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## Anaerobic biodegradation of $^{13}\text{C}_6$ -phenol: Analysis of bacteria population involved in two different degradation kinetics.

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### Abstract

Biodegradation of  $^{13}\text{C}_6$ -phenol until mineralisation was observed under mesophilic and thermophilic conditions during the degradation of municipal solid waste. In Mesophilic conditions, as well as in thermophilic, two types of degradation kinetics were obtained. One follows a first order kinetic and the other follows a lag-phase model. The pyrosequencing analysis shows that in the fastest kinetic *Clostridium* sp. and *Anaerobranca* sp. were the microorganisms majorly present under mesophilic and thermophilic conditions, respectively. These microorganisms have been already referenced in the literature as microorganisms involved in phenol degradation. Our results suggest that these differences could be explained by the proportions of microorganisms present in both kinetics.

### Keywords

$^{13}\text{C}_6$ -Phenol; Anaerobic digestion of MSW; Pyrosequencing; Operational taxonomic unit OUT; SSUrRNA.

## INTRODUCTION

The development of digestion has been promoted in recent years based on several important European guidelines: The production of renewable energy (directive EU 27/09/2001); augmentation of thermal production in 50% (Law POPE 13/07/05) and the provisioning of an organic amendment to fields in order to contributing to the maintenance of soil fertility (circular of 28th June 2001). In this context, it is necessary to make sure that the digested products do not contain any unwanted contaminants like phenol, which has significant mutagenic and adverse effects on health. The aim of this study is to monitor the fate  $^{13}\text{C}_6$ -phenol, to identify microorganism that could be involved in its degradation and their impact in the phenol degradation kinetic during the anaerobic digestion process.

## MATERIALS AND METHODS

**Microcosms' incubation:** The microcosms were reconstituted with the MODECOM standard French waste, we inoculated anaerobically with landfill leachates and then we incubated them, either under mesophilic or thermophilic conditions in the dark. Once the methanogenesis had stabilized (at 130 days), we injected 7mg/L of  $^{13}\text{C}_6$ -phenol 99% (Cambridge Isotope Laboratory - Andover, USA) in all the microcosms, before adding in one of them the biocide ( $\text{HgCl}_2$ ) with the aim to set an abiotic control. Throughout the incubation liquid and gas sampling were taken for subsequent analysis.

**$^{13}\text{C}_6$ -phenol quantification:** For two hundred days, we took liquid samples of each microcosm in order to monitor the remaining concentration of  $^{13}\text{C}_6$ -phenol in the incubations. After, each sample was derivatized with acetic anhydride, before being extracted by a PDMS-DVB SPME fibre as described by Limam et al., 2010. Each sample was analysed with a Trace Ultra gas chromatograph coupled to a mass spectrometer (Thermo Fisher Scientific, France) and quantification was performed on the selected ion monitoring mode (m/z 100 for  $^{13}\text{C}_6$ -phenol).

**Biogas isotopic monitoring:** Throughout the incubation time, we measured the gas composition in order to monitor the transformation of  $^{13}\text{C}_6$ -phenol into  $\text{CO}_2$  and  $\text{CH}_4$ . A gas chromatograph/combustion/isotope ratio mass spectrometer (GC/C/IRMS) system (Thermo Fisher Scientific, France) was used to measure  $\text{CH}_4$  and  $\text{CO}_2$  isotopic composition. The results are expressed in delta notation ( $\delta^{13}\text{C}$ ) relative to the standard V-PDB in  $^{13}\text{C}$  percentage.

