1   **When is a soil remediated? Comparison of biopiled and windrowed soils**

2   **contaminated with bunker-fuel in a full-scale trial**

3

4   Frédéric Coulon[1], Mohammed Al Awadi[2], William Cowie[2], David Mardlin[2,3], Simon

5   Pollard[1], Colin Cunningham[4], Graeme Risdon[5], Paul Arthur[3,6], Kirk T. Semple[6*] and

6   Graeme I. Paton[2,3]

7

8   [1]Centre for Resource Management and Efficiency, Sustainable Systems Department,

9   School of Applied Sciences, Cranfield University, Cranfield, MK43 0AL, UK

10   [2]Institute of Biological and Environmental Sciences, University of Aberdeen,

11   Cruickshank Building, St Machar Drive, Aberdeen AB24 3UU, UK

12   [3]Remedios Limited, Campus 3, Unit E2, Aberdeen Science and Technology Park,

13   Aberdeen, AB22 8GW

14   [4]CLARRC, John Muir Building, The Kings Buildings, The University of Edinburgh,

15   Edinburgh, EH9 3LJ

16   [5]TES Bretby, Bretby Business Park, Ashby Road, Burton upon Trent, DE15 0YZ

17   [6]Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK

18

19

20

21

[*] Corresponding author. E-mail: k.semple@lancaster.ac.uk

1   **Abstract**

2   A six month field scale study was carried out to compare windrow turning and biopile

3   techniques for the remediation of soil contaminated with bunker C fuel oil. End-point

4   clean-up targets were defined by human risk assessment and ecotoxicological hazard

5   assessment approaches. Replicate windrows and biopiles were amended with either

6   nutrients and inocula, nutrients alone or no amendment. In addition to fractionated

7   hydrocarbon analysis, culturable microbial characterisation and soil ecotoxicological

8   assays were performed. This particular soil, heavy in texture and historically

9   contaminated with bunker fuel was more effectively remediated by windrowing, but

10   coarser textures may be more amendable to biopiling. This trial reveals the benefit of

11   developing risk and hazard based approaches in defining end-point bioremediation of

12   heavy hydrocarbons when engineered biopile or windrow are proposed as treatment

13   option.

14

15

16   Capsule: Windrows outperform biopiles in the bioremediation of bunker oil contaminated

17   soils

18

20

21

1. Introduction

On site ex situ techniques such as windrow turning and biopiling rely on the management of the biodegradative potential of microorganisms to render hydrocarbons less toxic through mineralisation, biotransformation and assimilation (Barr, 2002). A benefit of bioremediation is the broad scope of petroleum fuel compounds amenable to the process, including branched and unbranched chain aliphatic and aromatic compounds (Chatterjee et al., 2008). Using windrow turning, a site contaminated with high concentrations of diesel range organics (DRO, C_6-C_{24}) was remediated from an initial TPH concentration of 10,000 mg kg^{-1} to less than 1,000 mg kg^{-1} within six months (Barr, 2002).

In this study the petroleum hydrocarbon was No. 6 fuel oil (referred to as bunker C fuel oil) a grade of residual fuel for marine engines. Bunker C has been reported to be the least susceptible to biodegradation in a study carried out by Walker *et al.*, (1976) where the biodegradation of two crude and two fuel oils were compared. However in a large-scale study, Comeau *et al.* (1991) reported the successful biological treatment of 21,475 m^3 of bunker fuel contaminated soil within 12 to 14 weeks under active management. Initial TPH concentrations of 6,000 mg kg^{-1} decreased to less than 1,000 mg kg^{-1} during that study. This observation is in contrast with Song *et al.*, (1990) who reported that bioremediation of bunker C was very slow and incomplete and that the initial steady rate of degradation ceased after 8 weeks. It was concluded that some bunker C constituents were structurally resistant to biodegradation.

Windrows require the frequent turning of soil to enhance aeration, homogenise pollutant sources, nutrients and degraders and to accelerate bioremediation. Biopiles require forced aeration and often use complementary suction of soil gas to feed air to the microbial community. Both are managed under similar regulatory frameworks and can be conducted on site but they require careful water management to optimise the degradation rate. Continuous monitoring of soil hydrocarbon concentration, oxygen level and qualitative and quantitative microbial community changes commonly complement management strategies (Chatterjee et al., 2008). To assess bioremediation as an active process, a measure of the number of hydrocarbon degraders present in a sample is required. Despite moving towards molecular approaches, there is still a great reliance upon culture based techniques including most probable number (MPN) and simple colony forming unit enumeration (CFU) (Coulon et al., 2004; Masak *et al*., 2003). Larger microbial populations have been correlated with enhanced rates of bioremediation (Lindstrom *et al*., 1991).

Soil ecotoxicity assays are based upon direct measurements of soil attributes and enable a rapid and real-time assessment of changes in the soil processes as a function of remedial treatment adopted (Paton *et al*., 2006). Microbial assays commonly used as indicators of hydrocarbon contamination include microbial biomass measurements (CFU, MPN, PLFA), microbial community diversity, function, size species response, and soil respiration (Delistraty, 1984; Van Beelen and Doelman, 1997; Phillips *et al*., 2000; Coulon et al., 2004). Such measurements serve to complement the enumeration of hydrocarbon degraders.

1

2 To monitor changes in the hazard of remediating soils, plant bioassays have been

3 commonly adopted either for plant growth or seed germination (Molina-Barahona *et al*.,

4 2005). When considering soil organisms, earthworms are used because they are sentinel

5 soil test organisms (Callahan *et al*., 1991; Dorn and Salanitro, 2000). Ecological

6 measurements enable an empirical evaluation of hazard assessment and the interpreted

7 response indicates the cumulative impact of potential harm in the soil (Dawson *et al*.,

8 2007). Dawson et al (2007) acknowledged the complexity of interpreting the response of

9 a battery of assays and proposed a ranked approach to selecting the most suitable method.

