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Enzymatic estimation of biosolids stability in aerobic digestion systems

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Abstract

This paper reports the results obtained in a study of the aerobic stabilization of sludge in a laboratory-scale reactor. A variety of parameters were measured including: physicochemical (pH, dissolved oxygen, temperature, volatile and total solids, chemical oxygen demand and hydrogen sulfide production); microbiological (fecal coliforms, *Escherichia coli*, viable biomass, and the relationship between active and total cells); and measurements of enzymatic activity (oxygen uptake rate, dehydrogenase activity and esterase activity).

From the results, it may be concluded 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 quotient between 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.

Currently, Europe produces in excess of 6.5 million tons 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 kECU/year is spent on the management of treated sludge, a further 150 million ECU/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 European Directive 91/271, it is expected that more wastewater treatment plants (WWTP) will be constructed. In Spain, where the main number of WWTPs is situated in cities with less than 50,000 people, the aerobic digestion process for the sludge's stabilization is the most employed. 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.

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

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 measurements, 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.

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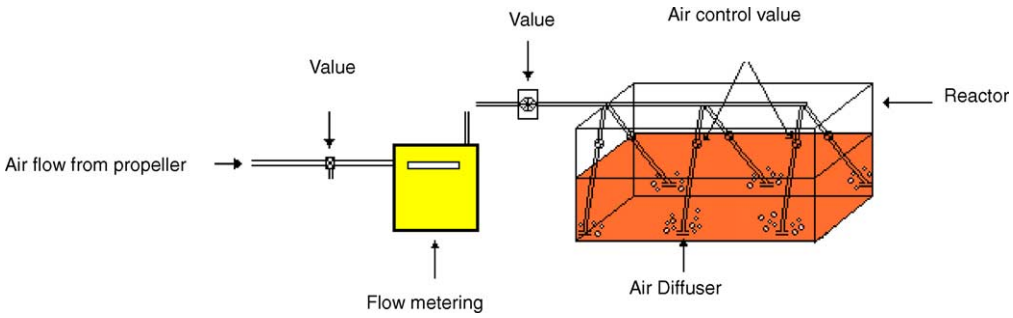

Fig. 1. Batch system diagram used during the experiment.

2. Material and methods

The stabilization assays were carried out in a 100-l capacity, benchtop reactor, which operated in batch mode for 135 days. Fig. 1 shows a schematic diagram of the laboratory reactor. The mixed, primary and biological sludge, came from the primary settling tank of "El Torno" Wastewater Treatment Plant in Chiclana de la Frontera (Cadiz). A control system was established, whereby four analyses per week were performed for the first 2 weeks, three per week in the following 3 weeks and two per week for the remainder of the trial. 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 with the 0.5 to 1.4 ± 0.1 ppm range.

2.1. Physicochemical parameters

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.

Oxygen and temperature levels were monitored with a WTW OXI 92 model oxygen meter, with membrane heads, 0.1 ppm oxygen precision and an in-built temperature sensor.

Hydrogen sulfide production was determined using a method based on the procedure proposed by Hartman et al. [1]. Five millilitres 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.

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 [2]. This method is valid for the determination of samples with COD readings of less than 2500 mgO_2/l.

Total (TS) and volatile solids (VS) were assessed in accordance with Standard Methods [2].

2.2. Microbiological parameters

The procedure recommended in Standard Methods [2] 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.

The active bacterial population was determined using the method according to Griebe et al. [3], using a tetrazolium salt (*5-cyano-2.3-ditolyl tetrazolium chloride* (CTC)) and epifluorescence microscopy, to distinguish the metabolically active bacteria from those, which are 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 involves incubation of the sludge samples with the tetrazolium salt at 4 mM concentration, for 2 h, at room temperature and in darkness. At the end of this time, the reaction is interrupted with the addition of 1 ml of 37% formol. Following incubation, and prior to their respective dilutions, the samples were treated with sonication (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 are effected on the total volume of 10 ml. On reaching the appropriate count density, 1 ml of DAPI (10 μg/ml) is added and is left to incubate at room temperature and in darkness for 15 min. Finally, the samples are filtered through a 0.2 μm pore size polycarbonate membrane; the filters were mounted in immersion oil and examined in a fluoresce micrcoscope (olympus Labophot2). The microscope with 200 W mercury burner was used with a 100× oil immersion fluorescence objective. The optical filter combination for optimal viewing of CTC-treated preparations consisted of a blue 400–480 nm excitation filter in combination with a 590-nm barrier filter. The CTC- and DAPI-stained bacteria in the same preparation could be viewed simultaneously with a 365-nm excitation filter, Y455 emision filter, and a 400-nm barrier filter. The number of microorganisms present in a millilitre of sample is calculated by applying the following conversion formula:

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

where N, number of microorganisms per millilitre; S, real area of filtration, 3.1416 cm^2; n, average number of microor-

ganisms per field of vision; *C*, real area of microscopic range (0.025 mm^2); *V*, volume of filtered sample (10 ml); *D*, sample dilution.

