Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil: process evaluation through composting and anaerobic digestion approach

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CERTIFIQUEM:
Que l'enginyer **Tahseen A. S. Sayara** ha realitzat sota la nostra dirección el treball que, amb el títol "Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil: process evaluation through composting and anaerobic digestion approach", es presenta en aquesta memòria, la qual contitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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DON'T let the past hold you back, you're missing the good stuff

IT IS OUR LIFE! WE CALL IT SOIL,,,

IT IS THE STUFF, IN WHICH WE TOIL,,,

FROM SOIL WE'VE SPRUNG, TO SOIL WE'LL GO,,,

PROTECT THE SOIL OF THIS EARTH SO WE CAN GROW,,,

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Last and surely not least, my wonderful and great family,,,
To the soul of my father, God bless him, I dedicate this work.
My beloved, honourable and great mother, the candle which was burnt to light the road for us, this is the real moment to thank for your infinite care from kinder till now.
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My Great FAMILY,,, Every Thing I DO I DO It FOR YOU ALL,,, May God Bless You All.

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Resume of the dissertation

Among the different available remediation technologies, it is well-known that bioremediation methods which mainly depend on microorganisms to degrade, transform, detoxify or break down the contaminants, they are recognized as cost-effective and environmental-friendly methods. In fact, microorganisms "engine of bioremediation process" carry out their normal duty under aerobic or anaerobic conditions, which without doubt extends and motivates the desires to make use of such abilities to reduce environmental threats caused by various contaminants. However, to achieve satisfactory results during any bioremediation process, providing optimal conditions for microorganisms is considered as an essential/crucial task. Composting as one of the applied bioremediation technologies used to remediate soils contaminated with organic contaminants like PAHs still needs more investigation although a valuable effort has been devoted to elucidate the behaviour of this process in the remediation of PAHs-contaminated soils. However, till recently, anaerobically treatment of PAHs-contaminated soil received less attention as it was believed that PAHs are poorly or even impossible to be degraded under such conditions. Therefore, the present study tried to touch both aerobically bioremediation of PAH-contaminated soil through composting and anaerobically treatment of the same soil under strict methanogenic conditions. Consequently, the study objective was mainly to shed light on the effects of several controlling factors that determine the processes behaviour and the PAHs degradation as a consequence.

Firstly, regarding the composting technology, screening of several organic co-substrates to select the most efficient one that is able to enhance the contaminants degradation (known as biostimulation) was considered as the first step to follow the process. The screened co-substrates included: raw organic fraction of municipal solid wastes (OFMSW), industrial compost from OFMSW composting (COFMSW), compost derived from home composting (HCOFMSW), anaerobically digested sludge (ADS), non-digested activated sludge (NDS) and centrifuged non-digested activated sludge (CNDS). Whereas,

Pyrene (1g/kg, dry matter) was used as representative of PAHs during this experiment. The obtained results indicated that stable compost derived from OFMSW was able to enhance the contaminant degradation rate to high extent (69%) compared with other used co-substrates. Further, this experiment drew attention to an important factor; compost stability degree that has not been investigated before as the degradation rate varied according to the variation in the stability degree of the same co-substrate. In this context and depending on the obtained results through screening experiment, compost originated from OFMSW was used as the sole organic co-substrate throughout the subsequent bioremediation-composting treatments and main focus was devoted to clarify systematically the impact of some factors which may influence the process. Systematic study of the process was performed applying experimental design technique through central composite design (CCD) methodology as this technique provides the ability to overview the effect of several factors and their interaction in the case of any dependent influence. Whereas, second-order polynomial model was used to fit the results.

In the first stage after co-substrates screening, and to visualize the process response under different conditions, bioremediation of pyrene-contaminated soil was investigated considering the impacts of three factors which namely were: Pyrene concentration (0.1-2 g/kg, dry matter), soil to compost mixing ratio (1:0.5-1:2 w/w) and compost stability measured as respiration index (0.78, 2.69 and 4.52 mg O_2 g^{-1} Organic Matter h^{-1}). The results indicated that stable compost is more capable to increase the degradation rate within short period, but prolongation of the treatment process was able to stabilize moderate to active compost, consequently, favourable results could be achieved. Nevertheless, increasing the soil to compost ratio (>1:1) within the treatment mixture did not have any significant effect on the contaminant removal, and higher amounts could inversely influence the process. In fact, this result is considered as a key point in the process design because feasibility (economics) of the composting technology based on the amount of the compost to be added to the contaminated soil. Additionally, it was clear that the degradation rate is proportional with the pyrene concentration such that high concentration enhanced the degradation rate. However, concentrations higher than 1.3g/kg began to exhibit an increased toxicity on the microbial activity, meanwhile low concentration (0.1g/kg) were unable to motivate degradation rate indicating that the contaminant concentration plays a major role in the whole process.

As normally contaminated site are found to have several creosote derivatives, whereas PAHs represent a major part of these contaminants. A mixture of PAHs (Flourene,

Phenanthrene, Anthracene, Flouranthene, Pyrene and Benzo(a)anthracene) simulated a real creosote sample was used as target contaminant during the soil composting-bioremediation process. Concurrently, PAHs concentration (0.1-2 g/kg, total PAHs concentration based on dry matter) and compost stability (0.37 - 4.55 mg O_2 g^{-1} Organic Matter h^{-1}) had been evaluated. The results confirmed again the particular importance of compost stability degree during the bioremediation process, more specifically during the first stage of the process. Stable composts enhanced the degradation levels in soil-compost mixture and degradation rate of 92% was achieved after 10 days, but only 40% was degraded with the less stable compost. Interestingly, humic matter as part of the compost organic matter was found to be increased with stability degree in the sense that, the more stable compost was the more humic matter observed. Accordingly, Humic matter was assumed to facilitate the desorption of the PAHs to be more available for degradation by the microbial activity and thus increasing their degradation rate. The PAHs concentration was also important during the process, since the degradation rates increased with the increase in the PAHs concentration. Moreover, all the individual PAHs demonstrated a considerable decrease in their concentration after the incubation period, but pyrene was degraded to lower levels in some treatments compared to others PAHs which highlights the influence of the contaminant properties and the microbial preferential behaviour during this process.

The previous results were all obtained through biostimulation of the indigenous microorganisms within the treatment mixture by introducing organic co-substrates. Furthermore, in a comparative study to investigate the impact of bioaugmentation by introducing exogenous microorganism and to compare it with biostimulation. White-rot fungi (Trametes versicolor ATCC 42530) was used in a PAHs (1g/kg, total PAHs on dry matter bases) contaminated soil spiked with Flourene, Phenanthrene, Anthracene, Flouranthene, Pyrene, Benzo(a)anthracene and Chrysene, with percentages simulating a real creosote sample. Evaluation of the process through different analysis included the dynamic respiration index (DRI), the cumulative respiration at five days (AT_5), the enzymatic activity and the fungal biomass demonstrated that the introduced *Trametes versicolor* did not enhance the PAHs degradation. On the contrary, biostimulation using compost of OFMSW was able to enhance the degradation rate where 89% of the total PAHs was degraded within 30 days of composting compared to only 29.5% that was achieved by the soil indigenous microorganisms without any co-substrate (unamended). Also, the results showed that the stable compost of OFMSW had a great potential to enhance the PAHs degradation compared to non-stable co-substrates such as rabbit food.

In the second part of the present study, anaerobically treatment of PAHs-contaminated soil has been investigated under strict methanogenic conditions. Similar as in the composting treatments, the effect of PAHs concentration and the stability of compost as an organic co-substrate were also systematically evaluated under mesophilic conditions (37˚C). PAHs degradation rate ranged between 31%-90% were obtained after 50 days of incubation, demonstrating the effectiveness of such biological treatment in this field. However, the process out put including PAHs degradation and biogas production found to be influenced by the two factors. For instance, PAHs degradation rate positively correlated with concentration increase and composts stability degree, but biogas production was fundamentally dependent of compost stability degree and inversely influenced by this factor. Interestingly, in some cases compost addition did not greatly improve the PAHs degradation which indicated that PAHs could be directly degraded by the inocula. Subsequently, the latter study was performed without co-substrates or nutrient except the contaminated soil and the inocula. Beside the influence of PAHs concentration (0.1-2 g/kg), the effect treatment operation temperature (mesophilic 37˚C and thermophilic 55˚C) and soil to inocula ratio (0.2-5:1, soil:inocula) were involved. Under both operation temperatures, and in spite of biogas inhibition, certain rate of degradation was achieved during the first 30 days of incubation, where this degradation rate was found to proportion with PAHs concentration and soil to inocula mixing ratio. Nevertheless, treatments prolongation up to 50 days and due to unclear reasons, reversible results were obtained as PAHs concentrations were increased, indicating the bioformation of PAHs under such oxygen-deficient conditions. Therefore, future work should be devoted to clarify the reasons behind this behaviour.

It is worth to highlight that part of the results presented in this thesis were obtained in collaboration with the investigation group of the laboratory of environmental biotechnology in the Academy of Science of the Czech Republic as part of research stay. Additionally, the results presented in the current thesis represent the beginning of a new research line in the research groups; Compostaje de Residuos Sólidos Organicos (2009 SGR 95) and Biodegradación de Contaminantes Industriales i Valurización de Residous (2009 SGR 656) of the Universitat Autònoma de Barcelona.

Resumen global de la disertación

Entre las diferentes tecnologías de remediación disponibles, es bien conocido que los métodos de biorremediación dependen principalmente de los microorganismos para degradar, transformar, detoxificar o descomponer los contaminantes, los que se reconocen como métodos rentables y respetuosos con el medio ambiente. De hecho, los microorganismos del "motor del proceso de biorremediación "desarrollan su actividad en condiciones aeróbicas o anaeróbicas, que sin duda se usan para la reducción ambiental de los contaminantes. Sin embargo, para lograr resultados satisfactorios durante cualquier proceso de biorremediación, es considerado como un elemento esencial proporcionar las condiciones óptimas para los microorganismos. El compostaje es una de las tecnologías aplicada y usada para la remediación de suelos contaminados con contaminantes orgánicos como; hidrocarburos aromáticos policíclicos (PAHs), aunque todavía se necesita más investigación para aclarar el comportamiento de este proceso en la remediación de suelos contaminados con PAHs. Sin embargo, hasta hace poco se creía que en condiciones anaeróbicas los PAHs eran imposibles de ser degradados en estas condiciones. Por lo tanto, el presente estudio trató dos modos de biorremediación de suelos contaminados con PAHs mediante compostaje y mediante tratamiento en condiciones anaerobias, se utilizo el mismo suelo bajo estrictas condiciones metanogénicas. El objetivo del estudio era determinar los efectos de varios factores controlables que determinaran el comportamiento y la degradación de los procesos de PAHs y sus consecuencias.

El primer paso para seguir el proceso, en relación con la tecnología de compostaje, fue el estudio de varios co-sustratos orgánicos para seleccionar el más eficiente y capaz de mejorar la degradación de contaminantes (conocido como bioestimulación). Los co-sustratos seleccionados fueron: la fracción orgánica de residuos sólidos urbanos (FORM), el compost industrial de compostaje de FORM (COFMSW), el compost derivado de compostaje doméstico (HCOFMSW), lodos digeridos anaeróbicamente (ADS), lodos activados no digeridos (NDS) y lodos activados no digeridos y centrifugados (CNDS).

Considerando que el pireno (1g/kg materia seca) fue utilizado como representante de los PAH durante esta serie de experimentos. Los resultados obtenidos indican que el compost derivado de la FORM fue capaz de obtener la mayor tasa de degradación de los contaminantes (69%) en comparación a otros co-sustratos utilizados. Además, en este estudio se detectó un factor importante; el grado de estabilidad del compost que no se había investigado antes, pues la tasa de degradación varía en función del grado de estabilidad. En este contexto y a raíz de los resultados obtenidos mediante la experimentación, el compost que se obtuvo a partir de la FORM se utilizó como único co-sustrato orgánico en los tratamientos posteriores de compostaje y biorremediación. El estudio se llevó a cabo con la aplicación de la técnica del diseño experimental a través del diseño de composición central (CCD). Como metodología de esta técnica está la posibilidad de la visión general del efecto de diversos factores y su interacción. Los resultados obtenidos se ajustaron a un modelo polinomial de segundo orden.

En primer lugar, para determinar la respuesta del proceso en diferentes condiciones, en la biorremediación de suelos contaminados con pireno se estudio con tres factores diferentes; la concentración de pireno (0.1-2 g/kg, materia seca), el porcentaje de suelo en la mezcla de compost (1:0.5-1:2 w/w) y la estabilidad como índice respirométrico (0.78, 2.69 and 4.52 mg O_2 g^{-1} Organic Matter h^{-1}). Los resultados indicaron que el compost es más estable, y es capaz de aumentar la tasa de degradación en un plazo corto de tiempo, pero la prolongación del proceso de tratamiento fue capaz de estabilizar el compost, por lo tanto, se podrían lograr resultados favorables. Sin embargo, el aumento de la cantidad de suelo en el compost (>1:1) no tiene ningún efecto significativo sobre la remoción de contaminantes, y en cantidades más altas podría influir inversamente en el proceso. De hecho, este resultado se considera un punto clave en el diseño del proceso, debido a la viabilidad económica de la tecnología de compostaje basada en la cantidad de compost añadido al suelo contaminado. Además, la tasa de degradación es proporcional a la concentración de pireno, así que altas concentraciones aumentan la tasa de degradación. Sin embargo, concentraciones superiores a 1.3 g/kg comenzaron a mostrar un aumento en la toxicidad sobre la actividad microbiana y en concentraciones muy bajas (0.1g/kg) se detectan muy bajas velocidades de degradación, hecho que indica que la concentración del contaminante juega un papel importante en todo el proceso de degradación.

Por lo general en los lugares contaminados se encuentran varios derivados en forma de creosota, mientras que los PAH representan una parte importante de estos contaminantes. Una mezcla de hidrocarburos aromáticos policíclicos (Flourene, Phenanthrene, Anthracene,

Flouranthene, Pyrene and Benzo(a)anthracene) se utilizan para simular una muestra real de la creosota utilizada como contaminante durante el proceso de compostaje del suelo-bioremediación. Al mismo tiempo, habían sido evaluadas las concentraciónes de hidrocarburos aromáticos policíclicos (0.1-2 g/kg, materia seca) y la estabilidad del compost (0.37 - 4.55 mg O_2 g^{-1} Organic Matter h^{-1}). Los resultados confirmaron la especial importancia del grado de estabilidad del compost durante el proceso de biorremediación, más concretamente durante la primera etapa del proceso. El compost estable mejora los niveles de degradación en la mezcla suelo-compost y la tasa de degradación alcanzó un 92% después de 10 días, pero solo el 40% se degradó con el compuesto menos estable. Curiosamente los compuestos húmicos aumentan con el grado de estabilidad, el compuesto más estable fue aquel con más compuestos húmicos. En consecuencia, se asumió que la materia húmica facilita la deserción de los PAH haciéndoles más disponibles para la degradación de la actividad microbiana y, por tanto aumentando su velocidad de degradación. La concentración de PAH también fue importante durante el proceso, ya que la tasa de degradación se incrementó con el aumento de la concentración de los PAH. Por otra parte, todos los PAH participantes demostraron una considerable disminución en su concentración después del periodo de tratamiento, pero el pireno fue degradado a niveles más bajos en algunos tratamientos en comparación con otros PAH, este hecho pone de manifiesto la influencia de las propiedades y el comportamiento de contaminantes microbianos durante este proceso.

Los resultados anteriores fueron obtenidos a través de todos los bioestimulos de los microorganismos dentro de la mezcla de tratamiento mediante la introducción de co-sustratos orgánicos. Además, en un estudio comparativo para la investigación del impacto de la bioaumentación mediante la introducción de microorganismos exógenos y compararla con bioestimulación, los hongos de podredumbre blancos (Trametes versicolor ATCC 42530) fueron utilizados para tratar el suelo contaminado con porcentajes de PAH similares a la muestra real de creosota. El proceso se evaluó a partir del análisis del índice respirométrico dinámico (DRI), la respiración acumulada durante 5 días (AT_5), la actividad enzimática y la biomasa fúngica. Se demostró que el hongo ligninolítico *Trametes versicolor* no aumentaba la degradación de los PAH. Por el contrario, el uso de compost de FORM en la bioestimulación fue capaz de aumentar la tasa de degradación, un 89% en un periodo de 30 días, en comparación con el 29.5% que se logró para el suelo sin ningún tipo de co-sustrato. Además, los resultados obtenidos mostraron que el compuesto estable de

FORM tiene un gran potencial para mejorar la degradación de los PAH en comparación con los co-sustratos no estables tales como substrato ligninocelulósico (comida de conejo).

En la segunda parte del estudio, el tratamiento anaerobio de los PAH del suelo contaminado se ha investigado bajo estrictas condiciones metanogénicas similares a los tratamientos de compostaje, el efecto de la concentración de hidrocarburos aromáticos policíclicos y la estabilidad del compost como co-sustrato orgánico también fueron sistemáticamente evaluados bajo condiciones mesofílicas (37˚C). La degradación de los PAH osciló ente 31%-90% valores que se alcanzaron después de 50 días de incubación, lo que demuestran la efectividad del tratamiento biológico. Sin embargo, el proceso de degradación de los PAH y la producción de biogas, se encuentran influenciados por los dos factores. Por ejemplo, la tasa de degradación de los PAH tiene una relación positiva con el aumento de la concentración y el grado de estabilidad del compost. Curiosamente, en algunos casos si no se adiciona compost se mejora en gran medida la degradación de los PAH, hecho que indica que los PAH podrían ser degradados directamente por el inoculo. Este último estudio se realizó sin la utilización de co-sustratos o nutrientes, excepto la tierra contaminada y el inóculo, los resultados dependieron de la concentración de hidrocarburos aromáticos policíclicos, de la temperatura y de la relación proporción suelo/inóculo. En ambas temperaturas de operación, y a pesar de la inhibición del biogás, se lograron ciertas tasas de degradación durante los primeros 30 días de incubación, y se ha encontrado que esta es directamente proporcional a la concentración de PAH. Sin embargo, con la prolongación del experimento hasta 50 días y por razones poco claras, los resultados obtenidos muestran un incremento de la concentración de PAH, este hecho indica la bioformación de PAH en condiciones deficientes de oxígeno. Por lo tanto, en los próximos estudios se debería aclarar las razones de este comportamiento.

Cabe destacar que parte de los resultados presentados en esta tesis se han obtenido en colaboración con el grupo de investigación del Laboratorio de Biotecnología Ambiental en la Academia de Ciencias de la República Checa. Además, los resultados presentados en la actual tesis representan el comienzo de una nueva línea de investigación para los grupos de investigación; Compostaje de Residuos Sólidos Orgánicos (2009 SGR 95) y Biodegradación de Contaminantes Industriales y Valorización de Residuos (2009 SGR 656) de la Universidad Autònoma de Barcelona.

Abbreviations

AFP: Air Filled Porosity.

ASE: Accelerated Solvent Extractor.

C:N: Carbon to Nitrogen ratio.

CCD: Central Composite Design.

DM: Dry Matter.

DRI: Dynamic Respiration Index.

EC: Electrical Conductivity.

GC/MS: Gas Chromatography-Mass Spectrometry.

GC: Gas Chromatography.

HPLC: High-Performance Liquid Chromatography.

MC: Moisture Content.

MSW: Municipal Solid Waste.

OFMSW: Organic Fraction of Municipal Solid Wastes.

OM: Organic Matter.

PAHs: Polycyclic Aromatic Hydrocarbons.

PLFA: Phospholipid Fatty Acids.

SFE: Supercritical Fluid Extraction.

SRI: Static Respiration Index.

TKN: Total Kjeldahl Nitrogen.

TOC: Total Organic Carbon.

TS: Total Solids.

USEPA: United States Environmental Protection Agency.

WHO: World Health Organization.

Chapter 1

General Introduction

"Unsustainable development results in the production of brownfields and derelict land"

(Simpson, 1996)

Background

The industrial revolution after the World War II has extremely changed the life norms. Modern lifestyles are largely depending on the benefits offered by the different industries, where "specifically", chemical industries and their derivatives are extensively contributed in the various life-branches. However, when the relation between modern lifestyles "civilization" and the environment is to be evaluated, disproportionally growth is to be appeared accompanied with great tension on the different environment components. Anthropogenic contaminations caused by human activities throughout the worldwide industries coupled with other natural contamination feed such tension and are seriously threatening the environment.

Soil is a major component of all terrestrial ecosystems, and is the most basic for all natural resources as it supports all the terrestrial life. However, soil resources of the world are finite, essentially non-renewable, unequally distributed in different ecoregions. Despite inherent resilience, soil is prone to degradation or decline in its quality due to misuse and mismanagement with agricultural uses, contamination with industrial uses, and pollution with disposal of urban wastes. Regarding to contamination caused by industrial activities, soil contamination is a typical side effect of such activities; consequently, contaminated land is a global concern and can be considered a major barrier to sustainable development. The rate of contamination is increasing, where still humble efforts are devoted to deal with

this problem. It is estimated that more than 1400000 contaminated sites in the European Union countries, where remediation of these sites is estimated to be €85 billion (ESB, 1998). However, in the United States, it has been estimated that contaminated sites treatment cost is about $ 1.7 trillion over the next 30 years (Mark et al., 1997). These values show the magnitude of the problem and the need for applying effective remediation technologies. The problem has increased with the increasing public awareness and concern about the presence of chemicals in the environment especially because many of these chemicals are toxic or carcinogenic to the humans and environment in the same time. Soil contamination has been identified as one of the major threats to soil function (support life systems) in Europe by the Communication from the European Commission "Towards a Thematic Strategy for soil Protection" (EC, 2002, 2006).

1.1 The need for soil remediation

Although awareness of prevention and sustainable development practices continues to grow, industrialization of developing countries as well as current solid waste disposal practices ensure that contaminated sites remain a continual environmental problem. Consequently, contaminated land is a global concern and can be considered a major barrier to sustainable development. Accordingly, there is a critical and urgent need to develop and implement effective remediation technologies to reduce the threats caused by such contaminants.

An adequate approach to deal with land contamination should have:

1. Comprehensive remedial plan for lands that are already contaminated.
2. Comprehensive strategy to prevent or minimize future contamination.

1.2 Remediation methods of contaminated soil

Different methods can be applied for soil remediation, where every method has its own operation and favourable conditions. However, in some case, the available circumstances require to apply a method although it is not the adequate one. The different remediation methods can be divided in seven generalised categories:

1. **Biological methods:** those which depend on the microbial activity to completely mineralize or transform the contaminant to a less toxic form. Generally, the biological

methods are recognized as the primary dissipation mechanism for most organic pollutants in the soil environment (Dua et al., 2002; Mohan et al., 2006). The process has the ability to destroy a wide range of organic compounds in a reasonable time. Nevertheless, in some case, the process end-point can be uncertain and difficult to gauge, and the treatment itself may be slow and not all contaminants are conducive to such treatment means. Factors like concentration, bioavailability, enzymatic activity, capability of microbial metabolism, etc., influence the process to great extent. In general, biological methods are normally considered as an attractive, environmental friend and cost-effective (Antizar-Ladislao et al., 2004; Hamdi et al., 2007).

2. **Chemical methods:** under chemical reaction, the contaminants are destroyed, fixed or neutralised; therefore, more recalcitrant organic contaminants can be easily destroyed or converted to other less harmful ones. The problem is when the contaminants can not completely destroy. In this case, the reagent itself may cause damage to the soil, in the same time an additional secondary treatment is needed (Evans and Furlong, 2003; Gan et al., 2009).

3. **Thermal methods:** depend on heat to destroy the contaminants through incineration, gasification and pyrolysis. Although the contaminants are most effectively destroyed, high energy cost is needed and the formation of other pollutants in forms more toxic one is probable. Other drawback is the probability to destroy the soil structure itself specifically the soil organic matter (USEPA, 1993b).

4. **Physical methods:** which depend on the removal of the contaminated soil to the landfill or the containment of the contaminated site. In fact, these methods are only moving the problem to another place or time (Evans and Furlong, 2003).

5. **Solidification/vitrification method:** solidification is the encapsulating of the contaminant within a monolithic solid of high structural integrity, with or without associated chemical fixation, when it is then termed “stabilization”. Vitrification uses high temperatures to fuse contaminated material. The advantage is that the contaminated material is rendered and become unavailable to the environment (Khan et al., 2004).

6. **Phytoremediation:** is an emerging remediation technology that uses plants to remove contaminants from soil and water. This technology has shown its potential for accumulating, immobilizing, and transformation a low level of contaminants. However, it has some limitations like long duration of time, potential contamination of the vegetation and food chain, and difficulty in establishing and maintaining vegetation at sites with high toxic levels (Gan et al., 2009).

7. **Integrated remediation methods:** when the applied remediation method can not accomplish the degradation of the contaminants by itself due to some limitations or drawbacks under certain conditions. Another method can be performed prior or posterior to overcome these drawbacks and to achieve better results regarding the contaminant removal. Various combinations of physical, chemical and biological treatments can be employed in conjunction with one another to treat PAH contaminants in soils. For example, a combined chemical (Fenton-like and ozonation) and biological treatment for the remediation of shale oil and transformer oil contaminated soil was applied (Goi et al., 2006).

Among the different remediation methods, biological treatments are often regarded as cost-effective as and environmentally friendlier than other treatments. In this concern, the present work is concerned to generally discuss this type of treatment and specifically the application of composting technology as aerobic treatment and on the other side strict methanogenic anaerobic digestion is to be applied as another biological treatment option.

1.3 Remediation method: Selection criteria

For treatment system design, treatment processes should be selected first by screening the alternatives. Many factors should be considered for the selection of proper treatment processes. Common selection criteria are implementability, effectiveness, cost, and regulatory consideration. In other words, an optimum process would be the one that is implementable, effective in removal of contaminants, cost efficient, and in compliance with the regulatory requirements (Kuo, 1999).

The important questions for full-scale bioremediation application are:

- What is the expected degradation/removal that can be achieved through the selected technology (bioavailability, microbial activity, etc.)?

- What is the fate of the contaminant (mineralization, biotransformation, evaporation, etc.)?
- How much time is needed to reach the set goal?
- What are the estimated costs? Is it a feasible choice?
- What is the size of any side-effects regarding the environment and humans (environmental impact assessment)?

1.4 Bioremediation strategies: *In-situ* versus *ex-situ*

Nowadays, several bioremediation techniques have been applied for contaminated soil treatment. For instance, these remediation techniques can be carried out either on the site or out of it. The selection of the most adequate technique presumably depends on the previously mentioned selection criteria (section 1.3).

1.4.1 *In-situ* bioremediation

Is the treatment of contaminant without removal/excavation of the contaminated soil (where it is). *In situ* methods are suited to instances where the contamination is widespread throughout, and often at some depth within, a site, and of low to medium concentration (Alexander, 1999; Evans and Furlong, 2003). This type of remediation is considered less expensive since it does not include the excavation fees. Moreover, dust release or volatilization of the contaminants could be avoided; however, it is characterized by its slow action especially. Some of *in-situ* bioremediation methods include:

Bioventing: is an *in-situ* remediation technology that uses indigenous microorganisms to biodegrade organic constituents adsorbed to soils in the unsaturated zone. Soils in the capillary fringe and the saturated zone are not affected. In bioventing, the activity of the indigenous bacteria is enhanced by inducing air (or oxygen) flow into the unsaturated zone (using extraction or injection wells) and, if necessary, nutrients can also be added to the soil to stimulate the growth and metabolism of the indigenous species. The process is similar to soil vapour extraction (SVE). However, while SVE removes constituents primarily through volatilization, bioventing systems promote biodegradation of constituents and minimize volatilization (generally by using lower air flow rates than for SVE) (Norris et al., 1994).

Bioaugmentation: This process involves the introduction of exogenous species or enzymes into a contaminated soil to stimulate the degradation of organic pollutants present in the

soil. The introduced culture from outside are assumed to have valuable specific degradation capacities or serving as donors of catabolic genes that accelerate the degradation rate within short period. The introduced microorganisms must remain viable and should compete with the microorganisms already existing in the system (Teng et al., 2010; Top et al., 2002). A number of inoculants which specifically degrade various xenobiotic compounds are commercially available.

Biostimulation: Biostimulation involves the introduction of nutrients or substrates such as fertilizers or different organic co-substrates, to stimulate the growth and metabolism of the indigenous species performing the biodegradation of pollutants. Substrates containing nitrogen and phosphorous are the most commonly used stimulants due to their electron acceptor capabilities. Also it may involve the addition of electron acceptors or electron donors to increase the numbers or stimulate the activity of indigenous biodegradative microorganisms (Sayara et al., 2009; Tejada et al., 2008; Widada et al., 2002)

1.4.2 *Ex-situ* Bioremediation

In this remediation technique, the contaminated soil is removed from its origin to another site for treatment. This description applied whether the material is taken to another venue, or simply to another part of the site (in site). These treatments could be better controlled and monitored; as a result, normally less time is required compared with *in-situ* treatments. Nevertheless, excavation and transport costs make it less cost-effective. These treatments include:

Bioslurry system (bioreactor): is accomplished by combining the excavated soil with water and other additives. The soil is treated in a controlled bioreactor where the slurry is mixed to keep the solids suspended and microorganisms in contact with the contaminant, where normally the biodegradation occurs at a rapid rate (Cassidy and Hudak, 2002; Khan et al., 2004).

Landfarming: involves the excavation of the contaminated soil and spreading it on thin layers (no more than 1.5m). Biodegradation of pollutants is stimulated aerobically by aeration and/or the addition of nutrients, minerals and water to promote the growth of the indigenous species (Evans and Furlong, 2003; Khan et al., 2004).

Biopiles: also known as bioheaps, compost cells or biocells, used for the remediation of excavated contaminated soil. This technology involves the piling of contaminated soil into piles or heaps and the stimulation of aerobic microbial activity either through aeration or the addition of nutrients, minerals or moisture. A typical height of biopiles can be up to 6m. Biopiles are similar to landfarming due to the fact that this technology also uses oxygen as a way to stimulate bacterial growth. But the later is aerated through tilling or blowing, where biopiles are aerated by forcing air to move by injection through perforated piping placed throughout the pile (USEPA, 2001; Khan et al., 2004).

Composting: composting bioremediation relies on the mixing of the contaminated soil with another organic amendment, wherein as the organic amendment matures, the pollutants are degraded by the active microorganisms within the matrix. In addition, the organic amendment provides nutrients for the soil indigenous microflora which along with the introduced microorganisms through the amendment will degrade the target contaminants (Antizar-Ladislao et al., 2004, 2006; Sayara et al., 2009; Semple et al., 2001). In fact, composting treatment holds the potential to serve as a low-cost method of treating hazardous waste with minimal environmental controversy; however, information is lacking regarding the treatability of various toxicants and optimum conditions for treatment. An illustration of the composting process is presented in Figure 1.1 where windrows were used.

Figure 1.1: Composting process (windrow) of contaminated soil.

1.5 Bioremediation of polycyclic aromatic hydrocarbons (PAHs)

1.5.1 PAHs: Properties and sources

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings (benzene ring) on a linear or angular layout. They are relatively neutral to stable with relatively low solubility in water, but are highly lipophilic, where most of them have low vapour pressure. The chemical properties of individual PAHs are dependent in part upon their molecular size (i.e., their number of aromatic rings) as well as their molecular topology (i.e., their pattern of aromatic linkage). An increase in the size and angularity of a PAH molecular generally results in an associated increase in their hydrophobicity and electrochemical stability, which contributes to its persistence (Kanaly and Harayama, 2000; Loick et al., 2009). In fact, PAHs are known to exhibit actually toxic effects or posses mutagenic, teratogenic, or carcinogenic properties; as a consequence, some are classified as priority pollutants by the US Environmental Protection Agency (USEPA) (Kanaly and Harayama, 2000; Loick et al., 2009). Figure 1.2 shows some PAHs with different molecular topologies, where Table 1.1 shows the 16-PAHs priority pollutants according to the USEPA.

PAHs are formed and introduced to environment either naturally or anthropogenically. Naturally, PAHs are mainly formed as a result of pyrolytic process, especially the incomplete combustion of organic materials during different human activities, such as processing of coal and crude oil, combustion of natural gas, forest fires, combustion of refuse, vehicle traffic, as well as in natural process such as carbonization and volcanic eruption (WHO Regional Office for Europe, 2000). However, these processes are believed to have relatively small contribution of PAHs in the terrestrial ecosystem. On the other side, anthropogenic sources which are notably increased with the world industrial extension especially during the 20th century. PAHs are introduced into the environment through accidental spillage, misguided disposal of petroleum and creosote wastes, and intensive combustion of fossil fuels, coal, wood preserving products and leaking from underground tanks, etc. (Dyke et al., 2003).

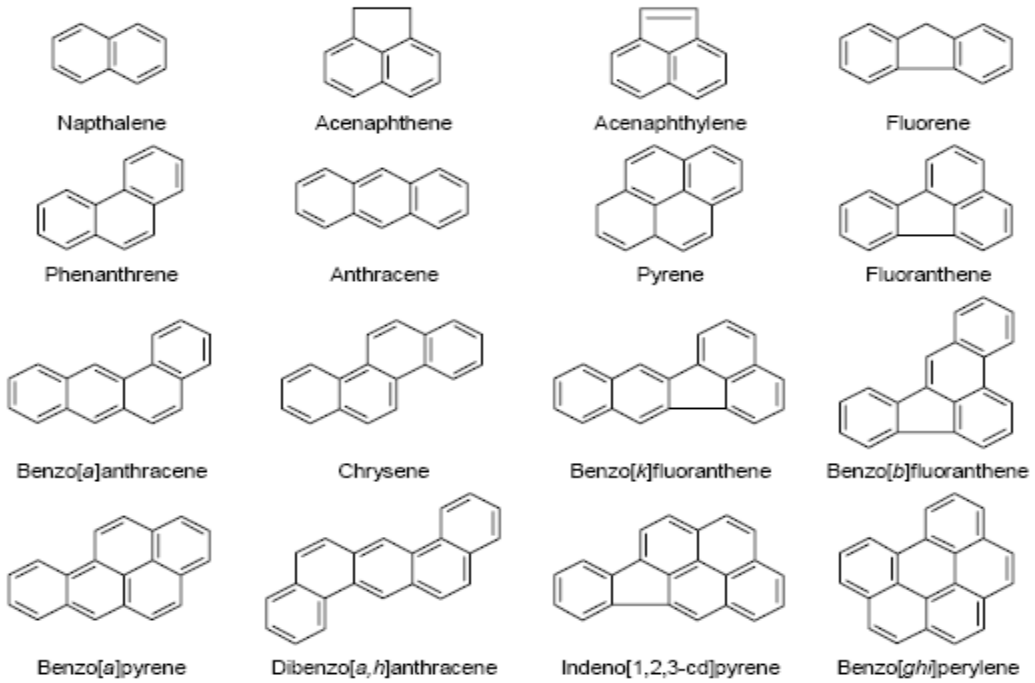

Figure 1.2: Structures of the 16 USEPA-PAHs.

Table 1.1: Selected properties of the 16 USEPA-PAHs (Mackay et al., 1992).

PAH	Number of rings	Molecular weight	Aqueous solubility (mg/l)	Vapor pressure. (Pa	Log K_{ow}
Naphthalene	2	128	31	$1.0*10^{2}$	3.37
Acenaphthylene	3	152	16	$9.0*10^{-1}$	4.00
Acenaphthene	3	154	3.8	$3.0*10^{-1}$	3.92
Flourene	3	166	1.9	$9.0*10^{-2}$	4.18
Phenanthrene	3	178	1.1	$2.0*10^{-2}$	4.57
Anthracene	3	178	0.045	$1.0*10^{-3}$	4.54
Pyrene	4	202	0.13	$6.0*10^{-4}$	5.18
Flouranthene	4	202	0.26	$1.2*10^{-3}$	5.22
Benzo(*a*)anthracene	4	228	0.011	$2.8*10^{-5}$	5.91
Chrysene	4	228	0.006	$5.7*10^{-7}$	5.91
Benzo(*b*)flouranthene	5	252	0.0015	-	5.80
Benzo(*k*)flouranthene	5	252	0.0008	$5.2*10^{-8}$	6.00
Benzo(*a*)pyrene	5	252	0.0038	$7.0*10^{-7}$	5.91
Dibenzo(*a,h*)anthracene	5	278	0.0006	$3.7*10^{-10}$	6.75
Indeno(*1,2,3-cd*)pyrene	6	276	0.00019	-	6.50
Benzo(*ghi*)perylene	6	276	0.00026	$1.4*10^{-8}$	6.50

1.5.2 Soil contamination with PAHs and European management

Contaminated land can implicitly define as "the presence of substances in the land which when present in sufficient quantity or concentration are likely to cause harm to the environment or human health". In a wider sense, however, contamination may be viewed as a condition whereby soil or water contains above background concentrations of substances which are not normally there (Evans and Furlong, 2003). Among the different contaminants, PAHs which are found in all surface soils (Kanaly and Harayama, 2000). However, overall estimates from the European Environment Agency (EEA) identify metals and mineral oil as the main soil contaminants in Europe. From almost 90% of the European sites for which information on contaminants is available, PAHs represents about 13% of these contaminants (EEA, 2007). Massive soil contamination with PAHs is recognized from extensive industrial activities using petroleum and coal, petroleum spills, wastes disposal (point sources), etc. whereas the contamination rate is decreased in regions with less industrial activities. Depending on the source of contamination, soils can contain PAH concentration ranging between 1 $\mu g\ kg^{-1}$ and 300 $g\ kg^{-1}$ total PAHs (Kanaly and Harayama, 2000; Loick et al., 2009; Bamforth and Singleton, 2005). As guidance, soil assessment according to their PAHs content is shown in Table 1.2. Unfortunately, in spite of the threats associated with soil contamination as shown in Figure 1.3, soil protection has not, to date, been subject to a specific legislative instrument at European Union (EU) level.

