Vol.48, n. 5 : pp. 841-849, September 2005
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY
AN INTERNATIONAL JOURNAL

Morphological Study of Biomass During the Start-Up Period of a Fixed-Bed Anaerobic Reactor Treating Domestic Sewage

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ABSTRACT

This work focused on a morphological study of the microorganisms attached to polyurethane foam matrices in a horizontal-flow anaerobic immobilized biomass (HAIB) reactor treating domestic sewage. The experiments consisted of monitoring the biomass colonization process of foam matrices in terms of the amount of retained biomass and the morphological characteristics of the cells attached to the support during the start-up period. Non-fluorescent rods and cocci were found to predominate in the process of attachment to the polyurethane foam surface. From the 10th week of operation onwards, an increase was observed in the morphological diversity, mainly due to rods, cocci, and Methanosaeta-like archaeal cells. Hydrodynamic problems, such as bed clogging and channeling occurred in the fixed-bed reactor, mainly due to the production of extracellular polymeric substances and their accumulation in the interstices of the bed causing a gradual deterioration of its performance, which eventually led to the system's collapse. These results demonstrated the importance and usefulness of monitoring the dynamics of the formation of biofilm during the start-up period of HAIB reactors, since it allowed the identification of operational problems.

Key words: Anaerobic digestion, biofilm, domestic sewage, fixed-bed reactor, polyurethane foam

INTRODUCTION

Anaerobic fixed-bed reactors have been increasingly used to treat domestic sewage in recent years. The conventional anaerobic filter configuration has been continuously improved with innovative configurations of reactors that provide good performance and stability, whose main contributing factors are long cellular retention times and high biomass concentrations. In this sense, the support utilized to immobilize the biomass plays an essential role in these systems, and is directly associated with the cellular retention time, biomass concentration and microbial diversity.

Polyurethane foam, which has been studied for many years for the adhesion of anaerobic microorganisms, has shown promising results (Huysman et al., 1983, Fynn and Whitmore, 1984; Calzada et al., 1984; Gijzen et al., 1988; Pascik, 1990; Varesche et al., 1997; Picanço et al., 2001; Ribeiro et al., 2003). However, although this support material provides a suitable environment for the adhesion of a mixed consortium of anaerobic microorganisms, its application in full-scale fixed-bed reactors must be carefully investigated, since it is a compressible material and the accumulation of solids or polymeric

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substances in the interstices of the bed can seriously impair the flow pattern in the reactor.
Hydrodynamic problems were detected by Zaiat et al. (2000) in a fixed-bed reactor, the horizontal-flow anaerobic immobilized-biomass (HAIB) reactor filled with polyurethane foam, after only 15 weeks of operation treating domestic sewage at 25°C and 2 $kg.m^{-3}.day^{-1}$. Although partial bed clogging was observed, the reactor's performance was not affected, since a back-washing procedure was applied in the bed. However, the effect of this problem on a long-term operation can impair the performance and stability of the anaerobic process. Therefore, studies of biofilm formation and biomass growth in HAIB reactors are important to shed light on the problems occurring in long-term operation, mainly relating to channeling and bed clogging. Such studies would also allow operating procedures to be adopted to prevent such problems from occurring.
The configuration of horizontal-flow anaerobic immobilized-biomass (HAIB) reactors was proposed by Foresti et al. (1995) as an innovative fixed-bed reactor for wastewater treatment. This reactor offers a potential alternative for full-scale application, as shown previously by the high performance of a bench-scale reactor treating paper industry effluent (Foresti et al., 1995), glucose-based substrate (Zaiat et al., 1997), and degrading toxic substances such as phenol, benzene, toluene, ethylbenzene, xylenes and formaldehyde (Bolaños, et al., 2001, Nardi et al., 2002, Oliveira et al., 2004).
This paper reports on the monitoring of anaerobic biofilm formation on polyurethane foam matrices in a fixed-bed reactor treating domestic sewage during the acclimatization period. Qualitative and quantitative analyses of the biomass were associated with the performance parameters obtained along time to infer the criteria to be used to establish the end of the start-up period.

