Degradation of Oleic Acid in Anaerobic Filters: The Effect of Inoculum Acclimatization and Biomass Recirculation

M.Alcina Pereira, Manuel Mota, Madalena M. Alves

ABSTRACT: The degradation of oleic acid in anaerobic filters was studied and the effect of an acclimated inoculum and biomass recirculation was evaluated. Three anaerobic filters (R1, R2, and R3) were operated in parallel. The anaerobic filters R1 and R2 were inoculated with nonacclimated biomass, whereas the anaerobic filter R3 was inoculated with acclimated biomass. In the anaerobic filters R2 and R3, biomass settling and recirculation were applied. The use of an acclimated inoculum and biomass recirculation (R3) was beneficial in terms of removal efficiency, which was 4 to 8% higher than in the anaerobic filters R1 and R2 when oleate was the sole carbon source fed to the reactors at an applied organic load of 12.5 kg of chemical oxygen demand (COD)/m^3·d, even with an oleate to calcium and magnesium ion molar concentration ratio of 6.8. Biomass recirculation significantly reduced the biomass washout and the toxic effect on the acetogenic and methanogenic populations. The use of an acclimated inoculum was beneficial in terms of methane yield, which was 50% greater than that observed for the reactors inoculated with nonacclimated inoculum for the highest applied organic loading rate (12.5 kg COD/m^3·d). At the end of the operation, the biomass was encapsulated by a whitish matter, which was well detected by microscopic examination. When this sludge was incubated in batch vials at 37 °C where no substrate was added, methane production from the adsorbed organic matter was evidenced, attaining a maximum value (at standard temperature and pressure) of 39.7 mL/g volatile solids·d for the biomass taken from R1. With stirring (150 r/min), the methane production rate was 13.8 times higher than under static conditions. When oleate was added to this sludge, methane production was delayed, suggesting that adsorbed matter can be an intermediate of oleate degradation such as stearic, palmitic, myristic, or other saturated acids. *Water Environ. Res.*, **73**, 1 (2001).

KEYWORDS: anaerobic filters, long-chain fatty acids, toxicity.

Introduction

Lipids are one of the major components of organic matter in wastewaters. Although domestic wastewater contains approximately 40 to 100 mg/L of lipids, industrial wastewaters are of greater concern when considering the potential toxicity of this type of effluent (Forster, 1992). Along with slaughterhouses and edible oil and fat refineries, dairy industries are important contributors to total lipid emissions (Rinzema, 1988). Although a considerable amount of lipidic matter can be removed by a physicochemical treatment of flotation, the remaining fraction can still be harmful to anaerobic treatment (Hwu et al., 1996).

The main problems associated with anaerobic treatment of lipid-containing wastewater are (1) the adsorption of a lipid layer around biomass particles causing biomass flotation and washout and (2) the acute toxicity of long-chain fatty acids (LCFA) against both methanogens and acetogens, the two main trophic groups involved in LCFA degradation (Koster and Cramer, 1987, and Rinzema et al., 1994).

During the past few years, anaerobic wastewater treatment technology has been markedly improved by the development of the upflow anaerobic sludge blanket (UASB) concept and its application worldwide, along with more recent designs of the expanded granular sludge bed and the internal circulation reactors (Habets et al., 1997). In such systems, biomass immobilization is achieved by self-granulation, a crucial requirement that, when unsuccessful, irreversibly affects the overall performance most of the time. Tentative application of granule-based digesters to lipid-containing wastewaters revealed that, although granular sludge is more resistant to LCFA toxicity than suspended or flocculent sludge, physical stability of granules is problematic for lipid-containing wastewaters. Hwu et al. (1998) studied the adsorption of oleic acid in relation to granular sludge flotation in a UASB reactor and concluded that granular sludge flotation occurred at concentrations far below the toxicity limit. This might suggest that complete washout of granular sludge would occur before inhibition. Furthermore, it is known that the addition of calcium salts to some extent prevents inhibition problems, but has no effect on flotation problems (Hanaki et al., 1981, and Rinzema, 1988).

Sam-Soon et al. (1991) used a UASB reactor to treat oleate as a unique carbon source and reported that the original inoculated granules suffered from disintegration and encapsulation by a gelatinous and whitish mass. In addition, after comparing the performance of several digester configurations treating effluents from an ice-cream factory, Hawkes et al. (1995) concluded that granulation was not viable with this kind of effluent and that the anaerobic filter would be the more appropriate configuration.

In an anaerobic fixed bed reactor, the support medium is a physical protection against washout and is potentially attractive for biomass retention in this particular kind of wastewater.

Alves et al. (2001) recently concluded that, to treat oleate-based effluents, it is advantageous to pre-expose the biomass to lipids. The observed increasing tolerance was interpreted as an acclimation. Therefore, the use of an acclimated inoculum when treating LCFA-based wastewaters should enhance reactor performance in terms of methane yield and resistance to shocks.

On the other hand, it is known that recycling induces a protective effect when dealing with toxic wastewaters (Young, 1991), improving the stability by decreasing the toxic concentration. Furthermore, for LCFA-based wastewaters, the benefit of using biomass recycling on digester performance was reported by Hwu et al. (1997), who found that the washed-out biomass exhibited a
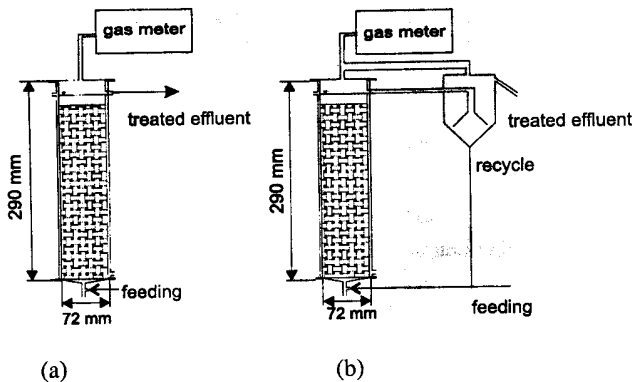
Figure 1—Schematic representation of the experimental set-up: (a) R1, and (b) R2 and R3.

greater oleate degradation capacity than the biomass remaining inside the reactor.

In this study, the degradation of oleic acid in anaerobic filters was studied. In addition, the use of an acclimated inoculum and biomass recirculation was evaluated. Sodium oleate was used as an LCFA model because, in general, it is the most abundant of all LCFA present in wastewater (Komatsu et al., 1991) and one of the more toxic (Galbraith et al., 1971).

Materials and Methods

Experimental Set-Up. Three anaerobic filters (R1, R2, and R3) with equal dimensions were constructed of Plexiglas; these are schematically described in Figure 1. The initial liquid volume was 1 L and the support matrix consisted of polyvinyl chloride Raschig rings 21 mm in size, with a specific surface area of 230 m^2/m^3 and a porosity of 92.5%. Settlers coupled to anaerobic filters R2 and R3 (Figure 1b) were constructed of Plexiglas and had a liquid volume of 200 mL. The substrate was stored at 4 °C to minimize acidification. Temperature was kept constant at 37 ± 1 °C.

