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Use of microbial activity parameters for determination of a biosolid stability index

J. Barragán Sánchez [a,b], J.M. Quiroga Alonso [a], M.D. Coello Oviedo [a,*]

[a] Department of Chemical Engineering, Food Technology and Environmental Technology, University of Cadiz, CASEM, Campus Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain
[b] AQUALIA, Avda. San Francisco Javier, 15-2° B, 41005 Sevilla, Spain

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Abstract

Variations in microbial activity during the aerobic digestion of sludge generated at wastewater treatment plants were studied. Results obtained by the measurement of enzymatic activity and microbiological parameters were compared with those determined by traditional methods (COD, suspended solids, etc.). Their variation with digestion time was monitored for batch digestion over a period of 135 days. The relationship between these measurements and control parameters of the sludge was also investigated.

It was found that the traditional physicochemical and microbiological parameters present a series of problems which detract from their usefulness. The enzymatic parameters dehydrogenase activity (primary metabolism) and esterase activity (secondary metabolism) are better able to characterise the process, and the ratio of these two variables may be used to estimate the degree of endogenesis and, consequently, the degree of stability of the aerobic sludge digestion. In addition, these techniques are swift and simple to employ.

Keywords: Aerobic stabilization; Endogenous respiration; Dehydrogenase activity; Esterase activity; Oxygen uptake rate; Enzymatic activity

1. Introduction

The integral treatment of wastewater necessarily implies the management and treatment of the sludge produced, and this represents more than 50% of the construction and operating costs of wastewater treatment plants (Metcalf and Eddy, 2003).

Currently, Europe produces in excess of 6.5 million tonnes of dry sludge each year and it is forecast that this amount will increase more rapidly over the coming years. In terms of cost, 1 million Euro/year is spent on the management of treated sludge, a further 150 million Euro/year is spent on the control and handling of sludge digestion, and a similar, as yet unknown figure, is required for the management of industrial sludge. With the definitive introduction of the European Urban Waste Water Treatment Directive (91/271/EEC), it is expected that more wastewater treatment plants will be constructed and, since the majority of these facilities will be of average size, the sludge digestion systems to be implemented will be primarily aerobic. The great demand for this resource—once stabilized for different purposes, such as agriculture—makes better knowledge essential, both of its properties and of the degree of stability achieved (Polo Gómez et al., 1998).

All of the above implies an improvement in the treatment processes, a greater knowledge of the degree of stability and of the parameters that should be used to measure this stability. Notwithstanding, there is no consensus to determine which tests or criteria may or should

* Corresponding author. Fax: +34956016040.
E-mail address: dolores.coello@uca.es (M.D. Coello Oviedo).

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be used as indices of sludge stability in the different treatments to which sludge is submitted.

Enhanced treatment, also referred to as "Advanced Treatment", is a term used to describe treatment processes which are capable of virtually eliminating pathogens which may be present in the original sludge. Enhanced treated sludge will be free from Salmonella and will have been treated so as to ensure that 99.9% of pathogens have been destroyed, (ADAS web site, 2001).

In this paper, three groups of parameters are compared. Two of these, the physicochemical and microbiological parameters, have been used traditionally; the third, enzymatic activity measurement, is beginning to be employed in the control of this type of process. From a comparison of these three parameter types, conclusions may be reached regarding which provides truer and more precise information in respect of the variation in stability during the aerobic digestion of mixed sludge.

2. Methods

The stabilization assays were carried out in a 100 l capacity benchtop reactor, operated in batch mode for 135 days (Fig. 1). The mixed primary and biological sludge came from the primary settling tank of "El Torno" Wastewater Treatment Plant in Chiclana de la Frontera (Cadiz). The rate of aeration was controlled by a group of compressors, which provided the system with a 0.05 m^3/h flow, allowing oxygen concentration within the reactor to be kept within the 0.5–1.4 ± 0.1 mg/l range according to Metcalf and Eddy (2003).

2.1. Physicochemical parameters

2.1.1. pH

In the evaluation of pH values, a CRISON Portable 506 pH/mV meter was employed, fitted with a CRISON electrode, reference Cat. No. 52-00.

