Measurement of Microbial Numbers and Biomass Contained in Thermophilic Anaerobic Reactors

R. Solera, L.I. Romero, D. Sales

ABSTRACT: This paper describes the determination of the microbial population in terms of the number, biomass, and composition of single- and two-phase, laboratory-scale thermophilic (55 °C) anaerobic reactors under steady-state conditions. Epifluorescence microscopy with 4',6-diamidine-2-phenylindole (DAPI) as fluorochrome was used to determine the total number of microorganisms in the reactors, and autofluorescence microscopy was used to determine the total methanogenic bacteria populations. The results obtained by the direct count methods were compared to the quantity of biomass contained in the system, which was determined by volatile suspended solids. The concentration of acidogenic bacteria was estimated by subtraction of the autofluorescence results from those of the DAPI epifluorescence microscopy. The viable bacterial population was determined by plating techniques using an anaerobic chamber.

The total bacterial and methanogenic populations of single-stage digesters increase when the hydraulic retention time decreases; nevertheless, the percentages of the principal bacterial groups (acidogenic and methanogenic) remain constant at 87% and 13%, respectively. In the two-stage reactors, the percentages of the acidogenic and methanogenic groups are 99% and 26% of the total population in the acidogenic and methanogenic reactors, respectively. In the single-stage reactors, biomass determinations can be used to estimate microbial concentrations, and vice versa, as there is a high positive correlation between microorganism concentration and biomass. The syntrophic relationship between the bacteria involved in the anaerobic process is a possible explanation for the low values of viable population obtained in the reactors studied. Nevertheless, there is a high correlation between direct counts by epifluorescence microscopy and viable plate counts for the combined system studied. *Water Environ. Res.*, **73**, 684 (2001).

KEYWORDS: acidogens, autofluorescence, epifluorescence, methanogens, plating count, thermophilic anaerobic digestion.

Introduction

The anaerobic treatment of industrial wastewater has a number of potential benefits including low energy consumption, low excess sludge production, and enclosure of odors and aerosols. In the mixed culture of an anaerobic reactor, several different degradation reactions take place. Complex kinetics, interactions, and different steps have been reported by numerous authors (McCarty and Smith, 1986). Anaerobic digestion can be considered a two-step process even though it really is a coupled sequence of microbiological interactions. First, complex organic materials are depolymerized and converted to fatty acids, carbon dioxide, and hydrogen. In the next step, methane is generated by reduction of carbon dioxide, by molecular hydrogen, or by other reduced fermentation products such as fatty acids. Two main groups of bacteria are considered to be involved: acidogenic and methanogenic. The stability of the system depends on the viable bacterial groups involved in the process. Understanding the functioning of anaerobic reactors requires quantitative information on microbial numbers, biomass, and activities of the bacterial groups involved in the process. Numbers, biomass, and activities represent distinct ecological parameters. Although normally correlated to each other, these parameters should not be used in an interchangeable manner. On occasion, when one parameter is required (e.g., the number of microorganisms) it is necessary to measure a less relevant parameter, such as biomass, because of technical problems and then to calculate the required parameter from this. For example, the lack of methods for determining the concentration and viability of the different bacterial groups involved in the anaerobic process is a significant limitation in studies on the kinetics of the process, development, operation, and monitoring of particular reactors because the measurement of biomass (as volatile suspended solids [VSS], principally) does not provide this information.

The main objectives of this paper are to quantify the microbial numbers and biomass content of thermophilic anaerobic reactors and to make a comparative analysis of the results obtained by different counting methods. Microbial numbers were determined by direct count procedures (microscopic counting methods) and indirect count procedures (plate counting methods). The count of the total bacterial population was performed by the epifluorescence microscopy method using fluorochrome as the stain agent (4',6-diamidine-2-phenylindole, DAPI). Methanogenic bacteria were counted by the autofluorescence microscopy method. The viable bacterial population was quantified by the solid-medium plating technique, which used an anaerobic chamber for the spreading onto plates and the subsequent incubation of inoculated plates. Biomass was determined by measuring VSS.

These techniques have been applied to the measurement of the microbial populations contained in both single- and two-stage, laboratory-scale reactors. In the single-stage process the main reaction steps, acidogenesis and methanogenesis, take place in the same reactor, while in the two-stage process they take place in separate reactors.