Substrate. Initially, the substrate was made by diluting skim milk with tap water and supplementing the solution with macro- and micronutrients. The macronutrient solution was composed of magnesium chloride ($MgCl_2 \cdot 6H_2O$)—25 g/L, potassium ortho-phosphate (KH_2PO_4)—28.3 g/L, and potassium chloride (KCl)—45 g/L. The solution was added at a rate of 0.6 mL/g of chemical oxygen demand (COD) fed. The micronutrient solution was composed of ferrous chloride ($FeCl_2 \cdot 6H_2O$)—2 g/L, zinc chloride ($ZnCl_2$)—0.05 g/L, cupric chloride ($CuCl_2 \cdot 2H_2O$)—0.038 g/L, manganese chloride ($MnCl_2 \cdot 4H_2O$)—0.5 g/L, aluminum chloride ($AlCl_3 \cdot 6H_2O$)—0.09 g/L, cobaltous chloride ($CoCl_2 \cdot 6H_2O$)—2 g/L, nickel chloride ($NiCl_2 \cdot 6H_2O$)—0.092 g/L, sodium selenite ($Na_2SeO_3 \cdot 5H_2O$)—0.164 g/L, ethylenediamine tetraacetic acid (EDTA)—1g/L, Resazurin—0.2 g/L, and hydrochloric acid (HCl, 36%)—1 mL/L. The composition of this solution was based on the work of Zehnder et al. (1980). Micronutrients were supplemented to the influent feed by adding 1 mL/L. To give suitable alkalinity, 5 g sodium carbonate/L of feed were added. Later on, skim milk was gradually replaced by sodium oleate. To achieve a COD/nitrogen/phosphorus ratio of 250:5:1.1, a nitrogen supplement of 98.9 g of ammonium chloride/L of macronutrient solution was added.

Routine Analysis. Routine reactor performance was monitored by measuring influent and effluent total and soluble (centrifuged

10 minutes at 15 000 r/min) COD influent flow rate, effluent volatile fatty acids (VFA), and methane production. Chemical oxygen demand was determined according to *Standard Methods* (APHA et al., 1989). Volatile suspended solids (VSS) concentration was estimated by the difference between total and soluble COD. The conversion from COD to VSS was based on the consideration that biomass can be expressed by the empirical equation $C_5H_7O_2N$, corresponding to 1 g of VSS to 1.42 g of COD. Volatile fatty acids (acetic, propionic, and butyric) were determined by high-performance liquid chromatography using a Chrompack (Middleburg, The Netherlands) column (300 mm × 6.5 mm) and a mobile phase of 5 mM sulfuric acid (H_2SO_4) at 0.7 mL/min. The column was set at 40 °C and the detection was spectrophotometric at 220 nm. Methane content of the biogas was measured by gas chromatography using a Chrompack Haysep Q column (0.149- to 0.177-mm openings), with nitrogen carrier gas at 30 mL/min and a flame-ionization detector. Temperatures of the injection port, column, and flame-ionization detector were 120, 40, and 130 °C, respectively.

Methanogenic Activity, Toxicity, and Biodegradability Tests. Methanogenic activity tests were performed using the pressure transducer technique (Coates et al., 1996, and Colleran et al., 1992). This test involves monitoring pressure increase developed in sealed vials fed with nongaseous substrates or pressure decrease in vials previously pressurized with gaseous substrates hydrogen and carbon dioxide (H_2/CO_2). The nongaseous substrates were acetate, propionate, butyrate, and ethanol. The working volume was always 12.5 mL and the total volume was 25 mL in the case of nongaseous substrates and 70 mL in the case of gaseous substrates. Strict anaerobic conditions were maintained. The hand-held pressure transducer was capable of measuring a pressure increase or decrease of 200 kPa over a range of −200 to +200 mV, with a minimum detectable variation of 0.5 kPa corresponding to 0.05 mL biogas in 10 mL of headspace. A sensing element consisting of a 2.5-mm square silicon chip with an integral sensing diaphragm is connected to a digital panel meter module and the device is powered by a 7.5-V direct current transformer. The anaerobic basal medium, which was made with demineralized water, was composed of cysteine–HCl (0.5 g/L) and sodium bicarbonate (3 g/L); the pH was adjusted to 7.0 to 7.2 with 8 N sodium hydroxide. The medium was prepared under strict anaerobic conditions by boiling and cooling under a gaseous atmosphere of nitrogen and carbon dioxide (80:20 v/v). Sludge and anaerobic buffers were delivered to the vial under strict anaerobic conditions by using a special pipette connected to the same gas and a manifold for flushing all the vials while they were opened. No calcium or trace nutrients were added.

Methanogenic toxicity tests were also performed using a pressure transducer technique (Colleran and Pistilli, 1994). The oleate concentration ranged from 100 to 900 mg/L and, for the toxicity tests, acetate was added as cosubstrate to evaluate the influence of oleate concentration on the acetoclastic toxicity. *Fifty percent inhibition concentration (IC_{50})* was defined as the oleate concentration that caused a 50% relative methanogenic acetoclastic activity loss. All batch tests were performed in triplicate assays.

Biodegradability tests were performed by adding increasing oleate concentrations (100, 300, 500, 700, and 900 mg/L) to the sludge in batch vials. The maximum methane production rate (MMPR), the percentage of methanization (PM) achieved, and the lag phases were all determined. Background methane production due to residual substrate was discounted in MMPR and PM values.

Table 1—Methanogenic activity of the acclimated and nonacclimated inocula.

	Methanogenic activity (mL CH_4/g VS·d)[a,b]				
Inoculum	Acetate	Propionate	Butyrate	Ethanol	H_2/CO_2
Nonacclimated	165.1 ± 23.8	0	0	77.3 ± 16.7	146.9 ± 16.6
Acclimated	20.6 ± 4.7	0	0	9.8 ± 2.0	52.0 ± 6.0

[a] Methane (CH_4) at standard temperature and pressure.
[b] ±95% confidence interval.

Biodegradability tests were performed in duplicate assays. The specific values of degradation rate and PM were obtained by dividing the methane production rates by the volatile solids (VS) content of each vial determined at the end of the experiment.

Seed Sludge Characterization. Two different seed sludges were tested. Anaerobic filters R1 and R2 were inoculated with biomass (25 g VSS/L) from an anaerobic filter fed with skim milk as substrate for more than 900 days. In anaerobic filter R3, the inoculum consisted of biomass (18.4 g VSS/L) acclimated in an anaerobic digester fed with lipids and oleate for more than 400 days (as described in Alves et al., 2001. In each reactor, a volume of 300 mL was inoculated.

Both inocula were characterized in terms of (1) methanogenic activity against acetate, propionate, butyrate, ethanol, and H_2/CO_2; (2) toxicity of oleate to acetoclastic bacteria; and (3) capacity of oleate biodegradation. Concerning the methanogenic activity against propionate, butyrate, and ethanol, it should be noted that, because these substrates are indirect methanogenic substrates, a valid measurement of the maximum specific methanogenic activity against these acids can only be obtained when the acetoclastic and hydrogenophilic activities are not rate-limiting (Dolfing and Bloemen, 1985). In this study, this condition prevailed for all samples. Table 1 summarizes the results of the specific methanogenic activity against the selected substrates.

As shown in Table 1, both inocula had no detectable activity against propionate and butyrate, whereas, with the other substrates, the nonacclimated inoculum exhibited a specific methanogenic activity that was 8 times higher in acetate and ethanol and 3 times higher in H_2/CO_2 than the activities in the acclimated biomass. However, concerning the toxicity of oleate against acetoclastic

bacteria, IC_{50} values of 40 and 250 mg/L were obtained for the nonacclimated and the acclimated inoculum, respectively, evidencing the better resistance of the acclimated inoculum to the toxicant studied.