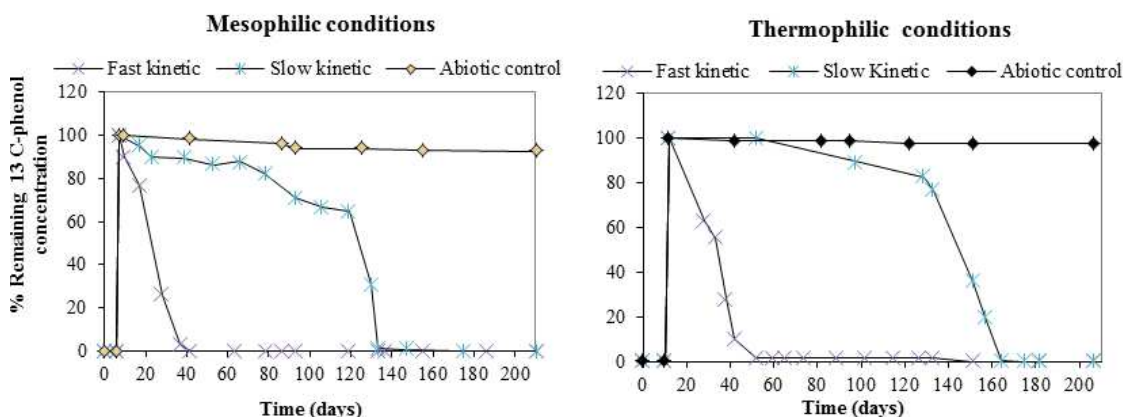
**DNA extraction and Pyrosequencing analysis:** Samples were collected from incubation at days 20 and 120, where degradation rate is higher, in order to identify the microorganisms present at that moment. Total DNA was extracted from the microcosms with the Power Soil DNA Isolation Kit (Mebio Laboratories Inc). After, DNA was quantified by Qubit (dsDNA HS Assay Kit, Invitrogen, Eugene) before checking its integrity by electrophoresis on agarose gel and EtBr staining. Pyrosequencing was performed as described in Smith, et al., 2010 by the Research and Testing Laboratory (Lubbock, TX) based on RTL protocols, and resulting data was analysed with the open source software package QIIME “Quantitative Insights Into Microbial Ecology” (Caporaso, et al., 2010). 16S DNA sequence reads were trimmed where quality score dropped lower than 25 and discarded if their final length was shorter than 150 nt. In addition, remaining reads where the longest homopolymer was greater than 6 nt or containing an ambiguous base were also discarded. Sequences were then aligned with PyNAST (Caporaso, et al., 2010) using the Silva 108 database core-aligned set formatted for QIIME as a template. Putative chimeric sequences were identified with ChimeraSlayer and removed from dataset. Remaining sequences were clustered in Operational Taxonomic Unit (OTUs) at 97% sequence similarity using uclust (Robert Edgar, unpublished, 2009). OTUs taxonomic assignment was then performed with the RDP classifier (Wang, et al., 2007, Cole, et al., 2009) at a 0.8 bootstrap cut-off using the most abundant sequence in each OTU as representative sequence. Finally, OTUs not corresponding to the expected phylogenetic domain were discarded.

## RESULTS AND DISCUSSION

### Phenol degradation under mesophilic and thermophilic conditions

The percentage of remaining concentration of  $^{13}\text{C}_6$ -phenol for mesophilic and thermophilic conditions is represented in Figure 1a and b respectively. For both conditions our results clearly suggest that  $^{13}\text{C}_6$ -phenol degradation is catalysed by microorganisms. Indeed, on the abiotic control microcosms, where the biocide was added, the concentration of  $^{13}\text{C}_6$ -phenol remains stable in time, whereas in the biotic microcosms, the  $^{13}\text{C}_6$ -phenol totally decreased (Fig.1). This biodegradation of  $^{13}\text{C}_6$ -phenol led to its mineralisation since an important increase of  $^{13}\text{C}$  percentage in  $\text{CO}_2$  and  $\text{CH}_4$  has been observed which supports our conclusion (data not shown). Under mesophilic conditions, we have identified benzoate as a degradation product of  $^{13}\text{C}_6$ -phenol which confirms the findings of Fang et al., 2004. Under thermophilic conditions, non metabolites were observed. Overall, our results agree with different studies where biodegradation was possible under mesophilic conditions but diverge from Leven et al. 2005, who did not observe the degradation of phenol under thermophilic conditions with a similar type of inoculum.

For each condition, both mesophilic or thermophilic, we have obtained two types of degradation kinetics. For mesophilic conditions, one kinetic follows a first order model (Fig.1a) where total degradation is obtained in 40 days, and the second type of kinetic follows a Lag-phase model where degradation is completed only after 133 days. For thermophilic, one follows a first order kinetic (Fig 1b) where degradation is completed after 60 days, and the second follows a Lag-phase model where degradation is completed after 160 days. In order to explain these significant differences between the phenol degradation kinetics for the same condition, we analysed and compared the microorganisms present in each experiment.