Methods

Experimental Procedure. The experimental protocol was designed to quantify the main bacterial population contained in a laboratory-scale continuously stirred tank reactor (CSTR) without recycling solids. In this reactor, the solids and liquid retention times are equal. Two types of system were used: a single-stage reactor and a two-stage reactor. Single-stage reactors were operated at two hydraulic residence times (HRTs): 4 and 10 days (digesters R4 and R10, respectively). In the two-stage system,

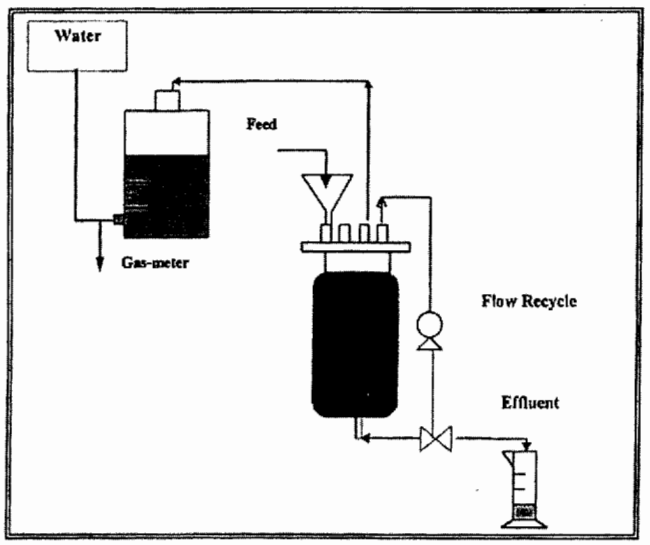

Figure 1—Schematic diagram of the single-stage experimental reactors.

HRTs were 1.7 and 4 days for the acidogenic phase (reactors $RA_{(1.7)}$ and $RA_{(4)}$, respectively), and 4 days for the methanogenic phase (reactors $RM_{(RA\ 1.7)}$ and $RM_{(RA\ 4)}$, for the corresponding HRT of the acidogenic phase).

Acidogenic and methanogenic bacteria differ mainly in their physiology, requirements for nutrients and pH, and their kinetics of growth and nutrient uptake. The bacteria also differ in their ability to support environmental changes. As a result of their differing growth characteristics, it is possible to select and enrich the different bacteria in the digester by inoculation of the medium contained in a single-stage reactor. The conditions selected for the first stage were those favoring the growth acid formers (i.e., a short HRT of 1.7 days and a low pH of 5.5); these conditions may be inhibitory to the methane formers. The methanogenic reactor was fed with filtered acidogenic effluent to suppress acidogenic bacteria in the feed.

Quantification assays were performed when reactors reached steady-state conditions during a period of 20 days. The attainment of steady state was verified after an initial period (three times the HRT) by checking whether the effluent characteristic values continued at the mean of the previous measurements. Quantification of total bacterial population was performed by epifluorescence microscopy using DAPI as the staining agent. The total methanogenic population in the reactors was counted by autofluorescence microscopy. The concentration of the nonmethanogenic population was estimated by subtracting the results of the autofluorescence microscopy from those of the DAPI epifluorescence. This method is considered to give a quantity of nonmethanogenic bacteria that corresponds closely to the population of acidogenic bacteria contained in the reactor (Zeikus, 1980; Morgan et al., 1991; and Ince et al., 1997).

The biomass contained in the system was determined by VSS. The direct microbial counts and biomass measurement were correlated and the number of bacteria per gram of biomass was estimated. The viable bacterial population was quantified by the plating technique, which used an anaerobic chamber for spreading onto solid medium and incubation of inoculated plates. These results were compared with the total bacterial population obtained by DAPI microscopy to estimate the accuracy of the count by plating, in comparison with that from epifluorescence microscopy. The two sets of results were correlated. All assays were carried out in duplicate and the results shown are the average values of all of them.

Experimental System. A schematic diagram of the laboratory-scale CSTR used in the study is shown in Figure 1. The stirred tank reactor consisted of a vertical cylinder tank (25-cm long and 10-cm i.d.). The active liquid volume was 2 L and empty volume was 2.4 L. Reactor temperature was maintained at 55 °C and the biogas generated was collected in a gas meter. The feed was added in a semicontinuous mode: one dose per day. Effluent recirculation was used to mix and homogenize the liquid in the system.

Figure 2 shows a schematic diagram of the laboratory-scale, two-phase system composed of two CSTRs in a series, the first with a volume of 1 L for the fermentative step and the second with a working volume of 5 L for the methanogenic step.

