

DIANA NOEMÍ GUILLÉN FERRARI

**ASSESSMENT OF COMPOST FOR BIOREMEDIATION OF  
POLYAROMATIC HYDROCARBON (PAH) AND  
PETROLEUM HYDROCARBON SOILS**



**UNIVERSIDADE DO ALGARVE**  
FACULDADE DE CIÊNCIAS E TECNOLOGIA  
2017

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**Erasmus Mundus MSc in Chemical Innovation and Regulation  
Mestrado Erasmus Mundus em Inovação Química e  
Regulamentação**

**Trabalho efetuado sob a orientação de:**

**Work supervised by:**

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Dr. Thomas J. Aspray (Heriot Watt University)

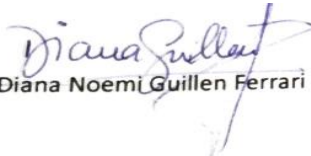


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Declaration of Authorship

I declare that I am the author of this work, which is original. The work cites other authors and works, which are adequately referred in the text and are listed in the bibliography.



Diana Noemi Guillen Ferrari

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## **Avaliação de composto para biorremediação de solos contaminados com hidrocarbonetos poliaromáticos (HAP) e hidrocarbonetos de petróleo**

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A biorremediação dos solos contaminados com hidrocarbonetos de petróleo e hidrocarboneto poli aromático (HAP) pode ser uma abordagem de tratamento econômica e eficiente. A introdução de emendas orgânicas que podem fornecer nitrogênio disponível e a potencial adição de microorganismos pode contribuir para melhorar o processo de biodegradação. No entanto, pouco trabalho considerou quais emendas orgânicas são melhores em termos de sua abundância relativa de degradadores para poluentes específicos. Neste trabalho, os compostos de resíduos verdes (GW) de diferentes estabilidades foram avaliados pelo potencial de biorremediação. Doze amostras de compostagem foram caracterizadas por parâmetros incluindo estabilidade (usando uma configuração modificada validada neste projeto), contagens de placas de *Pseudomonas* e *Actinomicetos* cultiváveis e abundância degradativa de gene (alkB, PAH-RHD GP e PAH-RHD GN). Estes são importantes para a degradação de alcanos e PAH, respectivamente. Comparando os compostos de resíduos verdes acabado e não acabado do mesmo site, os resultados mostraram que a abundância de alk B (codificando alcano monooxigenase) foi maior nas amostras de compostagem menos estáveis, o que corresponde à maior contagem de *Pseudomonas*. Resultados semelhantes foram obtidos para o PAH-RHD GN (que codifica a Dioxigenase Hidroxilante de hidrocarbonetos aromáticos poliaromáticos em bactérias Gram negativas), as amostras menos estáveis apresentaram maior abundância de genes degradantes. Para PAH-RHD GP (codificando a Dioxigenase Hidroxilante de hidrocarbonetos aromáticos poliaromáticos em bactérias Gram positivas), não foi possível ver uma diferença clara entre amostras acabadas e não acabadas. Dado que os compostos menos estáveis tendem a ter maior Nitrogênio disponível, isso apoiaria o uso desses compostos para a biorremediação de solos contaminados com hidrocarbonetos de petróleo alifáticos e com hidrocarbonetos poli aromáticos.

**Keywords:** biorremediação, composto, alk B, PAH RHD GN, PAH RHD GP, alcanos, HAPs, qPCR

## Assessment of compost for bioremediation of polyaromatic hydrocarbon (PAH) and petroleum hydrocarbon soils

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Bioremediation of petroleum and polyaromatic hydrocarbon (PAH) contaminated soils can be a cost effective and efficient treatment approach. The introduction of organic amendments which can provide available nitrogen, and potentially additional microorganisms, can contribute to improve the biodegradation process. However, little work has considered which organic amendments are best in terms of their relative abundance of degraders for specific pollutants. In this work, green waste (GW) composts of different stability were assessed for their bioremediation potential. Twelve compost samples were characterised for parameters including stability (using a modified set up validated in this project), culturable microbial *Pseudomonas* and *Actinomycetes* plate counts and degradative gene (alkB, PAH-RHD GP and PAH-RHD GN) abundance. These are important for the degradation of alkanes and PAH respectively. Comparing finished and un-finished green compost from the same site, results showed that the abundance of alkB (encoding alkane monooxygenase) was highest in the less stable compost samples, which corresponds with the higher *Pseudomonas* counts. Similar results were obtained for PAH-RHD GN (encoding Polycyclic Aromatic Hydrocarbon-Ring Hydroxylating Dioxygenase in Gram negative bacteria), the less stable samples presented higher abundance of degrading genes. For PAH-RHD GP (encoding Polycyclic Aromatic Hydrocarbon-Ring Hydroxylating Dioxygenase in Gram positive bacteria) abundance, it was not possible to see a clear difference between finished and un-finished samples. Given that less stable composts tend to have higher available N this would support the use of these composts for bioremediation of aliphatic petroleum hydrocarbon and Poly aromatic hydrocarbon contaminated soils.

**Keywords:** Bioremediation, compost, alk B, PAH RHD GN, PAH RHD GP, PAHs, alkanes, qPCR

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## **LIST OF ABBREVIATIONS OR ACRONYMS**

Alk B: Alkane monooxygenase B gene

BSI: British Standard Institute

CFU: Colony forming unit

DM: Dry matter content

DNA: Deoxyribonucleic acid

EC: Electrical conductivity

FGW: food and green waste

GW: Green waste

NCIMB: National Collections of Industrial, Marine and Food Bacteria.

OM: Organic matter content

PAH: Polyaromatic Hydrocarbons

PAH RHD GN: Polyaromatic Hydrocarbon Ring Hydroxylating Dioxygenase Gram Negative gene

PAH RHD GP: Polyaromatic Hydrocarbon Ring Hydroxylating Dioxygenase Gram Positive gene

PAS: Public Available Specification

PCR: Polymerase Chain Reaction

qPCR: quantitative Polymerase Chain Reaction

## 1. INTRODUCTION

Bioremediation can be defined as the use of bacteria or plants to degrade, remove or neutralise chemical compounds from soil or water (Collin, 2004). Two methods used for bioremediation of environmental pollutants using microbes are biostimulation and bioaugmentation. Biostimulation consists in the introduction of nutrients, such as nitrogen and/or phosphorus, to enhance the remedial activity of the microorganisms. On the other hand, bioaugmentation involves the introduction of organisms, that are known to degrade the contaminant chosen, into the contaminated soil. (Collin, 2004; Wu et al., 2016).

Polyaromatic hydrocarbons (PAH) are organic compounds that contain two or more fused benzene rings; they are toxic and persistent pollutants, some of them with carcinogenic or mutagenic potential (Gupta et al., 2015; White & Claxton, 2004). Their occurrence is natural and also anthropogenic, being the increase in the concentration in the environment caused by the latter. Petroleum is composed by a mixture of hydrocarbons; aliphatic (e.g. alkanes, cycloalkanes), aromatic, PAHs, and asphaltenes; these compounds are easily degraded by microorganisms, particularly the alkanes and low molecular PAHs, being the higher molecular PAHs and asphaltenes more recalcitrant to biodegradation. (Ward & Singh, 2003).

The introduction of organic amendments (e.g. composts, manure, municipal waste) which can adjust the organic matter content, providing available nitrogen, phosphorus and other nutrients to stimulate the growth of native bacteria, and additional microbial consortium instead of a single microorganism, can contribute to improve the biodegradation process (Namkoong et al., 2002; Semple et al., 2001). Generally, pollutant removal and its degradation efficiency, is positively related to the population of degrading microorganisms (Ghosal et al., 2016), which makes more interesting to explore the potential of compost to increase the population of degraders.

Bioremediation of petroleum and polyaromatic hydrocarbon (PAH), contaminated soils can be a cost effective and efficient treatment approach (Wu et al., 2013). The use of conventional technologies that include removal, modification or isolation (e.g. incineration, landfill) are expensive and could transfer the contaminant from one site to another, or between phases (Haritash & Kaushik, 2009; Ward & Singh, 2003).

Bioremediation has been used successfully for the degradation of PAH contaminated soils (Antizar-Ladislao et al, 2006; Chen et al., 2015; Kästner & Mahro, 1996) and of alkanes (Diplock et al., 2009; Namkoong et al., 2002; Taccari et al., 2012). Although many sites are

contaminated with both aliphatic and aromatic contaminants, few relatively articles deal with degradation of PAH and alkanes (Shahi et al., 2016; Wu et al., 2017).

### Compost Maturity and Stability

Maturity and stability in compost, have been used sometimes as synonyms, but are two different parameters that should be determined separately (Wichuk & McCartney, 2010; Wu & Martinez, 2000). BSI PAS 100:2011 defines mature compost as material with low rate of biodegradation, absence of pathogens and no phytotoxicity and stable compost as material with biodegradation rate lower than  $16 \text{ mg CO}_2 \text{ gOM}^{-1} \text{ d}^{-1}$ . For (Wichuk & McCartney, 2010) a mature compost is ready for a particular use. Many authors have mentioned the use of mature compost for biodegradation (Gomez & Sartaj, 2013; Kästner & Miltner, 2016; Taccari et al., 2012; G. Wu et al., 2013), but the use of phytotoxicity as a maturity indicator is not a good choice for compost applicated to bioremediation, because this material is going to be degraded even further, and the salts that contribute to phytotoxicity could be useful as nutrients in this process.

Being compost stability the measurement of the oxigen consumption,  $\text{CO}_2$  production or self-heating from the decomposition of the organic matter by the microorganisms (Barrena-Gomez, et al., 2006) and this decomposition is linked to the abundance of degradable organic matter and microorganisms, stability seems to be the key measure for the assesment of the potencial of compost for bioremediation.

To assess the bioremediation potential of green waste and green and food waste compost samples the aims of this research were:

- 1) Improve the robustness of ORG0020 compost stability determination.
- 2) Assess the relationship between compost stability with the abundance of PAH and alkane degraders using qPCR. PCR methods allow specific and sensitive detection of degrading genes in bacteria from different taxonomic group, including non-culturable microorganisms (Perez-de-Mora et al., 2010) and is less time consuming.
- 3) Assess the relationship between culturable microbial *Pseudomonas* and *Actinomycetes* count in compost and contaminates soil and the abundance of catabolic genes.
- 4) Assess the potential increment of alkane and PAH degraders of adding compost samples to known contaminated soil samples.

## 2. MATERIALS AND METHODS

### 2.1 Compost sample characterisation

Green, and food and green waste (GW and FGW respectively) compost samples, finished and post-sanitised, were collected from seven UK composting sites.

Table 2.1 Compost identification, type, source and status.

Sample	Site	Process type	Feedstock	Status
A	1	In-vessel	FGW	Finished
B	2	Ex situ	GW	Finished
C	3	In-vessel	FGW	Finished
D	4	Ex situ	GW	Post- sanitised
E	4	Ex situ	GW	Finished
F	1	In-vessel	FGW	Post sanitised
G	1	In-vessel	FGW	Finished
H	5	Ex situ	GW	Finished
I	5	Ex situ	GW	Post- sanitised
J	6	Ex situ	GW	Finished
K	7	Ex situ	GW	Post- sanitised
L	7	Ex situ	GW	Finished

*FGW: food and green waste, GW: green waste.*

The following assays were carried out on the 12 samples.

Dry matter was carried out according to BS EN 13040:2007 with the following modifications: weight of sample approximately 9 g, dried for 20 h, weighted, the tray was placed in the oven for an additional h, weighted, the difference between the two weights was not more than 0.1 g.

Determination of laboratory bulk density was carried out according to BS EN 13040:2007.

Organic matter content was carried out according to BS EN 13039:2011 with the following modifications: the sample was dried for 20 h at 103 °C, then placed in the desiccator and allowed to cool to room temperature, weighted, then placed again in the oven for 1 h more, placed back in desiccator and allowed to cool to room temperature, weighted, the difference between the two weights was not more than 0.01 g.

pH: It was carried out according to BS EN 13037:2011 (HI 8424 pH meter, Hanna Instruments, USA). Commercially available buffers pH 7 and 10 were used for calibration. (Hanna Instruments, UK).

Electrical conductivity: according to BS EN 13038:2011 (HI 99301 EC/TDS meter, Hanna Instruments, USA). Unfiltered water extracts were used.

## **2.2 Microbial plate counts**

Sample aliquots, 1 g fresh weight were suspended in 9 ml of 0.85% (w/v) NaCl and shaken for one min. Dilutions were made in decimal steps and cultured in triplicate on Actinomycetes Isolation Agar (Fluka-Sigma Aldrich, India) and Pseudomonas Isolation agar (Fluka- Sigma Aldrich, India) for 48 h at 25 °C. Colonies were counted manually with colony-forming units based on single dilutions, less than 300 CFU per plate.

## **2.3 Compost stability**

2.3.1 Compost stability was assessed using two set ups.

Eleven experiments were run, three (no. 9, 10 and 11) of which were done at low flow rate ( $\approx 25 \text{ ml min}^{-1}$ ) and nine (no. 1,2,3,4,5,7,12 and 13) at high air flow rate ( $45\text{-}50 \text{ ml min}^{-1}$ ).

Original set up (figure 2.1.A): It was done according to ORG0020 standard laboratory based test to measure compost stability by measurement of evolved carbon dioxide (Llewelyn, 2005). (Hailea V10 diaphragm pump, Guangdong Hailea Group Co., Ltd., China).

Standardisation procedure of 1M solutions of HCl and NaOH can be found in appendix.