Figure 1.3: Soil contamination with different types of contaminants.

References to soil protection can be found scattered throughout the European Community regulatory structure, establishing a number of instruments and measures that have a direct or indirect impact on the quality of soil (Rodrigues et al., 2009). For instance, the Waste Framework Directive (2006/12/EC) and the implementation of the Water Framework Directive (2000/60/EC) were indirectly/directly led to the recovery of contaminated areas and to the mitigation of certain soil contamination problem (Rodrigues et al., 2009).

Table 1.2: Standard limiting PAH content (μg kg^{-1}) in the soil surface layer (Malawska and Wilkoirski, 2001)

Total PAHs content	*Pollution class*	*Soil assessment*
<200	0	Unpolluted (natural content)
200-600	I	Unpolluted (increased content)
600-1000	II	Slightly polluted
1000-5000	III	Polluted
5000-10000	IV	Heavily polluted
>10000	V	Very heavily polluted

1.5.3 Health and environmental concern

The available data from animal studies indicate that several PAHs may induce a number of adverse effects. Carcinogens potential that can produce tumours has been observed in some organisms even at small doses. A number of other adverse effects such as immunotoxicity, genotoxicity, reproductive toxicity have been documented. However, the critical endpoint for the health risks evaluation is the well-documented carcinogenicity of several PAHs (IARC, 1983; WHO Regional Office for Europe, 2000). Human and non-human mammals can absorb PAHs by inhalation, dermal contact or ingestion. As a results environment contamination with this type of contaminants is considered a real threat for the several life aspects, and leads to the feed for a comprehensive strategy for environmental remedial action.

1.6 Bioremediation

Bioremediation, which will be broadly defined as a managed or spontaneous process in which biological catalysis acts on pollutants, thereby remedying or eliminating environmental contamination present in water, wastewater, sludge, soil, aquifer material, or gas streams holds great potential as a practical and cost-effective approach to solve a wide variety of contamination problems. Therefore, it is expected that bioremediation will play an increasingly important role in the cleanup of soils, sediments, and groundwater contaminated with hazardous organic chemicals (Alvares and Illman, 2006). For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. As a result of aerobic conditions, the degradation of the contaminant by microbial activity will produce carbon dioxide (CO_2) and water (H_2O) as shown in Figure 1.4, which is considered environmentally acceptable compared with the end products of other remediation methods which produce unacceptable products which may need second step of treatment.

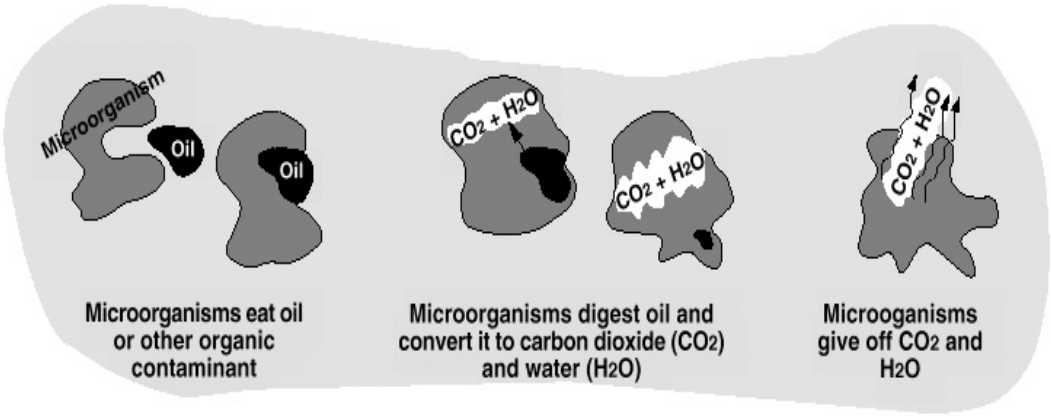

Figure 1.4: Microbial degradation of contaminants.

1.6.1 Bioremediation controlling factors

For any conceivable organic compound, there exists a microorganism that can degrade it under the right condition (Gale, 1952). If not, evolution and adaptation would produce such strain (Alexander, 1965; Alvares and Illman, 2006). This hypothesis can not be proved wrong, because failure to degrade a contaminant can be attributed to the researcher's failure to use the right strain under the right conditions, in other words "*the absence of evidence is not in itself evidence of absence*" (Alvares and Illman, 2006). Accordingly, the susceptibility of bioremediation process depends largely on different controlling factors. Indeed, as this process fundamentally depends on the microbial activity, the existence of a microbial population that is capable to synthesize the enzymes needed to degrade the target contaminants is considered the first and primary requirement (key component). Afterwards, and regardless of the bioremediation technology and the presence of the right microorganisms, PAHs biodegradability is usually influenced by several factors which can be divided into two broad groups: those which related to the contaminant itself, i.e., PAHs physicochemical properties and the others are influenced by environmental conditions. Regarding the physicochemical properties, PAHs water solubility is an important characteristic during the bioremediation process. It is believed that microorganisms can only degrade the PAHs fraction which is dissolved in the treatment matrix solution, and typically of a low-medium toxicity range. This dissolved fraction of PAHs is known as the bioavialable fraction. For instance, PAHs water solubility decreases with the increase in number of fused benzene-rings, and with angularity. Consequently, high molecular weight PAHs are less degradable compared with low molecular weight PAHs. The recalcitrance of PAHs to microbial degradation generally increase with their molecular weight and their octanol-water partitioning coefficient (log K_{ow}, Table 1.1), where many of high-molecular

weight PAHs are only degraded with difficulty or not at all, due to their low water solubility, high resonance energy and toxicity (Antizar-Ladislao et al., 2004; Cerniglia, 1992). The other physicochemical property is the volatility, especially during elevated temperatures that occur in composting (>50˚C) which facilitate the PAHs volatilization (Antizar-Ladislao et al., 2004). In fact, the volatilization of PAHs decreases with the increase of fused rings. In general, the number of aromatic rings, the structure of the PAHs, and the resonance energy might also affect the degradation rate of PAHs (Antizar-Ladislao et al., 2004; Kästner and Mahro, 1996). Environmental conditions like temperature, pH, moisture, nutrients, etc. should be within the favourable ranges that enhance the microbial activity. These environmental conditions can be summarized as shown in Table 1.3.

Table 1.3: Optimum environmental condition for microbial activity during bioremediation (Vidali, 2001).

Parameter	Optimum value
Moisture content	40-60 %
pH	6.5-8
Oxygen content	10-18 %
Nutrient content	C:N:P=100:10:1
Temperature (˚C)	20-30
Contaminants- Hydrocarbons	5-10 % of dry weight of soil
Heavy metals	700 ppm
Soil type	Low clay or silt content

1.7 Strategies to enhance bioremediation efficiency

When natural degradation processes can not proceed in contaminated environments due to variable conditions, the need for human intervention is necessary to stimulate biodegradation.

1.7.1 Biostimulation approach

Biostimulation is considered as an important strategy to enhance the bioremediation process by providing the degraders with the favourable environment under which they can effectively degrade the target contaminant. It involves the addition of nutrients (nitrogen, phosphorus and trace minerals), pH adjustment, providing proper moisture content, aeration, etc. to stimulate the indigenous microbial activity (Kästner and Mahro, 1996).

Moreover, temperature plays a major role in controlling the nature and extent of microbial hydrocarbon metabolism. Temperature increase leads to increase the diffusion rate of the organic compounds by decreasing their viscosity which leads to increase the bioavialability by increasing solubility, diffusion and reaction rate (Mohan et al., 2006; Northcott and Jones, 2001). However, increasing the solubility and volatilization due to elevated temperatures may enhance their toxicity which limits or inhibits the microbial activity (Leahy and Colwell, 1990). On the other side, several studies has demonstrated that mesophilic temperatures (<45˚C) are more favourable for the degradation of wide arrays of hydrocarbon contaminants compared with less degradation rates which were achieved within thermophilic ranges. In fact, temperature effect of the degradation rate has been contrasted among the available literature studies, but the majority is agreed that low temperatures are more favourable for such process (Baraniecki et al., 2002; Eriksson et al., 2001).

1.7.2 Bioaugmentation approach

Another strategy employed to enhance the degradation rate is bioaugmentation. Mainly it relies on introducing of single strains or consortia of microorganisms with desired catalytic capabilities. It involves the application of indigenous or allochthonous wide type or genetically modified microorganisms to polluted sites to accelerate the removal of undesired compounds (Dua et al., 2002; Mrozik and Piotrowska-Seget, 2009; Vogel, 1996). It is usually applied to tackle low biomass availability and the lack of the required contaminant degraders during the bioremediation process. In this case when the indigenous microorganisms are present in low number or even absent, the addition of such exogenous microorganisms may speed up the degradation process (Mohan et al., 2006; Van et al., 1998). For instance, the results of this strategy are not always favourable as the introduced microorganisms may encounter in different conditions that inhibit their activity. Normally the competition between the indigenous and exogenous microorganisms for the limited carbon sources as well as antagonistic interactions and predation by protozoa and bacteriophages. Also, native species diversity may act as a resistance barrier to the invasion of non-native species (Kennedy et al., 2002; Mrozik and Piotrowska-Seget, 2009).

1.7.3 Surfactants

Bioavialability of some contaminants like high molecular weight-PAHs is normally recognized as limiting factor during the bioremediation process because of their low water-solubility and strong sorption/sequestration in micropores or organic matter (Johnsen et al., 2005; Tang et al., 2005). To overcome this problem, surfactants like TritonX-102, Tween-80, Genapol X150, etc. can be added to improve the contaminants transport to degraders as they increase their water solubility by decreasing the interfacial tension between water and hydrophobic pollutants.

1.7.4 Co-metabolism

It can be defined as a non-specific enzymatic reaction, with a substrate competing with structurally similar primary substrate for the enzymes active site (Mohan et al., 2006). It has been used to enhance the degradation of many recalcitrant pollutants by introducing analogues compounds to the target pollutant and positive results were recorded under such conditions (Igwo-Ezikpe et al., 2010).

1.8 Advantages and disadvantages of the bioremediation process

In comparison with the other techniques that can be used for soil treatment, bioremediation has the following advantages:

1. Low cost compared to other remediation technologies.
2. Under right and controlled conditions, complete degradation of organic compounds to nontoxic by-products can be achieved.
3. In most technologies, minimum mechanical equipments are needed.
4. It can be implemented as in-situ or ex-situ process. In-situ bioremediation is safer since it does not require excavation of contaminated soils. Also, it does not disturb the natural surroundings of the site.
5. Environmentally sound with public acceptance. The possibility to treat large areas with low impact and disturbance.
6. Great potential to degrade wide arrays of organic compounds.

On the other hand, like any technology, bioremediation has some disadvantages or limitations which are often referred to the metabolic and physiological requirements of bacteria. These disadvantages can be summarized as:

1. Contaminants partial degradation to metabolites that are toxic (sometimes more toxic than the parent compound), and/or potentially more highly mobile compounds/or their accumulation in the environment.
2. Less tolerant to toxins where contaminants with high concentrations normally inhibit microorganisms. Additionally, a variation in the environmental conditions retards or inhibits the process. therefore, process successful and efficiency may vary considerably from one site to another,
3. In ex-situ bioremediation, the volatilization of organic compound could be difficult to be perfectly controlled.
4. Generally requires longer treatment time compared with other remediation technologies especially for in situ methods.

1.9 Composting technology

The traditional use of landfills for disposal of wastes becomes wasteful because both space and potential resources. Each landfill has a limited lifetime with relation to the available space, this means that another landfill must be constructed to absorb the generated wastes, or another alternative should be used to deal with these wastes. Accordingly, various technologies have been emerged and applied. Among the applied technologies to recover organic solid wastes like municipal solid waste (MSW) and sewage sludge derived from waste water treatment plants; Composting is recognized as efficient and cost-effective process. In the last decades, the application of the composting process has been extended to include other wastes like hydrocarbons-contaminated soils which represent a major part of this research.

1.9.1 Composting process

Composting is an aerobic process, which requires oxygen to stabilize the organic wastes, optimal moisture content and porosity, and their common control variables are temperature, oxygen and moisture (Haug, 1993). Thus, composting-bioremediation is the application of the composting technology for wastes and contaminants treatments.

1.9.2 Fundamentals of composting

1.9.2.1 Composting stages

When composting matrix is to be prepared, the different influenced parameters should be adjusted within the optimum values in attempt to facilitate and enhance the microorganisms duty, thereby, optimum results within a reasonable time can be achieved. Regarding the microbial activity during the composting process, two main stages are considered:

1. **Decomposition stage:** As the microbial population begins to degrade the most readily degradable material and the population increase, the heat generated by the microbial activity accumulates within the pile and the temperature continues to increase steadily passing form the mesophilic ranges (25-45˚C) to the thermophilic ones (more than 45 ˚C). Simultaneously, high rate of aeration is needed to ensure and maintain the microbial activity during this stage.

2. **Curing stage:** which take place at lower temperature. However, still many naturally occurring reactions take place during this stage although the microbial activity is relatively low compared with the previous stage as the nutrients pool has been depleted. One of the characteristics that take place in this stage is the material humification that is increasing at various stages of composting process (Hsu and Lo, 1999), which gives an interesting value to the produced compost especially for soil bioremediation as it will be explored in this work.

1.9.2.2 Composting variables

During the composting process, raw material of the compost mix is degraded by microorganisms to synthesize new cellular material and to obtain the energy for these catabolic processes. For instance, several chemical transformations take place which can be determined through several parameters including:

a) Temperature: temperature evolution during the composting process gives provision of the aerobic conditions as considerable amount of heat is released by aerobic decomposition of the organic material. Initially, the composting process is marked either by psychrophilic or mesophilic temperatures depending on the ambient and composting mixture temperature.

At the beginning of the process, a short lag period is typical for the development of the microbial population before the temperature begins to increase. Afterwards, the temperature rises rapidly to reach the thermophilic ranges during the decomposition of the easily degradable fraction, and then it begins to decrease gradually to reach ambient temperature during the curing phase. For instance, proper temperature is an important factor that should be followed to evaluate the process behaviour. Typical temperature evolution during the composting process is presented in figure 1.5.

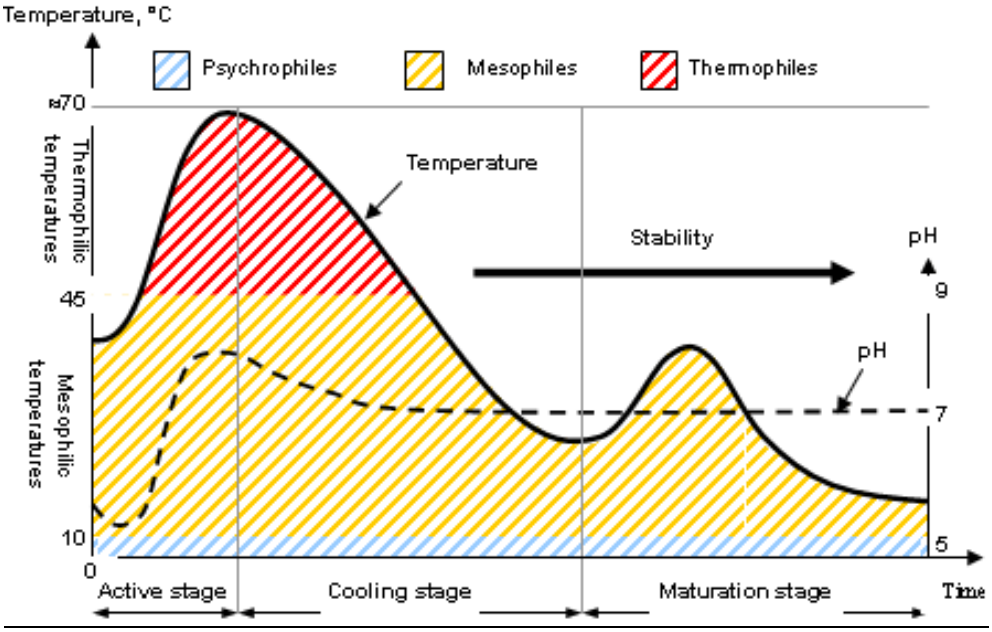

Figure 1.5: Typical temperature profile of a composing process.

b) Oxygen: as an aerobic process, maintaining adequate percentage of oxygen during the composting process is essential for microbial activity; otherwise, the process or parts of the treated material will turn to anaerobic condition. An oxygen concentration between 10-15% is considered adequate, although concentrations as low as 5% can be sufficient. Depending on the material activity, aeration rates and frequencies should be adjusted to coincide with different stages. In this concern, oxygen consumption can be determined through respiration techniques which give an indication about the material stability degree and the microbial activity within the composted materials (Barrena et al., 2009, Adani et al., 2003). Accordingly, materials with respiration index between 0.5-1.5 mg O_2 g^{-1} OM h^{-1} are considered stable, while values higher than 1.5 mg O_2 g^{-1} OM h^{-1} correspond to unstable materials.

c) Moisture: Microorganisms can only use the organic molecules for metabolism and new cells production if they are dissolved in water. Consequently, optimum moisture content should be provided during the composting process in order to maintain the microbial activity. Moisture content between 40-60% is recommended in this case (Davis and Cornwell, 1998; Haug, 1993). It should be noted that low moisture leads to slow the process, meanwhile; high moisture can reduce or even stop the oxygen transfer.

d) pH: Normally, microbial activity is limited with a narrow optimal range of pH and it could be inhibited in more acidic or alkaline conditions. Therefore, pH values between 6-7.5 are considered suitable for such biological process. pH variation during the composting process is illustrated in Figure 1.5, where an normally an initial decrease occurs during the decomposition phase as a result of acids accumulation. Afterwards, these acids are degraded with the microbial evolution and lead to increase the pH again. However, if pH rises to 9, the nitrogen is converted to ammonia and becomes unavailable to microorganism as a nutrient, also it will cause bad odour in the composting plant (Pagans et al., 2006).

e) Carbon to Nitrogen ratio (C:N): during the composting process, microbial activity can be maintained or enhanced by the availability of essential nutrients like carbon, nitrogen, phosphorous and potassium. However, in the composting process, carbon and nitrogen are usually considered as limiting factors for efficient decomposition (Richard, 1992). An initial carbon to nitrogen ration of 15 to 30 is recommended as an optimum value for composting (Haug, 1993). However, it is important to point out that the bioavailability of nitrogen and carbon should be considered in the calculation of C:N ratio. In fact, while the nitrogen presents in the majority of waste is mainly found in biodegradable form, carbon can be presented in non-biodegradable form (Sánchez, 2007; Zhang et al., 2004). The proportion of readily, moderately and low biodegradable organic matter will influence the process kinetics.

f) Particle size and air filled porosity: Small particle size promotes the microbial activity as more surface area can be found. Nevertheless, having small particle size is inversely proportion with required porosity needed for air flow due to compaction. In this regard, air filled porosity (AFP) which is the ratio between air volume to the total volume of the composted material, should be adjusted to be within 25-30% (Agnew and Leonard, 2003; Haug, 1993; Ruggieri et al., 2009).

It is worth to remark that several composting methods can be applied, and the selection of the method is dependent on the capital cost, labour cost, time, the availability of land, etc. these methods include:

1) Passive composting method in which aeration is accomplished through the passive movement of the air through the pile or the windrow.
2) Turned composting methods, are aerated by passive aeration through regularly turning to maintain the proper porosity, mixing the material, releasing heat, water vapour and gases.
3) Static aerated pile composting in which mechanical aeration is applied through air ducts, and aeration is achieved by blowing or drawing air through the composting material.
4) In-vessel composting using drums, silos or channels with high-rate controlled aeration system, designed to provide optimal conditions.

1.10 Bioremediation of PAHs-contaminated soil through composting

At present, employing the biochemical abilities of microorganisms is the most popular strategy for the biological treatment of contaminated soils and waters (Head, 1998). Microorganisms, more so than any other class of organisms, have a unique ability to interact both chemically and physically with a huge range of both man-made and naturally occurring compounds leading to a structural change to, or the complete degradation of the target molecule (Dua et al., 2002; Head, 1998; Semple et al., 2001).

Applying *in-situ* bioremediation of PAHs contaminated soil has been found inefficient for the removal of many PAHs from contaminated soil within a reasonable time periods. Inadequacies are due to strong absorption of PAHs to soil and low PAH aqueous solubility as well as temperature and soil type limitations (Loick et al., 2009; Wilson and Jones, 1993). Fortunately, treating the same soil using *ex-situ* methods was found to increase the degradation rate within a reasonable time as the controlling factors are easier to be adjusted and controlled.

Among the *ex-situ* technologies that have been employed to deal with contaminated soils is the composting technology that has been received more attention during the last decades because of its high efficiency to degrade various organic contaminants like PAHs, explosives, pesticides, chlorophenols, etc (Gandolfi et al., 2010; Sayara et al., 2009; Semple et al., 2001; Loick et al., 2009; Lemmon and Pylypiw, 1992; Breitung et al., 1996). Simply,

the process relies of the addition of compost's primary ingredient to the contaminated soil, wherein as the compost matures; the pollutants are degraded by the active microflora within the mixture (Semple et al., 2001).

The removal of PAHs by biodegradation has been investigated in several studies under a range of conditions and with different kinds of contaminated materials. In a composting process using municipal solid waste and fertilizers to treat PAHs with creosote-contaminated soil, substantial amounts of high molecular weight (81.63% for benzo(a)anthracene as minimum and 98.63% for flourene as maximum degradation rate, and in between for other PAHs) were removed after 15 days of incubation at 45˚C (Civilini, 1994). Antizar-Ladislao et al. (2006) investigated the composting of manufactured gas plan contaminated soil (total 16 USEPA PAHs 100.3 mg per kg dry soil) using silver-sand and green waste as amendments under different incubation conditions including different temperatures (38 ˚C, 55 ˚C and 70 ˚C), different moisture contents (40%, 60% and 80%) and different mixing ratios. A degradation rate of 82% was obtained with 60% moisture content, 0.35:0.35:1 mixing ration and 38˚C, indicating that low temperatures (mesophilic temperatures) were more adequate for PAHs removal. In another study conducted by Gandolfi et al. (2010), using compost as amendment was effective in enhancing biodegradation of diesel and four-ring PAHs as it is effectively influenced the microbial communities within the mixture and the reduction of some toxicity form the other side. Kästner et al. (1999) found that the addition of compost increased the mineralization of anthracene from 43 to 67%, while the amounts fixed to the soil and therefore not recoverable decreased from 45 to 21%.

It is important to remark that the degradation rate of PAHs using composting technology is influenced by various controlling factors. Accordingly, emphasis should be devoted on developing a standardized method to study the effects of different conditions by assessing the given conditions and characteristics of the contaminated material, including co-contaminations and indigenous microbial communities. This should then lead to developing methods to systematically treat contamination (Loick et al., 2009). In this regard, the present research tried to investigate some of the important parameters that presumably influence the overall process behaviour and the PAHs degradation rate as a consequence. Screening of various organic co-substrates and the impact of different controlling factors like PAHs concentration, co-substrate stability, mixing ration, co-metabolism, and bioaugmentation have been systematically investigated in attempt to provide more clear imagine about the process and its final outputs.

1.11 Anaerobic digestion

Anaerobic digestion is a biological process by which the organic matter is degraded in the absence of oxygen and the presence of anaerobic microorganisms. Biogas which mainly composed of methane (CH_4) and carbon dioxide (CO_2) is produced as a result of the organic matter digestion, and this produced biogas can be used for electricity generation.

Anaerobic digestion has been applied for the treatment of different wastes like municipal solid wastes and wastewater sludge (Ferrer et al., 2008). Recently, more attention has devoted to this technology as it provides two advantages regarding the environment; firstly: the treatment of solid wastes and secondly: the production of biogas which is recognized as a source of clean energy (Chynoweth et al., 2001; Schievano et al., 2008).

1.11.1 Anaerobic digestion stages

Anaerobic digestion is the consequence of a series of metabolic interactions among various groups of microorganisms. It occurs in four main stages (figure 1.6): hydrolysis, acidogenesis, acetogenesis and methanogenesis (Ponsá et al., 2008).

1. ***Hydrolysis:*** in the first stage of hydrolysis, the fermentative bacteria via its extracellular enzymes convert the undissolved complex organic matter like cellulose into simpler soluble compounds such as sugars, amino acids, fatty acids, alcohol and CO_2. This stage is important especially for high organic wastes and could represent a limiting factor for the digestion process.

2. ***Acidogenesis:*** in this stage, the first stage hydrolyzed compounds are converted to simple organic acid (fatty acids mainly composed of acetate, propionate and butyrate), hydrogen and carbon dioxide.

3. ***Acetogenesis:*** This involves the breakdown of fatty acids and other compounds to form acetic acid, carbon dioxide and hydrogen which will be used by methanogenic bacteria in the last stage.

4. ***Methanogenesis:*** all the simple compounds obtained in the previous stage (acetate, hydrogen, carbon dioxide) are converted to methane by the methanogenic microorganisms.

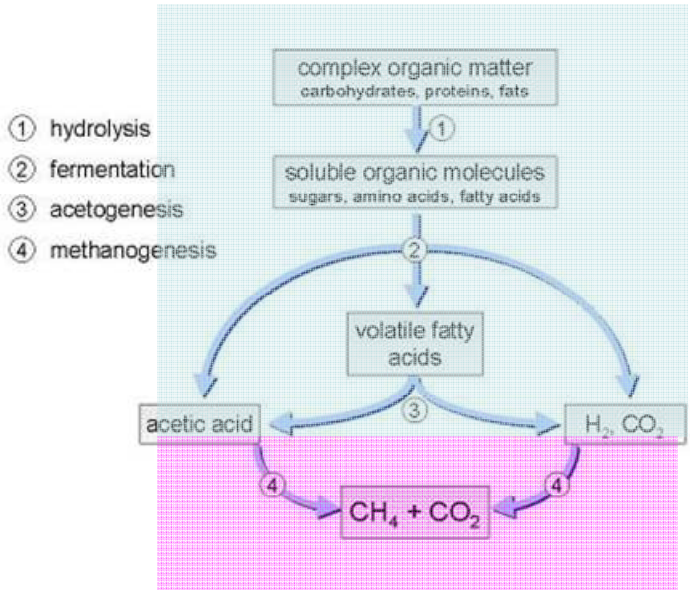

Figure 1.6: the different consecutive stages of anaerobic digestion process.

1.11.2 General operating parameters of anaerobic digestion

As any treatment process, several operating parameters should be controlled in attempt to enhance the anaerobic digestion process. Nevertheless, anaerobic digestion process is characterized by its sensitivity to any variation in the controlling parameters compared with other biological treatments like composting. Sometimes the process is totally inhibited as a result of parameters shock. The main parameters that should be taken into account are summarized as:

- **Temperature:** Anaerobic digestion is normally carried out under two mainly temperature ranges: mesophilic and thermophilic. The mesophilic range is between 20°C-40°C and the optimum temperature is considered to be 30°C-35°C. The thermophilic temperature range is between 50°C-65°C. Thermophilic ranges are more efficient regarding the degradation rate, biogas production and retention time, but the energetic requirements are also higher.

- **pH:** Anaerobic digestion is highly affected by the pH value. pH between 6.5-7.5 are considered optimal for such process. However, as the digestion process passes through consecutive stages, the two stages of acidification and methanogenesis require different pH levels for optimal process control. The retention time of digestate affects the pH value and in a batch reactor acetogenesis occurs at a rapid rate. Acetogenesis can lead to accumulation of large amounts of organic acids (normally volatile fatty acids) resulting in pH below 5. As

a result, excessive generation of acid can inhibit methanogens due to their sensitivity to acid conditions. For most case, instability of digestion reactors is recorded by a rapid increase of volatile fatty acids concentration.

- **Carbon to Nitrogen ratio (C:N):** C:N values between 20-30 are within the optimum ranges for right process. High ratio of C:N will lead to rapid consumption of nitrogen by methanogens and results in lower biogas production. On the other hand, lower C:N ratio leads to ammonia accumulation an pH values exceeding 8.5, which is toxic to methanogenic bacteria.

- **Retention time:** this factor is related to the feedstock composition, anaerobic digestion technology and operating temperature. For instance, the retention time under thermophilic conditions is about 14 days less than mesophilic treatments which normally range between 10-40 days.

- **Loading rate and mixing:** organic loading rate above the system sustainable capacity will result in low biogas yield due to the accumulation of inhibiting substance like fatty acids. On the other side, for process smooth operation, an adequate degree of mixing is needed to blend the fresh material with the digestate that contains microorganisms. A 60% reduction in the degree of mixing may cause as much as a 50% decrease in treatment efficiency. However, excessive mixing can disrupt the microorganisms aggregates.
- **Biogas production:** which is the most important product obtained from the anaerobic digestion. The biogas mainly comprise of methane (55-70%, by volume), carbon dioxide (30-45%, by volume) and other gases such as sulphur compounds.

1.12 PAHs degradation through anaerobic digestion

Although aerobic treatments are believed to be more effective for waste treatments in general and for PAHs-contaminated soil specifically, anaerobic digestion can also be applied for such treatments. For instance, controlled anaerobic treatments are more economically than aerobic ones as no oxygen is needed especially for deep contaminated sites. Applying anaerobic digestion for PAHs remediation is a promising process especially for accidental oil spills as well as remediation of water submerged soil such as paddy field and swamps where limiting oxygen flow presents due to soil pore saturation or clogging of

aggregates. Furthermore, this technology can be used to treat deep under ground soil as no oxygen is needed (Gan et al., 2009). Although anaerobic investigations to treat PAHs contaminants are still limited as few studies have been conducted in this field compared with aerobic treatments, some of the available studies demonstrated the capability of anaerobic microorganisms to degrade some of PAHs. In this regard, Ambrosoli et al. (2005) achieved removal percentages in the approximate range of 30–60% for various PAHs in treating PAH-contaminated soil under denitrifying conditions by inoculating with a mixed population of microorganisms obtained from a paddy soil. Furthermore, Chang et al. (2002) conducted a laboratory scale study using anaerobic PAH-adapted consortium culture which was incubated with spiked soil and amended with nutrients. After an incubation period of 90 days at 30 ˚C, it was found that the spiked PAHs were significantly degraded. Additionally the degradation of PAHs was shown to be enhanced by the addition of nutrient supplements such as acetate, lactate or pyruvate. In this research, anaerobic degradation of PAHs under methanogenic conditions was investigated. The process behaviour under different variables including PAHs concentration, different compost of OFMSW as amendments, themophilic and mesophilic conditions and different soil to inocula mixing ratio was evaluated.

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Chapter 2

Research objectives

The general objective of this research was to study the bioremediation of PAHs-contaminated soil through aerobic and anaerobic techniques. Consequently, the research content can be divided into two main sections: the first section which was the aerobic treatments carried out through applying a composting approach as an emerging and attractive way to deal with such like contamination. The second section was the anaerobically treatment of the same contaminated soil but under strict anaerobic methanogenic conditions. In this context, the general objective was performed through several consequent experiments in an attempt to clarify the influence of different parameters controlling the bioremediation process. Accordingly, the specific objectives regarding the bioremediation process can be summarized as following:

A) Specific objectives regarding the bioremediation process using composting approach:

- To select organic co-substrates from several organic amendments including sewage sludge, organic fraction of municipal solid wastes (OFMSW) and compost derived from OFMSW that can be applied for the bioremediation of PAHs-contaminated soil. The selection criteria were to adapt the co-substrate which can achieve higher degradation rate within a reasonable period compared with the others.
- To study systematically the composting-bioremediation process employing the experiment design technique. In this regard, the objective was to determine the impact of various factors to determine the optimum conditions to carry out such process and the relation between the studied factors. Concern was devoted to visualize the impact of the compost stability degree determined through the respiration index.

- To study the degradation of artificially complex mixture of PAHs simulating a real creosote sample. Co-metabolism, impact of some controlling factors and co-substrates properties were to be evaluated regarding the process performance and contaminants degradation rate.
- To compare and clarify the feasibility and effectiveness of bioaugmentation and biostimulation application as possible alternatives to enhance the bioremediation process.

B) Specific objectives regarding the bioremediation process using anaerobic digestion approach:

- To asses the feasibility of using the anaerobic digestion process for bioremediation of PAH-contaminated soil as an alternative biological treatment.
- To evaluate systematically the process efficiency under different factors including PAHs concentration and various types of OFMSW compost as possible organic co-substrates to enhance the process.
- To compare the efficiency of the process under different temperature, mainly; thermophlic and mesophilic ranges. Meanwhile, factors like PAHs concentration and soil to inocula were to be evaluated for both temperature regimens.
- To determine the bioavailable portion of the contaminants, and the evolution of the microbial culture during the digestion process under thermophilic and mesophilic temperature.

Chapter 3

Analytical methods and bioremediation approaches

This chapter is divided into three basic sections to provide: (i) summarized description of the common analytical methods which were employed but are not completely described through the research articles content; (ii) the bioremediation systems; (iii) and finally the experimental design technique which was used several times in the current research. However, information regarding the used materials including soil, organic co-substrates, inocula, etc., is provided within each corresponding chapter as several materials were employed.

3.1 Analytical methods

Analysis of common parameters was carried out according to the standard procedures described in "Test methods for the examination of composting and compost" (US Department of agriculture and US composting council, 2001). At least two representative samples were analyzed and the presented results are the mean values with standard deviation.

3.1.1 Moisture content (MC) and dry matter (DM)

Moisture content expressed on a wet basis denotes the moisture content as a percentage of the sample after it had been dried; this is useful for determining whether a composting mixture has the correct moisture for composting (40-60%). This parameter was done by drying the samples in a forced-air oven (stove) at 105˚C for 18-24h. The samples were weighted before and after drying using a digital balance of ±0.01g precision, then the moisture content is expressed as follow:

$$\%\text{Moisture Content (MC)} = \frac{\text{Wet weight - Dry weight}}{\text{Wet weight - Container weight}} * 100 \qquad \text{(Equation 3.1)}$$

$$\Rightarrow \%\ \text{Dry Matter (DM)} = 100\text{-}\%\text{MC} \qquad \text{(Equation 3.2)}$$

3.1.2 Total organic matter (OM)

This term is also equivalent to volatile solids content (VS). It was determined through sample ignition (combustion) in a muffle furnace for 2.5 hours at 550˚C and then the organic fraction is expressed as follows:

$$\%\ \text{Organic Matter (OM)} = \frac{\text{Dry weight - Final weight}}{\text{Dry weight - T}} * 100 \qquad \text{(Equation 3.3)}$$

Where T: is the weight of the capsule used to incubate the sample

$$\Rightarrow \%\ \text{Ash}=100\text{-}\%\text{OM} \qquad \text{(Equation 3.4)}$$

3.1.3 pH

Slurry of tested material and deionized water was blended at a ratio of 1:5 (v/v) basis. The sample was shaken (magnetic shaker) for 30 minutes at room temperature to allow the salts to solubilise in the deionized water. The pH was measured using an electrometric pH meter (Crison, micropH200) directly in the slurry or in the extracted solution.

3.1.4 Total Kjeldahl Nitrogen (TKN)

The sum of organic nitrogen plus ammonia nitrogen ($N\text{-}NH_4^+$) expressed as total Kjeldhal nitrogen (TKN) was determined using 0.5 g of the sample. The sample was digested for 1.5 hours at 400°C using 25 mL of concentrated sulphuric acid in 100 mL Kjeldhal tubes using a Bloc Digester equipped with six tubes (J.P. Selecta S.A., Barcelona, Spain). To speed up the digestion, a catalyst (Kjeltab®) was added. Each digestion block contained two blank tubes that contained the standard amount of acid described above and a catalyst tablet (Kjeltab®). After allowing the sample to cool, the sample was diluted using deionised water. A Büchi Distillation Unit K-355 (Flawil, CH) was used for sample distillation with an excess of NaOH (35%). The condensate was placed in flask with 100 mL of boric acid (4%) with mixed indicator. A colorimetric assay was used to measure the

amount of nitrogen formed by adding HCl and an acid indicator. TKN was calculated using Equation 3.5.

$$TKN = \frac{(V_i - V_0) \quad N * 1.4}{W_{wb}} \qquad \text{(Equation 3.5)}$$

Where:

TKN: total Kjeldhal nitrogen (%); V_i: HCl consumed volume (mL) in sample titration; V_0: HCl consumed volume (mL) in control titration; N: normality of the HCl used in determination; and W_{wb}: sample weight (g), wet basis.

3.1.5 Humic matter

10 g sample of oven dried compost was added to 100 ml of 0.1 N of NaOH (volume:mass; 10:1) and the headspace was replaced with N_2. The samples were shaken (130 rpm) for 24 hour at 25 ˚C. After that the samples were centrifuged at 10000 rpm for 10 minutes. The supernatant was decanted (collected) in polypropylene containers and another 100 ml of 0.1 N of NaOH was added to the residue of the original samples and they left for another 24 hours on the shaker (same as the first step). Afterward, the residue was suspended in 50 ml distilled water and then the washing was separated. The alkaline extract was acidified to pH 2 with 2 N HCl for 24 hours at 25 ˚C. To separate fulvic acid from humic acid, the soluble material was separated by centrifuged at 10000 rpm for 10 minutes by which the coagulant represent the humic matter and the soluble part contains the fulvic acids, then the two substances were freeze- dried (lyophilization) for purification and quantification but only humic matter water followed during this study (US Department of agriculture and US composting council, 2001).

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3.1.6 Organic matter stability

Organic matter stability which can be defined as the extent to which readily biodegradable organic matter has decomposed, is recognized as an important parameter to evaluate the evolution and progress of the biological treatment since stabilization of the organic matter through such treatments is principally the main goal. Among the suggested biological methodologies, aerobic respiration indices have been highlighted as the most suitable tool for biodegradability and/or stability assessment (Barrena et al., 2009).

