity, oxygen demand and eutrophication effect. Since optimal conditions for the relevant microorganisms can be maintained independent of the different hydraulic retention times in biofilm, biofilm processes have gained extensive applications in urban wastewater treatment, especially in the cases where nitrification is expected. Unfortunately, nitrification too frequently is unstable. The causes of instability include the very low maximum specific growth rate of nitrifiers and competition with heterotrophs for space and oxygen.

Urban wastewater contains carbohydrates, proteins, lipids, humic substances and nucleic acids as well as other organic compounds in highly variable quantities.^{1,2} These contaminants, which must be removed, are complex mixtures in particulate, colloidal and soluble forms that range in size from 0.001 to well over 100 µm.³ The effects of soluble organic carbon matter

on nitrification and on microbial spatial distribution as a result of oxygen and space competition within biofilms have been extensively investigated by many researchers.^{4–7} These results indicated that high C/N ratios induced interspecies competition for oxygen between ammonia-oxidizing bacteria and heterotrophic bacteria, which resulted in the reduction of ammonia oxidation. The effect of particulate organic matter on biofilm nitrification was studied in a rotating biological contactor,⁸ though suspended solids can be easily removed by various physical pretreatments. Particulate BOD restrained nitrification to the same extent as soluble BOD, and the authors suggested that total influent organic matter might be a better predictor of nitrification than soluble organic concentration.⁸ Although there is considerable colloidal organic matter present in wastewater, little is known about its effect on nitrification.

Although the proportions of the soluble, colloidal and particulate phases may also be variable,³ high proportions of colloidal organic matter, as much as 30% measured as COD,⁹ are present in urban

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wastewater. It has been reported that organic polymers with molecular weights greater than 10^6 Da cannot cross the extracellular polymeric substances (EPS) matrix forming the biofilm,¹⁰ and are even less likely to cross the bacterial membrane which is permeable only to monomers or oligomers of a few hundred Da.¹¹ Consequently, it is necessary to investigate the effect of colloidal organic matter on nitrification to optimize wastewater treatment operations, in particular simultaneous carbon oxidation and nitrification.

EPS are the predominant components in biofilms, reported to make up to 90% of the biofilm's organic carbon material. The composition of the EPS determines many important properties of biofilms, such as density, porosity, diffusivity, strength, elasticity, frictional resistance, thermal conductivity, and metabolic activity.^{12,13} Previous studies indicate that EPS generally contain heterogeneous polysaccharides and proteins as the major components. According to Larsen and Harremoës,¹⁴ extracellular enzymes in the EPS matrix mediated the hydrolysis of the biofilm's colloidal organic matter. The composition of EPS may, to some extent, be influenced by the degradation process, in soluble or colloidal form, of the substrate. Information about EPS components relevant to colloid degradation may provide a further valuable insight into biofilm structure and process control.

The aim of this study was to compare the effects of soluble and colloidal organic carbon on nitrification in an up-flow biological aerated filter (UBAF). Starch was chosen as a model of a colloidal substrate, while glucose was selected as the soluble organic substrate. Since previous research has shown that influent C/N ratio is an important factor in the nitrification of biofilms, it was also selected as a main parameter. In addition, the relationship between substrate hydrolysis and the characteristics of biofilm EPS was investigated.

2 METHODS AND MATERIALS

2.1 Reactors

Continuous-flow studies were performed in two identical bench-scale biofilters (denoted as filters A and B) made of Plexiglas with the dimensions 2.0 m

high and 5.5 cm inner diameter. The reactors were placed in a constant temperature-controlled room $25(\pm 1)^\circ\text{C}$. The filters were filled with burned clay particles up to 1.5 m in depth. The particles had a diameter of 2–4 mm, specific area of $3.99\text{ m}^2\text{ g}^{-1}$, porosity of 0.75, packed density of 890 kg m^{-3} and packed porosity of 0.45. Inlets for air and water influent were located at the bottom of the reactors. Six taps located at heights of 10, 40, 70, 100, 130 and 150 cm, respectively, were provided to allow for liquid and solid sampling and headloss measurement during operation. Liquid and gas flowed up through the filter concurrently.