Results from the biodegradability batch experiments demonstrated that acclimated inoculum caused the oleate to biodegrade faster. The maximum methane production rate and the lag phases were lower for the acclimated inoculum. Both sludges were able to convert at least 70% of oleate into methane in the range of concentrations being studied (Table 2). Thus, the acclimated inoculum was more resistant to the oleate toxicity and exhibited higher rates of methane production when this compound was the sole carbon source added in batch vials. However, its acetoclastic and hydrogenophilic activity was lower than that determined for the nonacclimated inoculum.

Operating Mode. During start-up, the reactors were fed with skim milk (period I). After this period, a mixture of skim milk with sodium oleate was used, with increasing oleate concentrations at a constant organic loading rate (period II), and, in period III, oleate was the sole carbon source fed to the digesters (Table 3). The recycle flow in anaerobic filters R2 and R3 (initially 15 L/d) was doubled after day 224.

Results and Discussion

Operating Performance. Figure 2 presents the operating performance during the three periods. Figure 3 presents the effluent values for acetate, propionate, and butyrate and Tables 4, 5, and 6 summarize the pseudo-steady-state values of the operating conditions and performance data for each period.

Start-up of the reactors was accomplished by gradually increas-

Table 2—Results from the biodegradability batch experiments: maximum methane production rate, percentage of methanization, and lag phases.

|	Oleate concentration in the batch vial (mg/L)				
Inoculum	100	300	500	700	900
Acclimated					
MMPR (mL CH_4/g VS·d)[a,b]	1 ± 2	17 ± 3	30 ± 4	38 ± 3	36 ± 4
PM (%)[b]	80 ± 18	74 ± 11	92 ± 7	79 ± 5	81 ± 3
Lag phases (h)	122	122	142	150	173
Nonacclimated					
MMPR (mL CH_4/g VS·d)[a,b]	1 ± 1	6 ± 1	19 ± 4	21 ± 7	33 ± 7
PM (%)[b]	69 ± 24	92 ± 11	89 ± 13	88 ± 4	83 ± 4
Lag phases (h)	127	132	192	220	295

[a] Methane (CH_4) at standard temperature and pressure.
[b] ±95% confidence interval.

## Table 3—Characteristic operating conditions of each period.

Period	Time (d)	Substrate content (mg COD/L) Skim milk	Substrate content (mg COD/L) Oleate	Comments[a]
I	0–44	2000	—	HRT decrease from 3 to 0.64 days.
	44–80	2000	—	Constant HRT.
	80–94	4000	—	Organic load increase.
II	94–105	3000	1000	
	105–119	2000	2000	Constant loading rate and HRT.
	119–150	1000	3000	
III	150–205	—	4000	
	205–224	—	8000	Oleate organic load increase.
	224–233	—	8000	Recycle flow increase.

[a] HRT = hydraulic retention time.

ing the organic loading until 6 kg COD/m³·d was achieved (80th day) (Figure 2a). From day 94 on, the substrate composition was gradually shifted from skim milk to oleate at a constant organic loading rate of 6 kg COD/m³·d (period II). During these periods, removal efficiencies greater than 90% and effluent acetate and propionate levels less than 130 mg/L were measured in the three
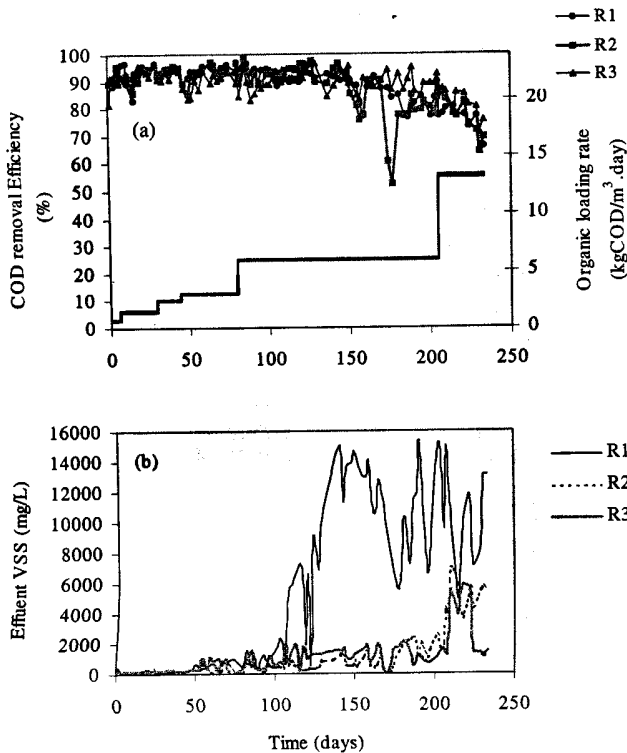
Figure 2—Reactor performance during the three operating periods.
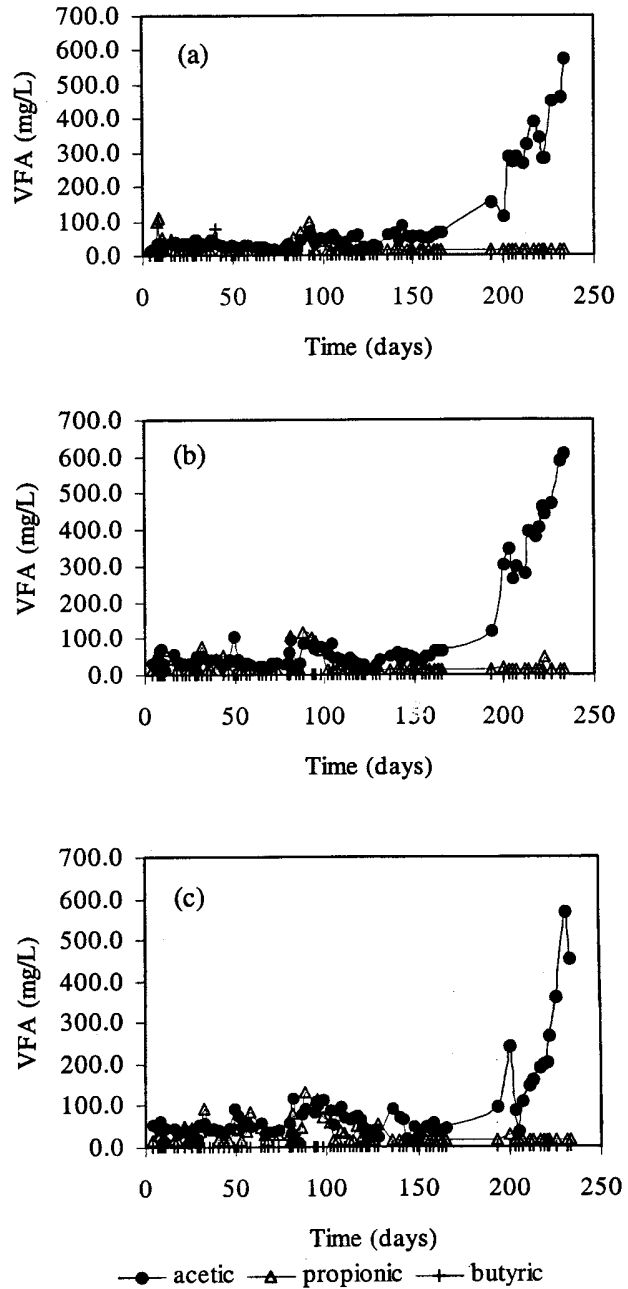
Figure 3—Time course of VFA evolution during the trial period: (a) R1, (b) R2, and (c) R3.

reactors. However, the effluent VS concentration in anaerobic filter R1 were significantly greater than the concentrations in anaerobic filters R2 and R3, attaining an average value as high as 12 000 mg/L at the end of period II. In anaerobic filters R2 and R3, (with biomass recirculation) maximum VSS values detected during the same period were 720 and 1400 mg/L in anaerobic filters R2 and R3, respectively.