MATERIALS AND METHODS

The pilot-scale HAIB reactor (Fig. 1), with a total volume of 237.5 liters was constructed using 14.5 cm diameter (D) commercial plastic tubes. The reactor, composed of five 2.88-m tube stages in series with a total length of 14.4 m, was filled with 1-cm side cubic particles of polyurethane foam (apparent density of 23 $kg.m^{-3}$), resulting in a bed porosity of 0.4. The reactor was operated at a hydraulic detention time (HDT) of approximately 4 hours and fed with domestic sewage. Before entering the HAIB reactor, the sewage was pre-screened using a 1-mm screen and stored in a tank.
The performance of the HAIB reactor was evaluated by monitoring total and filtered chemical oxygen demand (COD), volatile fatty acids (VFA) and bicarbonate alkalinity, according to the Standard Methods for Examination of Water and Wastewater (1995), while the gas composition was evaluated by gas chromatography with a thermal conductivity detector.
Stimulus-response hydrodynamic assays were performed using dextran blue (7.5 $g.l^{-1}$) and eosin Y (4.5 $g.l^{-1}$) as tracers. The tracer solution was injected as a pulse in the influent stream and samples were analyzed at 10-minute intervals using a DR-4000 Hach spectrophotometer.
The biomass colonization process was monitored based on the amount of retained biomass and the morphological characteristics of the cells attached to the support. Eight polyurethane cubes from each stage of the reactor were collected weekly over a 10-week period. To quantify the biomass, the cubes were placed in a 40-ml glass flask with 15 ml of water and approximately 5 g of glass beads and the flask was shaken for 20 minutes. The total volatile solids (TVS) content of the solution was measured according to the Standard Methods for the Examination of Water and Wastewater (1995).
It is worth pointing out that the reactor was operated without previous inoculation. Therefore, the bed was initially composed of clean polyurethane foam matrices, which were then colonized by the microorganisms present in the domestic sewage.
Samples for scanning electron microscopy (SEM) were subjected to the technique developed by Nation (1983) and adapted to microbial biofilms by Araújo (1994). These samples were fixed in 0.1 M phosphate buffer (pH = 7.3) containing 2.5% glutaraldehyde for 12 h at 4ºC. After fixation, the samples were rinsed three times in 0.1 M phosphate buffer (pH = 7.3) and dehydrated gradually after successive immersions in ethanol solutions of increasing concentrations (50%, 70%, 80%, 90% and 95%). Each rinsing and dehydrating step lasted 10 min. The samples were washed three times in 100% ethanol (PA grade) before immersion in hexamethyldisilazane for 30 seconds. The drying step was completed by holding the samples at 60ºC for 2 h. The particles were then coated with gold powder and stuck onto

microscope slides with silver glue. SEM analyses were performed using a Zeiss model DSM-960 digital scanning microscope.

For optical microscopy observations, polyurethane foam particles were rinsed with distilled water and drops of the resulting liquid were immediately examined under a phase-contrast microscope. Fluorescence was verified using a UV light source attached to an Olympus BHT 2 microscope.