3.1.6.1 Static Respiration Index (SRI)

A static respirometer which was built according to the original model described by Iannotti et al. (1993, 1994) followed by the modifications and recommendations given by The US Department of agriculture and The US Composting Council (2001) was used. The drop of the oxygen content in a flask containing compost sample was monitored with a dissolved oxygen meter (Lutron 5510, Lutron Co. Ltd. Taiwan) connected to a data logger, and the data logger is connected to computer that can represent the oxygen drop as a figure through a self-made software program.

The experiments were carried out at a fixed temperature (37˚C). The samples were incubated for 24 hours under this temperature, and during all the incubation periods, samples were aerated with previously humidified air. The aeration system consisted of a flask with a two-hole stopper and two glass delivery tubes. At the bottom tip, the delivery tube had an aquarium air-stone to produce small air bubbles. This sparger was immersed in water to humidify the air. The flask was always kept inside the incubation bath. The delivery tube was connected to a manifold that served to deliver air to the different flasks containing the samples. The manifold was constructed from plastic tubing connected by quick disconnect fittings. Individual valves were also fitted to each sample tube to regulate the air flow (Barrena et al., 2005). Respirometric configuration is shown in Figure 3.1.

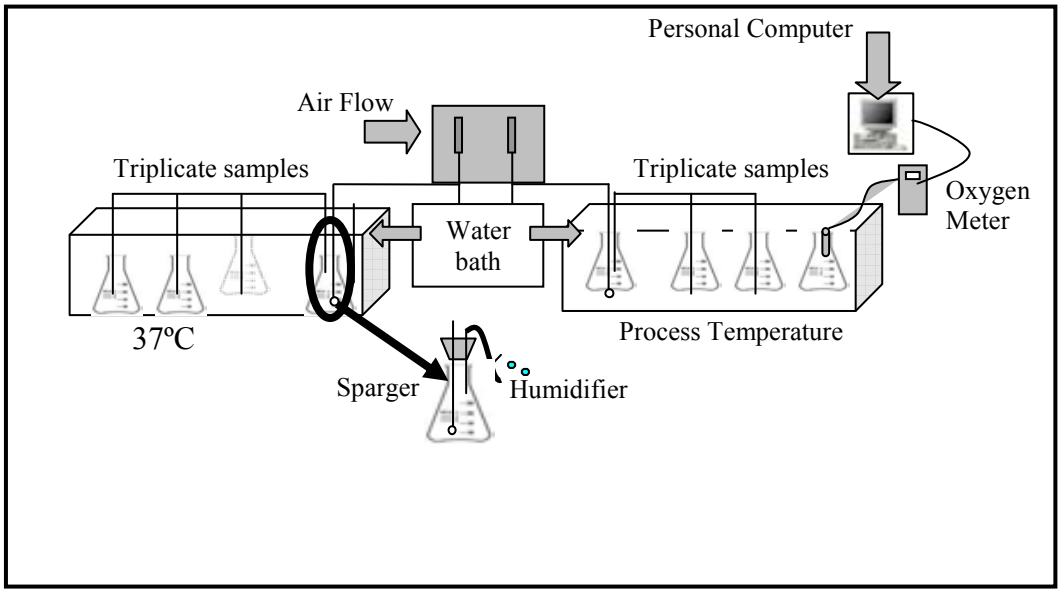

Figure 3.1: Static Respirometric scheme, (Barrena et al., 2005).

When the incubation period was finished, the dissolved oxygen (DO) sensor was assembled into the flasks without removing them from the water bath. The air pressure was then equilibrated with the outside pressure by inserting a hypodermic needle through the stopper. Oxygen level was then recorded every 15s for 90 minutes where in all experiments three replicates were used to determine RI_{37}. The respiration index of the sample referred to total organic matter content was calculated from the slope in a linear segment on the chart of O_2 (%) versus time using equation 3.6.

$$SRI = \frac{V \cdot P \cdot 32 \cdot m \cdot 60}{R \cdot T \cdot X \cdot DM \cdot OM} \qquad \text{(Equation 3.6)}$$

Where:

SRI: respiration index (mg O_2 g OM^{-1} h^{-1}); V: volume of air in the flask (ml); P: atmospheric pressure at elevation of measurement (atm); m: slope of change in percent O_2 saturation per minute divided by 100; R: ideal gas constant (0.08206L atm mol^{-1} K^{-1}); T: temperature in (K); X: wet weight of compost test aliquot (g); DM: fraction of total solids of a parallel sample aliquot (g DM g X^{-1}); OM: fraction of organic matter of a parallel sample aliquot in dry basis (g OM g DM^{-1}).

3.1.6.2 Dynamic Respiration Index (DRI)

Determination of DRI was based on previous works by Adani et al. (2003, 2004, and 2006) and Barrena et al. (2005). The detailed description and explanation of the used system can be found in Ponsá et al. (2010). Briefly, the system consists of an Erlenmeyer flask as reactors, containing a plastic net to support the organic waste and provide an air distribution chamber, placed in a water bath at 37ºC (Barrena et al., 2005). Airflow in the reactors is manually adjusted by means of an air flow controller (Bronkhorst Hitec, The Netherlands) to provide constant airflow, and modified when necessary to ensure minimum oxygen content in exhaust gases of 10% v/v (Leton and Stentiford, 1990). Airflow is previously humidified at the sample temperature to avoid the sample drying during the incubation period. According to the biodegradability of the samples, initial air flow selected was between 30-45 mL min^{-1} for active samples and 15-25 mL min^{-1} for more stable samples such as compost. Exhausted air from the reactors was sent to oxygen and carbon dioxide sensors prior dehumidification in a water trap. Both air flow meters and oxygen sensors were connected to a data acquisition system to continuously record these values. Data concerning air-flow, and O_2 and CO_2 content in the exhaust gas for all reactors is registered

at least once every 15 minutes. Experimental set up of dynamic respiration determination is presented in figure 3.2.

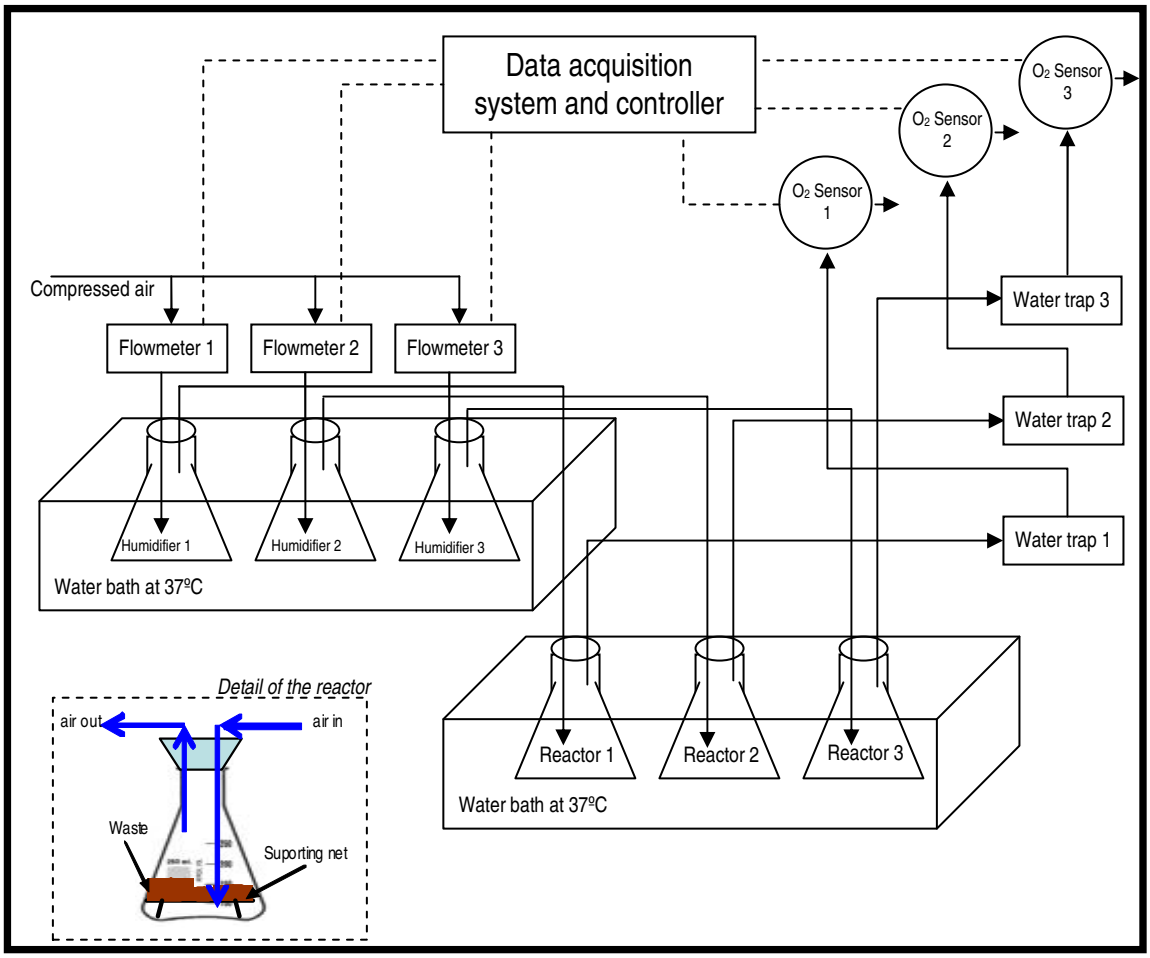

Figure 3.2: Experimental set up for dynamic respiration indices determination (Ponsá et al., 2010).

Dynamic respiration index can be calculated from oxygen and air flow data for a given time applying equation 3.7.

$$DRI_t = \frac{(O_{2,i} - O_{2,o}) \times F \times 31.98 \times 60 \times 1000^a}{1000^b \times 22.4 \times DM} \qquad \text{(Equation 3.7)}$$

Where:

DRI_t: Dynamic Respiration Index for a given time t, mg O_2 g^{-1} DM h^{-1}; $(O_{2,i}\text{-}O_{2,o})$: difference in oxygen content between airflow in and out the reactor at that given time, volumetric fraction; F: volumetric airflow measured under normal conditions (1 atm and 273 K), ml min^{-1}; 31.98: oxygen molecular weight, g mol^{-1}; 60: conversion factor,

minutes/hour; 1000^a: conversion factor, mg g^{-1}; 1000^b: conversion factor, mL L^{-1}; 22.4, volume occupied by one mol of ideal gas under normal conditions, L; DM: dry mass of sample loaded in the reactor, g.

A dynamic respiration index curve can be built from on-line collected data as shown in Figure 3. From these data, several respiration indices can be calculated like oxygen uptake rate indices and cumulative consumption indices.

Oxygen Uptake Rate Indices - DRI

- DRI_{max}: maximum DRI_t obtained.
- DRI_{1h}: average DRI_t in the one hour of maximum activity.
- DRI_{24h}: average DRI_{1h} in the 24 hours of maximum activity (Adani et al., 2003).

Cumulative Consumption Indices - AT

- AT_n: Cumulative oxygen consumption in n days calculated as shown in Equation 3.8.

$$AT_n = \int_{t_l}^{t_l+n} DRI_t \cdot dt \qquad \text{(Equation 3.8)}$$

Where t_l is time when lag phase finishes. Lag phase (Federal Government of Germany, 2001) ends when the oxygen uptake rate, expressed as a 3-hour mean, reaches 25% of the maximum uptake rate calculated as the average of three hours (Figure 3.3). The weight of the oxygen consumed during the lag phase is subtracted from the weight of the oxygen consumed throughout the entire test (lag phase + n days). Results are expressed as mg $O_2 \cdot$ g DM^{-1} or mg $O_2 \cdot$ g OM^{-1}. Both the mean and the standard deviation are to be listed. If possible, three replicates are recommended. However a minimum of two replicates must be always analyzed for each sample and a third replicate must be undertaken when deviation among duplicates is over 20%.

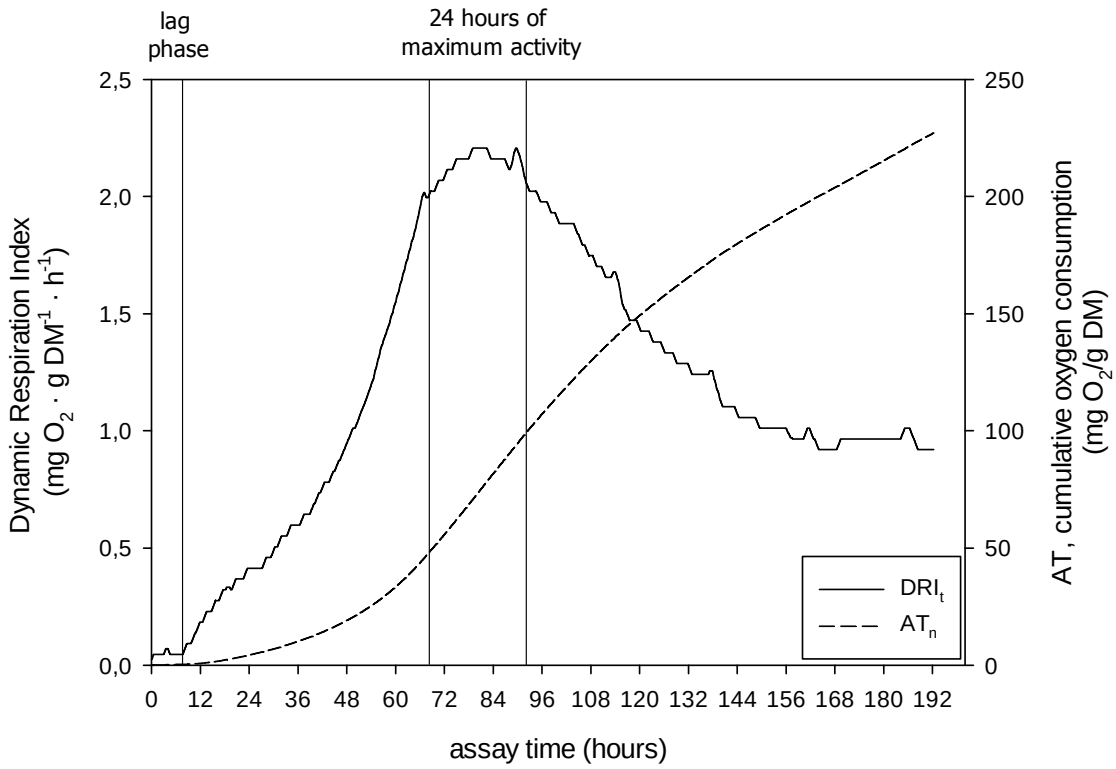

Figure 3.3: Typical curve for dynamic respiration indices evolution and calculation (Ponsá et al., 2010).

Interestingly, it is important to highlight that the main difference between the statics and dynamics methodologies is that SRI presents a single value of biological activity potential, while the DRI approach generates an activity profile which might permit a deeper analysis of organic materials biodegradability: this should include both total biodegradable organic matter content and information regarding the biodegradation rate (Barrena et al., 2009; Ponsá et al., 2010).

3.1.7 Anaerobic digestion: Biogas production

The biogas production during anaerobic digestion process is considered as an indicator about the biodegradability of the digested organic matter under that condition. Consequently, during the incubation period, the produced biogas pressure was followed and registered for biogas quantification and; meanwhile, biogas sample were analyzed using GC for biogas qualification as described in details elsewhere (Sayara et al., 2010). After each measurement time, the biogas was directly purged to minimize the any possible solubilisation of carbon dioxide. Blanks which contain only inocula were incubated in parallel with treatments in order to quantify the biogas gas produced by the inocula itself.

Consequently, when the biogas produced by each treatment was to be quantified, the quantity produced by the introduced inocula itself was directly subtracted from the total amount.

Quantification of the biogas was carried out based on the procedures described by the German Institute for Standardization (DIN, 1985) and Cossu and Raga (2008) which provides the parameter GB_{21} expressed as normal litres of biogas produced per kg of total solids (Nl kg DM^{-1}) under 273 K temperature and 1.01325 bar pressure during 21 days. A new analytical methods based on GB_{21} was developed such that the biogas production GB_n can be determined for n days of incubation as the treatments normally continuo until the biogas production is ceased. The procedures to determine the GB_n and/or the biochemical methane production (BMP_n) are as following:

1- The volume of biogas or methane produced at 37ºC and 1 atm in each experiment is calculated applying Equation 3.9.

$$V_{37ºC,n} = \frac{\left[B - \left(W/BD_w\right)\right] \times \sum_{i=0}^{n} P_i}{1.032502} \qquad \text{(Equation 3.9)}$$

Where:

$V_{37ºC,n}$: the volume of biogas (or methane) produced in a bottle after n days (litres); B: the bottle working volume (litres); W: the total wet weight of the mixture introduced in the bottle (kg); BD_w: the wet bulk density of the mixture (kg · L^{-1}) ; P_i: the pressure measured after pressure release (bar); n: is the days after experiment started; 1.032502 is the atmospheric pressure (bar).

2- The net volume of biogas (or methane) produced, after subtracting the biogas (or methane) produced by the blank is calculated applying Equation 3.10.

$$V_{net\ 37ºC,n} = \left[V_{37ºC,n}\right] - \left[\left(\sum_{i=0}^{3} V_{37ºC\ inoc,i}/W_{inoc,i}\right)/3\right] \times S_{inoc} \qquad \text{(Equation 3.10)}$$

Where $V_{net\ 37ºC,n}$: the net volume of biogas (or methane) produced in a sample bottle after n days (litres); $V_{37ºC\ inoc.,i}$: the volume of biogas (or methane) produced in each blank triplicate after n days (litres); $W_{inoc,i}$: the total wet weight of inoculum initially introduced in each blank triplicate (g); S_{inoc}: the wet weight of the inoculums used when making the initial mixture waste-inoculum (g).

3- The biogas production during n days (GB_n) and biological methane potential during n days (BMP_n) is finally determined using Equation 3.11

$$GB_n\ (BMP_n) = \left[\left(V_{net\ 37ºC,n}/Z\right) \times \frac{273.15}{310.15}\right] \qquad \text{(Equation 3.11)}$$

Where GB_n: the net volume of biogas produced from a waste sample after n days (NL biogas·kg DM^{-1}); BMP_n: the net volume of methane produced from a waste sample after n days (NL methane·kg DM^{-1}); Z: the amount of DM of sample initially loaded in the reactor (kg DM); 310.15 is the temperature measured in Kelvin at which is carried out the experiment (310.15 K) and equivalent to 37ºC; 273.15 is the temperature in Kelvin which corresponds to Normal conditions (273.15 K) and equivalent to 0ºC.

3.2 Bioremediation systems

3.2.1 Composting system

In all bioremediation treatments through composting, Dewar® vessels with 4.5 L working volume were used as reactors as illustrated in figure 3.4. These vessels were modified and conditioned to operate in batch-mode form to carry out the composting experiments. In fact, as they are thermally isolated, the composting process can be kept under the natural composting temperatures, and the influence of ambient temperature can be minimized or even ignored. Air was provided for the composting mixture through a pipeline connected to the bottom of the reactor where a plastic mesh is placed to insure a correct distribution of the air through the composting mixture, and the exhausted air exits the reactor through an outlet in the reactor cover. Oxygen concentration was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom), where the inlet of the sensor is connected to the reactor outlet and consequently the oxygen percentage was determined. Aeration rate and frequency were adjusted to prevent any limitation or excess in the oxygen percentage in the reactors. In this regard, electrical valves connected to digital timers were used to adjust the aeration frequencies, and flow meters to control the air flow rate into the reactor. The aeration system configuration was able to sporadically aerate the composting mixture such that the oxygen concentration was well maintained to ensure aerobic conditions (more than 10% of O_2). Temperature was monitored by Pt-100 sensors (Sensotran, Spain) connected to a data acquisition system (DAS-8000, Desin, Spain) that was connected to a personal computer. The software used (Proasis®Das-Win 2.1, Desin, Spain) also permit to monitor both the temperature and oxygen content in the reactors.

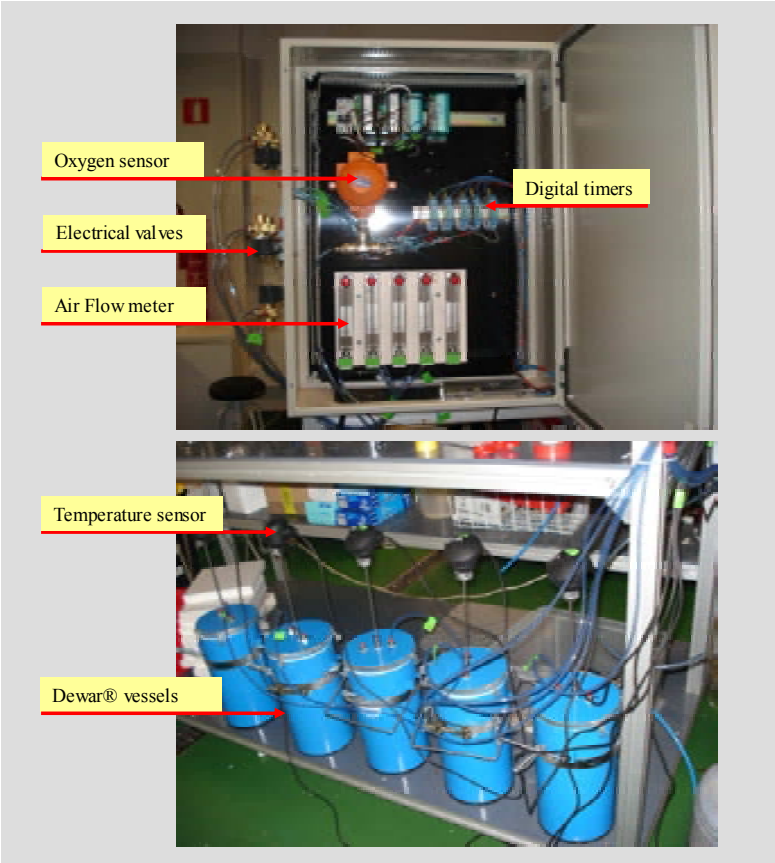

Figure 3.4: Composting reactors and controlling system.

Preparation of the contaminated soil was performed by artificially spiking the PAHs into soil according to the determined concentration values. The percentage of each compound when a PAHs mixture was applied was determined according to the results obtained by another group co-researcher using US EPA method 3611B, (1996) which principally depends on alumina column cleanup and separation of petroleum wastes. After that, the contaminated soil was mixed with the organic amendment to different ratios previously determined depending on the experiment goal (w:w, dry weight). The mixture then was mixed with bulking agent at ratio 1:1 (V:V) in attempt to provide proper porosity to maintain aerobic conditions. All entire composting treatments component were manually mixed according to the used ratios and then were introduced into the reactors as shown in Figure 3.5 which shows some phases of the bioremediation process. It is important to remark that the used bulking agent consisted of wood chips and pruning wastes that were not biodegraded under laboratory composting conditions. Additionally, water content of the composting mixtures was adjusted to be within the recommended value (50-60%) by adding tap water before incubation. The composting matrix was left under natural composting

temperatures. Aeration flow rate and frequency were monitored and adjusted during the process to avoid any limitation or excess in the oxygen concentration that may affect the process.

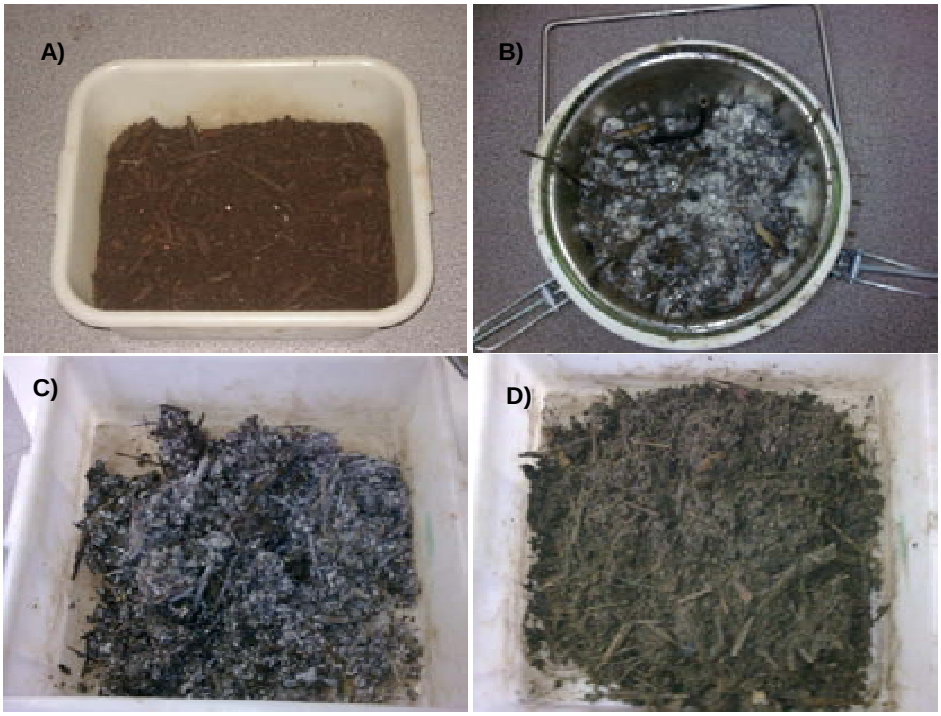

Figure 3.5: Laboratory bioremediation of PAHs-contaminated soil through composting,A: initial mixture; B: reactor-mixture incubation, C: reactor content during the sampling; D: final mixture after composting.

3.2.2 Anaerobic digestion system

The start-up of the anaerobic digestion process depends on preparing and mixing of the organic matter to be digested with inocula which presumably have the anaerobic microbial culture needed for carrying out the degradation. Two types of incula can be used depending on their temperature dependence; mesophilic which normally works at 37˚C and thermophilic which normally work at 55˚C. In the present study, both of them has been used and they were brought from a real full-scale anaerobic digester of OFMSW treatments plants (Barcelona, Spain) working under those different temperatures. Generally, the inocula consisted of a liquid fraction having a small part of dry matter and incubated for at least two week under the corresponding temperatures to remove any remaining easily biodegradable fraction before use.

Perfectly sealed aluminium bottles (Traveller SIGG®, Spain) of 1 litre working volume were used as reactors for carrying out the designed treatment assays. The mixture was prepared introducing the different components (inocula, contaminated soil, organic amendment and water) into the reactor. Afterwards, the bottles were sealed with a ball valve and the headspace was purged with nitrogen gas to ensure anaerobic conditions. The bottles were incubated under the corresponding temperature (mesophilic or thermophilic). Figure 3.6 gives some details about the used reactors.

Figure 3.6: Reactors used for anaerobic bioremediation treatments.

3.3 Experiment Design Methodology

Experiment design methodology is a structured, organized method that is used to determine the relationship between the different factors (Xs) affecting a process and the output of that process (Y). This involves designing a set of experiments, in which all relevant factors are varied systematically. When the results of these experiments are analyzed, they help to identify optimal conditions, the factors that most influence the results, and those that do not, as well as details such as the existence of interactions and synergies between factors (Deming and Morgan, 1987;Montgomery, 1991)

Among the different design techniques, Central Composite Design (CCD) which was proposed by Box and Hunter (Montgomery, 1991) is recognized as a useful technique

which can be calibrated much more easily using a second order polynomial model. Moreover, a response surface through the design matrix and its corresponding outputs can be created without using all the possible combination levels of the factors and consequently reducing the needed experiment runs. On the contrary, using a full factorial design generally requires more runs to accurately estimate model parameters.

For an experiment involving (*k*) factors, normally the studied factors are coded using the notation (0, ±1, ±α) where (±1): represent the axial point, (0): represents the central point, and (±α): represent the extreme values (low and high) as shown in figure 3.7.

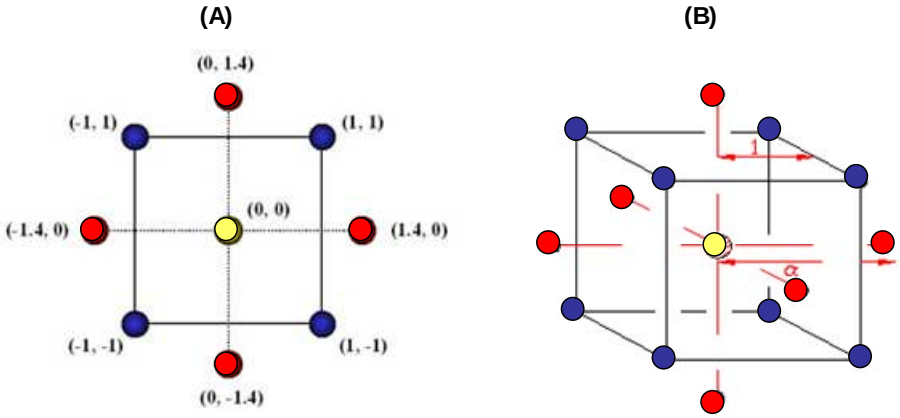

Figure 3.7: Central composite design representation for two factors (A) and three factors (B).

The design consists of 2^k factorial points representing all combinations of the coded values (±1), 2k axial points at a distance ±α from the origin, and at least 3 (triplicates) central points with the coded values set to zero in order to improve the resolution of the method. The value of α is determined according to the following equation:

$$\alpha = F^{1/4} \qquad \text{(Equation 3.12)}$$

Where $F=2^k$

According to this equation the value of α is ± 1.414, ± 1.66 for two factors and three factors respectively.

The determination of the natural values of the coded factors (0, ±1 and ± α) was then calculated using the following equation:

$$x_i^* = \left(\frac{x_i - c_x}{d_x}\right) * d^* \qquad \text{(Equation 3.13)}$$

Where:

X_i^* = coded value of the factor,

x_i= natural value of the factor,

c_x = the centre point of the experiment level,

d^*= the distance between the new extremes values,

d_x= the distance between the natural values.

The design matrix was built according to this design method and then the experimental part and analysis of the results was performed using the Sigmaplot® 8.0 software package (Systat Software Inc, San Jose, USA).

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Chapter 4

Bioremediation of PAHs-contaminated soil through composting

4.1 Published articles

This part contains the articles corresponding to bioremediation of PAH-contaminated soil through composting and that had been published in indexed international journals.

***Article I*:** Preliminary screening of co-substrates for bioremediation of pyrene-contaminated soil through composting.

***Article II*:** Optimization and enhancement of soil bioremediation by composting using the experimental design technique.

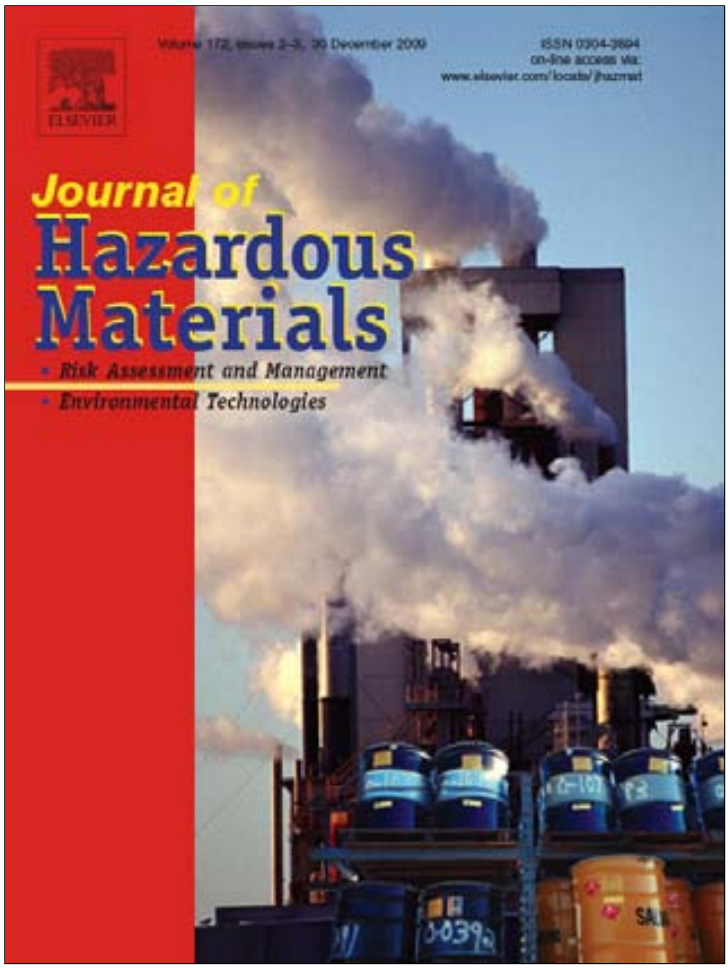
***Article III*:** Effects of compost stability and contaminant concentration on the bioremediation of PAHs-contaminated soil through composting.

Article I

Preliminary screening of co-substrates for bioremediation of pyrene-contaminated soil through composting.

Tahseen Sayara, Montserrat Sarrà, Antoni Sánchez.

Journal of Hazardous Materials. 2009. vol (172), p.1695-1698.



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Short communication

Preliminary screening of co-substrates for bioremediation of pyrene-contaminated soil through composting

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ABSTRACT

The feasibility of using different organic amendments of different origin and properties in the bioremediation of pyrene-contaminated soil by means of composting has been tested. The selected pyrene concentration was 1 g of pyrene per kg of dry soil. The organic amendments used include: raw organic fraction of municipal solid wastes (OFMSW), industrial compost from OFMSW composting (COFMSW), compost derived from home composting of OFMSW (HCOFMSW), anaerobically digested sludge (ADS), non-digested activated sludge (NDS) and centrifuged non-digested activated sludge (CNDS). The degradation rate was related to the amendment properties that directly affected the composting process. Raw OFMSW was not capable to enhance pyrene degradation in comparison to control, but stable HCOFMSW exhibited the highest removal rate (69%). The amendments stability and the temperatures reached as a consequence influenced the process, and thermophilic temperatures showed an inhibition effect on the microbial activity related to pyrene degradation. Some of the tested wastes need to be further investigated to find inexpensive organic amendments for soil bioremediation.

1. Introduction

At present, soil contamination by polycyclic aromatic hydrocarbons (PAHs) is of environmental concern. Many of these compounds have been shown to be acutely toxic, mutagenic and carcinogenic, and are of great concern with respect to both the environment and human health [1]. PAHs are introduced in the environment through accidental spillage, misguided disposal of petroleum and creosote wastes, combustion of fossil fuels, coal, wood preserving products and leaking from underground tanks [2].

Due to their hydrophobic nature, low aqueous solubility, low volatility and resistance to biological degradation, most PAHs bind to soil particles and sediments, which make them less available for biological uptake [3]. Thus, they finally accumulate in the environment. The treatment of PAH-contaminated sites is imperative according to new stringent regulations [4].

Since several microorganisms are capable of metabolizing PAHs [5–8], bioremediation may be a viable option for PAH-contaminated sites remediation. It has been demonstrated that bioremediation or composting can remediate soils contaminated with hydrocarbons, PAHs, chlorophenols, polychlorinated biphenyls and explosives [6–9].

Composting can be used for bioremediation purposes. However, there is still a need for more investigation in the bioremediation of hazardous wastes like PAHs using highly available and low-cost co-substrates [6–10]. The effectiveness of the composting process offers the potential for substantial cost saving over other chemical and physical methods; also it is a relatively simple process to implement and operate, as raw materials for composting are often organic wastes available everywhere.

Composting is a biological process in which microorganisms are responsible for mineralization and humification of organic matter under optimal conditions of temperature, oxygen availability and moisture, which can increase the enzyme kinetics involved in the degradation of PAHs, solubility and mass transfer rates of the contaminants [7,11]. In the process of composting a contaminated soil, organic amendments are often added to increase the amount of nutrients and the readily biodegradable organic matter present in soil [12]. Also, the application of organic wastes in the bioremediation of PAH-contaminated soil may provide ways for recycling and reutilization of these organic wastes. The resulting end-product of the composting process is of great interest in soil application as fertilizer or organic amendment. Therefore, it seems very important to examine the effect of different organic wastes on the degradation of PAH-contaminated soil.

Recent studies have demonstrated the efficiency of soil bioremediation by composting, but usually these studies only focus on one type of organic amendment, where nowadays various sources of these amendments are available. Carrying out a screening study

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Table 1
Characteristics of the soil and organic amendments used.

	Parameter/material[a]						
	Soil	OFMSW	COFMSW	HCOFMSW	ADS	NDS	CNDS
Moisture content (%)	11.4	54.4	49.7	48.2	83.4	79.5	68.1
Organic matter content (%, db)[b]	2.7	67.7	52.4	47.3	41.2	76.4	83.2
Total carbon (mg C g^{-1}) (db)	12.4	315.5	201.3	251.8	280.7	451.3	483.5
Total Nitrogen Kjeldahl (mg N g^{-1}) (db)	6.5	19.4	19.0	26.1	21.3	44.6	42.8
C/N ratio	1.9	16.3	10.6	9.6	13.2	10.1	11.3
Phosphorous (%) (db)	–	0.5	1.4	1.6	1.0	0.8	1.0
Potassium (%) (db)	–	0.8	2.0	2.5	0.17	0.33	0.12
pH	6.7	5.8	7.4	8.7	8.1	6.5	8.7
Electrical conductivity (mS/cm)	0.2	2.8	2.9	3.0	2.0	2.2	1.5
Static respiration index (mg O_2 g^{-1} OM h^{-1})[c]	0	5.0	2.6	1.7	1.4	2.9	2.8

[a] OFMSW: raw organic fraction of municipal solid wastes; COFMSW: industrial compost from OFMSW composting; HCOFMSW: compost derived from home composting of OFMSW; ADS: anaerobically digested sludge; NDS: non-digested activated sludge; CNDS: centrifuged non-digested activated sludge.
[b] db: dry basis.
[c] OM: organic matter.

to have a general idea about applicability of various organic wastes to PAHs composting should help to select future approaches to be adopted for the treatment of PAH-contaminated soils.