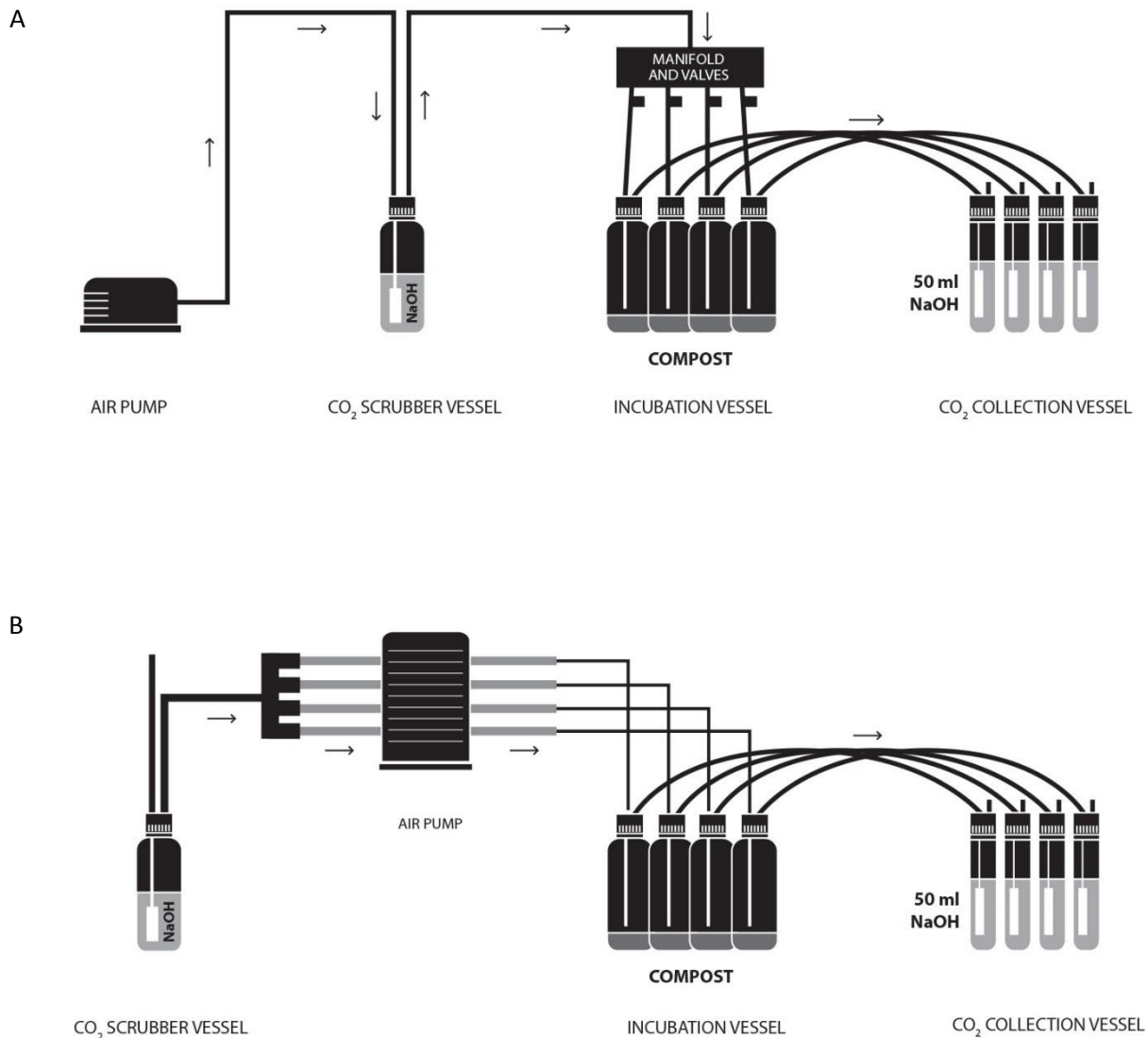
Modified set up (figure 2.1.B): Instead of the diaphragm pump a multichannel peristaltic pump head (205CA12, Watson Marlow Limited, UK.) connected to either a 502S or 520 Du pump (Watson Marlow Limited, UK) was used. Marprene manifold tubing 2.79 mm bore and 0.8 mm wall thickness, (part no 905.0027.008; Watson-Marlow Limited, UK) was used.

Monitoring and adjustment (as necessary) of flow rate were done daily before and after changing the NaOH traps in both setups. Monitoring was carried out at the exit pipe of the CO<sub>2</sub> traps using an Ellutia 7000 flow meter. The flow rate and time taken for monitoring and adjustment were recorded.

2.3.2 Compost stability modifying the flow

A short experiment (no. 3) only until day four using the original set up but modifying the flow on day two was run. The flow was adjusted to  $<10 \text{ ml/min}$  in three vessels, the other four (three replicates and one blank) were maintained within method range.

Figure 2.1 Compost stability set ups



## 2.4 Contaminated soil characterisation

Soil samples (31 in total) were provided by industrial contacts and collaborators from three contaminated sites from the U.K. and Switzerland.

pH: 1g fresh weight of contaminated soil sample was suspended in 5 ml of distilled water, shaken for 30 s then let settled for 30 min and shaken again for 30 s before pH was measured (HI 8242 pH meter, Hanna Instruments, USA). Commercially available buffers pH 7 and 10 were used for calibration (Hanna Instruments, UK).

## 2.5 Soil microbial plate counts

The method used for compost samples was used.

## **2.6 DNA extraction and quantification**

Standards: DNA was extracted from bacterial culture of *Pseudomonas putida* (NCIMB 9816, culture collection) and *Rhodococcus* sp. (NCIMB 12038, culture collection) in nutrient agar grown for 48 h at 30 °C, using QIAamp DNA Mini Kit (QIAGEN, Germany) according to manufacturer's manual and quantified with a NanoDrop 2000 (Thermo Scientific).

Compost: Genomic DNA was extracted from compost samples in duplicate (0.25 g fresh compost each) and then pooled, using PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to manufacturer's manual and quantified with a NanoDrop 2000 (Thermo Scientific).

Contaminated soil: Genomic DNA was extracted from soil samples (0.25 g fresh soil each) using PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to manufacturer's manual and quantified with a NanoDrop 2000 (Thermo Scientific).

## **2.7 Detection of alk B, PAH-RHD GN AND PAH-RHD GP genes**

To obtain the standards for Real time PCR, End point PCR was carried out with Veriti, 96 well thermal cycler (AB Applied Biosystems). PCR reactions were of 20 µl containing: 10 µl Quantitect SYBR green PCR mastermix (Qiagen Germany), 2 µl primer forward, 2 µl primer reverse (both Eurofins Genomic, 4.000 mM), 0.8 µl of bovine serum albumin (BSA), 20mg/ml, Roche), 3.2 µl Nuclease-free water (Ambion) and 2 µl of template DNA or Nuclease-free water (negative control).

The products were visualized and checked for a single band of the expected size alongside a 100 bp DNA ladder (Promega, USA) by agarose gel electrophoresis (1,5% agarose with gelred acid stain, (Biotium).

Standards' DNA clean up: End point PCR product DNA was cleaned using Wizard SV Gel and PCR Clean-Up System (Promega Corporation, USA) according with manufacturer's manual.

Real time PCR: Step One, Real time PCR System, AB Applied Biosystems, were performed in 20 µl volumes containing: 10 µl PerfeCTa SYBR green ROC (Quanta, USA) 2 µl forward primer (Eurofins Genomic, 4.000 mM), 2 µl reverse primer (4.000X mM), 0.8 µl of bovine serum albumin (BSA), 20mg/ml, Roche), 3.2 µl Nuclease-free water (Ambion) and 2 µl of template DNA or Nuclease-free water (negative control)

Table 2.2 Characteristics of the PCR primers used

Target gene	Sequence 5' to 3'	Amplicon size (bp)	Reference
<b>alkB F</b>	5'-AAYAC6GC6CAYGARCT6GG6CAYAA3'	550	(Kloos et al., 2006)
<b>alkB R</b>	5'-GCRTGRTGRTC6GARTG6CGYTG-3'		
<b>PAH-RHD GN F</b>	5'-GAGATGCATACCACGTKGGTTGGA-3'	292	(Cébron et al., 2008)
<b>PAH-RHD GN R</b>	5'-AGCTGTTGTTCCGGGAAGAYWGTGCMGTT-3'		
<b>PAH-RHD GP F</b>	5'-CGGCGCCGACAAATTYGTNGG-3'	306	(Cébron et al., 2008)
<b>PAH-RHD GP R</b>	5'-GGGGAACACGGTGCCRTGDATRAA-3'		

End point PCR for all genes and Real time PCR for alkB were carried out as follows: Step one initial denaturation at 95°C (10 min), followed by a touchdown PCR 5 cycles of 3 steps (45 s at 95 °C , 1 min at 62 °C and 45 s at 72 °C), followed by 40 cycles of 45s a 95 °C, 1 min at 57 °C , 45 s at 72 °C, data collection at 78 °C for 30 s (only Real time PCR), final extension was 7 min at 72 °C (for End point PCR only). (Wallisch et al., 2014)

Real time PCR for PAH-RHD GN AND PAH-RHD GP were done with the following temperature profiles: step one heated to 95 °C (30 s), followed by 50 cycles of 4 steps (30 s at 95 °C, 30 s at 56°C, 30 s at 72 °C and data collection at 80 °C for 10 s).



### 3. IMPROVEMENT OF ORG0020 SETUP FOR COMPOST STABILITY

#### DETERMINATION. RESULTS AND DISCUSSION

##### 3.1 Compost characterisation

Five finished and unfinished compost samples from four composting sites were used for the stability experiments. The dry matter (DM) and organic matter (OM) content were measured, as they are necessary for the calculation of compost stability, results are shown in table 3.1.

The compost samples stability ranged from 0.48 to 7.93 mg CO<sub>2</sub> g OM<sup>-1</sup>d<sup>-1</sup> and therefore fall within the PAS 100:2011 (BSI, 2011) limit of 16 mg CO<sub>2</sub> g OM<sup>-1</sup>d<sup>-1</sup>.

Table 3.1 Characterisation of samples used for compost stability experiments

Sample	Feedstock	Size (mm)	DM (%)	OM (%)	Stability (mgCO <sub>2</sub> gOM <sup>-1</sup> d <sup>-1</sup> )
A	FGW	0-20	54.63	36.04	0.48
B	GW	0-10	43.89	43.59	2.49
C	FGW	0-20	58.58	64.34	3.95*
D	GW	0-20	34.93	43.67	7.93
E	GW	0-20	42.85	33.74	2.39

*\*with low flow (25 ml/min), the rest of the samples were measured at high flow = 45-50 ml min<sup>-1</sup>. GW: green waste, FGW: food and green waste. The results presented of DM, OM and Stability are the average among three replicates.*

##### 3.2 Equilibration experiments

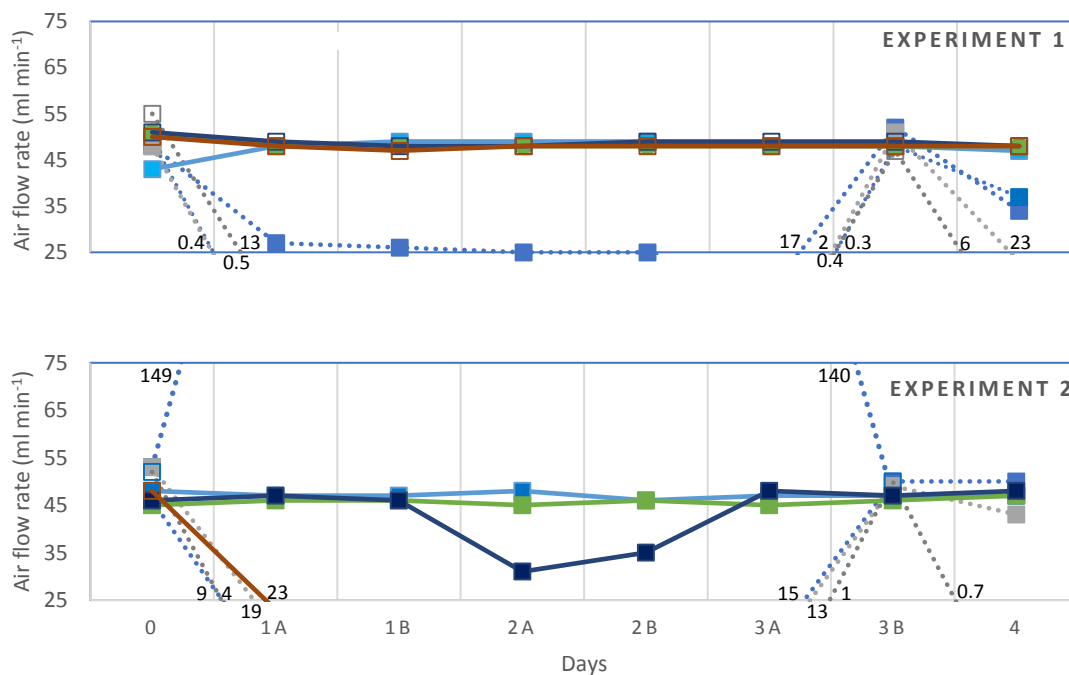
###### 3.2.1 Equilibration experiments with both set ups

To study the impact of the flow behaviour during the equilibration period, experiments were run until day 4, the air flow was measure twice a day for the four days. As shown in figure 3.1. the air flow of the original set up (dotted line) was variable, the vessels that don't appear in the graph fell out of range: airflow lower than the method's minimum (25 ml min<sup>-1</sup>) in experiments 1 and 2, and higher than the maximum (75 ml min<sup>-1</sup>) in experiment 2. The modified set up air flow rate (solid lines) was within method range throughout this period. The hollow squares represent the blanks of each set up.

The results of the CO<sub>2</sub> evolution after the equilibration period are shown in table 3.2., they correspond to the average of three replicates. In experiment 1 the standard deviation is significantly higher in the original set up than in the modified, this could be due to the

variation of the airflow during the equilibration period, falling out or range in the four vessels of the original set up. Less precision is also observed in the original set up of experiment 2 (table 3.2.), probably influenced by lower and higher flowrate in some vessels (figure 3.1).

