ANAEROBIC BIOREMEDIATION OF PAH–CONTAMINATED SOIL: ASSESSMENT OF THE DEGRADATION OF CONTAMINANTS AND BIOGAS PRODUCTION UNDER THERMOPHILIC AND MESOPHILIC CONDITIONS

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Abstract

The degradation of polycyclic aromatic hydrocarbons (PAHs) including fluorene, phenanthrene, anthracene, fluoranthene and pyrene were investigated under thermophilic and mesophilic anaerobic-methanogenic conditions. By using central composite design, the impact of PAH concentration and soil to inocula ratio were systematically studied for their influence on PAH removal and biogas production. During the first 30 days, PAH concentration decreased in all samples indicating that the inocula used were able to biodegrade the contaminants; although an inhibition effect was initially observed in biogas production. Phospholipids fatty acids analysis was used to monitor the microbial communities present during the process. These communities were more complex in samples containing moderate to high PAH contamination concentrations. After 50 days, the concentration of PAHs increased in the majority of samples indicating possible reversed biotransformation of these compounds.

Keywords: Anaerobic digestion, PAH-contaminated soil, thermophilic/mesophilic conditions, phospholipids fatty acids, central composite design

1. Introduction

Soil contaminated with polycyclic aromatic hydrocarbon (PAHs) is a global environmental problem that is continuing to increase because these compounds are widely present in many substances used as part of modern life (Agarwal, 2009). Warnings about the dangers and potential side effects of PAHs have motivated researchers to investigate their bioremediation and detoxification. It is well known that, for many organic contaminants, aerobic treatments are more efficient and widely used than anaerobic ones because of their broader catabolic range (Cajthaml et al., 2002). However, aerobic processes are not the only options that need to be considered because different types of contaminants can be present for which such processes are difficult to apply. High number of materials contaminated with PAHs could be treated by using anaerobic processes (Popescu et al., 2009; Soceanu et al., 2009), and therefore it is necessary to understand their fate under these conditions, as well as understand if anaerobic treatments could be applied as a biological alternative process.

Although anaerobic bioremediation of PAH-contaminated soil has been investigated under a number of different conditions relatively little is still known and more research is necessary (Zhang et al., 2000). Recently, various electron acceptors have been used for the anaerobic bioremediation of different compounds such as hydrocarbons (Callaghan et al., 2006; Dionisi et al., 2006; El-Hadj et al., 2006). Evidence of anaerobic degradation of various PAHs have been reported: phenanthrene and fluorene have been degraded by sulphate-reducing bacteria (Coates et al., 1997) and under nitrate-reducing conditions (Eriksson et al., 2003), and iron-reducing and methanogenic conditions promote the degradation of similar compounds (Chang et al., 2002; Chang et al., 2006; Meckenstock et al., 2004; Sayara et al., 2010). It is worth mentioning that a gradient of contaminant concentrations are found in different contaminated matrices, and these concentrations vary from low, such as wastewater sludge (µg/kg, dry matter (DM)), to high, such as soils or sediments (g/kg, DM) (Chang et al., 2003; Chang et al., 2008; Christensen et al., 2004; Trably et al., 2003).

Anaerobic digestion under methanogenic conditions can be carried out under different temperature including mesophilic (37˚C) and thermophilic (55˚C) regimes (De Baere, 2000). However, mesophilic treatments are considered more economical because less energy is required. As a result, most anaerobic full-scale plants work under mesophilic conditions (Ferrer et al., 2008) even though thermophilic treatments have a higher biogas production rate because of the associated faster reaction rates (Cecchi et al., 1991). Accordingly, the study of

these two different treatments to gain a full understanding of the mechanisms and behavior of this process is recognized as an alternative source for renewable and clean energy.

In a normal start-up of a batch anaerobic digester a certain amount of inoculum needs to be added together with the substrate to be digested to generate the required conditions and microorganisms needed to start the biological reactions (Liu et al., 2009). Theoretically, the degradation of organic matter including PAHs can be promoted by increasing the density of microbial activity either by favorable conditions or by increasing the amount of microorganisms present. Equally, the adaptability of the introduced microorganisms is also crucial for promoting the digestion process (Fernández et al., 2005). Consequently, the determination of the ratio between the treated material and inoculum is of great importance. In this regard, the minimum amount of inoculum required to provide sufficient activity with the maximum load of organic material is considered to be crucial for process design.

The role of anaerobic degradation for PAH-contaminated soil is an area in which little work has been reported. To our knowledge, there is a lack of information about the bioremediation of PAH-contaminated soil under strict methanogenic-anaerobic conditions. To date, there have been no reports of the behavior of such processes under various temperatures, and no information is available about the influence of the main factors that typically affect the process such as contaminant concentration and soil to inocula ratio.

Hence, the objective of the present study was to comparatively assess the process performance under different operating conditions by using two different types of inoculum requiring different temperature: one thermophilic and one mesophilic. Moreover, two main controlling factors such as the PAH concentration and the soil to inocula ratio were evaluated to assess their effect on the PAH degradation rate. The biogas production was measured as an indicator of process activity, whereas PAH degradation was evaluated by measuring the concentration decrease and the production of biodegradation metabolites using advanced techniques. Also, phospholipid fatty acid (PLFA) analysis was used to determine the evolution of the microbial communities present in each sample. In both the thermophilic and mesophilic case the experiments were systematically carried out by using an experimental design technique considering the different levels of the studied factors, to allow for statistical validation of the results obtained.

2. Experimental

2.1. Soil

The soil used in this study was an uncontaminated soil classified as sandy loam soil. It was collected from the surface horizon (0–30 cm) of an agricultural field located in Prades (Tarragona, Spain). The soil was air-dried and then sieved (2 mm) to remove any debris and kept at 4˚C until use. The soil texture consisted of 73.4% sand, 18.6% silt and 8% clay. No PAHs were detected in the soil before being used for experimental purposes. Other properties of the soil are shown in Table 1.

2.2. Contaminants

Fluorene, Phenanthrene, Anthracene, Fluoranthene and Pyrene, all of 98–99% purity, were selected as contaminants in order to evaluate their degradation under anaerobic methanogenic conditions. These compounds were obtained from Sigma-Aldrich (Barcelona, Spain). These five PAHs are included on the list of the 16 USEPA priority pollutants. The weight percentage of each compound as part of the total PAHs concentration (∑PAHs) was 33, 31, 10, 22 and 4%, respectively. These percentages were chosen to simulate a real creosote sample the percentages of which were determined following fractionation of a commercial creosote sample (Creosote lot: 42-13B, Chem Service, Sugelabor, Spain) in our laboratory by using the method 3611B of the USEPA, in which the volatile part was ignored. The PAHs were mixed in the appropriate proportions to create a stock solution. This stock solution was used to spike the soil to obtain the desired concentration according to the experimental design range (0.1–2 g/kg, DM) measured as the total concentration of the five PAHs.

2.3. Anaerobic inocula

In this study, two types of inoculum with different optimal temperatures, thermophilic (55˚C) and mesophilic (37˚C), were used. The mesophilic-anaerobic inocula were obtained from the digested effluent of an anaerobic digester after the solid-liquid separation in a waste treatment plant (Barcelona, Spain). The inoculum contained 10.02% of total solids, which is composed of the entire microbial consortium involved in the anaerobic digestion process, together with some difficult to degrade or inert solids. The thermophilic inoculum was obtained from a thermophilic treatment plant (Terrassa, Spain). The thermophilic inoculum was filtered through a 4 mm sieve to remove some big parts of the feedstock and 11.8% total solids remained. In both cases the treatment plants are fed with the source-selected organic fraction of municipal solid wastes. The two inocula were kept separately in plastic containers with a gallon capacity under strict anaerobic conditions and incubated in water baths at 37˚C

and 55˚C for about two weeks before use to remove any biodegradable materials. No contamination by PAHs was detected in the inocula prior to the experiments. Other characteristics of the inocula are presented in Table 1.