Feed Solution. Distillery wastewater (vinasses) obtained from an ethanol-producing wine distillery plant located in Tomelloso (Ciudad Real, Spain) was used. In general, the vinasses showed an adequate relationship between the different macronutrients and micronutrients with a favorable chemical oxygen demand (COD)/nitrogen/phosphorus ratio suitable for microbiological treatment. A complete study of the characteristics and properties of vinasses can be found in a previous paper by Sales et al. (1982). Before being used, vinasses were transported and maintained at 4 °C. The original substrate was diluted with tap water to attain the required COD concentration to be used in the feed for this experiment (approximately 15 g COD/L). Sodium hydroxide was added to the reactor to maintain a neutral pH and, in the case of the acidogenic reactor, pH 5. Vinasses biodegradation batch experiments (Pérez, 1995) indicated that this was a complex medium formed by two substrates of different nature and biodegradability: S_1, the easily biodegradable substrate fraction (80% of the total), and S_2, the recalcitrant substrate fraction. Initial COD of vinasses was 30 g COD/L, and the concentration of VSS and bacteria was negligible.

The methanogenic reactor was fed with acidogenic effluent, filtered using a Millipore filter (GVWP, Millipore Iberica S.A., Madrid, Spain) with a 0.22-μm pore size to retain the acidogenic microorganisms, and supplemented with sodium hydroxide to maintain pH at approximately 8.5.

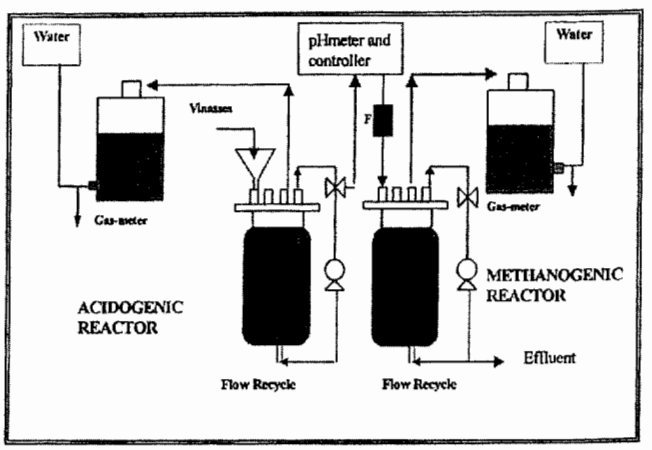

Figure 2—Schematic diagram of the two-stage experimental reactors (F = filter).

Table 1—Performance and operating parameters for the control of the anaerobic process during the period of study.[a]

Reactor	HRT (d)	OLR_o (g/L·d COD_o)	COD_r (%)	pH_e	Biogas (L/L·d)	CH_4 (%)	CO_2 (%)	H_2 (%)
R10	10	1.44	82.1	7.35	0.47	82	18	0
R4	4	3.75	80.1	7.60	0.80	85	15	0
$RA_{(1.7)}$	1.7	9.17	31.9	5.45	0.29	48	31	21
$RA_{(4)}$	4	3.79	30.1	5.53	0.18	66	29	7
$RM_{(RA\ 1.7)}$	4	2.43	61.5	7.70	0.32	94	6	0
$RM_{(RA4)}$	4	2.65	71.7	7.80	0.45	91	9	0

[a] OLR_o = organic loading rate, COD_c = organic removal efficiency (as percentage of initial COD_o), COD_o = initial COD, and pH_e = effluent pH.

Analytical Methods. The analytical determinations made in this study can be grouped in two categories: those used to monitor and control the anaerobic digestion process and those used to count the bacterial population contained in the reactors.

Analytical Determinations Used to Monitor and Control the Anaerobic Digestion Process. All analytical determinations were carried out according to *Standard Methods* (APHA et al., 1995). The parameters analyzed for liquid samples in both effluent and influent were pH and soluble COD, while, for gaseous samples, the volume of biogas produced at standard temperature and pressure and its composition (methane, carbon dioxide, and hydrogen) were analyzed. In the two-stage system, volatile fatty acids (acetic, propionic, and butyric) were analyzed by gas chromatography according to Fang et al. (1995).

Soluble COD was determined by the dichromate reflux method. The sample was first filtered using a Millipore AP4004705 filter with a 0.45-μm pore size; the filtrate was then used for the COD analysis. Gas production was measured continuously by water displacement. Gas composition (methane, carbon dioxide, and hydrogen) was determined using a modified gas chromatography method previously described by Nebot et al. (1995).

Analytical Determinations Used to Count Bacterial Population. Quantification of total bacterial numbers contained in the system was determined by epifluorescence microscopy with DAPI, according to Kepner and Pratt (1994) and *Standard Methods* (APHA et al., 1995). Immediately after sampling, 10 mL of the sample was diluted using phosphate buffer (pH = 7.2) to give a count of 10 to 15 cells per field, and homogenized with a vortex Heidolph Reax 2000 (Merck Eurolab, Switzerland) for 30 seconds. Bacterial cells were retained on a 25-mm black polycarbonate membrane filter with a 0.22-μm pore size (GTBO, Millipore). Cells were counted visually using a Nikon Labophot-2A/2 microscope (Izasa Laboratory, Madrid, Spain) fitted with a 100-W mercury lamp, Nikon UV-1A excitation and barrier filters, and a 100X oil objective. Those cells falling within the area of the ocular in randomly located fields were counted. Ten random fields per sample were counted and duplicate counts were performed for all samples.

