The use of microsensors to study the role of the loading rate and surface velocity on the growth and the composition of nitrifying biofilms

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ABSTRACT

The good composition and activity of biofilms are very important for successful operation and control of fixed-film biological reactors employed in liquid effluents treatment. During the last decade, microsensors have been applied to study microbial ecology. These sensors could provide information regarding the microbial activity concerning nitrification and denitrification that occur inside biofilms. Other techniques of molecular biology, such as fluorescence in situ hybridization (FISH), have also contributed to this matter because their application aids in the identification of the bacterial populations that compose the biofilms. The focus of this paper was to study the loading rate and surface velocity to promote the development of nitrifying biofilms in three distinct flow cells that were employed in the post treatment of a synthetic wastewater simulating the effluent from a UASB (Upflow Anaerobic Sludge Blanket) reactor. Using the FISH technique, it was found that the population of ammonia-oxidizing-bacteria was greater than that of nitrite-oxidizing-bacteria; this was also supported by the lower production of nitrate determined by physicochemical and microsensor analyses. It was verified that the loading rate and surface velocity that promoted the greatest nitrogen removal were 0.25 g N-amon·m⁻²·biofilm·day⁻¹ and 1 m h⁻¹, respectively.

Key words | biofilms, FISH, microsensors, nitrification, post-treatment

INTRODUCTION

Biofilms offer a series of advantages for microbial cells, such as protection against environmental factors, metabolic cooperation and the potential for acquisition of new genetic characteristics.

The biological systems that use biofilms for the treatment of liquid effluents include – but are not exclusive to – expanded/fluidized bed reactors, anaerobic biological filters, biodiscs, submerged aerated filters and anaerobic filters. The employment of expanded/fluidized bed biological reactors in the treatment of effluents has proven to be efficient in the conversion of organic biodegradable substrates and the removal of nutrients (Campos 1983; Polanco et al. 1996; Patel et al. 2006). The observed phenomena were justified by both the presence of an elevated concentration of active biomass that was gathered to the support material of the biological reactor and symbiosis among the microorganisms of the biofilm.

The use of molecular biology techniques, such as fluorescence in situ hybridization (FISH), allows for the specific identification, quantification, structural characterization and spatial distribution of the microbial community in flocs and biofilms (Amann et al. 1995). The use of FISH associated to microsensors can significantly improve operations monitoring because the combined use of these analytical tools allows for the observation of both the structure and relationship of microbial communities inside the biofilm (Okabe et al. 2003).

In this study, the development of a nitrifying aerobic biofilm was investigated using flow cell laboratory-scale reactors to obtain the concentration profiles of some chemical species related to the nitrifying activity of the biofilm. The system was monitored by microsensors for dissolved oxygen (DO), pH, ammonia ions (N-NH₄⁺) and N-NO₃⁻. Specific probes that target the 16S ribosomal DNA were
employed to quantify the nitrifying community in the biofilm formed on the submerged surface of the flow cells walls. The objective of this study was to define the best loading rate and the liquid surface velocity that will optimize the nitrogen removal from synthetic wastewater with similar characteristics to the UASB effluent.

**METHODS**

**Development of the biofilm**

**Flow cell operation**

Biofilms were developed in three flow cells constructed based on Jackson *et al.* (2001), each of them with a smooth base canal of polycarbonate (3.8 cm width, 4 cm depth) with a working volume of 150.0 mL (Figure 1). The system was inoculated with a sludge sample from the activated sludge reactor located after the UASB reactor of the Wastewater Treatment Plant located in Rio Claro (São Paulo, Brazil – Latitude: 22°35'54.55"S; Longitude: 47°33'50.76"W). This inoculum was chosen due to its high population of ammonium-oxidizing bacteria \(2.0 \times 10^5\) NMP/100 mL. This sludge was introduced in the flow cells and recirculated in the same ones for a period of 4 h. Later, this sludge was removed from the flow cells with a vacuum system and the feeding with the synthetic substrate was initiated. The synthetic substrate, which had sucrose as a carbon source, was composed to simulate a typical UASB effluent (Spinola 2009): 180 mg CaCO\(_3\)/L of alkalinity, 95 ± 5 mg L\(^{-1}\) of degradable COD, pH of 7.5 ± 0.1 and 40 ± 10 mg N L\(^{-1}\) of TKN-Total Kjeldahl Nitrogen. The synthetic substrate was pumped (25 ± 2 ºC) to the flow cells using a peristaltic pump and part of the effluent was recirculated back into the flow cell, to permit velocity variations.