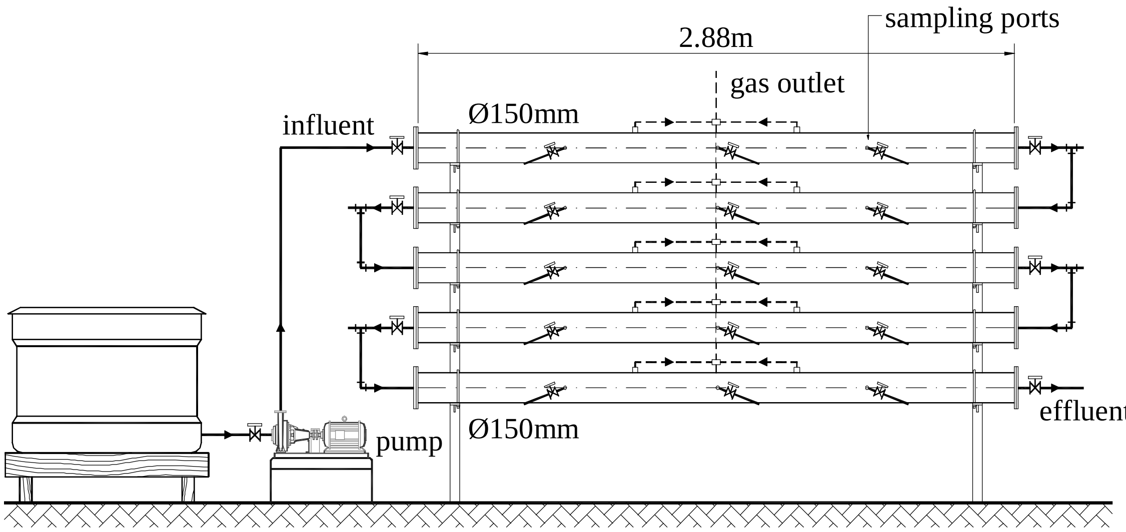

Figure 1 - Sketch of the pilot-scale HAIB reactor (Zaiat et al., 2000)

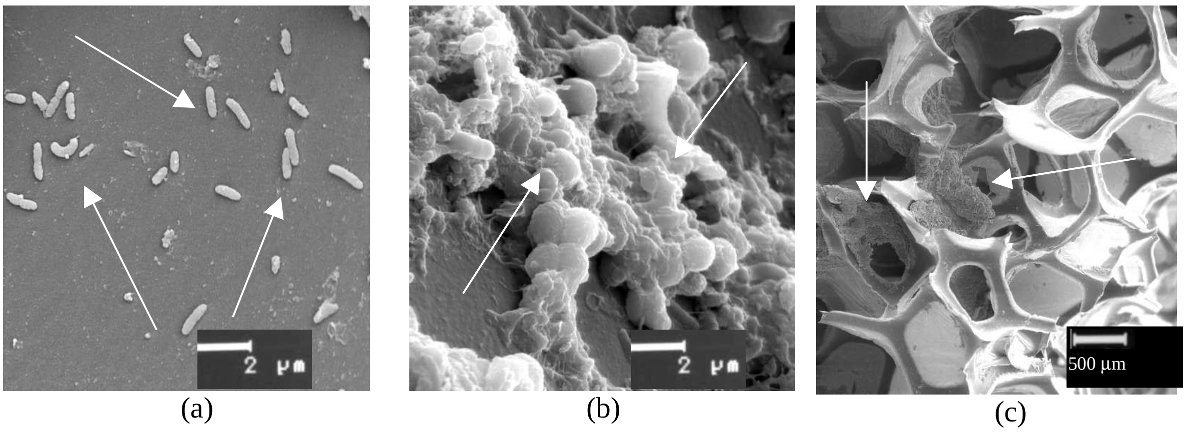

Figure 2 - Patterns of biomass immobilization. (a - randomly distributed cells individually attached to the polyurethane surface; b - cells attached to each other and linked by extracellular polymeric substances; c - micro-granules).

RESULTS AND DISCUSSION

During the acclimatization period, the COD removal efficiency was approximately 40%, with average influent and effluent COD of 370 ± 97 mg.l^{-1} and 214 ± 92 mg.l^{-1}, respectively. The pH values ranged from 7.0 to 7.5 and the effluent total volatile acids concentration was found to be 42 ± 10 mg HAc.l^{-1}. The VSS removal efficiency was 62% with an influent VSS concentration of 130 mg.l^{-1}. The temperature ranged from 11°C to 28°C.

The SEM analysis revealed three distinct patterns of biomass immobilization on the polyurethane surface similar to those described by Varesche et al. (1997), who worked with a HAIB reactor treating glucose. Fig. 2a shows randomly distributed cells individually attached to the polyurethane surface. Small multi-cellular structures attached to the inner polyurethane surface are visible in Fig. 2b. Micro-granules probably immobilized mechanically or originating from an advanced colonization phase, are shown in Fig. 2c. The micro-granules displayed mean size of 500 μm. A great diversity of morphologies was observed in all the samples examined. Nevertheless, no significant differences were observed in the distribution of morphologies along the sections of the HAIB reactor. In fact, a single cube of polyurethane foam contained a great variety of organisms, including acidogenic and acetogenic bacteria, besides methanogenic archaea. Thus, each matrix acted as a micro-reactor, in which a complete series of anaerobic process reactions occurred.