The methanogenic bacterial population was counted by autofluorescence direct counting using the same microscopy that was used for the epifluorescence method. To estimate the number of methanogens, UV light was used because these bacteria fluoresce under such light, as described by Doddema and Vogels (1978). The sample was fixed with a glutaraldehyde solution buffer (final concentration, 5%). Next, the sample was diluted and homogenized by the same procedure as described above, but, in this case, the dilution gave counts between 80 and 100 per field of view. A small (5 μL) drop of the sample was placed on the Neubauer improved "bright-line" chamber, which is portioned off by ridges into regular cubical chambers of known accurate volume (i.e., 2.5×10^{-5} mL). The Neubauer improved chamber has a depth of 0.1 mm and a field of vision area of 0.4 mm^2. The total number of bacteria in 80 of these small chambers was counted and averages were taken. Clumps of bacteria were counted as one organism and, to avoid errors, only those bacteria on the top and left graduation lines were counted in each small cube. A Nikon BV-1A filter was used for excitation at 420 nm.

Viable bacterial population was determined by plating count. An anaerobic chamber was used for the cultivation of anaerobic bacteria. Microorganisms were grown in a medium (essentially as described by Maestrojuan [1987]) containing the following components (g/L of distilled water): glucose, 4.50; triptone, 2; yeast extract, 2; sodium bicarbonate ($NaHCO_3$), 2.8; cysteine hydrochloride, 0.5; resazurin, 0.001; ammonium chloride (NH_4Cl), 1; magnesium chloride ($MgCl_2 \cdot 6H_2O$), 0.1; calcium chloride ($CaCl_2 \cdot 2H_2O$), 0.1; and dibasic potassium ortho phosphate ($K_2HPO_4 \cdot 3H_2O$), 0.4. The trace mineral solution (10 mL) was used contain-

Table 2—Microorganism concentrations and biomass in the studied reactors.

Reactor	Total population ($\times 10^8$ cell/mL)	Acidogenic population ($\times 10^8$ cell/mL)	Methanogenic population ($\times 10^8$ cell/mL)	VSS (g/L)
R10	8.06 ± 1.92	6.77 ± 2.07	1.29 ± 0.50	0.47 ± 0.09
R4	29.70 ± 7.15	25.10 ± 7.00	4.60 ± 0.80	1.57 ± 0.22
$RA_{(1.7)}$	3.06 ± 0.60	2.70 ± 0.60	0.36 ± 0.09	0.57 ± 0.04
$RA_{(4)}$	24.40 ± 2.40	24.20 ± 2.40	0.17 ± 0.05	1.05 ± 0.09
$RM_{(RA\ 1.7)}$	5.50 ± 2.69	4.49 ± 2.66	1.00 ± 0.58	0.66 ± 0.08
$RM_{(RA4)}$	10.39 ± 1.63	7.72 ± 1.56	2.68 ± 0.27	0.89 ± 0.15

Precision of counts: 95% confidence interval.

Table 3—Percentages of acidogenic and methanogenic groups and number of bacteria per gram of biomass in the studied reactors.

Reactor	Acidogenic percentage (%)	Methanogenic percentage (%)	Number of microorganisms (× 10^{12}/gVSS)
R10	83.18	16.82	1.86
R4	83.41	16.59	1.87
$RA_{(1.7)}$	87.96	12.04	0.49
$RA_{(4)}$	99.29	0.71	2.35
$RM_{(RA\ 1.7)}$	79.00	21.00	0.79
$RM_{(RA4)}$	73.84	26.16	1.20

Precision of counts: 95% confidence interval.