2.4. Experimental system

Experiments with both inocula were performed in triplicate by using sealed 1-L bottles (Traveller SIGG®, Spain) as reactors. They were tightly sealed and equipped with a ball valve that can be connected to a digital manometer to determine the pressure of biogas produced. The contaminants were prepared in a stock solution (5 g total PAHs in 500 mL dichloromethane) and used to spike the soil at different concentrations (0.1–2 g/kg total PAHs; approximately 300–6000 mg chemical oxygen demand (COD)/L) determined according to the experimental design technique. The solvent was allowed to evaporate at room temperature. Next, the soil and inoculum were mixed together based on the dry weight fraction at different ratios ranging from 0.2:1 to 5:1 (w/w, soil/inoculum). As inoculum and soil are different in their total solids content, distilled water was added to the samples to correct for this and ensure the same total solids content for both. Thus, all samples had the same amount of total solids equal to that corresponding in each inoculum (11.8 and 10.02%). All the bottles were then tightly sealed and purged several times with compressed nitrogen gas to ensure anaerobic conditions (Ponsá et al., 2011) before being incubated under strict anaerobic static conditions. The mesophilic samples were incubated in a temperature-controlled chamber at 37˚C, and the thermophilic samples were incubated in an oven adjusted to 55˚C. The total experimental time was set to 50 days in line with previous experiments (Sayara et al., 2010). After 30 days one sample from each set of experimental conditions was removed from the incubator to evaluate the process behavior. The remaining samples (in duplicate) were left for the full 50 days. A blank experiment (in triplicate) with only inoculum was used to evaluate the baseline of biogas production by an endogenous matter. These samples did not contain any additional nutrients or organic matter. All the results are expressed as the average measured after 30 and 50 days.

2.5. Analytical methods

2.5.1. Determination of biogas and analysis

The production of biogas was followed and determined quantitatively by measuring the pressure increase in the headspace of the sample bottle by means of a SMC (ISE30)

Pressure Switch manometer (1 MPa, 5% accuracy). The quantity of the produced biogas in each sample was determined by subtracting the biogas produced by the inoculum (blank experiment) from that produced in the sample. Because it is crucial to be able to accurately determine the differences between the production of biogas from the inoculum and the PAH experiments, the inoculum was maintained under anaerobic conditions for 15 days prior to use to remove the biodegradable organic matter and to reduce biogas production owing to endogenous activity (Ponsá et al., 2011). The net value of biogas produced was calculated and expressed under normal conditions (0˚C and 1 atm). Biogas analysis, particularly the CH_4 and CO_2 contents were determined by using a gas chromatograph (GC 5890 Capillary Hewlett Packard) as described by Sayara et al. (2010).

2.5.2. PAHs analysis

To determine the concentration of PAHs after 30 and 50 days of incubation, the contents of the sample bottles were dried by using a lyophilizer (Benchtop 5L, Virtis Sentry, NY). Lyophilized samples were extracted by using an ASE 200 System (Dionex, Voisins-le-Bretonneux, France). The extraction cell (11 mL) was loaded in an oven and extracted with hexane-acetone (3:1, v/v). The cell was heated (150 ˚C, 7 min) and subsequently extraction took place at 150 ˚C under 103.4 bar for 5 min. The cell was then flushed with fresh solvent (60% of total cell volume) and finally the solvent was purged from the cell with nitrogen gas for 60 seconds. For each sample the extraction cycle was performed three times. The resulting organic extracts were collected in 40 mL vials, dried under vacuum at room temperature and finally dissolved in acetonitrile for subsequent analysis. Reverse phase high-performance liquid chromatography analyses were performed by using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a LiChroCart column filled with LiChrospher® PAH (250 x 4 mm; particle size 5 µm; pore size 150 Å; Merck, Darmstadt, Germany), a 2996 diode-array detector and 2475 fluorescent detector (Waters, Spain). Separation of the PAHs was achieved with a gradient program, by using a mixture of methanol:acetonitrile (1:1 v/v, solution A) and Milli-Q water. After 5 min of isocratic elution with 70% of solution A, the eluent was changed slowly to be 100% solution A over 15 min and maintained constant for the following 20 min. PAHs were identified by using UV spectrophotometry and comparison of retention times to commercially available standards (Dr. Ehrenstorfer, Augsburg, Germany). The concentrations of the five PAHs were calculated according to the USEPA method 610. The compounds were quantified with the fluorescent detector under excitation/emission conditions: phenanthrene and anthracene at –250/390 nm;

fluorene, fluoranthene and pyrene at –280/340 nm. Calibration curves with the standards were prepared over a linear range (0.1–10 µg/ml) for each compound, and the recovery rate was approximately 95%.

2.5.3. Metabolite identification

Samples that had been previously extracted (See section 2.5.2) were analyzed by using gas chromatography (Agilent HP 6890 Series II) coupled to a mass selective detector under electronic impact ionization (Agilent HP 5973) with a HP5-MS (30 m x 0.25 mm x 0.25 µm; Agilent, Spain). The operating conditions of the chromatograph were as follows: injector (splitless 1 min) 320^{o}C, injection volume 1–3 µl (depending on the sample), oven temperature: 50°C (1 min), rate 7°C/min, final temperature 320^{o}C with helium as carrier gas at 0.7 ml/min. The detector remained in solvent delay mode for 3.2 min and the mass range measured was 40–400 (m/z). The products detected were identified by comparing the mass spectral data to the Wiley 7® library.

2.5.4. Volatile fatty acids

The total volatile fatty acid composition (acetate, propionate, *iso*-butyrate, *n*-butyrate, *iso*-valerate, *n*-valerate; (g/L)) was determined by using gas chromatography (GC). Samples were centrifuged for 10 minutes (10^4 rpm) then filtered through to remove any solid particle (0.25 µm). Known volumes of the filtrates were mixed equally (1:1, v/v) with pivalic acid as standard, before analysis by GC. Samples (1 µl) were injected into the GC (GC 5890 Capillary Hewlett Packard) equipped with a flame ionization detector (280˚C) and a splitless injector (260˚C). A HP-Innowax column (Crosslined polyethylene Glycol; 30 m x 0.53 mm x1 µm; Agilent) was used. The temperature was maintained at 80˚C for 1 min before increasing at a rate of 5˚C/min until 150˚C and then at 20˚C/min until 230˚C was attained.

