

Anaerobic degradation of PAHs in soil: impacts of concentration and amendment stability on the PAHs degradation and biogas production

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Abstract

In this study, the bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil under strict anaerobic-methanogenic conditions was systematically studied applying the central composite design approach. The effect of PAHs concentration and the stability of the compost as an organic amendment for anaerobic digestion were examined. In all assays, the used methanogenic consortium was able to degrade the PAHs although some inhibition effects were observed during the initial stage in some cases. The degradation rates varied between 31.4-90.6% during 50 days incubation period. The study demonstrated that the PAHs concentration influences the degradation rate where more degradation was observed by increasing the concentration of PAHs. However, the biogas production as a result of the digestion process was more influenced by the compost stability which also has its effect on the degradation rates as more degradation occurred with more stable compost, but more biogas was produced with less stable compost, which indicates that the biogas is mainly produced by the anaerobic digestion of the amended compost. Finally, it seems that compost addition is required to improve the process in some cases but in other circumstances it does not greatly improve the bioremediation of PAHs.

Keywords: Polycyclic aromatic hydrocarbons (PAHs); anaerobic digestion; soil bioremediation; compost stability; biogas.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are constituents of fossil fuel, crude oil and creosote. These petroleum products are some of the most widely used chemicals in our modern society (Holliger and Zehnder, 1996; Sarkar et al., 2005). Because of leakage from underground and aboveground storage tanks and pipelines, spills at production wells, refineries and distribution terminals, and improper disposal and accidents during transport, these compounds have become of the most frequently encountered pollutants in the ecosystem (Holliger and Zehnder, 1996). As a consequence, aquatic, sediments or soil microbial communities are exposed to a continuously growing of such diverse chemicals (Kobayashi and Rittmann, 1982). However, because of their toxicity, carcinogenicity and mutagenicity with respect to both the environment and human health, PAHs are considered of particular concern.

As biological degradation represents the major route through which PAHs and other organic chemicals are removed from contaminated environments (Chang et al., 2002; 2003), in recent years much attention has been dedicated to study these biological processes, specially focusing on optimizing their degradation potential for bioremediation purposes as these technologies are believed to be more economical compared to other technologies. Nevertheless, some PAHs physical properties such as their low aqueous solubility and their high solid-water distribution ratios, still stand against their ready microbial utilization and promote their accumulation in the solid phases of the terrestrial environment (Johnsen et al., 2005).

The bioremediation of PAHs contaminated soils using aerobic treatments has been studied and applied successfully (Beaudin et al., 1996; Antizar-Ladislao et al., 2004; Antizar-Ladislao et al., 2006; Haderlein et al., 2006; Sayara et al., 2009). On the other hand, bioremediation of contaminated soil by using anaerobic digestion has

received much less attention. More recently, anaerobic treatment process with different electron acceptors was investigated and remarkable results were obtained for both aliphatic and monoaromatic hydrocarbons (Callaghan et al., 2006), although in general few researches are available on the anaerobic biodegradation of PAHs (Zhang et al., 2000). Anaerobic degradation of PAH has been also demonstrated in several microcosm studies with nitrate, ferric iron, or sulphate as electron acceptors and under methanogenic conditions. Therefore, anaerobic digestion could be an interesting alternative for the bioremediation of PAHs contaminated sites. Anaerobic digestion simultaneously produces biogas that reduces the environmental impact produced by the combustion of fossil fuel as it is able to produce methane used in the production of energy (Chynoweth et al., 2001; Holm-Nielson et al., 2009). Meanwhile, it is one of the technologies used to reduce the volume of the produced organic wastes in modern societies.

Naphthalene and phenanthrene are reported to be degraded under sulfate-reduction (Coates et al., 1996a; 1996b; 1997; Meckenstock et al., 2000), also flourene and phenanthrene were reported to be degraded under nitrate-reducing, iron-reducing and sulfate-reducing conditions, where some degradation pathways were proposed for these compounds under such conditions (Eriksson et al., 2003; Ramsay et al., 2005; Tsai et al., 2009). However, to the best of our knowledge, no studies have been performed to systematically investigate the effect of compost addition (of different stability degrees) on the bioremediation of PAHs-contaminated soils under methanogenic conditions.