ing the following components (g/L of distilled water): nitrylacetic acid, 1.280; ferric chloride ($FeCl_3 \cdot 6H_2O$), 1.350; manganese chloride ($MnCl_2 \cdot 4H_2O$), 0.100; cobaltous chloride ($CoCl_2 \cdot 6H_2O$), 0.024; zinc chloride ($ZnCl_2$), 0.100; cupric chloride ($CuCl_2 \cdot 2H_2O$), 0.025; boric acid (H_3BO_3), 0.010; sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 0.024; sodium chloride (NaCl), 1.000; sodium selenite ($Na_2SeO_3 \cdot 5H_2O$), 0.026; and nickel chloride ($NiCl_2 \cdot 6H_2O$), 0.120. Noble agar (Difco Laboratories, Detroit, Michigan) at a final concentration of 2% (w/v) was used. Sterile monosodium sulfide ($Na_2S \cdot 9H_2O$) was added to the medium just before it was spread onto the plates. All components were assembled in glass-distilled water and sterilized by autoclaving at 120 °C for 20 minutes. Dispensing of solid medium, manipulations of cultures, inoculation, and incubation of plates were all performed in an anaerobic cabinet (model 1029, Forma Scientific, Marietta, Qhio) under an atmosphere of nitrogen, carbon dioxide, and hydrogen (85:15:5). The dilution medium contained the same constituents as the growth medium, except agar. The sample was diluted in an anaerobic cabinet and 0.1 mL of the selected dilutions was spread on the surface of the agar plates via gentle swirling. Each dilution was plated in triplicate. Plates were incubated inverted at 55 °C for 72 hours in an incubator inside the cabinet. Finally, biomass was determined by measuring the VSS contained in the digester medium, according to *Standard Methods* (APHA et al., 1995).

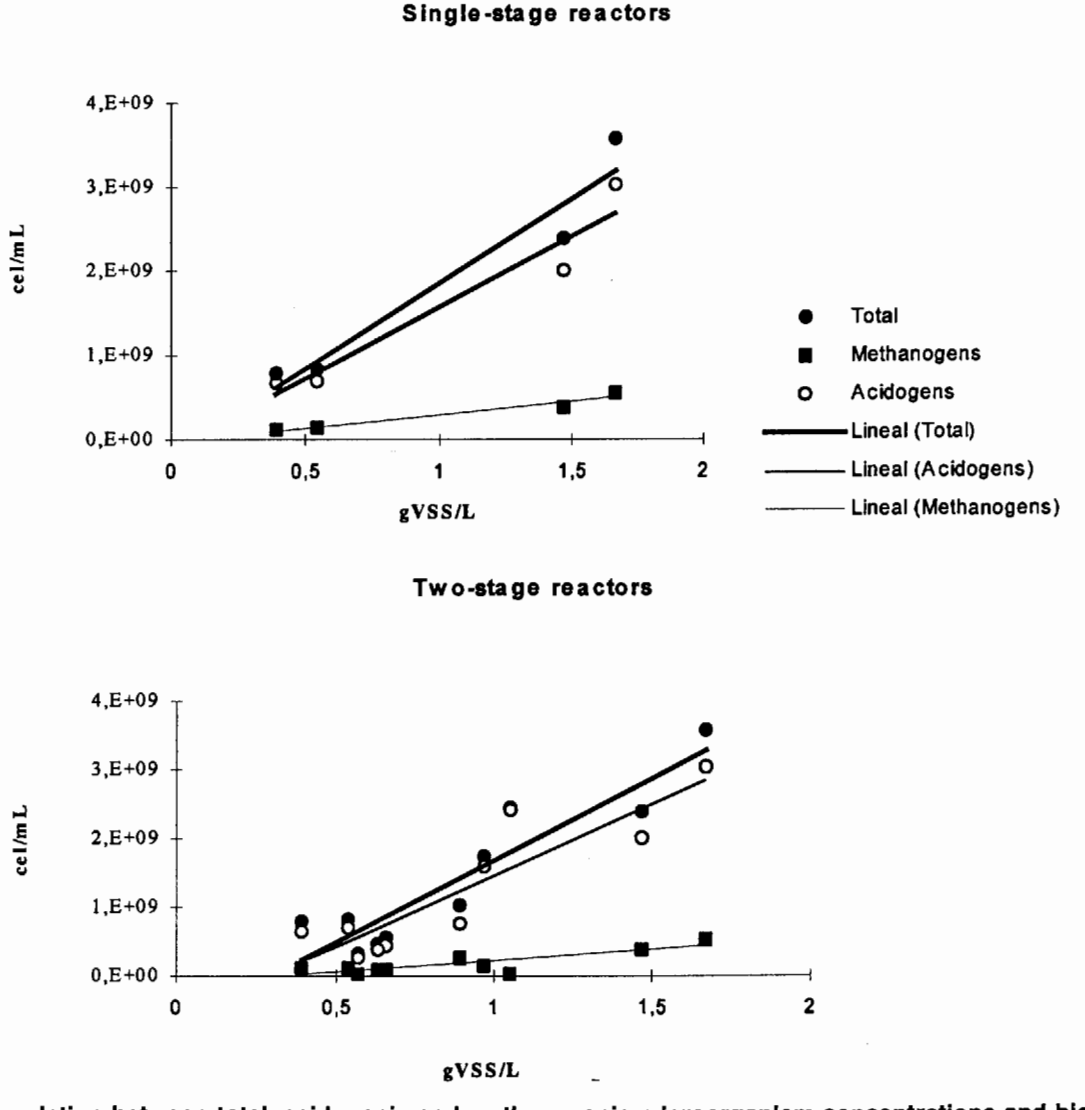

Figure 3—Correlation between total, acidogenic and methanogenic microorganism concentrations and biomass.