2.5.5. Evaluation of the bioavailability of PAHs

The bioavailable fractions of PAHs were estimated by using sequential supercritical fluid extraction (SFE) (Cajthaml and Šašek, 2005). The extraction was performed with a PrepMaster extractor (Suprex, Pittsburgh, USA) equipped with a VaryFlow restrictor operating at 40˚C with a downward stream of carbon dioxide (5.5 SFE/SFC, Supercritical fluid chromatography and extraction system, Messer Technogas, Prague, Czech Republic). The samples (1 g, dry basis) were extracted at 50 ˚C and 200 bar. Each extraction was carried out in duplicate and the compounds were collected after 5, 10, 20, 40, 60, 80, 120, 160, and

200 min. SFE represents a desorption model that generally assumes that the extraction is controlled by two rate constants differing in orders of magnitude (Williamson et al., 1998). The "F fraction", which represents the rapidly desorbed fraction of the target chemical from soil, is usually assumed to be representative of equilibrium release conditions, and the next, more slowly released, portion is considered to be kinetically rate-limited. The chemical release data is then modeled by an empirical two-site model, consisting of two first-order equations:

$$S_t = F\, S_0 \exp(-k_1 t) + (1-F)\, S_0 \exp(-k_2 t) \quad (1)$$

Where S_t is the pollutant concentration remaining in the soil after time t; F is the fraction of chemicals that is rapidly released; S_0 is the original concentration of the pollutant in soil; k_1 and k_2 are the first order rate constants. Prism version 5.0 (GraphPad, USA) was used to calculate the F values according to Equation 1.

2.5.7. Quantification of microbial biomass

Samples for PLFA analysis were extracted by using a mixture of chloroform, methanol and phosphate buffer (1:2:0.8; v/v/v). LiChrolut Si-60 solid-phase extraction cartridges (Merck, Whitehouse Station, NJ) were used for the separation of the extracts and phospholipid fractions were subjected to mild alkaline methanolysis (Šnajdr et al., 2008). Gas chromatography-mass spectrometry (GC-MS) was used for analysis of the free methyl esters of phospholipid fatty acids (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, USA). The GC instrument was equipped with a split/splitless injector and a DB-5MS column (J&W Scientific, Folstom, 60 m, 0.25 mm, 0.25 μm film thickness) was used for separation. The temperature program started at 60 °C and was maintained for 1 min in splitless mode. Then the splitter was opened and the oven heated to 160 °C at a rate of 25 °C min^{-1}. The second temperature ramp reached 280 °C at a rate of 2.5 °C min^{-1} and this temperature was maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectral data were recorded under electron impact at 70 eV, mass range 50–350 amu. Methylated fatty acids were identified according to their mass spectra and quantified by using their individual chemical standards (Sigma-Aldrich, Prague, Czech Republic and Matreya LLC, Pleasant Gap, USA). Fungal biomass was quantified based on 18:2ω6,9 content, bacterial biomass was quantified as a sum of i14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω7, 16:1ω9, 16:1 ω5, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1ω7, 18:1ω9, 10Me-18:0 and cy19:0. Biomass of Gram positive and Gram negative bacteria were estimated by using concentrations of i14:0, i15:0, a15:0, 15:0, i16:0 and 16:1ω7, 18:1ω7, 16:1

ω5, cy19:0 cy17:0, respectively. To evaluate the anaerobic PLFA markers the quantities of cy19:0, cy17:0 and 18:1ω9 were used (Oravec et al., 2004; Sampedro et al., 2009; Šnajdr et al., 2008).

2.6. Experimental design and statistical analysis

The effect of two factors (k = 2): the PAHs concentration (x_1) and the soil to inocula mixing ratio (x_2), on the bioremediation of PAHs-contaminated soil under anaerobic conditions were systematically studied by using the central composite design (CCD). More details about the experimental design technique and its application can be found and reviewed elsewhere (Deming and Morgan, 1987; Sayara et al., 2010). The design matrix is presented in Table 2, in which the coded and actual values of the two independent variables (x_1 and x_2) and the actual response of each combination regarding the biodegradation percentage (Y_D) and the biogas production (Y_G) are reported. In total, nine experiments (in triplicate) were carried out according to CCD principles. Control samples, containing only the corresponding inoculum were also carried out. Statistical analysis was performed for all the variables by using the Sigmaplot® 8.0 software package (Systat Software Inc, San José, USA) and according to the statistics recommended for CCD (Deming and Morgan, 1987).

3. Results and discussion

3.1. Response surface and statistical analysis

CCD was applied to study the process behavior under different variables. The degradation (D) percentage (%) and the biogas (G) production (L kg^{-1} TS) after 30 and 50 days were selected as functions to be optimized along with their parameters to represent the response of the process. Second order models for each variable are illustrated in Equations 2–9 under thermophilic (T) and mesophilic (M) conditions:

$$Y_{TD30} = 51.9 + 23.28x_1 - 6.69x_1^2 - 0.69x_2^2 - 1.9x_1x_2 \quad (2)$$

$$Y_{TD50} = 32.14 + 25.09x_1 + 6.62x_2 - 5.8x_1^2 - 2.18x_1x_2 \quad (3)$$

$$Y_{TG30} = 3.62x_1 - 7.03x_2 + 3.1x_1^2 + 1.7x_2^2 - 2.4x_1x_2 \quad (4)$$

$$Y_{TG50} = 8.18 + 7.4x_1 - 11.5x_2 - 0.75x_1^2 + 1.9x_2^2 \quad (5)$$

$$Y_{MD30} = 42.7 + 46.18x_1 - 13.23x_1^1 + 0.21x_2^2 - 1.36x_1x_2 \quad (6)$$

303 $$Y_{MD50} = 20.95 + 68.18x_1 - 22.77x_1^2 + 0.39x_2^2 \quad (7)$$

304 $$Y_{MG30} = -135.5x_1 + 6.3x_2 + 50.7x_1^2 + 8.7x_1x_2 \quad (8)$$

305 $$Y_{MG50} = -96.55x_1 + 6.02x_2 + 35.82x_1^2 + 5.6x_1x_2 \quad (9)$$

306

307 The correlation coefficients (R) and p values of the functions representing the degradation
308 percentage for both inocula after 50 days are shown in Table 3. The regression model that was
309 selected is considered to be adequate to describe the data and the relationship between the
310 independent and dependent variables. Conversely, the correlation coefficients and p values
311 obtained from the rest of the treatment models designed to represent the biodegradation
312 percentage and the biogas production were not favorable (data not shown). It is proposed that
313 the low organic matter content and the presence of the contaminants influenced the biogas
314 production. However, the values obtained from the statistical analysis of these samples do
315 give a general idea about the process performance when such conditions occur, especially in
316 the case of biogas production/inhibition owing to the presence of PAHs.

317

318 *3.2. Overall degradation of the contaminants*

319 The anaerobic degradation of the PAHs under the different conditions studied was
320 evaluated after 30 and 50 days incubation to monitor the process. Fig. 1 shows the results
321 obtained under thermophilic and mesophilic conditions. After 30 days, all samples showed
322 some degradation and the same trend under the two temperature conditions was observed. The
323 biodegradation observed seems to demonstrate that the native microorganisms of the
324 inoculum (thermophilic or mesophilic) have the catabolic capacity to degrade the PAHs used,
325 and it could be an indication that the methanogenic metabolism becomes coupled to the
326 anaerobic degradation of the PAHs, as observed in other studies (Chang et al., 2006; Yuan
327 and Chang, 2007). However, the samples containing low concentrations of PAHs (0.1g/kg)
328 resulted in the lowest degradation rate, suggesting that microbial activity is governed by the
329 pollutant concentration in which a minimum threshold level is required to initiate degradation
330 and catabolic induction in biodegrading microorganisms (Boethling and Alexander, 1979). It
331 is also worth noting that, at low concentrations, more surface area on the soil particles is
332 available for adsorbtion of the contaminant relative to high concentrations. Therefore, a
333 stronger interaction between the soil and contaminant could be formed making the
334 accessibility and biodegradation of the contaminant complex (Table 4) in which, for the most
335 part, the “F” fraction at low concentrations is lower than at the corresponding high

concentrations. In addition, a part of the biodegradation could be related to the soil's endogenous microorganisms because the used soil was not sterilized.