10 In the UK, and increasingly across the world the end-point of remedial activity is defined

11 not by the total concentration of the chemical of concern but by the concentration likely

12 to pose significant risk. The use of human derived risk-derived approaches can be related

13 to the intended end use of a given site and the target concentration to protect the target

14 receptor is adopted accordingly (Khan and Hussain, 2001).

15

16 For this study, risk assessment of each hydrocarbon fraction was carried out using the risk

17 based corrective action model: RISC4. The default parameters used were for a toddler

18 (residential) and adult (workplace) receptors and criteria were defined as pass or fail for

19 each fraction. There are three approaches that are often adopted in defining the remedial

20 targets for a site. These are: general action concentrations (GAC) soil clean-up target

21 levels (SCTL) and site specific assessment criteria (SSAC). GAC are derived using

22 largely generic assumptions about the characteristics and behaviour of contaminants,

23 pathways and receptors and apply to a range of sites. SCTL are back calculated risk

1 acceptable concentrations, but often generic. Where circumstances relevant to a specific

2 site scenario and receptor behaviour define the SSAC.

3

4 The aim of this work was to compare the performance of two bioremediation techniques

5 (windrows and biopiling) on bunker C Fuel oil impacted soils at the field scale.

6 Individual treatments were compared and assessed using hazard and risk criteria

7 associated with the soil. Furthermore the aim was to consider at what point soil may be

8 deemed to be remediated and how different clean-up treatments compared in meeting

9 these end-points.

10

11 **2. Material and Methods**

12 *2.1.   Regularly applied techniques*

13 *2.1.1. Site set up, sampling and sample preparation*

14 Soil (40 tonnes) was collected from a contaminated Bunker fuel site located at a former

15 dockyard in eastern Scotland. Soil was pre-screened through a >20mm sieve and field

16 screening was conducted to ensure that there was no co-contamination with heavy metals

17 and metalloids (with a Niton 700 XRF multi-element analyser). Basic   soil

18 characterisation was performed (pH, bulk density loss on ignition, extractable N and P

19 concentration) as Guicharnaud et al., 2009. For the experiment, duplicate 3 tonne masses

20 of soil were set up as either windrows or biopiles. These were constructed to have a 2.5

21 m^2 base and were tapered in a pyramidal shape to a peak ogf 1.4 m. Bulked amendments

22 were applied (nutrients and inocula, nutrients alone or no enhancement) using a six tonne

23 capacity hybrid agricultural silage mixer hydraulically powered by a tractor. Nutrients

1   were added in the form of ammonium nitrate and potassium orthophosphate to obtain a

2   C: N: P ratio of 100: 20: 2. The inocula, supplied by Remedios Limited (Aberdeen), was

3   an attenuated enrichment culture from No.6 oil impacted soil and the cell concentration

4   added was such as to give 5×10^7 CFU g^{-1} soil. After further mixing by a Bobcat

5   excavator, the treatments were split into two replicates. A control soil was set up which

6   was left fallow with no enhancement or physical remedial action. The windrows were

7   turned twice a week. All treatments and the fallow soil were maintained at between 70 %

8   and 80 % of their maximum water holding capacity. Air was pumped into the biopiles in

9   a 48 hour cycle to prevent oxygen limitation. Samples were collected from each windrow

10   treatment every two weeks for a period of 28 weeks. Microbial counts were made after 0,

11   4, 10, 16, 22 and 28 weeks. A strict sampling protocol was adhered to: five replicate

12   samples were taken from across each soil treatment, each of these individual replicates

13   being composed of homogenised composite bulked 500 g samples. Prior to analysis

14   samples were stored at 4 °C and analysis were performed on representative soils after the

15   bulked replicates had been coned and quartered. Analysis was always carried out within

16   eight days of sampling. For other analysis, the samples were collected and bulked at the

17   start, mid-point (12 weeks) and end of the experiment (28 weeks). All biological and

18   chemical analysis was performed on the five independent replicates for each biopile or

19   windrow at each sample time described.

20

21   *2.1.2. Total hydrocarbon determination*

22   Homogenised soil (5 g) was ground with anhydrous Na_2SO_4 and the sample placed in a

23   50 ml glass centrifuge tube. Dichloromethane (DCM)/acetone solution (1:1, 20 ml) was

1   added to the sample and sonicated for 30 minutes (applied energy on soil

2   suspension=1200 J ml^{-1}). Samples were then placed on an end-over-end shaker for 16

3   hours at 60 rpm then centrifuged at 1750 x g at 4 $^{\circ}$C for 20 min. After phase separation

4   with water to remove the polar fraction, a 10 ml aliquot of DCM/acetone extract was

5   collected and added to 40 ml glass Wheaton vials and the sample concentrated under N_2

6   gas. The sample was then suspended in 4.9 ml of hexane and sonicated for 2 minutes.

7   After the addition of copper turning (0.5 g), a squalane standard was added as an internal

8   standard. Analysis was performed by GC-FID as described by Dawson *et al.* (2007).

9

10   *2.1.3. Basal respiration*

11   Basal respiration was determined as described by Paton *et al.* (2006). Briefly, 2 g of

12   homogenised soil were weighed into gas vacuettes and allowed to stand for 6 hours in an

13   incubator at 25 $^{\circ}$C. A sample of headspace from the vial was injected into the GC

14   injection loop (250 µl) system with a nitrogen carrier gas (20 ml min^{-1}) onto a 80/100

15   mesh Poropak Q column (2 m × 1/8″OD×2 mm). The oven temperature (Chrompack CP

16   9001) remained constant at 100 $^{\circ}$C and the FID temperature was set at 250 $^{\circ}$C. Respiration

17   values were determined on a mg CO_2 g $soil^{-1}day^{-1}$ basis following subtraction of a blank

18   vial containing atmospheric CO_2 only.

