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Early stages in biofilm development in methanogenic fluidized-bed reactors

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Summary. Biofilm development in methanogenic fluidized-bed reactors with sand as the carrier was studied on a laboratory scale. The microorganisms present in consecutive layers of the biofilm of mature sludge granules were preliminarily characterized on the basis of their morphology, element composition and adhesion capacity and were compared to bacteria which take part in the initial colonization of sand. The early phase of biofilm development was monitored with reactors receiving waste-waters containing different mixtures of volatile fatty acids and inoculated with fluidized-bed reactor effluent for different lengths of time. The results obtained indicate that facultative anaerobic bacteria abundantly present in the outermost biofilm layers of mature sludge granules are probably the main primary colonizers of the sand. *Methanothrix* spp. or other methanogens were rarely observed among the primary colonizers. The course of biofilm formation was comparable under the various start-up conditions employed including variations in waste-water composition, inoculation and anaerobicity. However, omission of waste-water and thus of substrate resulted in rapid wash-out of the attached biomass.

Introduction

The disadvantage of the lower rate of purification of industrial waste waters in anaerobic as compared to aerobic systems can be overcome by the use of retained biomass systems. Examples of such systems are the upflow anaerobic sludge blanket reactor (Lettinga et al. 1980), the fixed-film reactor (Henze and Harremoës 1983) and the fluidized-bed (FB) reactor (Heijnen et al. 1986). In an FB reactor, bacteria are retained by biofilm formation on the mobile support material. The FB system has several advantages compared to other retained biomass systems, for instance a higher amount of biomass retention, less accumulation of inert sediment and better mass transfer to the biofilm (Heijnen et al. 1986).

Due to the very slow growth of the bacteria involved, start-up times of retained biomass reactors tend to be quite long, being in the range 2–9 months (Henze and Harremoës 1983; Switzenbaum and Jewell 1980; Switzenbaum et al. 1985). Start-up of FB reactors has been studied on a laboratory scale (Denac and Dunn 1988; Gorris et al. 1988, 1989) as well as on industrial scale (Enger et al. 1986; Heijnen 1983; Heijnen et al. 1986). These studies dealt with the impact of a number of physical and chemical factors (inoculum, waste-water composition, organic load, hydraulic retention time, toxic compounds) on the development of biofilms and on reactor performance under steady-state conditions.

The very first stages of biofilm development, starting with the colonization of the initially bare sand grains, have so far not been approached experimentally. This paper reports the results obtained by characterizing the bacteria involved in initial colonization and early biofilm formation.

Materials and methods

Experimental conditions. Start-up experiments were performed with lab-scale upflow FB reactors as described earlier (Gorris et al. 1988, 1989). The reactors used in the present study had a total volume of 100 ml and a sludge-bed volume of 80 ml. They received a synthetic waste-water at an organic loading rate of 1 g chemical oxygen demand (COD)/day. The organic fraction of the waste-water consisted of acetate, propionate and butyrate (3:1:1, w/v). Salts, minerals and vitamins were included as described previously (Gorris et al. 1989). Deviations from these standard conditions are given in the text. To initiate colonization of the bare sand grains, the starting reactors received 45 ml/h of effluent from a fully operative FB reactor for a given period of time and the same amount of synthetic waste-water. The effluent was replaced by tap-water when inoculation was stopped.

Analytical procedures. Quantification of the amount of biomass at-

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tached to sand grains in the developing FB reactors was by use of the lipid-phosphate assay of White et al. (1979a, b). As methane production of starting FB reactors is below the detection limit in direct gas-chromatographic analysis for at least the first 10 days of operation, the methanogenic activity of sludge sampled at this early stage was assessed indirectly by use of a batch assay, employing a mixture of volatile fatty acids as substrate according to Gorris et al. (1989).

Measurement of adhesion. Adhesion of bacteria was quantified by the assay of Walach and Pirt (1986), except that absorbance was measured at 420 nm. Also, the amount of cells adhering to the glass surface in the assay was determined directly by measuring the absorbance of suspensions obtained after refluxing ten times with 1% ethylenediaminetetraacetate (EDTA).

Mechanical treatment of sludge granules. Sludge granules were suspended in water and swirled on a Vortex for 10 s. After decanting (fraction 1) the granules were subjected to three consecutive treatments (fractions 2 to 4).