2.1.2. Dissolved oxygen

Oxygen and temperature levels were monitored with a WTW OXI 92 model oxygen meter, with membrane heads, 0.1 mg/l oxygen precision and an in-built temperature sensor. Units of dissolved oxygen are mg O_2/l.

2.1.3. Hydrogen sulphide production

Hydrogen sulphide production was determined using a method based on the procedure proposed by Hartman et al. (1978). Five ml of sludge was incubated at 20 °C for 24 h, in 12 ml capacity screw-top tubes. The gas produced was measured with a PacIII Dräger gas monitor, fitted with a catalytic H_2S sensor. Units of hydrogen sulphide concentration are mg H_2S/l.

2.1.4. Chemical oxygen demand (COD)

Chemical oxygen demand (COD) measurements were obtained by performing dichromatometry on the digestion samples and on the feed sludge from the digester, in accordance with Standard Method 5220C of the APHA (1992). This method is valid for the determination of samples with COD readings of less than 2500 mg O_2/l.

2.1.5. Total and volatile solids

Total solids (TS) and volatile solids (VS) were assessed in accordance with Standard Methods (APHA, 1992). Units of total and volatile solids are g/l.

2.2. Microbiological parameters

2.2.1. Fecal coliforms and Escherichia coli

The procedure recommended in Standard Methods (APHA, 1992) was employed in the analysis of fecal coliforms and *Escherichia coli*. The method is based on a statistical count using the most probable number technique (MPN), and requires three series of five tubes. The culture medium employed was DIFCO A-1 Medium.

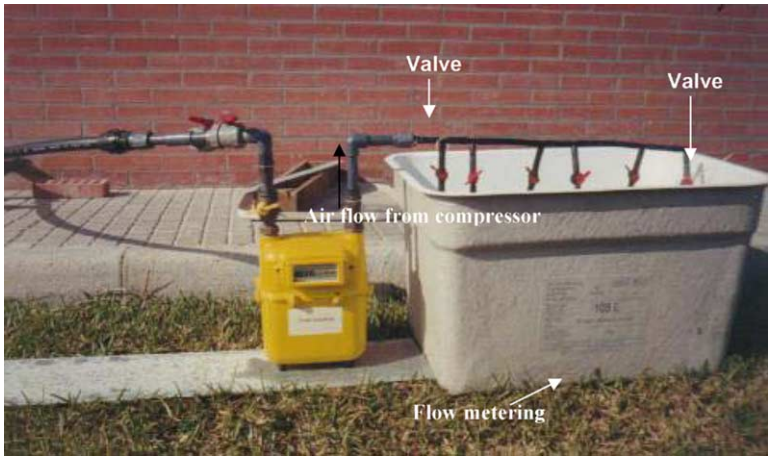

Fig. 1. Batch reactor system used during the experiment.

2.2.2. Active population

The active bacterial population was determined using the method according to Griebe et al. (1997), using a tetrazolium salt (5-cyano-2,3-ditolyl tetrazolium chloride (CTC)) and epifluorescence microscopy, to distinguish the metabolically active bacteria from those which were not. The same procedure was applied in the calculation of the total number of bacteria. On this occasion, epifluorescence microscopy was used in conjunction with fluorochrome 4,6-diamidin-2-phenylindol (DAPI). Briefly, this method involved incubation of the sludge samples with the tetrazolium salt at 4 mM concentration, for two hours, at room temperature and in darkness. At the end of this time, the reaction was interrupted with the addition of 1 ml of 37% formol. Following incubation, and prior to their respective dilutions, the samples were sonicated (ULTRASONS-H, Selecta) for 15 min in order to break up the flocs that prevent a reliable and efficient bacterial count. Thereafter, successive 1/10 dilutions were effected on the total volume of 10 ml. On reaching the appropriate count density, 1 ml of DAPI (10 μg/ml) was added and is left to incubate at room temperature and in darkness for 15 min. Finally, the samples were filtered through a 0.2 μm pore size polycarbonate membrane. Observation with different filters and at various wavelengths served to differentiate the samples stained with CTC from those stained with DAPI. The number of microorganisms present in a millilitre of sample was calculated by applying the following conversion formula:

$$N = \frac{S \times n}{C \times V} \times D$$

where N is the number of microorganisms per millilitre; S the real area of filtration: 3.1416 cm^2; n the average number of microorganisms per field of vision; C the real area of microscopic range (0.025 mm^2); V the volume of filtered sample (10 ml) and D the sample dilution.