19

20   *2.1.4. Determination of culturable heterotrophs and hydrocarbon-degraders*

21   Homogenised soil (2 g) was weighed into a glass Universal bottle and 20 ml of ¼

22   strength Ringer's solution was added. Samples were then vortexed for 30 seconds and

23   sonicated for 1 minute and allowed to stand for a further 2 minutes. A 100 µl aliquot of

1   soil suspension was removed and serially diluted in ¼ strength Ringer's solution to the

2   appropriate dilution factor (10^{-5} or 10^{-4} dilution factor). An aliquot of 10 μl of each

3   dilution series was added in triplicate to ¼ strength Luria Bertani medium to determine

4   heterotrophs and Bushnell-Hass with 1 % diesel as the sole carbon source for

5   hydrocarbon-degraders. No 6 Fuel oil was unsuited as a substrate due to the viscous

6   nature of the material. Samples were incubated at 25 °C for 24 - 48 hours thereafter and

7   colony-forming units (CFUs) enumerated. Results are expressed as CFU g^{-1} of dry soil.

8

9

1   *2.2.   Techniques conducted at the start, mid-point and termination of the experiment*

2   *2.2.1. Fractionated hydrocarbon (H-C) analysis*

3   The extraction procedure and GC-FID analysis settings for fractionated hydrocarbon

4   fractions have been previously described by Risdon *et al.* (2008). Briefly, 5 g of soil was

5   dried with 5g of anhydrous sodium sulphate (Na_2SO_4) and spiked with 1 mL of a solution

6   containing *o*-terphenyl (oTP), squalane (Sq), heptamethylnonane (HMN) and 2-

7   fluororbiphenyl (2-Fb) at a concentration of 200 µg ml^{-1} each in acetone. Soil samples

8   were extracted with 4 ml of acetone and sonicated for 2 minutes at 20˚C. Hexane and

9   acetone were added to the samples to achieve a 1:1 ratio. The samples were sonicated for

10   a further 10 minutes followed by manual shaking (twice) then followed by centrifugation

11   for 5 minutes at 1000 x g. After passing the supernatant through a filter column, a

12   sequential step series (including resuspension of samples in 10 ml of acetone: hexane

13   (1:1), sonication for 15 minutes at 20 ˚C, centrifugation for 5 minutes at 1000 x g and

14   then decanted into a filter column, this was performed and repeated twice). The final

15   extract volume was adjusted to 40 ml with a mixture of acetone: hexane (1:1) before

16   analysis. A silica gel column was used to separate the aliphatic and aromatic fractions.

17   Approximately 80 ml of RO water and a spatula of sodium chloride (baked at 400˚C for 4

18   hours) were added to the extracts partitioning out any acetone into the water and ensuring

19   the removal of the non-polar content. The split of the aliphatic/aromatic fractions was

20   achieved by eluting with 3 ml of hexane followed by 3 ml of DCM, respectively.

21

22   H-C content was quantified using a gas chromatograph fitted with a flame ionization

23   detector (GC-FID Agilent 7890). Quantification of H-C and sub-ranges of hydrocarbons

1 (Table 1) was made by integrating peak areas using Agilent Chemstation Software

2 Revision B.01.01 (164) SR1 (2001-2005), and by comparison against refined mineral oil

3 standards. External multilevel calibrations were carried out for both diesel/mineral oil

4 fractions and surrogates, quantification ranging from 0.5 to 2500 µg ml^{-1} and from 1 to 5

5 µg ml^{-1}, respectively.

6

7 *2.2.2. Bioluminescence microbial biosensor assay*

8 A methanol extract was used to extract bioavailable H–C for use in the bacterial

9 biosensor assays; 2 g of soil (dry weight) was extracted with 4 ml methanol (Bundy *et al.*,

10 2004). The biosensor *Escherichia coli* HMS174 (Selifonova & Eaton, 1996) was used to

11 measure the change in concentration of inducible hydrocarbon (as reflected by

12 isopropylbenzene, (Selifonova and Eaton, 1996). The toxicity of the soil extracts was

13 determined using 2 constitutively marked biosensors *Pseudomonas putida* F1 Tn5 and

14 *Escherichia coli* HB101 pUCD607 (Paton *et al.*, 2006).

15

16 Cells for each of the assays were previously freeze-dried using standard procedures

17 (Bundy *et al.*, 2001) and stored at -20 $^{\circ}$C. All standards and extracts were diluted to 5 %

18 methanol prior to analysis (Paton *et al,* 2009). Cells were resuscitated in 10 mL 0.1 M

19 KCl between 30-60 minutes on an orbital shaker at 200 rpm and 25 $^{\circ}$C before being

20 sequentially aliquotted (0.1 ml cell suspension) into 0.9 ml of test sample. All procedures

21 were carried out in 1.5 ml covered glass cuvettes and time exposure for the constitutive

22 promoted sensors and inducible sensor was 30 and 120 minutes, respectively. A reference

23 control for *Escherichia coli* HMS174 was prepared by adding 2.8 µl of isopropylbenzene

1   IPB) to 100 ml deionised water containing 0.4 ml methanol. All bioassays were carried

2   out in triplicate, with a total volume of 1 ml and light output was measured using a Jade

3   (Bio orbit 1251) portable luminometer. Results were expressed as a percentage of this

4   postitve reagent control.