Electron microscopy. Samples were suspended in water and vortexed. Droplets of the resulting suspensions were applied on copper grids and air dried. With granules collected from starting reactors, it was necessary to add glass beads while vortexing in order to remove the adhering cells adequately. Samples were examined with a Philips EM 201 (Philips, Eindhoven) transmission electron microscope (TEM).

X-Ray microanalysis. The analysis was carried out with a Philips EM 400 T, equipped with a scanning facility and an EDAX PV 9100/65 X-ray analysis system, as described earlier (Heinen and Lauwers 1988). The original data, recorded as counts per second, were expressed as percentages of the total counts of a punctual measurement (5–10 nm). Several elements, such as Al, Si, Mn and Fe, could not be registered since their concentrations in cytoplasm and cells inclusions were not higher than in background readings. Because these values are excluded and only the relevant elements are listed, the sum of the amounts given in Table 2 is less than 100%.

Results and discussion

Characterization of inoculum and mature biofilms

Effluent from a fully operative FB reactor, containing mature sludge granules, was used as inoculum in the start-up procedure. The reactor effluent contained bacteria torn loose by shear forces of the circulating wastewater within the inoculum reactor. Conceivably, these forces act mainly upon the outermost surface of the granules. Thus the inoculum contained essentially the material from that part of mature FB granules. Mechanical treatment was employed to obtain four suspensions representing consecutive layers of cells from the outermost surface to the innermost parts of the biofilm. Microscopic examination showed that the first two fractions consisted of a mixed bacterial population, with *Methanothrix* present only occasionally. Representative cell types of the outermost layers included rod-shaped cells with electron-dense inclusions at one or both ends, filamentous straight or spiralled forms, cells with dark inclusions in their outer layer and sickle- or double-sickle-shaped cells. In the third fraction, *Methanothrix* appeared to be the predominant organism, frequently accompanied by long, thin or ultrathin filaments, closely attached to filaments of *Methanothrix* cells. In the fourth fraction, cells were found to be increasingly more densely packed and embedded in electron-dense amorphous material, as a result of which individual cells and cell packages ultimately became indistinguishable.

The various fractions were characterized with respect to their methanogenic activity. It was found that the first and second fraction produced only 11%–13% and 17%–21%, respectively, of the amount of methane formed by the third and fourth fractions (0.54 μmol/h). Thus, in accordance with the microscopic observations, the fractions representing mainly the outermost layers of mature granules hardly contained any methanogenic bacteria.

Additional information on the bacterial composition of the outermost biolayer was obtained by using part of the first suspension to inoculate rich culture medium (Medium 1, Balch et al. 1979) either with or without penicillin and incubating these cultures either anaerobically or aerobically at 37° C for 16 h. Following incubation, the optical densities (OD) recorded at 420 nm were compared. In anaerobic medium containing penicillin, an OD_{420} of 0.14 was measured, while values of 0.81 and 0.83 were recorded for anaerobic incubations without penicillin and for aerobic incubations without penicillin, respectively. Apparently, the outermost layer of biofilm consists predominantly of facultatively anaerobic microorganisms.

Fraction 1 was screened for the presence of microorganisms capable of adherence. Only 13% of the total amount of cells was found to adhere to a glass surface (Table 1). A comparable percentage was measured when the non-adhering fraction was subjected to the test for a second time. Diluting the original suspension by about twice and repeating the test yielded adhesion percentages of 24% and 19%, respectively. Electron-microscopic examination revealed that the adhering fractions contained similar morphological forms as found in the outer biolayer. *Methanothrix* was observed very rarely.

Table 1. Adhesion of cells from the outer biofilm layers onto a glass surface

Microorganisms	Optical density at 420 nm						
	In 5-ml pipette (120 mm)[a]				In 10-ml pipette[b]		
	a	b	c	d	40′	80′	120′
Untreated suspension	0.45	0.39	0.21	0.16	0.23	0.23	0.23
Non-adhering fraction	0.39	0.34	0.16	0.13	0.17	0.15	0.14
Adhering fraction	0.06	0.05	0.05	0.03	0.06	0.08	0.09

[a] Test performed with (a) undiluted suspension; (b) non-adhering fraction; (c) sample diluted 2.1 times; (d) non-adhering fraction of the diluted sample

[b] Time dependence of test performed with the undiluted suspension

From these results it may be concluded that among the bacteria that constitute the outermost biolayers of the mature FB granules facultatively anaerobic bacteria predominate and many are capable of adherence.

