Image analysis to quantify morphological changes in granular sludge induced by nitrate in EGSB reactors

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Abstract Four EGSB reactors (R1 to R4) were operated at an organic loading rate of 10 kgCOD/(m^3.d), hydraulic retention time of 3.6 h, upflow velocity of 10 m/h and different influent nitrate concentrations (0, 20, 60 and 100 mgN-NO$_3^-$/L for R1, R2, R3 and R4, respectively. After about 20 days of operation, the granular sludge was characterized for: (i) morphology by quantitative image analysis; (ii) specific methanogenic activity in the presence of acetate, propionate, butyrate, ethanol and H$_2$/CO$_2$; (iii) settling velocity. LfA parameter, defined as the ratio of total filaments length to total projected area, was determined and was sensitive to quantify the different surface morphology, induced by nitrate to the granular sludge. Although the growth of filamentous structures was evident, no significant decrease on the settling velocity was observed, most likely because granules grew during the trial period. A higher washout occurred in R4, as compared to R1, R2 and R3.

Keywords Image analysis, EGSB reactors, granular sludge stability, nitrate

Introduction

The performance of UASB and EGSB bioreactors is highly dependent on the quality of the biomass used, regarding its specific methanogenic activity, its settling properties and its granular morphology. The application of that kind of reactors to carbon removal in wastewater treatment systems has increased greatly in the last few years, as so has the number of research works covering the development of integrated carbon and nutrient removal processes, including denitrification (Akunna et al., 1992. Hendriksen et al., 1996). From the operational viewpoint, it is important not only to account for the operational parameters, but also for the morphological and physical characteristics of the biomass. Certainly, granules deterioration or excessive growth of filamentous microorganisms can be an indicator of serious operational problems like washout or filamentous bulking. Image analysis allows the quantification of several of those morphological parameters, like the filamentous length, and the biomass aggregates area, among others. The LfA parameter (defined as the ratio between total filament length and total area of aggregates) has been used to estimate the state of the biomass during a granulation process (Araya Kroff et al., 2004). The presence of nitrate in the feed induces the growth of denitrifying bacteria with filamentous characteristics. These microorganisms can originate low-density aggregates, or destabilisation of dense-granule sludge beds (Bhatti et al., 2001). The aim of this work was to use image analysis to quantify morphological changes in anaerobic granules exposed to nitrate, in EGSB reactors. Specific methanogenic activity of the biomass in the presence of various individual substrates (acetate, propionate, butyrate, ethanol and H$_2$/CO$_2$) were also carried out.

Materials and Methods

Reactor operation. Four experiments were set up, using four identical 1,4 Liter EGSB reactors (R1 to R4). The reactors were constructed in plexiglas with 2,2 m height, 2.8 cm internal diameter and an external jacket for water circulation, to keep the temperature constant at 37ºC. The four reactors were inoculated with the same amount of biomass and were operated at identical organic and hydraulic conditions: organic loading rate of 10 kgCOD/(m^3.d), hydraulic retention time of 3.6 h.

The upflow velocity was kept at 10 m/h by means of an external recirculation. The substrate concentration was 1500 mg COD/L and was composed of 30% acetate, 60% propionate and 10% ethanol. Alkalinity was supplied in the form of Na_2CO_3 and $CaCO_3$, in a total of 3 g/L. In each experiment, the sole different operating condition applied was the presence of different nitrate concentrations in the feed (0, 20, 60 and 100 $mgN-NO_3$/L for R1, R2, R3 and R4 respectively). In order to keep a favourable environment for methanization over denitrification, the COD:N ratio was kept over a value of 15 .

Analytical Methods. Volatile fatty acids (VFA) were measured by HPLC. Specific methanogenic activity (SMA) were performed in batch assays using a pressure transducer technique described elsewhere (Colleran et al., 1992), and were evaluated against different individual liquid substrates: acetate, propionate, butyrate and ethanol, and H_2/CO_2 (80:20 v/v). Methane, CO_2 and N_2 were measured by gas chromatography with Helium as the carrier gas and a TCD detector. Volatile suspended solids (VSS) were determined according to Standard Methods (1989). Sequential Injection Analysis (SIA) was used to measure nitrate concentrations in all the experiments. The cadmium reduction method was used to determine nitrate. The method sensibility ranges from 5 $mgN-NO_3^-$ to 100 mg $mgN-NO_3^-$. Settling velocity was individually measured for 100 particles of each sample in a water column of 30 cm.