5

6   *2.2.3. Seed germination assay*

7   For this assay, 10 mustard seeds (*Brassica alba*) were added separately in 5 replicates to

8   120 ml wide mouth glass jars containing 20 g of soil re-wetted to 75% WHC. Lids were

9   loosely screwed on to reduce evaporation but allow aeration and the seeds were left to

10   germinate at 25 $^{\circ}$C, 80 % humidity and no light for 4 days. The number of seeds

11   germinated was recorded after 4 days. A non-contaminated control soil (Boyndie),

12   maintained at 44% (v/w), was used as the baseline to obtain a 100 % recovery in the

13   assays.

14

15   *2.2.4. Earthworm assays*

16   *Lumbricus terrestris* were obtained from worms direct. Five replicate earthworms were

17   exposed to 50 g of soil sample in 120 ml wide mouth glass bottles for 14 days (Dawson *et*

18   *al.*, 2007; Shakir Hanna & Weaver, 2002). Before and after exposure, the earthworms

19   were washed in tap water and placed in a plastic container lined with moist tissue paper

20   to depurate overnight. Individual earthworms were then rinsed, dried, weighed and placed

21   in a glass jar containing 50 g of soil re-wetted to 80% WHC. Earthworms were incubated

22   individually for 14 days at 15 $^{\circ}$C, 80 % humidity and no light. Lids were fitted loosely to

23   minimise evaporation but allow aeration. The control soil was Boyndie. Earthworms were

1 examined on days 1, 2, 3, 4, 7, 10 and 14 for lethality. Sublethal effects were assessed by

2 measuring the mass of the earthworms after exposure and then depuriating using standard

3 techniques as per Callahan et al (1991.)

4

5 *2.3.   Risk assessment derived remedial targets*

6 Fractionated H-C measurements were interpreted in a risk derived framework using the

7 risk based corrective action model: RISC4 to compare the estimated and the obtained

8 values (RISC4 Manual). Default parameters were used for toddler (residential) and adult

9 (workplace) receptors.

10

11 *2.4.   Data analysis*

12 Data analysis was performed using the five independent replicates for each biopile or

13 windrow at the stated timepoints described. Analysis of the treatments however were only

14 assessed by considering the duplicate treatment data which represents the replication at

15 the field scale. The H-C concentration, culturable heterotroph and degrader numbers were

16 analysed by one-way ANOVA (following where appropriate log transformation of data) a

17 non-parametric Mood median test was used as the alternative. Pearson correlation

18 coefficients and where appropriate multiple linear regression equations were calculated to

19 determine which parameters explained the most variation in the chemical and biological

20 responses (i.e. H-C concentration, heterotroph number, degrader number and ecotoxicity

21 tests). Results in this study represent mean and standard error of two independent

22 replicates (for treatments) but for individual determinants and corresponding bioassays

23 five replicates for comparative evaluation of techniques was performed. A multiple linear

1   regression was performed to assess the relationship between hydrocarbon fractions, time,

2   treatments and toxicity response. Significance is expressed at $p \leq 0.05$. All analyses were

3   conducted using MINITAB (Release 15).

1

3. Results

3

3.1 General soil attributes

The pH range of the soil remained between 6.0 and 7.0 for (optimal range for H-C degradation (Vidali, 2001) the duration of the experiment regardless of the treatment (data not shown) and this is amenable to an active hydrocarbon degrading microbial population (Atlas, 1981). The bulk density of the biopile treatments remained constant throughout the duration of the study (0.93 g cm^{-3}) but was significant reduced for the windrow treatments (0.78 g cm^{-3}) (further data not shown).

11

3.2 Total hydrocarbon determination

After 30 weeks, the total extractable petroleum hydrocarbon content in windrow treatments, including windrow, windrow with nutrients and windrow with nutrients and inocula, were reduced to 4%, 3% and 2%, respectively of their initial value (Figure 1). In contrast, extractable TPH in biopile treatments were only reduced to 22% of their initial values (Figure 2). The results reveal that biostimulation and bioaugmentation enhanced the decrease in TPH concentration throughout the duration of the experiment. The control values as represented by the fallow treatment decreased by only 19% during the field scale experiment.

21

1 *3.3 Basal Respiration*

2 Respiration values collected throughout the experiment for the windrow and biopile

3 remediation study are shown in Figure 3 and Figure 4, respectively. Relative to the other

4 treatments, the control had a significantly lower respiration value throughout the

5 experiment. However, both the nutrient alone and nutrient and inocula treatments with

6 both windrow and biopile management resulted in a significant increase ($p < 0.05$) in

7 respiration. The highest CO_2 production was reached during the first the first 5 and 12

8 days in windrow and biopile treatments, respectively. Throughout the study the windrow

9 treatments had significantly higher respiration values (up to 0.8 mg CO_2 g^{-1} soil) than the

10 biopile ones (up to 0.4 mg CO_2 g^{-1} soil).

11

12 *3.4 Total culturable heterotroph and degrader numbers*

13 The number of culturable heterotrophs in both windrows and biopile treatments generally

14 decreased by one order of magnitude with time (Table 1). In contrast, the number of

15 hydrocarbon degraders increased at least by one order of magnitude regardless the type of

16 treatments (Table 2).

17

18 *3.5 Fractionated hydrocarbons analysis*

19 Fractionation of the oil residues showed that the most prominent aliphatic and aromatic

20 fractions were C_{12} - C_{35} and C_{16} - $C_{35,}$ respectively (Tables 3). Overall, windrow

21 treatments out competed biopile treatments. Degradation of the aliphatic and aromatic

22 fractions in windrows was 6 and 4 times higher respectively than the biopile ones after 6

23 week of treatment. Nutrient addition and bioaugmentation of the biostimulated windrows

16

1   led to a 2 fold higher degradation rate of the aliphatic fraction whereas the aromatic one

2   was barely enhanced by the treatments (Table 3). Higher degradation rates were

3   registered for the aliphatic fraction followed by the aromatics. The mass fraction of

4   aromatics relative to aliphatic hydrocarbons increased by more than 20% in windrows

5   whereas in biopile the mass fraction remained nearly the same.