Characterization of the primary colonizers

To study the potentially colonizing bacteria present in the reactor content selectively, a glass rod was inserted into the sludge-bed of a starting reactor during the first weeks of operation. Subsequently, the rod was removed, whereupon the bacteria attached to it were collected. TEM observations indicated that the attached cells were predominantly of the same morphotypes as the adhering microorganisms in fraction 1.

To study the incipient colonization of sand grains in starting FB reactors, granules were sampled during the first 10 days of start-up and examined by TEM. The bacterial population attached to the surface of the sand grains was found to consist of a limited number of morphologically discernible cell-types. These cells appeared to be identical to those observed in the outer biolayers of mature granules and in the adhering fraction of the outer biolayer suspension. *Methanothrix* was sporadically observed in these samples. The relative frequency of occurrence was found to increase after 5–7 days of operation, although *Methanothrix* was never a prominent representative of the primary colonizers under the prevailing conditions.

Elemental characterization studies (X-ray microanalysis)

In the foregoing experiments, morphological similarities had been found between bacteria present in the outermost biofilm layer of mature granules, cells adhering to the inner surface of pipettes or on a glass rod inserted into the sludge bed and the initial colonizers of the sand grains in starting FB reactors. To obtain additional proof that these cell types were indeed identical, three of the typical morphological forms observed, i.e. rod-shaped cells with black and others with lighter inclusions and ultrathin filaments, were examined with respect to their element compositon by X-ray microanalysis. The sickle- (or double-sickle-) shaped forms and the spiralled filaments were not included in this test.

Table 2. Results of X-ray microanalysis of selected cell types encountered in mature biofilms and capable of adhering to surfaces

Cell-type[a]	Assay location	Sample source[b]	% of total counts of punctual measurement[c]					
			Na	Mg	P	S	K	Ca
A	Cytoplasm	1	10.3	1.3	21.8	—	7.6	—
		2	12.1	—	27.6	—	8.0	—
		3	7.1	—	25.9	—	7.2	—
		4	6.2	4.1	26.9	—	0.5	—
	Inclusion	1	1.6	7.1	43.7	—	8.2	—
		2	1.4	5.7	47.5	—	7.0	—
		3	0.7	9.7	48.9	—	5.7	—
		4	0.9	6.9	47.7	—	8.1	—
B	Cytoplasm	1	1.1	—	17.9	10.1	2.5	3.1
		2	0.8	—	19.7	12.4	2.9	2.7
		3	0.6	—	16.2	9.7	2.3	3.3
		4	1.2	—	18.5	10.7	1.9	2.7
	Inclusion	1	—	—	16.7	16.0	2.2	2.4
		2	—	—	17.5	19.3	2.7	2.5
		3	—	—	15.9	17.2	1.9	1.9
		4	—	—	16.2	16.8	2.4	2.7
C	Cytoplasm	1	3.9	—	8.4	2.4	3.7	—
		2	2.9	—	9.1	2.2	3.3	—
		3	4.2	—	7.9	1.9	2.9	—
		4	3.3	—	8.4	23.0	3.5	—
	Inclusion	1	40.1	—	—	1.4	0.6	—
		2	37.9	—	—	2.3	0.8	—
		3	41.2	—	—	1.9	0.7	—
		4	38.8	—	—	1.2	0.9	—

[a] A, short rods with black inclusions; B, rod-shaped cells with lighter inclusions; C, long, ultrathin (0.2 μ) filaments with less electron-dense inclusions

[b] Suspensions of cells (1) present in the outer layer of mature FB-granules; (2) adhering to a glass rod inserted into the sludge-bed; (3) able to adhere to the inner surface of a pipette; (4) adhering to the surface of sand grains

[c] Elements which were recorded in comparable amounts in all cell types are not listed; —, not detectable

Table 3. Determination of the conversion factor for calculation of biomass values from lipid-phosphate values