The first and the second section of the reactor appeared to present higher concentrations of microorganisms and abiotic material, as well as the presence of extracellular polymeric substances (EPS). Moreover, the samples taken from the third and fourth section were cleaner than those taken in the previous stages. The incidence of morphologies was quantified according to the number of organisms observed by optical microscopy and classified as predominant, frequent, less frequent and rare (Table 1).

The microscopic analyses of samples taken in the first week showed an initial colonization step in which rods and vibrio, cocci, filaments, rods in chains and spirochetes predominated. These microorganism morphologies are generally associated with the hydrolytic and fermentative phases of anaerobic degradation. No methanogenic-like microorganism morphology was found in these samples, nor was a significant reduction of the filtered COD or the presence of methane in the biogas found during this phase. Therefore, the total COD removal achieved was attributed to the physical retention, solubilization and hydrolysis of suspended solids.

Fig. 3a illustrates the initial phase of biofilm formation. In this phase, rods attached directly to the nude surface of polyurethane foam predominated. Cocci attached to the rods were also found (Fig. 3b). Wherever rods were attached to the polyurethane surface (Fig. 3c), the presence of extracellular polymeric substances (EPS), or a similar material, was clearly observed. This fact led to the conclusion that such material participates in the first phase of biofilm formation. These results were similar to those observed by Ribeiro et al. (2003), who used horizontal differential reactors fed with glucose-based wastewater.

Rods and cocci were the predominant morphologies observed from the 2nd to the 6th week, mainly in the regions of the first phase of colonization. On the other hand, filaments, vibrios, and cocci in chains were often found, in regions in advanced stages of colonization, showing highly diverse morphologies. Archea-like cells were rarely found, probably due to the low filtered COD removal efficiency (10%) and high volatile fatty acids concentration (52 mg HAc.l^{-1}).

Rods, cocci-like archaeal cells, showing F420 auto-fluorescence, were observed in the samples taken from the 7th week onwards. Rods and cocci were found mainly in regions where the biofilm was in an advanced stage of development. Spirochetes, vibrios and oval rods were observed attached to the polyurethane surface. With regard to the biomass immobilization patterns, micro-granules were often found retained physically on the pores of the polyurethane foam. At this time, the reactor displayed a high morphological diversity and an increase in the COD removal efficiency (20%). Moreover, the methane content in the biogas was approximately 50% and the VFA concentration decreased to 40 mg HAc.l^{-1}.

The morphological diversity had increased by the 10th week of operation. Rods (Fig. 3d), *Methanosaeta* sp.-like archaeal cells (Fig. 3e), cocci (Fig. 3f), spirochetes, filaments, fluorescent rods and cocci were present. *Methanosaeta* sp.-like archaeal cells predominated over *Methanosarcina sp.*-like archaeal cells. This finding is congruous with that of Gujer and Zhender (1983), who observed that specific growth rates of *Methanosaeta* sp.-like cells were higher than those of *Methanosarcina* sp-like cells when the acetate concentration in the environment was lower than 55 mg.l^{-1}. Such a VFA concentration was never reached during the monitoring period of the HAIB reactor reported here. The microorganism morphologies observed in the 10th week of operation were the same as those found on day 90 (approximately 13th week), when an apparently dynamic equilibrium was reached.

Table 1 - Morphological incidence during the experimental phase

Morphologies	Time (Week)								
	1	2	3	4	5	6	7	8	10
Rods	P	P	P	P	P	P	P	P	P
Cocci	F	F	F	P	P	P	P	P	P
Filaments	LF	F	F	F	F	F	F	F	P
Vibrio	LF	LF	F	F	P	F	F	F	F
Cocci in chains	-	LF	LF	LF	LF	LF	LF	LF	LF
Methanosaeta sp.- like	-	-	-	-	-	-	-	R	P
Methanosarcina sp.- like	-	-	-	-	-	-	R	-	LF
Spirochetes	-	-	-	-	-	-	LF	F	F
Fluorescent rods	-	-	-	R	LF	LF	F	F	F
Fluorescent cocci	-	-	-	-	-	R	F	F	F

Incidence: (P) Predominant; (F) Frequent; (LF) Less Frequent; (R) Rare; (-) Not observed

Additional morphological studies were conducted on samples taken at the end of the first and second year of operation of the HAIB reactor and the diversity and patterns of biomass immobilization were the same as those found up to 10 weeks of operation. Therefore, this period of time sufficed to engender the formation of a highly diversified and structured biofilm. In fact, Zaiat et al. (2000) observed a start-up period of 8 weeks in this HAIB reactor treating domestic sewage. Fluctuations in the COD effluent values occurred up to the 8th week of operation, after which they decreased continuously and the methane percentage in the biogas remained stable at 67 ± 2%.