2.3. Measurements of biological activity

2.3.1. Specific oxygen uptake rate (SOUR)

The *specific oxygen uptake rate* (SOUR) was determined in accordance with the recommendations of Standard Methods (APHA, 1992). Notwithstanding, and due to the high concentration of solids present in the samples, an initial dilution was performed, using purified water previously filtered through a 0.45 μm pore size membrane filter and then autoclaved (the use of this water prevents sudden osmotic changes in the microorganisms, and does not constitute an additional oxygen demand). The respirometric activity of the microbial population was evaluated using a 500 ml bottle containing the dilute sample, which was homogenised with the aid of a magnetic agitator. Oxygen evolution and consumption throughout the trial were monitored by an OXI92 WTW oxygen meter. The specific oxygen uptake rate (SOUR) was calculated in accordance with the following expression, employed by Awong et al. (1985).

2.3.2. Esterase activity

The procedure followed in determining *esterase activity* was a modification of the method described by Fontevielle et al. (1992), using FLUKA-manufactured fluorescein diacetate (FDA) as reagent. The sample was diluted twice: 1/220 and 1/440, respectively, and at the same time, a buffer phosphate control was prepared. Measurements were taken on a final 5 ml volume. The volume of added reagent was 100 μl of fluorescein diacetate, prepared by adding 2 mg of FDA per millilitre of acetone. The samples were incubated in the dark for a period of 10 min, after which the reaction was interrupted by adding 1 ml of mercuric chloride to a 400 ppm concentration. Thereafter, the sample was centrifuged at 1500*g* for 10 min, and the optical density of the supernatant was measured at 490 nm in the spectrophotometer. The activity value was calculated by applying the following expression:

$$\mathrm{EA} = \frac{\Delta \mathrm{abs}}{\varepsilon} \times \frac{1}{t} \times v \times F \times \frac{1}{C}$$

where EA is the esterase activity (μmoles of hydrolysed FDA min^{-1} g VS^{-1}); Δabs the absorbance or optical density at 490 nm; t the incubation time (min); v the volume of $HgCl_2$ (ml); F the dilution factor; C the concentration of volatile solids (g/l) and ε the extinction coefficient of fluorescein acetate.

2.3.3. Dehydrogenase activity

Dehydrogenase activity was evaluated with the method proposed by López et al. (1986). This method is based on the measurement of the colour produced on reduction of the original substrate, INT 2-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride), to INT-formazan, by the oxidative effect of the dehydrogenase enzymes. The samples were diluted to a final concentration of 1 g of VSS/l. At a final volume of 5 ml, 0.5 ml of INT reagent was added at 3.95 mM and was then incubated for 30 min in darkness. The reaction was stopped by the addition of 1 ml of 37% buffered formaldehyde. Extraction was effected by placing 2 ml of the INT mixture/sample in a test tube, which was then centrifuged at 1500*g* for 15 min. The supernatant was separated and, in order to extract the formazan, 5 ml of 95% ethanol was added to the pellet which has formed and ultrasound was employed to encourage dilution. Finally, the extract was centrifuged once more and the absorbance of the supernatant was measured at 480 nm wavelength. INT-dehydrogenase activity was calculated in equivalent oxygen units using the following equation (López et al., 1986):

$$\mathrm{DHA} = \frac{1024 \times D_{480} \times V}{v \times C \times t \times F}$$

where DHA, INT-dehydrogenase activity (mg O_2 g VS^{-1} day^{-1}); 1024 the conversion factor (López et al., 1986); D_{480} the absorbance at 480 nm wavelength; V the final volume of solvent used to extract formazan (ml); v the volume of reagent used and sample treated (ml); C the concentration of volatile solids (g/l); t the incubation time (min) and F the dilution factor.