2.2 Operating conditions

Throughout the experiments the two reactors were fed with synthetic wastewater with a constant ammonia concentration of $50\text{ mg NH}_4^+-\text{N dm}^{-3}$ and the C/N ratio was increased stepwise from 0.5 to 1.0, 2.0, 4.0 and finally 6.0. Synthetic concentrated wastewaters contained nitrogen, inorganic and organic carbon sources as well as other micro-elements (Table 1). In filter A (the control) glucose was used as the sole soluble organic substrate, while both glucose and starch were used in equal amounts in terms of COD in filter B (the study) as soluble and colloidal organic substrate, respectively. The concentrated wastewaters were stored at 4°C and diluted with tap water to the desired concentration before being fed to the reactors. The liquid and air flow rates were fixed at $1.42\text{ dm}^3\text{ h}^{-1}$ and $24\text{ dm}^3\text{ h}^{-1}$, giving superficial velocities of 0.6 m h^{-1} for the liquid and 10 m h^{-1} for the gas.

During start-up, both reactors were continuously fed with synthetic wastewater containing ammonia and other nutrients but no organic substrate. No additional seed was used. Liquid and air superficial velocities were maintained at 0.6 m h^{-1} and 10 m h^{-1} . Start-up lasted about 100 days and finally ammonia entering the reactors could be completely oxidized into nitrate. Consequently, it is reasonable to suppose that at the beginning of the study, the filters were colonized by a majority of autotrophic nitrifying bacteria. During the last month the NH_4^+-N , NO_2^--N , NO_3^--N and volatile attached solids (VAS) profiles of different liquid and solid samples remained unchanged. The headloss of the two biofilter's did not increase

Table 1. The composition of concentrated synthetic wastewater

Constituent	Concentration (g dm^{-3})	
	Filter A	Filter B
NaHCO_3	10	10
$(\text{NH}_4)_2\text{SO}_4$	4.97	4.97
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.16	0.16
CaCl_2	0.02	0.02
KH_2PO_4	0.0015	0.0015
K_2HPO_4	0.04	0.04
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.0005	0.0005
Glucose	Between 0.50 and 6.00	Between 0.25 and 3.00
Starch	0	Between 0.24 and 2.88

significantly. Meanwhile, the values of pH and dissolved oxygen concentration along the two biofilters remained constant.

Both reactors were initially fed with synthetic wastewaters containing $25 \text{ mg COD dm}^{-3}$, corresponding to a C/N ratio 0.5. When a steady-state was reached, the influent COD concentration was increased to 50 mg dm^{-3} , and subsequently increased stepwise to 100, 200 and 300 mg dm^{-3} . The steady-state was considered to be attained when the effluent total organic carbon (TOC), ammonia, nitrite and nitrate concentrations remained constant for at least a week. Backwashing was employed manually when a fixed headloss of a 100 cm water column was reached. Backwashing conditions were: after shutting off the feed to the reactor, in a first washing step clean water and air were introduced for 10 min at the bottom of the filter with superficial velocities of 25 m h^{-1} and 55 m h^{-1} , respectively. Then in a second step the airflow was stopped but clean water continued to enter the filter for 5 min at the same velocity as in the first step.

2.3 Extraction method of biofilm EPS

When the influent COD concentration was increased to 300 mg dm^{-3} , the biofilm EPS was extracted to examine the effects of substrate conditions on its composition. Biofilm samples were taken from different heights of the two reactors and analysed in triplicate. EPS extraction was achieved by centrifugation with formaldehyde.¹² EPS extracts were kept overnight at 4°C prior to chemical analysis.