2.3. Measurements of biological activity

The *oxygen uptake rate* (OUR) was determined in accordance with the recommendations of Standard Methods [2]. Notwithstanding, and due to the high concentration of solids present in the samples, an initial dilution was performed, using purified water previously filtered through 0.45 μm 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 was 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. [4]:

$$\text{SOUR} = \frac{60 \times R}{C}$$

where SOUR, specific oxygen uptake rate (mgO_2/g TS h); 60, coefficient that converts minutes into hours; *R*, oxygen uptake rate (mgO_2/l min); *C*, sample biomass concentration (gTS/l).

The procedure followed in determining *esterase activity* is a modification of the method described by Fontevielle et al. [5], 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 centrifugeg at 3000 rpm 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:

$$\text{EA} = \frac{\text{ABS}}{81.5} \times \frac{1}{t} \times v \times F \times \frac{1}{C}$$

where EA, esterase activity (μmoles of hydrolysed FDA $min^{-1}\,g^{-1}$ VS); ABS, absorbance or optical density at 490 nm; *t*, incubation time (min); *v*, volume of $HgCl_2$ (ml); *F*, dilution factor; *C*, concentration of volatile solids (g/l); 81.5, extinction coefficient of fluorescein acetate.

Dehydrogenase activity was evaluated with the method proposed by Lopez et al. [6]. 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 3000 rpm 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.

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

where DHA, INT-dehydrogenase activity (mgO_2/gVS d); D_{480}, absorbance at 480 nm wavelength; *V*, final volume of dissolvent used to extract formazan (ml); *v*, volume of reagent used and sample treated (ml); *C*, concentration of volatile solids (g/l); *t*, incubation time (min); *F*, 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 reflected 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 [7], 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 probably to the process that takes place in the reactor by the oxidation of easily biodegradable substances and when the respiration rate starts to fall.

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

T^a (°C)	20
pH	6.1
TS (g/l)	60.4
VS (g/l)	59.98
COD (mgO_2/l)	77388
H_2S (mg/l)	100
FC MPN/g TS	9000
EC NMP/g TS	579.2
CTC/DAPI	0.08
SOUR (mgO_2/h/gTS)	3.08
DHA (mgO_2/gVS d)	31.6
EA (μmoles FDA/min gSV)	14.5
DHA/EA (Mol O_2/μmol FDA)	49.6

Table 2
pH, volatile solids and sulfide production tendency with the experiment

	Day													
	0	2	4	7	16	25	30	38	46	53	60	79	106	135
pH	6.10	6.52	6.83	6.71	6.87	7.29	7.39	6.93	6.95	7.31	7.50	7.81	7.73	7.65
%VS	60.00	63.90	61.80	62.00	58.60	59.50	58.80	58.20	55.80	55.20	52.40	48.90	43.00	39.70
COD (gO_2/l)	77.39	69.65	69.53	71.66	67.86	76.64	71.34	55.49	55.96	50.60	48.79	43.12	38.40	37.31
H_2S (mg/l)	100	100	100	90	50	30	–	35	–	40	24	12	3	0

Table 2 also reveals the tendency linear reduction experienced by the VS (regression coefficient, $r^2 = 0.87$), due to the endogenous respiratory process, taking place in the reactor, which provokes a gradual decrease in the percentage of solids and stabilization of the sludge. A removal rate of 33.7% was achieved after 135 days.

The decline in COD recorded during the assay may be due to the process of degradation in the system and to the endogenous respiratory processes. 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 remain practically constant, indicating that the system is stable and that the remaining, non-degraded COD is due either to organic material, which is highly resistant to biodegradation or to other, non-biodegradable inorganic compounds, which can not degradable by microorganism.

With regards to the evolution in hydrogen sulfide production, this 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 the 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. 2 represents the evolution displayed by fecal coliforms and *Escherichia coli*. It may be observed that, after an exponential decrease over the first 30 days, the number of these microorganisms remains constant until the end of the assay. In Table 3, some results obtained during the digestion process are shown.