Two preliminary tests were performed in order to verify the gathering of the biofilm to the support media, estimate an adequate time for operation of the flow cells and determine the maximum loading rate. In these tests, in which only one flow cell was used per experiment, the following loading rates and surface velocity were adopted: 1.5 g N-amon·m\(^{-2}\) biofilm·day\(^{-1}\) and 1.0 m h\(^{-1}\) (first test) and 0.5 g N-amon·m\(^{-2}\) biofilm·day\(^{-1}\) and 8.0 m h\(^{-1}\) (second test).

Based on the observations made in the two prior tests, the rates of nitrogen loading and surface velocities of the three final experiments were defined. For each of these experiments, three flow cells were operated simultaneously under three different surface velocities. In the first experiment, the loading rate was 0.5 g N-amon·m\(^{-2}\) biofilm·day\(^{-1}\), and the surface velocities were 1; 8 and 4 m h\(^{-1}\). In the second and third experiments, the loading rates were 1.0 g N-amon·m\(^{-2}\) biofilm·day\(^{-1}\) and 0.25 g N-amon·m\(^{-2}\) biofilm· day\(^{-1}\), respectively, and the surface velocity from the first experiment was maintained. The average operational time of the flow cells was nine days, which is the time necessary for the concentrations of dissolved oxygen (DO) to approach 0 mg L\(^{-1}\) in the most internal zones of the biofilm.

**Monitoring**

DO was monitored daily in the bulk media and inner of the biofilm using the DO microsensors according to previously published methods (Gonzalez 2009). Alkalinity, pH and TKN were measured every two days, in the influent and effluent of the flow cells, NO\(_3^-\) and NO\(_2^-\) were analyzed by the flow injection method (FIA) (APHA 2005).

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**Figure 1 | Illustration of the flow cell (measured in cm).**
To obtain images of the biofilm structure, the flow cells were positioned under an inverted microscope (Olympus® IX51). The images were acquired using a camera (Samsung®-SDC313) that was connected to a microscope and a microcomputer (40x magnification). Visualization of the structures was done using the personalized software, Pixer View®.

Microsensors

Concentrations of DO, N-NH₄⁺, N-NO₃⁻ and H⁺ (pH) were obtained inside the biofilms by applying the microsensors constructed at the Laboratory of Microsensors in the School of Engineering of São Carlos (USP). Clark-type DO microsensors with 20 μm tips were prepared and calibrated as previously described (Revsbech 1989) (Figure 2). Liquid-ion exchange membrane microsensors (LIX-liquid ion-exchanger) with tips measuring 15 μm were also constructed, according to previous literature (de Beer et al. 1997), for measuring concentrations of the N-NH₄⁺ and N-NO₃⁻ ions and pH. The microsensors for N-NH₄⁺ and N-NO₃⁻ were calibrated separately with solutions of the reference ions diluted to concentrations between 10⁻¹ and 10⁻⁵ mol L⁻¹, which were prepared with the media utilized in the experiment. Calibration of the pH microsensors was done by using pH standard solutions in the range of 4.0–10.0. After calibration, each microsensor was positioned in the micro-manipulator, and its vertical displacement was controlled by a computer. Concentration profiles of the various chemical species were obtained by introducing the microsensors to the biofilms gathered on the surface of the flow cells as previously described (de Beer et al. 1993).