According to the results of biomass retention (Fig. 4), the first stage of the reactor contained a larger amount of biomass than the others, probably due to the higher concentration of substrate and the physical retention of suspended solids inside the polyurethane foam pores. The framework of the polyurethane foam and its high porosity appear to provide good conditions for biofilm development, particularly due to the high superficial area and protection against shear stress.

The first and the second stages contained larger amounts of abiotic material and with the apparent presence of extracellular polymers. This material and the increased biomass retention contributed to hydrodynamic problems that resulted in the reduction of the useful volume and affected the reactor's overall performance. After 10 weeks of operation, the biomass retained in the first stage increased to 8.6 mg TVS.ml^{-1} foam, while values of less than 3 mg TVS.ml^{-1} were observed in stages II, III and IV. At the end of the first and second year of operation, the maximum amount of biomass retained in the polyurethane foams was approximately 35 mg TVS.ml^{-1} foam.

By the 90th day of operation, a high percentage of methane in the biogas (75%), high COD removal efficiency (80%) and high alkalinity production were observed. In this operational phase, the effluent COD values were about 100 mg.l^{-1} and 75 mg.l^{-1}, respectively, for non-filtered and filtered samples, with a VSS concentration of 20 mg.l^{-1}. The apparent dynamic equilibrium was characterized according to the values of methane in the biogas, COD removal efficiency and VSS in the effluent, which presented oscillations of less than 15%. However, after two years of operation, the non-filtered COD in the effluent was 170 mg.l^{-1} and the effluent VSS concentration was 30 mg.l^{-1}.

To discover what was happening, a hydrodynamic test was performed involving stimulus-response assays, using eosin Y and blue dextran as tracers. The main cause for the HAIB reactor's reduced performance after a period of long-term operation was found to be associated with hydrodynamic problems caused by bed clogging. A 75% decrease in the useful volume in relation to the bed without biomass was observed. This clogging led to a reduction in the operational autonomy, requiring back-washings to correct the problem. However, this bed-cleaning procedure failed to restore the reactor to its previous performance levels.

Electronic microscopy analyses revealed an increased amount of abiotic material and EPS during the period corresponding to the reactor's reduced performance. Therefore, the decrease in performance was due not only to the reduction of useful volume but also to the accumulation of EPS and abiotic material, causing mass transfer and kinetic limitations.

A mass balance showed that clogging was caused mainly by the production of solids inside the reactor and, to a much lesser extent, by the entrance of suspended solids in the reactor. Studying the influence of the substrate on the anaerobic biofilm formation on polyurethane foam matrices, Ribeiro (2001) observed that the extracellular polymer production was related with the source of the substrate. This author also stated that the production of extracellular polymers per volume of bed increased even when the retention of biomass remained constant, especially in the case of complex synthetic wastewater with protein, carbohydrate and lipids. Therefore, bed clogging after the HAIB reactor's long-term operation seemed to be related mainly with the excretion extracellular polymers. This material accumulated in the bed interstices, causing reduction of the working volume, channeling, and other hydrodynamic anomalies.

The concentration of biomass after two years of operation (35 mg TVS.ml^{-1} foam) seemed to be unrelated with these operating problems. Bolaños et al. (2001) and Oliveira et al. (2004), working with bench-scale HAIB reactors treating phenol and formaldehyde, respectively, observed neither clogging-related problems nor the production of extracellular polymers. The biomass concentrations were 48 and 44 mg TVS.ml^{-1} foam in the reactors treating phenol and formaldehyde, respectively. Those values were higher than that observed in this study after two years of operation.