6

7   *3.6 Bioluminescence microbial biosensor assay*

8   Response of the constitutive biosensor *Escherichia coli* HB101 pUCD607 in both

9   windrow and biopile treatments increased by more than 40% and 55%, respectively

10   during the experiment (Table 4a). This trend was also observed for *Pseudomonas putida*

11   F1 Tn5 and the controls. In contrast, response of the inducible sensor, *Escherichia coli*

12   HMS174 (Table 4b), showed that the luminescence decreased over time, indicating a

13   reduction in the concentration of the target analyte or metabolites to this biosensor. The

14   results are expressed as the percentage of the maximum inducible luminescence from a

15   saturated sample of isopropyl benzene. It has been shown that this biosensor is responsive

16   to a wide range of analogues of this compound and these results do not reflect a reduction

17   in the concentration of this specific analyte. In general, the results ranged between 15 –

18   20% luminescence at the start of the experiment to 1.4% or less luminescence at the end

19   of the experimental time.

20

21   *3.7 Seed germination assays*

22   The control sample germination rate at the start of the experiment increased to double

23   that rate at the end of the experimental time (Table 5). Windrow control and windrow

1   with nutrients had a similar increased germination rate as the control, while windrow with

2   nutrients and inocula had about triple the increase in germination rate of the control. For

3   the biopile treatments, a lower increase in seed germination rate was observed for both

4   biopile and biopile with nutrients. Biopile with nutrients and inocula had tripled in

5   germination rate at the end of the experiment.

6

7   *3.8 Earthworm assays*

8   At the end of the experiment, there was significantly greater survival than at the start of

9   the treatment or the mid point (Table 6). The windrow treatments resulted in a lower

10   survival counts compared with the control with the windrow with nutrients and inocula

11   having the lowest results. For the biopile treatments, the survival rate was different from

12   the windrow treatments compared with the control. Biopile and biopile with nutrients had

13   a significant increase in survival counts. The biopile with nutrients and inocula also had a

14   small increase in survival at the end of the experiment.

15

16   *3.9 Risk assessment derived remedial targets*

17   By the end of the experiment, the results obtained from the fractionated analysis in the

18   soil (Table 3) were compared to that in the RISC 4 criteria and a pass / fail mark was

19   given to each fraction and treatment. An overall risk assessment (RA) mark was awarded

20   to indicate if the site had passed the criteria at that time point (Table 7).

21

22   **4. Discussion**

18

Song *et al*. (1990) in a study of the biodegradation of bunker oil reported that it took 48 weeks of incubation to degrade fifty percent of TPH even under optimal nutrient conditions. It was reported that most of the bunker oil components were structurally resistant to biodegradation and they concluded that "bioremediation has only very limited beneficial effects on bunker oil elimination from soil".

In this study, the amendment of treatments with nutrients increased significantly the hydrocarbon degradation at the initial stages of the experiment. This was further increased at the initial stages by the addition of the inocula. It may be inferred, as many papers report, that the microbial population in the control soils was nutrient limited but that there was a capable microbial population present (Atlas, 1981; Coulon et al. 2004; Bamforth and Singleton, 2005; Delille and Coulon, 2008).

In the control soil, the indigenous population was capable of degrading the available hydrocarbon without any further treatment, but the rate was slower and hence there is a requirement for the remediation management team to relate the need for speed of degradation with the cost of amendment (Khan et al, 2004). Throughout the study the degrader numbers were higher than the total heterotrophic numbers. This is contrary to the observation of Adako and Orugbani (2007) who reported a corresponding increase in heterotrophic and petroleum degrading bacteria during hydrocarbon degradation. However, the effective remediation management (aeration, sieving and nutrients) could have liberated additional substrates (mainly hydrocarbons) and resulted in an increase in the total bacterial population (Townsend *et al*., 2000). It has been documented that culturable heterotrophic numbers can decrease in fuel oil amended soils (Turco *et al*.,

1   1995) as a response to physical and chemical changes to the soil which may affect

2   aeration and nutrient diffusion.

3

4   Respiration has been identified as a key indicator of bioremediative activity (Diplock et

5   al., 2009). In this study, by week 28, the nutrient treatment for both the windrow and

6   biopile techniques was observed to have the highest respiration rate corresponding with

7   an increased number of hydrocarbon degraders. This could be a result of a shift in the

8   assimilation efficiency of the cells due to the addition of nutrients and hence the change

9   of the metabolic quotient. Alternatively, it could be simply that a higher cell number

10   correlates with increased degradation (Coulon et al., 2004; Margesin et al., 2007).

11

12   A measure of the luminescence of biosensors is a surrogate for metabolic activity of soil

13   bacteria (Steinberg et al., 1985; Bundy et al., 2004). This may be used to assess the

14   burden of pollution on the potential metabolic activity of key soil organisms and this may

15   in turn aid in decision making for bioremediation (Trott et al., 2006; Dawson et al.,

16   2007). Dawson et al. (2008) compared the response of a constitutive and monoaromatic

17   hydrocarbons induced biosensor in a site contaminated with benzene, toluene, ethyl

18   benzene and xylenes. The results are similar to those in this study, in that the decline in

19   hydrocarbon concentration caused a reduction in bioluminescence of the induced sensor

20   while the constitutive sensor increased in bioluminescence. Bundy *et al.* (2001) also

21   noted that at the start of the remediation activity, the presence of more water soluble

22   intermediates caused an increase in toxicity and this was also evident in this study.