Notwithstanding, and given the values of the parameters obtained in our experiment, it may not be concluded that the process reaches stabilization during this residence time, because, as reported elsewhere in the bibliography [8], 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 USA legislation related to sludge reutilization classifies the biosolids in two different groups: class A biosolids and Class B biosolids. Class A biosolids must comply with one of the following: fecal coliform is less than 1000 most probably number (MPN) per gram of total solids (dry weight) or *Salmonella* sp. bacteria density is less than 3 MPN per 4 g total solids (dry weight). Moreover, enteric virus density must be less or equal than one plaque forming unit (PFU) per 4 g of total dry weight solids and viable helminth ova density, less than 1 per 4 g total solids. Class B biosolids present a fecal coliform concentration under 2×10^6 MPN per gram of total solids.

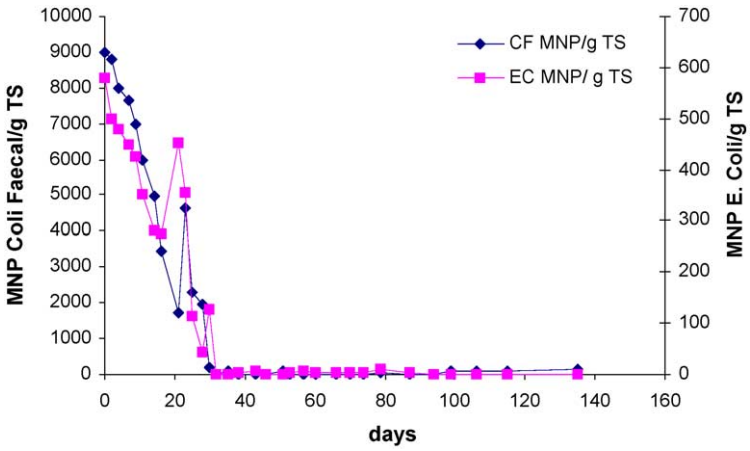

Fig. 2. Faecal coliforms and *Escherichia coli* concentrations.

Table 3
Concentration of C. faecal and *E. coli* in the experiment

Day	MNP C. faecal/g TS	NMP *E. coli*/g TS
0	9000.00	579.18
2	8800.00	500.00
4	8000.00	480.00
7	7650.00	450.00
9	7000.00	425.00
11	6000.00	350.00
14	5000.00	280.00
16	3446.85	275.75
25	1882.78	144.14
28	1943.78	134.86
30	204.95	126.12
35	117.53	1.54
38	20.77	3.46
46	9.72	1.30
53	20.77	3.46
66	4.62	2.12
70	6.22	2.33
74	2.47	2.47
87	22.93	2.20
99	104.70	1.25
106	95.00	0.80
135	130.98	0.70

Biosolids obtained in the assay could not be classified according to USA legislation: although fecal coliform's MPN allows to considerate this biosolid as Class A, both enteric virus density and viable helminth ova are unknown at the end of the experience.

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. 3 shows how, in the first few days of the assay, the number of active cells is relatively small, increasing to a maximum on day 16, due to the favourable conditions of oxygenation and the presence of substrate. It should be pointed out that, initially, the sludge is anoxic, due to the design of the wastewater treatment plant, and, according to other authors [9], the determination of active cells using the CTC fluorochrome technique is not suitable for measuring the activity of microorganisms when these are exposed to anaerobic conditions; this may explain the low values obtained at the start of the experiment. Around day 30, a marked downward trend becomes evident, and this continues 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.

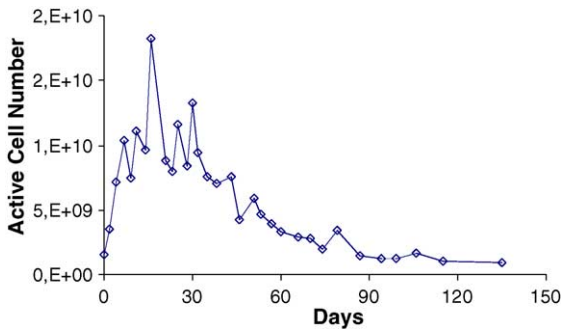

Fig. 3. Changes in the active cells number (CTC).

