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Determination of the Microbial Population in Thermophilic Anaerobic Reactor: Comparative Analysis by Different Counting Methods

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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 DAPI (4′,6-diamidine-2-phenylindole) as fluorochrome was used to determine the total number of micro-organisms in the reactors, and autofluorescence microscopy for the number of the autofluroescent methanogenic populations. The results obtained by the direct count methods were compared to the quantity of biomass contained in the system, determined by volatile suspended solids. The viable bacterial population was determined by plating techniques using an anaerobic chamber. The total bacterial and F420 autofluorescent populations of single-stage digesters increase when the hydraulic retention time decreases; nevertheless, the percentages of the autofluorescent methanogens remain constant at 13%. In the two-stage reactors, the percentages of this group are 99% and 26% of the total population in the acidogenic and methanogenic factors, 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. It was obtained a high correlation between direct counts by epifluorescence microscopy and viable plate counts for the combined system studied.

Introduction

The anaerobic treatment of industrial wastewater has a number of potential benefits, including low energy consumption, low excess sludge production, and enclosure of odours and aerosol. In the mixed culture of anaerobic reactor, several different degradation reactions take place. Complex kinetics, interactions and different steps have been reported by numerous authors [1–6]. Anaerobic digestion can be considered a two-step process, even though it is really a coupled sequence of microbiological interactions. Firstly, complex organic materials are depolymerized and converted to fatty acids, CO_2 and H_2. In the next step, methane is generated by reduction of CO_2, by

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molecular hydrogen or by other reduced fermentation products, such as fatty acids. There are considered to be two main groups of bacteria 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. Though normally correlated to each other, these parameters should not be used in an interchangeable manner. On occasion, when one parameter is required, such as number of micro-organisms, it is necessary because of technical problems to measure a less relevant parameter, such as biomass, 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, since the measurement of biomass (as volatile suspended solids, principally) does not provide this information.

Direct count procedures by microscopic methods yield the highest estimates of members of micro-organisms and are occasionally used for indirect calculation of biomass. Epifluorescence microscopy with fluorometric stains are widely used for direct counting of bacteria, since it does not require culturing [7–9]. In particular, DAN-specific fluorescent stains 3,6-bis (dimethylamino) acridinium chloride (acridine orange) and 4′,6-diamidine-2-phenylindole (DAPI) are most often used [10].

A characteristic peculiarity of methanogens is their UV-induced blue-green autofluorescence which permits counting by autofluorescence microscopy [11–12]. However, this method is subjective under following conditions: it only shows methanogens with a high content of F420 such as hydrogen-utilising methanogens; acetate-utilising methanogens belonging to the genus *Methanosaeta* can not be counted at all and genus *Methanosarcina* are found in clumps made up of many individual cells. Nevertheless, it is one frequently used method to count autofluorescent methanogens in anaerobic reactors [13–17].

There are, however, several drawbacks to direct observational methods, including the inability to distinguish living from dead micro-organisms and the inability to perform further studies on the observed micro-organisms. Because of this, an important avenue of research has been the development and utilization of molecular techniques. Molecular techniques have successfully been applied for the direct detection and identification *in situ* of individual microbial cells and have therefore been used to monitor the spatial distribution of micro-organisms in environmental samples and treat system. However, it must be kept in mind that the physiological state (living or non-living) is operationally-defined and based on the general properties of a particular stain, and dormant or extremely slow-growing cells cannot be detected. Thus, the interpretation of results is often ambiguous [10]. Therefore, molecular techniques are expensive in comparison with direct count methods by microscopy (i.e. confocal laser scanning microscopy, target molecular probes). Nevertheless, DAPI epifluorescence methods is usually used to obtain a total count even when molecular methods are applied [18].

The viable count procedures by plate count methods permit the estimation of numbers of particular types of microorganisms in the total viable microbial biomass within anaerobic reactors. The real advantage of a viable plate count is that conditions can be adjusted for detecting some particular groups when these constitute a significant portion of the microbial community. In some cases, positive correlation between direct fluorescence microscopic counts and viable plate counts in high; in other cases, correlation is low [7]. The differences between direct counts and plate counts may simply reflect the proportion of living, dead or injured micro-organisms, the selectivity of the media and incubation conditions used.

As a consequence of the reasons mentioned above, it is obvious that various methods can be used to quantify micro-organisms into reactors. No universal method can be applied, since micro-organisms in anaerobic reactors are extremely diverse and all of these methods have advantages and disadvantages. The diversity of micro-organisms requires the use of a variety of a methodological approaches.