The objective of this work was to asses the feasibility of employing anaerobic digestion process in the bioremediation of PAHs-contaminated soil. The impact of two factors was investigated during the experimental work: introducing various types of composts with different stability degree as organic co-substrates in the digestion

process, whereas the other factor was the effect of different levels of PAHs concentrations. Moreover, the rate of the biogas production and its components as a result of such process was assessed. The influences and interactions among the studied factors were systematically clarified applying the experimental design technique through the central composite design methodology.

2. Materials and methods

2.1. Soil

An uncontaminated soil classified as sandy loam soil was obtained from Prades (Tarragona, Spain), collected from the surface horizon (0-30 cm). The soil was air-dried and then was sieved to 2 mm to remove any debris and maintained at 4°C until use. The soil consists of 73.4% sand, 18.6% silt and 8% clay. No PAHs were detected in the soil before being prepared for the experiments. Other properties of the soil are presented in Table 1.

2.2. Contaminants

A group of PAHs listed among the 16 USEPA priority PAHs pollutants were obtained from Sigma-Aldrich (Barcelona, Spain). The used PAHs include: Fluorene, Phenanthrene, Anthracene, Fluoranthene and Pyrene with 98-99% purity, which were used as contaminants. The weight percentage of each compound as a part of the total PAHs (\sum PAHs) was 33%, 31%, 10%, 22% and 4%, respectively. These percentages were determined according to the results of a fractionation process of a commercial creosote sample (Creosote lot: 42-13B, Chem Service, Sugelabor, Spain) in our laboratory using the method 3611B of the US Environmental Protection Agency, taking into account that the volatile part was ignored. This was done to simulate a real creosote

sample. PAHs contaminants were combined together using their percentage in a stock solution and then were spiked into the soil to have the desired concentration according to the experimental design set (0.1-2 g/kg, on dry matter basis) measured as total amount of PAHs. The applied concentration was selected to represent low to high concentration, although in the real contaminated sites, the contamination level may exceed or be lower than these values.

2.3. Co-substrates

As co-substrates for anaerobic digestion, five types of compost derived from the organic fraction of municipal solid wastes (OFMSW) were used during the experiment treatments. These composts have different degrees of stability that refers to the resistance of compost organic matter to follow rapid degradation and it was directly determined by the dynamic respirometric index (DRI), which was selected as a measure of stability. The composts were obtained from composting plants located in Barcelona (Spain) except compost B that was obtained from a home composter located in the University Autònoma of Barcelona. These composts are characterized by different degrees of stability ranging from full-stable to unstable compost (Ponsá et al., 2008; Ruggieri et al., 2008). This provides the ability to understand their effect as co-substrates on the degradation of the used PAHs as major objective, and also to assess the biogas production as a consequence. The main characteristics of the used composts are presented in Table 1. These composts were free from any PAHs.

2.4. Inoculum preparation

A mesophilic-anaerobic inoculum was obtained from the digested effluent of the anaerobic digester after the solid-liquid separation in a real waste treatment plant

(Barcelona, Spain), which is fed with the OFMSW and uses the Valorga process. The inoculum is a methanogenic culture consisting of 10.2% of total solids. It was conserved in a plastic gallon under strict anaerobic conditions and incubated in water bath at 37°C for about one month before use for the removal of remaining biodegradable organic matter. No PAHs were detected in the inoculum before the experiment run.

2.5. Experimental system

The experiments were conducted using 1-L bottles (traveller SIGG[®], Spain). The soil and compost were mixed together based on dry weight fraction 1:1 (w:w), and this fraction was the same for all the batches. The bottles were filled with the inoculum and the mixture (contaminated soil and compost) at a ratio of 1:1 (w:w) after diluting the mixture with distilled water until reaching the same total solid content for all the assays, which was 10.2%. Afterwards, the bottles were purged with N₂ and perfectly sealed. The bottles were closed and incubated under strict-static anaerobic conditions in a temperature-controlled chamber at 37°C for 50 days until no detectable biogas production was observed.

Blanks with only inoculum were used to determine the biogas production due to indigenous matter (inoculum). Moreover, controls that have inocula with only contaminated soil (1 g/kg) were used to determine the capacity of the anaerobic consortium to degrade the contaminant in the absence of the organic amendments. All the batch assays, blanks and controls were carried out in triplicates and the results are expressed as average of the triplicates.