In general, degradation capacities under mesophilic conditions were higher than those obtained under thermophilic conditions. However, thermophilic conditions resulted in better rates of biodegradation when low concentrations of PAHs were used. This phenomenon can be explained by high temperatures, which, in most cases, increase the desorption of PAHs and their solubility, as observed in the determination of the bioavailable portion of the contaminants, relative to the corresponding mesophilic conditions (Table 4). Moreover, under thermophilic temperatures the mass transfer and reaction kinetics are enhanced. Nevertheless, other studies have reported contradictory results. For instance, Chang et al. (2002) found that a temperature of 30˚C enhanced PAH degradation better than at 40˚C.

Under the different combinations and conditions, and according to the obtained results regarding the contaminants bioavailability (Table 4), the biodegradation of the contaminants was controlled by their characteristics such as water solubility, number of benzene rings and structural conformation (shape) of the molecules. However, no reliable trend or order in biodegradation is apparent.

Analysis of selected components of PLFA demonstrated that anaerobic bacteria were present in all samples, but their abundance in the thermophilic samples was higher than in the mesophilic ones (Table 5). Furthermore, moderate to high PAH concentrations significantly ($p<0.05$) altered the microbial community structure by changing anaerobic communities in such a way that more anaerobic microorganisms were observed at high PAH concentrations under both conditions, and better degradation rates of PAHs were consequently achieved. The PLFA profiles were in agreement with the rates of PAH degradation (Fig.1) under both conditions with less degradation observed at low PAH concentrations. According to Donald et al. (1998), the microbial community responded to PAHs contamination at both the phenotypic and genotypic levels, which is also in agreement with the obtained results.

As expected for biological treatments of PAHs, their concentration decreases with time as a result of the microbial activity, or no change occurs in the case of the process failure or absence of optimal conditions for microbial activity. However, in this study the results were completely contradictory to what was expected. For the majority of samples under both conditions (37 and 55˚C), it was found that the PAH concentrations after 50 days of incubation were higher than those obtained after 30 days. We therefore propose the following hypotheses: a) sorption or occlusion of the contaminants (PAHs) in the organic matter during the first 30 days and release after depletion of biodegradable organic matter, b) new PAHs

bioformed under these conditions. To examine the potential impact of sorption on the results, representative samples (in duplicate) were extracted twice under extreme conditions: high temperature (200˚C), pressure (206.84 bar) and 4 static cycles (10 min). The same samples were also extracted by using a Soxhlet and analyzed as explained elsewhere (Sayara et al., 2010). The extraction and analysis methods resulted in values that corroborated an increase in the PAH concentration. Indeed, Thiele et al. (2002) demonstrated that PAHs were formed in soil under oxygen deficient conditions. In that study, the concentration of high molecular weight PAHs (more than 3 aromatic rings) increased considerably, whereas for compounds with three rings the concentration decreased, probably owing to anaerobic biodegradation. Furthermore, the incubation of edible oil in closed containers at room temperature led to the formation of PAHs (Guillen et al., 2008) indicating that anaerobic conditions are likely responsible for such a phenomenon.

3.3. Metabolites

The observed initial decreases in the concentration of PAHs show that the inocula were able to degrade the target contaminants. Anaerobic biodegradation of PAHs was also monitored by analyzing the metabolites produced as a result of microbial activity in the samples (Table 5). In fact, several compounds were identified by GC-MS. These compounds included: phenol, hydroxyfluorene, benzeneacetic acid, cresol, pyridine, fluorenone, fluorenol, carbazole, anthracenone, anthracenedione, biphenyl and anthrone. These compounds are known metabolites generated when PAHs are degraded by microorganisms through several oxidization pathways. Consequently, the metabolites found in this study provide evidence of the microbial capacity to degrade PAHs under the conditions studied. PAH degradation pathways under anaerobic conditions are still not clear, and various hypothesis or arguments are found in the literature. Meckenstock et al. (2004 and Zhang and Young (1997) argued that carboxylation is the initial step in PAH biotransformation under sulphate reducing conditions. On the contrary, Bedessem et al. (1997) proposed hydroxylation as the initial step in PAH biotransformation under the same conditions. Vogel and Grbic-Galic (1986) argued that the anaerobic transformation of benzene and toluene to CO_2 and CH_4 occurred through hydroxylation, when phenol and cresol were identified as intermediates of benzene and toluene, respectively. Because these compounds have been identified in the present study, it would be possible to assume that hydroxylation was the initial step occurring during the biotransformation of PAHs in this case. Most likely, PAHs undergo initial ring reduction followed by hydrolytic ring cleavage to yield aliphatic acids for cell growth.

Moreover, naphthalenol was detected as metabolite from naphthalene biotransformation in sulfidogenic sediments (Bedessem et al., 1997). All of these observations support the hypothesis that PAHs degrade under methanogenic conditions and hydroxylation is the initial step in their biotransformation. Unfortunately, there are no studies that investigate PAH biodegradation under anaerobic conditions, at thermophilic temperatures.

3.4. Biogas production

The cumulative biogas production under thermophilic and mesophilic conditions is shown in Fig. 2. It is clear that the methanogenic inocula under both temperature conditions were exposed to inhibition effects because the PAHs were detrimental for the microorganisms. Usually, under similar conditions, an adapted ecosystem is used to avoid this inhibition. Consequently, as shown in Fig 2, an adaptation period of at least 20 days was needed in most cases except for Runs 2 and 8 under thermophilic conditions. The inhibition rate (negative values indicate that biogas production in the control samples is higher than in the samples containing PAHs) gradually increased with time indicating an increase in toxicity of the samples. However, in most samples, after 20 days' incubation, biogas production changed and inhibition decreased over time, although not completely eliminated in the mesophilic samples. In addition, process recovery was noted with prolonged incubation times and methane production was observed and increased significantly following the inhibition stage (data not shown). This observation demonstrates that both of the inocula were not previously exposed to similar contaminants and agree with the preliminary analysis that showed that they were free from PAHs. Interestingly, the study clearly indicated that although the presence of PAHs considerably inhibits biogas production, PAH degradation is not affected. Indeed, Fuchedzhieva et al. (2008) reported similar behavior for fluoranthene biodegradation, which continued despite cell growth inhibition in the presence of a biosurfactant complex. In general, better results were obtained for the samples treated under thermophilic conditions relative to mesophilic ones, because the higher temperatures facilitate the reaction rates and enhance enzymatic activity. However, microbial activity can be hampered by the presence of toxic metabolites resulting from degradation of the parent compounds. Indeed, analysis showed high rates of toxicity in all samples relative to a non-toxic control (data not shown). This toxicity is likely owing to the PAHs themselves and their metabolites as a result of microbial biodegradation. Additionally, the ratios of saturated to monounsaturated PLFAs (S/M) and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre) (Table 5) were significantly higher in the mesophilic samples relative to