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 total count was performed by the DAPI epifluorescence microscopy method. Autofluorescent methanogens were counted by the autofluorescence microscopy method. The viable bacterial population was quantified by the solid-medium plating technique, employing an anaerobic chamber for spreading onto plates and the subsequent incubation of inoculated plates. The biomass was determined by measuring volatile suspended solids (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—take place in the same reactor, whilst in the two-stage process they take place in separate reactors.

Materials and Methods

Experimental plan

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 liquids 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 retention time (HRT): 4 and 10 days (digesters R4 and R10, respectively). In the two-stage system, 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. They 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 favouring the growth acid-formers, i.e. a short HRT (1.7 days) and a low PH (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 the 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 autofluorescent methanogens in the reactors were counted by autofluorescence microscopy. The concentration of the non-methanogenic population was estimated by subtracting the results of the autofluorescence microscopy from those of the DAPI epifluorescence.

The biomass contained in the system was determined by volatile suspended solids. The direct microbial counts and biomass measurement were correlated and the number of cells per gram of biomass was estimated.

The viable bacterial population was quantified by the plating technique, employing an anaerobic chamber for spreading onto solid medium and incubation of inoculated plates. These results were compared with the total 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 vertical cylinder tank (25 cm length and 10 cm internal diameter). The active liquid volume was 2 L. Reactor temperature was maintained at 55°C and the biogas generated was collected in a gas-meter. The feed was added in a semi-continuous mode: one dose per day. Effluent recirculation was used to mix and homogenise the liquid in the system.

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 macro- and micro-nutrients with a favourable BOD : N : P ratio (91 : 4 : 1) suitable for

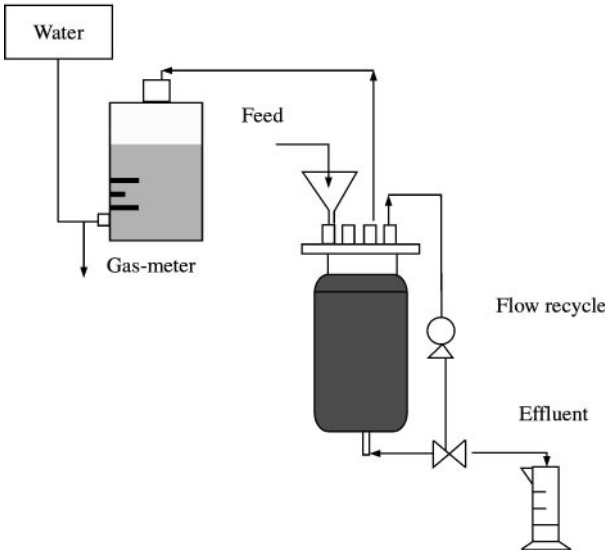

Figure 1. Schematic diagram of the experimental CSTR.

microbiological treatment. A complete study of the characteristics and properties of vinasses can be found in a previous paper by the authors [19]. Before their utilization, vinasses were transported and maintained at 4°C. The original substrate was diluted with tap water to attain the required chemical oxygen demand (COD) concentration to be used in the feed for this experiment (around 15 g/COD/L). This was supplemented with sodium hydroxide to maintain a neutral pH, and pH 5 in the case of the acidogenic reactor. Vinasses biodegradation batch experiments [20] 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, and the concentration of volatile suspended solid (VSS) and bacteria was negligible.

The methanogenic reactor was fed with acidogenic effluent, filtered using a Millipore GVWP filter with a 0.22 μm pore size to retain the acidogenic microorganisms, and was supplemented with sodium hydroxide to maintain a pH of around 8.5.

Analytical methods

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

1) Analytical determinations utilised to monitor and control the anaerobic digestion process. All analytical determinations were carried out according to the 'Standard Methods' [21]. The parameters analysed for liquid samples in both effluent and influent were pH and soluble COD, while for gaseous samples the volume of biogas produced at STP conditions and its composition (CH_4, CO_2 and H_2) were analysed.

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 (CH_4, CO_2 and H_2) was determined using a modified gas chromatography method previously described by Nebot [22].

2) Analytical determinations utilised to count bacterial population. Quantification of total numbers contained in the system was determined by epifluorescence microscopy with DAPI, according to Kepner et al. [8]. Immediately after sampling, 10 mL of the sample diluted using phosphate buffer (pH 7.2) to give a count of 10–15 cells per field and homogenised with a vortex Heidolph Reax 2000, for 30 s. Bacterial cells were retained on a 25-mm black polycarbonate membrane filter with a 0.22 μm pore size (Millipore GTBO). Cells were counted visually using a Nikon Labophot-2A/2 microscope fitted with a 100 W mercury lamp, Nikon UV-1A excitation and barrier filters and an ×100 oil objective. Those cells falling within the area of the ocular in randomly located fields were counted.