2.6. Analytical methods

2.6.1. Stability

The co-substrate (compost) stability was determined using the dynamic respirometric index (DRI), determined according to Adani et al. (2002) and Barrena et al. (2009). Briefly, this index represents the oxygen consumption of a known sample of organic matter incubated under optimal conditions with a continuous air supply. Moisture content, organic matter content (OM), Kjeldahl nitrogen, total carbon content, humic acids content, pH and electrical conductivity were determined according to standard methods (The US Department of Agriculture and The US Composting Council, 2001).

2.6.2. Quantification and analysis of biogas

Biogas samples were periodically taken from assay bottles for analysis of the produced biogas. Quantitative biogas production was followed by measuring the pressure increase in the headspace by means of a SMC (ISE30) Pressure Switch manometer (1 MPa, 5% accuracy) at 37°C. Biogas production of blank (inoculum only) batches was subtracted from biogas production of each treatment to obtain the net resulting value of biogas production and then was expressed under normal conditions (0°C, 1 atm). The characterization of the biogas was performed using a gas chromatograph (GC 5890 Capillary Hewlett Packard) to determine the levels of CH₄ and CO₂ in the biogas as a result of the anaerobic process. Biogas samples of 1 ml were injected in the GC equipped with Porapak Q, 3 m 1/8" column, where helium was used as carrier gas. The initial temperature was maintained at 30°C for 3 min, and then it was increased at 10°C/min until 70°C and maintained for 5 min.

2.6.3. PAHs analysis

At the end of the incubation period (50 days), the mixture was centrifuged for 30 min at 10000 rpm. Samples from the supernatant were analyzed for the soluble part of

the PAHs, where the remaining solid part was dried using a lyophilizer (Benchtop 5L, Virtis Sentry, NY) for later PAHs quantification.

PAHs were extracted using a Soxhlet extraction process, and then they were identified and quantified by gas chromatography. Samples were extracted using acetone/dichloromethane (1:1 v/v) as solvent during two hours. For instance, Soxhlet extraction is an adequate method for PAHs extraction from soil (Saim et al., 1997) compared with other methods. After extraction the solvent was left to evaporate to atmosphere and then the remaining residue (extract) was dissolved in 10 ml of dichloromethane. A 1- μ l extract of this solution was injected in a gas chromatograph (GC8690N, Agilent, Spain) equipped with flame ionization detector (FID) and a splitless injector. A Zebron ZB-5HT Inferno column (Agilent, Spain) was used. Initial temperature was maintained at 50°C for 1 min, and then it was increased at a rate of 7°C/min until 320°C, then another rate of 20°C/min until 400°C was applied and maintained at this final temperature for 5 min to clean the column of any organic for the next sample. The concentrations of PAHs were determined after the calibration of the method with standard PAHs samples of different concentrations. Also, as quality control, some standard samples (at least 3) were introduced simultaneously to be analyzed during PAHs analyses. Remaining PAHs percentages were calculated by dividing the PAHs residue concentration into the PAHs original concentration.

2.7. Experimental design methodology and statistical analysis

Central Composite Design (CCD) methodology with two variables ($k=2$) was applied to investigate the effect of the contaminant concentration (x_1) and the compost stability (x_2) on the bioremediation of PAHs-contaminated soil under anaerobic conditions. This methodology is commonly used in process optimization and allows the

estimation of a full quadratic model for each response. CCD can calibrate the model much more efficiently without using all the possible combination levels of the factors and consequently reducing the experiment runs, and also permits to statistically distinguish between the role of the factors and the random error associated to the experiments. On the contrary, using a full factorial design generally requires more runs to accurately estimate model parameters. When CCD methodology is to be applied with two variables, the value of α ($\alpha = F^{1/4}$, where $F = 2^k$) is 1.414; this value (α) represents the extreme values (low and high) of the factors involved within the design, and then the factors values were normalized within the decided design values (0.1-2 g/kg). The design consist of 2^k factorial points representing all combinations of coded values (± 1), $2k$ axial points at a distance $\pm\alpha$ from the origin, and at least 3 (triplicates) central points with the coded values set to zero. More details of the experiment design technique and its application can be found and reviewed elsewhere (Deming and Morgan, 1987).

Design matrix is presented in Table 2, where the coded and actual values of the two independent variables x_1 , x_2 , and the actual response of each combination regarding to the degradation rate (Y_1) and the biogas production (Y_2) are also reported.