During period III, when oleate was the sole carbon source fed to the digesters, a progressive decrease of the removal efficiency and an increase in the levels of VFA, predominantly acetate, was verified in the three reactors (Figures 2 and 3). During this period,

Table 4—Pseudo-steady-state operating conditions and performance data of anaerobic filter R1 during each operating period (±95% confidence interval).

Period	Time (d)	Hydraulic retention time (days)[a]	Influent COD (mg/L)[a]	Organic loading rate (kg COD/m³·d)[a]	Effluent soluble COD (mg/L)	Methane (%)[b]	Biogas (m³/m³·d)[b]	Effluent VSS (mg/L)
I	1-6	3.15 (±0.06)	2200 (±73)	0.70 (±0.03)	198 (±54)	ND	ND	228 (±108)
	6-29	1.54 (±0.02)	↓	1.43 (±0.05)	162 (±60)	48.9 (±11.9)	ND	124 (±63)
	29-44	0.99 (±0.01)		2.22 (±0.08)	146 (±18)	61.5 (±1.2)	ND	186 (±26)
	44-80	0.64 (±0.01)		3.44 (±0.13)	118 (±19)	60.7 (±2.8)	1.22 (±0.10)	649 (±145)
	80-94	↓	3839 (±65)	6.00 (±0.14)	209 (±94)	60.5 (±1.4)	2.39 (±0.14)	798 (±350)
II	94-105		↓	↓	254 (±22)	60.1 (±1.3)	2.57 (±0.11)	1770 (±345)
	105-119				339 (±26)	55.9 (±4.3)	2.26 (±0.07)	5687 (±1681)
	119-150				312 (±25)	65.5 (±3.0)	1.04 (±0.05)	12019 (±1801)
III	150-205	↓		↓	634 (±77)	70.5 (±2.7)	1.05 (±0.09)	11457 (±1296)
	205-233	↓	7979 (±61)	12.47 (±0.22)	2027 (±309)	66.7 (±2.7)	1.22 (±0.06)	9908 (±2050)

[a] Vertical arrow indicates that the last values were kept through the corresponding period.
[b] ND = Not determined.

Table 5—Pseudo-steady-state operating conditions and performance data of anaerobic filter R2 during each operation period (±95% confidence interval).

Period	Time (d)	Hydraulic retention time (d)[a]	Influent COD (mg/L)[a]	Organic loading rate (kg COD/m³·d)[a]	Effluent soluble COD (mg/L)	Methane (%)[b]	Biogas (m³/m³·d)[b]	Effluent VSS (mg/L)
I	1-6	3.29 (±0.08)	2200 (±73)	0.67 (±0.03)	165 (±37)	ND	ND	145 (±39)
	6-29	1.56 (±0.02)	↓	1.41 (±0.05)	181 (±51)	38.4 (±4.1)	ND	184 (±60)
	29-44	1.01 (±0.01)		2.18 (±0.08)	133 (±19)	55.4 (±8.1)	ND	190 (±13)
	44-80	0.64 (±0.01)		3.44 (±0.13)	121 (±19)	60.7 (±3.8)	1.04 (±0.36)	312 (±184)
	80-94	↓	3839 (±65)	6.00 (±0.14)	324 (±78)	60.5 (±6.6)	2.36 (±0.27)	316 (±139)
II	94-105		↓	↓	298 (±45)	71.1 (±3.8)	2.82 (±0.25)	435 (±76)
	105-119				204 (±15)	64.8 (±2.7)	2.32 (±0.19)	457 (±165)
	119-150				257 (±75)	67.2 (±2.5)	1.55 (±0.14)	723 (±214)
III	150-205		↓	↓	707 (±91)	68.5 (±3.9)	1.45 (±0.16)	1501 (±371)
	205-224		7979 (±61)	12.47 (±0.22)	1564 (±195)	68.3 (±6.7)	1.33 (±0.09)	5642 (±1083)
	224-233	↓	7979 (±61)	12.47 (±0.22)	2403 (±275)	64.8 (±2.5)	1.40 (±0.10)	5259 (±697)

[a] Vertical arrow indicates that the last values were kept through the corresponding period.
[b] ND = Not determined.

Table 6—Pseudo steady-state operating conditions and performance data of anaerobic filter R3 during each operation period (±95% confidence interval).

	Time (d)	Hydraulic retention time (d)[a]	Influent COD (mg/L)[a]	Organic loading rate (kg COD/m³·d)[a]	Effluent soluble COD (mg/L)	Methane (%)[b]	Biogas (m³/m³·d)[b]	Effluent VSS (mg/L)
I	1–6	3.19 (±0.13)	2200 (±73)	0.69 (±0.04)	169 (±44)	ND	ND	151 (±53)
	6–29	1.52 (±0.02)		1.45 (±0.05)	143 (±25)	33.7 (±12.4)	ND	139 (±21)
	29–44	1.00 (±0.01)		2.20 (±0.08)	180 (±40)	53.8 (±5.0)	ND	191 (±48)
	44–80	0.64 (±0.01)	↓	3.44 (±0.19)	205 (±43)	57.1 (±5.0)	1.60 (±0.05)	507 (±213)
	80–94		3839 (±65)	6.00 (±0.14)	500 (±92)	57.6 (±9.0)	2.25 (±0.02)	555 (±349)
II	94–105				372 (±99)	65.0 (±9.0)	2.53 (±0.30)	630 (±123)
	105–119				340 (±22)	67.7 (±4.9)	2.44 (±0.14)	1414 (±562)
	119–150				287 (±95)	68.7 (±2.9)	1.80 (±0.18)	1344 (±205)
III	150–205		↓	↓	423 (±55)	73.4 (±1.8)	1.40 (±0.13)	1312 (±250)
	205–224		7979 (±61)	12.47 (±0.22)	1264 (±257)	72.1 (±3.1)	1.89 (±0.31)	5223 (±591)
	224–233	↓	7979 (±61)	12.47 (±0.22)	1809 (±307)	73.1 (±4.5)	1.48 (±0.08)	1404 (±254)

[a] Vertical arrow indicates that the last values were kept through the corresponding period.
[b] ND = Not determined.

the efficiency of anaerobic filter R3 was persistently greater than that observed for anaerobic filters R1 and R2 (Figure 2a and Tables 4, 5, and 6). This behavior can be explained by the higher resistance to oleic acid toxicity exhibited by the biomass inoculated in anaerobic filter R3.

Because of experimental limitations, the operation was stopped on day 167 for a period of 15 days and the digesters were stored at 4 °C. The restart was performed under the same operating conditions and, with the exception of anaerobic filter R2, it proceeded without significant disturbance. Anaerobic filter R2 exhibited a sudden decrease in COD removal efficiency (achieving only 50%) but returned to its previous performance after 5 days.

On day 224, the recycled flow of anaerobic filters R2 and R3 was increased to 30 L/d. No significant effect was detected, except for a decrease in the effluent VSS concentrations on anaerobic filter R3 (Table 6).