2.4 Analytical methods

Liquid samples, except effluent, were filtered through a $0.45 \mu\text{m}$ pore size membrane filter before analysis. Solid samples were taken from the filters before backwashing. Ammonia, COD and alkalinity were measured using standard methods.¹⁵ Because of the thermal resistance of a burned clay particle, the VAS of solid samples was directly measured gravimetrically. TOC was determined by a Tekmar-Dorhmann TOC analyser (model Apollo 9000). Nitrite and nitrate concentrations were analysed simultaneously by a Dionex ion chromatograph (series 3500 i). Starch was assayed using the starch-iodine complex (SIC) method of San Pedro *et al.*¹⁶ A phenol-sulfuric acid method was used to quantify carbohydrate, with

Table 2. The frequency of backwashing of both biofilters during the experiments

Influent COD (mg dm^{-3})	Frequency of backwashing (day^{-1})	
	Filter A	Filter B
25	nb ^a	nb ^a
50	10–15	10–15
100	5–7	6–9
200	3	4
300	1	2

^a nb—No backwashing.

glucose as standard.¹⁷ Protein concentrations were determined using the Bradford Coomassie Blue method,¹⁸ with Bovine Serum Albumin (BSA) as standard. DNA was determined with the DAPI method with calf thymus DNA as standard.¹⁹

3 RESULTS AND DISCUSSIONS

3.1 Backwashing and biomass growth

Backwashing was performed once the set headloss of 100 cm water was reached. Influent COD and the frequency of backwashing are listed in Table 2. No backwashing was carried out when the influent C/N ratio was 0.5. The headloss increased significantly when both filters were fed with $50 \text{ mg COD dm}^{-3}$. The frequency of backwashing was higher when using increased amounts of feed organic matter, and was probably a consequence of the more rapid growth of heterotrophs at higher contents of organic carbon. It can be observed that the frequency of backwashing of filter A was always greater than that of filter B, which implied the more rapid accumulation of microorganisms in the filter fed with glucose (filter A). Biomass growth in both filters was quantified by the changes of VAS of the solid samples taken from different filter heights (Table 3). For both filters, biomass growth rates diminished gradually from the bottom to the top. In the lower parts, the biomass of filter A was slightly greater than that of filter B, which may be partially explained by the differences in cell growth per unit organic carbon oxidized between the two reactors.

The surface characteristics of biofilms formed in

Table 3. The evolution of biomass under steady state as a function of the influent COD concentration

Influent COD (mg dm^{-3})	Biomass (mg VAS g^{-1} substratum) at different filter heights											
	Filter A						Filter B					
	0.1m	0.4m	0.7m	1.0m	1.3m	1.5m	0.1m	0.4cm	0.7m	1.0m	1.3m	1.5m
25	10.1	9.5	7.9	6.3	6.0	5.2	9.7	8.5	8.0	7.0	6.4	6.1
50	13.3	11.2	10.6	8.2	6.0	4.7	12.7	10.1	10.2	8.3	6.7	5.7
100	15.2	13.1	11.1	9.8	7.9	7.9	14.2	11.8	10.3	10.3	9.3	8.5
200	15.8	14.2	11.9	10.0	8.7	8.1	14.5	11.9	11.1	11.6	9.6	9.0
300	17.4	13.1	12.1	10.1	10.2	9.7	15.5	13.1	14.3	13.3	9.1	9.9

Figure 1. Surface morphological characteristics of biofilms taken from 10cm port of both filters using scanning electron microscopy (left from filter A and right from filter B).

colloidal and soluble substrate degradation were examined by scanning electron microscopy. The typical microbial structure of the solid samples taken from the 10 cm port of both filters at day 143 is shown in Fig 1. Rod-shaped bacteria dominated in both biofilms while filamentous organisms were rarely present. Moreover, distinct differences in morphological characteristics could be observed. The microorganisms fed with glucose existed in a loose and separated state whilst the microorganisms fed with glucose and starch were embedded in gel-shaped pillars of extracellular biopolymers. There were a variety of visible channels between the tight networks entwined by these bacteria–biopolymer pillars in the biofilm of filter B, suggesting that substrate composition could, to some extent, affect biofilm morphology.