Table 4—Correlation between direct microbial counts and biomass.

Population	Parameter[a]	Reactor: Single-stage	Reactor: Two-stage
Total	Equation	$2.00 \times 10^9 X - 1.52 \times 10^6$	$2.38 \times 10^9 X - 6.91 \times 10^8$
	R^2	0.9354	0.8524
Acidogens	Equation	$1.70 \times 10^9 X - 1.36 \times 10^8$	$2.06 \times 10^9 X - 5.93 \times 10^8$
	R^2	0.9319	0.7930
Methanogens	Equation	$3.05 \times 10^8 X - 1.06 \times 10^7$	$3.19 \times 10^8 X - 1.00 \times 10^8$
	R^2	0.9532	0.6475

[a] R^2 = correlation coefficient.

Results and Discussion

Performance and operating parameters for controlling the anaerobic process are shown in Table 1. Microorganism concentrations, obtained by direct count and determinations of biomass, are shown in Table 2. Percentages of the principal groups of microorganisms and the number of cells per gram of biomass are shown in Table 3. All the results shown represent the average values for the total days of the study.

Principal population percentages (acidogenic and methanogenic) remain practically constant (83:17) in the single-stage digesters operated under different HRTs. Nevertheless, operation under the shorter HRT assists the microbial population to reach a greater size in the steady-state condition. The results of correlation between direct microbial counts and biomass are shown in Figure 3 and Table 4.

Independent of the operated HRT, the positive correlation between microorganisms concentration and biomass is high because the percentages of the principal groups remain constant and, therefore, the number of cells per gram of VSS remains constant (see Table 3). In these cases, biomass determinations can be used to estimate microbial concentrations in the reactors and vice versa.

In the two-stage reactors, the percentages of the main groups remained as previously described for the single-stage reactors in the initial assays. However, the acidogenic and methanogenic percentages are slightly higher in each corresponding phase of the reactor than those obtained in the single-stage system. The size of population in the acidogenic reactor is smaller when operated under an HRT of 1.7 days. This HRT is short enough to "wash out" slow-growing microorganisms. Moreover, the system does not work under stable conditions, as shown by the molecular hydrogen content of the biogas (Table 1) and levels of volatile fatty acids (Figure 4).

Microbial number and biomass results are greater when the two reactors are operated under an HRT of 4 days. Acidogenic microorganisms constitute more than 99% of the total population in the first reactor phase, and methanogenic bacteria more than 26% in the second reactor phase. Nevertheless, an effective separation of acidogenic and methanogenic phases was not obtained. Thus, some methanogenic bacteria remained in the acidogenic reactor and vice versa. The fact that molecular hydrogen utilizer methanogens require less strict growth conditions than the acetogenic type and, hence, can resist the operating conditions imposed (low pH and short HRT) may explain why they remain in the acidogenic reactor. Microscopic observations under autofluorescence revealed a different methanogenic morphotype (long rod) compared with those of the other reactors studied (short and medium-length rod).

The correlation between direct microbial counts and biomass for the two-stage reactors is not good (Figure 3 and Table 4) because the principal population percentages are substantially different in this case; population contributions to the total biomass also vary substantially. Hence, the worst correlation was obtained for the methanogens, which presented the lowest percentage in the reactors.

There is no correlation between direct counts and biomass because the principal group percentages do not remain constant when the reactors are operated under different HRTs. In this case, biomass may increase or decrease without a corresponding change of methanogenic numbers. Therefore, in the two-stage digesters, biomass cannot be used reliably to estimate the concentration of microorganisms, at least for the methanogenic type.

Viable concentrations and plating efficiency are shown in Table 5. The correlation between direct count by DAPI fluorescence microscopy and viable plate count for the single- and two-stage reactors are shown in Figure 5.

Counts obtained by direct epifluorescence microscopy are 2 orders of magnitude higher than counts obtained by culture techniques. These results are in accordance with those previously reported by Atlas and Bartha (1993). The syntrophic relationship between the microorganisms involved in the anaerobic process may explain the low values of viable population obtained in the reactors studied because growth by the plating method makes it difficult to maintain this relationship. In addition, plating procedures are more effective for quantifying a microbial population that constitutes a significant proportion of the microbial community in the reactor, but microorganisms constituting low proportions of the community can be overlooked by plating procedures.

Nevertheless, there is a high correlation between direct counts by fluorescence microscopy and viable plate counts in all of the studied systems. This high correlation may confirm that the selected growth medium and incubation conditions used are suitable. Using this count procedure, a viable count representative of the main groups contained in the reactors and, consequently, representative of total population in the digesters can be obtained.