2. Materials and methods

2.1. Soil

An uncontaminated agricultural soil classified as sandy loam soil was collected from the surface horizon (0–30 cm) in Prades (Tarragona, Spain). The soil was air-dried, sieved to 2 mm and kept at 4 °C until use. The soil consists of 73% sand, 19% silt and 8% clay. No PAHs were detected in the soil. The main properties of the soil are presented in Table 1.

2.2. Contaminant

Pyrene (98% purity, Sigma–Aldrich, Spain), composed of four benzene rings fused together was selected as PAH contaminant to be monitored during soil composting treatments. Pyrene is a toxic, recalcitrant PAH commonly found in soil and considered as an indicator of monitoring PAH-contaminated wastes, and it is listed among the 16 USEPA priority PAHs pollutants [13]. Normally PAHs with four or more fused aromatic rings are recalcitrant to microbial attack and are not easily degraded [5]; therefore it can be considered a good alternative for organic amendments' screening studies on bioremediation. This contaminant was spiked into the soil to obtain the desired concentration (1 g of pyrene per dry kg of soil). This concentration can be considered relatively high according to previous studies [5–7] although it has been previously used in bioremediation processes.

2.3. Co-substrates (organic amendments)

Several organic amendments were tested to identify their effect as composting co-substrates in the bioremediation of pyrene-contaminated soil. These organic co-substrates include: raw organic fraction of municipal solid wastes (OFMSW), industrial compost from the OFMSW composting (COFMSW), compost derived from home composting process of the OFMSW (HCOFMSW), anaerobically digested sludge (ADS), non-digested activated sludge (NDS) and centrifuged non-digested activated sludge (CNDS). OFMSW and COFMSW were obtained from a composting plant (Barcelona, Spain) and HCOFMSW was obtained from a home composter in the University Autònoma of Barcelona, with wastes of the same origin and constituents than those used in industrial composting. ADS, NDS and CNDS were obtained from different wastewater treatment plants (Barcelona, Spain) with different operation technologies. Main characteristics of soil and organic amendments are presented in Table 1. Heavy metal contents are of Class A (the lowest content) for all the organic amendments according to National Spanish Ministry of Agriculture (http://www.mapa.es/agricultura/pags/fertilizantes/documentos/RD824_2005.pdf).

2.4. Preparation of composting experiments

Firstly, the contaminated soil was manually mixed with the organic amendment at a ratio of 1:1 (w/w, wet weight). The mixture was then mixed with bulking agent at a ratio of 1:1 (v/v) to provide a proper porosity to maintain aerobic conditions. The bulking agent consists of wood chips and pruning wastes and it can be considered not biodegradable under laboratory composting conditions. Water content of the composting matrix was adjusted to be within the recommended value (40–60%) by adding tap water before composting. The final wet weight ratio of soil to amendment was dependent on the biodegradation of organic matter, but as a general rule, it was within 1:1 to 1:0.5, once bulking agent was sieved. All the composting experiments were carried out in duplicates.

2.5. Laboratory reactors

4.5-L Dewar vessels were modified and conditioned to operate in a batch operation mode in the composting process [14]. Temperature was monitored by Pt-100 sensors (Sensotran, Spain) connected to a data acquisition system (DAS-8000, Desin, Spain). Aeration was provided sporadically to the reactors according to the process performance, where oxygen concentration was maintained between 15–18% to ensure aerobic conditions. Oxygen concentration was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom). Composting time was set at 25 days because in this type of reactors this is a sufficient time to cover completely the active decomposition stage of the composting process [14]. The volatilization of pyrene during the composting process was considered negligible since blank experiments with sterilized soil showed that the contaminant concentration remained constant during more than 25 days.

2.6. Analytical procedures

Stability of organic amendments was determined using the static respirometric index determined according to Barrena et al. [15]. This index is equivalent to the oxygen uptake rate of the material. Moisture content, organic matter content (OM), total Kjeldahl

nitrogen, total phosphorous, total potassium, total carbon content, pH and electrical conductivity were determined according to standard methods [16].

Sampling was performed by opening the reactor and mixing well its content to get two representative grab samples (about 20–30 g). Afterwards, 10 g from each sample were extracted using acetone/dichloromethane (1:1, v/v) as solvent during 2 h. After this extraction the solvent was left to evaporate during 24 h and then the remaining residue (extract) was dissolved in 10 ml of dichloromethane. A 1-μl extract of this solution was injected in a gas chromatograph (GC8690N, Agilent, Spain) equipped with flame ionization detector (FID) and splitless injector. A Zebron ZB-5HT Inferno column (Agilent, Spain) was used for pyrene identification. Initial temperature was maintained at 50 °C for 1 min, 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. The concentration of pyrene was determined after the calibration of the method with standard pyrene samples (0.010–2 g/kg, correlation coefficient $R^2 = 0.9983$). Quality assurance and quality control data indicated that both the extraction system and gas chromatography are within the acceptable level according to international methods. Results are presented as average of two samples with standard deviation.

2.7. Statistics

Anova test was performed to compare different degradation values obtained from duplicate experiments. If Anova test resulted in statistically significant differences, Tukey test was performed in pairwise comparisons. 95% confidence level was selected for all statistical comparisons. Statistical tests were conducted with SPSS 15.0.1 (SPSS Inc., USA).

3. Results and discussion

Table 1 summarizes the characteristics of the materials used. All the organic wastes proposed as amendments were characterized by a high content of organic matter in comparison to soil. This makes them good candidates for composting purposes as nutrient sources to encourage the growth of the populations already present in the soil. Also these amendments are suggested to provide valuable populations of microorganisms, which along with the indigenous ones can degrade the contaminant. In terms of nutrient composition, it is evident that both composts from OFMSW presented higher levels of P and K, which could enhance the biological activity. Moreover, values of pH and electrical conductivity are different to some extent among them, which can also affect the process. Normally, microorganisms function within a narrow optimal range of pH and their activity is inhibited in more acidic or alkaline conditions. As expected, the main difference observed was the respiration index that presented high values for non-stabilized wastes (NDS, CNDS and specially raw OFMSW) and lower values for biologically treated materials (ADS and HCOFMSW), as reported in previous studies [17]. The only abnormal value of stability was found for industrial COFMSW, which was probably due to an incomplete curing process. However, no data about the composting process at full-scale was available. Although the identification of microorganisms was not performed, the respiration index is considered a suitable index to measure the overall aerobic microbial activity of the used amendments [15,18].

Fig. 1 presents the results obtained for the pyrene removal when the composting experiments were carried out. Although a certain removal was achieved in the control experiment (non-amended soil), it was evident that the percentage of pyrene degradation significantly increased in the presence of some organic amendments.

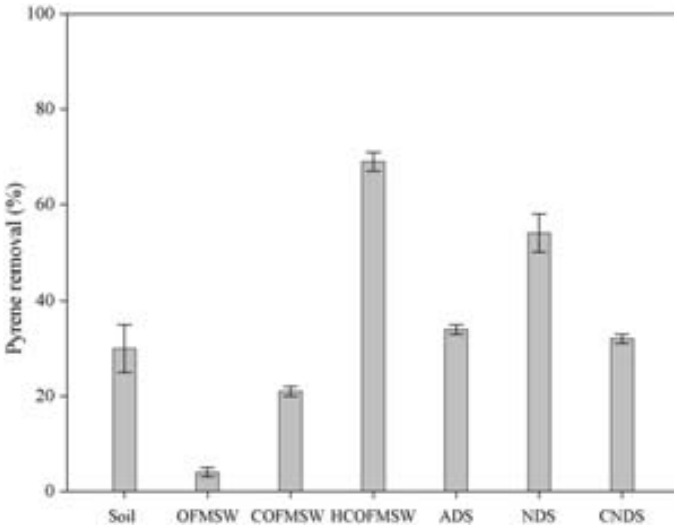

Fig. 1. Removal of pyrene in the soil after composting with organic wastes following 25 days of treatment. Results are presented as average of two samples with standard deviation. OFMSW: raw organic fraction of municipal solid wastes; COFMSW: industrial compost from OFMSW composting; HCOFMSW: compost derived from home composting of OFMSW; ADS: anaerobically digested sludge; NDS: non-digested activated sludge; CNDS: centrifuged non-digested activated sludge.

Specifically, degradation using OFMSW of several stability degrees (OFMSW, COFMSW, HCOFMSW) or several types of sludge (ADS, NDS, CNDS) were statistically different among them and different from control with soil. According to this, the stimulation of the indigenous microorganisms by adding suitable organic amendments could be a viable option, although the selection of the organic amendment is crucial to enhance PAHs biodegradation.

For instance, raw OFMSW was inefficient to enhance pyrene removal although most factors related to a successful composting process were in the optimal ranges [19]. In this case, temperature rapidly reached the thermophilic range and it was maintained in this range for six days (Fig. 2). In fact, it is observed that a there is a positive correlation between the maximum temperature achieved in the composting experiments and the level of respiration index (Table 1). Accordingly, respiration index can be determined for

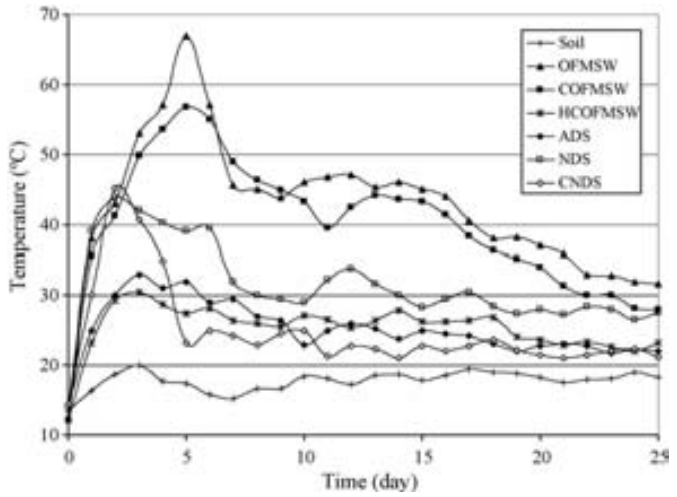

Fig. 2. Temperature profiles of the composting process with organic wastes. Temperature differences between duplicate experiments were lower than 5 °C. OFMSW: raw organic fraction of municipal solid wastes; COFMSW: industrial compost from OFMSW composting; HCOFMSW: compost derived from home composting; ADS: anaerobically digested sludge; NDS: non-digested activated sludge; CNDS: centrifuged non-digested activated sludge.

predicting the temperature to carry out a bioremediation process (thermophilic or mesophilic). This is of special relevance for PAH biodegradation. Although it is reported that high temperatures are supposed to increase the desorption of hydrophobic contaminant, to improve the mass transfer rates and to enhance the enzymatic kinetics involved in the biodegradation process [20], the results obtained in this study demonstrated that these conditions were not adequate for microorganisms responsible for pyrene biodegradation. This could be explained by the preferential degradation of easily degradable material observed in some organic amendments rather than PAHs as more decrease in the organic matter (11%) was observed in less stable amendments when comparing to those presenting a high degree of stability (Table 1). Another hypothesis can be the negative effect of high temperatures on specific microorganisms responsible for PAHs degradation, as reported in other studies [21]. A similar behaviour was observed with COFMSW, which is biologically active according to respiration index (Table 1). In fact, when considering only wastes whose source is OFMSW, it seemed to be an exponential positive correlation between waste stability and pyrene removal (Fig. 1) in the sense that, the more stable a waste is, the more pyrene removal is observed. Although only three stability values were available, for this waste this correlation presented a correlation coefficient of 0.95 (Eq. (1)):

$$\text{Pyrene removal}\,(\%) = 621\exp(-1.24\text{RI}) \quad (1)$$

where RI is the static respiration index expressed in mg $O_2\,g^{-1}\,OM\,h^{-1}$.

Other studies have shown that the fraction of humic acids present in stable compost enhance the desorption process of hydrophobic organic components from soil [22], which increases the contaminant bioavailability, thus increasing the rate of the degradation. Furthermore, it has been suggested that the sorption of both microorganisms and PAHs to the colloidal surfaces of humic matter stimulate their biodegradation [23]. Unfortunately, the humic characterization of the organic amendments used in this study was not carried out, although it can be the focus of future studies.

Experiments with several typologies of wastewater sludge as organic amendment showed different results. Pyrene removal was similar to that of soil for ADS and CNDS, and higher for NDS. In this case, no clear correlation could be found among stability and pyrene degradation since stability values were similar (especially NDS and CNDS) and the temperature reached was in the mesophilic range during all the composting process. Although sewage sludge has been reported to enhance the degradation of hydrocarbons in soil-compost mixtures [24,25], no results have been found on the use of different typologies of sludge to enhance PAHs biodegradation. In this case, the bioavailability of the contaminants can be affected by the sludge type and, consequently, the degradation rate. Oleszczuk [25] noted a great difference in the bioavailable fraction of PAH depending on the stage of the experiment and the sludge type.

4. Conclusions

The preliminary results presented in this work show that low-cost and easily available organic wastes can be used as organic amendments to enhance the biodegradation of pyrene-contaminated soils. In particular, stable home compost from OFMSW was found to improve significantly the pyrene removal. Further studies should be focused on the importance of organic amendments' stability for PAHs biodegradation in composting processes and in the knowledge of whether stability influence is related to chemical composition (for instance, presence of humic acids) or the microbiological communities that are active at each stability level. Nevertheless, it is important to mention that the use of stable amendments for soil bioremediation will inherently produce an end-product of high stability, which can be used as organic amendment or fertilizer.

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Article II

Optimization and enhancement of soil bioremediation by composting using the experimental design technique.

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ORIGINAL PAPER

Optimization and enhancement of soil bioremediation by composting using the experimental design technique

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Abstract The objective of this study was the application of the experimental design technique to optimize the conditions for the bioremediation of contaminated soil by means of composting. A low-cost material such as compost from the Organic Fraction of Municipal Solid Waste as amendment and pyrene as model pollutant were used. The effect of three factors was considered: pollutant concentration (0.1–2 g/kg), soil:compost mixing ratio (1:0.5–1:2 w/w) and compost stability measured as respiration index (0.78, 2.69 and 4.52 mg O_2 g^{-1} Organic Matter h^{-1}). Stable compost permitted to achieve an almost complete degradation of pyrene in a short time (10 days). Results indicated that compost stability is a key parameter to optimize PAHs biodegradation. A factor analysis indicated that the optimal conditions for bioremediation after 10, 20 and 30 days of process were (1.4, 0.78, 1:1.4), (1.4, 2.18. 1:1.3) and (1.3, 2.18, 1:1.3) for concentration (g/kg), compost stability (mg O_2 g^{-1} Organic Matter h^{-1}) and soil:compost mixing ratio, respectively.

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Keywords Soil bioremediation · Compost stability · Experimental design · Pyrene · Municipal solid waste

Introduction

In recent years, the development of chemical processes has contributed to the increase of environmental pollution (Harrison 2001). Simultaneously, the widespread of society's environmental concern and the publication of strict environmental regulations have helped in reducing the levels of pollutants in the environment. However, many pollutants are still released and some of them are eventually deposited in soil, which has received less attention than other media such as water resources or atmosphere. Consequently, contaminated soils constitute one of the major environmental issues that needs to be resolved (Boopathy 2000; Ohura et al. 2004).

Polycyclic aromatic hydrocarbons (PAHs) constitute an important group among the different pollutants that are introduced to the environment either by natural or anthropogenic sources (Johnson et al. 2005; Ohura et al. 2004). Their composition of fused aromatic rings and their specific physical properties such as low aqueous solubility and high solid–water distribution ratios stand against their straightforward microbial utilization and promote their accumulation in the solid phases of the terrestrial environment

(Johnson et al. 2005). PAHs with low molecular weigh such as naphthalene, phenanthrene and anthracene are relatively easy to degrade. However, PAHs with four or more fused aromatic rings are more recalcitrant to microbial attack and are not easily degraded (Cerniglia 1992; Wilson and Jones 1993; Serrano Silva et al. 2009).

Bioremediation techniques have been developed and improved to remediate soils polluted with hazardous chemicals (Romantschuk et al. 2000; Bento et al. 2005). In this framework, composting appears to be particularly useful in the bioremediation of petroleum hydrocarbons, especially the PAH fraction (Antizar-Ladislao et al. 2004, 2006; Haderlein et al. 2006).

Although the concept of treating PAH-contaminated soil by means of co-composting with organic materials or by mixing soil with finished compost has been reviewed (Semple et al. 2001; Antizar-Ladislao et al. 2004) and has proved to be effective in the degradation of PAHs (Beaudin et al. 1996), composting of contaminated soil can be still considered an emerging ex situ biotreatment (Antizar-Ladislao et al. 2006). Specifically, there is a lack of knowledge about the optimal conditions for PAH biodegradation and the properties that a given compost should present to enhance a rapid pollutant removal. In addition, it is necessary to develop a systematic approach that permits to determine the optimal conditions for PAH bioremediation in soil.

According to previous studies, several organic amendments have been used in the bioremediation of contaminated soil, and different biodegradation rates have been obtained. Clearly, when the organic amendment is preferred over the target compound, microbial activity for degrading the target pollutants may be inhibited (Cookson 1995). Moreover, the application of bioremediation for soil decontamination may be further limited by a large number of environmental parameters such as temperature, pH, oxygen availability, nutrients and salinity. Thus, the microbial activity that is necessary for the transformation of organic contaminants in soil (Marín et al. 2005) should be maintained at adequate levels during the bioremediation process to degrade the target contaminant. Accordingly, the addition of compost can facilitate the degradation of organic contaminants because compost adds supplement nutrients and carbon into the contaminated soil (Namkoong et al. 2002; Oleszczuk 2007; Anastasi et al. 2009). Nevertheless, little or no research has been conducted regarding the effect of compost stability on the composting of PAH-contaminated soils, a point that is crucial to understand the real role of organic amendments in soil, as stability is directly related to organic matter composition and biological activity (Said-Pullicino and Gigliotti 2007; Barrena et al. 2009).

Finally, the ratio of the contaminated soil to compost should be also determined since an inappropriate ratio may retard or inhibit microbial activity (Thomas et al. 1992; Namkoong et al. 2002). On the other hand, the economics of a composting technology is based on the amount of the compost to be added to soil. Thus, the overall cost of the system is decreased upon increasing the amount of the contaminated soil ratio to the compost where an equivalent microbial activity and efficiency may be maintained (In et al. 2007).

Since biological and biochemical models are not currently available for a detailed description of soil bioremediation processes, statistical experimental design techniques appears as a suitable and feasible method that can be used to obtain relevant and reproducible information for process optimization, data processing, calibration, quality control and organization of the analytical process. These techniques have been largely applied to other research areas (Deming and Morgan 1987; Sánchez et al. 2000; Delgado-Moreno et al. 2009). However, its application to soil bioremediation is scarce, and often the factors affecting biodegradation in soils are studied separately, which adds an important uncertainty to the conclusions presented and difficulties for results interpretation.

The objective of this work is to systematically study the effect of contaminant concentration, soil:compost mixing ratio and compost stability on the optimization of soil bioremediation using a low cost material for the composting process such as compost from the Organic Fraction of Municipal Solid Waste (OFMSW). To the best of our knowledge, no reports are available in literature regarding the influence of the compost properties on the bioremediation of PAH contaminated soil, and the relation among compost stability, contaminant concentration and soil:compost mixing ratio. Pyrene was used as PAH model and composting experiments

were conducted at laboratory-scale reproducing the conditions found at full scale.

Materials and methods

Soil

The soil used in this study was an uncontaminated soil classified as sandy loam soil obtained from Prades (Tarragona, Spain). The soil was collected from the surfaces horizon (0–30 cm). The soil was air-dried, sieved to 2 mm and kept at 4°C until use. The soil consists of 73% sand, 19% silt and 8% clay. Other characteristics of the soil are shown in Table 1. PAHs concentrations were below the detection limit in soil.

Contaminant

Pyrene (98% purity, Sigma–Aldrich, Spain) was selected as model PAH to be monitored during soil composting experiments. This contaminant was dissolved (5 g/l) in a volatile organic solvent (dichloromethane) and homogenously spiked into the soil to obtain the desired concentration according to the experimental design values (range of 0.1–2 g/kg).

Compost

Three types of compost coming from OFMSW with different levels of stability were used during the experimental design to evaluate the effect of these composts on the degradation of pyrene-contaminated soil. Compost A was obtained from a home composter in the University Autònoma of Barcelona. Compost B and Compost C were obtained from two composting plants located in Barcelona (Spain). Their levels of stability can be considered as full-stable for Compost A, moderately stable for Compost B and unstable for Compost C. Their levels of stability are selected to cover the full range of organic matter stability that can be found in OFMSW compost and related materials, whose range is from 0.5 to 1 mg O_2 g^{-1} Organic Matter h^{-1} for stable OFMSW compost to 7–8 mg O_2 g^{-1} Organic Matter h^{-1} for raw OFMSW (Ponsá et al. 2008; Ruggieri et al. 2008). The main characteristics of the three composts used are also presented in Table 1. Preliminary analysis showed that they are essentially free of PAHs.

Laboratory-scale composting system

Dewar® vessels (4.5 l) were modified and conditioned to operate as batch-mode reactors for the composting experiments. These reactors were thermally isolated, so the influence of ambient temperature could be ignored, besides being perfectly closed. These conditions were similar to that of composting at full-scale, where the isolating properties of organic matter make the composting process to occur under quasi-adiabatic conditions (Barrena et al. 2006).

The air was provided through a pipeline connected to the bottom of the reactor were a plastic mesh is placed to insure a correct distribution of air through the composting mixture. The exhaust air exits the composter through a hole in the reactor cover and then oxygen concentration is monitored. Oxygen concentration was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom). Aeration was sporadically provided to the reactors according to the

Table 1 Characteristics of the soil and composts used in the experimental design

Parameter	Compost A	Compost B	Compost C	Soil
Respiration index (mg O_2 g^{-1} OM h^{-1})	0.78	2.69	4.52	–
Organic matter content (%, db)	53.9	66.7	52.7	2.7
Moisture content (%, wb)	30.0	30.0	40.6	11.4
Kjeldahl nitrogen (mg N g^{-1} DM)	27.3	40.0	19.7	6.45
Total carbon (mg C g^{-1} DM)	255.6	315.9	205.4	12.4
Electrical conductivity (ms/cm)	6.46	7.36	7.13	0.2
pH	8.63	8.41	7.61	6.7

OM organic matter, *DM* dry matter, *db* dry basis, *wb* wet basis

process performance, when oxygen concentration was considered limiting for aerobic conditions (lower than 10% in exhaust air). Temperature was monitored by Pt-100 sensors (Sensotran, Spain) connected to a data acquisition system (DAS-8000, Desin, Spain) that was connected to a personal computer. The software used (Proasis®Das-Win 2.1, Desin, Spain) also permitted to monitor both the temperature and oxygen content in the reactors.

Soil-compost composting system

Mixtures to be composted were prepared as follows. First, the soil was artificially contaminated by spiking the contaminant (pyrene) at different concentrations ranging between 0.1 and 2.0 g/kg according to the experimental design explained later. Then the soil was mixed with composts A, B or C at different mixing ratios (1:0.5–1:2 w/w) and finally the resulting mixture was mixed with bulking agent at a ratio of 1:1 v/v to provide a proper porosity to maintain aerobic conditions. This ratio can be considered typical and non-limiting for the composting of OFMSW (Ruggieri et al. 2009). The bulking agent used consisted of wood chips and pruning wastes that were not biodegraded under laboratory composting conditions. If necessary, water content of the composting mixture was adjusted to be within the recommended value (40–60%) by adding tap water at the beginning of each composting experiment.

Composting mixtures were manually prepared according to the values proposed from the experimental design technique (Table 2) as explained below.

Experimental design methodology

The initial conditions for pyrene degradation in composting experiments were selected according to a Central Composite Design (CCD). This approach is adequate since it implies a reduced number of experiments in comparison to a full factorial design.

Briefly, the percentage of pyrene degradation was statistically modeled as a function of three fundamental operating variables, which are the factors of the CCD, that is: initial pyrene concentration (x_1), compost stability measured as respiration index (x_2) and soil:compost mixing ratio (x_3). The factor levels

Table 2 Experimental design matrix with the corresponding normalized coded levels for each factor considered

Run	Concentration (g/kg)	Stability (mg O_2 g^{-1} OM h^{-1})	Mixing ratio (soil:compost)	Degradation (%)
R01	1	1	1:1	89.80
R02	1	−1	1:1	92.80
R03	1	1	1:−1	90.27
R04	1	−1	1:−1	86.72
R05	−1	1	1:1	69.05
R06	−1	−1	1:1	81.99
R07	−1	1	1:−1	65.00
R08	−1	−1	1:−1	76.63
R09	0	0	1:0	98.05
R10	0	1	1:0	91.90
R11	0	−1	1:0	99.31
R12	0	0	1:α	97.02
R13	0	0	1:−α	94.34
R14	α	0	1:0	96.60
R15	−α	0	1:0	87.32
R16[a]	0	0	1:0	98.86
R17	0	0	1:0	98.70
R18	0	0	1:0	98.01
R19	0	0	1:0	98.53
R20	0	0	1:0	98.32

[a] R16–R20 are the replicates carried out at the central point to validate statistically the experimental design

Table 3 Natural values of the levels of the factors considered in the experimental design

Factor	$-\alpha$	-1	0	1	α
Concentration (g/kg)	0.1	0.5	1.05	1.6	2
Stability (mg O_2 g^{-1} OM h^{-1})	–	0.78	2.70	4.52	–
Mixing ratio (soil:compost)	0.5	0.83	1.24	1.66	2

were normalized and coded with the notations ($-\alpha$, -1, 0, 1, α). The value of $\alpha = 1.68$ is determined according to the number of the factors and levels to be studied. A detailed description of the CCD experimental design and its application can be found elsewhere (Deming and Morgan 1987; Rigas et al. 2005).

Table 3 shows the normalized coded levels and the natural values of these factors set in the statistical experiment, whereas Table 2 presents the experimental design matrix and the factor combinations along with the replicates which were run at the central point for statistical validation. A total number of 20 experiments were carried out to represent the system, where a full second-order polynomial model was selected to fit each response function (Y, representing pyrene biodegradation) to the values of the factors considered according to Eq. 1:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

For each sampling day (10, 20 and 30 days of process), the model parameters b_i were estimated from experimental values using a multi regression software (Sigmaplot® 8.0, Systat Software Inc, San Jose, USA).

Beside the 20 experiments, control reactor which has only contaminated soil (1 g/kg) and bulking agent was used to follow the degradation of the contaminant by indigenous microorganisms already exist in the soil to be compared with the other amended reactors.

Sampling

Initial concentration of the contaminated soil was measured in three reactors where two grab fresh samples (20–30 g) were taken to be sure that the concentrations were homogeneous and within the required values. The differences among theoretical and actual values were less than 3%.

Samples were collected after 10, 20 and 30 days of composting. The reactors were opened and the reactor contents were mixed well to get homogenous and representative samples. Then two grab fresh samples (20–30 g) were taken, manually grinded, dried, sieved (2 mm) for analysis. During each sampling, moisture content was adjusted, if necessary.

Analytical procedures

Compost stability defined as the rate of organic matter degradation as a result of microbiological activity was determined using the dynamic respirometric index (DRI), determined according to Barrena et al. (2009). Briefly, this index represents the oxygen consumption of a known sample of organic matter incubated under optimal conditions and with a continuous air supply. Moisture content, organic matter content (OM), total carbon content, Kjeldahl nitrogen, pH and electrical conductivity were determined according to standard methods (The US Department of Agriculture and the US Composting Council 2001).

Pyrene in the composting mixture was extracted using a Soxhlet extraction process, then it was determined by gas chromatography. Duplicate 10 g samples were extracted using acetone/dichloromethane (1:1 v/v) as solvent during 2 h. After extraction the solvent was left to evaporate and then the remaining residue (extract) was dissolved in 10 ml of dichloromethane. A 1-µl extract of this solution was injected in a gas chromatograph (GC8690 N, 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, 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. The concentration of pyrene was determined after the calibration of the method with standard pyrene samples. The recovery of pyrene using this method was higher than 95%. Blank abiotic experiments showed no significant volatilization of pyrene by directly analyzing gas samples from composting exhaust gases.

Data statistical analysis

Statistical analysis was performed for all variables using the Sigmaplot® 8.0 software package (Systat Software Inc, San Jose, USA). The replication of experiments at central point permits the statistical validation of results according to CCD experimental design (Deming and Morgan 1987). The optimization of the proposed polynomial function to obtain the optimal conditions corresponding to the three factors considered was solved by using a self-made program using C-language.

Results and discussion

Characterization of materials

Table 1 summarizes the characteristics of the raw materials used in the experimental design. The soil low content of organic matter (2.7%), which is typical of Mediterranean soils (Cayuela et al. 2009), is a good indication of the weakness of the soil by itself to support the activity of microorganisms for any bioremediation process, which is typical of Mediterranean soils (Cayuela et al. 2009). Contrarily, the three composts have an important percentage of organic matter that can be a suitable source for the carbon and nutrients needed for the remediation process to stimulate the microbial activity and consequently the contaminant degradation (Haderlein et al. 2006). The most significant difference found in the three composts used in this study is the degree of stability, whereas pH and electrical conductivity are relatively similar. Compost stability is an important factor related to organic matter composition (Said-Pullicino and Gigliotti 2007). In addition, other authors have pointed that, although stable compost inherently implies low microbial activity, it contents a high percent of humic matter (Gourlay et al. 2003; Plaza et al. 2009), which is strongly related to the possibilities of solubilize PAHs (Quadri et al. 2008). Therefore, the study of compost stability as a controlling factor for the bioremediation of contaminated soil can be of great interest since a waste treatment plant can easily produce compost of different stability by adjusting the conditions and duration of the biological process (Ponsá et al. 2008; Barrena et al. 2009).

Response surface analysis and interpretation

Experimental design methodology was applied to investigate the response of composting process of pyrene-contaminated soil under the effect of the previously mentioned factors. The percentage of pyrene degradation (Y) in the process was measured at 10, 20 and 30 days. The second-order polynomial model was able to fit the obtained results and to statistically represent the process response. The values of the b_i coefficients of the fitted model and the correlation coefficients (R^2) corresponding to each time (10, 20 and 30 days) are presented in Table 4. As can be observed, the values of R^2 and p demonstrate the suitability of the model to fit the experimental results. Specifically, and according to R^2 and p values, the second-order model was adequate to represent the results after 20 and 30 days of composting process; however, experimental values obtained at 10 days were not completely adjusted even when other more complex polynomial models were tested, which means that, at short process times, the studied factors only explain partially the response of the system. Other authors have found similar results when using second-order models to represent the biodegradation of petroleum hydrocarbons (Vieira et al. 2009), although in that case a composting process was not used.

Table 4 Values of b_i coefficients, R^2 and p obtained after 10, 20 and 30 days according to the fit of experimental values to Eq. 1

Coefficient	Time		
	10 days	20 days	30 days
b_0	26.3	16.3	38.35
b_1	69.4	49.96	29.03
b_2	0.05	6.15	13.79
b_3	16.3	60.02	35.8
b_{11}	−20.1	−16.18	−11.09
b_{22}	0.13	−2.99	−3.2
b_{33}	−2.61	−17.06	−10.6
b_{12}	−3.59	4.45	3.0
b_{13}	−5.8	−10.70	−2.4
b_{23}	−0.36	0.26	−1.31
R^2	0.69	0.96	0.85
P	0.086	<0.0001	0.0038

Pyrene degradation

In Figs. 1, 2, and 3, the responses obtained for pyrene degradation according to the fitted model are presented as contour lines as a function of both the concentration and mixing ratio where stability is fixed for Composts A, B and C, which represents a different degree of stability from highly stable (A) to unstable (C). With this methodology the responses shape and the optimal conditions of the studied factors for bioremediation can be precisely described.

Figures 1, 2, and 3 shows that the three types of compost enhanced pyrene removal although the degradation rate was different. The rate of degradation is strongly correlated to the stability degree, especially in the first stage of composting (10 days, Fig. 1a). These results are in agreement with other research studies on PAHs degradation (Laor et al. 1999; Haderlein et al. 2006; Tejada et al. 2008).

During the entire composting process that took place in 30 days, a high degradation rate was observed during the first 10 days in comparison to the final period that exhibited a low rate of degradation. These observations have been previously reported in other studies (Namkoong et al. 2002; Wan et al. 2003), where a rapid degradation of total petroleum hydrocarbons (TPH) was observed in the early stage (within 15 days) of reaction in all experiments. Following the rapid degradation of TPH within 15 days, residual TPH was slowly degraded. Also, Lee et al. (2008) observed two differentiated phase in compost-amended soil during the degradation process, where about the 82% of the overall degradation occurred in the first stage.

In this study, the degradation rate in the last days decreased, which might be explained by the low content of contaminant and nutrients. Anyway, it is worthwhile to mention that a practically complete pyrene removal was observed by the end of the process with both Compost A and Compost B (Fig. 3). Since, to our knowledge, no such complete removal has been previously reported for a wide range of pollutant concentrations, this fact confirms the suitability of the composting technology for the bioremediation of contaminated soil when the controlling factors are systematically studied and suitable amendments are provided.

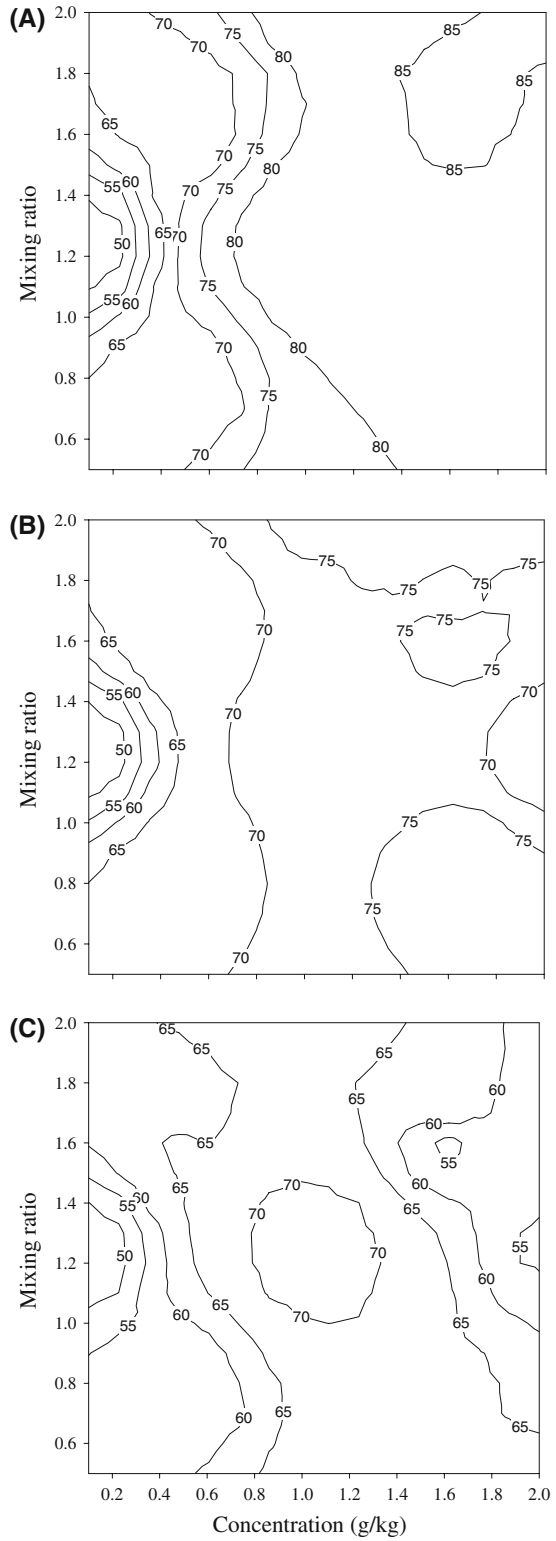

Fig. 1 Percentage of pyrene degradation after 10 days of process. **a** Compost A; **b** Compost B; **c** Compost C

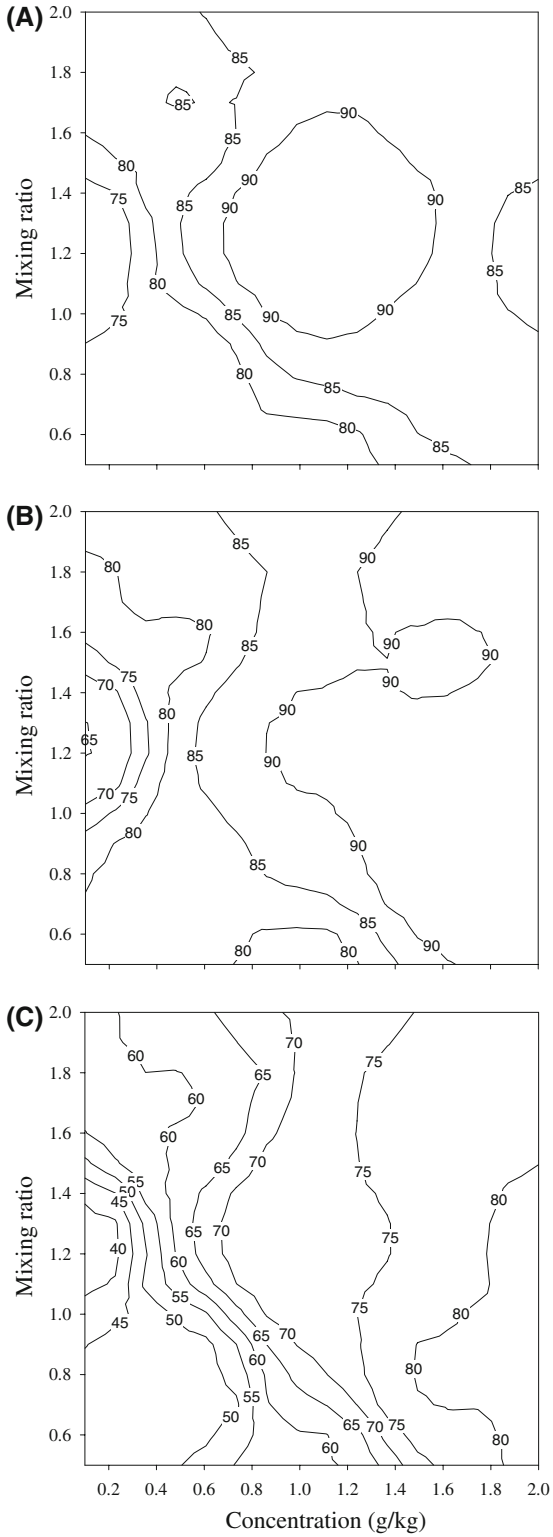

Fig. 2 Percentage of pyrene degradation after 20 days of process. **a** Compost A; **b** Compost B; **c** Compost C

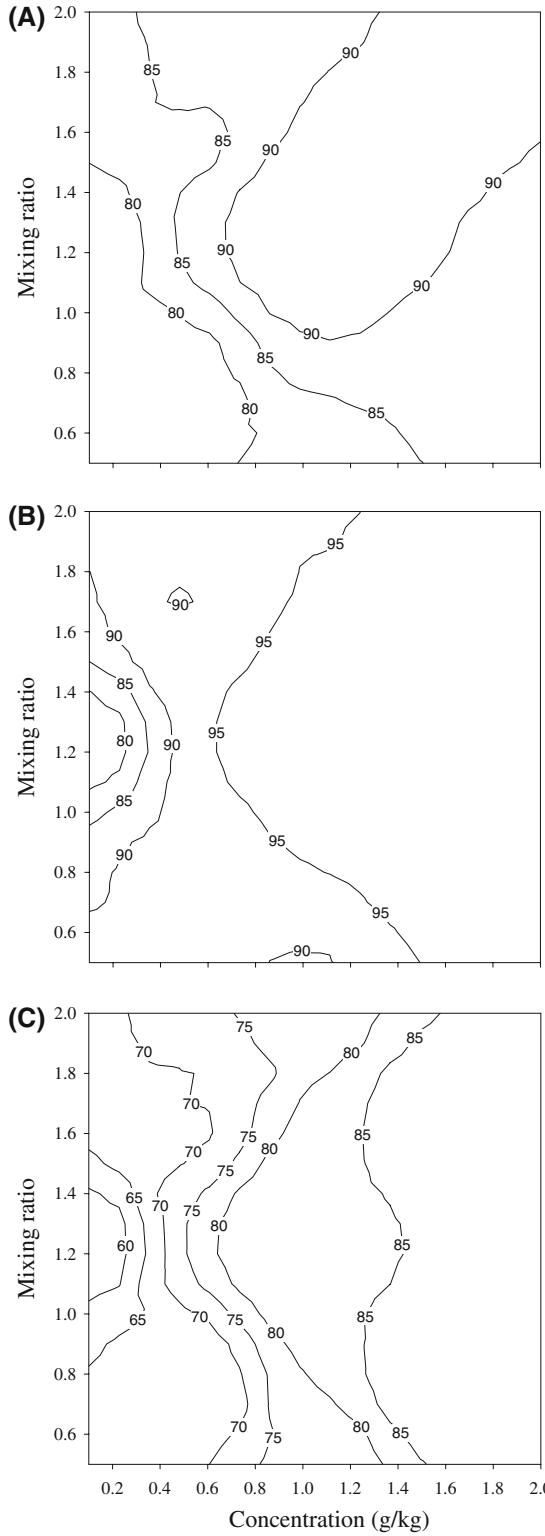

Fig. 3 Percentage of pyrene degradation after 30 days of process. **a** Compost A; **b** Compost B; **c** Compost C

Effect of compost stability on the composting process

As expected, compost stability (Table 1) strongly influenced the composting process, where the most active compost (C) followed by moderately stable compost (B) presented a high microbial activity during the first 10 days. The increase in the temperatures observed and the high rate of aeration needed (data not shown) are evidences of such activity. After a lag period of about 2 days the temperature began to increase to reach thermophilic ranges (>45°C), which were maintained for 1 week. Contrarily, experiments with Compost A were always in the mesophilic range of temperature. It is important to note that, although Compost C presented a high microbial activity, this was not favorable for pyrene degradation.