438 the thermophilic ones, indicating microbial community stress under such conditions (Moore-
439 Kucera and Dick, 2008). However, analysis of volatile fatty acids showed that there is no
440 inhibition caused by such acids because they were not detected in any sample after 30 or 50
441 days. The pH values of the sample mixtures after 30 and 50 days were within 7.3–7.9, which
442 is around the optimal values for anaerobic digestion. Therefore, the fluctuation of biogas
443 production observed was more likely owing to some PAH metabolites that were formed
444 transiently during their anaerobic biodegradation. The oscillation in the biogas production
445 (Fig. 1, thermophilic run 8) clearly shows this effect.
446
447 *3.5. Process response for PAH degradation*
448 The variations in PAH degradation (%) under the conditions studied factors are shown
449 in Fig. 3 and Fig. 4 for thermophilic and mesophilic inoculum, respectively. Under mesophilic
450 conditions, it is clear that the degradation capacity is always proportional to PAH
451 concentration, whereas the soil to inocula mixing ratio was found to influence PAH
452 degradation at low concentrations (less than 1 g/kg). For instance, low (0.2:1) or high (5:1)
453 mixing ratios enhanced the degradation rate, but intermediate ratios negatively affected the
454 process. The same behavior was observed under thermophilic conditions during the first 30
455 days, although PAH concentration influenced the degradation capacities to a smaller extent
456 (Fig. 3A). Nevertheless, after 50 days the degradation capacity response demonstrated that the
457 process is proportional to both factors, with the highest degradation (75%) being obtained at
458 the highest concentration and mixing ratio. The increased toxic potential of higher PAH
459 concentrations on the inocula can explain why degradation decreases as inoculum ratio
460 increases. Similar results were seen with the increase of the inoculum ratio under anaerobic
461 conditions (Chang et al., 2002).
462 In both cases the PAH degradation was clearly proportional to the soil to inocula
463 mixing ratio and the best results were obtained when using the highest ratio (5:1). These
464 results are in accordance with those obtained by Hernandez et al. (2008) who reported that
465 increasing the concentration of substituted phenolic compounds enhanced the anaerobic
466 digestion process, but that at higher concentration the total biodegradation decreased. The
467 sequential decrease in biogas production (Fig. 2) observed during the first three weeks
468 demonstrates the difficulties the microflora had to overcome to adapt their biodegradation
469 potential. Here, time was an essential factor to allow for effective microbial adaptation under
470 these conditions. Although PAHs and their metabolites had an inhibitory effect on the
471 methanogenic communities, other anaerobic microbes were able to continue the degradation

process. Interestingly, PAH degradation increased when a high soil to inocula ratio was used for both cases. This observation is fundamental when considering an economical evaluation because larger quantities of contaminated soil could be treated with smaller quantities of inoculum. Because no other nutrient sources were available to the microorganisms, it is likely that the organic matter of the soil served as the nutrient source, and that, because the organic matter content was low, this nutrient limitation motivated the microorganisms to use PAHs. However, the addition of an organic matter source, such as compost, could stimulate microbial activity and, consequently, accelerate PAH degradation. This effect was clearly demonstrated in previous studies (Darush et al., 2006; Sayara et al., 2010).

3.6. Process response for biogas production

The response of biogas production under thermophilic and mesophilic conditions is shown in Fig. 5. The value for the biogas produced by a sample was calculated by subtracting the value obtained from the control experiment (only inoculum present) from the value obtained from the contaminated soil sample. The negative values indicate that the background biogas production was higher than for the contaminated samples owing to inhibition. The process behavior was completely different under thermophilic and mesophilic conditions, with no biogas being produced under mesophilic conditions, whereas certain amounts were produced under thermophilic conditions in several samples. A soil to inoculum mixing ratio of almost 3:1 was the inflection point in relation to the applied concentrations (0.1–2 g/kg). A soil to inocula ratio higher than 3:1 was gently influenced by the variation in PAH concentration. The biogas production rate slightly increased with increasing PAH concentration under thermophilic conditions, whereas no variation was seen with mesophilic inocula (stagnation state). At mixing ratios below 3:1, different behaviors were observed demonstrating various interactions between the studied variables within the two temperatures domains. Up to a point (almost 1.4g /kg), increasing the concentration of PAHs enhanced the biogas production rate under thermophilic conditions. Thereafter, further increases in PAH concentration negatively affected the microbial activity because of their toxic properties. Moreover, biogas production was enhanced at low soil to inoculum ratios when additional inoculum was introduced. Nevertheless, increasing inoculum quantity negatively affected the biogas production under low PAH conditions (<0.2g/kg). Under mesophilic conditions, the rate of inhibition gradually decreased as the soil to inocula ratio increased until a ratio of 3:1 was achieved. Above this ratio, the rate of inhibition plateaued. PAH concentrations below plateau conditions (mixing ratio less than 3:1) influenced the process in two different ways:

an increase in PAH concentration was followed by an increase in the inhibition rate until a certain concentration was reached (almost 1g/kg) after which the process changed its behavior and a decrease in inhibition was observed.

4. Conclusions

A comparative study on the anaerobic bioremediation of PAH-contaminated soil under methanogenic conditions, at thermophilic and mesophilic temperatures, has been conducted. This study indicates that inhibition of biogas production is related to high concentrations of PAHs. Although a high rate of biodegradation was achieved during 30 days, a prolonged incubation time for both thermophilic and mesophilic samples showed an increase in the PAH concentration. This increase was probably caused by the reverse bioformation of these compounds. Further research is necessary to clarify this effect and to determine exactly the optimal times required for biodegradation.

Acknowledgments

Financial support was provided by the Spanish Ministerio de Educación y Ciencia (Project CTM2009-14073-C02-01) and by the Czech Science Foundation (Grant No. 525/09/1058).

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Figure captions

Fig. 1. Percentage (%) of PAHs remaining after 30 and 50 days incubation. (A) Thermophilic conditions and (B) mesophilic conditions

Fig. 2. The cumulative biogas production (L/kg TS) after 50 days. (A) Thermophilic conditions and (B) mesophilic conditions

Fig. 3. Response of PAHs degradation (%) under thermophilic conditions. (A) After 30 days and (B) after 50 days

Fig. 4. Response of PAHs degradation (%) under mesophilic conditions. (A) After 30 days and (B) after 50 days

Fig. 5. Response of the total biogas production (L/kg TS) after 50 days. (A) Thermophilic conditions and (B) mesophilic conditions

660 **Fig. 1**

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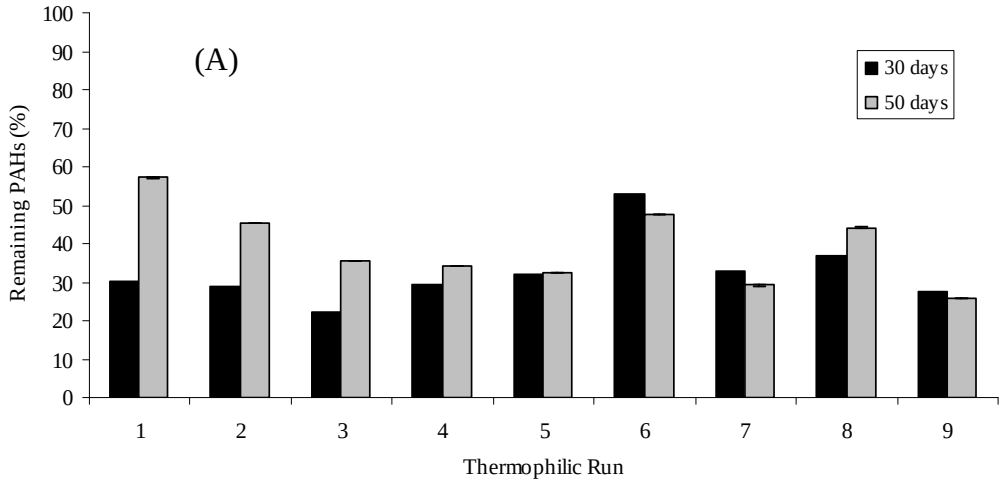