The microsensors had been introduced vertically in biofilms until their tips reached the supported surface of the flow cells. The measurement with microsensors was made in this way because by doing this we knew that all the biofilm thickness had been analyzed with the applied microsensors.

Microbiological monitoring

Fluorescent in situ hybridization (FISH)

The specific oligonucleotide probes for the 16S rRNA sequences were the following: Nso190, which hybridizes with the majority of the β-Proteobacteria ammonia oxidants (Mobarry et al. 1996); Nit3, which is specific for Nitrobacter (Wagner et al. 1996); and Ntspa662, which is specific for Nitrospira (Daims et al. 2001). All probes were marked with Cy3 at the 5' end. Hybridization was performed in 30% formamide for 90 min (46 °C), and then samples were washed for 15 min (48 °C) in a 112 mmol/L NaCl buffer. After hybridization, the samples were stained with 10 μL of a 10 μg mL⁻¹ DAPI solution (4,6-diamidino-2-phenylindole) and observed under a fluorescence microscope (Olympus BX-50) equipped with a color camera (QcolorR5C). The percentage of cells hybridized with the Nso190 probe was obtained by calculating the number of hybridized cells in relation to the total number of cells stained with DAPI.

RESULTS AND DISCUSSION

Tests

In the first test, it was verified that the concentrations of DO within the biofilm rapidly decreased in the first operation day of the flow cell. The formation of gas bubbles was also observed, which is likely due to the anaerobic activity related to the degradation of organic compounds, as a result of the high loading rate (1.5 g N-amon·m⁻² biofilm⁻¹ day⁻¹) and low surface velocity (1 m h⁻¹) that had contributed to a formation of a thicker biofilm with low concentrations of dissolved oxygen in its deeper zones (where the anaerobic degradation occurred with the formation of methane, sulfidric and carbonic gas). Although we observed the formation of gases within the biofilm, analyses for identification of heterotrophic bacteria had not been done in the present research.

In the second test, a lower loading rate (0.5 g N-amon·m⁻² biofilm⁻¹ day⁻¹) and higher surface velocity (8 m h⁻¹)
were used, and the occurrence of a gradual decrease of the DO concentrations was verified inside the biofilm; concentrations near 0 mg L\(^{-1}\) were measured on the ninth day of flow cell operation. The biofilms verified in the beginning of the two tests are presented in Figure 3, in which can be observed the presence of denser aggregates in the beginning of the first experiment and the opposite for the second experiment.

We conclude, by means of the accomplishment of these tests that, a higher superficial velocity takes the acceleration of the biofilm development, whereas low superficial velocity leads to slow biofilm development. This happened due to the occurrence of an increase in the mass transfer resistance when the biofilm was submitted to a low superficial velocity. In accordance with these results, we defined the necessary time for the growth of the biomass and the rates of nitrogen loading and surface velocities of the three final experiments. The defined time was nine days, which was considered short in accordance with the low growth rate of the nitrifying bacteria. This had happened because the sludge sample that served as the inoculum of the flow cells already contained biofilm (grown prior to the experiments), which was further developed adhering to the support surface of the same cells.

**First experiment**

On the sixth day after starting the experiment, distinct DO values were determined in the deepest zones of the gathered biofilms in the three flow cells; the DO concentrations were 1; 3 and 6 mg L\(^{-1}\) in cells 1; 2 and 3, respectively. The high concentration in the developed biofilm of flow cell 3 was due to the detachment of biofilms from the support, which may have been due to activity of anaerobic microorganisms and consequent gas production in the deepest regions.

The profiles of the N-NO\(_x\)\(^-\) in the biofilms of the three flow cells indicate an increase in the ion concentration in the liquid media in the interior of the biofilm. Here it is assumed that the oxidation of ammonia ions to nitrite ions occurred due to the action of the ammonia oxidizing bacteria, which was identified by hybridization with the Nso290 specific probe.