In relation to process temperature, it is clear that mesophilic range is preferable for microorganisms responsible for PAHs biodegradation, although high temperatures are supposed to increase the desorption and mass transformation of the contaminant and to enhance the enzyme kinetics (Pignatello and Xing 1996). The effect of temperature is in agreement with other studies that show the inactivation of bioremediation of PAHs in contaminated soil by thermophlic temperatures (Potter et al. 1999; Antizar-Ladislao et al. 2004; Haderlein et al. 2006; Hesnawi and McCartney 2006). In addition, stable compost are believed to have a significant portion of humic acids, which were found to enhance the desorption of hydrophobic organic components from soil (Janzen et al. 1996; Quadri et al. 2008), which increases the contaminant bioavailability. Furthermore, it has been suggested that the sorption of both the microorganisms and the PAHs to the colloidal surfaces of humic matter stimulate their biodegradation (Laor et al. 1999).

In our study, it is evident that stable compost (A) resulted in high removal percentages at short process times, but it is also important to note that with more time, Compost B achieves similar results. It can be hypothesized that the stabilization of organic matter that is inherent to any composting process finally results in the same bioremediation results, if enough time is given. These observations are supported by other studies (Plaza et al. 2009), which suggest that the changes undergone by the humic acids fraction during composting may be expected to contribute to facilitate microbial accessibility to PAHs, whereas other works point out that the application of compost to PAH-contaminated soil is expected to increase the affinity of soil humic acids for PAHs (Senesi et al. 2007; Plaza et al. 2009).

Effect of mixing ratio

Figures 1, 2, and 3 show the effect of the different mixing ratios on the process performance. This factor is especially important since the determination of the minimum quantity of the amendment that could support and maintain the desired activity to have a high degradation rate is directly related to the process economics. In this study, the minimum mixing ratio soil:compost used (1:0.5) was able to enhance the microbial activity to degrade the contaminant up to 80% with Compost A, but the maximum rate was found when a mixing ratio of 1:1.5 was used during the first 10 days for both Compost A and B. In the remaining incubation period the mixing ratio effect was almost the same for Compost A and B, whereas a lower ratio of Compost C showed good results when compost progressively stabilize (Fig. 3c). In fact, in the case of active compost (C), high mixing ratio showed low degradation rate, which may be caused by a preferential degradation of easily degradable materials. Namkoong et al. (2002) and Chang et al. (2009) reported that while the addition of organic supplements increased the rate of contaminant degradation, excessive supplementation could eventually inhibit degradation.

Another important aspect shown in Figs. 1, 2, and 3 is the relationship between the mixing ratio and the pollutant concentration, since it is especially important to observe that when low concentrations were tested, more amount of compost was needed to accelerate the degradation rate (Figs. 1a, 2a, 3a).

Effect of pollutant concentration

In this study, high degradation rate was achieved when high pollutant concentration is available in both Compost A and Compost B in the first 10 days (Fig. 1). In Compost C, concentration within 0.8–1.3 g/kg was found to be suitable for biodegradation. Concentration above this value inhibits the activity, while the low concentration may be not sufficient to support the microbial activity. According to JØrgensen

et al. (2000), usually the degradation of hydrocarbons is governed by first-order kinetics, where the degradation rate of a compound is proportional to its concentration. In this study, a complete kinetic analysis of degradation rate showed that both first and second order kinetics can represent the experimental data. First-order rate constant was within 0.07–0.15 day^{-1}, whereas second-order rate constant was within 0.10–0.30 $(g/kg)^{-1}$ day^{-1}. These rate constants are significantly higher than those found in the bioremediation of total petroleum hydrocarbons using a composting strategy by adding grass clippings and sheep manure to soil (Mihial et al. 2006). Moreover, a clear trend was observed between the value of rate constant and the compost stability (Fig. 4). This fact again empathizes the influence of stability on PAH biodegradation rate.

Process optimization

The obtained function Y from the experimental design after 10, 20 and 30 days of composting process was used to determine the optimal combination of initial conditions for the implementation of the composting process. Table 5 shows the optimal values of the three studied factors. The results obtained confirmed again that Compost A gives better results than the other used compost at short times. With this compost, 86% of pyrene removal was reached for experiment runs R01 and R11. The same results were obtained using the second-order model function. At longer process times (20 and 30 days of composting), Compost A and Compost B approximately presented the same result with a practically complete removal of pyrene. In fact, the optimized values showed that if compost has a degree of stability of about 2.18 mg O_2 g^{-1} OM h^{-1}, a 100% degradation can be achieved in 30 days. From this point of view, it is essential to study the stability of any organic amendment that it is intended to be used for soil bioremediation.

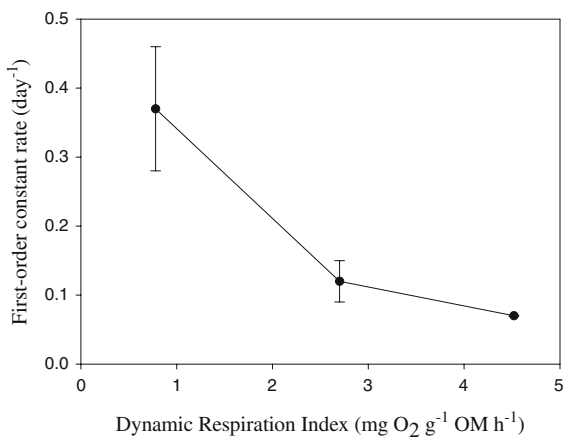

Fig. 4 Influence of compost stability on first-order rate constant

Conclusions

The results obtained in this research can provide important indications for the improvement of bioremediation processes using the composting technology. The general conclusions are summarized in the following points:

- A systematic approach is necessary to optimize the biodegradation of PAHs in soil if the composting technology has to be applied, since a large number of factors can severely affect the process performance.
- The experimental design technique seems a suitable approach to study the factors affecting the bioremediation process. A CCD technique seems a good compromise for statistical validation with an assumable number of experiments.
- CCD permits to obtain reliable values of the optimal conditions for PAH biodegradation, with the aim to minimize the cost of the process.
- Stability is the most important property when an organic waste or compost is used to enhance soil bioremediation. As this property can be easily

Table 5 Natural values of the experimental design factors for the optimal responses after 10, 20 and 30 days of process

Day	Concentration (g/kg)	Stability (mg O_2 g^{-1} OM h^{-1})	Mixing ratio (soil:compost)	Pyrene degradation (%)
10	1.4	0.78	1.4	86.1
20	1.4	2.18	1.3	97.7
30	1.3	2.18	1.3	100

changed when compost is produced, it seems a good parameter to consider in bioremediation planning.
– Further work is needed in the bioremediation with more recalcitrant PAHs or when mixtures of several PAHs are to be biodegraded.

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Article III

Effects of compost stability and contaminant concentration on the bioremediation of PAHs contaminated soil through composting.

Tahseen Sayara, Montserrat Sarrà, Antoni Sánchez.

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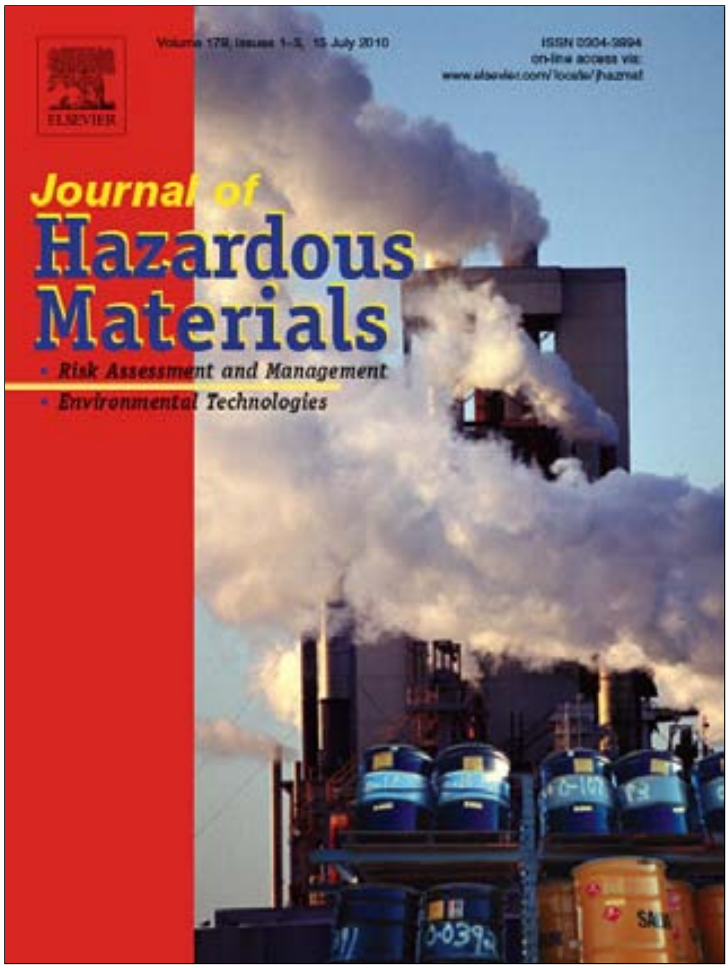

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Effects of compost stability and contaminant concentration on the bioremediation of PAHs-contaminated soil through composting

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ABSTRACT

The objective of this study was to investigate the effect of two factors: the stability degree (0.37–4.55 mg O_2 g^{-1} Organic Matter h^{-1}) of different composts derived from the organic fraction of municipal solid wastes and the concentration of a complex mixture of PAHs including flourene, phenanthrene, anthracene, flouranthene, pyrene and benzo(a)anthracene in the bioremediation of soil. The two factors were systematically studied applying central composite design methodology. The obtained results demonstrated that compost stability degree was particularly important during the first stage of the process. Stable composts enhanced the levels of degradation in soil–compost mixture and a degradation rate of 92% was achieved in this period, but only 40% was degraded with the least stable compost. The PAHs concentration was also important during the process, since the degradation rates increased with the increase in the PAHs concentration. Moreover, all the individual PAHs demonstrated a notable decrease in their concentrations after the incubation period, but pyrene was degraded to lower levels in some treatments compared to others PAHs.

1. Introduction

The large range of hazardous chemicals with different structures and different toxicity levels that are continuously released from several anthropogenic sources are continuously causing environmental pollution [1]. Polycyclic aromatic hydrocarbons (PAHs) are one of the most encountered pollutants in the ecosystems; as a consequence, soil contamination with these contaminants is a matter of major concern as they can be introduced to the soil by various sources, where these compounds are categorized as toxic for both humans and environment [2]. Indeed, with more concern regarding the ecosystem and more strict regulation like the EU landfill directive (1999/31/EC), which tries to reduce the amount of wastes that can be sent to the landfill, transforming the contaminated soil to such landfills has become limited. Furthermore, the restoration of many contaminated sites is preferable as the available agricultural areas are gradually degraded with time [3]. Accordingly, there is a critical need to develop and implement an effective remediation technology to reduce the threats caused by such contaminants and create a sustainable reuse of soil.

Bioremediation can be regarded as an attractive technology that results in the partial or complete biotransformation of many organic contaminants to microbial biomass and stable innocuous end-product. Moreover, this technology is believed to be cost-effective and environmentally accepted [4,5]. The contaminated soil is normally deficient in nutrients that are necessary to support the indigenous microorganisms to develop themselves, or sometimes the microorganisms are only available at low levels that makes the bioremediation process progress at very slow rates [6]. To overcome these conditions, normally the bioremediation of hydrocarbon contaminated soils often rely upon the addition of nutrients or microorganisms (biostimulation and bioaugmentation) [7,8]. Composting as a remediation tool has been considered a suitable technology in the bioremediation of contaminated soils, and it has been used to mitigate these limiting factors, as it also improves the soil properties [9]. On the other hand, applying composting technology provides a sustainable reuse of the organic biodegradable fraction of wastes, which is both microbial and nutrient rich. However, contaminants bioavailability is an important factor affecting microbial degradation rates in soil and sediments as the microorganism are able to attack the target contaminant only when it is dissolved within the materials [10]. Thereby, the selected organic amendment for the bioremediation process should serve to improve and overcome any deficiencies or limitations that influence the process efficiency. One challenge with this type of research is that composting feedstock composition can vary widely from one facility to another; this can affect the chemical and microbial conditions in the amendments [11]. Bioremediation of contaminated soils using composting process depends on a number of physical, chemical, and biological factors that determine the microbial accessibility to the target contaminants [10,12,13], where the amendment properties are of great role in determining the pro-

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cess behavior. Although various amendments have been applied during the composting of contaminated soil, still much specialized research is needed. Compost stability is one of these factors as this parameter is related to microbial activity within the organic material and it also can be related to the compost composition like humic matter [6]. Until now, no studies were reported that explore this important parameter and its influence on the degradation of PAHs in the soil. For an efficient treatment process, the microbial activity, which is considered the main factor in the bioremediation process, should be maintained at adequate levels [14]. The contaminants concentration is one of the factors that influence the microbial activity as these contaminants may exhibit some toxic or inhibition influence when they exist at high levels. However, low concentration could be below the required level needed to provoke the microbial enrichment to attack this contaminant [15].

In this research, we tried to investigate the bioremediation process of soil contaminated by a complex mixture of PAHs using the composting technology, where the mixture of PAHs simulated a real creosote sample. Different composts derived from the organic fraction of municipal solid wastes were suggested as organic amendments considering the effect of their different degrees of stability as an objective to be clarified. We believe that although the development and widespread application of bioremediation process are in a good research position, it still is limited by a lack of full understanding of such important factors (compost stability and pollutant concentration). For this reason, this two factors were studied systematically using central composite design methodology. All the experiments were scheduled and carried out under laboratory-scale level that is representative of real composting conditions.

2. Materials and methods

2.1. Soil

The soil used in this study was collected from the surface horizon (0–30 cm) in Prades (Tarragona, Spain). The zone where the soil was collected is an agricultural one; hence it is free from any PAHs contamination and preliminary analysis confirmed that there are no PAHs in the used soil or their concentration are below the detection limits. This soil is classified as sandy loam soil and consists of 73% sand, 19% silt and 8% clay. For the experimental purpose, the soil was air-dried, sieved to 2 mm and kept at 4 °C until use. Some characteristics of the soil were determined and are presented in Table 1.

2.2. Chemicals

Different compounds of PAHs were purchased from (Sigma–Aldrich, Spain). These PAHs include: flourene, phenanthrene, anthracene, flouranthene, pyrene and benzo(a)anthracene with 98–99% purity. According to the US Environmental Protection Agency (USEPA), these PAHs are classified as priority pollutants as they are toxic and carcinogens. As a group of contaminants to be followed during the experiment course, the abovementioned six PAHs were mixed together in a stock solution that was spiked into the mixture soil–compost with a concentration based on total PAHs. The percentage of each individual PAH compound as a part of the total PAHs concentration ($\sum$PAHs) was 30%, 29%, 9%, 20%, 3.5%, 8.5%, respectively. These percentages were determined according to the results of fractionation process of a creosote sample (Creosote lot: 42-13B, Chem Service, SUGELABOR S.A., Spain) in our laboratory using the method 3611B of the USEPA, where the volatile part was ignored. A stock solution of the used PAHs was prepared using the previous percentages. Afterwards, they were spiked into the soil to obtain the desired concentrations according to the experimental design matrix (0.1–2 g/kg) as total PAHs. For instance, the low to high concentrations were applied to understand the performance of the process under such conditions considering PAHs concentration as an important factor in a remediation technology. Also the same stock solution was used to calibrate the instrumentation for the PAHs determination.

2.3. Organic co-substrates

As an organic co-substrate (amendment) to be added for stimulating the composting process, composts derived from the organic fraction of municipal wastes (OFMSW) were applied during the bioremediation experiments. In this study, five types of OFMSW composts were used during the experimental treatments. The main difference between these composts was the degree of stability "the rate of organic matter decomposition as a result of the microbiological activity" and it is mainly related to the availability of readily degradable substrates. The different levels of stability were determined using the Dynamic Respirometric Index (DRI). Only compost B was obtained from a home composter in the *University Autònoma of Barcelona*, where the others were obtained from composting plants located in the Barcelona area (Spain). These composts were selected to be characterized by a different degree of stability ranging from full-stable to unstable compost [16,17], which would provide the ability to examine their effects on the degradation of the used PAHs and to relate and predict the effect of their major components as a consequence on the bioremediation process. The main characteristics of the used composts are also presented in Table 1.

2.4. Composting reactors and monitoring instruments

The used reactors were Dewar® vessels (4.5-l), which were modified and conditioned to operate in a batch-mode way for the composting experiments. These reactors are thermally isolated, so the process can be kept under the natural composting tempera-

Table 1
Characteristics of the used composts and soil.

Parameter/material	Soil	Compost A	Compost B	Compost C	Compost D	Compost E
Moisture content (%, wb)[a]	6.64 ± 0.01	38.64 ± 0.22	30.1 ± 0.42	53.8 ± 0.25	32.63 ± 0.08	40.55 ± 0.35
Organic matter content (%, db)[b]	3.68 ± 0.35	44.48 ± 0.39	53.96 ± 0.11	61.63 ± 1.83	44.59 ± 0.35	51.85 ± 1.2
Total organic carbon (%, db)[b]	1.26 ± 0.02	18.52 ± 1.14	24.29 ± 2.79	31.75 ± 0.28	19.43 ± 1.2	20.44 ± 0.28
Total Kjeldahl nitrogen (%, db)[b]	0.65 ± 0.14	2.67 ± 0.45	2.62 ± 0.06	4.09 ± 0.12	3.09 ± 0.15	1.94 ± 0.12
pH	6.7 ± 0.02	8.07 ± 0.08	8.63 ± 0.04	8 ± 0.16	8.11 ± 0.01	7.61 ± 0.01
Electrical conductivity (mS/cm)	0.2 ± 0.01	4.91 ± 0.13	6.46 ± 0.18	5.27 ± 0.14	6.01 ± 0.0	7.13 ± 0.04
Humic acids (%, db)[b]	1.5	10.12	11.62	14.6	8.95	4.72
Dynamic Respiration Index ($mg\,O_2\,g^{-1}\,OM\,h^{-1}$)	–	0.37 ± 0.02	0.58 ± 0.4	1.71 ± 0.13	3.07 ± 0.3	4.55 ± 0.1

[a] wb: wet basis.
[b] db: dry basis.

tures, and the influence of ambient temperature can be minimized. Aeration was provided through a pipeline connected to the bottom of the reactor where a plastic mesh is placed to insure a correct distribution of the air through the composting mixture, and the exhausted air exits the reactor through an outlet in the reactor cover. Oxygen concentration was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom), where the inlet of the sensor is connected to the reactor outlet and consequently the oxygen percentage in air was determined. Aeration rate and frequency were adjusted to prevent any limitation or excess in the oxygen percentage in the reactors, consequently, sporadically aeration mode was used and the oxygen concentration was well maintained to insure aerobic conditions (more than 10%). Temperature was monitored by Pt-100 sensors (Sensotran, Spain) connected to a data acquisition system (DAS-8000, Desin, Spain) that was connected to a personal computer. The software used (Proasis®Das-Win 2.1, Desin, Spain) also permits to monitor both the temperature and oxygen content in the reactors. These two parameters (temperature and oxygen concentration) are useful to control and follow the process during the different phases.

2.5. Experiments set-up

The PAHs were mixed together according to their percentages to be introduced as the target contaminants during the composting process. These contaminants were spiked into soil to have the initial concentration expressed as total of PAHs according to the values determined by the experimental design technique that were decided to be from 0.1 g/kg to 2 g/kg (dry matter). After this, the contaminated soil was mixed with the organic amendment at ratio of 1:1 (w:w, dry weight). The mixture was then mixed with bulking agent at a ratio 1:1 (V:V) in an attempt to provide proper porosity to maintain aerobic conditions. All the components of the composting treatments were manually mixed according to the aforementioned ratios, resulting in about 3.5 kg that were used in each reactor. The used bulking agent consisted of wood chips and pruning wastes that were not biodegraded under laboratory composting conditions. Water content of the composting mixtures was adjusted to be within the recommended values (50–60%) by adding tap water before incubation. The composting matrix was left under natural composting temperatures. Aeration flow rate and frequency were monitored and adjusted during the process to avoid any limitation or excess in the oxygen concentration that may affect the process. All the composting mixtures were manually prepared according to the proposed values of the experimental design technique (Table 2) and were incubated for 30 days.

2.6. Sampling

During the incubation period, the performance of the process was monitored and samples were collected after 10, 20 and 30 days of composting in order to measure the degradation rate in these periods. For sampling, the reactors were opened and the reactor contents were manually well mixed. Then duplicate grab samples (20–30 g) were taken. During each sampling, moisture content was adjusted, if necessary. Thus, the composting mixture was moistened with tap water and remixed well to maintain water content within the optimum values (50–60%).

2.7. Analytical procedures

2.7.1. Co-substrates stability degree

Compost stability was determined using the Dynamic Respirometric Index (DRI), determined according to Barrena et al. [18]. Briefly, this index represents the oxygen consumption by microorganisms to degrade the easily degradable organic matter of a sample within a unit time (h) when it is incubated under optimal and controlled conditions and with a continuous air supply. About 150 g duplicated samples were incubated in 500 ml Erlenmeyer glass flasks provided with a plastic mesh placed in the bottom to support the incubated sample and to ensure equally distributed air. The flasks are perfectly sealed with a rubber pieces where the aeration and exhausted air tubes passing through. The samples are incubated in a thermostatic water bath adjusted at 37 °C where the respirometer system is supplied with an oxygen sensor, a control cabinet and air supply system based on mass flow-meters and personal computer unit. A constant air flow was supplied to ensure that the oxygen concentration in the exhausted air is greater than 10%. As a result DRI provides an accurate measure of the biological stability of the organic matter contained in the biomass in form of the maximum respiration activity (Oxygen Uptake Rate) of the samples. The complete details of this analysis can be found elsewhere [19,20].

2.7.2. PAHs analysis

The content of the PAHs in the composting mixture was determined after extraction using a Soxhlet extraction process. Duplicated 10 g samples were extracted using acetone/dichloromethane (1:1 v/v) as solvent during 2 h. Afterwards, the solvent was left to evaporate and then the remaining residue (extract) was dissolved in 10 ml of dichloromethane. A 1-μl extract of this solution was injected in a gas chromatograph (GC8690N, Agilent, Spain) equipped with flame ionization detector (FID) and a splitless injec-

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

Run	Factor levels				Response[a]		
	Coded		Actual		Y_{10}	Y_{20}	Y_{30}
	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	81.47	88.26	96.38
2	+1	−1	1.74	0.58 ± 0.04	92.21	96.47	96.53
3	−1	+1	0.38	3.07 ± 0.29	67.55	79.01	89.81
4	+1	+1	1.74	3.07 ± 0.29	75.26	89.09	96.56
5	0	0	1.05	1.71 ± 0.13	73.79	81.82	84.39
6	$-\alpha$	0	0.1	1.71 ± 0.13	18.61	57.44	45.84
7	$+\alpha$	0	2.0	1.71 ± 0.13	66.51	78.26	80.98
8	0	$-\alpha$	1.05	0.37 ± 0.02	70.40	90.79	86.46
9	0	$+\alpha$	1.05	4.55 ± 0.01	40.13	77.28	76.11
10	0	0	1.05	1.71 ± 0.13	75.61	79.29	78.68
11	0	0	1.05	1.71 ± 0.13	75.07	90.28	85.58
12	0	0	1.05	1.71 ± 0.13	69.35	75.42	85.21

[a] The response (Y) represents the degradation percentage (%) after 10, 20 and 30 days of composting.

tor. 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. The concentration of the PAHs was determined after the calibration of the method with standard PAHs samples.

To investigate the volatilization of the PAHs during the composting process, samples from the exhausted air were collected using Tedlar bag of known volume [21], and then samples of 1 ml of that air were analyzed using the same GC methodology. However, this test was simply used to check if part of the PAHs decrease is caused by volatilization, but the actual amount of the volatilized PAHs could not be determined as only small amounts of some low molecular weight PAHs were detected.

2.7.3. Other characteristics

The other characteristics of both soil and organic co-substrates including: moisture content, organic matter content (OM), pH, electrical conductivity, organic carbon, Kjeldahl nitrogen and humic matter fraction were determined on collected samples according to standard methods [22].

2.8. Experimental design methodology

For bioremediation of PAHs-contaminated soil through composting, the influence of PAHs concentration (x_1) and compost stability (x_2) were processed and statistically validated through setting up a central composite design (CCD) technique and a second-order model was produced to correlate the studied factors. A series of experiments were carried out at different points as follow: four experiments using the extreme points ($\alpha = 1.414$), four experiments corresponding to a two factors complete factorial design and two experiments at central points. For the validation of the design, 2 more experiments were conducted at the central points and so 12 experiments as total were carried out and 5 values for each independent variable were tested.

To simplify the recording of the conditions and processing of the experimental data, the factor levels were coded with the notations ($-\alpha$, -1, 0, $+1$, α). The value of $\alpha = 1.414$ was determined according to the number of the studied factors (2). The experiment design technique was carried out as it is explained in the literature [23,24]. Table 2 presents the design matrix, where the coded and the actual values of the studied factors and their combination are described, also the actual response of the process (Y) after 10, 20 and 30 days is also shown.

2.9. Data statistical analysis

Statistical analysis was performed for all variables using the Sigmaplot® 8.0 software package (Systat Software Inc., San Jose, USA). The replication of experiments at central point permits the statistical validation of results according to CCD experimental design [23].

3. Results and discussion

3.1. Soil and co-substrates characterizations

In this study several types of OFMSW compost were evaluated as an organic amendment during the composting of PAHs-contaminated soil. Obviously, the available high content of organic matter in these composts in comparison with the available amount in the soil (Table 1) is thought to be needed to support and develop the microbial activity during the bioremediation process. Meanwhile, these composts are suggested to provide valuable populations of microorganisms that presumably can degrade the contaminant [6]. One of the most important characteristics of the applied amendments that play a major role in the remediation process is the humic matter portion [24,25]. Analysis of the composts showed that humic matters are available among them, but their portions are different according to their stability degree. Indeed, humic matter as part of the compost organic matter was found to be increased with stability degree in the sense that, the more stable the compost was the higher humic matter content was observed. However, compost C deviated from this fact to a small extent, which might be attributed to its high content of organic matter (Table 1). The other characteristics are almost considered within the acceptable levels for such process. Generally, the growth factors in the used compost are better than those in the soil; consequently, these organic amendments may have a major role in improving the degradation of the contaminants.

3.2. Response surface and statistical analysis

The obtained percentage of PAHs degradation (Y) after 10, 20 and 30 days were used as functions to correlate the studied factors (x_1 and x_2), where second-order polynomial model was used to fit these values as shown in the following equations:

$$Y_{10} = 75.2 + 3.36x_2 + 2.16x_1^2 - 2x_2^2 - 1.59x_1x_2 \quad (1)$$

$$Y_{20} = 82.33 + 6.57x_1 - 2.3x_1x_2 \quad (2)$$

$$Y_{30} = 106.33 - 24.59x_1 - 4.52x_2 + 8.5x_1^2 + 2.4x_1x_2 \quad (3)$$

Depending on these equations the regression coefficients (R) of Y_{10}, Y_{20} and Y_{30} were 0.83, 0.53 and 0.6, respectively. The obtained regression coefficient after 10 days represents a good regression model, whereas those of 20 and 30 day represent a non-perfect regression. In all cases the P values describing the significance levels were not concluding ($P > 0.05$). However, although these statistical values are not within the preferable values to describe the process, the constant variance test indicated that these can be used to predict the degradation rates within ±10%.

3.3. The composting process

Temperature variations of the composting materials with time in some experimental runs are illustrated in Fig. 1. A lag phase was observed even though its duration varied among the treatments, but it was clear in run 9 where about two days were needed to stimulate the microorganisms. As a result of the microbial activity, temperature began to rise to thermophilic ranges that were achieved during the first week in the treatments when less stable composts were used, but it was always in the mesophilic ranges when more stable composts. It is believed that the achieved

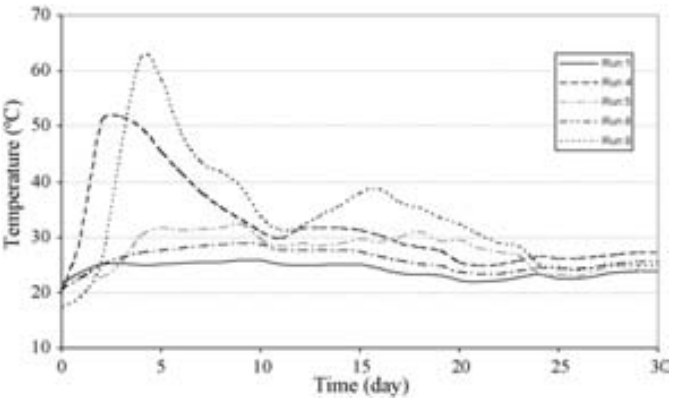

Fig. 1. Temperature profiles of the composting materials over time.

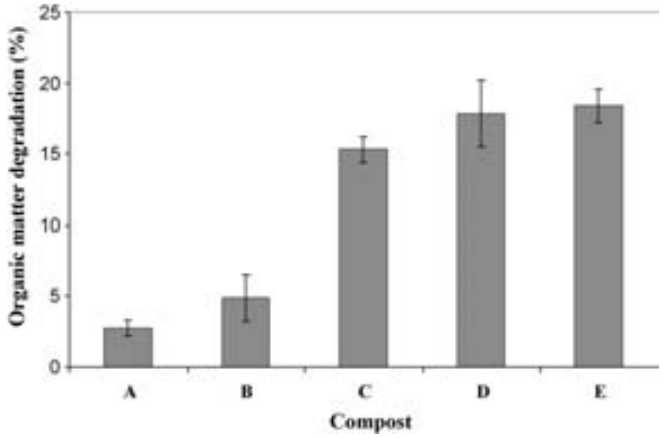

Fig. 2. Percentage of organic matter degradation after 30 days of composting.

thermophilic temperatures in the beginning of composting were attributed to the sufficient amounts of easily degradable materials [19,26]. This assumption agreed with results obtained regarding the organic matter degradation (Fig. 2) that showed a notable decrease in this stage for the less stable composts. Moreover, high aeration and frequencies were needed during the first stage of the process especially in the less stable composts (data not shown), which implied that the microbial activity was intense. However, as the materials became more stable and the process entered to the cooling phase, less amount of air was needed. The temperature increases as well as the reduction in the organic matter fraction during the whole process are the most important evidences of the process [17,26].

3.4. PAHs degradation

The degradation of the PAHs was assessed during the entire incubation period (30 days). Fig. 3 presents the remaining PAHs throughout the different runs. By the end of the incubation period, high rates of degradation (76.11–96.53% as total PAHs) were achieved among all the experiments except the run 6 where only 45.8% was achieved. For instance, during the first 10 days the highest rate of degradation (92%) was observed in run 2, whereas the lowest rate (18%) was in run 6 during this period, and in the other runs it was within 40–80%. During the remaining period, a low rate of degradation was observed. The contrast among the different treatments could be clearly visualized during the first 10 days of incubation as different degradation rates were obtained. Thus, the compost stability appeared as an effective factor especially when treatments with the same concentration are to be compared (runs 5, 8 and 9). In run 9, which had the most active compost, almost 40% of the PAHs were degraded during that period (10 days). However, almost the double degradation rates were obtained when more stable compost was used. It is worthy to indicate that less stable composts got stabilized with more incubation time. Consequently, degradation rates with these composts improved with time, for instance, degradation rate of 76% after 30 days was obtained in run 9. Nevertheless, this rate of degradation was still less than those obtained under the same conditions when more stable compost was supplied. On the other hand, the rates of degradation were found to be varied under the different concentrations, which imply that the degradation process is also influenced by this factor. In general, the PAHs degradation was influenced by the two factors, where the used amendments were able to enhance the degradation process to a great extent.

Regarding the degradation pattern of the individual PAHs, they demonstrated a notable rate of degradation in the treatments except in the case of pyrene, which had a recalcitrant behavior especially in the first 10 days where a low rate of degradation was observed in almost all the experiments. However, with more incubation time, its concentrations was decreased but not as the other PAHs. The PAHs degradation order was the same among the experiments and it was, in general: flourene > phenanthrene > anthracene > flouranthene > benzo(a)anthracene > pyrene. However, in run 6 it was different and was: flouranthene> phenanthrene>flourene>benzo(a)anthracene>anthracene> pyrene. Clearly, neither the solubility nor the octanol–water partition coefficient (K_{ow}) order of these PAHs controlled the degradation. According to [27], the molecular conformation prevails over other parameters in controlling the degradation when the degradation trends for each hydrocarbon are similar under all conditions.