Organism	Dry weight of cells (mg)	Phosphate (μg)	Conversion factor
Bacillus caldolyticus	2.72	12.93	0.21
B. flavothermus	4.95	24.63	0.20
B. pumilus	5.25	8.66	0.61
B. subtilis	2.73	11.88	0.23
Pseudomonas fluorescens	2.40	28.40	0.09
P. putida	2.00	17.65	0.11
Streptococcus aureus	3.86	19.80	0.20
Methanosarcina barkeri	4.94	15.24	0.32
Methanobacterium formicicum	7.14	13.70	0.52
M. thermoautotrophicum	5.75	18.53	0.31
Methanothrix soehngenii	7.37	19.80	0.37
Mature FB granules	5.26	16.04	0.33

The analytical data obtained in characterizing separately the cell cytoplasm and the intracellular inclusion of the three cell types present in the four different suspensions are compiled in Table 2. Comparing the individual cell types, convincing element similarities are indicated, with the exception of a very low K value in one sample (cell type A, cytoplasm, suspension 4). On the other hand, there appear to be sufficient element differences between the three types examined to differentiate between them. The short rods characterized by black terminal inclusions typically contained low amounts of Na and K but high amounts of P in their cytoplasm. The inclusions were low in Na, high in Mg and K and extremely high in P content and most probably represent polyphosphate granules. The rods with the lighter inclusions contained very little P, some Ca and quite high amounts of S. Also, there were low but persistent amounts of Cl detectable (not shown in Table 2). The inclusions differed from the cytoplasmic site mainly by a total absence of Na and by higher S values. The thin filaments could be recognized by the findings that the concentration of all elements assayed for, most typically P, was low, that Cl was present (not shown) and that Ca was absent. The inclusions were characterized by a total absence of P and by extremely high Na values. None of the elements detected by X-ray microanalysis was present in sufficient amounts to function as the matching counterpart for the Na, so that the composition of these inclusions cannot be deduced.

In conclusion, the morphological similarities observed between several of the cell types present in the four samples are corroborated by the data on their element composition.

Quantitative aspects of initial biofilm formation

Because the options approached thus far supplied descriptive information only, quantitative aspects of biofilm development during the start-up phase were assessed by the lipid-phosphate assay. White et al. (1979a, b) originally employed this assay for enumeration of microorganisms in sediments. To calculate biomass data from the results of the assay, they used a conversion factor which describes the ratio between the lipid-phosphate content and the dry weight of the bacterial cells. By analysing known amounts of a number of different pure microbial cultures, a ratio of 0.21 mg dry weight/μg phosphate was obtained. Since this ratio was obtained by analysing mainly soil microorganisms, it might not be valid for bacteria in the anaerobic biofilm, the majority of which are methanogenic in the case of mature granules (Gorris et al. 1988, 1989). A number of pure cultures of anaerobic methanogenic strains, some other bacteria not commonly prevalent in soil samples as well as typical soil bacteria were therefore analysed (Table 3).

Although the deviations were rather substantial, the non-methanogens examined indeed yielded a mean conversion factor of, on average, 0.21. The results obtained with the methanogenic species were more consistent, rendering a conversion factor of 0.37 as the mean value. The latter value is probably the appropriate conversion factor when samples from mature methanogenic FB reactors are under examination. Since our studies were directed towards the early stages of biofilm development in which methanogens apparently do not play a major role, as indicated by the results discussed above, we employed the value of 0.21 to calculate the biomass data in the following experiments.

The lipid-phosphate assay was applied to investigate the course of biomass accumulation during the first 2 weeks of start-up of FB reactors on the synthetic waste-water containing different mixtures of volatile fatty acids. The amount of biomass immobilized on the sand increased steadily over the first 10 days of operation, which was the period of continuous inoculation with mature FB reactor effluent (Fig. 1). This indicates that bacteria present in the inoculum actually adhered to the sand grains. Feeding the starting reactors a mixture of acetate and propionate instead of the mixture of acetate, propionate and butyrate had little effect, but with a mixture of acetate and butyrate, biofilm develop-