The results of the hybridizations with the Nso190 probe are shown in Figure 4. The percentages presented in the graph refer to the number of cells hybridized with the Nso190 probe for ammonia oxidants, in relation to the total number of cells stained with DAPI. The Nit3, specific for Nitrobacter (Wagner et al. 1996), and Ntspa662, specific for Nitrospira (Daims et al. 2001), presented no signal, which indicates a lower population of nitrite-oxidizing bacteria.

Based on the profile of N-NO\(_x\)\(^-\) ions and the results of the physicochemical analyses, which showed that the nitrite concentrations were higher than the nitrate (Figure 5), it was concluded that oxidation of ammonia nitrogen to nitrite ions occurred inside the biofilms in the three flow cells when the oxidation of the nitrite ions to nitrate was less expressive. The pH varied within the range 6.8 to 8.0. According to previous literature (Paredes et al. 2007), the optimal pH range for nitrification is between 7.0 and 8.0, in which the lower pH values favor the growth of nitrite oxidizing bacteria. Greater concentrations of nitrate ions in the effluent of the flow cell were observed at the end of the
experiment when the pH values were lower. It is noted that the decline in pH began after the third day, which indicated nitrification activity.

**Second experiment**

The DO concentrations decreased rapidly in a relatively short time (Figure 6), and it was inferred that the low oxygen concentration was not enough to promote the growth and stabilization of the nitrifying population, which results in a low removal rate of ammonia ion. The factor that may have most influenced this scenario was the relatively high loading rate (1.0 g N·m⁻²·biofilm⁻·day⁻¹) applied to the flow cells, which resulted in thick biofilms after a few days of operation. The limitation of DO diffusion in thick biofilms may cause the formation of anaerobic zones in most internal regions (Araujo 2003).

The absence of nitrate ions was verified, and the concentrations of nitrite ions detected in the three flow cell effluent were relatively small (inferiors to 2 mg N·NO₂⁻·L⁻¹) (Figure 7). According to Villaverde et al. (2001), the accumulation of nitrite mainly occurs due to the slower growth of nitrite-oxidizing organisms compared to that of the ammonia-oxidizing bacteria.

**Third experiment**

The DO profiles, on the sixth day of operation, were similar in the three flow cells. The DO concentrations were between 1.25 and 3.5 mg L⁻¹, inside the biofilms, and between 3.5 and 5.0 mg L⁻¹, in the bulk. From the pH profiles, it was verified that, in flow cells 2 and 3, there was a considerable reduction in pH values from the bulk to the biofilm. A decrease in the concentration of N-NH₄⁺ ions, followed by an increase in the concentration of N-NOₓ⁻ ions, was verified in all flow cells. High concentrations of N-NOₓ⁻ ions were also observed in the bulk. From the profiles it can be verified that the conditions of the medium were favorable for the occurrence of nitrification because DO was consumed. Figures 8–10 show that the pH remained near 7, the concentration of N-NH₄⁺ ions decreased in the liquid media–biofilm direction, and the concentration of N-NOₓ⁻ ions increased inside the biofilm.

The physicochemical analysis results corroborate with those using the microsensors, i.e. it was verified in the first analyses that there was a reduction in the TKN values in all cells during the third experiment; it was also observed that the cell that presented greatest removal/conversion of TKN (near 100%) was that with the lowest surface
velocity (1 m h\(^{-1}\)). The above infers that elevated surface velocities probably provoke the dragging of nitrifying microorganisms. Additionally, from the physicochemical analyses for the detection of nitrite and nitrate ions, it was verified that the concentration of nitrite ions in the effluents of the three flow cells peaked on the fourth day of the experiment with values varying between 23 and 28 mg L\(^{-1}\); the concentrations of nitrate ions, which peaked on the sixth day of testing (5 to 10 mg L\(^{-1}\)), were lower than the nitrite ions during the entire experimental period.