The autofluorescent methanogens were counted by autofluorescence direct counting, using the same microscopy as that used for the epifluorescence method as described by Doddema and Vogels [11]. The sample was fixed with a glutaraldehyde solution buffer (final concentration, 5%). Next, the sample was diluted and homogenised using the same procedure as described above, but in this case, the dilution gave counts of between 80 and 100 per field of view. A very small drop (5 μL) 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. Micro-organisms were grown in an essential medium as described by Maestrojuan [23–24], containing the following (g/litre of distilled water): glucose, 4.50; triptone, 2; yeast extract, 2; $NaHCO_3$, 2.8; cysteine hydrochloride, 0.5; resazurin, 0.001; NH_4Cl,1; $MgCl_2{\cdot}6H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.1; $K_2HPO_4{\cdot}3H_2O$ 0.4. The trace mineral solution (10 mL) used is shown in Table 1. Noble agar (Difco Laboratories, Detroit, MI, U.S.A.) at a final concentration of 2% (wt/vol) was used. Sterile Na_2S-$9H_2O$ was added to the medium just before it was spread onto the plates. All components were assembled in glass-distilled water and sterilised by autoclaving at 120°C for 20 min. Dispensing of solid medium, manipulations of cultures, inoculation and incubation of plates were all performed in an anaerobic cabinet (model 1029, Forma Scientific) under an atmosphere of N_2, CO_2 and H_2 (85 : 15 : 15). 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 with gentle swirling. Each dilution was

plated in triplicate. Plates were incubated inverted at 55°C for 72 h in an incubator inside the cabinet.

Biomass was determined by measuring the volatile suspended solids contained in the digester medium, according to 'Standard Methods' [21].

Results and Discussion

Single-stage reactors

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

Autofluorescent methanogens remain practically constant (17%) in the single-stage digesters operated under different HRTs. Nevertheless, operation under the shorter HRT assists the bacterial population to reach a greater size in the steady-state.

Independently of the operated HRT, the positive correlation between micro-organism concentration and biomass is high and, consequently in these cases biomass determinations can be used to estimate microbial concentrations in the reactors, and vice versa (Figure 2).

Viable concentrations and plating efficiency are shown in Table 4. The correlation between direct count by DAPI fluorescence microscopy and viable plate count for the single-stage reactors is shown in Figure 3.

Counts obtained by direct epifluorescence microscopy are two orders of magnitude higher than counts

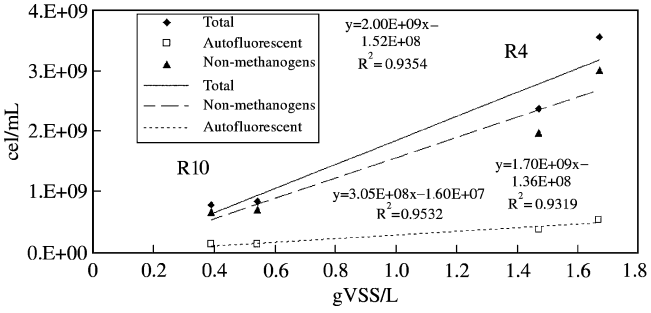

Figure 2. Correlation between micro-organism concentrations by direct count and biomass as VSS, for the single-stage reactors.

Table 1. Trace mineral content of solution used with solid growth medium

Component	g/litre of distilled water
Nitrylacetic acid	1.280
$FeCl_3.6H_2O$	1.350
$MnCl_2.4H_2O$	0.100
$CoCl_2.6H_2O$	0.024
$ZnCl_2$	0.100
$CuCl_2.2H_2O$	0.025
H_3BO_3	0.010
$Na_2MoO_4.2H_2O$	0.024
NaCl	1.000
$Na_2SeO_3{\cdot}5H_2O$	0.026
$NiCl_2.6H_2O$	0.120

Table 4. Viable concentrations and plating efficiency in the single-stage reactors

Reactor	Viable population ($\times 10^7$)(1)	Plating efficiency(2)
R10	0.19 ± 0.06	0.25
R4	6.69 ± 0.91	2.30

(1)CFU/mL; (2) CFU/DAPI.100.