Image acquisition and analysis. Filament image acquisition was accomplished through phase contrast microscopy on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100x magnification. Images used to quantify aggregates larger than 0.2 mm in equivalent diameter were acquired through visualisation on an Olympus SZ 40 stereo microscope (Olympus, Tokyo) with a 40x magnification. Images used to quantify aggregates smaller than 0.2 mm in equivalent diameter were acquired through visualisation on a Zeiss Axioscop microscope (Zeiss, Oberkochen) with a 100x magnification. All the images were digitised and saved with the help of a CCD AVC D5CE Sony video camera (Sony, Tokyo) and a DT 3155 Data Translation frame grabber (Data Translation, Marlboro), with a 768 x 576 pixel size in 8 bits (256 grey levels) by the Image Pro Plus (Media Cybernetics, Silver Spring) software package. 100 images in average per sample were acquired. Three programs, developed by Amaral (2003) for image analysis and processing, are detailed in Araya Kroff et al. (2004): 1) The Filament program that determines the total filament length present in images acquired through microscopic observation with a 100X magnification. The dispersed bulk filaments, but also those that are attached to an aggregate were quantified. 2) The Micro-aggregates program that determines the Feret diameter of aggregates smaller than 0.2 mm, also applied to images acquired through microscopic observation with 100X magnification, and 3) the Macro-aggregates program that determines the Feret diameter of aggregates larger than 0.2 mm, present in images acquired through visualization on a stereo microscope (15X magnification). LfA parameter (defined as the ratio between total filament length and total area of aggregates) already used to estimate the state of the biomass during a granulation process (Araya Kroff et al., 2004) was applied to the granular sludge characterization.

Results and discussions
During the operation of R1 and R2, the COD removal efficiency was stable around 98% for both reactors, while for R3 and R4 it decreases in the case of acetate and propionate: around 85-90% at the beginning and 90% at the end of the operation. This is in concordance with the SMA results that showed a reduction in biomass activity in presence of these substrates. Biogas composition was similar for R1 and R2, but a significant difference was observed in the Nitrogen content of R3 (30%) and R4 (40%), indicating the development of a denitrification process inside the reactors, more evident in R4 that was fed with the highest nitrate concentration. Furthermore, it showed that the denitrification process was developing at different degrees regarding to the nitrate concentration in the feed. Nitrous oxides were not detected in the gas phase, during all the experiments. The SMA of the biomass showed variations during the experiments. Table 1 summarizes the values of SMA in the presence of different substrates for samples of biomass taken from the inoculum and from the four reactors at the end of operation. All the values are presented in $mLCH_{4@PTN}$/gVSS.d.

Table 1 - SMA values obtained for the inoculum and for the sludge at the end of the operation of R1, R2, R3 and R4 (mLCH4$_{@PTN}$/gVSS.d).

	Acetate	Propionate	Butyrate	Ethanol	H$_2$/CO$_2$
Inoculum	773,8±22,5	117,4±12,1	51,5±6,5	1074,3±17,0	1327,5±312,4
R1	777,3±24,6	175,5±19,1	79,4±14,1	1132,9±55,5	1184,3±35,4
R2	676,5±115,0	121,7±23,3	90,2±5,7	921,4±20,3	1119,9±31,0
R3	429,6±52,8	114,0±17,3	60,4±20,0	476,9±12,2	732,9±111,9
R4	394,8±4,0	122,0±10,8	31,7±5,2	575,3±38,4	807,2±28,8

The changes in the SMA values could be related with the development of the denitrification process, although the COD:N ratio was kept over 15. SIA analysis revealed a nitrate removal around 80% for R3 and R4 reactors (data not shown). A clear decrease in the specific acetoclastic activity was observed with the increase in the nitrate concentration in the feed. This can be due to the establishment of a denitrifier population that out-compete with the methanogens for acetate in the reactors, giving rise to a consortium with a lower methanogenic acetoclastic activity. A similar pattern was observed for ethanol and H$_2$/CO$_2$, but was not so evident when in presence of propionate and butyrate. The fact that the activities changed in different ways may suggest that the phenomena involved were more complex than a simple competition for substrate. Some kind of inhibition by nitrate, nitrite or other intermediates (although not detected) could be responsible for the differentiated response of the different trophic groups to nitrate.