23

1   The other ecotoxicity assays suggest recovery as the total hydrocarbon concentration

2   declines. This finding is supported by previous studies including Dawson *et al.*, (2007)

3   who reported the indicators that differentiated the extent of soil remediation were

4   respiration, earthworm toxicity and mustard seed germination. Salanitro *et al.* (1997)

5   reported that bioremediated soils were neither toxic to earthworms, inhibitory in the

6   Microtox assay, nor deleterious to seed germination after months of treatment.

7

8   Although the response of the biosensor correlated both with that of the earthworms and

9   seed germination there was little linkage to the human risk assessment criteria. One of the

10   key problems is determining the point at which recovery has been met or where hazard is

11   deemed acceptable and then relating this to risk derived criteria. While LD_{50} and EC_{50}

12   values are often used, these may be insensitive to predicting ecological protection.

13   Furthermore NOEC and LOEC values require considerable data sets for accurate

14   estimations and in light of the nature of these experiments this may not be practicable

15   (Dawson et al., 2007). In the case of the most effective treatments (as assessed by

16   degradation) there was a considerable improvement on the recovery of the ecotoxicity

17   assays (Dawson *et al.,* 2007; Salanitro *et al.,* 1997). This could be interpreted, in the

18   context of this study, to mean that the hazard assessment criteria were such that the soils

19   were all acceptable (regardless of treatment) at the end time point.

20

21   There is no doubt that a measure of the TPH could be misleading as it is the component

22   fractions of the hydrocarbon that will pose a hazard or potential risk to defined receptors.

23   In the case of this study, despite exhaustive regression analysis no single fraction could

be deemed as the causal agent for the measured ecotoxicological response (TPHCWG.

1998). In part this could be because the adopted banding may not reflect the relative

sensitivity of the receptors under investigation (TPHCWG. 1998). These fractions are not

individual compounds and there is very limited dose response characterisation of these

receptors used to the fractions measured. Furthermore, these fractions do not occur in

isolation and it is difficult to factor out the relative impact of these grouped compounds.

The discipline as a whole will benefit from consideration of fractions and their dose

dependency responses to relevant soil ecotoxicity assays. It should also be remembered

that polar metabolites that may be more bioassimilable and potentially more toxic than

the non polar hydrocarbons particular are likely to remain undetected in this study

(TPHCWG. 1998).

Hydrocarbon fractions are however, widely used in human risk assessment. Placed in the

context of human risk assessment, fractionation quantification reveals an interesting

pattern relative to treatment adopted. At the end-point for GAC only WN passes while for

SSAC values W, WN and WNI all pass the criteria (Table 7). This means perhaps that

depending on the selection of an ecotoxicity acceptable dose factor, the risk to a toddler

from ingesting soil is greater than the hazard derived from earthworm, seed germination

and biosensor applications to the soil itself. Each receptor has a different response to the

substance of interest.

In the future, comparative evaluations of defined receptors will become more important,

because although the risk assessment models exist to protect humans and water courses

1 under a range of different conditions, this is not the case with ecological receptors. There

2 is currently a need to carry out empirical assays to quantify the response and then

3 consider using these data to derive ecologically protective doses.

4

5 Hazard and risk approaches are key in defining the status of soil in the context of being

6 "fit for purpose". Such approaches are of more meaning than a total hydrocarbon

7 measurement and may also place the relative potency of fractions in a suitable context.

8

9 **5. Conclusion**

10 Active management enhanced the biodegradation of bunker fuel oil in soil in a controlled

11 field-scale trial. The addition of nutrient and inocula accelerated the degradation rate for

12 the period of the study. Microbial measurements used in conjunction with chemical

13 analysis increased our understanding of field-based bioremediation. For this heavily

14 textured soil, windrow turning was more effective than biopiling because the resultant

15 soil was more friable. For coarser textured soils, biopiling may perform better. The likely

16 decision as to which technique to adopt would be determined by remediation managers.

17 The end-point of remediation needs to be defined relative to the receptor that requires

18 protection. In this study, soil that showed a significant ecological recovery was still

19 impaired with respect to human risk criteria and *visa versa*. There is a need to carry out

20 more comparative studies to better assess the relationship and relative sensitivity of

21 receptor-based end-points.

22

7

8

1   **References**

2   Adoki, A., Orugbani, T., 2007. Removal of crude petroleum hydrocarbons by

3   heterotrophic bacteria in soils amended with nitrogenous fertilizer plant effluents. African

4   Journal of Biotechnology 6, 1529-1535.

5   Atlas, R. M., 1981. Microbial degradation of petroleum hydrocarbons: an environmental

6   perspective. Microbial Reviews 45, 180-209

7   Andreoni, V., Gianfreda, L. 2007. Bioremediation and monitoring of aromatic-polluted

8   habitats. Applied Microbiology and Biotechnology 76, 287-308

9   Bamforth, S.M., Singleton, I., 2005. Review: Bioremediation of polycyclic aromatic

10   hydrocarbons: current knowledge and future directions. Journal of Chemical Technology

11   and Biotechnology 80, 723-736

12   Barr, D., 2002. Biological methods for assessment and remediation of contaminated

13   land: case studies. Construction Industry Research and Information Association, London.