Conclusions

In the single-stage reactors, the relative proportions of the acidogenic and methanogenic populations remain constant (83:17) in single-stage thermophilic reactors operated under different HRTs. Steady-state bacterial populations reached greater size when operated HRT was reduced. Further, there was a high correlation between direct count by DAPI epifluorescence microscopy and biomass expressed as VSS. Consequently, measurement of micro-

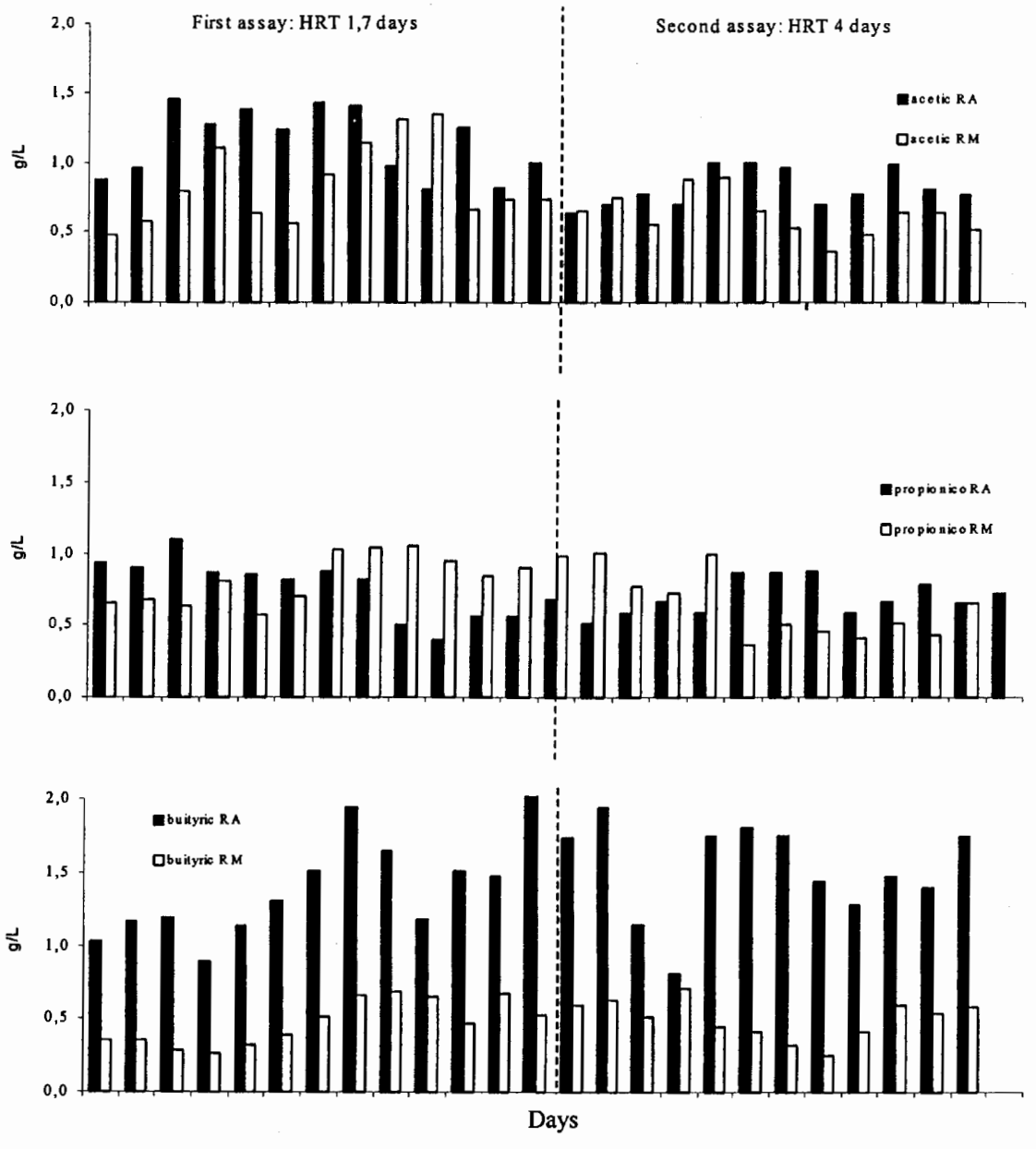

Figure 4—Levels of volatile fatty acids in the two-stage reactors.

Table 5—Viable concentrations and plating efficiency in the studied reactors.