3. Results and discussion

The mixed liquor from the primary decanters was markedly anaerobic, due to the hydraulic residence time spent in these decanters. The initial characteristics of the sludge are summarised in Table 1.

3.1. Physicochemical parameters

Table 2 shows some of the most representative values of the different physicochemical parameters analysed during the assay. The pH readings reflect those obtained by Al-Ghusain and Hao (1995), who also reported an increase in pH values over the course of time, with an ultimate stabilization at the end of the assay. The fall in pH levels between days 30 and 50 is due to the nitrification process that took place in the reactor following the oxidation of easily biodegradable substances and when the respiration rate started to fall.

Table 2 also reveals the linear reduction of VS arising from the endogenous respiratory process taking place in the reactor, which led to a gradual decrease in the percentage of solids and stabilization of the sludge. A removal of 33.7% was achieved after 135 days.

The decline in COD recorded during the assay may have been due to the process of degradation in the system and to the endogenous respiratory processes (Fig. 2). From an initial value of 77.39 g/l, COD levels fell to 37.31 mg/l by the end of the trial, representing a 48.2% reduction. From day 70 of the experiment, COD values remained practically constant, indicating that the system was stable and that the remaining, non-degraded COD was due either to organic material which was highly resistant to biodegradation or to other, non-biodegradable inorganic compounds.

Table 1
Composition of the sludge at the beginning of the experiment

Parameters	Value	Parameters	Value
pH	6.1	EC, NMP/g TS	579.2
TS, g/l	60	CTC/DAPI	0.08
VS, g/l	59.98	SOUR, mg O_2/g VS d	3.08
COD, mg O_2/l	77,388	DHA, mg O_2/g VS d	31.6
H_2S, mg/l	100	EA, μmoles FDA/min · g SV	14.5

TS: total solids; VS: volatile solids; COD: chemical oxygen demand; H_2S: hydrogen sulphide; EC: *Escherichia coli*; CTC/DAPI: ratio active cell/total cell; SOUR: specific oxygen uptake rate; DHA: dehydrogenase activity; EA: esterase activity.

Hydrogen sulfide production decreased in proportion to the length of time the sludge was retained in the reactor. This decrease was exponential at the start of the assay and was asymptotic at the end. This is due to the fact that aeration prevents anaerobic processes from producing H_2S, and these processes are significant in sludge which has been exposed to anaerobic conditions prior to the onset of the assay; in addition, as time goes by, there are fewer resources available for use by the anaerobic microorganisms in the production of H_2S.

3.2. Microbiological parameters

Fig. 3 presents the evolution of fecal coliforms and *E. coli*. It may be observed that, after an exponential decrease over the first 30 days, the number of these microorganisms remained constant until the end of the assay. Notwithstanding, and given the values of these parameters, it may not be concluded that the process reached stabilization during this residence time, since, as reported elsewhere in the bibliography (USEPA, 1992; Droste and Sanchez, 1983), this type of parameter is indicative only of the general trend in the stabilization process but is not indicative of stabilization itself, since subsequent revitalisation of the pathogenic microorganisms may occur.

The relationship between the number of active and total cells, calculated with the aid of fluorochromes CTC and DAPI, provides additional information regarding the state of the active population with respect to the total number of bacteria present. Fig. 4 shows how, in the first few days of the assay, the number of active cells was relatively small, increasing to a maximum on day 16 due to favourable conditions of oxygenation and the presence of substrate. It should be pointed out that, initially, the sludge was anoxic, due to the design of the wastewater treatment plant, and, according to other authors (Smith and McFeters, 1997), the determination of active cells using the CTC fluorochrome technique is not suitable for measuring the activity of microorganisms when these are exposed to anoxic conditions; this may explain the low values obtained at the start of the experiment. Around day 30, a marked drop in cells became evident, and this continued to the end of the assay, which reflects the trends observed in the enzymatic activity measurements and is also in line with the dynamics of the sludge stabilization process.