When comparing the third experiment with the two previous ones, in which greater nitrogen loading rates were applied, the third experiment showed the greatest efficiencies of TKN removal. Nevertheless, the rate of nitrogen loading applied in the third experiment was lower than that encountered in the literature for reactors with adhered growth used for nitrification (Metcalfe & Eddy 2005).

Gjaltema et al. (1997) observed that, when biofilms with elevated surface areas are submitted to low loading rates, the result is the formation of dense and compact layers, which lead to better operation of the reactor and corroborate the phenomena observed in this experiment.

**CONCLUSIONS**

The efficiencies of nitrogen removal expressed as a percentage and obtained as a function of the nitrogen loading rate and surface velocity applied to the studied biofilms are expressed in Figure 11.

In the experiment in which the surface nitrogen loading rate was 0.5 g N·m\(^{-2}\) biofilm\(\cdot\)day\(^{-1}\), it was concluded that the oxidation of ammonia nitrogen to nitrite ions was favored but not that of nitrite ions to nitrate ions. When the nitrogen loading rate was increased to 1.0 g N·m\(^{-2}\) biofilm\(\cdot\)day\(^{-1}\), it was verified that the

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**Figure 8** Profile of pH, DO, N-NH\(_4\)\(^+\) and N-NO\(_x\)\(^-/\) ions of flow cell 1 on the 6th operation day with a loading rate of 0.25 g N·m\(^{-2}\) biofilm\(\cdot\)day\(^{-1}\) and surface velocity of 1.0 m h\(^{-1}\) (\(T = 25 ± 2\) C).

**Figure 9** Profile of pH, DO, N-NH\(_4\)\(^+\) and N-NO\(_x\)\(^-/\) ions of flow cell 2 on the 6th operation day with a loading rate of 0.25 g N·m\(^{-2}\) biofilm\(\cdot\)day\(^{-1}\) and surface velocity of 8.0 m h\(^{-1}\) (\(T = 25 ± 2\) C).

**Figure 10** Profile of pH, DO, N-NH\(_4\)\(^+\) and N-NO\(_x\)\(^-/\) ions of flow cell 3 on the 6th operation day with a loading rate of 0.25 g N·m\(^{-2}\) biofilm\(\cdot\)day\(^{-1}\) and surface velocity of 4.0 m h\(^{-1}\) (\(T = 25 ± 2\) C).

**Figure 11** Efficiencies of TKN removal (percentage) as a function of the loading rate and surface velocity (\(T = 25 ± 2\) C).
DO concentrations decreased rapidly in a short time period, which caused both the aerobic cycle to be insufficient for growth and stabilization of the nitrifying population and a low rate of ammonia nitrogen removal.

In the flow cells operated with the lowest loading rates \(0.25 \text{ g N-ammon·m}^{-2}\text{biofilm·day}^{-1}\), the profiles obtained when using the microsensors indicated that the conditions of the media were favorable for nitrification; the pH remained close to neutral, the concentration of \(N-\text{NH}_4^+\) ions decreased in the liquid-biofilm medium and the concentration of \(N-\text{NO}_2^-\) ions increased in the biofilm. The best efficiencies of TKN removal were obtained at the lowest loading rate employed \(0.25 \text{ g N-ammon·m}^{-2}\text{biofilm·day}^{-1}\), which is lower than the values mostly suggested in the technical literature for fixed film reactors using nitrification which are of 0.5 to 2.5 \(\text{ g N-ammon·m}^{-2}\text{biofilm·day}^{-1}\) (Metcalfe & Eddy 2003). Considering the flow cells with the lowest loading rate, the cell that presented TKN removal near 100% was that submitted to the lowest surface velocity \(1 \text{ m h}^{-1}\). These systems presented a greater production of nitrite than nitrate when measured via physicochemical and microsensor analyses; this observation was supported by the FISH technique, which showed that the population of ammonia-oxidizing-bacteria was greater than that of nitrite-oxidizing-bacteria.

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