In general, the rate of degradation during the first 10 days was faster compared to the rest incubation period where less degradation occurred. In this sense, the organic contaminants could be sequestrated into the matrix of the soil as this process is generally a function of time [28]. Analysis of exhausted air samples indicated that very small amounts of some low molecular weight PAHs were volatilized during the thermophilic stages (especially for temperatures over 50 °C), thus a portion of these PAHs reduction is due to volatilization but this amount is so small compared to that resulting from the biodegradation as these elevated temperatures remained for about one week in the reactors with less

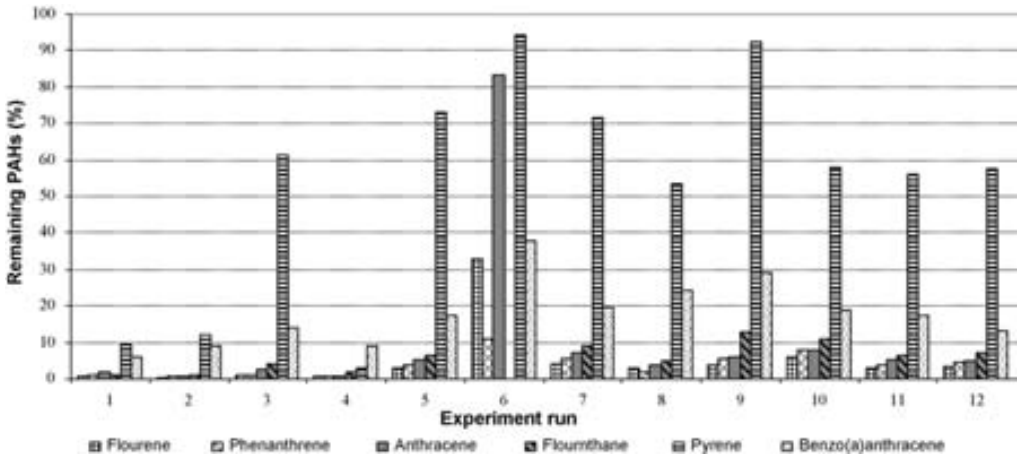

Fig. 3. Percentage of the remaining PAHs after 30 days of composting.

stable compost. However, the majority of the experiments were in the mesophilic ranges (Fig. 1) where no or negligible volatilization occurred. It was also reported that although a portion of the total petroleum hydrocarbons reduction is due to volatilization, abiotic loss has been reported to be generally less than 10% at 25 °C in the first 30 days [29].

3.5. Effect of compost stability on PAHs degradation

The response of the PAHs degradation percentages under different composts stability degrees denoted by DRI (0.37–4.55 mg O_2 g^{-1} OM h^{-1}) are illustrated in Fig. 4. As shown, the process response is changed when different composts were used as well as during the different composting stages indicating that the compost stability is one of the factors that influence the process performance. This effect was significant during the first stage of the composting process (10 days) as the applied composts had to pass different stages according to their composition and dominant microbial enrichment. With compost E (run 9), which is the most active one, only 40.13% of the total PAHs was degraded comparing to the other composts (A and C) under the same conditions (runs 5 and 8) where 70.4% and 73.79% of degradation were achieved, respectively. The same fact was also observed in runs 1 and 3 which demonstrated more degradation as more stable compost was used. Without doubt, these observations are of great interest when the efficiency of the composting technology is to be evaluated with other technologies. However, the highest rate of degradation (92.2%) was observed in run 2 indicating that the PAHs concentration has its influence in this case. For instance, Oleszczuk [30], observed that the influence of the composting process on the contribution of the potentially bioavailable fraction of the PAH depended on the stage of the experiment.

Regarding the composting process and as commented before, the thermophilic temperatures are thought to be not suitable for the microorganisms needed to attack the PAHs contaminants as the lowest degradation rate was observed (run 9). These temperatures were reported to inhibit the degradation process [31–33]. Contrarily, the mesophilic temperatures obtained under the same conditions gave higher rates of degradation indicating that these temperatures and the dominant microorganisms under these conditions are preferable for degradation of such compounds. However, other studies are not coincident with these observations [34].

The organic matter decrease was proportional with the stability degree, where more reduction occurred in compost E. This reduction shows that these types of composts still have a considerable amount of easily degradable matter which was preferable by the microorganisms rather than other sources of nutrients like PAHs. Accordingly, the mass loss after composting is a suitable evidence indicating the bio-oxidation of the composting matrix.

Among the most important properties correlated with the compost stability is the available amount of the humic matter as part of the organic matter. This matter was found to increase with stable compost (Table 1) where more stable composts had more humic matter. Sorption of the organic contaminants with the soil particles usually decreases the degradation rates as these contaminants become less accessible to the microorganism. However, it was found that the humic matter increases the bioavailability of the organic compounds and it can behave as surfactant during the remediation process, which reduces the bond between the soil and PAHs. The more degradation rates with more stable compost confirm this hypothesis and the PAHs were easily desorbed from the soil particles thereby the degradation was stimulated. Plaza et al. [25], demonstrated that during the composting process, the changed underwent by the humic matter are expected to facilitate the microbial accessibility to PAHs. These experiments

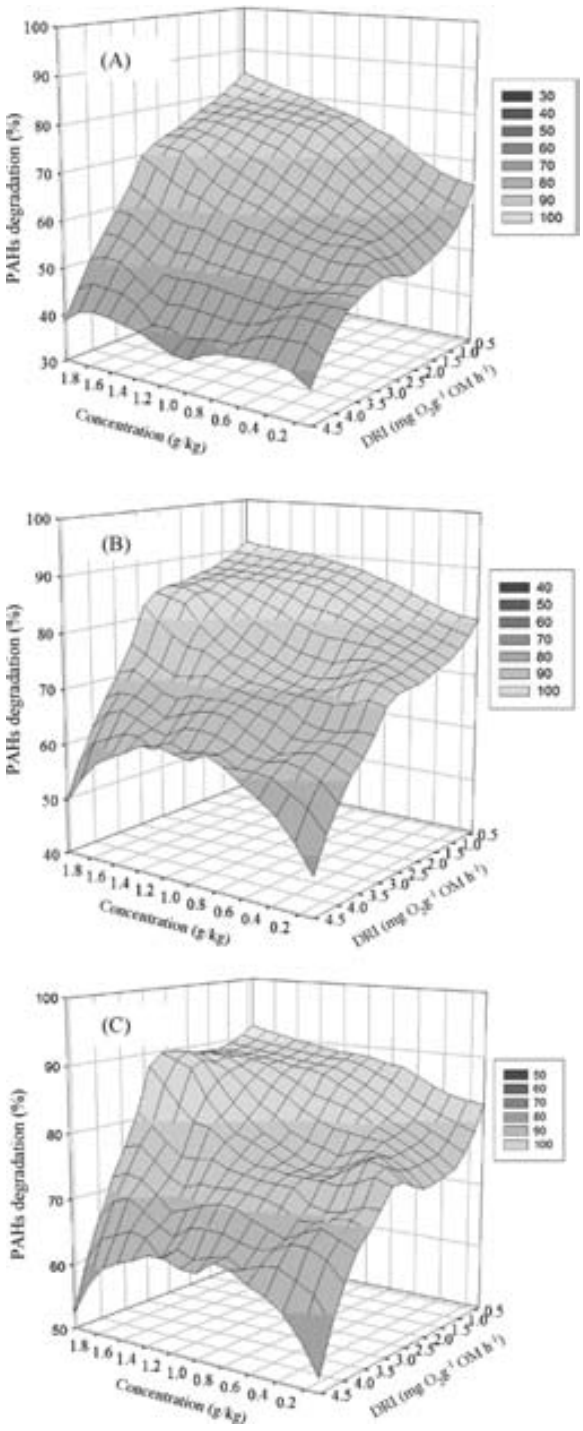

Fig. 4. The response of the PAHs degradation (%) under different concentrations and DRI (mg O_2 g^{-1} OM h^{-1}) during the incubation period where (A) corresponds to 10 days, (B) 20 days and (C) 30 days.

concurred well and confirm such suggestions. It was clear in the remaining composting period that the degradation process continued among all the experiments and better results had been obtained with more incubation time as the organic matter got more stabilized and the microorganisms were more acclimatized especially with less stable compost. The humic matter found to be more effective to increase the degradation rates than the high temperatures although it is well known that the produced high temperatures usually increase the kinetics and desorption of such compounds.

3.6. Effect of PAHs concentration

PAHs concentration influenced the degradation rate as observed in the different experiments. Fig. 3 presents the percentages of the remaining PAHs after 30 days of incubation, where Fig. 4 presents the response of the process under different concentrations. The lowest degradation rate (18%) after 10 day of incubation was observed in 6th run that has the lowest concentration (0.1 g/kg), where the highest rate (92.21%) was obtained in the 2nd run that has a concentration of 1.7 g/kg, where degradation rate of 66.5% was achieved with the highest used concentration (2 g/kg) during the same incubation period. By the end of the process (30 days), the degradation rate in the 6th run (0.1 g/kg) still maintained its order as the lowest achieved rate (45.8%), where it was able to achieve 80.9% with the highest applied concentration (2 g/kg). However, when comparing the rate obtained with highest concentration, it was less than the other obtained rates where less concentration were used, therefore, the concentration levels are considered crucial when composting process is to be used. For this reason, when low concentrations are present, these concentrations are thought to be below the levels that are assumed to begin the degradation process as the microorganisms start with easily available materials and as these materials depleted quickly before the degradation take place, it will be difficult to keep the required activity. These results agreed with those obtained in [15], where concentration of low PAH did not degrade even when the system was supplanted with additional carbon sources. Furthermore, JØrgensen et al. [35] argued that the degradation of hydrocarbons is governed by first-order kinetics, where the degradation rate of a compound is proportional to its concentration. However, this argument may be validated to some limits as the microbial activity could be affected (retardation or inhibition) when high concentration is available. In this study when the results of high concentrations are compared to other presenting lower concentrations, it is better to assume that retardation conditions were noted.

4. Conclusions

During the composting process, it was clear that the potentially available PAHs are influenced by the compost stability and the composting stages as a consequence. Accordingly, the following conclusions were deduced:

1. The observed different behaviors during the first 10 days of composting demonstrate that less stable compost is not adequate for this type of remediation, but more stable ones can promote the degradation quickly when the process is well controlled.
2. Humic matter was assumed to facilitate the desorption of the PAHs to be more available for degradation by the microbial activity. Indeed, humic matter was more effective to accelerate the degradation rates than the high temperatures.
3. By the end of the process, experiments with the less stable compost were able to improve their behavior as the composted materials were more stable, but their results were still less favorable than those obtained with more stable compost.
4. PAHs concentration was found to influence the process mainly when low concentrations are available, where the lowest degradation rate was obtained.
5. Both of the studied factors (compost stability and PAHs concentration) had a direct effect on the process behavior; therefore, before carrying out the composting process, initial knowledge about the available conditions may help to have an estimation about the process performance and consequently the expected degradation rates.

Acknowledgements

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4.2 Complementary documents

This part includes the unpublished work, but actually, it has been submitted for publication and it is under revision.

Article IV: Bioremediation of PAHs-contaminated soil through composting: influence of bioaugmentation and biostimulation on the contaminants biodegradation.

Article IV

Bioremediation of PAHs-contaminated soil through composting: influence of bioaugmentation and biostimulation on the contaminants biodegradation.

Tahseen Sayara, Eduard Borràs, Gloria Caminal, Montserrat Sarrà and Antoni Sánchez.

Chemosphere, submitted: September, 2010.

Bioremediation of PAHs-contaminated soil through composting: influence of bioaugmentation and biostimulation on the contaminants biodegradation

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Abstract

The degradation of several polycyclic aromatic hydrocarbons (PAHs) in soil through composting was investigated. The selected PAHs included: Flourene, Phenanthrene, Anthracene, Flouranthene, Pyrene, Benzo(a)anthracene and Chrysene, with their percentages simulating a real creosote sample. The degradation of PAHs (at an initial concentration of 1 g/kg of total PAHs) was assessed applying bioaugmentation with the white-rot fungi *Trametes versicolor* as an exogenous microorganism, and biostimulation using compost of source-selected organic fraction of municipal solid waste (OFMSW) and rabbit food as organic co-substrates. The evaluation of the process through different analysis included the dynamic respiration index (DRI), the cumulative respiration at five days (AT_5), the enzymatic activity and the fungal biomass. They revealed that the introduced *Trametes versicolor* did not enhance the degradation process of PAHs. However, biostimulation was able to improve the degradation rate where 89% of the total PAHs was degraded within 30 days of composting compared to only 29.5% that was achieved by the soil indigenous microorganisms without any co-substrate (unamended). Indeed, the obtained results showed that the stable compost of OFMSW had a great potential to enhance the PAHs degradation compared to non-stable co-substrates such as rabbit food. These observations justify that composting as remediation technology is an effective process that more likely depends on the co-substrates stability.

Keyword: Composting; Polycyclic Aromatic Hydrocarbons (PAHs); Bioaugmentation; Biostimulation; Stability.

1. Introduction

According to the Environmental Protection Agency (EPA), polycyclic aromatic hydrocarbons (PAHs) are recognized as priority pollutants. In fact, these types of pollutants are normally found in the environment as a result of natural or anthropogenic activities (Johnson et al., 2005). Their deleterious properties like high toxicity and carcinogenicity for both humans and other living organisms have made their remediation a critical need for environmental sustainability. Today, several technologies are available to deal with these environmental contaminants. However, each technology has its own conditions. Normally, the cost of a remediation technology is crucial for a feasible choice when the implementation is not a barrier. For instance, biological treatment is considered as very promising among the different applied technologies regarding to its efficiency and cost-effectiveness. Nevertheless, for PAHs biodegradation; factors such as the presence of specific degraders, the toxicity, the concentration, the bioavailability and the nutrients content are believed to play a major role in the process performance (Antizar-Ladislao et al., 2004; Gandolfi et al., 2010; Sayara et al., 2010a; 2010b).

For an effective PAHs bioremediation, the overall degradation and removal rate of the contaminants must be accelerated above naturally occurring microbial process (Mohan et al., 2008). For this reason, bioremediation of contaminated soil is usually carried out either by stimulation of the indigenous organisms by providing nutrients needed for increasing the microbial activity or by bioaugmentation through introducing consortia (indigenous or exogenous) to improve the biodegradation capacity. In some cases, both biostimulation and bioaugmentation are applied simultaneously (Hamdi et al., 2007; Mrozik and Piotrowska-Seget, 2009).

In the literature many studies have reported that several bacteria and filamentous fungi species are able to detoxify and degrade PAHs (Boonchan et al., 1998; Hamdi et al.,

2007). Nevertheless, the use of white-rot fungi as PAHs degraders has been extensively studied in liquid cultures; however, the applicability for contaminated soil treatments is scarcer. Also, not all white-rot fungi are able to colonize polluted soil due to the competition with other strains of fungi and indigenous bacteria. For bioaugmentation purpose, a wide range of white rot fungi have been employed to remediate soils contaminated with PAHs. Their ability to decompose several compounds including PAHs is due to their non-specific enzymatic system, where the ligninolytic enzymes and the cytochrome P450 are assumed to be responsible for the capacity of the white-rot fungi in the bioremediation process, and therefore they are proposed to carry out such process (Hamdi et al., 2007; Borràs et al., 2010).

It is noteworthy that using fungi in the bioremediation process receives more attention as these types of microorganisms are rapidly incorporated by the soil matrix. Also, they have the ability to grow in environments with low nutrient concentration, low humidity and acidic conditions (Mollea et al., 2005). Several studies have described the successful application of bioaugmentation in soil remediation process with different organic contaminants (Mohan et al., 2008; Teng et al., 2010). Synergistic degradation by white-rot fungi and bacteria can also occur during the bioremediation of PAH-contaminated soil since fungi can initially cleave the aromatic ring and bacteria then are able degrade the metabolites formed. However, other studies show different results by demonstrating the failure of the introduced microorganisms to degrade or enhancing the degradation of the target contaminants for several reasons (Karamalidis et al., 2010; Silva et al., 2009; Wiesche et al., 2003).

The present study investigates the impact of bioaugmentation and biostimulation on the bioremediation of PAHs-contaminated soil to compare and clarify the feasibility and effectiveness of these two applications. Bioaugmentation was carried out using a white-rot

fungi (*Trametes versicolor* ATCC 42530), where biostimulation was performed using two organic co-substrates; compost of OFMSW and rabbit food. These two organic co-substrates were used as they were characterized by different stability degrees measured through the respiration index (DRI) and their different nutrients content. The selected co-substrates are proposed to visualize the relation between these two characteristics (i.e., stability degree and nutrients content) on the process performance and the contaminants degradation rate.

2. Materials and methods

2.1 Soil

The soil used in this experiment is an agricultural soil collected from the surface horizon (0-30 cm) in Prades (Tarragona, Spain). Texture analysis demonstrated that it consists of 73.4% sand, 18.6% silt and 8% clay. Accordingly, it is classified as sandy loam soil. It was air-dried and sieved to 2 mm to remove any debris and kept at 4˚C until the experimental use. The soil was uncontaminated as no PAHs were detected before being used for the experiments. Other properties of the soil are presented in Table 1.

2.2 PAHs Contaminants

Contaminated sites are commonly found to be polluted with several types of creosote components. Consequently, different PAHs listed among the 16 USEPA priority pollutants that are normally found in contaminated soils were obtained from Sigma-Aldrich, (Spain) to be employed as target soil-contaminants. These PAHs include: Flourene, Phenanthrene, Anthracene, Flouranthene, Pyrene, Benzo(a)anthracene and Chrysene. They were mixed together and used as contaminants during the experiment. The percentage of

each compound as a part of the total PAHs (∑PAHs) was 30%, 28%, 9%, 20%, 3.5%, 3% and 6.5%, respectively. These percentages were determined according to the results obtained by a fractionation process of a real creosote sample (Creosote lot: 42-13B, Chem Service, SUGELABOR S.A, Spain) in the laboratory using the Method 3611B of the US Environmental Protection Agency, taking into account that the volatile part was ignored. A stock solution of the used PAHs contaminants was prepared after mixing them according to their percentages, and then they were spiked into the soil to reach a concentration of 1 g/kg (dry matter) as total PAHs for all experiments.

2.3 Organic co-substrates

Compost derived from the source-selected organic fraction of municipal solid wastes (OFMSW) and rabbit foods in the form of pellets (composition (% weight): alfalfa 30%, wheat husk 30%, barley 9%, soy 8%, beet 4% and impurities 11%) were used as organic co-substrates during the experiment treatments. The compost was obtained from a composting plant located in Barcelona (Spain), where rabbit food was obtained from a commercial market (Suprem, Barcelona). These two co-substrates were selected to determine the effect of the organic matter stability degree measured via the dynamic respiration index (DRI) on the bioremediation processes. In fact, both co-substrates represent extremely different stability degrees: stable and totally active organic co-substrates. Moreover, the two co-substrates presented great differences in their organic matter content. The main characteristics of the used organic co-substrates are presented in Table 1.

Table 1: Characteristics of the used organic amendments and soil.

Parameter/Material	Soil	Compost	Rabbit food
Moisture content (%, wb) *	6.64±0.01	32.82±0.2	11.23±0.12
Organic matter content (%, db) **	3.68±0.35	43.54±0.16	91.54±0.07
Total Organic Carbon (%, db) **	1.26±0.02	25.27±0.33	48.78±2.36
Total Kjeldahl Nitrogen (%, db)**	0.65±0.14	2.14±0.51	3.13±0.33
pH	6.7±0.02	8.37±0.01	6.01±0.14
Elec. conductivity (mS/cm)	0.2±0.01	4.92±0.13	3.99±0.23
Dynamic Respiration Index (mg O_2 g^{-1} OM h^{-1})	-	1.12±0.08	6.52±0.11

* wb: wet basis.
** db: dry basis.

2.4 Fungal strain preparation

The fungus *Trametes versicolor* ATCC 42530 was acquired from the American Type Culture Collection. The strain was maintained by subculturing every 30 days on 2% malt extract (w/v) agar slants (pH 4.5) at 25 °C. Fungal mycelial suspensions were obtained by blending the mycelium grown for 7 days on a malt extract medium that contained 20 g/L malt extract, and the pH was adjusted to 4.5.

2.5 Laboratory-scale composting reactors

The composting reactors used were Dewar® vessels with an operation capacity of 4.5 L. The vessels were modified and conditioned to operate as batch-mode reactors for composting purpose. Indeed, these reactors proved their efficiency in simulating real composting processes. More details about the reactors operation and monitoring systems can be reviewed in detail elsewhere (Sayara et al., 2009).

2.6 Composting experimental system

The contaminants were mixed together in a stock solution having the seven PAHs compounds, and then they were spiked into soil, where the applied concentration was 1 g/kg (dry matter). Afterwards, the contaminated soil was manually mixed with the proposed organic co-substrates at a ratio 1:0.25 (soil:co-substrate, dry weight). In treatments where the bioaugmentation was to be evaluated, the inoculum (*Trametes Versicolor*) was introduced (1 ml/3g of co-substrate) within the mixture. The porosity of the mixture was modified to ensure aerobic conditions introducing a bulking agent that consists of wood chips at a ratio of 1:1 (v/v). This bulking agent was considered as non-biodegradable under laboratory conditions. In all treatments, tap water was added during the preparation of the composting mixture to modify the water content according to the recommended values for composting process (50-60%). All the composting experiments were carried out in duplicates during 30 days of incubation. The experiment program was as follows:

Treatment (1): contaminated soil + *Trametes versicolor* + compost + bulking agent

Treatment (2): contaminated soil + *Trametes versicolor* + rabbit food + bulking agent

Treatment (3): contaminated soil + compost + bulking agent

Treatment (4): contaminated soil + *Trametes versicolor* + sterile compost + bulking agent

Also, a duplicated Control (C) treatment, which only had contaminated soil (1g/kg), was used to follow the PAHs degradation by the indigenous microorganism without any additives.

2.7 Sampling

During the composting process, samples from the reactors were drawn at distinct periods (5, 10, 20 and 30 days) in order to follow up the contaminants degradation during the different stages of the process. The reactors content was manually mixed to obtain homogenous and representative samples where about 30-40 g were taken and used for

carrying out further analysis. If necessary, water content of composting treatments was modified during sampling time.

2.8 Analytical methods

Moisture content, organic matter content (OM), Kjeldahl nitrogen, total carbon content, pH and electrical conductivity were determined according to standard methods (The US Department of Agriculture and The US Composting Council, 2001). All the results are presented as average of duplicates with standard deviation.

2.9 Respiration indices

They are used to evaluate and compare the microbial activity within the applied co-substrates, and the several composting mixtures. Specifically, a dynamic respirometer was built as described by Adani et al. (2006). Briefly, about 150 g of sample were placed in a 500 ml Erlenmeyer flask and incubated in a water bath at 37˚C. Meanwhile previously-humidified air was continuously supplied to the sample to ensure aerobic conditions. Two respiration indices were calculated from the oxygen vs. time curve:

I) Dynamic respiration index (DRI): this value represents the average oxygen uptake rate during the 24 h of maximum activity.

II) AT_5: it represents the cumulative oxygen consumption during 5 days of maximum respiration activity without considering the lag phase.

Both DRI and AT_5 are expressed in mg O_2 g^{-1} OM h^{-1} and mg O_2 g^{-1} OM, respectively. More details about the respiration test and the system configuration can be reviewed elsewhere (Ponsá et al., 2010a)

2.10 PAHs analysis

To determine the PAHs concentration in the composting treatments, the samples were firstly dried using a lyophilizer (Benchtop 5L, Virtis Sentry, NY). Then, 10 g of dry subsamples were extracted using a Soxhlet extraction process for two hours using acetone/dichloromethane (1:1 v/v) as solvent. After extraction, the solvent was left to evaporate to atmosphere and then the remaining residue (extract) was dissolved in 10 ml of dichloromethane. The PAHs were identified and quantified by gas chromatography. A 1-µl of the extract 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 (Phenomenex, USA) 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. The concentrations of PAHs were determined after the calibration of the method with standard PAHs samples of different concentrations. Remaining PAHs percentages were calculated by dividing the PAHs residue concentration into the PAHs original concentration.

2.11 Laccase extraction and quantification

The extraction of laccase was carried out according to a modified method described by Lang et al. (1998) where 30 mL sodium acetate buffer (0.16 M, pH 5) were added to 3 g of a homogenized sample and shaken for 30 min at 4°C; 1.5 mL of the extracts were transferred to Eppendorf vials and centrifuged at 15000 g for 15 min. The supernatant was then analyzed. Laccase activity was measured using the first step of the method for determination of manganese peroxidase (MnP) (Wariishi et al., 1992), where 2,6-dimethoxy phenol (DMP) is oxidized by laccase, even in the absence of a cofactor. One activity unit

(AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient is 24800 $M^{-1}cm^{-1}$.

2.12 Ergosterol extraction and quantification

Ergosterol was analyzed in homogeneously-mixed samples of the soil-phase cultures employing a modified method described by Novotny et al. (1999). An amount of 0.5-0.8 g from each sample was removed and placed in a test tube to be extracted with a mixture of 1 ml cyclohexane and 3 ml KOH-methanol solution (10% w/v) for 90 min at 70°C. Ultrasonication was applied for the first 15 min (Selecta, Spain). Then 1 ml distilled water and 2 ml cyclohexane were added; the tube was vortexed for 30 s and centrifuged at 3500 rpm for 5 min. The organic phase was recovered and the aqueous phase was washed twice with 2 ml cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen gas. The dry sterol residue was dissolved in 1 ml methanol for 15 min at 40 °C, vortexed for 30 s and centrifuged in Eppendorf vials at 6000 rpm for 3 min. Finally the resulting solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC equipped with an UV detector at 282 nm, using a reverse phase Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm). Methanol was isocratically supplied at 1 ml/min. The ergosterol content was expressed in micrograms per gram of solid dry weight (μg/g, dry weight).

2.13 PAHs metabolites identification: GC/MS

Samples were analyzed using gas chromatography (Agilent HP 6890 Series II) coupled to a mass selective detector by electronic impact ionization (Agilent HP 5973) using a HP5-MS (30 m x 0.25 mm x 0.25 μm) (Agilent). 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), ramp 7°C/min, final temperature 320°C, carrier gas He at 0.7 ml/min. The detector worked at solvent delay mode (3.2 min) and the mass range measured was 40-400 (m/z). The detected products were identified by comparing the mass spectra with data in the Wiley 7® library.

3. Results and discussion

3.1 Characteristics of organic co-substrates

As shown in Table 1, the used organic co-substrates have a valuable source of nutrients that can support the microbial activity needed for the bioremediation process. However, it is noteworthy that rabbit food is richer in organic mater (91.4%) than compost (43.5%), where total organic carbon represents 48.78% compared to 25.25%, respectively. These contents were clearly reflected and contributed in the contrasted respirometric indices. Indeed, rabbit food is a very active organic co-substrate (DRI 6.5 mg O_2 g^{-1} OM h^{-1}) implying the abundance of a great portion of easily degradable matter. Instead, the compost is considered stable (DRI 1.12 mg O_2 g^{-1} OM h^{-1}) as it finished the different stages of the composting process in the treatment plant. It is interesting to remark that the stability degree of an organic substrate may determine the availability of some chemical components like humic matter within the organic substrate. Such property is believed to play a major role in soil bioremediation process (Sayara et al., 2010b).

3.2 The composting process

Fig. 1 shows the evolution of the temperature profiles during the composting process. As it is the usual behavior in such process, the temperature began to increase in all treatments, indicating the oxidation process caused by the microbial activity in the composted materials (Ruggieri et al., 2008). Temperature profile varied among the different

treatments typologies during the first 10 days, but they presented almost the same pattern after that period. Clearly, the temperature evolved in distinct profiles especially in treatment 2 that was in the thermophilic range (first week) compared to the others that where always in the mesophilic range. This behavior was controlled by dominant components in these materials regarding to the availability of easily degradable materials (Table 1). However, as the available organic matter was gradually depleted as a result of the microbial activity, this was followed by a decrease in the temperature, and the composted materials passed through the maturation phase. Treatment typologies with compost as co-substrate varied among them especially during the first 10 days as mentioned before. In this context, temperature in treatments 1 and 3 were closed to each other, where in treatment 4 with sterilized compost, temperature was increased to a less extent compared with non-sterilized compost (treatments 1 and 3), which is attributed to the absence of microorganisms within the compost itself because of sterilization. For instance, the values obtained by the respiration test agreed with the activity encountered in each material (Fig. 2). The temperature profiles agreed with DRI values, indicating the adequacy of this test in determining the stability degree of organic materials (Ponsá et al., 2010b). In general, no inhibitory effects were observed as a result of the presence of the PAHs, which indicated the high capacity and tolerance of the microflora to adapt to present circumstances and the interest to follow their degradation.

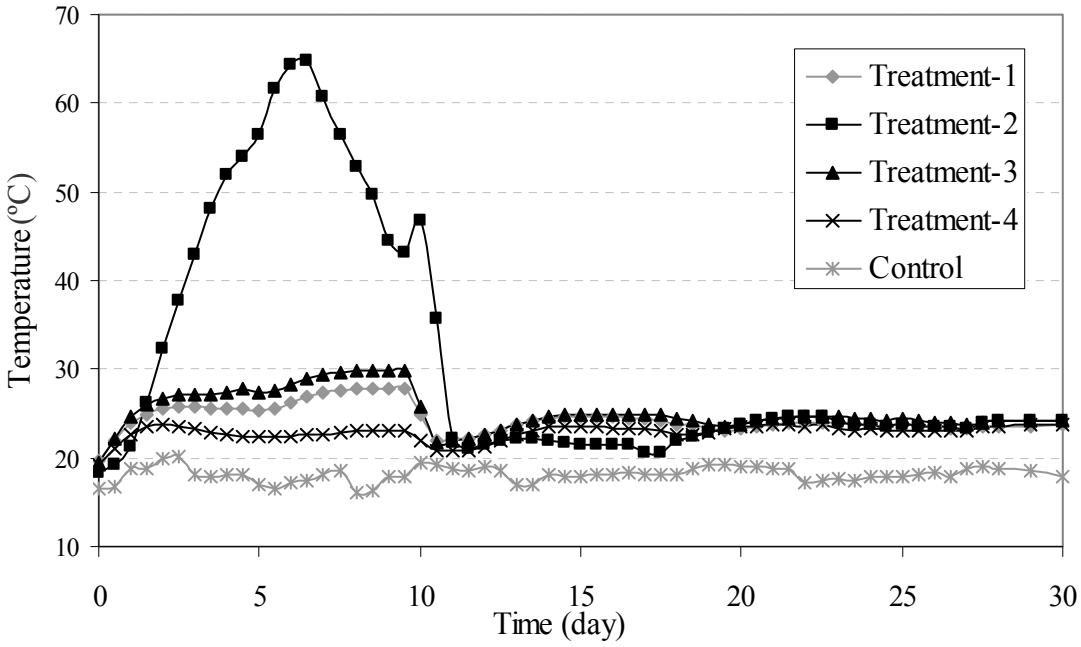

Figure 1: Temperature profiles during the composting process.

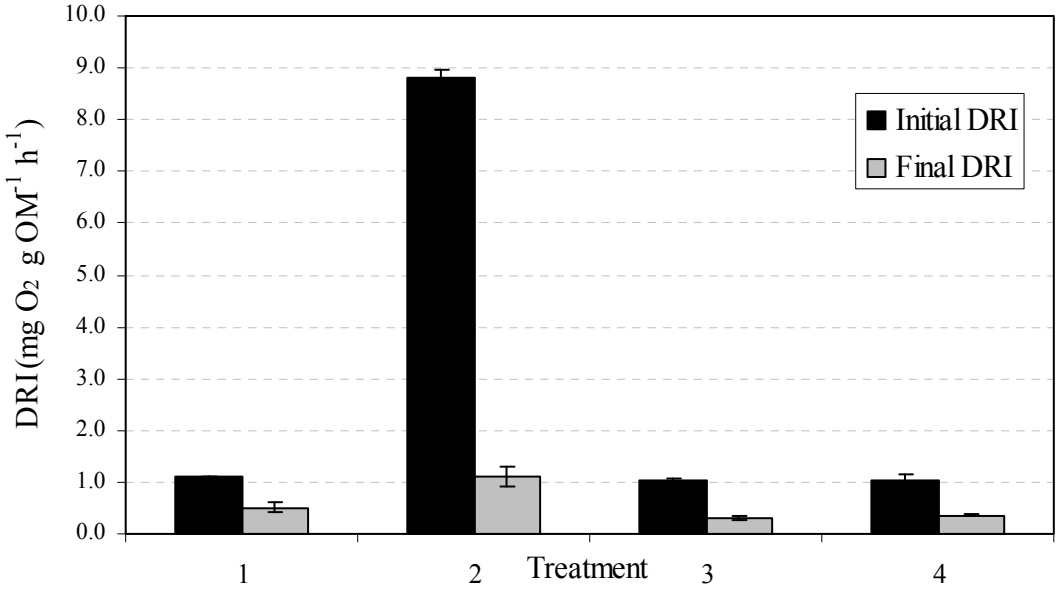

Figure 2: Initial and final values of the dynamic respiration index (DRI).

3.3 Degradation of PAHs

The different configurations investigated in this study demonstrated the high potential of microorganisms to degrade the PAHs contaminants. Fig. 3 shows the remaining PAHs (as total PAHs) after 5, 10, 20 and 30 days of composting. Also the degradation after 5 days of incubation under controlled conditions including fixed temperature (37˚C) and continuous aeration (AT_5) are presented in the same figure. Degradation rate of 89 % was achieved after 30 days in treatments with compost as co-substrate. Nevertheless, degradation rate of 71% was achieved in treatments with rabbit food as co-substrate, but only 29.5% of the PAHs were degraded in the control (C). Obviously, treatments with compost as co-substrate followed the same trend either they were augmented with *Trametes versicolor* or not, indicating the inefficiency of such modification in enhancing the remediation process. Moreover, in treatment 4, the degradation rate during the first 10 days was lower than those obtained in the other treatments (1 and 3), but this can be attributed to the absence of microorganisms within the compost as it was sterilized before. However, it is thought that it was able to be re-colonized to support the indigenous microflora necessary to degrade PAHs; consequently 86% of the PAHs was finally degraded after 30 days. These results are in accordance with previous results that demonstrated the capacity of stable OFMSW-compost in enhancing the degradation of such contaminants (Gandolfi et al., 2010; Sayara et al., 2009).

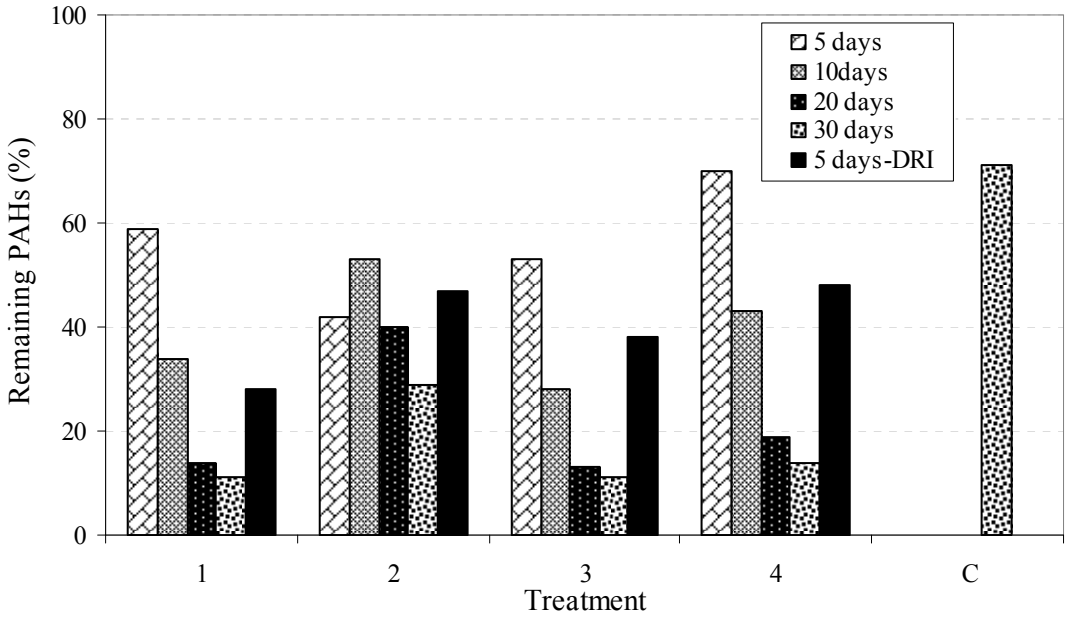

Figure 3: Remaining PAHs (%) after 5, 10, 20 and 30 days of composting, and in the experiment of dynamic respiration index (DRI) after 5 days. C corresponds to the control experiment.

Results that draw attention are those obtained in treatment 2, as degradation was almost equal to other treatments during the first 10 days, but later it was less efficient. The equality during the first 10 days was caused by the degradation of low molecular weight PAHs. Nevertheless, the difference was noted in degrading high molecular weight-PAHs. Additionally, the temperature profile in this treatment was different from others (Fig. 1), which was in the thermophilic range during the first period. The temperature increase to high levels (> 55˚C) might inhibit further degradation by the fungus and microflora from soil. In this regard, the applied co-substrate effect arise as an important factor and it can be concluded that the bioremediation efficiency is more likely to rely on the selectivity of components and properties of the co-substrate rather than its nutrients load as it is clear when comparing compost and rabbit food.

Although the indigenous soil microorganisms were able to degrade some of the PAHs as noticed in the control experiments (29.5%), they were unable to degrade the more

recalcitrant compounds like chrysene and benzo(a)anthracene. Accordingly, stimulation of these microorganisms is considered fundamental for the process enhancement. It is interesting to highlight that the PAHs degradation rate was found to be fast during the first 10 days, but during the last stage it followed a slow removal rate, which most likely attributed to the depletion of the nutrients needed for the microbial activity. The same behavior has been documented in previous works (Hafidi et al., 2008; Hamdi et al., 2007; Margesin et al., 2000; Sayara et al., 2010b; Silva et al., 2009).

Moreover, comparing the different treatments, it can be observed that the addition of *Trametes versicolor* did not enhance the remediation process as they followed the same trend especially for those where compost was applied. The obtained result are in accordance with some previous studied that used different kinds of fungi in an attempt to enhance the PAHs degradation, but no positive results were obtained (Baheri and Mysami, 2002; Karamalidis et al., 2010; Silva et al., 2009; Wiesche et al., 2003). Indeed, native microorganisms presented in the soil and organic amendments were more effective than the inocula as they were more adapted to this particular environment. In addition, the application of stable organic co-substrates that are believed to have a great portion of humic matter as part of their organic matter fraction seem to be more efficient to stimulate and enhance the degradation rate as they facilitate the desorption of PAHs (Gandolfi et al., 2010; Karamalidis et al., 2010; Sayara et al., 2010a; 2010b; Tejada et al., 2008).