3.3. Biological activity parameters

Table 2 shows how, during the first part of the experiment, and as a result of the initial anoxic conditions of the sludge, values of the specific oxygen uptake rate

Table 2
Results from traditional measurements (pH, percentage VS, COD and H_2S) and enzymatic activities (SOUR, DHA and FDA) during the experiment

Time, d	pH	%VS	COD, g O_2/l	H_2S, mg/l	SOUR, mg O_2/g TS h	DHA, mg O_2/g VS d	FDA, μmol FDA/min g VS
0	6.10 ± 0.01	60.0 ± 0.02	77.38 ± 0.02	100	3.08 ± 0.05	31.65 ± 0.05	14.58 ± 0.04
2	6.52 ± 0.01	63.9 ± 0.01	69.65 ± 0.02	100	4.24 ± 0.05	26.36 ± 0.06	11.96 ± 0.03
4	6.83 ± 0.02	61.8 ± 0.01	69.53 ± 0.01	100	5.21 ± 0.04	17.78 ± 0.04	7.17 ± 0.03
7	6.71 ± 0.01	62.0 ± 0.01	71.67 ± 0.01	90	6.23 ± 0.05	17.57 ± 0.04	12.32 ± 0.06
16	6.87 ± 0.01	58.6 ± 0.01	78.64 ± 0.01	50	8.19 ± 0.05	20.07 ± 0.04	15.850 ± 0.05
25	7.29 ± 0.01	59.6 ± 0.01	76.64 ± 0.01	30	6.15 ± 0.01	18.02 ± 0.04	12.94 ± 0.02
30	7.39 ± 0.01	58.8 ± 0.03	71.34 ± 0.01	nd	7.17 ± 0.04	23.09 ± 0.05	16.71 ± 0.03
38	6.93 ± 0.01	58.2 ± 0.03	55.49 ± 0.03	35	8.07 ± 0.04	23.10 ± 0.05	25.66 ± 0.03
46	6.95 ± 0.02	55.8 ± 0.03	55.96 ± 0.01	nd	14.50 ± 0.04	26.52 ± 0.03	34.90 ± 0.04
53	7.31 ± 0.01	55.2 ± 0.01	50.60 ± 0.01	40	7.94 ± 0.05	17.96 ± 0.03	24.63 ± 0.04
60	7.50 ± 0.01	52.4 ± 0.01	48.80 ± 0.02	24	6.50 ± 0.05	15.76 ± 0.03	12.00 ± 0.03
79	7.81 ± 0.02	48.9 ± 0.01	43.12 ± 0.01	12	2.87 ± 0.05	11.58 ± 0.03	17.79 ± 0.03
106	7.73 ± 0.01	43.0 ± 0.03	45.05 ± 0.01	3	2.02 ± 0.05	7.29 ± 0.05	11.27 ± 0.03
135	7.65 ± 0.01	39.7 ± 0.01	37.32 ± 0.01	0	1.13 ± 0.03	6.58 ± 0.04	10.10 ± 0.03

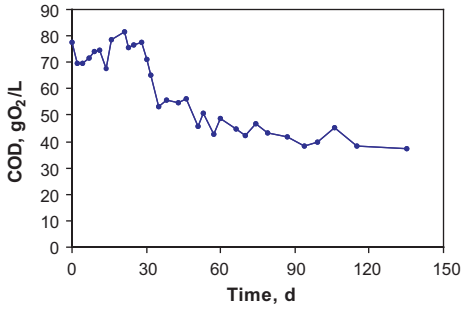

Fig. 2. Evolution of COD during the experiment.