Figure 3.1 Air flow in equilibration experiments with both set ups



### 3.2.2 Equilibration experiments: modifying the flow, only original set up

After observing this rise in the standard deviation of the CO<sub>2</sub> evolved on experiments 1 and 2 (table 3.2.), when during the equilibration period the air flow rate was lower than method's range; an experiment using the original set up but modifying the flow on day 2 was run, figure 3.2. The flow was adjusted to <10 ml/min in 3 vessels, the other 4 (3 replicates and 1 blank) were maintained within method range.

The stability results are shown in table 3.2. Higher CO<sub>2</sub> evolution, about 20%, was observed for the vessels with the modified flow than with the original set up. Therefore, it seems to be a relation between airflow within method range and accuracy of CO<sub>2</sub> evolution measurement.

Figure 3.2 Air flow in experiment modifying the flow

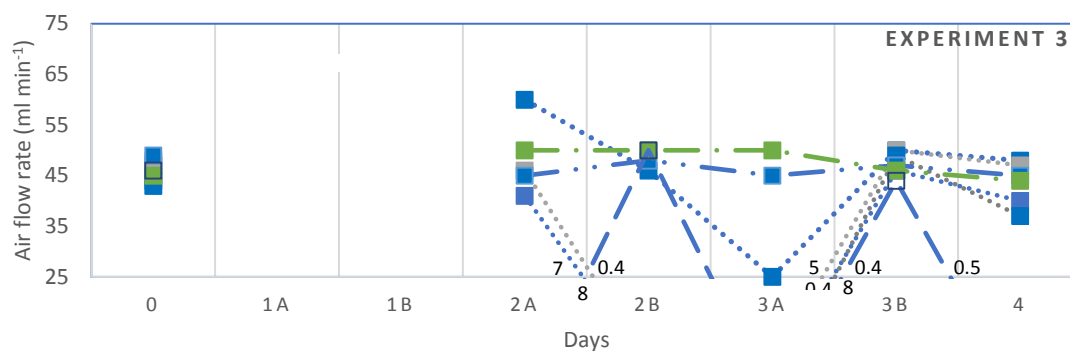


Table 3.2 CO<sub>2</sub> evolution after equilibration period with standard deviations in parentheses

Experiment number	1	2	3
Compost sample	B	B	D
	Original	1.97 (0.54)	8.99 (0.44)
Day four (mgCO <sub>2</sub> gOM <sup>-1</sup> )	Original*	n/a	10.90 (0.24)
	Modified	2.74 (0.22)	n/a

high flow = 45-50 ml min<sup>-1</sup> per vessel. n/a: not applicable. Original\*: original set up with modified air flow.

### 3.3 Comparison between full length compost stability experiments

In figure 3.3. it is possible to observe five full length experiments in which the flow was variable after the equilibration period in the original set up. In these experiments, the flow during equilibration was not measured, following the standard protocol. Experiments 4, 6, and 7 were run in triplicate plus one blank in both set ups. Experiments 12 and 13 were run with five replicates (one outlier on both set ups was eliminated) and one blank.

In all the experiments shown, there was variation of the airflow rate during the equilibration period in the original set up. In most of the cases, experiments 7,12, and 13, this is translated in higher standard deviation of the CO<sub>2</sub> evolved in day four than with the modified set up (table 3.3.). The airflow in the modified set up appears to have remained within range. At the end of the experiments, the average production of CO<sub>2</sub> was more precise with the modified set up: experiments 4, 6, 12 and 13.

Figure 3.3 Air flow in full length experiments with high flow

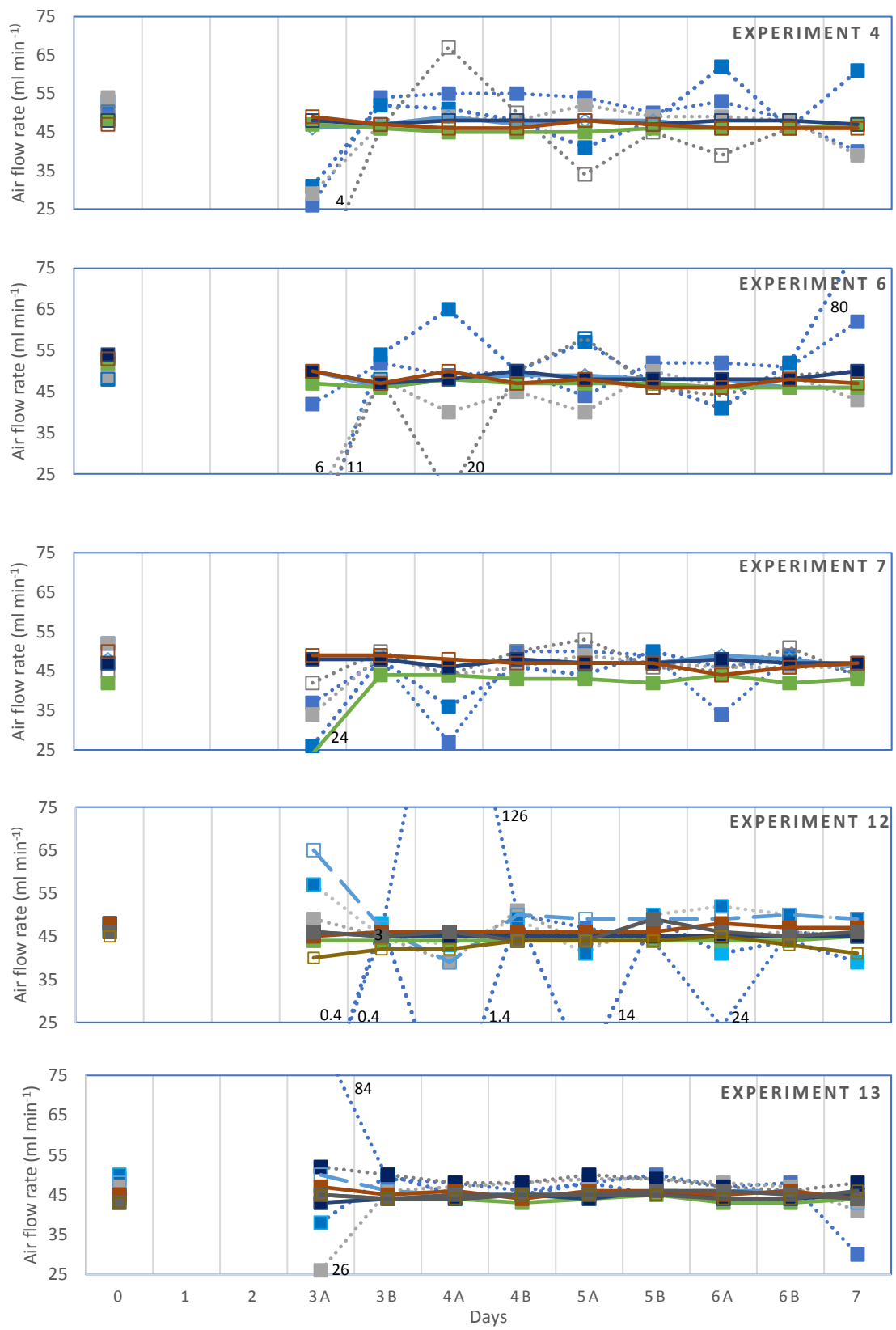


Table 3.3 CO<sub>2</sub> evolution per day and compost stability with high flow, with standard deviations in parentheses

Experiment number		4	6	7	12	13
Compost sample		B	A	D	D	D
Day four	Original	2.96 (0.31)	1.04 (0.07)	8.49 (0.63)	12.35(5.72)	7.03 (0.94)
(mgCO <sub>2</sub> gOM <sup>-1</sup> )	Modified	2.41 (0.60)	0.78 (0.004)	8.45 (0.51)	7.87 (0.30)	5.67 (0.36)
Day five	Original	2.76 (0.19)	0.75 (0.07)	8.05 (0.09)	7.65 (1.54)	6.33 (0.62)
(mgCO <sub>2</sub> gOM <sup>-1</sup> )	Modified	2.50 (0.13)	0.30 (0.06)	9.03 (0.65)	7.40 (0.36)	4.48 (0.36)
Day six	Original	2.10 (0.18)	0.60 (0.13)	8.44 (0.65)	6.15 (1.12)	5.91 (0.69)
(mgCO <sub>2</sub> gOM <sup>-1</sup> )	Modified	2.25 (0.17)	0.41 (0.06)	8.69 (0.98)	7.00 (0.20)	4.93 (0.39)
Day seven	Original	2.67 (0.30)	0.52 (0.07)	7.56 (1.10)	6.19 (0.96)	5.39 (1.00)
(mgCO <sub>2</sub> gOM <sup>-1</sup> )	Modified	2.48 (0.37)	0.45 (0.23)	8.30 (0.55)	6.63 (0.24)	5.17 (0.46)
Stability	Original	2.70 (0.22)	0.73 (0.08)	8.14 (0.56)	8.09 (1.78)	6.16 (0.79)
(mgCO <sub>2</sub> gOM <sup>-1</sup> d <sup>-1</sup> )	Modified	2.41 (0.12)	0.48 (0.04)	8.62 (0.67)	5.78 (0.18)	5.34 (0.38)

\*high flow = 45-50 ml min<sup>-1</sup> per vessel. Values correspond to the average of three replicates for experiments 4, 6 and 7 and of four replicates for experiments 12 and 13. Standard deviation shown in parenthesis.

### 3.4 Experiments with low flow

To compare how both set ups perform with lower airflow ( $\approx 25$  ml min<sup>-1</sup>), experiments were run in this condition, experiments 9, 10, and 11, data and graphs can be found in appendix. The standard deviation of the CO<sub>2</sub> evolved is higher for the original than for the modified set up, as happened for the higher airflow. Being the modified method more precise.

### 3.5 Precision and accuracy

(Aspray et al., 2015) found differences in stability results between two laboratories in five active samples by factors of 1.2 to 2.2, suggesting that the test is not robust enough for active samples. (Llewelyn, 2005) reported an in-house precision of 0.906 mg CO<sub>2</sub> gOM<sup>-1</sup> d<sup>-1</sup>, this value was assessed using a water bath at 25°C with 8 replicates, since the method's bath temperature is 30°C and samples are run in 3 replicates, the value of precision doesn't seem appropriate to assess the performance of the OG0020 test.

Sample homogeneity and the measurements of dry matter and volatile solids are important factors influencing the precision of this method (Llewelyn, 2012). However, in the experiments of this work, the value of dry matter and volatile solids used were applied consistently to both set ups, therefore, the factors influencing the precision would be sample homogeneity and air flow rate within range. When the flow was lower than the required by

the method there could have been a CO<sub>2</sub> accumulation in the vessels during the equilibration period, and once the airflow was restored to the value needed this CO<sub>2</sub> was collected in the NaOH traps which could affect the accuracy of the measurement.

## 4. ASSESSMENT OF THE BIOREMEDIATION POTENTIAL OF COMPOST BASED IN THE RELATIONSHIP BETWEEN COMPOST STABILITY AND CATABOLIC GENE ABUNDANCE. RESULTS AND DISCUSSION

### 4.1 Compost characterisation

Characterisation results of ten finished and post sanitised compost samples from six composting sites are shown in table 4.1. The pH range is between 7.11 and 8.65 for samples falling within the PAS 100:2011 stability limit of 16 mg CO<sub>2</sub> gOM<sup>-1</sup> d<sup>-1</sup>. By comparing the more active samples F and G, one unfinished and one finished, the pH is 6.59 and 6.19 respectively, which could be explained by the abundance of organic acids during the first stages of decomposition, lowering the pH, as degradation proceeds organic acids are neutralized and ammonia mineralized (Beck-Friis et al., 2003) and mature compost can have pH between 7 and 9. Food compost samples tend to have higher concentration of nitrate which can lead to lower pH and higher EC ( sample G) (Sanchez-Monedero, et al. , 2001). As the aim of this work is to study the relationship between compost stability and abundance of

Table 4.1 Compost characterisation

Sample	Feedstocks	pH	EC (mS/cm at 25°C)	DM (%)	OM (%)	Stability (mgCO <sub>2</sub> gOM <sup>-1</sup> d <sup>-1</sup> )
A	FGW	Nd	Nd	54.63	36.04	0.48
B	GW	7.41	0.92	43.89	43.59	2.49
C	FGW	8.32	1.16	58.58	64.34	3.95*
D	GW	8.10	0.81	34.93	43.67	7.93
E	GW	8.43	1.15	42.85	33.74	2.39
F	FGW	6.59	1.86	49.61	49.80	25.93
G	FGW	6.19	3.20	54.92	59.14	25.81
H	GW	7.92	1.16	49.50	46.19	3.25
I	GW	7.11	0.30	48.20	41.27	6.50
J	GW	8.65	1.56	54.13	33.91	3.62
K	GW	8.52	1.28	43.93	31.55	14.69
L	GW	7.17	1.18	51.00	33.86	2.11

\*with low flow (25 ml/min), the rest of the samples were measured at high flow = 45-50 ml min<sup>-1</sup>. GW: green waste, FGW: food and green waste. Nd: not determined. Data represents mean among 3 replicates. EC: electrical conductivity, DM: dry matter content, OM: organic matter content.

degraders, and samples F and G, from the same site, have approximately the same stability value, although one of them is finished and the other un finished, they are discarded from the study.

#### 4.2 Quantification of degrading genes: alk B, PAH RHD GN and GP

Real time PCR was run to quantify the abundance of the alk B, PAH RHD GN and GP genes. Alk B gene is an indicator of the presence of alk B degraders (Kloos et al., 2006) (Guermouche M'rassi et al., 2015) and PAH RHD genes of PAH degraders (Cébron et al., 2008) respectively.