Table 2. Performance and operating parameters for the control of the anaerobic process during period of study

Reactor	HRT	OLRo	CODr	pH_e	Biogas	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

HRT (days); organic loading rate (OLRo) as $g.L^{-1}{\cdot}d^{-1}$ CODo; organic removal efficiency (as percentage of initial COD); pH; volumetric biogas production as $L{\cdot}L^{-1}{\cdot}d^{-1}$ digester; percentages of CH_4, CO_2, and H_2 in the biogas.

Table 3. Microorganism concentrations by direct count, biomass by VSS and number of bacteria per gram of biomass, in the single-stage reactors

Reactor	Total population ($\times 10^8$)(1)	Non-methanogenic population ($\times 10^8$)(1)	Non methanogenic percentage	Autofluorescent methanogens ($\times 10^8$)(1)	Autofluorescent methanogenic percentage	VSS (g/L)	Number of bacteria $gVSS^{-1}$ ($\times 10^{12}$)
R10	8.06 ± 1.92	6.77 ± 2.07	83.18	1.29 ± 0.50	16.82	0.47 ± 0.09	1.86
R4	29.70 ± 7.15	25.10 ± 7.00	83.41	4.60 ± 0.80	16.59	1.57 ± 0.22	1.87

Precision of counts: 95% confidence interval.
(1) Microorganisms/mL.

obtained by culture techniques. These results are in accordance with those previously reported by Atlas [7]. There is a high correlation between direct counts using fluorescence microscopy and viable plate counts in the single-stage system. The high correlation may confirm that the selected growth medium and incubation conditions used are suitable to enumerate the groups that constitute a significant portion of the microbial community into single-stage anaerobic reactors.

Two-stage reactors

Performance and operating parameters for the control of the anaerobic process are shown in Table 5. Micro-organism concentrations, obtained by direct count, and determinations of biomass, are shown in Table 6. Percentages of the autofluorescent methanogens and number of cells per gram of biomass are shown in Table 6. As with the single-stage reactors, all the results shown are the average values for the total number of days of the study.

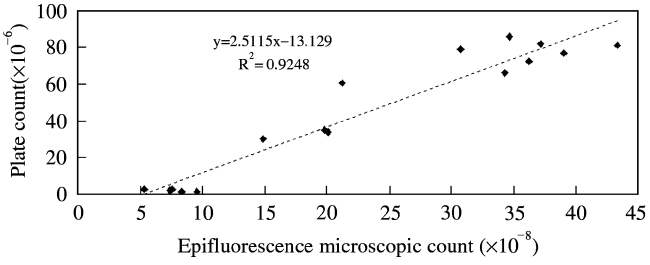

Figure 3. Correlation between epifluorescence microscopy count and viable plate count for the single-stage reactors.

In the initial assays, the percentages of autofluorescent methanogens remained as described above for the single-stage reactors. However, they are, respectively, slightly lower and higher in each acidogenic and methanogenic 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 a HRT of 1.7 days. This HRT is short enough to make 'wash-out' of slow growing micro-organisms possible. Moreover, the system does not work under stable conditions, as shown by the H_2 content of the biogas (see Table 5).

Bacterial number and biomass results are greater when the two reactors are operated under an HRT of 4 days. Autofluorescent methanogens constitute less than 1% of the total population in the first reactor phase, and 26% in the second reactor phase. Nevertheless, an effective separation of acidogenic and methanogenic phases was not obtained, thus some methanogens remained in the acidogenic reactor and vice versa.

The correlation between direct microbial counts and biomass for the two-stage reactors are not good (Figure 4). In this case, biomass may increase or decrease without a corresponding change of cell numbers. Therefore, in the two-stage digesters, biomass cannot be used reliably to estimate the concentration of micro-organisms, at least for the methanogenic type.

Viable concentrations and plating efficiency are shown in Table 7. The correlation between direct count by DAPI fluorescence microscopy and viable plate count for the two-stage reactor is shown in Figure 5. Counts obtained by direct epifluorescence microscopy are two orders of magnitude higher than

Table 5. Performance and operating parameters for the control of the anaerobic process during period studied

Reactor	HRT	OLRo	CODr	pH_e	Biogas	CH_4	CO_2	H_2
$RA_{(1.7)}$	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
$RM_{(RA\ 4)}$	4	2.65	71.7	7.80	0.45	91	9	0

HRT (days); organic loading rate (OLRo) as $g{\cdot}L^{-1}{\cdot}d^{-1}$ CODo; organic removal efficiency (as percentage of initial COD); pH; volumetric biogas production as $L{\cdot}L^{-1}{\cdot}d^{-1}$ digester; percentages of CH_4, CO_2, and H_2 in the biogas.