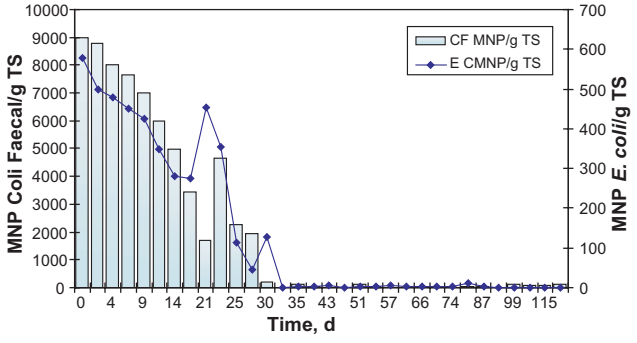

Fig. 3. Faecal coliforms and *Escherichia coli* count in aerobic sludges in the experiment.

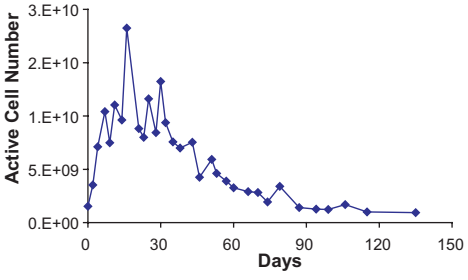

Fig. 4. Changes in the active cell numbers (CTC).

(SOUR) were relatively low compared to those typically recorded in activated sludge units (Coello, 1996). As the experiment progressed, and as oxygenation and feeding conditions stabilized, the microorganisms evolved and the consumption rate increased, from an initial value of 3.08 mg O_2/g TS h to a maximum value of 14.5 mg O_2/g TS h on day 46. From this point onwards, this parameter registered an exponential decrease, indicating a substantial reduction in respiratory activity. North American legislation establishes that sludge which has been aerobically stabilized may only be classified as 'category A' if it registers a value less than 1.5 mg O_2/g TS h (USEPA, 1992); these SOUR values were reached after day 110 of the assay.

Dehydrogenase activity, evaluated with spectrophotometry using INT fluorochrome, is a clear indicator of the primary activity of microorganisms, since it is related to cellular respiratory processes, whether or not this activity is aerobic (Maurines-Carbonell et al., 1989). In Fig. 4, it may be observed that the initial activity value of the mixed sludge was 32 mg O_2/g VS d. Thereafter, the first few days witnessed a drop in this value, due fundamentally to the adaptation of the microorganisms to the new environmental conditions. Once acclimatisation was complete, a slight increase in activity occurred and continued to day 46, after which there was a marked decrease in microorganism activity.

Esterase activity, evaluated using the spectrophotometer and the FDA fluorochrome, is representative of secondary metabolic processes, or in other words, the consumption of energy reserves. Fig. 5 shows how there was an initial decrease in this activity, probably because there were sufficient resources in the environment and no need to use energy reserves. Subsequently, when the microorganisms had become acclimatised, there was a marked increase, up to day 46, when the maximum value was recorded. Thereafter, values fell throughout the stabilization process. The evolution of

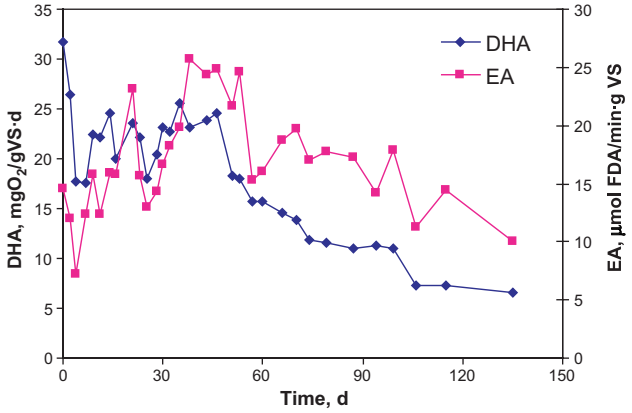

Fig. 5. Variations of dehydrogenase activity (DHA) and esterase activity (EA) into the experiment.