3.2 Performance of reactors

The performance of both reactors expressed in terms of TOC oxidation and ammonia removal is shown in Figs 2 and 3, respectively. TOC removal efficiency changed with increases in the influent COD concentration. For example, TOC removal efficiencies of 57.4% for filter A and 63.0% for filter B were obtained

with a COD influent concentration of 25 mg dm^{-3} whilst efficiencies of 85.2% and 88.6% for both reactors could be achieved with an elevated influent COD value of 100 mg dm^{-3} . Nevertheless, the effluent TOC concentrations increased slightly with each increment of influent COD. The effects of substrate conditions on TOC removal efficiencies were different with respect to influent COD concentration. At low COD concentration, the TOC removal efficiency of filter B was comparatively stable and greater than that of filter A. However, this difference between the two reactors was reduced at elevated influent COD concentrations. When both filters were fed with $300 \text{ mg COD dm}^{-3}$, the TOC removal efficiencies were similar. Since the organic oxidation of both filters occurred in the low part of filters with low influent COD and the upper parts played little role in organic matter removal, it is probable that the organic matter in the effluent consisted mainly of soluble microbial products.

The influent COD concentration imposed insignificant influence on ammonia oxidation in both reactors when the COD concentration was less than 200 mg dm^{-3} , ie $C/N < 4$. Moreover, ammonia removal efficiency remained stable and was little affected by back-

Figure 2. Total organic carbon (TOC) removal efficiency of both filters during operation.

Figure 3. Ammonia removal efficiency of both filters during operation.

washing. Nevertheless, ammonia removal efficiency was less than 100% in all runs where synthetic wastewater COD concentration was increased up to 200 mg dm^{-3} for filter B. For filter A, full ammonia removal could be accomplished while filter B gave full ammonia removal during the first two days but the value decreased to about 90% and 80% on the third and fourth days, respectively. Some authors have suggested that the reactor initially colonized by nitrifiers could resist the increase in the C/N ratio to four without losing nitrification efficiency when sucrose was used as the sole organic carbon source in a pilot-scale UBAF.⁴ In this study, nitrite was seldom detected in the effluents of either reactor.

In filter B full ammonia removal could be restored after backwashing. The ammonia removal efficiency deteriorated gradually during the filter operation. Ohashi *et al*⁵ emphasized the importance of backwashing in BAF nitrification. Their results showed that the biofilm remaining after backwashing also contained a higher fraction of nitrifiers than did the backwashing solids stripped from the outer layers of the biofilm, which consisted mainly of active heterotrophs. Furthermore, when the influent C/N ratio increased to 6.0, ammonia removal efficiency was severely restrained. Backwashing was performed daily for filter A and every two days for filter B. The ammonia removal rate reached only 80% and 70% for filter A and filter B respectively.

The profile of starch concentration across the filter as a function of influent C/N ratio is shown in Fig 4. Since the SIC method can detect amylose of chain length greater than eight,¹⁶ the figure shows that hydrolysis of starch into smaller molecules mostly took place in the 40 cm bottom of the filter bed. Within the range of starch concentration examined, starch was rapidly hydrolysed, indicating that the hydrolysis of starch into smaller molecules could not be the limiting step of its degradation. In an activated sludge study, Guellil *et al* suggested that hydrolysis could not be the limiting reaction in the use of organic matter because of the huge number of viable bacteria in activated sludge flocs.²⁰

The evolution of nitrogen up the filters as a function

Figure 4. Profiles of starch along filter height as functions of influent COD concentration.