As the first stage of composting process (decomposition stage) is normally characterized by an intensive microbial activity, high temperature (in the case of active materials) and degradation of PAHs occurred simultaneously. To determine the effect of temperature, subsamples were incubated under ideal conditions. The obtained results after 5 days of incubation (AT_5) gave more favorable results compared to those obtained under natural composting process and the same period (Fig. 4). Accordingly, fixed temperature of

37 ˚C was found to enhance the degradation process to a notable extent as observed in treatment 1 where probably the exogenous and indigenous microorganisms synergistically degraded the contaminant although no enzymatic activity was observed during the incubation period. This conclusion was proposed as treatment 3, which produced less favorable results compared to treatment 1 as no exogenous microorganisms were introduced. On the contrary, in treatment 2 and under the same conditions, less degradation was obtained in spite of the rich-nutrients availability, which again confirmed the suitability of more stable organic co-substrate for this bioremediation process (Sayara et al., 2010a; 2010b).

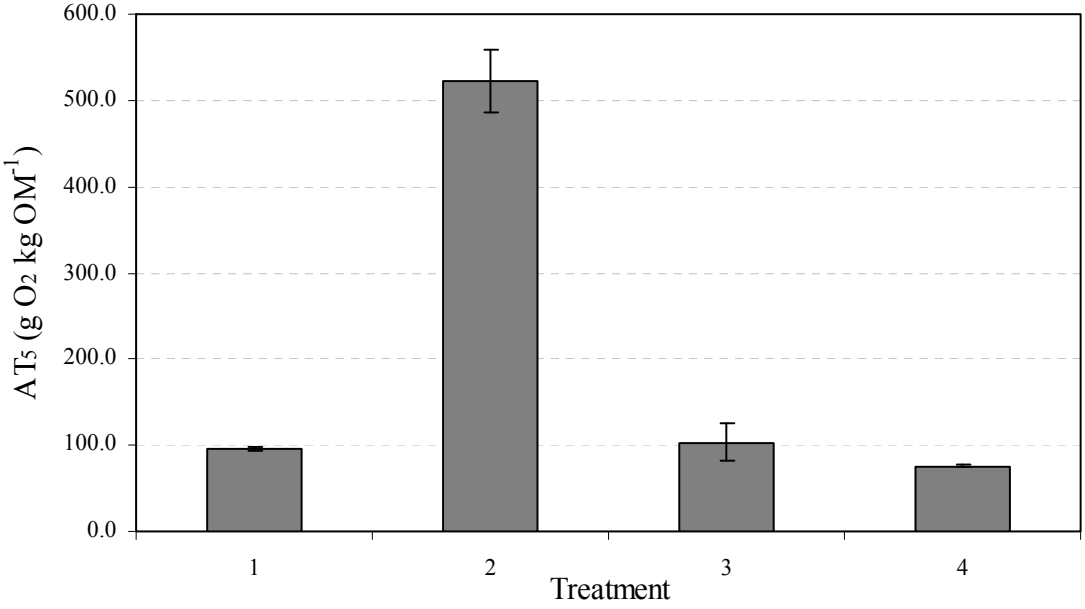

Figure 4: Values of the cumulative oxygen consumption during 5 days (AT_5).

3.4 Respiration tests

Microbial activity in each treatment was followed by measuring its DRI at the beginning and the end of the experiments (after 30 days) as presented in Fig. 2. AT_5 was also determined. Fig. 4 shows the average amount of the consumed oxygen in each treatment under these conditions. Indeed, this type of controlling helps to follow the microbial activity encountered within the treatments and the changes occurred in the treated

materials. Moreover, the effect of the temperature on the process can be detected especially because different temperatures where developed in the reactors during the first 5 days. It is clear that treatments with rabbit food were too active compared to other treatments (Fig. 2 and Fig. 4). However, the composting period was able to stabilize it. It is important to remark that bioaugmentation did not provoke any effect on the microbial activity as shown in Fig. 2 and Fig. 4, since treatments 1, 3 and 4 have almost the same initial DRI. Additionally, AT_5, which represents the amount of the oxygen consumed during 5 days clarifies again the absence of any influence of such bioaugmentation. Excluding AT_5 of treatment 2, the PAHs degradation rates after 5 days are in accordance with AT_5 values, where treatments 1 and 2 presented almost the same results. Nevertheless, it is noteworthy that treatment 4 was less active (Fig. 4) due to the sterilized compost, which has been reflected in its AT_5 value and as consequence in the degradation rate (30%) during that period. Normally, as the first period of bioremediation is characterized by a rapid decrease in the contaminants concentration, especially those of low molecular weight, (Hamdi et al., 2007; Sayara et al., 2009; Silva et al., 2009), the values of AT_5 were a reliable measure of the biological activity within the composted materials.

3.5 Enzymatic activity and fungal growth

As some of the studied contaminants are known as low bioavailable contaminants (Chrysene, Benzo(a)anthracene), the introduction of specialized microorganisms like *Trametes versicolor* with special degradation mechanisms (extracellular enzymatic system) was investigated in attempt to enhance their degradation. On the contrary, the enzymatic activity analysis demonstrated that laccase was not available for any bioaugmented treatment in any of the sampling times although visual observation after 5 days (first sampling day) clearly indicated the presence of fungus within the treatments mixture.

Consequently, the obtained degradation probably occurred due to the indigenous microorganisms and those that were introduced with the compost itself. However, in this study, it should be taken into account that the first sampling time was after 5 days, which complicates the interpretation of the inoculum effects before this time.

In fact, introducing exogenous microorganism is not always effective and always is remarked with some risks specially the case of white-rot fungi that are not soil microorganisms. For instance, introducing an adequate co-substrate is usually more efficient as observed by the obtained results that more probably demonstrate that the compost was simultaneously able to act for both bioaugmentation and biostimulation. According to Mrozik and Piotrowska-Seget (2009), several biotic factors can influence the bioaugmentation process, normally the competition between the indigenous and exogenous microorganisms for the limited carbon sources as well as antagonistic interactions and predation by protozoa and bacteriophages. Also, native species diversity may act as a resistance barrier to the invasion of non-native species (Kennedy et al., 2002). These factors play an essential role in the bioaugmentation process and its final results.

Regarding to the fungal biomass, which was measured in terms of ergosterol per grams of soil, an important indicator of the status of the soil vitality regarding its fungal biomass, it was found in all soil treatments but varied largely during the first 10 days depending on the used amendment. In treatment 2, it can be seen that the biomass content was quickly increased because of the availability of high content of easily degradable organic matter (Table 1) and an adequate aeration which are favorable fungal growth conditions. The intensive microbial activity was clearly noticed in the temperature profile which easily reached the thermophilic ranges in that period (Fig. 1). Additionally, the oxygen consumption as represented in Fig. 4 confirmed the intensive activity. However, as the available nutrient pool had been depleted, it was followed by a decrease in the fungal

biomass during the rest of the incubation period. Also high temperatures might influence the microbial growth especially because temperatures above 55˚C normally disturb the microorganisms. In the remaining treatments; treatments 1 and 3 had almost the same fungal biomass, although treatment 1 had a little more than treatment 3. This may be caused by the competitive influence that induced the indigenous microorganisms growth, or to the bioaugmentation despite of the absence of their enzymatic activity as discussed before. This is more probably because in treatment 4, the first 5 days was similar to treatment 1 and greater than treatment 3 although it had sterilized compost. After 10 days of composting, all treatments had almost the same fungal biomass except treatment 3 as it had sterilized compost that needed more time to be recolonized. Regarding the degradation rate, it was clear that treatments with compost as organic co-substrate continued the same trend along the incubation period but in treatment 2 where rabbit food was used, the degradation rate was slower. In this context, the material stability again remarked its influence on the process even though the microbial biomass was almost the same during the last period (Fig. 5).

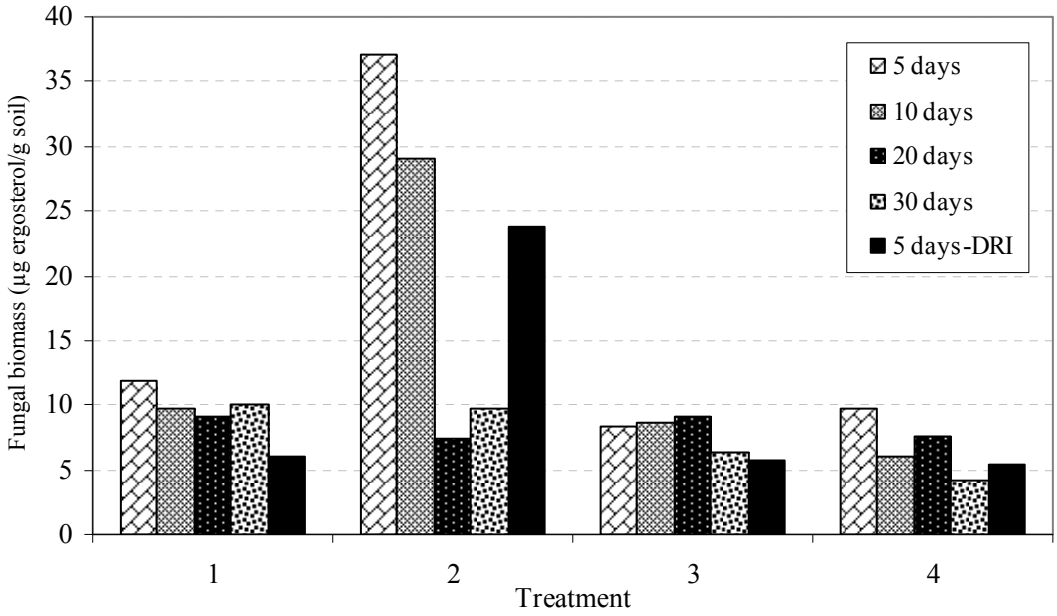

Figure 5: Fungal biomass evolution during the composting process and in the experiment of dynamic respiration index (DRI) after 5 days.

3.6 Identification of degradation products

Aerobic biodegradation of the studied PAHs was additionally followed by analyzing the produced metabolic intermediates. The presence of these intermediate products is considered a real indicator of such remediation process. Polar metabolites (Table 2) were identified from degradation of anthracene and fluorene in all treatments. The main product of fluorene was 9H-fluorenone that appeared at days 5 and 10. At day 20, 9H-fluorenone was not detected and 9H-fluorenol was also detected. These two metabolites have been described as metabolites from degradation of fluorene by white-rot fungi (Bezalel et al., 1996). Two major metabolites were detected from anthracene degradation: anthrone and anthraquinone. Both products have been extensively described as by-products during metabolism of anthracene by white-rot fungi (Hu et al., 2009). Moreover, dibuthylphtalate was also detected in samples corresponding to DRI analyses. This metabolite has been described to be implicated in the degradation of several PAHs. Products from degradation of the other PAHs could not be detected although its concentration decreased in the soil. One possibility is that degradation products were bonded to organic matter of soil or were further degraded by the fungus itself or the native microflora.

Table 2: Identification of metabolites compounds during the composting process.

Retentio time (mi	Molecular weigh	m/z of fragment ions	Structural suggestion
20.087	180	180.0, 152.0, 126.0, 120.9, 110. 98.0, 76.0, 63.0, 54.9, 50.0	9-fluorenone
20.138	182	181.0, 165.1, 152.0, 139.0, 126. 120.9, 115.0, 109.9, 105.0, 90.8 76.0, 69.5, 63.0, 57.0, 51.0, 44.	9-H-fluoren-9-ol
25.542	208	208.0, 180.0, 152.0, 126.0, 112. 103.8, 90.0, 85.0, 76.0, 63.0, 50. 44.0	9,10-Anthraquinone

4. Conclusions

The effect of biostimulation and bioaugmentation on PAHs-contaminated soil through composting has been evaluated. Results did not show any improvements due to bioaugmentation. On the contrary, biostimulation using different organic co-substrates was able to improve the degradation rate. The obtained results demonstrated that PAHs bioremediation through composting mainly relies on the co-substrate stability rather than the nutrients load. In fact, such results are interesting as alternative applications for compost derived from OFMSW are being explored. In this context, when the different organic co-substrates are to be evaluated, stable OFMSW compost would achieve several advantages like fast and high rate of degradation, low cost and less aeration due to its own stability.

Acknowledgements

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Chapter 5

Bioremediation of PAHs-contaminated soil through anaerobic digestion

5.1 Published articles

This part contains the articles corresponding to bioremediation of PAH-contaminated soil through anaerobic digestion and that had been published in indexed international journals.

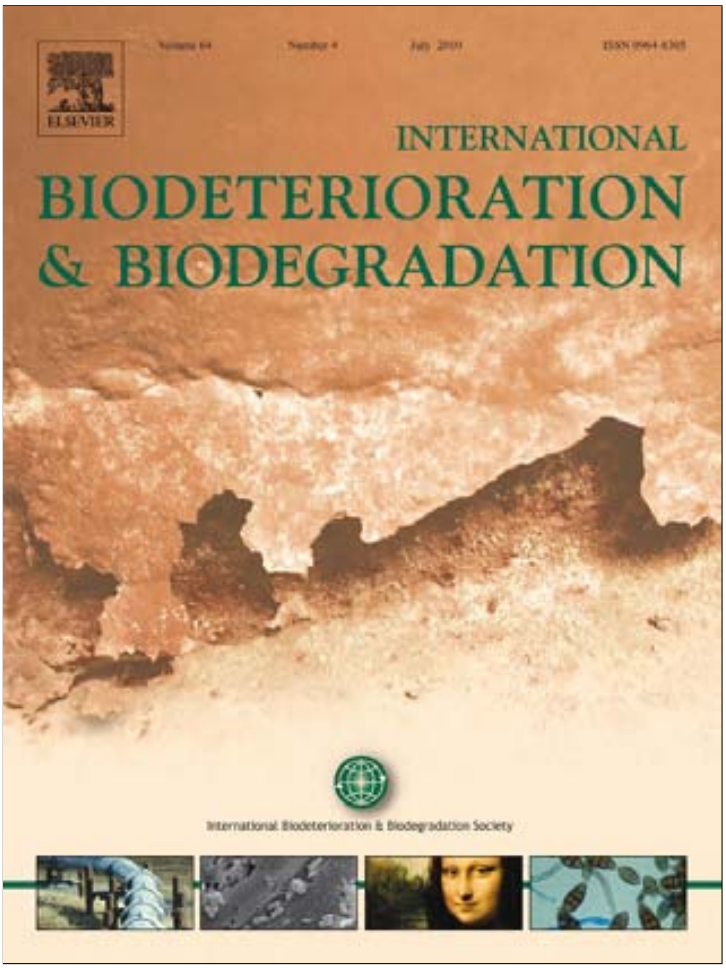
Article I: Anaerobic degradation of PAHs in soil: Impacts of concentration and amendment stability on the PAHs degradation and biogas production

Article I

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

Tahseen Sayara, Michele Pognani, Montserrat Sarrà, Antoni Sánchez

International Biodeterioration and Biodegradation. 2009. vol (64), p. 286-292.



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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 and 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.

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, 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 sulfate 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-Nielsen et al., 2009). Meanwhile, it is one of the technologies used to reduce the volume of the produced organic wastes in modern societies.

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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: Flourene, Phenanthrene, Anthracene, Flouranthene 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 asses 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 N_2 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.

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)[a]	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)[b]	3.7 ± 0.4	44.5 ± 0.4	53.9 ± 0.1	62 ± 2	44.6 ± 0.3	52 ± 1
Total organic carbon (%,db)[b]	1.26 ± 0.02	18 ± 1	24 ± 3	31.7 ± 0.3	19 ± 1	20.4 ± 0.3
Total Kjeldahl Nitrogen (%, db)[b]	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)[b]	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

[a] wb: Wet basis.
[b] db: Dry basis.

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 an 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 CH_4 and CO_2 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 10 000 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 2 h. 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-μl 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

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[a] (%)	Y_2[a] (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

[a] The response represents the degradation percent (Y_1) and the biogas production (Y_2) after 50 days of incubation.

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 et al., 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

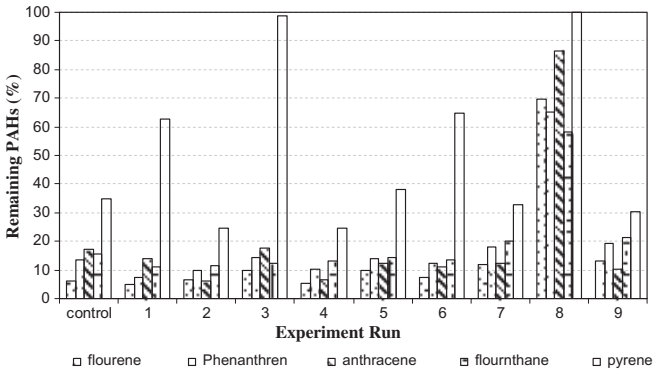

Fig. 1. Percentage of the remaining PAHs after 50 day of incubation.

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 ($\sum$PAHs) degradation was between 31.45% and 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

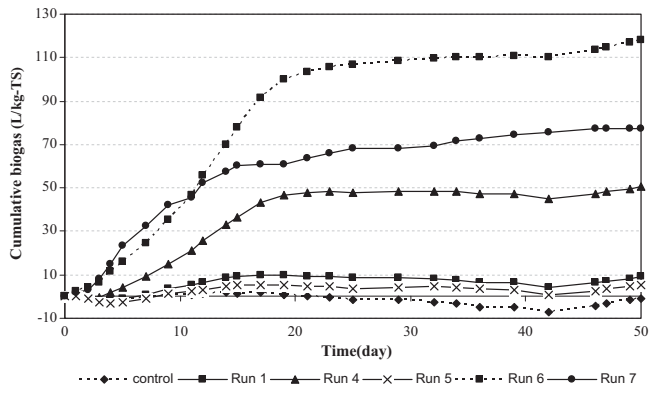

Fig. 2. Examples of the cumulative biogas production (L/kg-TS) during the 50 days incubation period.

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 and 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 O_2 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

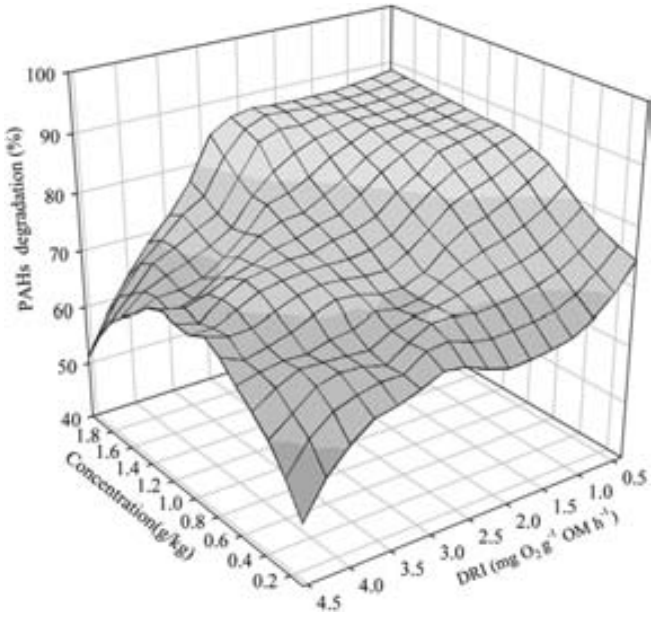

Fig. 3. Response of PAHs degradation (%) under different PAHs concentration and compost stability degrees measured by the dynamic respiration index (mg O_2 g^{-1} OM h^{-1}).

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

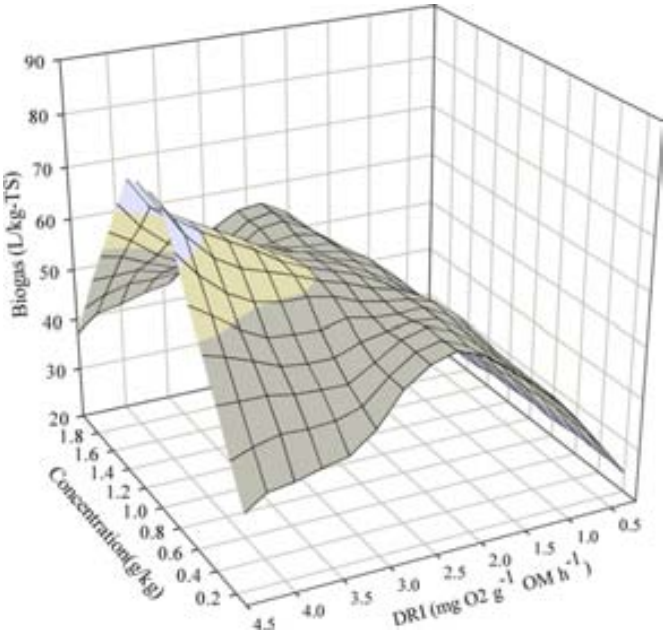

Fig. 4. Response of the biogas production (L/kg-TS, 0 °C, 1 atm) under different PAHs concentrations and stability degrees measured by the dynamic respiration index (mg O_2 g^{-1} OM h^{-1}).

in sediments with higher petroleum contamination were 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 mg O_2 g^{-1} 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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5.2 Complementary documents

This part includes the unpublished work, but actually, it has been submitted for publication and it is under revision.

Article II: Anaerobic bioremediation of PAHs-contaminated soil: assessment of the contaminants degradation and biogas production under thermophilic and mesophilic conditions.

Article II

Anaerobic bioremediation of PAHs-contaminated soil: assessment of the contaminants degradation and biogas production under thermophilic and mesophilic conditions.

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Anaerobic bioremediation of PAHs-contaminated soil: assessment of the contaminants degradation and biogas production under thermophilic and mesophilic conditions.

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Abstract

The degradation of polycyclic aromatic hydrocarbons (PAHs) including: Flourene, Phenanthrene, Anthracene, Flouranthene and Pyrene were investigated under thermophilic and mesophilic anaerobic-methanogenic conditions. The impact of PAHs concentration and soil to inocula mixing ratio on the process performance of the PAHs removal and the biogas production were systematically studied applying the central composite design technique. During the first 30 days of incubation, PAHs concentration was decreased in all treatments indicating that the applied inocula were able to degrade the contaminants although an inhibition effect was initially observed regarding the biogas production. In fact, PLFA microbial community structures was higher in treatments with moderate to high concentrations compared with low concentrations, which is in agreement and confirms the obtained results during that period. However, after 50 days of incubation, the PAHs concentration was increased in almost all studied cases indicating the reversed biotransformation of these compounds under such deficient conditions.

Keywords: anaerobic digestion; PAH-contaminated soil; thermophilic/mesophilic conditions; PLFA.

1. Introduction

Soil contamination with polycyclic aromatic hydrocarbon (PAHs) is an environmental problematic issue that is universally increasing as these compounds are widely contribute in many substances which are used in the modern life societies (Agarwal, 2009; Orecchio, 2010). Warnings about their danger and potential side effects have motivated researchers to pay more attention for their remediation and detoxification. It is well-known that for most organic contaminants; aerobic treatments are more postulated in their efficiency and convenient than anaerobic because of their broader catabolic range (Cajthaml et al., 2002; Corseuil et al., 1998). However, aerobic processes are not the only applicable option as different contamination typologies are encountered where such processes are difficult to be applied. High number of materials contaminated with PAHs are exposed to anaerobic process (e.g sludge after wastewater treatment, sediments, etc.), and therefore it is necessary to understand their fate under these conditions, and if anaerobic treatments could be of relevance and applied as biological-alternative process.

Anaerobic bioremediation of PAHs contaminated soil has been investigated under some different conditions. However, knowledge obtained from the conducted researches in this field still very poor and need more attention (Zhang et al., 2000). Recently, various electron acceptors were employed for anaerobic bioremediation of different compounds like hydrocarbons and pesticides (Callaghan et al., 2006; Kupper et al., 2008). Evidences of anaerobic degradation of various PAHs have been presented. In this context, phenanthrene and flourene has been degraded by sulphate-reducing bacteria (Coates et al., 1997; Tsai et al., 2009), or under nitrate-reducing conditions (Eriksson et al., 2003). Moreover, iron-reducing and methanogenic conditions were reported to degrade such components (Meckenstock et al., 2004; Ramsay et al., 2005; Sayara et al., 2010).

In fact, anaerobic digestion under methanogenic conditions can be carried out under different temperature ranges including mesophilic (37˚C) and thermophilic (55˚C) temperatures (De Baere, 2000). However, mesophilic treatments are considered more economical as less operation-energy is required; as a result, the majority of anaerobic plants are working under such conditions (Ferrer et al., 2008). Nevertheless, thermophilic treatments are characterized by their higher biogas production rate because of the faster reaction rates (Cecchi et al., 1991). Accordingly, considering these factors require deep understanding of the process behavior under different running temperature for achieving proper outputs, especially because this process is recognized as an alternative sources for renewable and clean energy.

In a normal started-up of a batch anaerobic digester, certain amount of inoculum should be added together with the substrate to be digested in order to provide the required microorganisms needed to start reactions (Liu et al., 2009). Theoretically, the degradation of organic matter including PAHs can be promoted by increasing the density of microbial enrichment activity either by favorable conditions, or by introducing sufficient amount of microorganisms. Consequently, determination of the ratio between the treated material and inocula is of great importance. In this concern, the minimum amount of inocula that could provide sufficient activity and the maximum amount of the loaded treated material are considered to be crucial for process design.

The role of anaerobic treatments of PAHs-contaminated soil is still an area where not much work has yet been documented. In this regard and to the authors knowledge, there is a lack of information about the bioremediation of PAHs contaminated soil under methanogenic-anaerobic conditions. To date, no reports have been published regarding the process behavior under different temperature-dependence, and no information is available about the influence of main factors that normally affect the process as contaminants

concentration and soil to inocula ratio. Hence, the objective of the present work was to comparatively asses the process performance under different operation systems employing two different types of inocula that have distinct temperature-dependence (thermophilic and mesophilic). Moreover, two controlling factors namely: PAHs concentration and soil to inocula ratio had been evaluated to asses their effect on the PAHs degradation rate. The direction and extent of the PAHs degradation was followed through measuring the biogas production as an indicator of the process activity where PAHs degradation was evaluated measuring the decrease in their concentrations and degradation metabolites compounds. In both cases and in order to have a comprehensive image about the process, the experiments were systematically carried out using experiment design technique employing different levels of the studied factors.

2. Materials and methods

2.1 Soil

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

2.2 Contaminants

Various PAHs including: Flourene, Phenanthrene, Anthracene, Flouranthene and Pyrene with 98-99% purity were selected as contaminants to evaluate their degradation under anaerobic methanogenic conditions. All PAHs were obtained from Sigma-Aldrich

(Barcelona, Spain), considering them as priority pollutants listed among the 16 USEPA. The percentage of each compound as a part of the total PAHs concentration (∑PAHs) was 33%, 31%, 10%, 22% and 4%, respectively. These percentages were determined to simulate a real creosote sample after 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, where the volatile part was ignored. The PAHs were combined together using their percentage in a stock solution and were spiked into the soil to have the desired concentration according to the experimental design set (0.1-2 g/kg, dry matter) measured as total concentration of the 5 PAHs.

2.3 Anaerobic inocula

In this study, two types of inocula (methanogenic consortia) with different temperatures-dependence; thermophilic (55˚C) and mesophilic (37˚C) where employed. 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 inocula contain methanogenic cultures consisting of 10.02% of total solids. On the other hand, thermophilic inocula were brought from another treatment plant (Terrasa, Spain), which works under thermophilic conditions. The thermophilic inocula were filtered through 4 mm sieve to remove big parts of the feedstock, where 11.8% as total solids remained. Both of the treatment plants are fed with source-selected organic fraction of municipal solid wastes (OFMSW). The two inocula were separately conserved in plastic gallons under strict anaerobic conditions and incubated in water baths at 37˚C and 55˚C for about two weeks before their use to allow for the activation and growth of methanogenic microorganisms and to remove any biodegradable materials. No PAHs were detected in the inocula before the experiment run. Other characteristics of the two inocula are presented in Table 1.

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

Inocula/parameter	TS	OM	TOC	TKN	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 soilids (%); OM: Organic matter (%); TOC: total organic carbon (%); TKN: total kehjlad nitrogen (%); EC: electrical conductivity (mS/cm).

2.4 Experimental system

Experiments with both inocula were performed in triplicates using 1-L bottles (Traveller SIGG®, Spain) as reactors. They were perfectly sealed and equipped with a ball valve that can be connected to a pressure digital manometer to determine the produced biogas pressure. The contaminants were prepared in a stock solution (5 g total PAHs in 500 ml dichloromethane) and spiked into the soil at different concentrations (0.1-2 g/kg, total PAHs) determined according to the experimental design technique. The solvent was let to evaporate under room conditions. Afterwards, the soil and inocula were mixed together based on dry weight fraction at different ratios ranging between 0.2-5:1 (w:w, soil:inocula). As inocula and soil are different in their total solids content, and in order to have the same total solids content for both of them, distilled water was added for treatments in order to modify it. Thereby, all treatments had the same amount of total solids which is equal to that corresponding to each inocula (11.8% and 10.02%). After that and to ensure anaerobic conditions, the bottles perfectly sealed were purged with N_2. The treatments of each inocula were incubated under strict- anaerobic static conditions. Mesophilic treatments were incubated in a temperature-controlled chamber at 37˚C, where thermophilic treatments were incubated in a stove adjusted to 55˚C. The total experiment time was set to be 50 days

according to previous experiments (Sayara et al., 2010). Nevertheless, after 30 days of incubation, a batch of the 3 bottles, representing the triplicate soil treatments were removed from the incubator and emptied to evaluate the process behavior after one month. The remaining duplicated treatments were left to continue until reaching 50 days of incubation. Furthermore, blanks (triplicates) with only inocula were used to evaluate the biogas production by indigenous matter (inocula). The treatments were performed without the addition of nutrients or any organic amendments. All the results are expressed as an average of duplicated samples measured after 30 and 50 days.

2.5 Analytical methods

2.5.1 Biogas quantification and analysis

Quantitative biogas production was followed by measuring the pressure increase in the headspace by means of SMC (ISE30) Pressure Switch manometer (1 MPa, 5% accuracy). The quantity of the produced biogas in each treatment was determined by subtracting the biogas produced by the inoculum itself (Blanks) form that produced in each treatment. Consequently, the net value of the produced biogas was obtained and then it was expressed under normal conditions (0˚C and 1 atm). Biogas quality i.e. identification and quantification of its components, particularly CH_4 and CO_2 was carried out using gas chromatograph (GC 5890 Capillary Hewlett Packard) as described by Sayara et al. (2010).

2.5.2 PAHs analysis

For PAHs determination after 30 and 50 days of incubation, the bottles content was dried using a lyophilizer (Benchtop 5L, Virtis Sentry, NY). Lyophilized samples were extracted using ASE 200 System (Dionex, Voisins-le-Bretonneux, France). The extraction cell (11 mL) was loaded into the oven and extracted with hexane-acetone (3:1, v/v). Static heating was applied to the vessel (150 ˚C, 7 min) and subsequent extraction was performed

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 by nitrogen for 60 s. 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. RP-HPLC analysis were performed 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). Separation of the PAHs was achieved with a gradient programme, using (A) a mixture of methanol:acetonitrile (1:1 v/v) and (B) Milli-Q water. After 5 min of isocratic elution with 70% A, the ratio was raised to 100% A in 15 min and kept constant for the subsequent 20 min. PAHs were identified on the basis of both UV spectra and match of retention times with commercially available standards. (Dr. Ehrenstorfer, Augsburg, Germany). Concentrations of the 5 PAHs out of the 16 compounds according to the US EPA method 610 were determined. The detected compounds were quantified with the fluorescent detector under following excitation/emission conditions: phenanthrene and anthracene - 250/390 nm; fluorene, fluoranthene and pyrene -280/340 nm. Calibration curves with the standards were prepared over a linear range (0.1-10 µg/ml) within the concentration of each compound.

2.5.3 Metabolites identification

Samples were analyzed using gas chromatography (Agilent HP 6890 Series II) coupled to a mass selective detector under electronic impact ionization (Agilent HP 5973) using a HP5-MS (30 m x 0.25 mm x 0.25 µm; Agilent). 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°C, carrier gas He at 0.7 ml/min. The detector worked at solvent delay mode (3.2 min) and the mass range measured was 40-400 (m/z). The detected products were identified by comparing the mass spectra with data in the Wiley 7® library.

2.5.4 Volatile fatty acids

Total volatile fatty acids (Acetate, Propionate, *iso*-Butyrate, *n*-Butyrate, *iso*-Valerate, *n*-Valerate; (g/L)) were determined by gas chromatography. Samples were centrifuged for 10 minutes at 10^4 rpm, then they were filtered through 0.25 μm. known volume of the filtrated sample was equally mixed (v:v) with pivalic acid as standard then samples were introduced for GC analysis. 1 μl sample was injected in GC (GC 5890 Capillary Hewlett Packard) with a flame ionization detector (280˚C) and Splittless injector (260˚C). HP-Innowax column (Crosslined polyethelene Glycol; 30 m x 0.53 mm x1 μm; Agilent) was used. Initial temperature was maintained at 80˚C for 1 min, then it was increase at a rate of 5˚C/min until 150˚C, then another rate of 20˚C/min until 230˚C was applied.

2.5.5 Toxicity test

Toxicity assays were evaluated according to the standardized Microtox methods (Microbics Corporation. 1992) using the bioluminescence bacteria *Vibrio fischeri* in frozen-dried form (SDI-USA) and activated prior to use by reconstitution solution. The light emission of the test organisms obtained by their direct contact with the sample was measure using the Microtox 500 analyzer (SDI, USA). The EC_{50} values of organics were evaluated using the basic test. The EC_{50} values and the corresponding 95% confidence range for 5 and 15 minute contact time were evaluated using the MicrotoxOmni software (SDI, USA).

2.5.6 Estimation of PAHs bioavailability

Bioavailable fractions of PAHs were estimated using sequential supercritical fluid extraction (SFE) (Cajthaml and Šašek, 2005). The extraction was performed with a PrepMaster extractor (Suprex, Pittsburgh, PA) equipped with VaryFlow restrictor operating at 40˚C and with a downward stream of carbon dioxide (5.5 SFE/ SFC, Messer Technogas, Prague, Czech Republic). The samples (1 g) were extracted at 50 ˚C and 200 bar. Each extraction was carried out in duplicates and the compounds were collected after 5, 10, 20, 40, 60, 80, 120, 160, and 200 min. Sequential supercritical fluid extraction represents a desorption model presuming generally that the extraction is controlled by the two rate constants differing by orders of magnitude (Williamson et al., 1998). ‘‘F fraction’’ which represents the rapidly desorbed fraction of the target chemical from soil (Williamson et al., 1998), it is usually assumed to be representative of equilibrium release conditions, and the subsequent, slowly released portion is considered to be kinetically rate-limited. The chemical release data was modeled by an empirical two-site model, consisting of two first-order equations (Williamson et al., 1998) as follow:

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

Where S_t is the pollutant concentration remaining in the soil after time t; F is the fraction of chemical 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 for calculating the “F” values according to the aforementioned equation.

2.5.7 Quantification of microbial biomass

Samples for phospholipid fatty acid (PLFA) analysis were extracted by a mixture of chloroform-methanol-phosphate buffer (1:2:0.8; v/v/v). LiChrolut Si-60 solid-phase extraction cartridges (Merck, Whitehouse Station, NJ) were used for separation of the

extracts and phospholipid fractions were subjected to mild alkaline methanolysis (Šnajdr et al., 2008). The 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, CA, USA). The GC instrument was equipped with split/splitless injector and a DB-5MS column (J&W Scientific, Folstom, CA, 60 m, 0.25 mm i.d., 0.25 μm film thickness) was used for separation. The temperature program started at 60 °C and was held 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 was up to 280 °C at a rate of 2.5 °C min^{-1}, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra 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 using their individual chemical standards obtained from Sigma-Aldrich, Prague, Czech Republic and Matreya LLC, Pleasant Gap, PA, 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 (G+) and Gram negative (G–) bacteria were estimated 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. As anaerobic PLFA markers, amounts of cy19:0, cy17:0, 18:1ω9 were used (Oravec et al., 2004; Sampedro et al., 2009, Šnajdr et al., 2008).