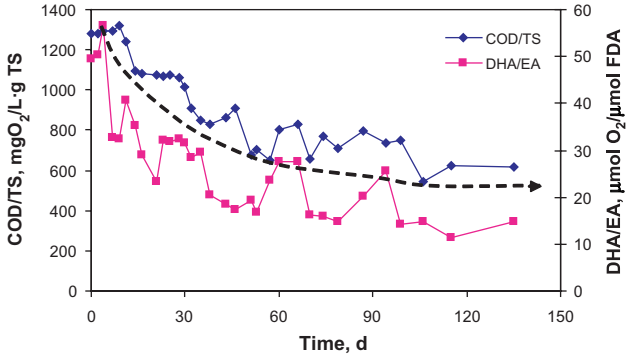

Fig. 6. Evolution of DHA/EA and COD/TS.

this parameter reflects the evolution of SOUR values, and coincides with dehydrogenase activity. Notwithstanding, and although both curves developed in the same way, in the first part of the experiment, esterase activity increased to a greater extent than dehydrogenase activity, although this difference became less marked during the stabilization period. This effect may have been due to the fact that, as time passed and the availability of energy resources in the reactor diminished, an endogenous phase was entered, during which the energy reserves, principally lipids, were consumed; this increase in the consumption of reserves is reflected in increased esterase activity.

3.3.1. Comparative analysis of the various parameters

The evolutionary pattern revealed by dehydrogenase and esterase activity levels differs from the trend shown by COD and TS measurements.

The COD/TS ratio provides information regarding the amount of substrate available per gramme of biomass. It must be borne in mind that not all of the substrate represented by COD readings is biodegradable. Nevertheless, a value may be identified for this quotient, above which microorganisms are unable to sustain life and a corresponding decline in biomass activity is observed.

The DHA/EA ratio provides information about the processes occurring between primary metabolism (respiratory processes, etc.) and secondary metabolism (consumption of energy reserves), since, if the quotient is small, it may be concluded that the endogenous processes predominate in the system and vice versa.

Fig. 6 shows the developmental pattern traced by both quotients. The figure reveals how, initially, when the sludge was not stabilized and the COD/TS ratio was high, the DHA/EA ratio was also high. As the experiment progressed and the sludge became stabilized, this quotient was gradually reduced, so that when the ratio was equivalent to 17 moles of O_2/µmol of hydrolysed FDA, around day 46, the evolution of the curve stabilized, indicating that the metabolic processes measured through esterase activity (EA) now enjoyed a more favourable environment compared to those processes linked to dehydrogenase activity (DHA); in other words, secondary metabolic activity was enhanced at the cost of primary activity and, consequently, the process entered a more markedly endogenous phase. It may also be observed how the increase in the enzymatic ratio (DHA/EA) from day 53 to day 66 coincided with an increased COD/TS quotient, which would indicate that the amount of substrate available to the microorganisms, expressed in terms of the COD/TS ratio, exerted a significant influence on the extent to which the process of endogenesis developed.

It would follow, therefore, that one way of analysing the evolution of sludge stabilization might be to study the relationship between dehydrogenase and esterase activity (DHA/EA).

A correlation coefficient of 0.7 was recorded between both curves over the course of the assay, implying that the amount of resources available to the microorganisms directly determines the degree of endogenesis achieved, and that this may be represented by the ratio between dehydrogenase and esterase activity.

4. Conclusions

Based on the results obtained from the pilot scale reactor, the following conclusions may be made:

- Enzymatic activities, and in particular the relationship between dehydrogenase and esterase activities, may be considered to be parameters capable of indicating the metabolic state of mixed sludge stabilization. In addition, the latter parameter may be used as an efficient tool in the routine control of aerobic sludge digesters, due to its simplicity, speed and low cost.
- The physicochemical parameters COD, TS and VS and the COD/TS ratio provide information about the efficiency of the stabilization process, but do not

reflect the evolution followed by the microorganisms, on which the treatment is based, nor the factors which affect them. Total solids have the drawback of producing a result after 24 h.

- Microbiological parameters have proved to be a useful tool in determining the bacterial concentration and population (fecal coliforms and *Escherichia coli*) but do not reflect the degree of endogenesis occurring in the system. The nature of the sludge floc, the high concentration thereof and the changes it undergoes, restrict the use of the epifluorescence microscopy technique in the routine control of the sludge population and sludge activity.
- Activity measurements are conditioned by the concentration of substrate existing in the system which is available to the microorganisms.

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