Figure 5. The evolution of nitrogen up the filters as a function of the influent COD concentration.

of influent organic carbon concentrations is shown in Fig 5. As mentioned above, the ammonia removal efficiency deteriorated with the increase in influent COD concentration. For example, both filters achieved complete ammonia removal at a COD concentration of 25 mg dm^{-3} , and the ammonia entering the filters was converted into nitrate in the effluent. However, ammonia removal efficiencies of 77.3% and 72.2% were achieved with $300 \text{ mg COD dm}^{-3}$ for filter A and filter B, respectively. Similarly, the nitrifying ability of both filters, shown by the profiles of $\text{NO}_x\text{-N}$ (the sum of $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$), diminished as the amount of organic carbon in the influent increased. Figure 5 shows that full nitrification was obtained with $25 \text{ mg COD dm}^{-3}$, whilst 64.7% and 58.9% ammonia were oxidized into nitrate with $300 \text{ mg COD dm}^{-3}$ for filter A and filter B, respectively. It is observed that the increase in organic matter in the influent resulted in the displacement of ammonia removal and nitrification from the bottom to the upper part of filter bed. The data (Fig 5) indicated that the filter bed can be segregated into three zones: (1) the lower part, the 40 cm bed from the bottom, (2) the upper part, the bed from 100 to 150 cm, and (3) the middle part, the bed from 40 to 100 cm.

The lower part of the filter bed, to some extent, made a substantial contribution to total ammonia removal. For an influent COD concentration of 25 mg dm^{-3} about 40–50% ammonia removal was obtained, while for $300 \text{ mg COD dm}^{-3}$ only 20% of ammonia was removed in this part. It seems that the ammonia removal efficiency of this part can be attributed to two processes: nitrogen uptake by assimilation and nitrification by autotrophic nitrifiers. On the one hand, biological nitrification took place when the organic carbon content was comparatively low, which was verified by the presence of nitrite and nitrate in samples from the 10 and 40 cm ports. The values of $\text{NO}_x\text{-N}$ concentration (shown in Fig 5) demonstrated that the more than 90% ammonia removal could be attributed to nitrification with an influent COD concentration of 25 mg dm^{-3} . The contribution of nitrification was of less importance at higher influent COD concentrations. When the influent COD concentration was increased to 300 mg

dm^{-3} , some 40% of the ammonia nitrogen removed was oxidized by nitrifiers. A low concentration of nitrite was found in this part, and the inhibition of nitrite oxidation caused by free ammonia at higher pH value may account for the nitrite accumulation. On the other hand, the ammonia removal by cell growth was shown by the nitrogen loss in this part. The contribution of assimilation became more significant as the influent organic carbon concentration increased owing to the larger amount of cell growth. Because of the competition for oxygen and space between heterotrophic aerobes and autotrophic nitrifiers in biofilm, nitrifying activity was restrained with high organic carbon concentration. Fdz-Polanco *et al* reported that the combined oxidation of organics and ammonia was only observed when TOC concentration was less than 30 mg dm^{-3} .⁴

In contrast, in the middle and upper parts of the filters where the organic matter concentration was much lower, the contribution of assimilation to ammonia removal was insignificant. Ammonia removal in the middle part first increased and finally decreased with the increase of influent organic matter. For example, 40%, 50% and 20% of ammonia was removed in the middle part of the filters when the organic concentrations fed into the reactors were 25, 100 and $300 \text{ mg COD dm}^{-3}$, respectively. The nitrification activity was displaced to the upper part of both filters as the organic matter in the influent increased. With an influent COD concentration of 25 mg dm^{-3} , in both reactors less than 10% of the ammonia removed was nitrified in the top 50 cm of the filters, whereas at influent $300 \text{ mg COD dm}^{-3}$ up to 60% of the ammonia removed was oxidized in the top 50 cm.

Figure 5 shows that there were some differences in ammonia removal and nitrification between filter A and filter B. The nitrification efficiencies of filter A at various C/N ratios were greater than that of filter B. In other words, COD present as colloidal organic matter imposed a greater reduction of nitrification than soluble COD at the same COD concentration in biological aerated filters. It is also clear that the difference in ammonia removal between both filters strongly correlated with the difference in nitrification. This result indicated that there was insignificant difference in nitrogen uptake by assimilation between colloidal and soluble organic matter.