Figure 4 presents the average values during periods II and III of: (a) effluent VFA–COD concentration, (b) nonacidified substrate concentration, (c) methane yield, and (d) retained organic loading rate. The effluent VFA concentrations result from the balance among β-oxidation and acetogenesis and methanogenesis. Methanogenic and acetogenic inhibition decrease the degradation rate of VFA, thereby increasing their concentration in the digester. On the other hand, β-oxidation inhibition decreases production of VFA, thereby increasing the concentration of nonacidified substrate. The bacteria involved in β-oxidation of LCFA, although not well studied, seem to be particularly sensitive to LCFA toxicity (Hanaki et al., 1981). During period II, effluent VFA–COD concentrations decreased continuously with increasing oleic acid concentrations

(Figure 4a). However, when oleate was the sole carbon source fed to the digesters (period III), a significant increase of VFA was observed, especially from anaerobic filters R1 and R2. Anaerobic filter R3 exhibited a lower VFA–COD accumulation. In all cases, the predominant volatile acid was acetate (Figure 3).

A slight increase of the nonacidified substrate with increasing oleate concentration at a constant organic loading rate (period II) can be observed in anaerobic filters R1 and R2. Further increase of the oleate organic load (period III) induced a significant increase of the nonacidified substrate in all digesters, with the effect being more important in anaerobic filter R1 than in anaerobic filters R2 and R3 (both have biomass recirculation). This fact may suggest that recirculation induced a lower β-oxidation inhibition because of the dilution of the toxic concentration by the recycle flow.

Figure 4c shows that the digester without biomass recirculation (R1) exhibited the lowest methane yield during periods II and III. This fact may also be related to the dilution of the toxic load, giving rise to a lower inhibition of the methanogenic bacteria. On the other hand, the higher liquid velocity resulting from recirculation can promote biogas release from biomass aggregates. For the three digesters, the fraction of COD removed that was used for methane production decreased with the increase in the oleate organic load as shown in Figure 4d, where the retained organic loading rate is plotted against the oleate loading rate. The retained organic load is the difference between removed organic loading and the loading rate corresponding to methane production. The observed increase along the trial period suggests that part of the oleate being fed was also being retained in the reactor and not biodegraded. This accumulation was slightly higher in anaerobic
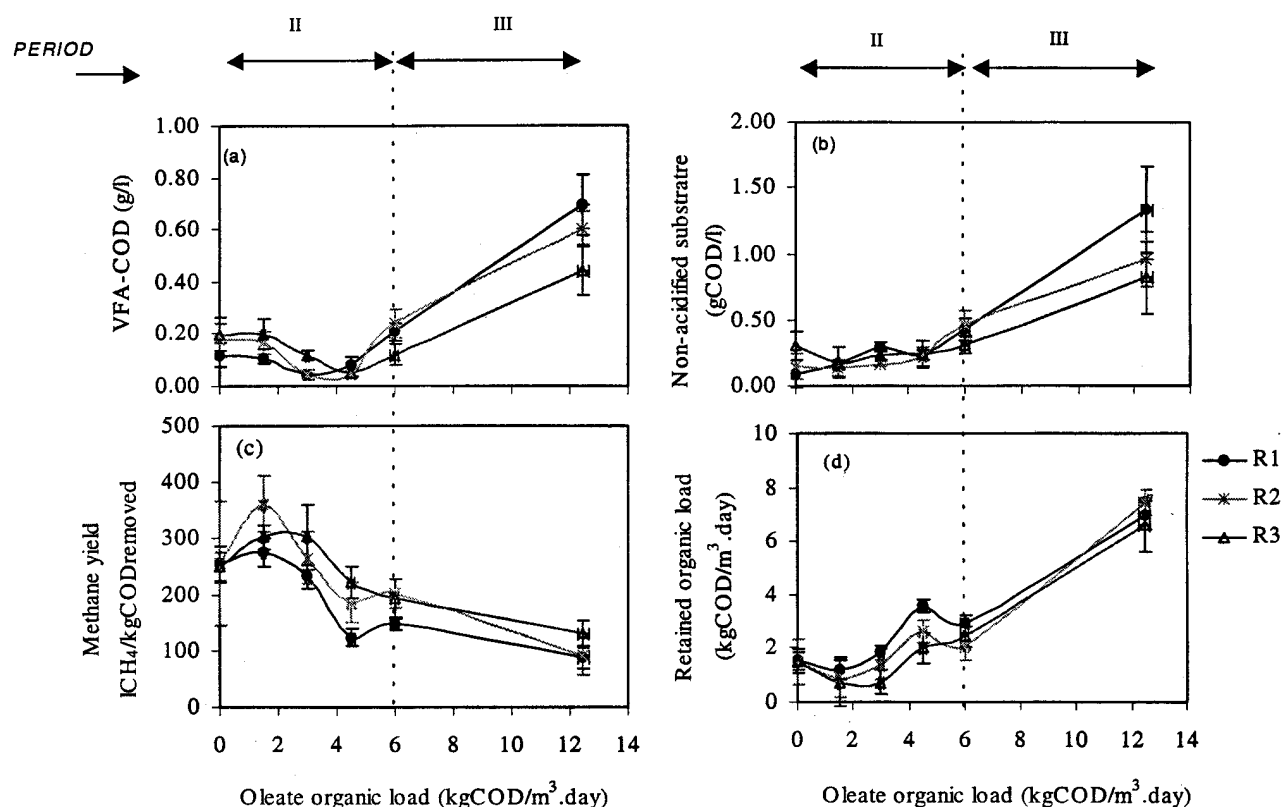
Figure 4—Average operating results in relation to the applied oleate loading rate during periods II and III for (a) effluent VFA–COD, (b) nonacidified substrate, (c) methane yield, (d) and retained organic load. Bars represent 95% confidence intervals.

filter R1 for most of the oleate loading rates. This finding is in accordance with Sayed et al. (1987), who found a low methane yield in a UASB treating an effluent with high lipid content.

Molar Ratio of Oleate to Calcium and Magnesium Ion. In this work, an oleate concentration of 8 g COD/L was fed to the digesters as the sole carbon source. However, it is known that the presence of calcium ions can lead to a decrease of LCFA inhibitory effect by lowering their soluble concentration through the production of a calcium–LCFA precipitate. The "free" oleate concentration depends on oleate and calcium concentration in the feed and the solubility product of the calcium oleate salt (Roy et al., 1985). Because each divalent ion can theoretically precipitate two oleate molecules, a total precipitation of the existing oleate would be possible for a molar ratio less than 2. Considering that magnesium ions can exhibit a similar effect, the molar ratio of oleate to calcium and magnesium ions ($Ca^{2+}+Mg^{2+}$) was determined during the trial period (Table 7).

These values were calculated considering the contribution of the calcium content present in the skim milk and tap water and the magnesium supplied in the macronutrients. Excluding the period from day 95 to 105, values exceeding the stoichiometric value 2 were observed and a maximum of 6.8 mol oleate/mol ($Ca^{2+}+Mg^{2+}$) was achieved in period III. Thus, even if all the calcium and magnesium ions had stoichiometrically precipitated the oleate, the concentration remaining in the medium highly exceeded the IC_{50} values of the inocula (40 and 250 mg/L for the nonacclimated and acclimated inocula, respectively), especially after the end of period II. However, it is important to note that the

influent oleate concentrations achieved in this work are significantly greater than those typically present in real dairy industries. According to Hanaki et al. (1981), in general, 44% (in terms of COD) of milk is fat and hydrolysis of this fat to LCFA does not reduce the COD load. Considering an average value of 4000 mg/L COD for the dairy effluents, a lipidic content of nearly 1700 mg COD/L would be present in those effluents. In this study, this value would correspond to an oleate organic load of 3 kg COD/m³·d. Under these operating conditions, the removal efficiency was greater than 90% and the methane yield was between 250 and 300 L CH_4/kg COD removed.