2.6 Experimental design methodology and statistical analysis

The effect of two factors (k=2), namely: 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 using Central Composite Design (CCD) methodology. More details about the experiment design technique and its application can be found and reviewed elsewhere (Deming and Morgan, 1987; Sayara et al., 2010). 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 the degradation percentage (Y_D) and the biogas production (Y_G) are also reported. As shown in Table 2, nine experiment runs (triplicates) were carried out according to the experiment design technique; meanwhile blank runs which only have the corresponding temperature-dependence inocula were also lunched in parallel. 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

3.1 Response surface and statistical analysis

Central composite design has been applied to delineate the process behavior under different levels of the previously mentioned factors. The degradation (D) percentage (%) and the biogas (G) production (L/kg-TS) after 30 and 50 day have been selected as functions to optimize the parameters and to represent the response of the process. The second order models for each considered variable are illustrated in equations 1-8 for thermophilic (T) and mesophilic (M) treatments.

$$Y_{TD30} = 51.9 + 23.28x_1 - 6.69x_1^2 - 0.69x_2^2 - 1.9x_1x_2 \quad (1)$$

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

$$Y_{TG30} = 3.62x_1 - 7.03x_2 + 3.1x_1^2 + 1.7x_2^2 - 2.4x_1x_2 \quad (3)$$

$$Y_{TG50} = 8.18 + 7.4x_1 - 11.5x_2 - 0.75x_1^2 + 1.9x_2^2 \quad (4)$$

$$Y_{MD30} = 42.7 + 46.18x_1 - 13.23x_1^1 + 0.21x_2^2 - 1.36x_1x_2 \quad (5)$$

$$Y_{MD50} = 20.95 + 68.18x_1 - 22.77x_1^2 + 0.39x_2^2 \quad (6)$$

$$Y_{MG30} = -135.5x_1 + 6.3x_2 + 50.7x_1^2 + 8.7x_1x_2 \quad (7)$$

$$Y_{MG50} = -96.55x_1 + 6.02x_2 + 35.82x_1^2 + 5.6x_1x_2 \quad (8)$$

Regarding the correlation coefficients (R) and *P*- values of the functions representing the degradation percentage for both inocula after 50 days (Table 3), the selected regression model is considered to be adequate to describe the data and the relation between the independent and dependent variables. On the other hand, the correlation coefficients and *P*-values obtained form the rest of the treatment models representing the degradation percentage and biogas production were not within the favorable domain, indicating that the process is more probably influenced by other factors that should be taken into account. In this study, the low organic matter content and the presence of the contaminants influenced the biogas production. However, the obtained values regarding the statistical analysis with the corresponding treatments could be used to have an impression about the process performance when such conditions are available, especially for biogas production/inhibition in the case of PAHs presence.

Table 2. Design matrix including Factor levels (coded and actual) and their response values for the 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 reperent the incubation time (day).

Table 3. Correlation coefficients (R) and *P*-values of each function (equation 1-8) 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

3.2 Overall degradation of the contaminants (PAHs)

Anaerobic degradation of PAHs under the different studied conditions was evaluated after 30 and 50 days of incubation in attempt to follow the process evolution with time. Figure 1 illustrates the obtained results under thermophilic and mesophilic conditions. After 30 days of incubation, all treatments showed a certain potential of degradation and almost the same trend under the two temperature conditions was observed. The observed degradation demonstrates that native microorganisms of the applied inocula either thermophilic or mesophilic have the catabolic capacity to degrade the used PAHs, and methanogenic metabolism is coupled to anaerobic PAHs degradation (Chang et al., 2006, Yuan and Chang, 2007). However, treatments with low PAHs concentrations (0.1g/kg) gave the lowest degradation rate at all; suggesting that microbial activity is driven by the pollutant concentration where a minimum threshold or trace level is required for maintaining degradative sequences and catabolic induction in degrading microorganisms (Boethling and Alexander, 1979). Additionally, in the case of low concentration, more surface areas of the soil particles are available for the contaminant to be adsorbed on compared with high concentrations; therefore stronger interaction could be formed, which complicates the accessibility and degradation of the contaminant as shown in Table 5, where “F” fraction of low PAHs concentration is lower in most cases than those

corresponding to high concentrations. Relatively, degradation capacities under mesophilic conditions were higher than those obtained with thermophilic. However, thermophilic condition gave better results especially when low PAHs concentrations were used. Probably, this is because high temperature are supposed to increase the desorption of the PAHs and their solubility as it was observed in the determination of the bioavailable portion of the contaminants, which was in most cases higher than those corresponding to mesophilic conditions (Table 5). Moreover, under thermophilic temperatures the mass transfer and reaction kinetics are more pronounced. Nevertheless, Chang et al., (2002) found that 30˚C enhanced PAHs degradation better than 40˚C and their presence together also enhanced the degradation. Under the different combinations and conditions the applied contaminants (individuals) followed a similar degradation trend that was almost in accordance with obtained results regarding the bioavailability (Table 5). In this context, It was found that the PAHs degradation followed this order: Flouren > Anthracene > Phenanthren > Flouranthene > Pyrene. Parameters like water solubility, number of benzene rings and structural arrangement (shape) of the molecules were suggested to drive the degradation.

Analysis of selected components of PLFA demonstrated that anaerobic bacteria are within all treatments assays, but their abundance in the thermophilic treatments was higher than mesophilic ones (Table 4). Furthermore, moderate to high PAHs concentrations significantly ($P<0.05$) altered microbial community structure mainly for anaerobic communities. Obviously, PLFA profiles are in agreement with the obtained PAHs degradation (Fig.1) under both conditions as less degradation rates were achieved with the lowest PAHs concentrations. According to Donald et al. (1998), the microbial community responded to PAHs contamination at both the phenotypic and genotypic levels that is in agreement with the obtained results.

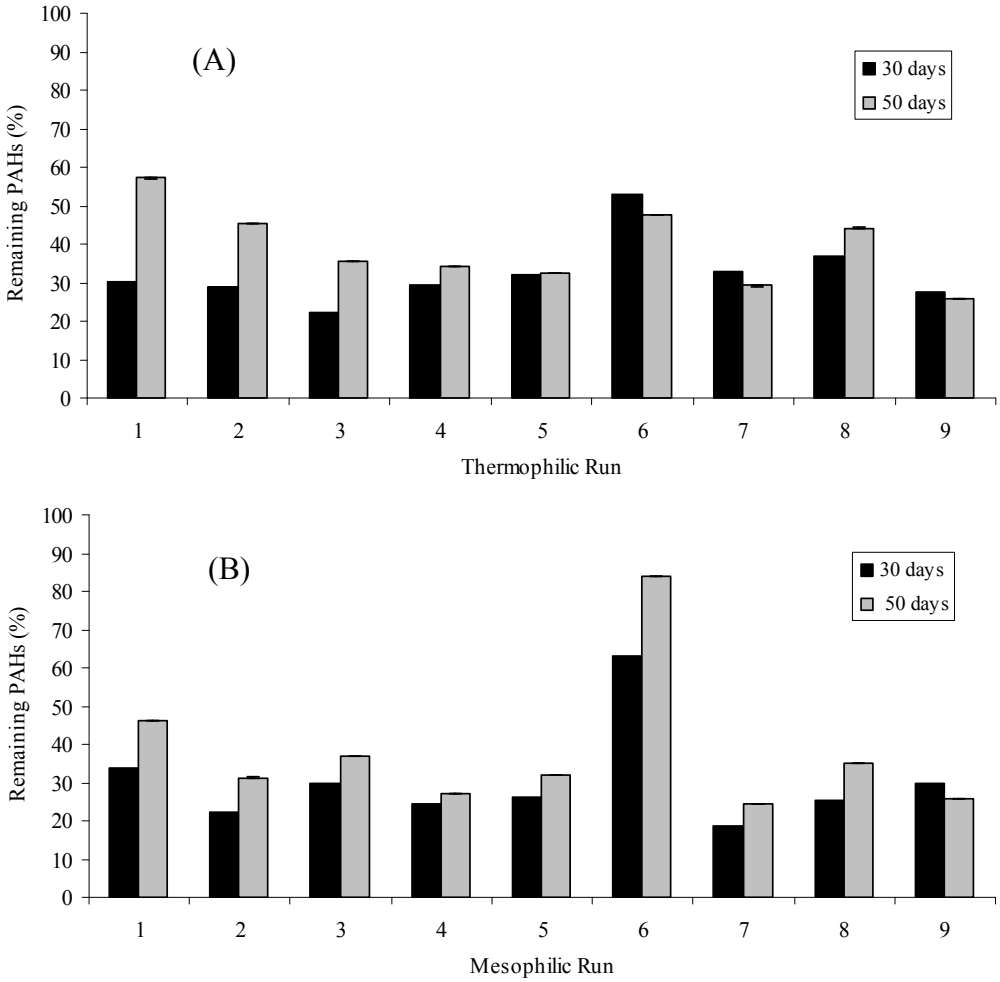

Figure 1. Remaining (%) of PAHs after 30 and 50 days of incubation; (A) thermophilic conditions and (B) mesophilic conditions.

As normally expected with biological treatments of PAHs, the concentration is decreased with time as a result of the microbial activity, or no change occurred in the case of the process failure or absence of optimal conditions for microbial activity. However, in this study the results were completely contradicting with this expectation. For the majority of treatments under both conditions (37˚C and 55˚C), it was found that the PAHs concentrations after 50 days of incubation are higher than those obtained after 30 days. For such case, two hypotheses could be assumed: A) sorption or occlusion of the contaminants (PAHs) to the organic matter during the first 30 days and since it is substantially changed

during the treatment period; therefore could also release the PAHs latter again. B) The PAHs had been bioformed under these conditions. In this regard and in order to clarify the sorption impact; duplicated representative sample were extracted twice using ASE 200 under extreme conditions (more than those explained in section 2.5.2-PAHs analysis) including high temperature (200˚C), pressure (206.84 bar) and 4 static cycles (10 min). Moreover, the same samples were extracted using Soxhlet extraction and analysed as explained elsewhere (Sayara et al., 2010). Unfortunately, all the used extraction and analysis methods gave the same results which demonstrated an increase in the PAHs concentration. Accordingly, it is more probably to assume the reversed biotransformation of the PAHs under such conditions as no other PAHs were found. Indeed, Thiele et al., (2002) demonstrated that the PAHs were formed in soil under oxygen deficient conditions. The concentration was increased significantly for those PAHs with high molecular weight (more than 3 rings), but for compounds with 3 rings, the concentration was decreased, which was probably due to anaerobic biodegradation. Furthermore, the incubation of edible oil in closed containers under room temperature led to the formation of PAHs (Guillen et al., 2008) indicating that anaerobic condition are more probably responsible about such phenomenon. Nevertheless, in the present study no new PAHs have been detected except those which were introduced in the initial mixture suggesting the re-biotransformation of these PAHs.

3.3 Metabolites

The observed decreases in the PAHs concentration lead to conclude that the used inocula were able to degrade the target contaminants. In this regard, anaerobic biodegradation of the used PAHs was followed by analysing the metabolites which are thought to be caused by the microbial activity found within the treatments (Table 4). In fact, several compounds had been identified with GC/MS analysis as a result of the proposed

activity. These compounds include: Phenol, hydroxyflourene, benzeneacetic acid, cresol, pyridine, flourenone, flourenol, carbazole, anthracenone, anthracenedione, biphenyl, anthrone. The detected compounds are known to be as metabolic derivatives generated when PAHs are degraded by microorganisms through several oxidization pathways. Consequently, the detected metabolites found in this study provided evidence for the microbial capacity to degrade PAHs under the studied conditions, while the different stages of the biodegradation process could lead to variation of these metabolites. PAHs degradation pathways under anaerobic condition still are not clear, where various hypothesis or arguments are found in the literature. Meckenstock et al, (2000) and Zhang and Young, (1997) argued that carboxylation is the initial step in PAH biotransformation under sulphate reducing conditions. On the other side, Bedessem et al., (1997) proposed that hydroxylation as the initial step in PAHs 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 was proceeding by hydroxylation reaction, where phenol and cresol were indentified as intermediate from benzene and toluene, respectively. In this concern, as those compounds have been identified in the present study, it would be more probably to assume that hydroxylation was the initial step during the biotransformation of the PAHs. It is more probably that PAHs compounds undergo an 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 were degraded under methanogenic conditions and hydroxylation was the initial step in their biotransformation. However, until recently, there are no studies that clearly investigated the PAHs degradation under such conditions.

Table 4. PLFA structure of the microbial biomass for treatments 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 (G+)	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 (G-)	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 biomas	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

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

	T-5	**T-6**	**T-7**	**M-5**	**M-6**	**M-7**
Flourene	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
Flouranthene	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)

3.4. Biogas production

The cumulative biogas production under thermophilic and mesophilic conditions is illustrated in Figures 2. It is clear that methanogenic inocula in both treatments were exposed to inhibition effects. Consequently, an adaptation period of at least 20 days was needed in most cases except run 2 and 8 under thermophilic conditions. The inhibition rate, i.e. "negative values which indicate that even the background gas production relative to blanks" was gradually increased with time indicating the increase of the toxicity within the treatments. However, in most treatments, it can be seen that after almost 20 days of incubation, biogas production curves began to change their trends such that the inhibition decreased over time but was not eventually eliminated especially for mesophilic treatments. Evidently, the process recovery upon prolonged incubation was also noticed as methane production was observed and increased significantly after this 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 emphasized that they were free from any PAHs, which complicated their activity till being adapted with these circumstances. Interestingly, the study clearly indicated that although the presence of PAHs

considerably inhibits the biogas production, the PAHs degradation was not affected. Indeed, Fuchedzhieva et al., (2008) indicated a similar behavior as flouranthene degradation continued in spite of the cell growth inhibition in the presence of biosufactant complex. Treatments under thermophilic condition achieved better result compared with mesophilic ones, which can be attributed to the high temperatures that facilitate the reaction rates and enzymatic activity. However, microbial activity could be disrupted by several toxic metabolites resulted from parent compounds degradation which was believed to be more toxic. Indeed, toxicity analysis (data not shown) showed high rate of toxicity in all treatments compared with non toxic control, which is proposed to be caused by the PAHs themselves and their metabolites products due to microbial degradation. Additionally, the ratios of saturated to monounsaturated PLFAs (S/M) and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre) (Table 4) were significantly greater in mesophilic treatments compared with the thermophilic, which clearly pointed out the stress present within them as elevated levels of these ratios indicate great levels of microbial community stress (Moore-Kucera and Dick, 2008). On the other hand, analysis of volatile fatty acids showed that there is no inhibition caused by such acids as they were not detected in all treatments after 30 and 50 days. Only a tiny (neglected) amount was found in run 1 of the thermophilic treatments after 30 days of incubation. Additionally, pH values of treatments mixture after 30 and 50 days was found to be between 7.3-7.9, which is within the optimum values for anaerobic digestion process. Thereby, the fluctuation encountered in treatments was more probably due to such PAH-metabolites which formed transiently during the anaerobic biodegradation of the PAHs. The vacillation in the biogas production (Thermophilic run 8) delineated clearly this effect. In fact, when the microorganisms adapted with the available ambient, more biogas was produced, however in the following

days, their activity was inhibited or disrupted from these new compound, and then they recover their activity showing their ability to proceed in transforming the contaminants.

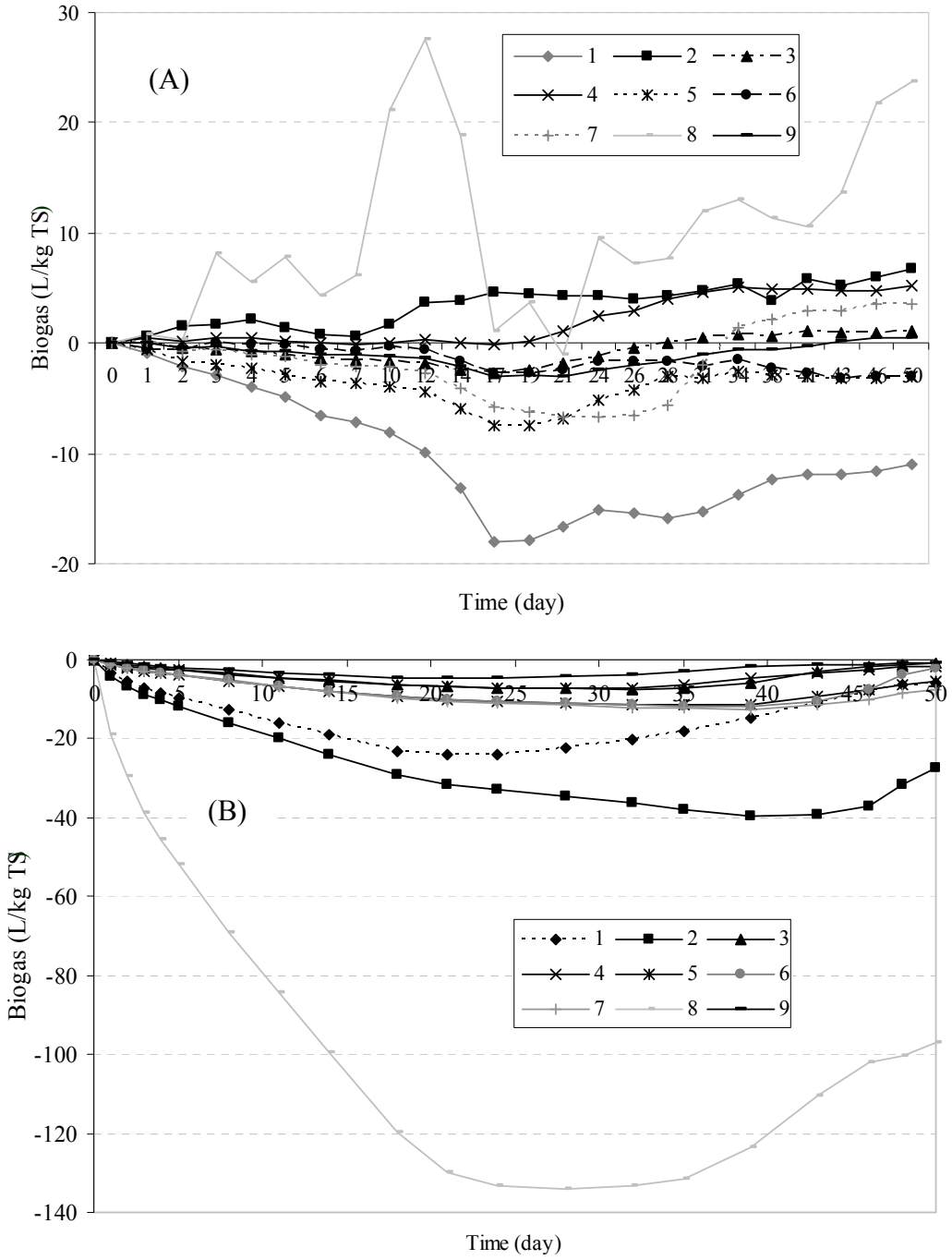

Figure 2. The cumulative biogas production (L/kg TS) after 50 days; (A) thermophilic conditions and (B) under mesophilic conditions.

3.5 Process response for PAHs degradation

The response of the PAHs degradation (%) under the studied factors is illustrated in Figure 3 and Figure 4 for thermophilic and mesophilic inocula respectively. Under mesophilic conditions, it is clear that the degradation capacity is always proportional with PAHs concentration as more degradation was observed with higher PAHs concentrations. However, soil to inocula mixing ratio was found to influence PAHs degradation under low concentrations (less than 1 g/kg), such that low (0.2:1) or high (5:1) mixing ratios enhanced the degradation rate, but intermediate ratios negatively affected the process. Almost the same behavior was observed under thermophilic conditions during the first 30 days although the PAHs concentration influenced the degradation capacities but to a smaller extent (Fig. 3A). Nevertheless, after 50 days the degradation capacity response demonstrated that the process is proportional with both factors, such that higher degradation (75%) was obtained with highest concentration and mixing ratio. The increased toxic potential of higher concentrations of the inocula may explain why degradation decrease as inocula ratio increased. Similar conclusions have been drawn with the increase of the amendment amount under anaerobic treatments (Chang et al., 2002).

In both cases the PAH-degradation was clearly proportional with soil to inocula mixing ratio where better results were obtained with the highest ratio (5:1). These observations are in accordance with the results obtained by Hernandez et al., (2008) where increasing substituted phenolic compounds concentration leaded to enhance the biomethanization process, but at higher concentration the total biodegradation was decreased. The observed sequential decrease in the biogas production (Fig. 2) during at least the first three weeks clarified the difficulties that the microflora had to overcome in order to follow up their degradation function. In this point, the time factor was essential for microbial adaptation under these conditions. Nevertheless, although PAHs and their

metabolites were found to be inhibitory to methanogens, other anaerobic microbes had the ability to follow up in the degradation process. An interesting result was the increase of PAHs degradation when high soil to inocula ratio was employed for both cases. This observation is considered fundamental for economical evaluation as more contaminated soil amount could be treated with fewer quantities of inocula. As no other nutrients sources were available for the microorganisms, it is more probably that low content of organic matter encountered in the soil components served as nutrient source for microbial activity, and this nutrients limitation motivated the degrader to grow and latter to use the PAHs as nutrients. Nutrients low content and PAHs were stressed the microbial community; thereby the addition of organic source like compost might stimulate microbial activity and, consequently, accelerate the PAHs degradation. This was clear in previous work were higher rates of degradation were obtained (Sayara et al., 2010).

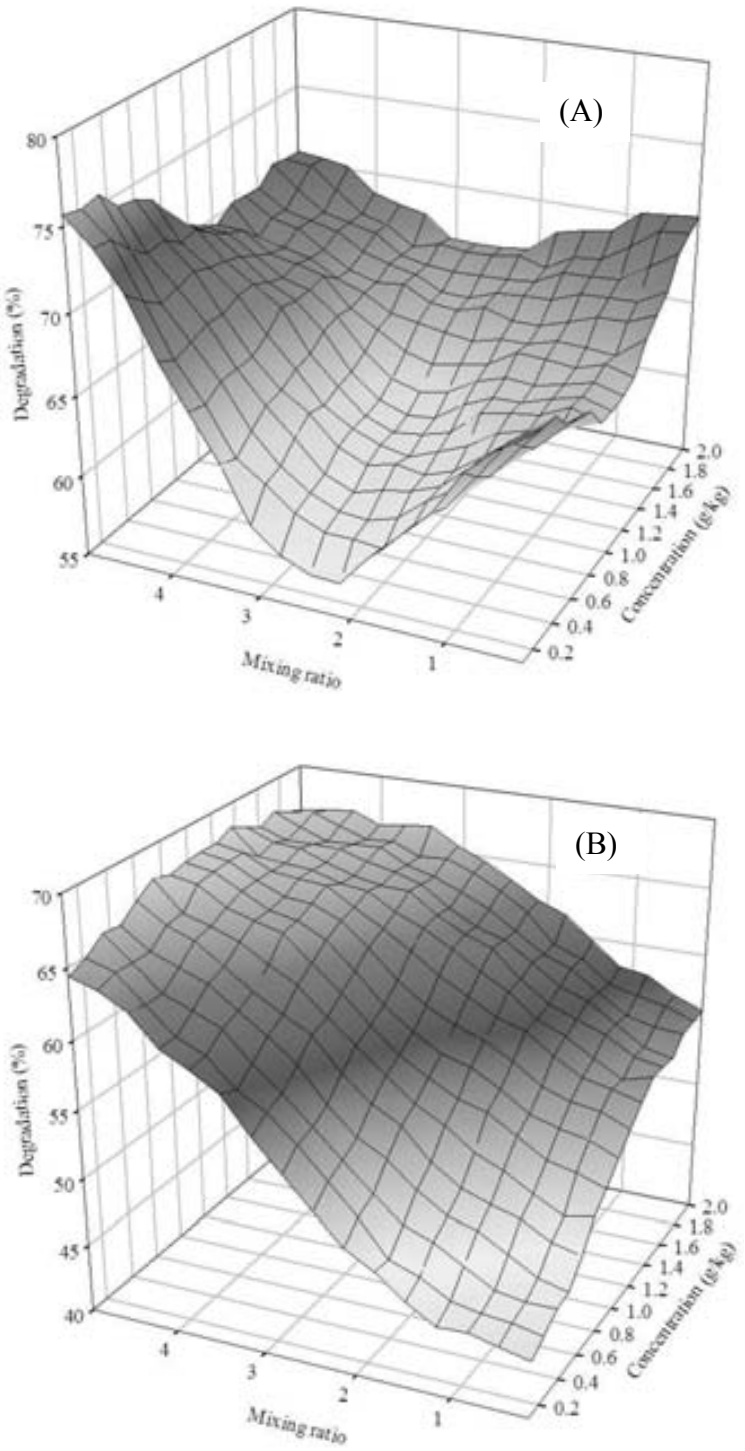

Figure 3. Response of PAHs degradation (%) under thermophilic conditions; (A) after 30 days and (B) after 50 days.

Figure 4.

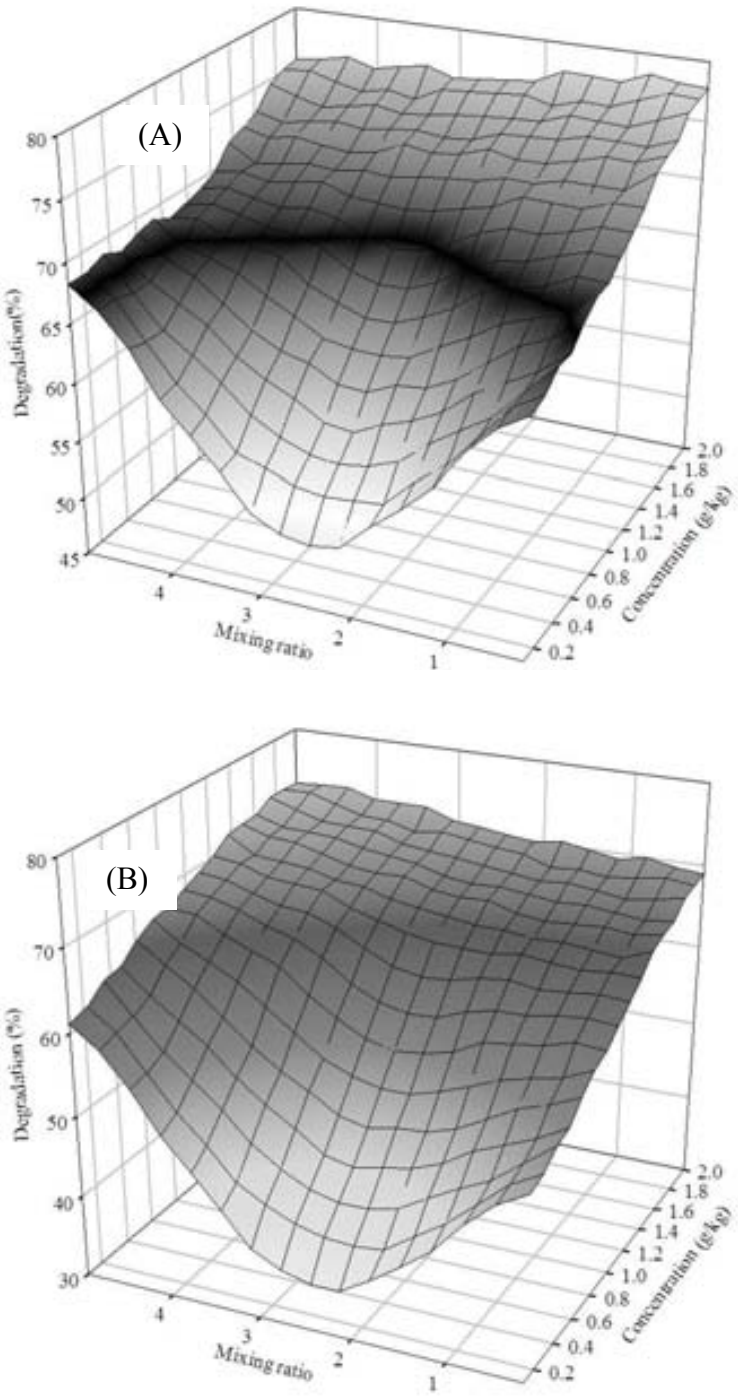

Figure 4. Response of PAHs degradation (%) under mesophilic conditions; (A) after 30 days and (B) after 50 days.

3.6 Process response for biogas production

The response of biogas production under thermophilic and mesophilic conditions is illustrated in Fig. 5. The produced biogas is referring to that obtained from the contaminated soil after subtracting that produced by the inocula itself. Whereas, negative values indicate that even the background biogas production relative to blanks is inhibited by the contaminants. As it can be seen, the process behavior was totally different among them as no biogas was produced under mesophilic conditions, while under thermophilic conditions; certain amounts were produced under specific conditions. Considering the soil to inocula mixing ratio, a ratio of almost 3:1 could be visualized as inflection point in relation with the applied concentrations (0.1-2 g/kg). Specifically, soil to inocula ratio higher than 3:1 was quietly influenced by the PAH concentration variation. The biogas production rate slightly increased with increasing PAHs concentration under thermophilic condition, meanwhile no variation has been depicted with mesophilic inocula under the same conditions (stagnation state). For mixing ratio below 3:1, different behaviors were observed that demonstrate various interactions among the studied factors within the two temperatures domains. The biogas production rate was positively increased by increasing the concentration under thermophilic conditions. This increase was valid until reaching specific concentration (almost 1.4g /kg) where the increment began to influence negatively the microbial activity demonstrating that the methanogeses could not tolerate with high concentration because of their toxic properties. Moreover, biogas enhancement was noticed with low soil to inocula ratio as more inocula were introduced. Nevertheless, increasing inocula quantity negatively affected the biogas production under low PAHs concentrations (<0.2g/kg). The scenario was different with mesophilic conditions as the rate of inhibition was gradually decreased by increasing the soil to inocula ration until reaching 3:1 where plateau condition were obtained. However, PAHs concentration under those conditions (mixing ratio less than 3:1)

influenced the process in two different matters. Increasing PAHs concentration followed by an increase in the inhibition rate until reaching a certain concentration which was almost 1g/kg where the process changed its behaviour and a decrease in the inhibition was observed.

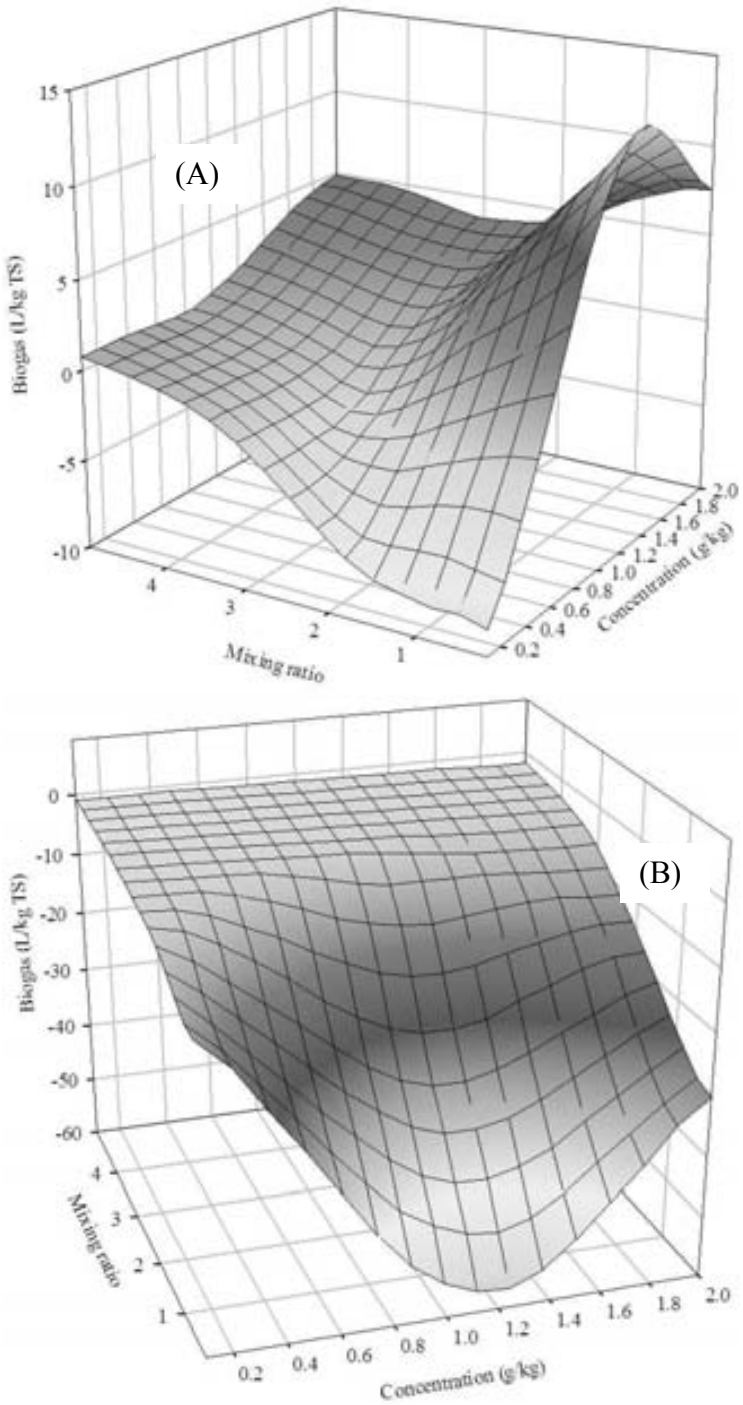

Figure 5. Response of the total biogas production (L/kg TS) after 50 days; (A) thermophilic conditions and (B) under mesophilic conditions.

4. Conclusions

This is the first time where a comparative study on anaerobic bioremediation of PAHs-contaminated soil under methanogenic conditions including thermophilic and mesophilc temperatures that has been conducted to evaluate the removal of PAHs. Additionally, this study may have been the first to conclusively demonstrate the inhibition of biogas production by PAHs. Although certain rate of degradation was achieved during 30 days, prolonged incubation for most treatments showed an increase in the PAHs concentrations which probably caused through reversed bioformation of these compounds under such conditions. Consequently, applying this technology needs more investigation to perfectly clarify and diagnose this effect.

Acknowledgment

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Chapter 6

General conclusions

Remediation of PAHs-contaminated soil has been studied applying composting technology and strict anaerobic-methnogenic conditions as two possible biological-alternatives to deal with such type of soil contamination. Indeed, the two-applied technologies were able to degrade the target contaminants, even though the degradation rate varied depending on the available conditions. Consequently, considering these technologies for PAHs-contaminated soils is recognized as a promising and viable option.

Through the research study, several conclusion were drawn which are considered important for future applying of these treatments. The main conclusions can be summarized as follow:

I- Composting approach

- Composting is an indisputably advantageous technology for bioremediation of PAH-contaminated soils. The process is cost-effective, low-risk and easier to be controlled compared with other technologies which may produce unfavourable out puts.
- The process depends on the addition of available low-cost co-substrates like compost, which provides an alternative use of such co-substrates rather than the agricultural-traditional use.
- Among the different screened organic co-substrates, compost derived from OFMSW is recognized as a suitable co-substrate for bioremediation-composting of PAHs soil. However, the effectiveness of this compost is highly correlated with its stability degree. In fact, through out the present study, stability degree was a crucial parameter that influences the degradation rate. The relation between stability degree and the presence of humic matter within the compost are well correlated also. This is believed to significantly influence the

bioavailability of the contaminants and its degradation rate as a consequence when stable compost with appreciable humic mater content is used.

- Experiment design technique is a reliable approach for studying the factors which influence the bioremediation process. It allows having a general outlook about the process behaviour under different factors with different levels. Subsequently, optimum value for treatments operation could be obtained, hence, costs and time could be saved.
- During the composting process, increasing the soil to compost mixing ratio does not necessarily increase the degradation rate. On the contrary, due to preferential behaviour, microorganisms will leave the contaminants in the case of available easy-degradable nutrients and thus reducing or slowing the degradation rate.
- Contaminants degradation rate is highly dependent on their concentrations. High concentration (2 g/kg, as in this present study) exhibited some rate of toxicity that inhibited or stressed the microbial activity. Nevertheless, low concentrations resulted in low degradation rates. This behaviour points out that there is a low limit of concentration that is needed to motivate the microbial activity. Accordingly, more attention should be paid when low concentration-contaminated sites are to be treated with this approach, or some additives are to be introduced to facilitate their degradation.
- Bioaugmentation of the composting mixtures with exogenous microorganisms did not add any enhancements on contaminants degradation. Really, introducing such microorganisms is not totally guaranteed as they encountered within environments different from those optimal where they were cultivated. However, biostimulation through introducing suitable co-substrate will parallely be able to provide wide arrays of microorganisms and nutrients for soil indigenous microorganisms as it was observed.
- Mesophilic temperature were observed to be more favourable for the degradation of PAHs during the composting- bioremediation processes.

II- Anaerobic-methanogenic approach

- Anaerobic-methanogenic consortia had the capacity to degrade PAHs. These findings spotlight on such less applicable methods as generally was believed that these approaches are incapable to handle these types of contaminants. On the contrary, more attention should be devoted to extend the knowledge about their behaviour as they can be applied for in-situ treatments.
- Although biogas production was found to be inhibited by PAHs, the anaerobic consortia proceeded in its activity regarding their degradation which indicates the diversity of the microorganisms in the introduced inocula.
- The degradation process was influenced by several studied factors. PAHs concentration was usually a crucial factor and the degradation rate proportioned with it. Moreover, high rate of soil to inocula pronounced the degradation which is an economical- remarkable factor.
- As small amounts of biogas were produced during the digestion process, because of the small amount of organic matter, mesophilic inocula are more adequate to be applied when the main goal is degrading the contaminants, especially from the economical point view.

In general, both composting and anaerobic digestion can be viewed as an optimal approach to ensure environmental sustainability and soil treatment when optimal conjunctive operation is applied. However, the obtained results through composting treatments were more favourable compared to those obtained through anaerobic treatments. In deed, composting treatments achieved acceptable and satisfactory results within short period when optimum conditions were provided. Nevertheless, anaerobic treatments needed more time and the yield results were less favourable. Furthermore, even the bioformation of PAHs is not totally justified (need further research), if prolongation of the incubation period would result in adverse performance, this factor should be considered with much attention during these treatments.

As an extension of the current research, the following recommendations are proposed for future work which may add more knowledge about the two presented approaches of soil bioremediation:

1. Characterization of the microbial activity within the treatments mixture in attempt to identify those microorganisms spectra with high capacity to degrade such organic environmental contaminants.
2. Evaluation the efficiency of surfactants as additives to increase the availability of more recalcitrant PAHs.
3. Studying the effect of bioaugmentation using a consortium of several exogenous microorganisms.
4. Studying the effect of soil properties on the process behaviour.
5. Evaluating the process behaviour in the case of the availability of real contaminated soil considering the optimum conditions obtained throughout the research under laboratory scale.
6. Studying the possibility to reduce/eliminate the lag phase during the anaerobic treatments providing nutrients sources or other additives.
7. Studying the possibility of bioformation of PAHs under anaerobic condition, and reasons behind this behaviour.