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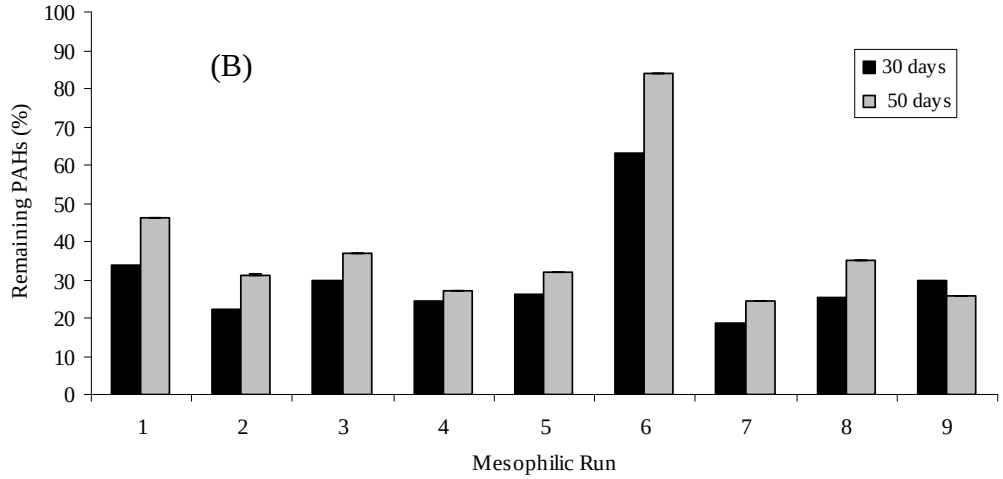

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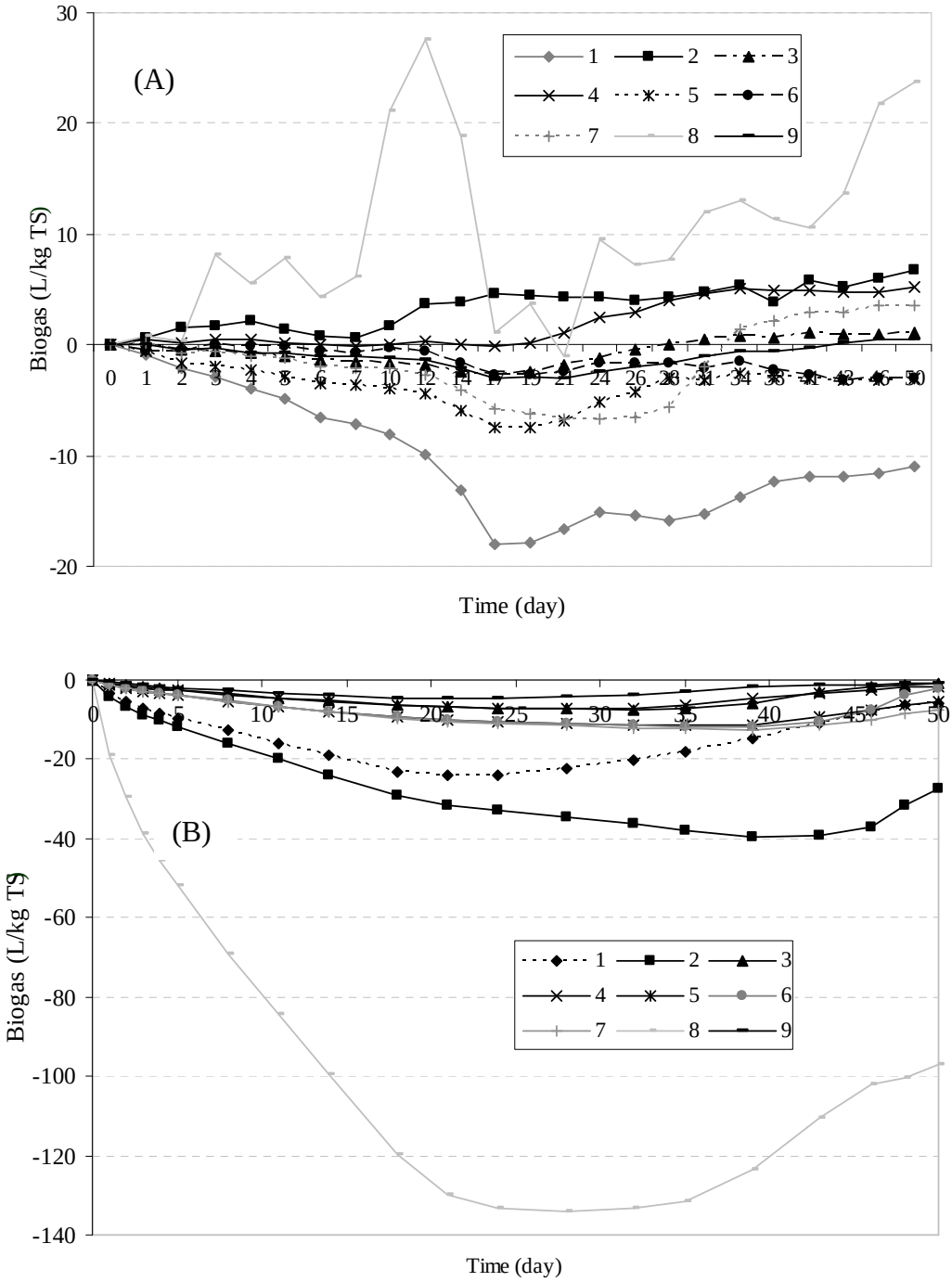
Fig. 2



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674 **Fig. 3**

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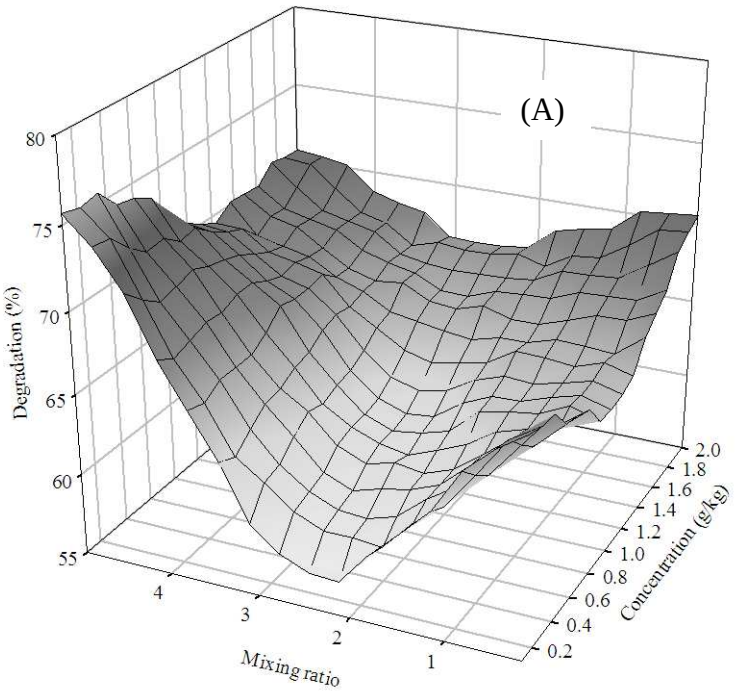