Methane Production Due to Adsorbed Substrate. The evidence of substrate accumulation suggests the adsorption of a

Table 7—Molar ratio of oleate to calcium and magnesium ions during the trial period.

Period	Time (days)	Molar ratio oleate/ (Ca^{2+} + Mg^{2+})	Remaining oleate concentration (*) (mg/L)
II	95–105	1.0	0.0
	105–119	2.0	10.5
	119–150	3.1	355.0
III	150–205	4.2	701.0
	205–233	6.8	1948.0

(*) Theoretical values.

Whitish
matter

(a)

Reduction of autofluorescence duo to the
presence of the whitish matter

Autofluoresce of methanogenic
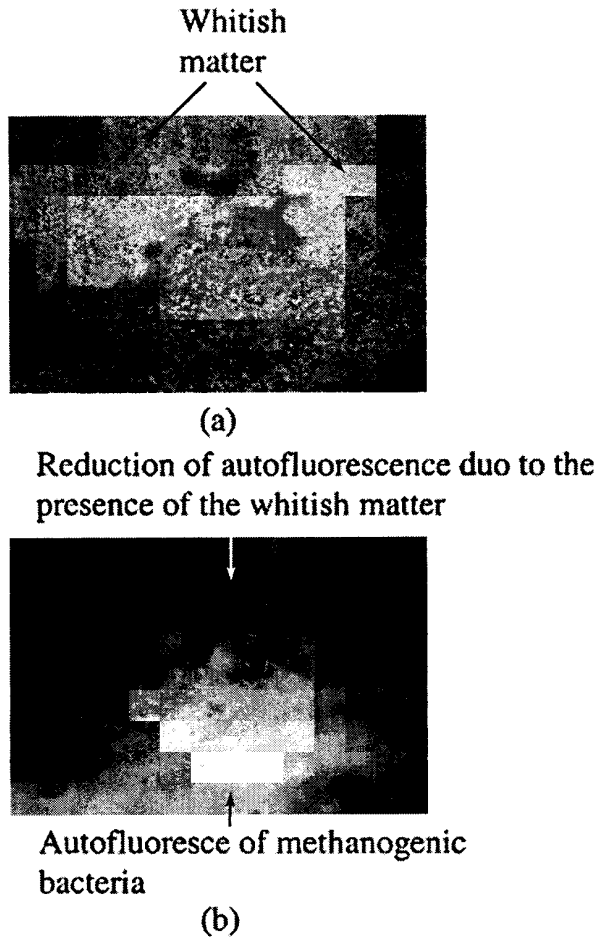bacteria

(b)

Figure 5—Microphotographs (400X) of the biomass present in R1: (a) phase contrast, and (b) same field observed under fluorescence at 420 nm.

considerable fraction of the fed oleate or a product of its degradation onto the biomass. The adsorption phenomenon was described in previous works. Rinzema (1988) found that, besides the accumulation of an LCFA precipitate in a UASB reactor, biomass aggregates coated with the same precipitate were observed. In addition, Hanaki et al. (1981) observed that LCFA resulting from the degradation of a lipidic substrate adhered onto the biomass in less than 24 hours. Hwu et al. (1998) studied the adsorption of LCFA in granular sludge and concluded that, after adsorption, a partial desorption promoted by biogas release was observed. Alves et al. (2001) observed that after feeding a reactor with oleate as the sole carbon source, the biomass, after being washed several times with anaerobic buffer, still exhibited high methane production due to residual substrate when incubated in batch vials at 37 °C. In this study, the biomass encapsulation was suggested by microscopic inspection (Figure 5). When observed under a phase contrast microscope, the biomass present in the digesters exhibited white zones (Figure 5a) that seem to act as a light emission barrier decreasing the visible autofluorescence of the methanogenic population (Figure 5b).

The accumulation of nondegraded substrate onto the biomass can hinder the transfer of substrate and products, reducing the rate

of methane production. However, according to a previous work (Alves et al., 2001), the adsorbed organic matter can be easily degraded to methane as long as no oleate is externally added to the medium. If oleate was added to the encapsulated sludge in batch vials, lag phases of up to 250 hours were observed. In this study, a similar result was observed in the sludge taken from anaerobic filters R1, R2, and R3 at the end of the trial period. Figure 6 represents the specific methane production when the biomass from the reactors was incubated in batch vials at 37 °C and 150 r/min in the presence of 0, 100, 300, 500, 700, and 900 mg/L oleate and after several washings with an anaerobic buffer.

As shown, the sludges from anaerobic filters R1 and R2 exhibited the maximal methane production in the blank vials (without any added oleate), suggesting that the adsorbed organic matter was easily degraded to methane if no external oleate was added to the
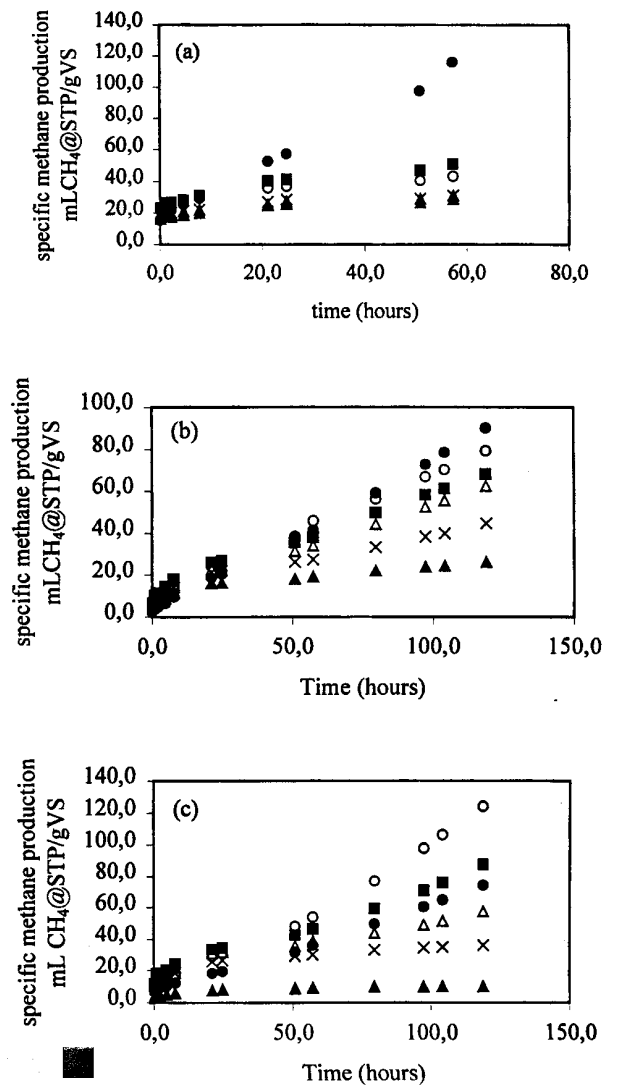

Figure 6—Oleate biodegradability batch assays with sludge taken from (a) R1, (b) R2, and (c) R3 at the end of the trial period. Methane production for added oleate concentrations of: 0-blank (•), 100 mg/L (O), 300 mg/L (■), 500 mg/L (△), 700 mg/L (×), and 900 mg/L (▲).