##### 4.2.1 Melt curve analysis and amplification efficiency

Melt curve analysis is an important quality control aspect for SYBR green based qPCR. The analysis of the alk B curves showed only one peak present in all the samples. The melting temperature of the product of the standards, made with pure cultures, is slightly lower than of the product of the environmental samples. In absence of samples, it seems there is some primer dimer amplification (negatives). The standard curve was linear, data shown in table 4.2. and the efficiency close to other study (where reported) using the same primers and method (Schulz et al., 2012; Wallisch et al., 2014). These primers were used for contaminated soil amended with compost (Wallisch et al., 2014) and in soil and maize and pea plant litter (Schulz et al., 2012), they have performed well in compost samples.

Table 4.2 Real time PCR standard curves data and efficiency

Gene	Slope	R <sup>2</sup>	Efficiency (%)
Alk B	≤ - 3.695	≥0.966	≥78.37
PAH RHD GP	≤ - 4.385	≥0.941	≥60.07
PAH RHD GN	≤ - 4.57	≥0.929	≥52.46

In the PAH RHD GN melt curve analysis samples D, the one with higher abundance (table 4.3), K and L showed a single peak (data not shown). Samples A, B, C, E, F, G, H, I and J presented multiples peaks, one of them in the melting temperature of the product desired and the rest around 74 °C. Multiple peaks were also observe for the standard at low concentration 103 and in absence of samples (negatives). Although the standard curve was linear (table 4.2.), the efficiency was low (≥52.46). The curve for PAH RHD GP showed one peak for samples A, B, C, D, E, G, H, I, J, K and L in the same temperature of the products of the

standards, and multiple peaks for sample F (lowest abundance) and for the negatives (absence of sample). The standard curve was linear with low amplification efficiency, but better than for PAH RHD GN. (Cébron et al., 2008) reported variation in DNA amplification efficiency due to presence of PCR inhibitor substances (e.g. humic acids) in soil and sediments samples, this could explain the low efficiency showed in this study for compost samples. Because the amplification efficiency is low, the values of the concentration of PAH RHD GN and GP could be higher if they were corrected measuring the value of inhibition.

#### 4.2.2 Catabolic genes' abundance

The abundance of alk B, PAH RHD GN and GP genes can be found on table 4.3 (the data represents the mean between two replicates). The alk B gene presence range was from  $1.23 \times 10^5$  to  $1.53 \times 10^8$  copies  $g^{-1}$  compost dw. The PAH RHD GN presence range from  $1.59 \times 10^5$  to  $6.75 \times 10^6$  copies  $g^{-1}$  compost dw. The PAH RHD GP presence range from  $1.96 \times 10^5$  to  $3.39 \times 10^7$  copies  $g^{-1}$  compost dw. Standard deviation between replicates was done as part of the qPCR program (CT st deviation < 0.50), for alk B all samples were within range; for PAH RHD GN there was high standard deviation between replicates in samples B, I, J and L, and for PAH RHD GP in sample G. In all samples, the difference between replicates was lower than one order of magnitude. This could be due to Ct values higher than 30, or error in pipetting.

#### 4.3. Relationship between compost stability and degrading gene abundance

Plots of catabolic gene abundance vs compost stability were made to explore if there is a correlation. As we can observe in figures 4.1, 4.2 and 4.3, apparently there is no linear correlation between these variables. Further analysis considered whether there was a correlation for specific feedstocks (GW and FGW) derived compost. Samples A, C and J (FGW) have no apparent correlation in any of the degrading genes. Samples B, D, E, H, I, K and L (GW) didn't show any linear correlation either (data not shown). Because compost composition changes depending on when the process started (availability of feedstocks, temperature), to study if there is a correlation it was necessary to reduce variables, the approach taken for this was comparing gene abundance and compost stability between finished and un-finished samples from the same site.

Figure 4.1 Alk B abundance vs compost stability

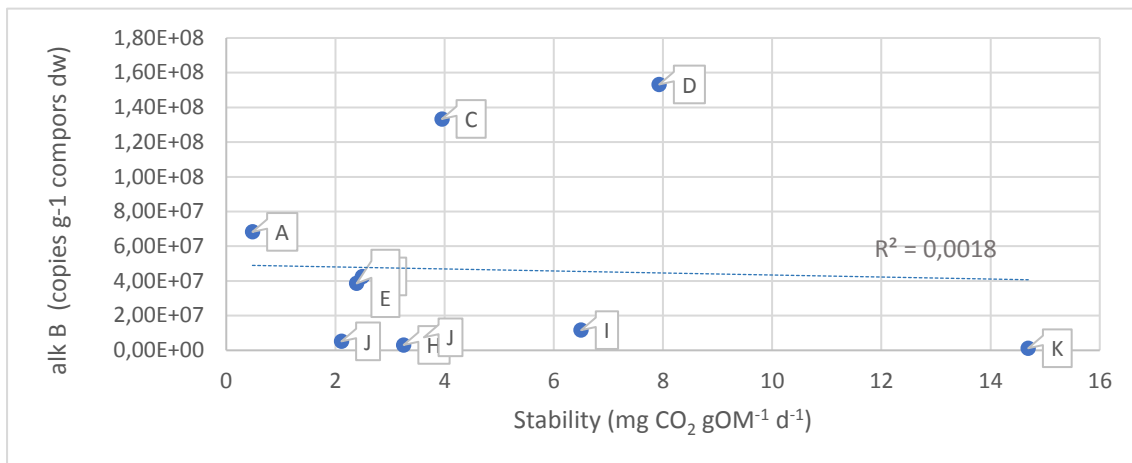


Figure 4.2 PAH RHD GP abundance vs compost stability

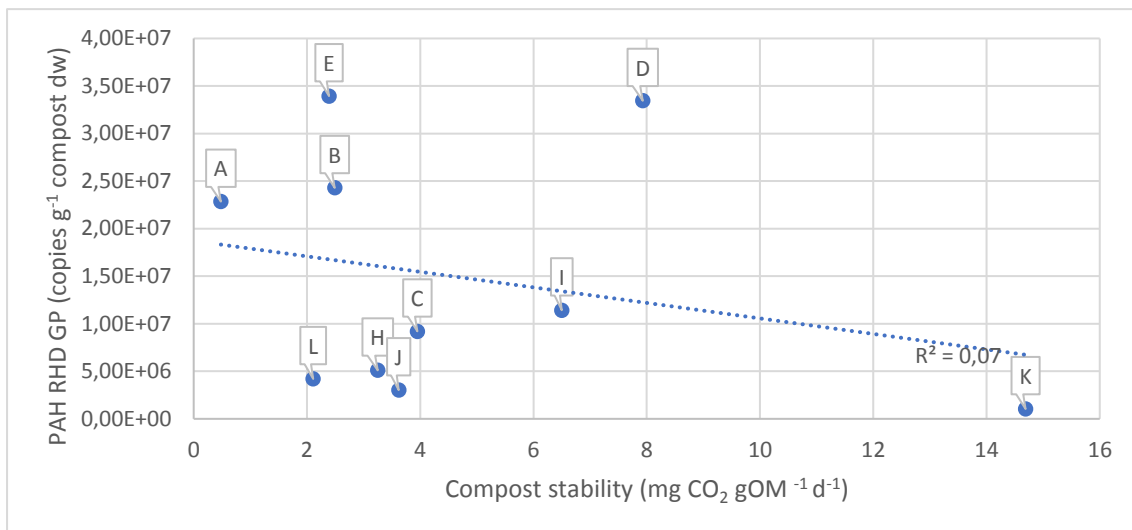


Figure 4.3 PAH RHD GN abundance vs compost stability

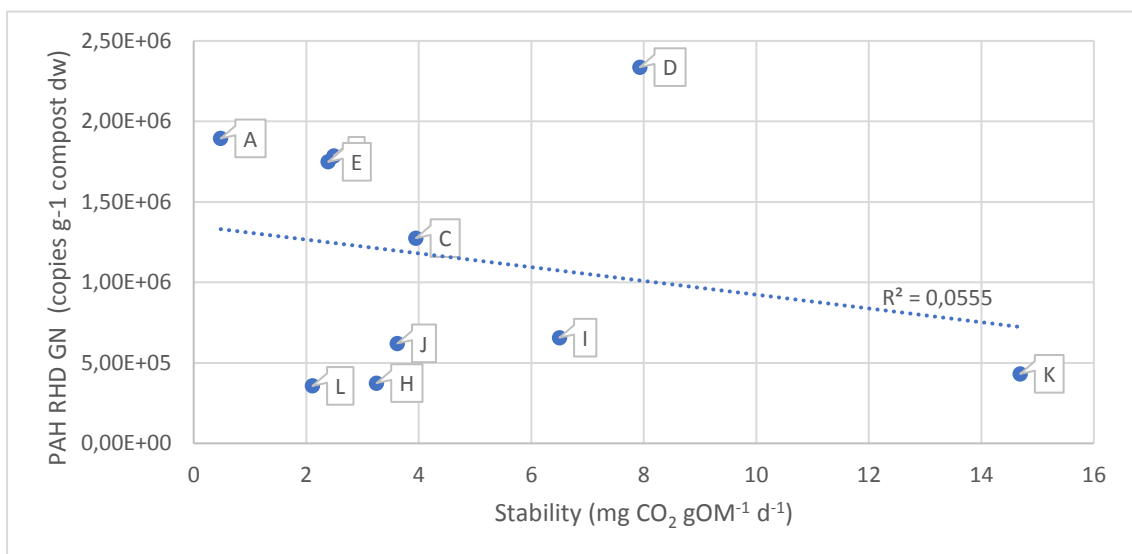


Table 4.3 Compost stability and alk B, PAH RHD GN and GP gene abundance

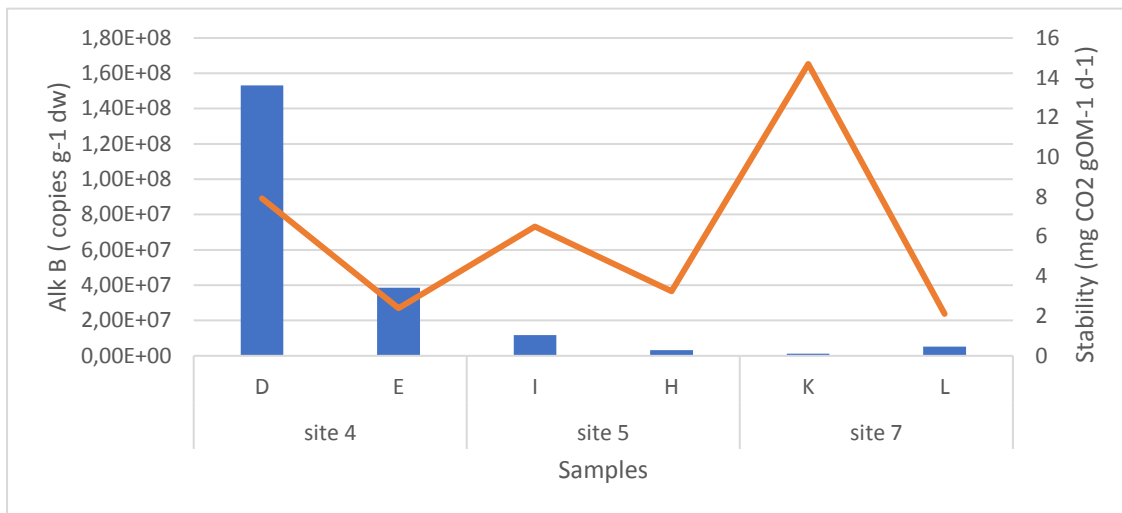
Site	Feedstocks	Sample	Status	Stability (mgCO <sub>2</sub> g OM <sup>-1</sup> d <sup>-1</sup> )	alk B	PAH RHD GP (copies g <sup>-1</sup> compost dw)	PAH RHD GN
4	GW	D	Un finished	7.93	1.53 x 10 <sup>8</sup>	3.34 x 10 <sup>7</sup>	2.34 x 10 <sup>6</sup>
	GW	E	Finished	2.39	3.86 x 10 <sup>7</sup>	3.39 x 10 <sup>7</sup>	1.75 x 10 <sup>6</sup>
5	GW	I	Un finished	6.50	1.16 x 10 <sup>7</sup>	1.14 x 10 <sup>7</sup>	6.75 x 10 <sup>6</sup>
	GW	H	Finished	3.25	3.12 x 10 <sup>6</sup>	5.10 x 10 <sup>6</sup>	3.75 x 10 <sup>6</sup>
7	GW	K	Un finished	14.69	1.22 x 10 <sup>6</sup>	1.04 x 10 <sup>6</sup>	4.32 x 10 <sup>5</sup>
	GW	L	Finished	2.11	5.19 x 10 <sup>6</sup>	4.20 x 10 <sup>6</sup>	3.57 x 10 <sup>5</sup>
1	FGW	A	Finished	0.48	6.83 x 10 <sup>7</sup>	2.28 x 10 <sup>7</sup>	1.89 x 10 <sup>6</sup>
2	GW	B	Finished	2.49	4.26 x 10 <sup>7</sup>	2.43 x 10 <sup>7</sup>	1.79 x 10 <sup>6</sup>
3	FGW	C	Finished	3.95	1.33 x 10 <sup>8</sup>	9.20 x 10 <sup>6</sup>	1.28 x 10 <sup>6</sup>
6	GW	J	Finished	3.62	7.50 x 10 <sup>6</sup>	3.02 x 10 <sup>6</sup>	6.21 x 10 <sup>5</sup>

GW: green waste, FGW: food and green waste.

#### 4.3.1. Relationship between compost stability and alk B abundance

Comparing GW finished and un-finished samples from sites 4 and 5 (figure 4.4), the unfinished and more active (D, I) have higher quantity of alk B genes (one order of magnitude) than the finished and less active (E, H). A similar result has been shown by (Wallisch et al., 2014) who found significantly higher gene numbers (one order of magnitude) in contaminated soil treated with immature compost compared to that with mature compost. (Franke-Whittle et al, 2009) have also found that in GW compost, the numbers of known compost microorganisms were higher for 2-week compost than 16-week samples, however, they didn't characterise them as alk B degraders. There is also the possibility of presence of alkane degraders that don't present the alk B gene, these microorganisms wouldn't be detected by this method. For site 7, samples K and L the abundance of alk B is higher in sample L, the less active, finished compost. The alk B abundance in the samples from this site are two and one orders of magnitude lower than in the sites 4 and 5 respectively.