While no significant changes in the granules morphology or in the overall reactors performance were detected in R1 and R2, a clear change of the granules surface was evident in R3 and R4: from a sharp defined surface (Figure 1A) to an irregular one (Figure 1B) due to the growth of filamentous structures (Figure 1C). Image analysis results showed an increase in the filamentous structures in both reactors, but more evident in R4 were a higher nitrate load was applied. The growth of these filamentous structures provoked changes in the LfA from 11.4 mm^{-1} for the inoculum, to 25.2 mm^{-1} and 50.6 mm^{-1} for the sludge taken at the end of R3 and R4 operation, respectively. Changes in the total area and filament length were also detected, varying in different ways in R3 and R4. Both reactors showed an increase in the diameter of the granules, which was more evident in R4. The LfA was a good indicator of the changes induced to the biomass during the operation of the reactors.
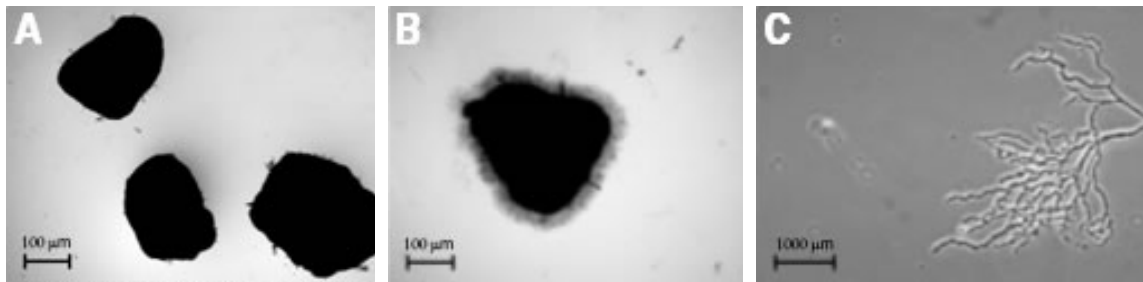

Figure 1 – (A) Stereomicroscope image (15x) from granules on the inoculum used for all the experiments. (B) Stereomicroscope image (15x) of a granule from R4. (C) Microscope phase contrast image (100x) of the filament structures developed in the biomass of R4.

LfA values showed a raise accordingly to the increase in effluent VSS content in R3 and R4. Although the effluent VSS values in R1 and R2 were negligible, in R3 and R4 they ranged from 0.02 to a maximum of 0.07 gVSS/L and from 0.02 to a maximum of 0.14 gVSS/L, respectively. Table 2 summarises the average effluent VSS concentrations of each reactor as well as the LfA values and the corresponding settling velocities. The settling velocities profile for reactors R1, R2 and R3 did not differ much from the inoculum profile. R4 showed a different profile with smaller values of maximum and mean values. It also showed a reduction in the percentage of granules with higher settling velocities, probably due to the change in granule density provoked by the growth of

the filamentous structures. Figure 2 shows settling velocity histograms for the inoculum and the sludge in R4 at the end of the operation.

Table 2 – Nitrate concentration, LfA values range of effluent VSS concentrations and maximum, minimum and average values of the settling velocity.

Reactor	Nitrate (mg/L)	LfA (mm^{-1})	VSS out (gVSS/L)	$V_{settling}$ (m/h)		
				Mean	Max	Min
Inóculum	-	11,4	-	51,5	107,8	12,0
R1	0	13,9	-	52,0	103,3	14,8
R2	20	45,5	-	52,7	114,2	17,3
R3	60	25,2	0.02-0.07	55,6	107,9	15,5
R4	100	50,6	0.02-0.14	45,9	97,5	13,0
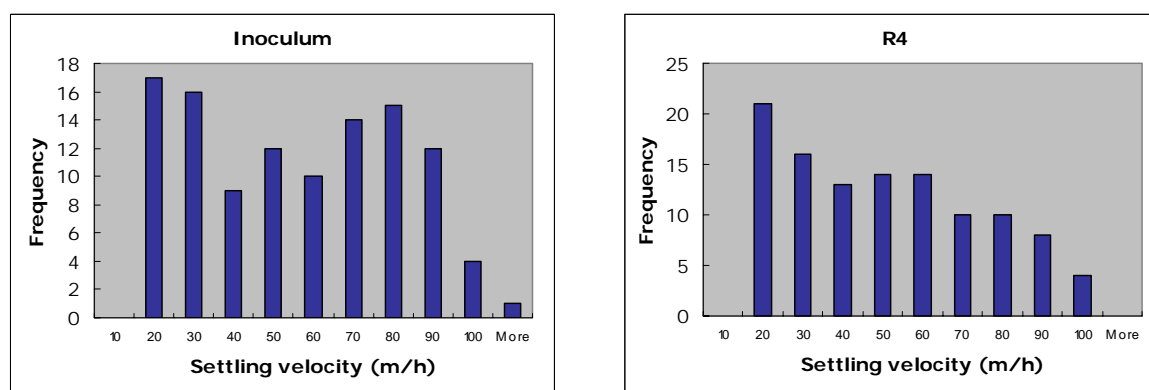
Figure 2 – Settling velocity histograms for the inoculum and for the sludge R4 at the end of the operation. Profiles were calculated over measurements of 100 granules of each sample.

Conclusions

The SMA measurements showed that the presence of nitrate affected in different ways different trophic groups of the anaerobic consortia.

The filaments length to aggregates area, LfA parameter, was sensitive to detect the morphological changes in the granular sludge, induced by the presence of nitrate at different concentrations. Although the growth of filamentous structures was evident, no significant decrease on the settling velocity was observed, most likely because granules grew during the trial period. A higher washout occurred in R4, as compared to R1, R2 and R3.

References

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