As shown in Table 2, nine triplicate experiment runs were carried out according to the experiment design technique, also a blank run (inoculum only) and a control run (contaminated soil and inoculum) were run in parallel. All the experiment runs were carried out in triplicates and the results are presented as average of the triplicates. Statistical analysis was performed for all variables using the Sigmaplot® 8.0 software package (Systat Software Inc, San Jose, USA) and according to the statistical recommended for CCD (Deming and Morgan, 1987).

3. Results and discussion

3.1. General characteristics of the inoculum and co-substrates

The used co-substrates are characterized by a high organic matter content (Table 1) that can play a major role for supplementing the enrichment culture with the needed nutrients for being active during the incubation period (Forster-Carneiro, 2007). On the other hand, these substrates have different degrees of stability according to the DRI that could affect the process performance as this parameter is related to microbial activity of the substrate and the characteristics and the content of readily biodegradable organic matter (Scaglia et al., 2007). Moreover, the used soil is characterized by low organic matter compared to the used substrates.

3.2. Response surface and statistical analysis

To investigate the response of the process under different values of the studied factors, the experimental design methodology was applied through the CCD technique. The percentage of PAHs degradation (Y_1) and the amount of the produced biogas (Y_2) after 50 days were used as objective functions to correlate these variables using a second-order polynomial model to fit them as explained in equations (1) and (2).

$$Y_1 = 53.43 + 66.9x_1 - 6.38x_2 - 25.4x_1^2 + x_2^2 + 0.9x_1x_2 \quad (1)$$

$$Y_2 = 1.62 - 5.59x_1 + 33.6x_2 + 2.4x_1^2 - 2.7x_2^2 + x_1x_2 \quad (2)$$

The best regression coefficients (r) of Y_1 and Y_2 were 0.76 and 0.80 respectively. Although these values do not correspond to a perfect correlation, they were the best way to describe the experimental data compared with the other traditional known models. Moreover, the significance levels of both models were not conclusive ($P > 0.05$), which indicates that other factors rather than the studied might affect the process and should be considered in terms of enhanced biodegradation with the addition of compost.

3.3. Anaerobic degradation of PAHs

Fig.1 presents the remaining PAHs after incubation of 50 days under anaerobic conditions. As pointed out, the PAHs concentrations were significantly decreased in all experiments except that in the 8th run according to the experiment design matrix (Table 2). Moreover, all the individual PAHs demonstrated a notable decrease in their concentration after the incubation period, but pyrene presented some recalcitrance behaviour compared with others PAHs especially in runs 1, 3, 5, 6 and 8. In general, flourene showed the highest rate of degradation among the other PAHs in the control and all the assays except the 8th one, where the other compounds (phenanthrene, anthracene, flouranthene) practically had the same rate of degradation. The observed degradation suggested that PAHs degraders were within the inocula microflora; however, it was supposed that these microorganisms represented a small fraction in the microbial community since less biogas production was observed especially in the initial days. However, the microorganisms were able to be adapted and responded with increasing their activities. It is thought that the adaptation may be the result of internal changes in the predominant species of methanogens, or due to a shift in the methanogenic population (Zeeman et al., 1985).

In the amended assays (contaminated soil, inoculum and compost), the overall PAHs (Σ PAHs) degradation was between 31.45% to 90.65% (run 8 and run 4 respectively) within a 50-day incubation period, where it was about 87.23% in the control which contains only contaminated soil (1 g/kg) and the inoculum. The used enrichment culture was able to acclimate with the new ambient although it was not previously exposed to such components, whereas some studies demonstrated that prior exposure to PAHs is a key factor determining whether the microbial community is

adapted for anaerobic PAH degradation (Hayes et al., 1999). The observed degradation in the control reactor is a good evidence for that ability to degrade the encountered PAHs. However, as it can be seen from the daily observation of the biogas production (Fig.2), an inhibition effect was observed in some assays during the first week of incubation, which indicates that PAHs are toxic to the microorganisms to some extent. Kroeker et al. (1979) demonstrated that the material may be inhibitory when it causes an adverse shift in the microbial population or inhibition of bacterial growth. Inhibition is usually indicated by a decrease of the steady-state rate of biogas production and the accumulation of organic acids (Chen et al., 2008). In this study the decrease in the biogas production was the best evidence for such inhibition during the incubation period. Also, it is worthwhile to mention that by the end of the process, samples from the supernatant liquid after centrifugation were analyzed for PAHs. The analysis demonstrates that no PAHs were in the liquid phase. Therefore, all the non-degraded PAHs were adsorbed onto the solid particles of the mixture.