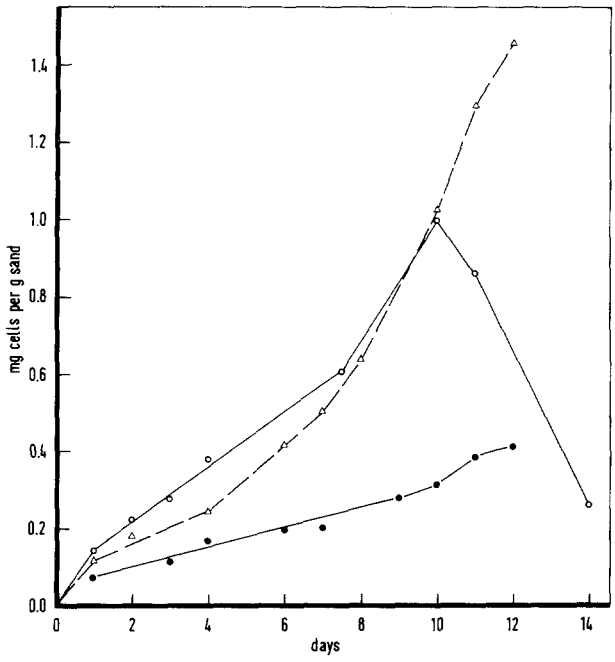

Fig. 1. Biomass increase in starting reactors continuously inoculated with effluent from a steady-state methanogenic FB reactor containing full-grown granules. The reactors received a synthetic waste-water with either (●) a mixture of acetate and butyrate (3:1, w/v), (△) a mixture of acetate and propionate (3:1, w/v) or (○) a mixture of acetate, propionate and butyrate (3:1:1, w/v). In the latter case, the inoculation was stopped at day 10, while the waste-water was replaced by tap-water

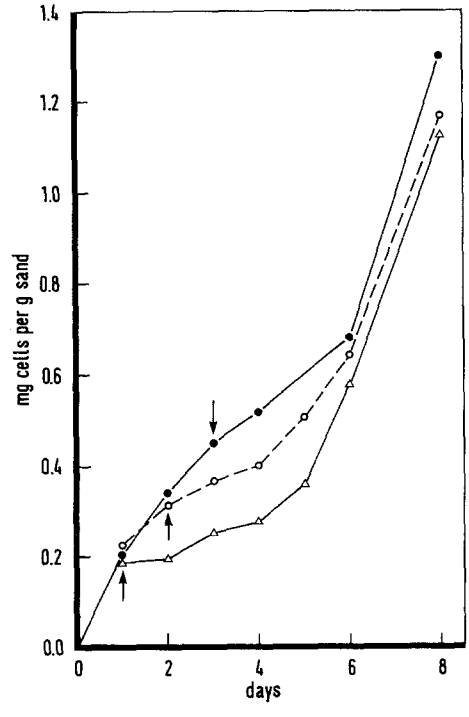

Fig. 2. Effect of terminating the inoculation with FB reactor effluent on the course of biofilm development in reactors fed a waste-water with acetate, propionate and butyrate. Inoculation was ended after (△) 1, (○) 2, or (●) 3 days of operation (*arrows*)

ment was clearly delayed. When the supplies of inoculum and waste-water were stopped and replaced by water only, the biomass immediately diminished. It was almost entirely washed out after 4 days. Further experiments indicated that this was due to a lack of substrate and not to the omission of inoculum (Fig. 2) because stopping the inoculation after 1, 2, or 3 days but maintaining the supply of substrate yielded a comparable pattern of biomass increase in all three reactors. However, a small period of delay, which was shortest after the 3-day inoculation and longest after the 1-day inoculation, was noticed at the moment the inoculum was omitted.

In the preceding experiments, no special precautions were taken to ensure strictly anaerobic conditions during reactor start-up, because the reactor content is probably kept constantly anaerobic by the action of facultatively anaerobic bacteria. However, during the early start-up phase of new reactors there is no detectable methane formation. To investigate whether this might be due to small amounts of oxygen present in the synthetic waste-water, which may affect the colonization process negatively, start-up was performed under strictly anaerobic conditions (Table 4). The colonization rate appeared to be identical under the strictly anaerobic conditions and under the usual start-up conditions.

Although the lipid-phosphate determinations rendered useful data to describe the early phase of biofilm development of starting FB reactors and the TEM examination indicated which cell morphotypes are involved in incipient colonization, both approaches did not address the question of whether the initial build-up of the biofilm is accomplished by methanogenic, non-methanogenic or a combination of both types of bacteria. Yet, this is a major fundamental question with important consequences for large-scale application of the FB concept.