3.3 Composition of EPS in biofilm

The compositions of the EPS extracted at different heights are listed in Table 4. The low nucleic acid contents in EPS extracts indicate the low degree of cell disturbance during the extraction process. It appeared that in all samples polysaccharide was the primary constituent, while protein was a minor component, less than 40 mg g^{-1} VAS. In contrast, previous studies on EPS analysis of activated sludge^{21,22} and biofilm^{23,24} showed that the predominant EPS material was protein. The low protein yields in this study may be associated with the extraction method adopted; formaldehyde may have interfered with protein determination.¹³ The protein content in filter B decreased from the bottom to the upper layer of the filter bed, which strongly correlated with the hydrolysis of starch. The highest protein yield was found at the point 10 cm above the entrance in filter B. More than 70% of the starch entering the reactor was hydrolysed in the first 10 cm. Dignac *et al*²⁵ reported that a portion of the extracellular protein could be exo-enzymes that participate in the hydrolytic activity of microbial aggregates. Moreover, the important role the EPS protein played in the adhesion process during biofilm formation has been demonstrated.²⁶ The polysaccharide content of the biofilms of filter A was greater than that of filter B taken from the same filter height. The total amount of material extracted from EPS (sum of polysaccharide and protein) in the biofilm sample taken from the 10 cm port of filter B was greater than from filter A.

4 CONCLUSIONS

This paper describes the effects of colloidal and soluble organic carbon on nitrification and extracellular polymeric substances in two UBAF reactors. The main conclusions drawn from this work are:

- (i) The biomass of the filter fed with glucose at the lower part was slightly greater than that of the filter fed with glucose and starch, which resulted in higher frequency of backwashing of the filter fed with soluble organic matters.
- (ii) The starch hydrolysis took place mainly in the bottom 40 cm of the filter bed, which implies that hydrolysis of colloidal organics into smaller mol-

Filter height (cm)	EPS component (mg g^{-1} VAS)					
	Filter A			Filter B		
	Polysaccharide	Protein	DNA	Polysaccharide	Protein	DNA
10	110 ± 10	8.0 ± 2.1	7.6 ± 4.4	110 ± 8	32.6 ± 2.2	8.5 ± 1.8
40	161 ± 8	12.7 ± 2.3	2.0 ± 1.2	76 ± 7	20.3 ± 1.8	7.6 ± 0.9
70	112 ± 13	26.1 ± 1.6	6.0 ± 3.4	53 ± 8	17.6 ± 1.6	12.4 ± 3.1
100	74 ± 14	13.4 ± 2.0	7.3 ± 1.6	24 ± 5	13.8 ± 0.1	5.1 ± 1.3
130	77 ± 10	6.4 ± 1.2	13.9 ± 3.6	63 ± 6	14.2 ± 3.3	12.4 ± 4.5
150	102 ± 13	10.3 ± 0.9	9.2 ± 2.7	43 ± 5	14.1 ± 1.2	11.6 ± 3.3

Table 4. The composition of EPS extracts in biofilm samples

ecules could not be the limiting step of the oxidation of colloidal organic matter in the biofilm.

- (iii) The organic matter oxidation was accomplished in the lower part of each filter bed throughout the experiment, while the nitrification was displaced gradually to the upper part of both filters with increasing influent COD concentration. The ammonia removal and nitrification efficiencies reduced as the influent organic matter concentration increased.
- (iv) COD present as colloidal organic matter imposed greater reduction of nitrification than soluble COD at the same COD concentration in biological aerated filters.
- (v) The colloidal substrate significantly affected the morphological characteristics and EPS compositions in biofilms. Polysaccharide was the primary constituent in EPS extracts while protein was the minor component. The protein contents in EPS diminished from the bottom to the top of the filter fed with starch, which was correlated with the hydrolysis of colloidal organic matter.

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