3.4. Biogas production

Fig.2 presents some examples of the cumulative biogas produced during the incubation period (50 days). As shown in this figure, an initial lag phase was observed in some assays and was extended up to one week. Following this lag phase, the biogas production began to increase markedly indicating that the microorganisms responded with an increase in their activity. The used enrichment already began to adapt with the new ambient, and the rate of the biogas production increased indicating an increasing in the density and the activity of the microbial population. In general, the quantity of the produced biogas is inversely proportional to the compost stability. In the less stable compost applied more biogas was produced: run 6 (117.9 L/kg-TS), which was the most

active compost, whereas in run 5 only 5.04 L/kg-TS were produced since this compost was completely stable. Clearly, compost stability has a determinant influence as it is shown in equation (2). These results agreed with that explained in the literature (Al-Masri, 2001; Schievano et al., 2008) where higher biogas-potential was obtained with more active materials like the OFMSW. However, since the PAHs degradation is the main objective of this study, the biogas production was left as an indicator of the process behaviour and microbial activity.

The analysis of the produced biogas demonstrated that methane gas represents the major part (around 60-70%) of its components, where the rest is mainly carbon dioxide. As methane production was observed in all of the experiment assays this directly infer that a methanogenic consortium was the predominant one during all the anaerobic process.

The biogas production is an evidence of the microbial activity which probably support the argument that PAHs decrease in the soil was the result of this microbial action. The gradual increase in the biogas production and the difference in the amount produced according to the different combinations of the experimental design reinforce such hypotheses. Moreover, the chromatographic analysis of the treated samples after the incubation period detected some new compound (peaks) which might be produced as a result of the biotransformation or metabolism processes occurred during the degradation process. Unfortunately, these compounds could not been identified.

3.5. Effect of PAHs concentration and compost stability on the process

Fig.3 presents the surface response of PAHs degradation and Fig.4 represents the surface response of the cumulative biogas production after 50 days of anaerobic digestion, where the response was carried out by transforming the results obtained from

the experimental design matrix according to CCD. From these figures, it is evident that the studied factors had a direct effect on the process behaviour and the objective functions. Considering the degradation rate, more than 80% of PAHs were degraded except in the 8th run. The obtained results demonstrate the capacity of the used enrichment to degrade such components. According to Fig.3, a degradation rate between 85-90% can be achieved under these anaerobic conditions when the PAHs concentration is greater than 1 g/kg and the compost stability degree is less than 2 mg O₂ g⁻¹ OM h⁻¹. It was clear from the results that the PAHs concentration is the controlling factor during the process (Fig.2 and equation 1). Small concentration of total PAHs (0.1 g/kg) gave the lowest degradation rate among the performed experiments which indicates that this concentration is not sufficient to support the growth and the development of the microorganisms needed to degrade these components. On the other hand, when the concentration of 2 g/kg was applied, the rate of degradation was slightly lower than other runs containing lower concentration. This observation could be caused by a toxicity or inhibitory effect. This hypothesis would be clarified if higher concentrations were used, although they are difficult to find in real environments. In literature, the same results were observed regarding the PAHs concentration. PAHs were anaerobically biodegraded in the Boston Harbor and the Chelsea River site that was less heavily contaminated, but at a slower rate than in the most heavily contaminated sediments (Hayes et al., 1999). Also higher rates of anaerobic PAHs degradation in sediments with higher petroleum contamination was observed in sediments from San Diego Bay (Coates et al., 1996a).

The compost stability had its influence on the process especially with low concentrations where more degradation occurred with more stable compost (run 1 and run 3). However, with high concentrations, its effect was not completely clear as the

incubation period was presumably sufficient to stabilize the material. However, Fig.3 shows that less stable compost is less capable to support the degradation process. This can be justified as preferential behaviour by the microorganisms, where they normally prefer easily degradable materials rather than complex ones, consequently; the contaminants are degraded afterwards when easily degradable material is available in such less stable composts. In consequence, the addition of compost can have a positive or a negative effect on PAHs biodegradation according to the compost stability.

The efficiency of the compost could be attributed to its components especially the humic acid as a part of the total organic matter. These humic acids were found to be coincident with the compost stability degree, where more stable compost has higher humic acids content (Table 1) as previously reported (Huang et al., 2006). In fact, it is believed that humic acids increase the bioavailability of the PAHs as desorption rate is increased and consequently their degradation rates are enhanced (Janzen et al., 1996; Plaza et al., 2009). Indeed, when the degradation rate of PAHs is to be enhanced, co-substrate properties that increase the bioavailability (humic matter) are of concern. However, the increase of such properties is obtained after stabilizing materials like the OFMSW. Favouring conditions for PAHs degradation enhancement are in contradiction with the biogas production as stable materials are not favourable in this case.