Table 6. Micro-organism concentrations by direct count, biomass by VSS number of bacteria per gram of biomass in the two-stage reactors

Reactor	Total population ($\times 10^8$)(1)	Non-methanogenic population ($\times 10^8$)(1)	Non methanogenic percentages	Autofluorescent methanogens ($\times 10^8$)(1)	Autofluorescent methanogenic percentages	VSS (g/L)	Number of bacteria $gVSS^{-1}$ ($\times 10^{12}$)
$RA_{(1.7)}$	31.0 ± 0.60	2.70 ± 0.60	87.96	0.36 ± 0.09	12.04	0.57 ± 0.04	0.49
$RA_{(4)}$	24.40 ± 2.40	24.20 ± 2.40	99.29	0.17 ± 0.05	0.71	1.05 ± 0.09	2.35
$RM_{(RA\ 1.7)}$	5.50 ± 2.69	4.49 ± 2.66	79.00	1.00 ± 0.58	21.00	0.66 ± 0.08	0.79
$RM_{(RA\ 4)}$	10.39 ± 1.63	7.72 ± 1.56	73.84	2.68 ± 0.27	26.16	0.89 ± 0.15	1.20

Precision of counts: 95% confidence interval.
(1) Micro-organisms/mL.

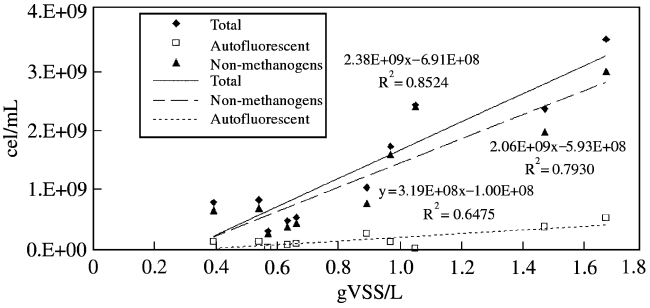

Figure 4. Correlation between micro-organism concentrations by direct count, and biomass as VSS, for the two-stage reactors.

Table 7. Viable concentrations and plating efficiency in the two-stage reactors

Reactor	Viable population ($\times 10^7$)(1)	Plating efficiency (2)
$RA_{(1\text{-}7)}$	—	—
$RA_{(4)}$	3.95 ± 0.77	1.64
$RM_{(RA\ 1\text{-}7)}$	—	—
$RM_{(4)}$	0.30 ± 0.11	0.30

(1) CFU·mL^{-1}; (2) CFU·DAPI^{-1}·100.

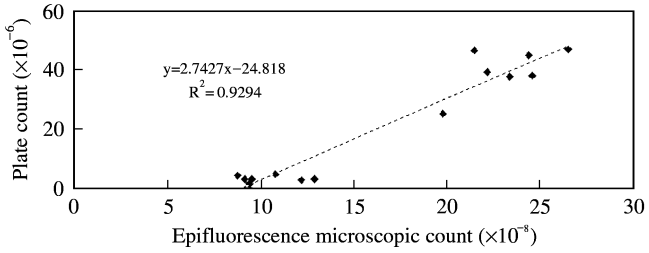

Figure 5. Correlation between epifluorescence microscopy count and viable plate count, for the two-stage reactors.

counts obtained by culture techniques. There is a high correlation between direct counts by fluorescence microscopy and viable plate counts in the two-stage system. These results are similar to those obtained in the single-stage reactors and therefore the conclusions previously drawn are also valid for two-stage reactors.

Conclusions

In the single-stage reactors:

1. There is a high correlation between direct count by DAPI epifluorescence microscopy and biomass expressed as VSS. Consequently, measurement of microbial number and biomass can be used in an interchangeable manner.
2. The autofluorescent methanogens constitute 17% of the total microbial community in single-stage thermophilic reactors operated under different HRTs.

In the two-phase reactors:

1. There is no correlation between direct count and biomass parameters, so that in this case biomass changes without a corresponding change of microbial numbers, particularly of autofluorescent methanogenic numbers. In this case, measurement of microbial number and biomass should not be used in an interchangeable manner.
2. Autofluorescent methanogens constitute less than 1% of the total population contained in the first phase of the reactor, and more than 26% of the total in the second phase. Therefore, the effective separation of the two phases is not obtained.

Viable plate count

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

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