Table 8—Initial methane production rate for the sludges from anaerobic filters R1, R2, and R3 when incubated in batch vials without any added substrate.

Sludge source	Methane production rate (mL $CH_{4(STP)}$/g VS·d)
R1 (150 r/min)	39.7
R2 (150 r/min)	17.2
R3 (150 r/min)	13.1
R1 (static)	2.9

medium. When external oleate was added to the medium, the methane production rate decreased.

The sludge from anaerobic filter R3 (the reactor inoculated with the acclimated inoculum) showed a different pattern. For oleate concentrations of 100 and 300 mg/L, the methane production rate was greater than that observed for the blank vial, suggesting that this sludge was able to biodegrade oleate if fed in such low concentrations.

Sludge from anaerobic filter R1 was also incubated under static conditions without any added substrate. Under these conditions, the initial methane production rate was significantly lower than the methane production rate under stirring conditions. Table 8 summarizes methane production rates from the different sludges under the conditions tested.

As the substrate was in intimate contact with the biomass, the effect of stirring improved only the external mass transfer of products (biogas). Sludge from anaerobic filter R1 produced methane from the adsorbed matter at a rate 13.8 times higher under stirring than under static conditions, suggesting that biogas release was significantly improved by the stirring. The experiment run under static conditions revealed that the biomass that originally formed a floating layer on the top of the reactor gradually deposited in the bottom and changed its appearance from whitish to dark.

The higher methane production rate obtained with the sludge from anaerobic filter R1 can be explained by the different operating conditions of the reactor during the trial period. The lower upflow velocity that was applied compared with anaerobic filters R2 and R3 justifies why more organic matter could have been adsorbed under low shear stress conditions. This agrees with the previously referred to result that, for most of the applied oleate loading rates, anaerobic filter R1 exhibited the highest degree of oleate retention as shown in Figure 4d. Apparently, the higher amount of adsorbed matter can explain the high methane production rate that was exhibited by this biomass.

These results concur with those obtained in a previous work in which an encapsulated sludge taken from a reactor fed with oleate as a sole carbon source exhibited a background methane production (washed, but without any added substrate) of 90 mL $CH_{4(STP)}$/g VS·d under stirring conditions (Alves et al., 2001). As shown in this study, the background methane production was delayed when oleate was added to the batch vials, suggesting that the adsorbed matter could be an intermediate of oleate degradation, such as stearic, palmitic, myristic, or other saturated acids, which are less toxic than oleate. The identification of this compound is the next step of the current research study.

These results suggest that suppressing the feeding after a feeding period under low shear stress conditions can induce degradation of the adsorbed matter. Therefore, it is advantageous to run

sequential cycles of adsorption–degradation to optimize LCFA degradation to methane. The rate of methane production in the degradation cycle is greatly enhanced by applying stirring conditions.

Conclusions

The combined effect of using biomass recirculation and an acclimated inoculum was beneficial for treating an oleate-based synthetic effluent. Comparing these conditions with an upflow anaerobic filter without biomass recirculation or an acclimated inoculum showed that the methane yield was 48% higher, the concentrations of VFA were 36% lower, and the removal efficiency was 10% higher when feeding oleate as the sole carbon source at a loading rate of 12.5 kg COD/m^3·d, even with an oleate/(Ca^{2+}+Mg^{2+}) molar concentration ratio of 6.8. Biomass recirculation was also found to be beneficial because of to biomass washout minimization and dilution of the toxic organic load, giving rise to a lower inhibition both on the acetogenic and methanogenic populations. The difference between soluble COD removal and methane production suggested that part of the oleate fed was retained in the reactor. Microscopic examination of the biomass at the end of the trial period revealed that white zones surrounded the aggregates, resulting in a significant decrease on the detectable autofluorescence of the methanogenic bacteria.

Methane production from the adsorbed organic matter was evidenced in batch assays where no extra substrate was added. Sludge taken from anaerobic filter R1 at the end of the operation that was washed and incubated in that condition showed a maximum degradation rate of 39.7 mL CH_4/g VS·d. With stirring (150 r/min), the methane production rate was 13.8 times higher than under static conditions. When oleate was added to the batch vials, this background methane production was delayed, suggesting that the adsorbed matter could be an intermediate of oleate degradation, such as stearic, palmitic, myristic, or other saturated acids, which are less toxic than oleate. The identification of this compound is the next step of this research work.

Based on current results, it can be concluded that, for treating effluents with high lipid content, operation in cycles of adsorption under low shear stress conditions and degradation under stirring conditions should be advantageous compared with the continuous operation.

Authors. Alcina Pereira is a Ph. D. student, Manuel Mota is a full professor, and Madalena Alves is an assistant professor in the Department of Biological Engineering of the University of Minho, Portugal.

Correspondence should be addressed to Madalena M. Alves, Centro de Engenharia Biológica-IBQF, Universidade do Minho, Campus de Gualtar, 4710-057 Braga codex Portugal; e-mail: madalena.alves@deb.uminho.pt

References

Alves, M.M.; Mota Vieira, J.A.; Álvares Pereira, R.M.; Pereira, M.A.; and Mota, M. (2001) Effects of Lipids and Oleic Acid on Biomass

Development in Anaerobic Fixed Reactors. Part II: Oleic Acid Toxicity and Biodegradability. *Water Res.* (G.B.), **35**, 264.

American Public Health Association; American Water Works Association; and Water Pollution Control Federation (1989) *Standard Methods for the Examination of Water and Wastewater.* 17th Ed., Washington, D.C.

Coates, J.D.; Coughlan, M.F.; and Colleran, E. (1996) Simple Method for the Measurement of the Hydrogenotrophic Methanogenic Activity of Anaerobic Sludges. *J. Microbiol. Methods,* **26**, 237.

Colleran, E.; Concannon, F.; Goldem, T.; Geoghegan, F.; Crumlish, B.; Killilea, E.; Henry, M.; and Coates, J. (1992) Use of Methanogenic Activity Tests to Characterize Anaerobic Sludges, Screen for Anaerobic Biodegradability and Determine Toxicity Thresholds Against Individual Anaerobic Trophic Groups and Species. *Water Sci. Technol.* (G.B.), **25**, 7, 31.

Colleran, E., and Pistilli, A. (1994) Activity Test System for Determining the Toxicity of Xenobiotics Chemicals to the Methanogenic Process. *Ann. Microbiol. Enzimology,* **44**, 1.

Dolfing, J., and Bloemen, W.G.B.M. (1985) Activity Measurements as a Tool to Characterize the Microbial Composition of Methanogenic Environments. *J. Microbiol. Methods,* **4**, 1.

Forster, C.F. (1992) Oils, Fats and Greases in Wastewater Treatment. *J. Chem. Technol. Biotechnol.,* **55**, 402.

Galbraith, H.; Miller, T.B.; Paton, A.M.; and Thomson, J.K. (1971) Antibacterial Activity of Long Chain Fatty Acids and the Reversal with Calcium, Magnesium, Ergocalciferol and Cholesterol. *J. Appl. Bacteriol.* (G.B.), **34**, 803.

Habets, L.H.A.; Engelaar, A.J.H.H.; and Groeneveld, N. (1997) Anaerobic Treatment of Inuline Effluent in an Internal Circulation Reactor. *Water Sci. Technol.* (G.B.), **35**, 10, 189.

Hanaki, K.; Matsuo, T.; and Nagase, M. (1981) Mechanisms of Inhibition Caused by Long Chain Fatty Acids in Anaerobic Digestion Process. *Biotechnol. Bioeng.,* **23**, 1560.