The initial appearance of methanogens in the biofilm community was timed by determination of the methanogenic activity of the developing sludge during the first weeks of operation. Equal amounts of sludge were sampled on consecutive days, beginning 24 h after termination of inoculation from three reactors fed the usual waste-water but inoculated during different

Table 4. Development of biofilm with and without ensuring strictly anaerobic start-up conditions

Days	Biomass (mg dry weight/g sand)	
	Non-anaerobic	Anaerobic
0	0.054	0.073
1	0.121	0.109
3	0.248	0.460
7	0.617	0.780
8	0.708	0.919
9	0.823	0.931
11	1.028	0.956
14	1.228	1.173

lengths of time (2, 3 and 5 days). Methane production was measured after an incubation period of 10 days for the first three samples and 5–7 days for the later ones (Fig. 3). It was found that the samples taken up to day 4 after disconnection failed to produce methane. Most of the samples taken after this time exhibited some methanogenic activity. In all cases, a 5–7 day delay in the onset of methanogenesis was recorded. The rate and total amount of methane formation of the samples taken from the different reactors varied considerably, due to which any direct correlation between the duration of the inoculation period and the time gap between the start of colonization and the onset of methane production was not revealed.

General conclusions

Using a fully operative FB reactor as inoculum source in the start-up procedure, the majority of the microorganisms introduced into a new reactor were found to be facultative anaerobes. These organisms formed the major part of the outer biofilm layers of mature granules in steady-state FB reactors. Morphologically similar organisms were observed among the primary colonizers of the bare sand.

The acetotrophic methanogen *Methanothrix* apparently did not take part in primary colonization although in previous studies (Gorris et al. 1988, 1989) 60%–70% of the total biofilm of granules matured under comparable conditions were found to consist of *Methanothrix* spp. The rather limited role of *Methanothrix* in early biofilm formation may have been due to several causes: (a) small amounts of oxygen present in the synthetic waste-water might have been detrimental for this strictly anaerobic organism, (b) *Methanothrix* might not be able to adhere to the carrier surface directly but instead adheres only to other microbes already attached or (c) since *Methanothrix* is a slow-growing organism, it may have had little chance to establish itself on the sand grains in the presence of fast-growing competitors presumably present in excess in the inoculum. The former two causes are not very likely since facultatively anaerobic organisms were abundant in the inoculum and it was found previously (Gorris et al. 1988) that the biofilm can consist solely of *Methanothrix* spp. when granules mature on a waste-water containing acetate as the sole source of carbon.

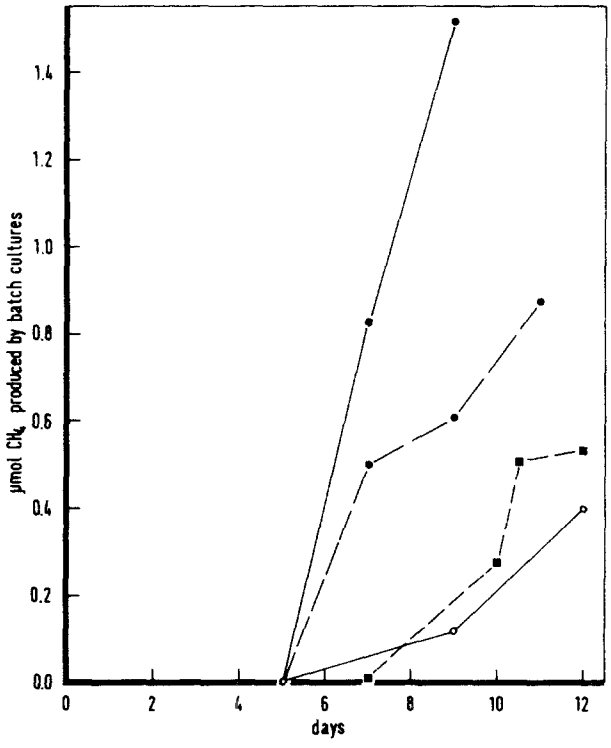

Fig. 3. Methane formation in batch cultures inoculated with sludge sampled from starting FB reactors, R1 to R3, which had received effluent from steady-state reactors for 2, 3 and 5 days, respectively. Sampling started 24 h after ending inoculum supply. (○—○) and (●—●) represent different samples from R1; (●----●) sample from R2 and (■----■) from R3

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