Biogas production was also affected by the two studied factors. Obviously, the PAHs concentration had almost no effect when compost with stability degree less than 3 $\text{mg O}_2 \text{ g}^{-1} \text{ OM h}^{-1}$ was applied. As expected, the biogas production increased with the increase in the DRI values. This may explained as more stable compost has less easily biodegradable materials (Barrena et al., 2009). Moreover, the PAHs are supposed to be available for the microorganisms as the humic acids exist (Janzen et al., 1996). PAHs affect the biogas production when less stable compost is to be used even though the

highest amount of the biogas can be produced with these unstable composts. High concentration or low concentrations are crucial in this matter. High concentration represented a negative effect on the microorganisms through inhibiting their activity, where low concentrations are not sufficient to stimulate and increase the decomposition process.

4. Conclusions

Anaerobic digestion can be another alternative for the remediation of PAHs contaminated soil. In this study, the applied methanogenic consortium was capable to degrade PAHs to a high percentage. The results demonstrated that the PAHs concentration seems to have an important role in the process performance since more degradation was observed as PAHs concentration was increased. However, the concentration effect need to be more investigated as some other inhibitory effects were observed. Furthermore, adding different co-substrates also had its influence on the process regarding the degradation, which can be improved or diminished according to the compost properties, especially its stability.

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Legends to Figures

Fig.1. Percentage of the remaining PAHs after 50 day of incubation.

Fig.2. Examples of the cumulative biogas production (L/kg-TS) during the 50 days incubation period.

Fig.3. Response of PAHs degradation (%) under different PAHs concentration and compost stability degrees measured by the dynamic respiration index ($\text{mg O}_2 \text{g}^{-1} \text{OM h}^{-1}$).

Fig.4. Response of the biogas production (L/kg-TS, 0°C , 1 atm) under different PAHs concentrations and stability degrees measured by the dynamic respiration index ($\text{mg O}_2 \text{g}^{-1} \text{OM h}^{-1}$).