The different behavior presented by the HAIB reactor treating domestic sewage and toxic substrates indicated that the production of extracellular polymers should be higher when the HAIB reactor was fed with easily degradable substrates. The production of extracellular polymer associated with filamentous microorganisms and suspended solids that remained in the interstices of the polyurethane foam were the causal factors of clogging in the fixed-bed reactor.

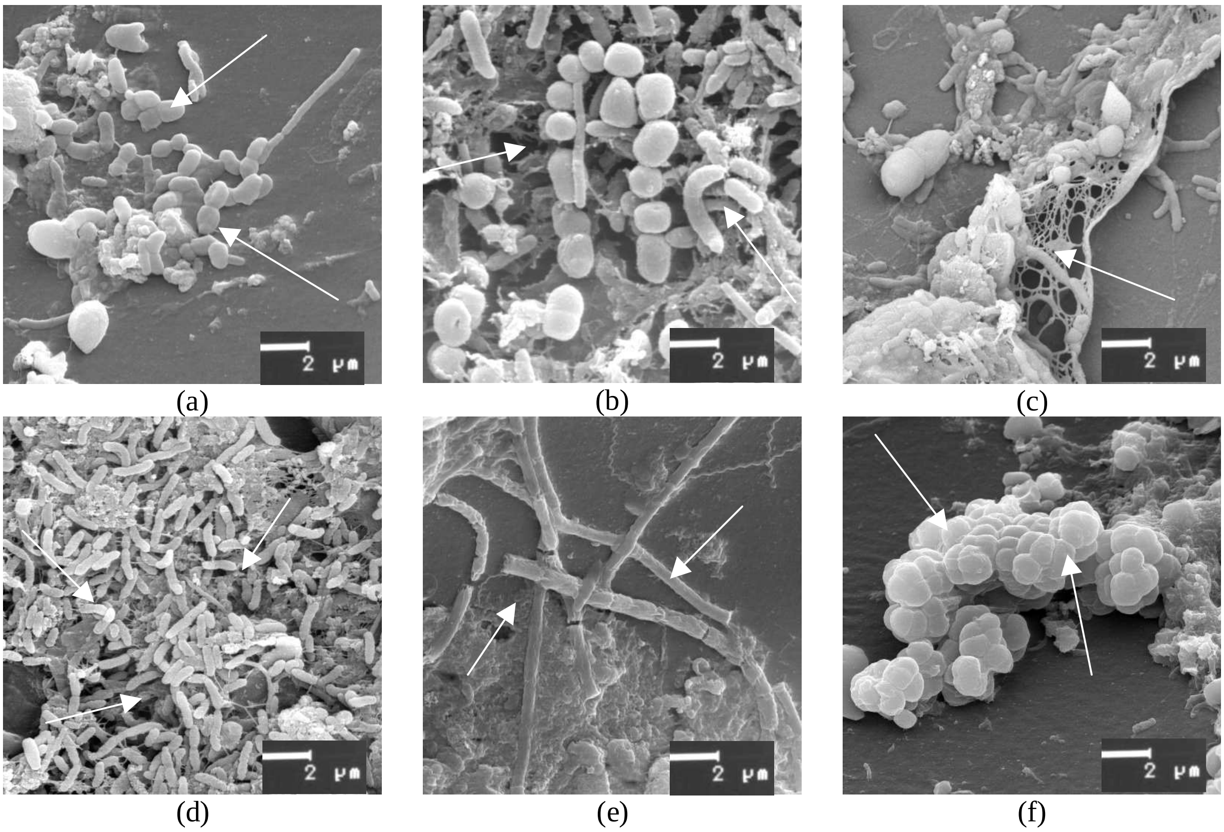

Figure 3 - Morphological incidence. (a - rods attached individually to the polyurethane surface; b - cocci attached to rods; c - Extracellular polymeric substance; d - rods; e - *Methanosaeta*-like, f - cocci).