Figure 4.4. Finished and un-finished compost Alk B abundance vs compost stability



#### 4.3.2 Relationship between compost stability and PAH RHD GN and GP

Comparing site 4 samples D and E the PAH-RHD GP abundance is similar in both. The PAH RHD GN abundance is higher in the un-finished, sample D. In site 5 samples I and J the abundance of PAH RHD GP and GN are higher in the un-finished compost (sample I). Comparing site 7, samples K and L the PAH-RHD GP abundance is higher in the finished sample (L) and the PAH-RHD GN abundance is higher in the un-finished and more active sample K.

The values of PAH RHD GP and GN abundance for these samples of compost, is within range of the values ( $4.4 \times 10^4 (\pm 2.4 \times 10^4)$ ) to  $4.7 \times 10^7 (\pm 2.4 \times 10^7)$  copies g<sup>-1</sup> soil or sediment dw) found in contaminated soil samples by (Cébron et al., 2008).

Figure 4.5. Finished and un-finished compost PAH RHD GP abundance vs compost stability

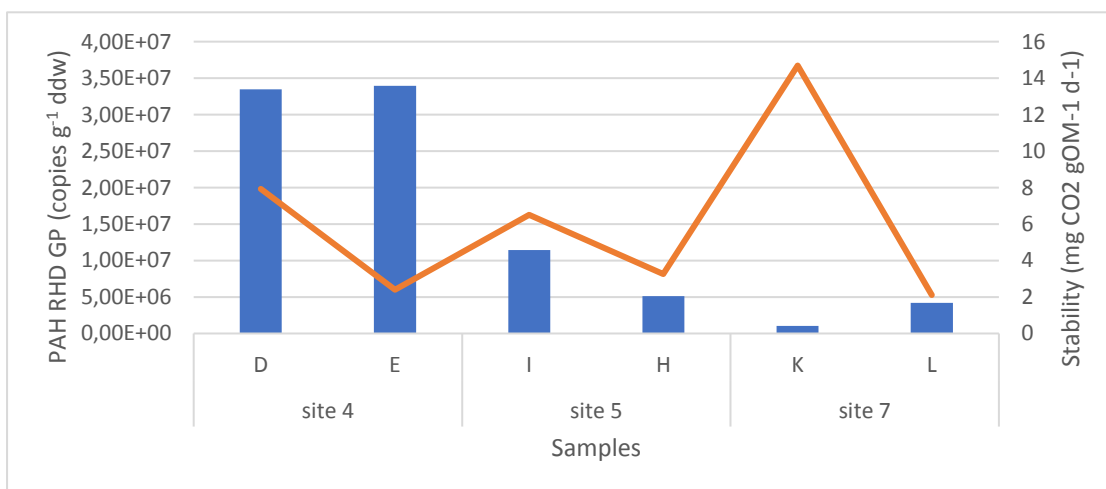
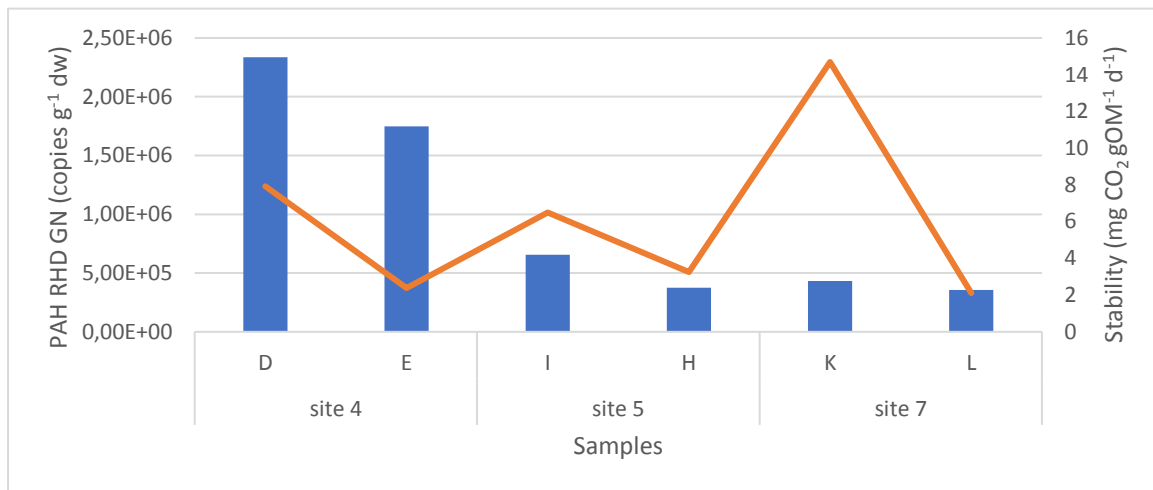


Figure 4.6. Finished and un-finished compost PAH RHD GN abundance vs compost stability



#### 4.4 Assessment of bioremediation potential

The results showed that there is apparent relationship between compost stability and Alk B and PAH RHD GN abundance, in more active samples the number of degrading genes is higher, which would support the use of less stable (immature) composts for bioremediation of PAH and petroleum hydrocarbon soils. Nevertheless, future work should prove if this correspond to abundance of expressed genes and therefore, the potential of augmenting the degraders population on soil, and as a result if the biodegradation rate is enhanced.

#### 4.5 *Pseudomonas* and *Actinomyces* microbial plate count

Since most of the alk B and PAH known degraders correspond to the genera *Pseudomonas* and *Actinomyces*, cultural microbial plate were counted, data in table 4.4.

Comparing the microbial count between samples D and E, from site 4, the actinomycetes count is higher in samples D (un-finished). In sites 5 and 7, the CFUs is higher in the samples H and K (less active, finished) than in I and L (more active, immature), this finding agrees with (Franke-Whittle et al., 2009), who found higher number of actinomycetes in mature compost (16-week) than in fresher samples (2-week) and it's consistent with (Antil & Raj, 2012) who includes the increase of actinomycetes count at the end of the composting process, as one of the indicators of compost maturity.

The pseudomonas count in sites 4 and 7 is one order of magnitude higher in the more active samples D and L than in the finished samples E and K. For samples I and H of site 5, the pseudomonas count is almost one order of magnitude higher in the finished sample (H), but the standard deviation (89.80) of the measurement in this sample, is too high to be reliable. This results are comparable to (MacCready et al., 2013) who have found higher numbers of

pseudomonas in the mesophilic phase, their numbers decreased after the temperature rises higher than 55°C, finding lower quantity in finished compost. However, the unfinished samples in this study are post-sanitised (heated to 65°C for 7 days).

Table 4.4. Compost stability and microbial plate count

Site	Sample	Status	Stability (mgCO <sub>2</sub> g OM <sup>-1</sup> d <sup>-1</sup> )	Pseudomonas (CFU g <sup>-1</sup> compost dw)	Actinomycetes
4	D	Un-finished	7.93	4.70 x 10 <sup>5</sup> (74.03)	1.56 x 10 <sup>8</sup> (12.90)
	E	Finished	2.39	1.84 x 10 <sup>4</sup> (25.19)	6.10 x 10 <sup>7</sup> (2.73)
5	I	Un-finished	6.50	1.92 x 10 <sup>6</sup> (17.83)*	1.96 x 10 <sup>8</sup> (26.88)
	H	Finished	3.25	1.55 x 10 <sup>7</sup> (89.80)	7.59 x 10 <sup>8</sup> (25.25)
7	K	Finished	14.69	4.26 x 10 <sup>6</sup> (10.27)	2.09 x 10 <sup>9</sup> (51.98)*
	L	Un-Finished	2.11	2.06 x 10 <sup>7</sup> (20.10)*	1.70 x 10 <sup>8</sup> (4.20)

The CFU count was done in three replicates (n=3), \*the CFU count was done using two replicates. Standard deviation in parentheses.

As was mentioned before a great number of alk B and PAH RHD harbouring bacteria correspond to the genera *Actinomycetes* and *Pseudomonas*. However, there is no clear relationship between the abundance of the gene and the microbial plate count, figures 4.7; 4.8 and 4.9., this could be explained by previous studies that showed that bacteria can harbour more than one alk B gene operon per cell (Heiss-Blanquet et al., 2005), similar conclusions have been made by (Cébron et al., 2008) about the PAH RHD gene, as bacteria can possess different number of genes per cell according to its genera.

Figure 4.7 Alk B gene abundance vs *Pseudomonas* (P) and *Actinomycetes* (A) plate count

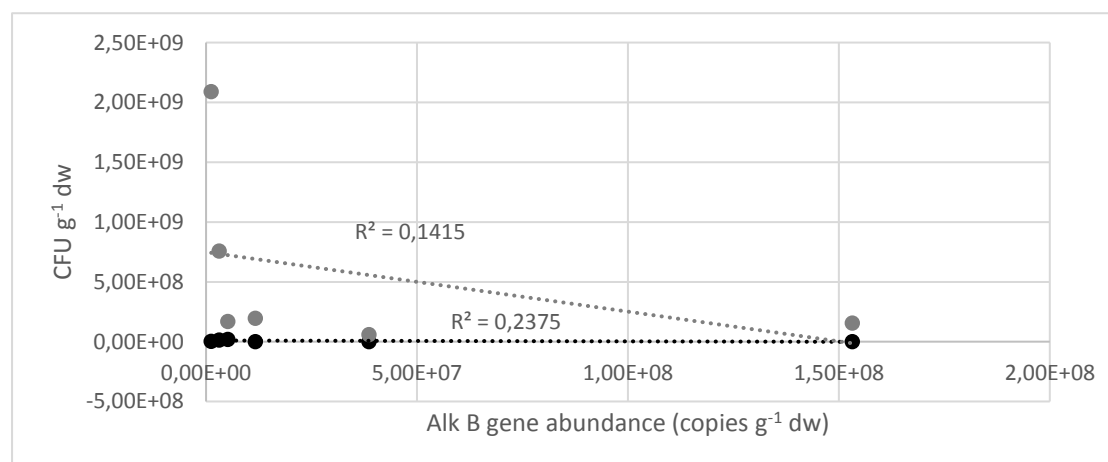


Figure 4.8 PAH RHD GP abundance vs *Pseudomonas* (P) and *Actinomyces* (A) plate count

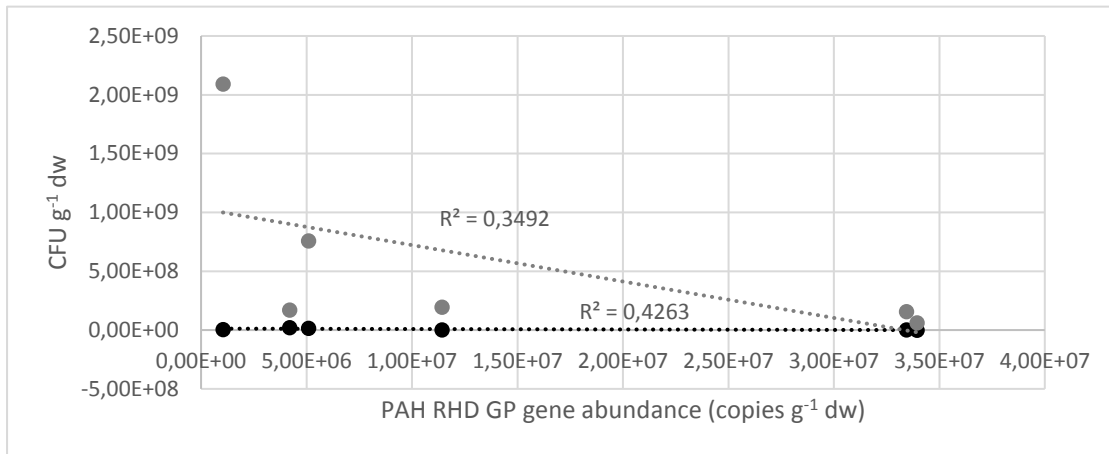
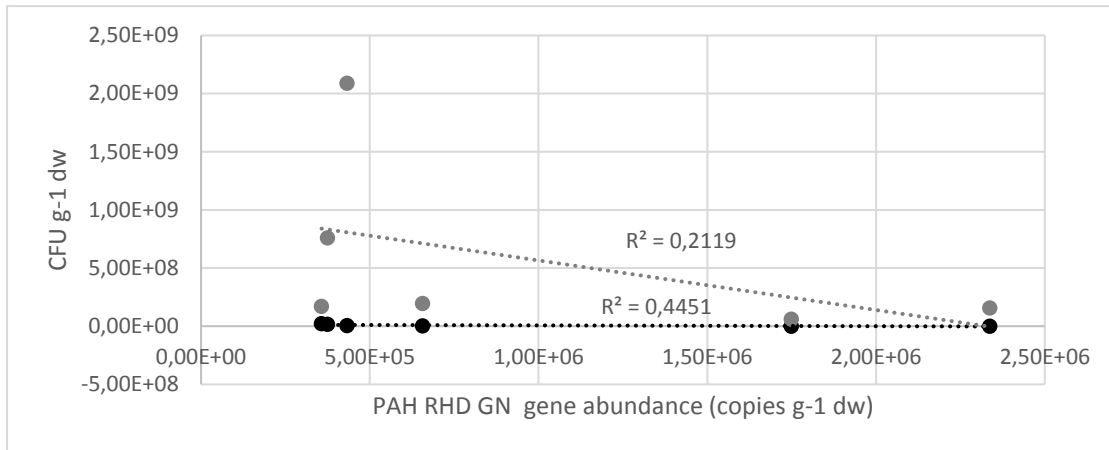


Figure 4.9 PAH RHD GN abundance vs *Pseudomonas* (P) and *Actinomyces* (A) plate count





## 5. COMPARISON OF ALK B, PAH RHD GP AND PAH RHD GN GENE ABUNDANCE IN PAH CONTAMINATED SOILS AND COMPOSTS. RESULTS AND DISCUSSION.