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Fig.1

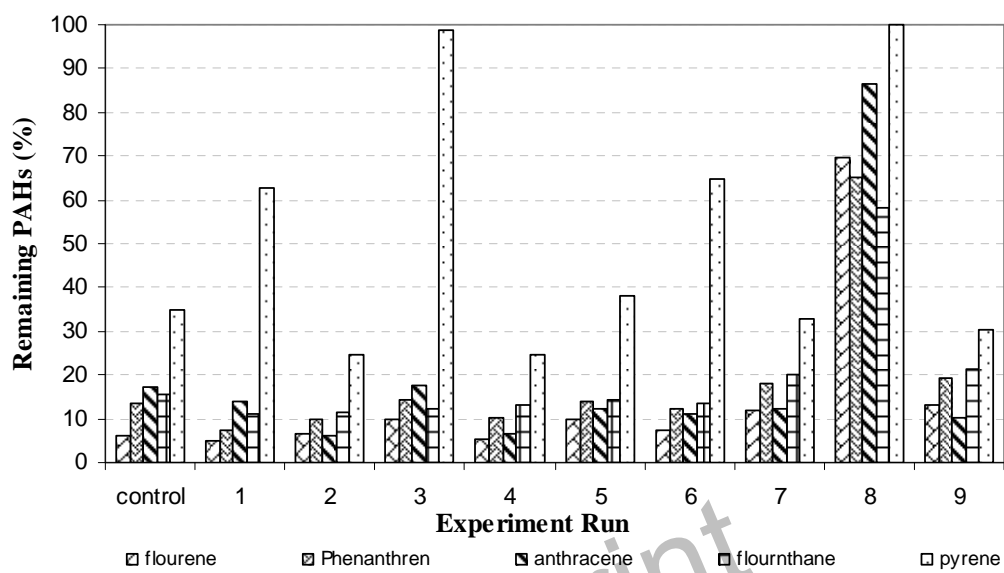


Fig.2

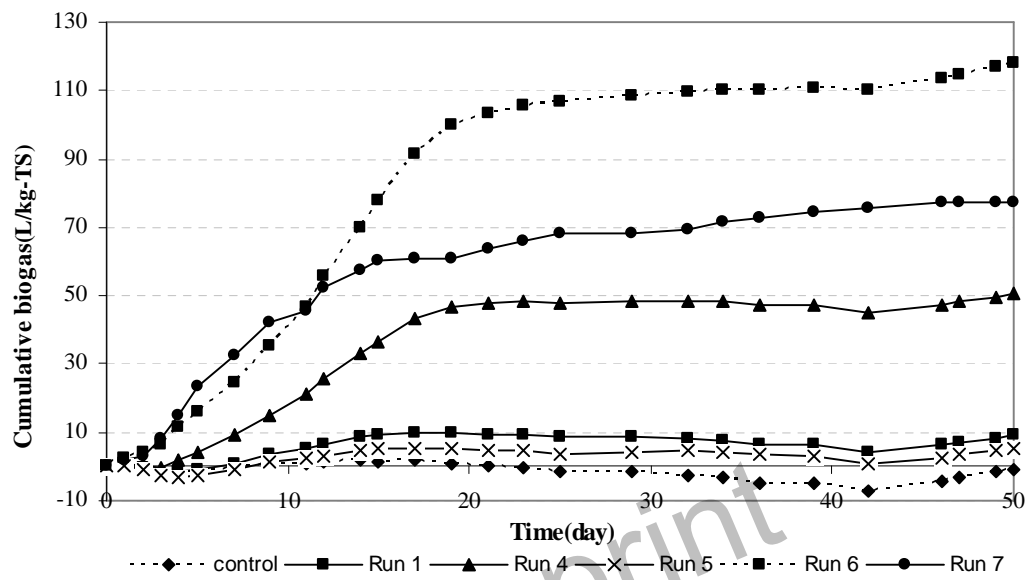


Fig.3

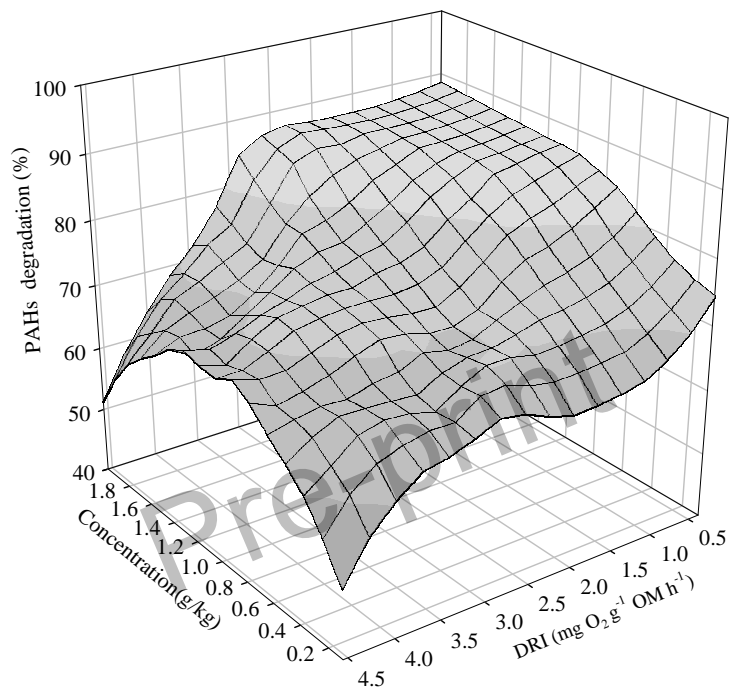
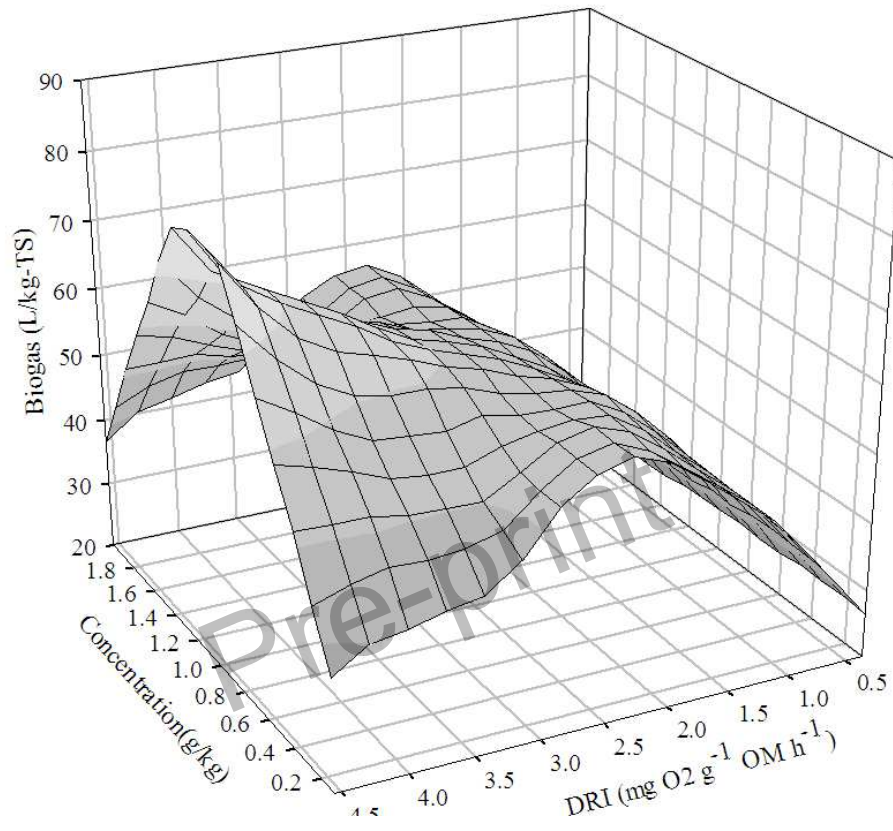