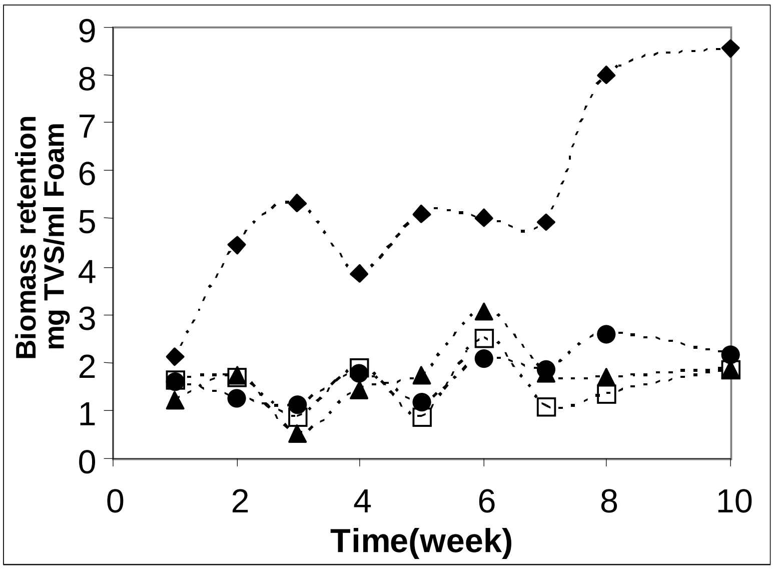

Figure 4 - Biomass retention during the start-up period (Stages of the HAIB reactor ♦ I; • II; ▲ III; □ IV).

The operation of the HAIB reactor for domestic sewage treatment must be reevaluated in order to increase the useful life of the polyurethane foam bed, since the bed-cleaning procedures adopted in this work proved ineffective. Operating with alternating stages could prove to be a successful procedure. For example, assuming a five-stage reactor, the first stage is changed every month successively from the second to the fifth stage. Thus, after five months of operation, the first stage, which shows the greatest accumulation of material, becomes the fifth stage and the accumulated organic material can be biologically degraded, since the concentration of substrate at the end of the reactor is very low.

Hydrodynamic problems such as bed clogging and channeling occurred in the fixed-bed reactor, mainly due to the production of extra cellular polymeric substances and accumulation in the interstices of the bed. This material accumulated mainly in the first and second stages of the reactor, resulting in the deterioration of its performance, which leads to the system's subsequent collapse.

The first stage presented the highest biomass retention, 8.6 mg $TVS.ml^{-1}$ foam in the 10^{th} week, while values lower than 3 mg $TVS.ml^{-1}$ were observed in the other stages. After two years of operation, a maximum biomass retention value of 35 mg $TVS.ml^{-1}$ was recorded.

CONCLUSIONS

These results demonstrated the importance and potential usefulness of monitoring the dynamics of biofilm formation during the start-up period. Rods and cocci were the main organisms attached directly to the foam and responsible for the initial colonization phase. An increase in the morphological diversity was observed around the 10^{th} week of operation. This diversity was an important indication of the end of the start-up period, which was also confirmed by the process monitoring parameters such as COD, methane content in the biogas and effluent volatile fatty acids concentration.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support received for this work from FAPESP- Fundação de Amparo a Pesquisa do Estado de São Paulo (Brazil) and the grant received from CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brazil).

RESUMO

Este trabalho apresenta um estudo morfológico de microrganismos aderidos à espuma de poliuretano

em reator anaeróbio horizontal de leito fixo (RAHLF), aplicado ao tratamento de esgoto sanitário. O processo de colonização do suporte pela biomassa anaeróbia e as características morfológicas das células aderidas foram monitorados durante o período de partida do reator. Bacilos e cocos não fluorescentes foram predominantes no processo de aderência direta à espuma de poliuretano. Aumento na diversidade biológica foi observado a partir da 10ª semana de operação do reator, com predominância de bacilos, cocos e arqueas metanogênicas semelhantes a *Methanosaeta*. Problemas hidrodinâmicos, tais como formação de canais preferenciais e colmatação do leito, foram observados no reator, principalmente devido ao acúmulo de polímeros extracelulares nos interstícios do leito. Este material acumulou principalmente nos estágios iniciais do reator, causando a diminuição gradativa do desempenho do processo anaeróbio de conversão da matéria orgânica. Os resultados demonstraram a importância e utilidade do monitoramento da dinâmica de formação do biofilme durante o período de partida de reatores, com resultados positivos para identificação de problemas operacionais.

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Received: March 25, 2004;
Revised: November 05, 2004;
Accepted: August 05, 2005.