### 5.1. Quantification of degrading genes: alk B, PAH RHD GN and GP.

In order to compare gene abundance in contaminated soils to those in composts as an approach to bioremediation, quantification of the abundance of degrading genes alk B, PAH RHD GN and GP in contaminated soils sample was done using Real time PCR. As was mentioned in chapter 4, Alk B gene is an indicator of the presence of alk B degraders (Kloos et al., 2006) (Guermouche M'rassi et al., 2015) and PAH RHD genes of PAH degraders (Cébron et al., 2008) respectively.

#### 5.1.1. Melt curve analysis

To check if the right product was amplified and the performance of the primers and the method, a melt curve analysis was made.

##### 5.1.1.1 Contaminated soil site 1

The analysis of the Alk B melt curve showed only one peak in all the samples, at around the same temperature of the products of the standards. The standard curve was linear, data shown in table 5.1, and the efficiency lower than others studies (Schulz et al., 2012; Wallisch et al., 2014), this could be because environmental samples are sensitive to inhibition.

In the melt curve analysis of PAH RHD GN one peak appeared for samples: 1, 2, 3, 4, 5, 6, 8, 9, 12, 14, 15 16, 17 and 18. Multiple peaks were present for samples: 10 (lowest abundance, table 5.3), 11 and 13 and for low concentration standard  $10^3$ . The standard curve was linear, and the efficiency was low, but higher than the one with the compost samples. PAH RHD GP melt curve showed multiple peaks for all the samples 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18. The standard curve was linear, and the efficiency was low, but higher than with the compost samples. Low efficiencies with these primers and its possible causes were discussed in section 4.2.1.

Table 5.1 Contaminated soil site 1, amplification curve data and efficiency

Gene	Slope	R <sup>2</sup>	Efficiency (%)
Alk B	-4.371	≥0.947	≥69.34
PAH RHD GP	-4.712	≥0.959	≥63.015
PAH RHD GN	-4.975	≥0.941	≥58.51

### 5.1.1.2 Contaminated soil sites 2 and 3

The analysis of the melt curve for Alk B gene showed one peak for samples: 1, 2, 3, 5, and 6. Multiples peaks were present samples 4, 7, 8, 9, 10, 11, 12, 13, 14. The standard curve was linear, data in table 5.2 and the efficiency was lower than in other studies (Wallisch et al., 2014)

For PAH RHD GN gene all the samples presented one peak. The standard curve was linear, data in table 5.2 and the efficiency low, even lower than in chapter 4 with compost samples. As mentioned before, this could be due the presence of inhibitors in the environmental samples (Cébron et al., 2008). The analysis of the melt curve for PAH RHD GP showed single peaks for samples 1, 2, 3, 4, 5, 6, 7 and 8. Multiple peaks were present for samples 9, 10, 11, 12, 13 and 14. The standard curve was linear, and the efficiency comparable as the ones obtained with the compost samples.

Table 5.2 Contaminated soils sites 2 and 3, amplification curves data and efficiency

Gene	Slope	R <sup>2</sup>	Efficiency (%)
Alk B	-4.282	≥0.908	≥71.21
PAH RHD GP	-4.71	≥0.999	≥63.048
PAH RHD GN	-6.142	≥0.857	≥45.486

### 5.1.2. Abundance of alk B, PAH RHD GN and PAH RHD GP catabolic genes.

#### 5.1.2.1 Contaminated soil site 1

The abundance of alk B, PAH RHD GN and GP genes can be found on table 5.3 (the data represents the mean between two replicates). The alk B gene presence range was from  $2.92 \times 10^5$  to  $1.52 \times 10^8$  copies  $g^{-1}dw.$ , high standard deviation for samples 1,9, 10, 12, 16, and 18. The PAH RHD GN presence range from  $6.05 \times 10^5$  to  $1.13 \times 10^9$  copies  $g^{-1}$  soil dw, high standard deviation for samples 1, 4, 9, 10, 13, 14 and 18. The PAH RHD GP presence range from  $2.54 \times 10^4$  to  $7.46 \times 10^5$  copies  $g^{-1}$  soil dw and showed high standard deviation for the samples 8, 10, 12 and 13. Lower values of PAH RHD genes were obtained by (Cébron et al., 2008)  $4.4 \times 10^4 (\pm 2.4 \times 10^4)$  to  $4.7 \times 10^7 (\pm 2.4 \times 10^7)$  copies  $g^{-1}$  soil or sediment dw for highly contaminated soils.

### 5.3 PAH concentration and degrading gene abundance in contaminated soil site 1

Sample ID	PAH Concentration (mg Kg <sup>-1</sup> soil dw)*	alk B	PAH RHD GP (copies g <sup>-1</sup> soil dw)	PAH RHD GN
1	14856.5	6.96 x 10 <sup>5</sup>	4.24 x 10 <sup>5</sup>	6.26 x 10 <sup>6</sup>
2	22619.6	8.87 x 10 <sup>5</sup>	2.28 x 10 <sup>5</sup>	1.13 x 10 <sup>9</sup>
3	15853.7	6.50 x 10 <sup>5</sup>	4.79 x 10 <sup>5</sup>	2.91 x 10 <sup>8</sup>
4	19225.3	5.75 x 10 <sup>5</sup>	7.46 x 10 <sup>5</sup>	3.35 x 10 <sup>6</sup>
5	19694.8	6.32 x 10 <sup>5</sup>	5.18 x 10 <sup>5</sup>	6.93 x 10 <sup>8</sup>
6	48135.6	6.51 x 10 <sup>5</sup>	4.92 x 10 <sup>5</sup>	3.63 x 10 <sup>6</sup>
8	6321.4	5.07 x 10 <sup>7</sup>	5.16 x 10 <sup>5</sup>	3.01 x 10 <sup>8</sup>
9	60461.2	3.53 x 10 <sup>5</sup>	4.15 x 10 <sup>5</sup>	1.71 x 10 <sup>6</sup>
10	63666.7	3.49 x 10 <sup>5</sup>	2.25 x 10 <sup>5</sup>	9.10 x 10 <sup>5</sup>
11	34687	5.20 x 10 <sup>5</sup>	3.44 x 10 <sup>5</sup>	2.74 x 10 <sup>6</sup>
12	18784	6.62 x 10 <sup>7</sup>	2.54 x 10 <sup>5</sup>	5.18 x 10 <sup>8</sup>
13	26388.7	2.92 x 10 <sup>5</sup>	5.24 x 10 <sup>4</sup>	6.05 x 10 <sup>5</sup>
14	5533.4	1.52 x 10 <sup>8</sup>	5.61 x 10 <sup>5</sup>	5.01 x 10 <sup>8</sup>
15	271.5	9.64 x 10 <sup>7</sup>	6.15 x 10 <sup>5</sup>	4.44 x 10 <sup>8</sup>
16	27099.9	3.33 x 10 <sup>5</sup>	2.44 x 10 <sup>5</sup>	3.58 x 10 <sup>6</sup>
17	460.6	3.04 x 10 <sup>7</sup>	3.99 x 10 <sup>5</sup>	1.02 x 10 <sup>9</sup>
18	53670.9	4.25 x 10 <sup>5</sup>	4.69 x 10 <sup>5</sup>	1.48 x 10 <sup>6</sup>

\*data provided by industrial contacts and collaborators

5.1.2.2 Contaminated soil sites 2 and 3 The abundance of alk B, PAH RHD GN and GP genes for sites 2 and 3 can be found on table 5.4 (the data represents the mean between two replicates). The alk B gene presence range was from 4.3 x 10<sup>6</sup> to 7.70 x 10<sup>7</sup> copies g<sup>-1</sup>dw and 2.62 x 10<sup>6</sup> to 1.93 x 10<sup>7</sup> copies g<sup>-1</sup> dw for sites 2 and 3 respectively, no high standard deviation error was present. The PAH RHD GN presence range from 1.32 x 10<sup>7</sup> to 5.33 x 10<sup>7</sup> copies g<sup>-1</sup> soil dw and 9.32 x 10<sup>6</sup> to 4.76 x 10<sup>7</sup> copies g<sup>-1</sup> soil dw, for sites 2 and 3. high standard deviation for samples 11 and 13 (lowest abundance). The PAH RHD GP presence in site 2 range from 3.65 x 10<sup>5</sup> to 2.62 x 10<sup>6</sup> copies g<sup>-1</sup> soil dw and in site 3 from 1.70 x 10<sup>5</sup> to 1.15 x 10<sup>6</sup> copies g<sup>-1</sup> soil dw and showed high standard deviation for the samples 1 and 11. Similar results were obtained by (Cébron et al., 2008) for highly contaminated soils, 4.4 x 10<sup>4</sup> (±2.4×10<sup>4</sup>) to 4.7 x 10<sup>7</sup> (±2.4 x 10<sup>7</sup>) copies g<sup>-1</sup> soil or sediment dw.

Table 5.4 Degrading gene abundance in contaminated soils site 2 and 3

Sample	Site	alk B	PAH RHD GP (copies g <sup>-1</sup> soil fw)	PAH RHD GN
1	2	7.70 x 10 <sup>7</sup>	7.21 x 10 <sup>5</sup>	2.58 x 10 <sup>7</sup>
2		7.62 x 10 <sup>7</sup>	1.92 x 10 <sup>6</sup>	5.33 x 10 <sup>7</sup>
3		3.69 x 10 <sup>7</sup>	1.68 x 10 <sup>6</sup>	1.95 x 10 <sup>7</sup>
4		7.49 x 10 <sup>7</sup>	2.62 x 10 <sup>6</sup>	2.16 x 10 <sup>7</sup>
5		4.38 x 10 <sup>7</sup>	1.54 x 10 <sup>6</sup>	1.32 x 10 <sup>7</sup>
6		8.27 x 10 <sup>7</sup>	1.80 x 10 <sup>6</sup>	4.79 x 10 <sup>7</sup>
7		2.45 x 10 <sup>6</sup>	6.11 x 10 <sup>5</sup>	2.17 x 10 <sup>7</sup>
8		2.59 x 10 <sup>6</sup>	5.97 x 10 <sup>5</sup>	2.60 x 10 <sup>7</sup>
9		4.03 x 10 <sup>6</sup>	4.99 x 10 <sup>5</sup>	1.61 x 10 <sup>7</sup>
10		23.94 x 10 <sup>6</sup>	3.65 x 10 <sup>5</sup>	1.83 x 10 <sup>7</sup>
11	3	23.86 x 10 <sup>6</sup>	1.70 x 10 <sup>5</sup>	1.10 x 10 <sup>7</sup>
12		2.62 x 10 <sup>6</sup>	4.32 x 10 <sup>5</sup>	3.01 x 10 <sup>7</sup>
		1.93 x 10 <sup>7</sup>	1.15 x 10 <sup>6</sup>	9.32 x 10 <sup>6</sup>
		1.28 x 10 <sup>7</sup>	9.73 x 10 <sup>5</sup>	4.76 x 10 <sup>7</sup>

## 5.2 Assessment of the bioaugmentation potential

Previous studies showed that the range of compost added for bioremediation of PAH and alkane contaminated soils is between 7 to 25% w/w (Antizar-Ladislao et al., 2006b; Taccari et al., 2012; Wu et al., 2013). This value is a fine balance between providing optimal quantities of nutrients and microorganisms, and keeping the amount of extra contaminated material to a minimum.

Comparing compost's D, E, I and H abundance of alk B, PAH RHD GP and GN genes and the range of these genes abundance in site 1 (table 5.5) we can conclude that the amendment with these composts would increase the degrading genes in this site. For samples K and L and site 1 the augmentation would occur for genes alk B and PAH RHD GP, for PAH RHD GN this would not be the case due since the abundance of this gene in these samples falls out of the range of the abundance in site 1.

In site 2 none of the compost samples would increase the abundance of PAH RHD GN, the range of these genes is higher in the soil than in the compost samples. For the alk B gene samples D, E, I and L could increase the abundance. For the PAH RHD GP gene all of the compost samples would increase the abundance of the gene.

In site 3 samples D, E, I, H, and L would increase the quantity of alk B gene, for PAH RHD GP all of the compost samples would contribute to the augmentation of the degrading genes, this is not the case for PAH RHD GN, where none of the samples could increase the abundance on the contrary samples K and L could decrease it.

Considering finished and un-finished compost for all the sites compost sample D (un-finished) would increase more the abundance of the catabolic genes than sample E (finished). Between samples I and H, sample I (un-finished) would increase the catabolic gene abundance. For samples K and L, none of them would increase the catabolic gene abundance.

Table 5.5 Catabolic gene abundance in compost samples and contaminated soil sites

Sample	Alk B	PAH RHD GN (copies g <sup>-1</sup> dw)	PAH RHD GP
<i>Compost samples</i>			
D	1.53 x 10 <sup>8</sup>	2.34 x 10 <sup>6</sup>	3.34 x 10 <sup>7</sup>
E	3.86 x 10 <sup>7</sup>	1.75 x 10 <sup>6</sup>	3.39 x 10 <sup>7</sup>
I	1.16 x 10 <sup>7</sup>	6.75 x 10 <sup>6</sup>	1.14 x 10 <sup>7</sup>
H	3.12 x 10 <sup>6</sup>	3.75 x 10 <sup>6</sup>	5.10 x 10 <sup>6</sup>
K	1.22 x 10 <sup>6</sup>	4.32 x 10 <sup>5</sup>	1.04 x 10 <sup>6</sup>
L	5.19 x 10 <sup>6</sup>	3.57 x 10 <sup>5</sup>	4.20 x 10 <sup>6</sup>
<i>Contaminated soil sites</i>			
1	2.92 x 10 <sup>5</sup> – 1.52 x 10 <sup>8</sup>	6.05 x 10 <sup>5</sup> – 1.13 x 10 <sup>9</sup>	2.54 x 10 <sup>4</sup> – 7.46 x 10 <sup>5</sup>
2	4.30 x 10 <sup>6</sup> – 7.70 x 10 <sup>7</sup>	1.32 x 10 <sup>7</sup> – 5.33 x 10 <sup>7</sup>	3.65 x 10 <sup>5</sup> – 2.62 x 10 <sup>6</sup>
3	2.62 x 10 <sup>6</sup> – 1.93 x 10 <sup>7</sup>	9.32 x 10 <sup>6</sup> – 4.76 x 10 <sup>7</sup>	1.70 x 10 <sup>5</sup> – 1.15 x 10 <sup>6</sup>

### 5.3 Comparison of gene abundance in site 1 and concentration of PAH

Plots of PAH concentration vs catabolic gene abundance were made to explore if there is a relationship between these variables. As we can observe in figures 5.1, 5.2 and 5.3 there appear to be no linear correlation. For alk B and PAH RHD GN it seems to be a maximum concentration where the abundance of these genes is the highest and after this they start to decrease and then remain at about 10<sup>5</sup> copies g<sup>-1</sup> dw, this could be related to high concentration of PAH which could inhibit its degradation (Providenti et al., 1993). For PAH RHD GP there seem to be no linear correlation.