Fig.4



Tables

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Table 1: Characteristics of the used compost and soil (mean values and standard deviation).

Parameter/Material	Soil	Compost A	Compost B	Compost C	Compost D	Compost E
Moisture content (% , wb) *	6.64 ±0.01	38.6 ±0.2	30.1 ±0.4	53.8 ±0.2	32.63 ±0.08	40.5 ±0.3
Organic matter content (% , db) **	3.7 ±0.4	44.5 ±0.4	53.9 ±0.1	62 ±2	44.6 ±0.3	52 ±1
Total Organic Carbon (% ,db) **	1.26 ±0.02	18 ±1	24 ±3	31.7 ±0.3	19 ±1	20.4 ±0.3
Total Kjeldahl Nitrogen (% ,db) **	0.7 ±0.1	2.6 ±0.4	2.62 ±0.06	4.1 ±0.1	3.1 ±0.1	1.9 ±0.1
pH	6.7 ±0.02	8.07 ±0.08	8.63 ±0.04	8.0 ±0.1	8.11 ±0.01	7.61 ±0.01
Elec. conductivity (mS/cm)	0.2 ±0.01	4.9 ±0.1	6.5 ±0.2	5.3 ±0.1	6.01 ±0.01	7.13 ±0.04
Humic Acids (% ,db) **	1.5	10.1	11.6	14.6	8.9	4.7
Dynamic Respiration index (mg O ₂ g ⁻¹ OM h ⁻¹)	-	0.37 ±0.02	0.6 ±0.4	1.7 ±0.1	3.0 ±0.3	4.55 ±0.1

* wb: wet basis.

** db: dry basis.

Table 2

Design matrix including factor levels (coded and actual) and their response values for the two factors, (mean values and standard deviation).

Run	Factor levels				Y ₁ (%)	Y ₂ (L/kg-TS)
	coded		actual			
	Concentration (x ₁) (g/kg)	Stability (x ₂) (mg O ₂ g ⁻¹ OM h ⁻¹)	Concentration (g/kg)	Stability (mg O ₂ g ⁻¹ OM h ⁻¹)		
1	-1	-1	0.38	0.58 ±0.04	89.8	9.05
2	+1	-1	1.74	0.58 ±0.04	90.6	7.84
3	-1	+1	0.38	3.1 ±0.3	83.9	47.4
4	+1	+1	1.74	3.1 ±0.3	90.7	50.5
5	0	-α	1.05	0.37 ±0.02	86.5	5.04
6	0	+α	1.05	4.55 ±0.01	87.2	117.9
7	0	0	1.05	1.7 ±0.1	83.6	77.1
8	-α	0	0.10	1.7 ±0.1	31.5	70.9
9	+α	0	2.00	1.7 ±0.1	82.8	75.5

*The response represents the degradation percent (Y₁) and the biogas production (Y₂) after 50 days of incubation.