Table 4
SOUR values

Day	SOUR (mgO_2/h/g TS)
0	3.08
2	4.24
4	5.21
9	6.23
16	8.19
25	6.15
38	8.07
46	14.5
53	7.94
60	6.50
79	2.87
106	2.02
135	1.13

3.3. Biological activity parameters

Table 4 shows how, during the first part of the experiment, and as a result of the initial anoxic conditions of the sludge, the specific oxygen uptake rate (SOUR) presented relatively low values compared to those typically recorded in active sludge units [10]. As the experiment progressed, and as oxygenation and feeding conditions stabilized, the microorganisms evolved and the consumption rate increased, reaching its maximum value on day 46. From this point onwards, this parameter registered an exponential decrease, indicating reduced 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 mgO_2/h/TS [8]; these SOUR values are 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 [11]. In Fig. 4, it may be observed that the initial activity value of the mixed sludge is 32 mg $O_2 g^{-1}$VS day^{-1}. Thereafter, the first few days witness a drop in this value, due fundamentally to the adaptation of the microorganisms to the new environmental conditions. Once acclimatisation is complete, a slight increase in activity is produced and continues to day 46, after which there is a marked decrease in microoganism 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. 4 shows how there is an initial decrease in this activity, probably because there are sufficient resources in the environment and no need to use energy reserves. Subsequently, when the microorganisms have become acclimatised, there is a marked increase, up to day 46, when the

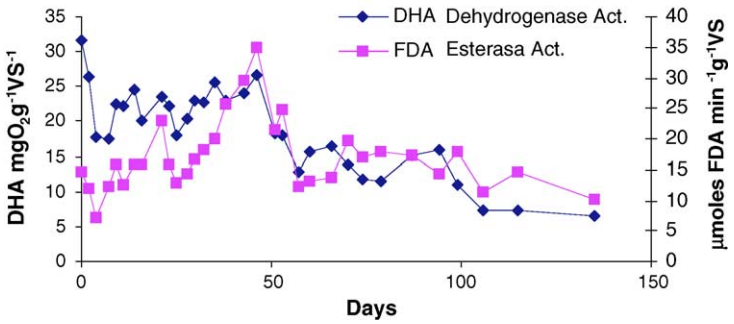

Fig. 4. Variations of dehydrogenase activity and esterase activity.

maximum value is recorded. Thereafter, values fall throughout the stabilization process. The evolution of this parameter reflects the evolution of SOUR values, and coincides with dehydrogenase activity. Notwithstanding, and although both curves develop in the same way, in the first part of the experiment, esterase activity increases to a greater extent than dehydrogenase activity, although this difference becomes less marked during the stabilization period. This effect may be due to the fact that, as time passes and the availability of energy resources in the reactor diminishes, an endogenous phase is entered, during which the energy reserves, principally lipids, are 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, as indicated in our discussion of section relating to the physicochemical parameters.

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, . . .) 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. 5 shows the developmental pattern traced by both quotients. The figure reveals how, initially, when the sludge is not stabilized and the COD/TS ratio is high, the DHA/EA ratio is also high. As the experiment progresses and the sludge becomes stabilized, this quotient is gradually reduced, so that when the ratio is equivalent to 17 moles of O_2/µmol of hydrolysed FDA, around day 46, the evolution of the curve stabilizes, indicating that the metabolic processes measured through esterase activity (EA) now enjoy a more favourable environment compared to those processes linked to dehydrogenase activity (DHA); in other words, secondary metabolic activity is enhanced at the cost of primary activity and, consequently, the process enters a more markedly endogenous phase. It may also be observed how the increase in the enzymatic ratio (DHA/EA) from day 53 to 66 coincides 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, exerts a significant influence on the extent to which the process of endogenesis may develop.

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).

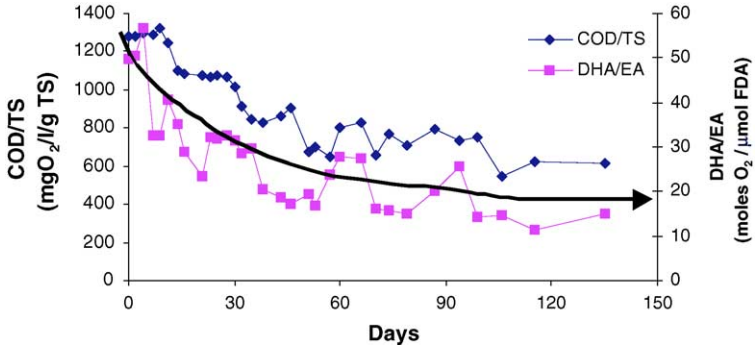

Fig. 5. Evolution of quotient DHA/EA and COD/TS.

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:

- The enzymatic activity measurements, and in particular, the relationship between dehydrogenase and esterase activity, may be considered a parameter capable of indicating the metabolic state of mixed sludge stabilization. In addition, this 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.
- The 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.
- The activity measurements are conditioned by the concentration of substrate existing in the system and which is available to the microorganisms.

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