14   Bundy, J.G., Campbell, C.D., Paton, G.I., 2001. Comparison of response of six different

15   luminescent bacterial bioassays to bioremediation of five contrasting oils. Journal of

16   Environmental Monitoring 3, 404-410

17   Bundy, G.J., Paton, G.I., Campbell, C.D., 2004. Combined microbial community level

18   and single species biosensor responses to monitor recovery of oil polluted soil. Soil

19   Biology and Biochemistry 36, 1149-1159

20   Callahan, C. A., Menzie, C. A., Burmaster, D. E., Wiilborn, D. C., Ernst, T., 1991. On-

21   site methods for assessing chemical impact on the soil environment using earthworms: A

22   case study at the Baird and McGuire superfund site, Holbrook, Massachussets.

23   Environmental Toxicology and Chemistry 10, 817-826

1 Chaillan, F., Chaîneau, C., Point, V., Saliot, A., Oudot, J., 2006. Factors inhibiting

2 bioremediation of soil contaminated with weathered oils and drill cuttings. Environmental

3 Pollution 144, 255-265

4 Chatterjee, S., Chattopadhyay, P., Roy, S., Sen, S.K., 2008. Bioremediation: a tool for

5 cleaning polluted environments. Journal of Applied Biosciences 11, 594 – 601

6 Comeau, C., Mahapfey, W.D., Parras, L., 1991. Full-scale bioremediation of

7 contaminated soil and water, in: Environmental Biotechnology for Waste Treatment.

8 Blackburn, J. W., Fox, R., Sayler, G. S., (Eds.), third ed. Plenum Press, New York, pp.

9 91-110.

10 Coulon F., Pelletier E., St Louis R., Gourhant L., Delille D., 2004. Degradation of

11 petroleum hydrocarbons in two sub-Antarctic soils: influence of an oleophilic fertilizer.

12 Environmental Toxicology and Chemistry 23, 1893-1901.

13 Dawson, J.J., Godsiffe, I.P., Thompson, T.K., Ralebitso-Senior, Killham, K.S., Paton,

14 G.I., 2007. Application of biological indicators to assess recovery of hydrocarbon

15 impacted soils. Soil Biology and Biochemistry 39, 164-177

16 Dawson, J.J.C., Iroegbu, I., Maciel, H., Paton, G.I., 2008. Application of luminescent

17 biosensors for monitoring the degradation and toxicity of BTEX compounds in

18 soils. Journal of Applied Microbiology 104, 141–151

19 Delille, D., Coulon, F., 2008. Comparative mesocosm study of biostimulation efficiency

20 in two different oil-amended sub-Antarctic soils. Microbial Ecology 56, 243-252.

21 Delistraty, D.A., 1984. Bioluminescent toxicity assay of synfuel by-product waters,

22 Bulletin of Environmental Contamination and Toxicology 32, 613–620

1 Diploick, E.E., Mardlin D.P., Kilham K.S., Paton G.I., 2009. Predicting bioremediation

2 of hydrocarbons: laboratory to field scale. Environmental Pollution 157, 1831-1840

3 Dorn, P.B., Salanitro, J.P., 2000. Temporal ecological assessment of oil contaminated

4 soils before and after bioremediation. Chemosphere 40, 419-426

5 Khan, F.I., Hussain, T., 2001. Risk-based monitored natural attenuation - a case study.

6 Journal of Hazardous Materials B85, 243–272

7 Khan, F.I., Husain, T., Hejazi, R., 2004. An overview and analysis of site remediation

8 technologies. Journal of Environmental Management 71, 95-122

9 Lindstrom, J.E., Prince, R.C., Clark, J.C., Grossman, M.J., Yeager, T.R., Braddock, J.F.,

10 Brown, E.J., 1991. Microbial populations and hydrocarbon biodegradation potentials in

11 fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. Applied and

12 Environmental Microbiology 57, 2514-2522

13 Margesin, R., Hämmerle, M., Tscherko, D., 2007 Microbial activity and community

14 composition during bioremediation of diesel-oil-contaminated soil: Effects of

15 hydrocarbon concentration, fertilizers, and incubation time. Microbial Ecology 53, 259-

16 269

17 Masak, J., Machackova, J., Siglova, M., Cejkova, A., Jirku, V., 2003. Capacity of the

18 bioremediation technology for clean-up of soil and groundwater contaminated with

19 petroleum hydrocarbons. Journal of Environmental Science and Health Part A Toxic /

20 Hazardous Substances and Environmental Engineering 10, 2447–2452

21 Molina-Barahona, L., Vega-Logo, L., Guerrero, M., Rameriez, S., Romero, I., Vega-

22 Jarquin, C., Albores, A. 2005. Ecotoxicological evaluation of diesel contaminated soil

23 before and after a bioremediation process. Environmental Toxicology 20, 100–109

Paton, G.I., Viventsova, R.E., Kumpene, J., Wilson, M.J., Weitz, H.J., Dawson, J.J., 2006. An ecotoxicity assessment of contaminated forest soils from Kola Peninsula. Science of the Total Environment 355, 106-117

Paton, G.I., Reid, B.J., Semple, K.T., 2009. Application of a luminescence-based biosensor for assessing naphthalene biodegradation in soils from a manufactured gas plant. Environmental Pollution 157, 1643-1648

Phillips, T.M., Seech, A.G., Liu, D., Lee, H., Trevors, J.T., 2000. Monitoring biodegradation of creosote in soils using radiolabels, toxicity tests, and chemical analysis. Environmental Toxicology 15, 99–106

RISC 4 model manual (http://www.groundwatersoftware.com/risc.htm)

Risdon, G., Pollard, S.J.T., Brassington, K.J., McEwan, J.N., Paton, G.I., Semple, K.T., Coulon, F., 2008. Development of an analytical procedure for weathered hydrocarbon contaminated soils within a UK risk-based framework. Analytical Chemistry 80, 7090-7096