Figure 5.1 PAH concentration vs alk B abundance in contaminated soils samples of site 1

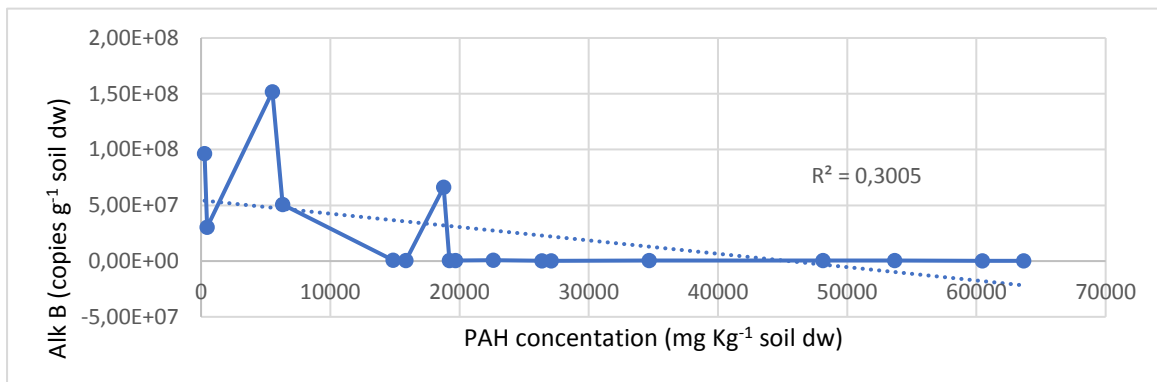


Figure 5.2 PAH concentration vs PAH RHD GP abundance in contaminated soils samples of site 1

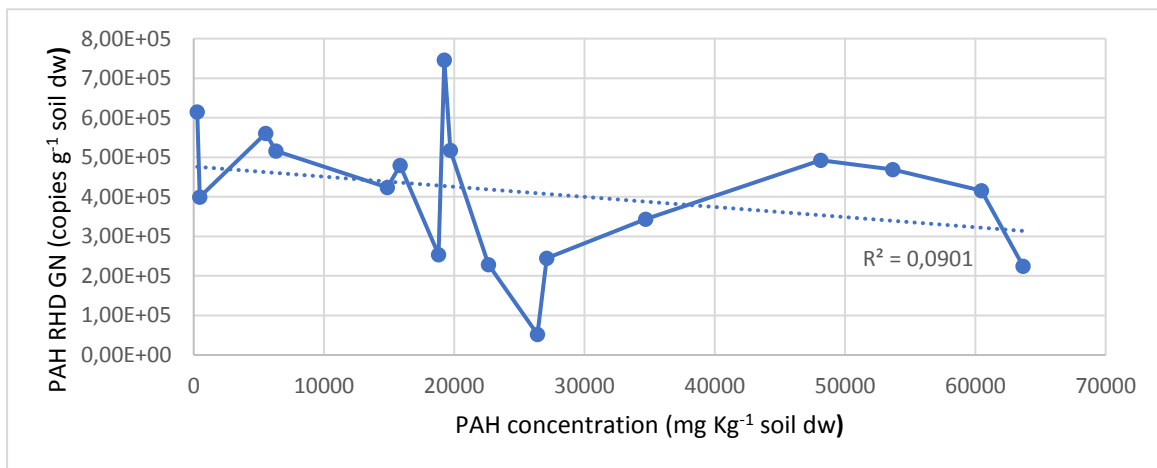
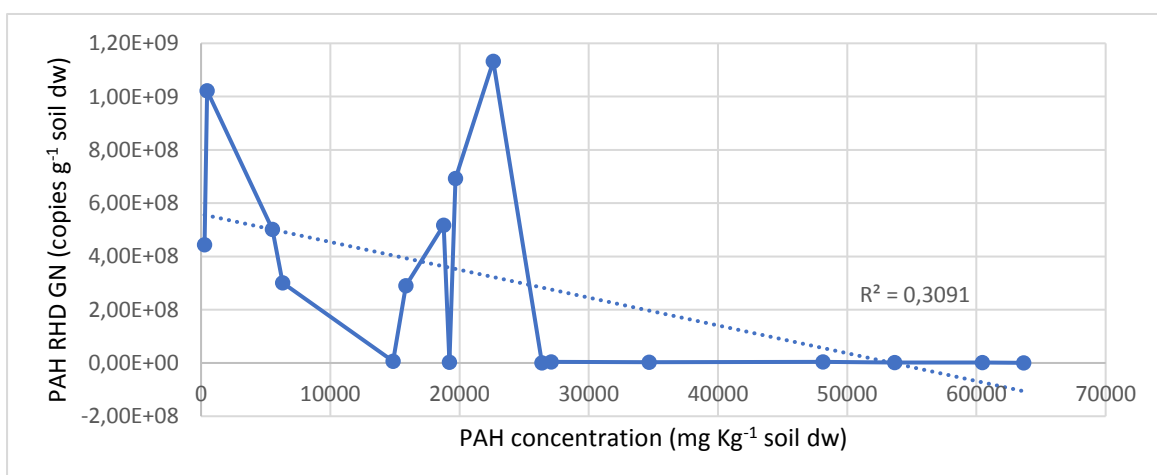


Figure 5.3 PAH concentration vs PAH RHD GN abundance in contaminated soils samples of site 1



#### 5.4 *Pseudomonas* and *Actinomyces* microbial plate count

Three random samples of site 1, 1 of site 2 and 2 of site 3 were chosen for *Actinomyces* and *Pseudomonas* culturable plate count, data in table 5.6. These results show that while the abundance of catabolic genes is similar in the contaminated soil samples and in compost samples, the microbial plate count is lower (one or two orders of magnitude for *Pseudomonas*, and three orders of magnitude for *Actinomyces*). This could suggest that the microorganisms present harbour more than one gene operon per cell (Cébron et al., 2008; Heiss-Blanquet et al., 2005), and also, that unculturable bacteria of this genera could be present.

Table 5.6 *Pseudomonas* and *Actinomyces* plate count in contaminated soil sites 1, 2, and 3

Site	Sample	<i>Pseudomonas</i>	<i>Actinomyces</i>
		(CFU g <sup>-1</sup> soil dw)	
1	6	<1000	2.21 x 10 <sup>4</sup> *
	11	<1000	3.48 x 10 <sup>4</sup>
	15	5.63 x 10 <sup>6</sup>	6.69 x 10 <sup>5</sup>
2	5	2.50 x 10 <sup>3</sup>	6.56 x 10 <sup>5</sup>
3	12	6.90 x 10 <sup>3</sup>	1.68 x 10 <sup>6</sup> *
	14	1.21 x 10 <sup>6</sup>	6.04 x 10 <sup>5</sup>

The CFU count was done in three replicates (n=3), \*the CFU count was done using two replicates.



## 6. CONCLUSIONS AND FUTURE WORK

In this work twelve compost samples of different type and age were characterised in order to analyse their bioremediation potential comparing compost stability and abundance of alk B, PAH RHD GN and PAH RHD GP genes. By comparing different types of compost, too many variables were present which made impossible to find a clear trend, therefore the potential was only analysed in Green compost, comparing finished and un-finished samples from the same site.

- A modified setup of the compost stability measurement ORG0020 was developed and found to be more precise than the original setup. With new set up, also the accuracy has been improved.
- Three primers have been tried in compost samples, the alk B having good results and the PAH RHD GN and GP with apparent inhibition, sample dilution or other methods to reduce the interference could be used to get more accurate results.
- Given that the results showed higher alkB and PAH RHD GN degrading gene abundance in less stable GW composts, and that these composts tend to have higher available N, this would support the use of these composts for bioremediation of aliphatic petroleum hydrocarbon and polyaromatic hydrocarbon (PAH) contaminated soils.
- For PAH RHD GP gene abundance and compost stability there was no clear relationship, the abundance values were similar between finished and un-finished samples.
- While this study shows that the abundance of degrading genes (alk B and PAH RHD GN) is higher in less stable compost, future work should prove if this correspond to abundance of expressed genes and therefore, the potential of augmenting the degraders population on soil, and as a result if the biodegradation rate is enhanced.
- Post-sanitised compost shouldn't contain pathogen microorganisms, to assure the unfinished (more active) compost doesn't spread *E. coli* or *Salmonella*, a microbial count and presence test should be made.
- Another approach it would be to do next generation sequencing or DGGE to identify the microorganisms to check if they have been proved to be alkane or PAH degraders or to reveal unknown or uncultured bacteria.

- Comparing the abundance of alk B, PAH RHD GN and GP in know contaminated soils and compost, the potential of the increment of alkane and PAH degraders is higher with un-finished composts.
- Pseudomonas plate count was higher in the more active composts, similar to results found in other studies.

## REFERENCES

- Antil, R. S., & Raj, D. (2012). Chemical and microbiological parameters for the characterization of maturity of composts made from farm and agro-industrial wastes. *Archives of Agronomy and Soil Science*, 58(8), 833–845. <https://doi.org/10.1080/03650340.2011.554402>
- Antizar-Ladislao, B., Lopez-Real, J., & Beck, A. J. (2006a). Bioremediation of polycyclic aromatic hydrocarbons (PAH) in an aged coal-tar-contaminated soil using different in-vessel composting approaches. *Journal of Hazardous Materials*, 137(3), 1583–1588. <https://doi.org/10.1016/j.jhazmat.2006.04.056>
- Antizar-Ladislao, B., Lopez-Real, J., & Beck, A. J. (2006b). Degradation of polycyclic aromatic hydrocarbons (PAHs) in an aged coal tar contaminated soil under in-vessel composting conditions. *Environmental Pollution*, 141(3), 459–468. <https://doi.org/10.1016/j.envpol.2005.08.066>
- Aspray, T. J., Dimambro, M. E., Wallace, P., Howell, G., & Frederickson, J. (2015). Static, dynamic and inoculum augmented respiration based test assessment for determining in-vessel compost stability. *Waste Management*, 42, 3–9. <https://doi.org/10.1016/j.wasman.2015.04.027>
- Barrena-Gomez, R., Vazquez, F., & Sanchez, A. (2006). The use of respiration indices in the composting process : a review. *Waste Management*, 37–47. <https://doi.org/10.1177/0734242X06062385>
- Beck-Friis, B., Smårs, S., Jönsson, H., Eklind, Y., & Kirchmann, H. (2003). Composting of Source-Separated Household Organics At Different Oxygen Levels : Gaining an Understanding of the Emission Dynamics. *Compost Science & Utilization*, 11(1), 41–50. <https://doi.org/10.1080/1065657X.2003.10702108>
- British Standards Institution. (2008). *BS EN 13040:2007: Soil improvers and growing media- Sample preparation for chemical and physical test, determination of dry matter content, moisture content and laboratory compacted bulk density*. London, United Kingdom: BSI.
- British Standards Institution. (2011). *BS EN 13037:2011: Soil improvers and growing media - Determination of pH*. London, United Kingdom: BSI.
- British Standards Institution. (2011). *BS EN 13038:2011: Soil improvers and growing media - Determination of electrical conductivity*. London, United Kingdom: BSI
- British Standards Institution. (2011). *BS EN 13039:2011: Soil improvers and growing media - Determination of organic matter content and ash*. London, United Kingdom: BSI.
- British Standards Institution. (2011). PAS 100:2011 Specification for Composted Materials. *British Standards Institution, London, UK*, 1–68. <https://doi.org/10.1128/AEM.71.9.4951>
- Cébron, A., Norini, M. P., Beguiristain, T., & Leyval, C. (2008). Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHD $\alpha$ ) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *Journal of Microbiological Methods*, 73(2), 148–159. <https://doi.org/10.1016/j.mimet.2008.01.009>
- Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., & Zhang, J. (2015). Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. *Biotechnology Advances*, 33(6), 745–755. <https://doi.org/10.1016/j.biotechadv.2015.05.003>
- Diplock, E. E., Mardlin, D. P., Killham, K. S., & Paton, G. I. (2009). Predicting