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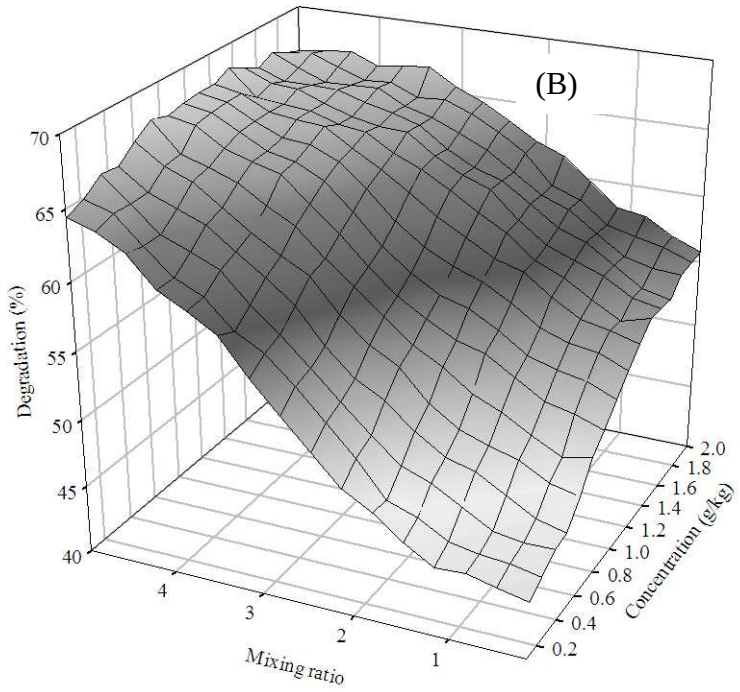

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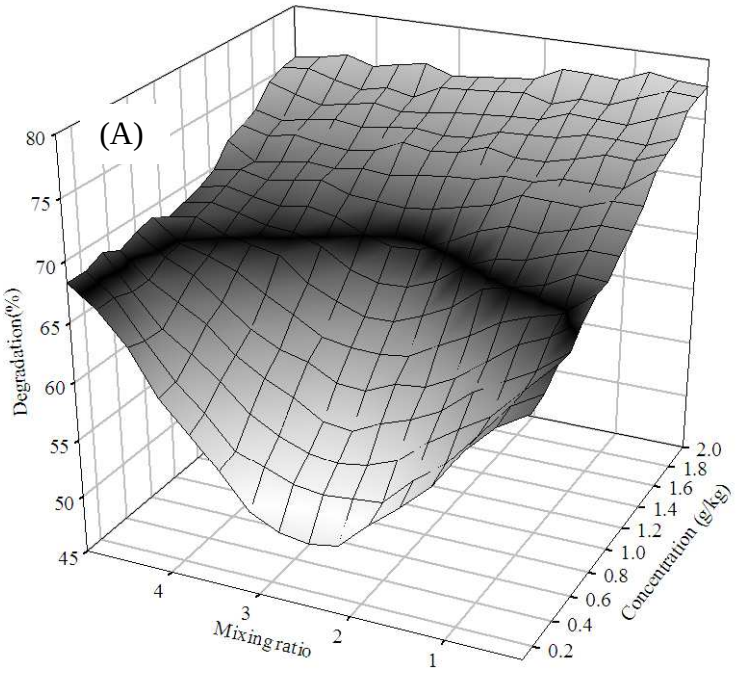
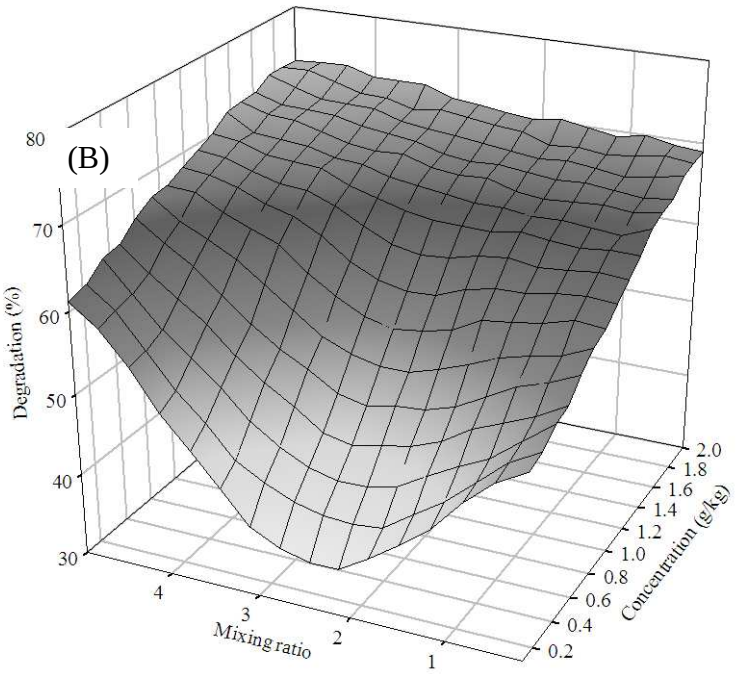
Fig. 4


(A)
Degradation(%)
80
75
70
65
60
55
50
45
4
3
2
1
Mixing ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Concentration (g/kg)


(B)
Degradation (%)
80
70
60
50
40
30
4
3
2
1
Mixing ratio
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Concentration (g/kg)

690

691 **Fig. 5**

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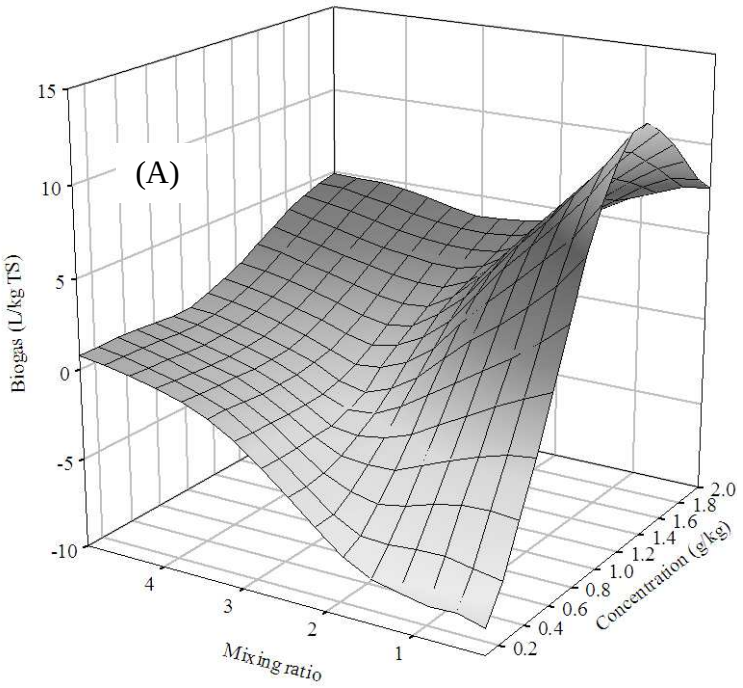