Hawkes, F.R.; Donnely, T.; and Anderson, G.K. (1995) Comparative Performance of Anaerobic Digesters Operating on Ice-cream Wastewater. *Water Res.* (G.B.), **29**, 525.

Hwu, C.-S.; Donlon, B.; and Lettinga, G. (1996) Comparative Toxicity of Long-chain Fatty Acid to Anaerobic Sludges from Various Origins. *Water Sci. Technol.* (G.B.), **34**, 5–6, 351.

Hwu, C.-S.; Tseng, S.-K.; Yuan, C.-Y.; Kulik, Z.; and Lettinga, G. (1998) Biosorption of Long-chain Fatty Acids in UASB Treatment Process. *Water Res.* (G.B.), **32**, 1571.

Hwu, C.-S.; van Lier, J.B.; and Lettinga, G. (1997) Thermophilic High-rate Anaerobic Treatment of Wastewater Containing Long-chain Fatty Acids: Effect of Washed Out Biomass Recirculation. *Biotechnol. Lett.,* **19**, 5, 453.

Komatsu, T.; Hanaki, K.; and Matsuo, T. (1991) Prevention of Lipid Inhibition in Anaerobic Processes by Introducing a Two-phase System. *Water Sci. Technol.,* **23**, 7/9, 1189.

Koster, I.W., and Cramer, A. (1987) Inhibition of Methanogenesis from Acetate in Granular Sludge by Long-chain Fatty Acids. *Appl. Eviron. Microbiol.,* **53**, 2, 403.

Rinzema, A. (1988) Anaerobic Treatment of Wastewater with High Concentration of Lipids or Sulfate. Ph.D. thesis, Wageningen Agricultural University, Wagningen, The Netherlands.

Rinzema, A.; Boone, M.; van Knippenberg, K.; and Lettinga, G. (1994) Bactericidal Effect of Long Chain Fatty Acids in Anaerobic Digestion. *Water Environ. Res.,* **66**, 40.

Roy, F.; Albagnac, G.; and Samain, E. (1985) Influence of Calcium Addition on Growth of Highly Purified Syntrophic Cultures Degrading Long Chain Fatty Acids. *Appl. Eviron. Microbiol.,* **49**, 3.

Sam-Soon, P.; Loewenthal, R.E.; Wentzel, M.C.; and Marais, G.V.R. (1991) A Long-chain Fatty Acid, Oleate, as Sole Substrate in Upflow Anaerobic Sludge Bed (UASB) Reactor Systems. *Water SA,* **17**, 1, 31.

Sayed, S.; Van Campen, L.; and Lettinga, G. (1987) Anaerobic Treatment of Slaughterhouse Waste Using a Granular Sludge UASB Reactor. *Biol. Wastes,* **21**,11.

Young, J.C. (1991) Factors Affecting the Design and Performance of Upflow Anaerobic Filters. *Water Sci. Technol.,* **24**, 8, 133.

Zehnder, A.J.B.; Huser, B.A.; Brock, T.D.; and Wuhrmann, K. (1980) Charaterization of an Acetate-Decarboxylating, Non-Hydrogen-Oxidizing Methane Bacterium. *Arch. Microbiol.* (Ger.), **124**, 1.
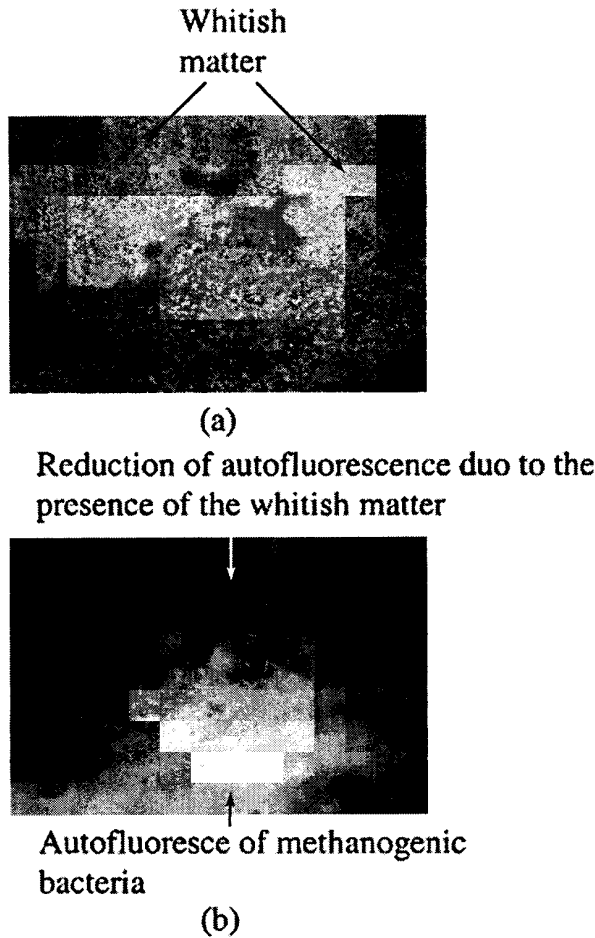
Whitish matter

(a)

Reduction of autofluorescence duo to the presence of the whitish matter

Autofluoresce of methanogenic bacteria

(b)

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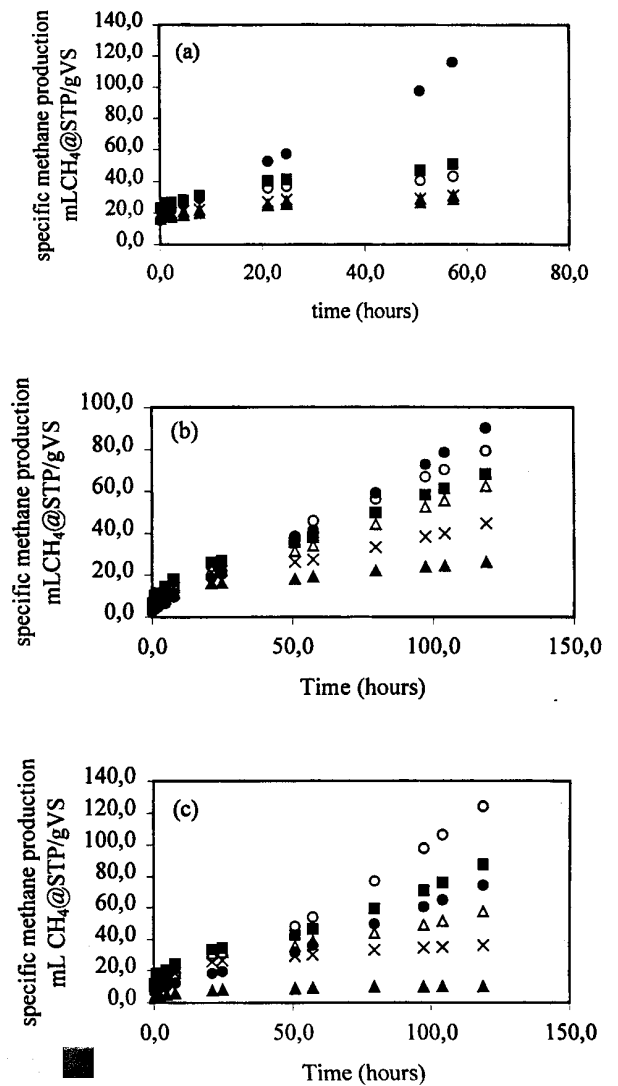

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