Reactor	Viable population ($\times 10^7$)[a]	Total population ($\times 10^8$)[a]	Plating efficiency[b] (%)
R10	0.19 ± 0.06	8.06 ± 1.92	0.24
R4	6.69 ± 0.91	29.70 ± 7.15	2.25
$RA_{(4)}$	3.95 ± 0.77	24.40 ± 2.40	1.61
$RM_{(RA4)}$	0.30 ± 0.11	10.39 ± 1.63	0.29

[a] Colony forming units (cfu)/mL.
[b] cfu/DAPI × 100.

bial number and biomass can be used in an interchangeable manner.

In the two-phase reactors, acidogenic bacteria reached more than 99% of the total population contained in the first phase of the reactor, and methanogens reached more than 26% of the total population in the second phase. Therefore, the effective separation of the two phases was not obtained. It is possible that the molecular hydrogen utilizer methanogens remained in the acidogenic reactor. Because there was no correlation between direct count and biomass parameters, biomass changed without a corresponding change of microbial numbers, particularly methanogenic numbers. In this case, measurement of microbial number and biomass should not be used in an interchangeable manner.

The relationship between epifluorescence microscopy and viable count results are in accordance with those reported by Atlas and Bartha (1993) for these parameters. The high correlation

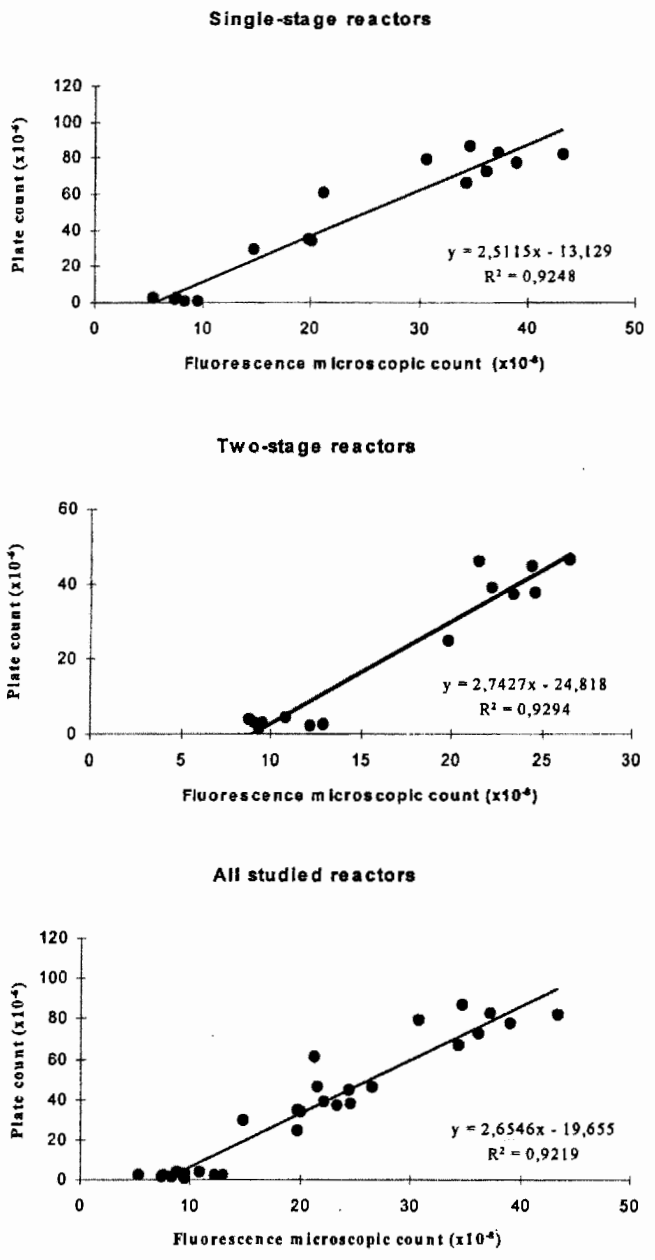

Figure 5—Correlation between direct microbial count by DAPI and viable plate count.

between epifluorescence microscopy and viable count may confirm that the protocol selected for this study is suitable for counting the main bacterial groups contained in reactors and, consequently, gives a representative count of total population in the digesters. The syntrophic relationship between the microorganisms involved in the anaerobic process may explain the low values of viable population obtained in this study.

Acknowledgments

Authors. R. Solera, L.I. Romero, and D. Sales are with the Department of Chemical Engineering, Food Technology and Environmental Technology, Faculty of Marine Sciences, University of Cádiz, Spain. Correspondence should be addressed to Rosario Solera, Professor of Environmental Technology, Department of Chemical Engineering, Food Technology and Environmental Technology, Faculty of Marine Sciences, University of Cádiz, Polígono Rio San Pedro s/n, 11510 Puerto Real, Cádiz, Spain

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