**Figure 1.** Remaining percentage of <sup>13</sup>C<sub>6</sub>-phenol. Mesophilic (left) and Thermophilic (right) degradation kinetics in function of time.

**Microorganisms involved in <sup>13</sup>C<sub>6</sub>-Phenol degradation** Pyrosequencing results of both conditions and kinetics were clustered into OTUs based on their sequence similarity and then used for taxonomic identification. The most representatives OTUs for our incubations are represented in table 1. We observe that the slow and fast incubations, under the same conditions, share most of the species. Thus, regarding microbial taxa content, the incubations under mesophilic conditions are similar among them but different from the thermophilic ones. In the same manner, thermophilic incubations are similar only among them. Despite of the species' similarities shared for the incubations under the same temperature, the proportions for these populations are different between the slowest and the fastest kinetics. This leads us to think about the influence of some populations on the degradation kinetics, and especially when the degradation takes place early.

**Table 1.** Most representative genus present in incubations: Left side, mesophilic conditions fast and slow, kinetic. Right side, thermophilic conditions fast and slow, kinetic

Genus	Mesophilic		Genus	Thermophilic	
	Fast kinetic	Slow kinetic		Fast Kinetic	Slow Kinetic
<i>Clostridium</i>	20.28 %	8.97 %	<i>Gelria</i>	14.9 %	33.8 %
<i>Sporosarcina</i>	14.6 %	0 %	<i>Anaerobranca</i>	28.4 %	0.8 %
<i>Incertae sedis</i>	28.1 %	66.9 %	<i>Opb 54</i>	3 %	37.8 %
<i>Bacillus</i>	12.8 %	0.2 %	<i>Shbz 1548</i>	19.0 %	0 %
<i>Paenibacillus</i>	5.1 %	1.0 %			

For instance, under mesophilic conditions, one of the microorganisms present in the fastest kinetic corresponds to *Clostridium* sp., which represents 20 % of total population versus 8.97% present in the slower kinetic. This microorganism has been already referenced as a phenol degrader by Fang et al., 2004, Fang et al., 2006 and Zhang et al., 2005, who also suggest that under methanogenic conditions *Clostridium* sp. could be responsible for the conversion of phenol to benzoate. Our finding confirms the Fang et co-workers' results since we have detected the benzoate and we have identified *Clostridium* sp in the kinetics. Among the minor population *Paenibacillus* sp, is present and it has been also referenced as a phenol

degrader by Singh et al., 2009. Under thermophilic conditions the differences are even stronger. The analysis shows that about 28% of the total population corresponds to *Anaerobranca* sp. as the major microorganism present in the faster kinetic, versus 0.89% for the slower kinetic. In our opinion, *Anaerobranca* sp. could have a positive influence in the degradation kinetics since it can synthesize cyclodextrin glycotransferases under thermophilic conditions to catalyze cyclodextrins (Thiemann et al., 2004). The cyclodextrins are known as oligosaccharides produced by the enzymatic degradation of cellulose or starch, which are able to enhance the biodegradation of phenol (Allan et al., 2007). The cyclodextrin mechanism lies in its structure, in fact, the interior of the cyclodextrin is hydrophobic, which forms a host-guest complex for hydrophobic molecules, like phenol. Conversely, the exterior of the cyclodextrin is hydrophilic, which enhances the solubility of the complex in the liquid phase and turns the complex cyclodextrin-phenol more bioavailable to degradation. This underlines the importance of *Anaerobranca* sp. and the cyclodextrins in phenol degradation.

## CONCLUSION

The  $^{13}\text{C}_6$ -phenol biodegradation is possible under thermophilic and mesophilic conditions during the anaerobic digestion of solid waste. Moreover, it was possible to demonstrate the degradation until mineralization into  $\text{CO}_2$  and  $\text{CH}_4$  thanks to the isotopic approach  $^{13}\text{C}$ . Under both conditions, two kinetics were obtained: one follows a first-order kinetic and the second follows a phase-lag model. Under mesophilic the fastest kinetic goes through the benzoic acid pathway and *Clostridium*.sp. could have an important role in the biodegradation process. Under thermophilic conditions, the results suggest that *Anaerobranca* sp. could enhance the phenol's degradation rate, by synthesizing cyclodextrins, which are known to increase phenol bioavailability.

## ACKNOWLEDGEMENTS

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