- bioremediation of hydrocarbons: Laboratory to field scale. *Environmental Pollution*, 157(6), 1831–1840. <https://doi.org/10.1016/j.envpol.2009.01.022>
- Franke-Whittle, I. H., Knapp, B. A., Fuchs, J., Kaufmann, R., & Insam, H. (2009). Application of COMPOCHIP microarray to investigate the bacterial communities of different composts. *Microbial Ecology*, 57(3), 510–521. <https://doi.org/10.1007/s00248-008-9435-2>
- Ghosal, D., Ghosh, S., Dutta, T. K., & Ahn, Y. (2016). Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons ( PAHs ): A Review, 7(August). <https://doi.org/10.3389/fmicb.2016.01369>
- Gomez, F., & Sartaj, M. (2013). Field scale ex-situ bioremediation of petroleum contaminated soil under cold climate conditions. *International Biodeterioration and Biodegradation*, 85, 375–382. <https://doi.org/10.1016/j.ibiod.2013.08.003>
- Guermouche M'rassi, A., Bensalah, F., Gury, J., & Duran, R. (2015). Isolation and characterization of different bacterial strains for bioremediation of n-alkanes and polycyclic aromatic hydrocarbons. *Environmental Science and Pollution Research*, 22(20), 15332–15346. <https://doi.org/10.1007/s11356-015-4343-8>
- Gupta, S., Pathak, B., & Fulekar, M. H. (2015). Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review. *Reviews in Environmental Science and Biotechnology*, 14(2), 241–269. <https://doi.org/10.1007/s11157-014-9353-3>
- Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons ( PAHs ): A review, 169, 1–15. <https://doi.org/10.1016/j.jhazmat.2009.03.137>
- Heiss-Blanquet, S., Benoit, Y., Marechaux, C., & Monot, F. (2005). Assessing the role of alkane hydroxylase genotypes in environmental samples by competitive PCR. *Journal of Applied Microbiology*, (December). <https://doi.org/10.1111/j.1365-2672.2005.02715.x>
- Kästner, M., & Mahro, B. (1996). Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of compost. *Applied Microbiology and Biotechnology*, 44(5), 668–675.
- Kästner, M., & Miltner, A. (2016). Application of compost for effective bioremediation of organic contaminants and pollutants in soil. *Applied Microbiology and Biotechnology*, 100(8), 3433–3449. <https://doi.org/10.1007/s00253-016-7378-y>
- Kloos, K., Munch, J. C., & Schloter, M. (2006). A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. *Journal of Microbiological Methods*, 66(3), 486–496. <https://doi.org/10.1016/j.mimet.2006.01.014>
- Llewelyn, R. H. (Direct L. S. L. (2005). *Development of standard laboratory based test to measure compost stability – Annex A* (Vol. 2005). The Waste & Resources Action Programme.
- Llewelyn, R. H. (Direct L. S. L. (2012). *Development of standard laboratory based test to measure compost stability – Annex A* (Vol. 2005).
- MacCready, J. S., Elbert, N. J., Quinn, A. B., & Potter, B. A. (2013). An Assessment of Bacterial Populations in a Static Windrow Compost Pile. *Compost Science & Utilization*, 21(2), 110–120. <https://doi.org/10.1080/1065657X.2013.837272>
- Namkoong, W., Hwang, E. Y., Park, J. S., & Choi, J. Y. (2002). Bioremediation of diesel-contaminated soil with composting. *Environmental Pollution*, 119(1), 23–31.

[https://doi.org/10.1016/S0269-7491\(01\)00328-1](https://doi.org/10.1016/S0269-7491(01)00328-1)

- Perez-de-Mora, A., Schulz, S., & Schloter, M. (2010). MNP- and Real-Time-Based PCR Method for the Quantification of Alkane Monooxygenase Homologous Genes (alkB) in Environmental Samples. In *Bioremediation, Methods in Molecular Biology* (Vol. 599, pp. 59–68). <https://doi.org/10.1007/978-1-60761-439-5>
- Providenti, M. A., Lee, H., & Trevors, J. T. (1993). Selected factors limiting the microbial degradation of recalcitrant compounds, *12*, 379–395.
- Sanchez-Monedero, M. A., Roig, A., Paredes, C., & Bernal, M. P. (2001). Nitrogen transformation during organic waste composting by the Rutgers system and its effects on pH, EC and maturity of the composting mixtures. *Bioresource Technology*, *78*, 301–308.
- Schulz, S., Giebler, J., Chatzinotas, A., Wick, L. Y., Fetzer, I., Welzl, G., ... Schloter, M. (2012). Plant litter and soil type drive abundance, activity and community structure of alkB harbouring microbes in different soil compartments. *The ISME Journal*, *6*(9), 1763–1774. <https://doi.org/10.1038/ismej.2012.17>
- Semple, K. T., Reid, B. J., & Fermor, T. R. (2001). Impact of composting strategies on the treatment of soil contaminated with organic pollutants. *Environmental Pollution*, *112*, 269–283.
- Shahi, A., Aydin, S., Ince, B., & Ince, O. (2016). The effects of white-rot fungi *Trametes versicolor* and *Bjerkandera adusta* on microbial community structure and functional genes during the bioaugmentation process following biostimulation practice of petroleum contaminated soil. *International Biodeterioration and Biodegradation*, *114*, 67–74. <https://doi.org/10.1016/j.ibiod.2016.05.021>
- Taccari, M., Milanovic, V., Comitini, F., Casucci, C., & Ciani, M. (2012). Effects of biostimulation and bioaugmentation on diesel removal and bacterial community. *International Biodeterioration and Biodegradation*, *66*(1), 39–46. <https://doi.org/10.1016/j.ibiod.2011.09.012>
- Wallisch, S., Gril, T., Dong, X., Welzl, G., Bruns, C., Heath, E., ... Schloter, M. (2014). Effects of different compost amendments on the abundance and composition of alkB harboring bacterial communities in a soil under industrial use contaminated with hydrocarbons. *Frontiers in Microbiology*, *5*(MAR), 1–10. <https://doi.org/10.3389/fmicb.2014.00096>
- Ward, O., & Singh, A. A. (2003). Accelerated biodegradation of petroleum hydrocarbon waste, 260–270. <https://doi.org/10.1007/s10295-003-0042-4>
- White, P. A., & Claxton, L. D. (2004). Mutagens in contaminated soil: A review. *Mutation Research - Reviews in Mutation Research*, *567*(2–3 SPEC. ISS.), 227–345. <https://doi.org/10.1016/j.mrrev.2004.09.003>
- Wichuk, K., & McCartney, D. (2010). Compost stability and maturity evaluation - a literature review. *Canadian Journal of Civil Engineering*, (April). <https://doi.org/10.1139/L10-101>
- Wu, G., Kechavarzi, C., Li, X., Sui, H., Pollard, S. J. T., & Coulon, F. (2013). Influence of mature compost amendment on total and bioavailable polycyclic aromatic hydrocarbons in contaminated soils. *Chemosphere*, *90*(8), 2240–2246. <https://doi.org/10.1016/j.chemosphere.2012.10.003>
- Wu, L., & Martinez, G. A. (2000). Comparison of Methods for Evaluating Stability and Maturity of Biosolids Compost. *Journal of Environmental Quality*, *29*, 424–429.
- Wu, M., Li, W., Dick, W. A., Ye, X., Chen, K., Kost, D., & Chen, L. (2017). Bioremediation of hydrocarbon degradation in a petroleum-contaminated soil and microbial population

and activity determination. *Chemosphere*, 169, 124–130.  
<https://doi.org/10.1016/j.chemosphere.2016.11.059>

## APPENDIX

### Chapter 2. Materials and methods

#### 2.3. SOP: Standardisation of solutions

Solutions 1M of HCl were prepared adding 83.6 ml of commercial solutions of HCl 37% (Fisher Scientific, UK) and diluting with distilled water in a volumetric flask to reach the 1l. Because HCl is not a primary standard, this solution is only approximately 1M, which makes it necessary to standardise it. Na<sub>2</sub>CO<sub>3</sub> (100%, Fisher Scientific UK limited) was used. Because we didn't use Na<sub>2</sub>CO<sub>3</sub> standard, the titrations of the HCl were done to ensure consistency in the concentration, not proper standardisation.

#### Procedure

- Na<sub>2</sub>CO<sub>3</sub> was dried for 1 hour at 270-300 °C
- Weight exactly about 2 and 2.5 g of Na<sub>2</sub>CO<sub>3</sub> in to and 250 ml Erlenmeyer flask, add 50 ml of distilled water.
- Add 2 or 3 drops of phenolphthalein solution
- Titrate with the HCl ≈ 1M solution, slowly and with constant agitation until the colour changes
- Add 2 or 3 drops of methyl orange 0.1%
- Continue the titration until the colour changes from yellow to red-orange.
- Write down the volume used
- Calculate the concentration:

$$\text{HCl molar concentration} = \frac{(\text{g}) \text{ weighted Na}_2\text{CO}_3 \times 2}{(\text{g})\text{MW Na}_2\text{CO}_3 \times (\text{l})\text{Volume HCl used}}$$

Solutions 1 M of NaOH were prepared dissolving 40gr of NaOH (Fisher Scientific UK) and diluting to 1litre in a volumetric flask. As HCl solutions are stable, they were used to standardise the prepared NaOH solution.

### Chapter 3. Improvement of ORG0020 setup for compost stability determination.3.4.

#### Experiments with low flow.

To compare how both set ups perform with lower airflow ( $\approx 25 \text{ ml min}^{-1}$ ), experiments were run in this condition, air flow shown in figure 3.4. In table 3.4. we can observe that for experiments 10 and 11 the standard deviation, on days 4 to 7, of the  $\text{CO}_2$  evolved is higher for the original than for the modified set up, as happened for the higher airflow.

Figure 3.4. Air flow in full length experiments with low flow.

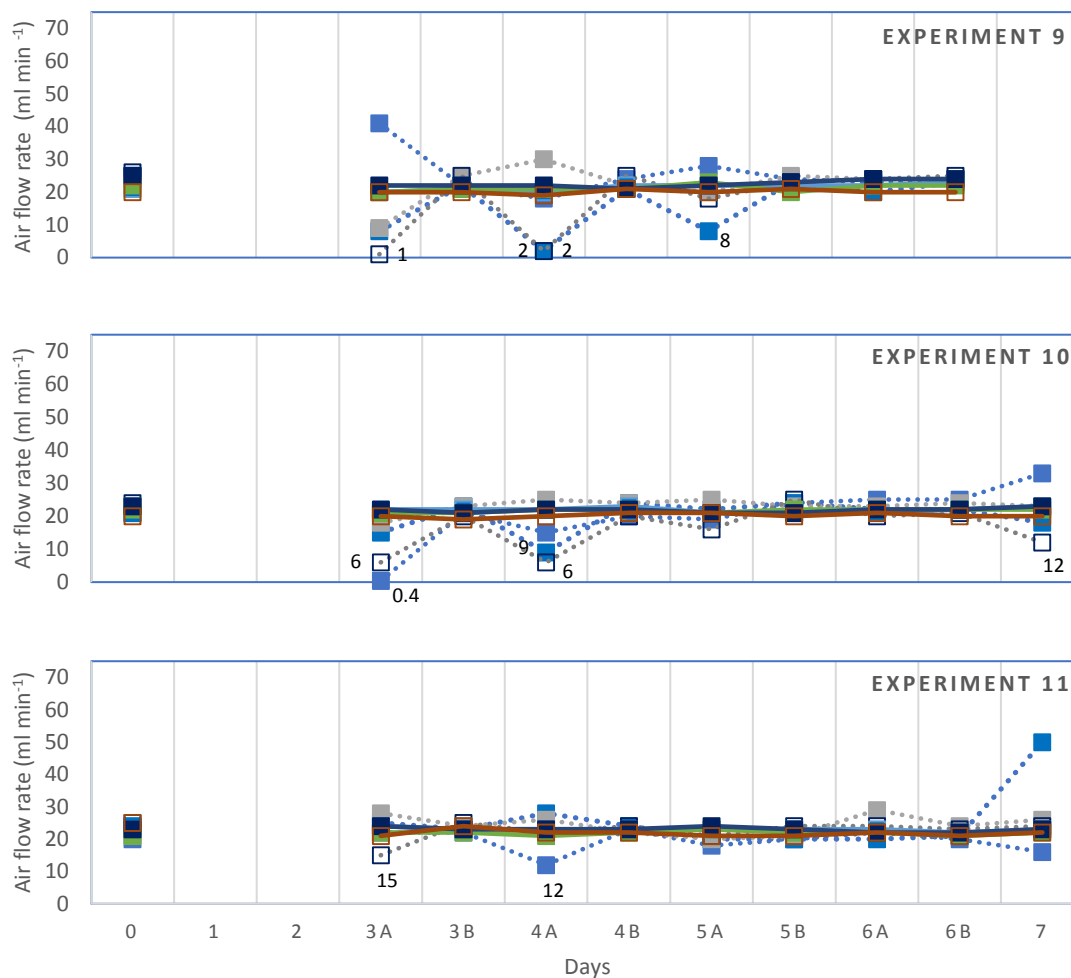


Table 3.4. CO<sub>2</sub> evolution and compost stability with low flow with standard deviations in parentheses.

Experiment number		9	10	11
Compost sample		E	D	C
Day four (mgCO <sub>2</sub> gOM <sup>-1</sup> )	Original	3.08 (1.09)	6.03 (2.06)	3.50 (1.05)
	Modified	2.45 (1.76)	8.29 (0.29)	4.00 (0.26)
Day five (mgCO <sub>2</sub> gOM <sup>-1</sup> )	Original	4.10 (0.83)	6.68 (2.30)	3.46 (0.91)
	Modified	3.61 (0.11)	8.47 (0.49)	3.86 (0.02)
Day six (mgCO <sub>2</sub> gOM <sup>-1</sup> )	Original	4.20 (0.48)	6.22 (1.90)	3.92 (0.46)
	Modified	3.87 (0.21)	7.16 (0.06)	4.11 (0.16)
Day seven (mgCO <sub>2</sub> gOM <sup>-1</sup> )	Original	3.72 (0.38)	6.21 (1.55)	3.92 (0.54)
	Modified	3.32 (0.15)	8.19 (0.37)	3.82 (0.23)
Stability (mgCO <sub>2</sub> gOM <sup>-1</sup> d <sup>-1</sup> )	Original	3.78 (0.23)	6.29 (1.86)	3.70 (0.70)
	Modified	3.31 (0.48)	8.03 (0.27)	3.95 (0.14)

\* low flow = 22-25 ml min<sup>-1</sup> per vessel