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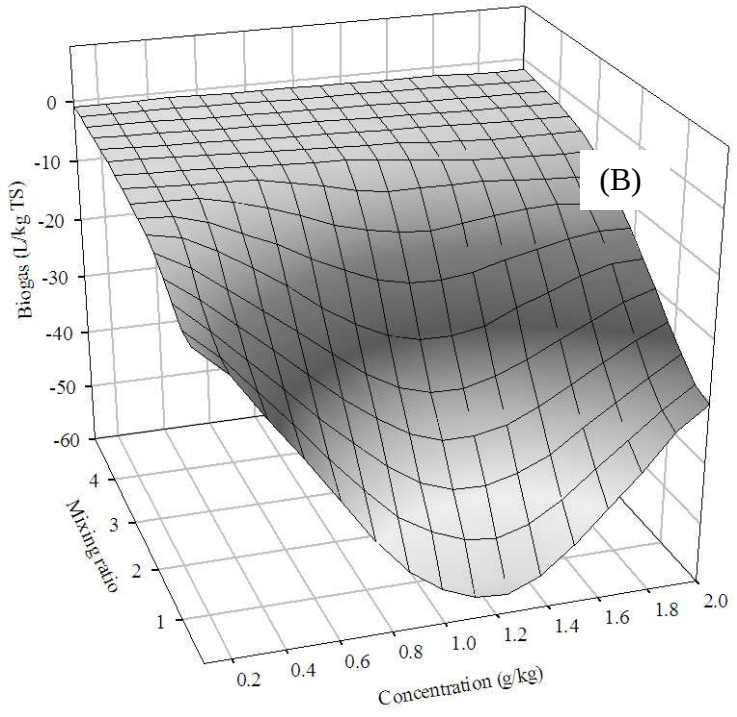

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Tables

Table 1. Characteristics of inocula and soil used in the experiments

Inoculum/parameter	***TS (w.b.)***	***OM (d.b.)***	***TOC (d.b.)***	***TKN (d.b.)***	***pH***	***EC***	***Density (g/L)***
Thermophilic	11.8	37.7	20.9	2.03	7.8	8.8	1113
Mesophilic	10.02	39.1	32.8	2.5	7.9	29.9	1013
Soil	93.3	3.7	1.26	0.7	6.7	0.2	-

TS: total solids (%); OM: organic matter (%); TOC: total organic carbon (%); TKN: total Kjeldahl nitrogen (%); EC: electrical conductivity (mS/cm); w.b.: wet basis; d.b.: dry basis.

Table 2. Design matrix including factor levels (coded and actual) and the response values for these two factors

Run	Factor levels				Y_{TD30}	Y_{TD50}	Y_{MD30}	Y_{MD50}	Y_{TG30}	Y_{TG50}	Y_{MG30}	Y_{MG50}
	Coded		actual									
	Concentration (x_1) (g/kg)	Mixing ratio (x_2) (soil:inoculum)	Concentration (g/kg)	Mixing ratio (soil:inoculum)								
1	-1	-1	0.38	0.9:1	69.96	42.89	66.36	53.82	-15.2	-10.9	-20.2	-5.9
2	+1	-1	1.74	0.9:1	71.25	54.78	77.75	68.83	4.8	6.8	-36.4	-27.6
3	-1	+1	0.38	4.3:1	77.58	64.55	70.12	63.12	0.4	1.1	-6.8	-0.9
4	+1	+1	1.74	4.3:1	70.71	65.87	75.43	72.92	4.5	5.3	-6.3	-1.7
5	0	0	1.05	2.6:1	67.91	67.43	73.92	67.90	-3.2	-3.0	-11.4	-5.4
6	-α	0	0.1	2.6:1	47.26	52.57	36.8	16.04	-2.1	-3.1	-11.5	-2.1
7	+α	0	2.0	2.6:1	67.17	70.83	81.23	75.57	-1.9	3.6	-12.1	-7.7
8	0	-α	1.05	0.2:1	62.91	55.82	74.57	65.00	12	23.7	-133.3	-97.0
9	0	+α	1.05	5:1	75.59	74.21	70.00	74.17	-1.0	0.4	-3.8	-0.70

**The response represents the degradation (D) percentage (Y_D) and the biogas (G) production (Y_G) after 30 and 50 days of incubation. Where; T: thermophilic; M: mesophilic and 30 or 50 represents the incubation time (day)*

Table 3. Correlation coefficients (R) and p-values of each function (cf. Equations 2–9) representing the treatment process

	Thermophilic				***Mesophilic***			
	Y_{TD30}	Y_{TD50}	Y_{TG30}	Y_{TG50}	Y_{MD30}	Y_{MD50}	Y_{MG30}	Y_{MG50}
R	0.65	0.93	0.65	0.70	0.86	0.89	0.78	0.79
P	0.63	0.06	0.61	0.5	0.17	0.03	0.16	0.15

Table 4. Values of "F" fraction (%) for each component after 50 days for samples 5, 6 and 7 under thermophilic (T) and mesophilic (M) conditions

	T-5	***T-6***	***T-7***	***M-5***	***M-6***	***M-7***
Fluorene	96.0	100.0	97.15	88.94	100.0	77.75
Phenanthrene	89.1	100.0	98.65	74.15	98.38	79.08
Anthracene	69.7	77.09	81.38	70.89	93.33	73.58
Fluoranthene	83.24	70.68	94.81	86.45	91.98	90.25
Pyrene	68.17	ND	92.94	70.23	4.31	78.75

ND: not detected (below the detection level)

Table 5. PLFA structure of the microbial biomass for samples 5, 6 and 7 after 30 and 50 days under thermophilic (T) and mesophilic (M) conditions

Biomass/Treatment	***T-5-30***	***T-6-30***	***T-7-30***	***M-5-30***	***M-6-30***	***M-7-30***	***T-5-50***	***T-6-50***	***T-7-50***	***M-5-50***	***M-6-50***	***M-7-50***
Fungi	2376.38	404.45	1031.77	1139.98	894.50	1256.78	1233.92	1034.08	1664.30	814.67	1139.79	804.89
Bacteria	83862.03	1399.42	2241.85	10669.97	11112.80	11264.75	2751.07	3575.27	3818.31	13201.81	13485.32	10294.19
Actinobacteria	455.42	134.46	272.89	665.29	632.97	669.43	290.03	380.47	329.70	544.23	712.66	600.43
Gram positive	2521.82	916.95	1153.36	8035.50	8616.16	8570.36	1625.29	2253.64	2357.06	10825.81	10476.29	8111.61
Gram negative	1202.15	283.71	736.54	1768.22	1648.93	1822.75	701.02	760.37	974.36	1591.95	2021.35	1396.72
Anaerobic bacteria	145317.61	24332.03	77354.29	42321.83	35682.23	44730.48	80674.27	83980.56	91905.50	34616.08	46113.00	33856.22
Total microbial biomass	163930.30	29044.05	87662.33	60262.10	53775.34	63563.36	91590.98	97311.69	105652.35	55498.66	67997.12	50700.74
Sum (cy17:0, cy19:0)=*Cy*	145.02	59.26	116.15	475.91	439.12	485.66	106.21	136.97	111.12	422.25	525.20	349.95
Sum(16:1w7,18:1w7)=*Pre*	1028.51	214.61	605.59	1250.36	1168.43	1287.89	580.19	602.89	843.19	1130.94	1456.30	1012.51
Ratio (*Cy/Pre*)	0.15	0.27	0.20	0.38	0.38	0.38	0.18	0.23	0.13	0.37	0.36	0.34
Saturated PLFA (S)	12191.59	3029.16	7229.61	6782.45	6712.44	6976.20	7163.70	9020.58	8519.43	7499.67	8024.28	6256.48
Monosaturated PLFA (M)	146229.72	24497.22	77858.54	43138.23	36452.92	45581.91	81162.86	84467.00	92657.62	35363.52	47083.95	34553.03
S/M	0.08	0.12	0.10	0.16	0.19	0.15	0.09	0.11	0.09	0.21	0.17	0.18
Anaerobic bacteria/total biomass	0.89	0.84	0.88	0.70	0.66	0.70	0.88	0.86	0.87	0.62	0.68	0.67