Rojas-Avelizapa, N.G., Roldán-Carrillo, T., Zegarra-Martínez, H., Muñoz-Colunga, A.M., Fernández-Linares, L.C., 2007 A field trial for an ex-situ bioremediation of a drilling mud-polluted site. Chemosphere 66, 1595-1600

Salanitro, J.P., Dorn, P.B., Huesemann, M.H., Moore, K.O., Rhodes, I.A., Rice Jackson, L.M., Vipond, T.E., Western, M.M., Wisniewski, H.L., 1997. Crude Oil Hydrocarbon Bioremediation and Soil Ecotoxicity Assessment. Environmental Science and Technology 31, 1769–1776

Selifonova, O.V., Eaton, R.W., 1996. Use of an *ifb–lux* fusion to study regulation of the isopropylbenzene catabolism operon of *Pseudomonas putida* RE204 and to detect

hydrophobic pollutants in the environment. Applied and Environmental Microbiology 62, 778-783

Shakir Hanna, S.H., Weaver, R.W., 2002 Earthworm survival in oil contaminated soil. Plant and Soil 240, 127–132

Song, H., Wang, X., Bartha, R., 1990. Bioremediation potential of terrestrial fuel spills. Applied and Environmental Microbiology 56, 652-656

Steinberg, S.M., Poziomek, E.J., Engelmann, W.H., Rogers, K.M., 1995. A review of environmental applications of bioluminescence measurements. Chemosphere 30, 2155-2197

Townsend R.T., Bonner, J.S., Autenrieth, R.L., 2000. Microbial dynamics during bioremediation of a crude oil-contaminated coastal wetland. Bioremediation Journal 4, 203–218

TPHCWG., 1998. Volumes 1 - 5 on Total Petroleum Hydrocarbons, Amherst Scientific Publishers, Amherst, MA.

Trott, D., Dawson, J. J. C., Killham, K. S., Miah, Md. R. U., Wilson, M. J., Paton, G. I., 2006. Comparative evaluation of a bioluminescent bacterial assay in terrestrial ecotoxicity testing. Journal of Environmental Monitoring 9, 44–50

Turco, R.F. and Sadowski, M.J. 1995. The Microflora of Bioremediation. H.D. Skipper and R.F. Turco (ed), Bioremediation, Science and Applications. 87-103. Soil Science Society of America Special Publication 43.

Van Beelen, P, Doelman, P., 1997. Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment. Chemosphere 34, 455-499

1   Vidali, M., 2001. Bioremediation, An overview. Pure and Applied Chemistry 73, 1163–

2   1172

3   Walker, J., Petrakis, L., Colwell. R., 1976. Comparison of the biodegradability of crude

4   and fuel oils. Canadian Journal of Microbiology 22, 598-602

5

6

Table 1 Culturable heterotroph numbers (CFUs g^{-1} dry soil) for windrow and biopile treatments (observations annotated with the same letter are not significantly different p$\leq$ 0.05)

Windrow treatments

Time	C	W	WN	WNI
0	3.11E+07^a	2.58E+07^a	3.71E+08^b	3.78E+07^b
4	2.17E+08^b	2.95E+07^a	3.43E+08^b	2.78E+08^b
10	4.05E+07^a	2.10E+07^a	1.25E+07^a	1.48E+08^b
16	1.26E+08^b	5.38E+07^a	1.46E+08^b	1.01E+08^b
22	1.85E+06^a	2.39E+06^a	8.02E+06^a	6.66E+06^a
28	3.54E+06^a	4.46E+06^a	1.74E+07^a	5.64E+07^a

Biopile Treatments

Time	C	B	BN	BNI
0	3.11E+07^a	2.56E+08^b	2.75E+08^b	2.91E+08^b
4	2.84E+08^b	2.39E+08^b	2.78E+08^b	2.61E+08^b
10	4.88E+07^a	4.95E+06^a	7.60E+07^a	2.04E+08^b
16	1.86E+08^b	1.07E+08^b	1.30E+08^b	9.61E+07^a
22	1.59E+06^a	6.92E+06^a	2.79E+07^a	1.75E+07^a
28	2.31E+06^a	9.98E+06^a	1.29E+07^a	4.12E+07^a

C Control
W Windrow   B Biopile
W+N Windrow + Nutrient   B+N Biopile + Nutrient
W+N+I Windrow + Nutrient + Inoculum   B+N+I Biopile + Nutrient + Inoculum

Table 2 Culturable hydrocarbon degrader numbers (CFUs g^{-1} dry soil) for windrows and biopile treatments (observations annotated with the same letter are not significantly different p≤ 0.05)

Windrow treatments

Time	C	W	WN	WNI
0	2.44E+07^a	2.67E+07^a	2.71E+07^a	2.88E+07^a
4	2.84E+08^b	6.37E+07^a	5.99E+08^b	4.82E+08^b
10	4.34E+08^b	2.15E+07^a	2.15E+07^a	2.15E+07^a
16	3.05E+08^b	3.98E+08^b	1.69E+08^b	1.67E+08^b
22	4.42E+08^b	1.39E+07^a	4.96E+08^b	1.95E+09^c
28	1.32E+08^b	2.31E+08^b	1.42E+09^c	2.18E+09^c

Biopile Treatment

Time	C	B	BN	BNI
0	2.15E+07^a	2.80E+07^a	3.41E+07^a	4.31E+07^a
4	3.16E+08^b	4.91E+07^a	4.67E+08^b	1.91E+08^b
10	5.67E+08^b	1.67E+07^a	1.08E+07^a	1.51E+07^a
16	1.09E+08^b	1.09E+08^b	1.18E+08^b	2.76E+08^b
22	5.19E+08^b	2.09E+07^a	2.91E+08^b	6.22E+08^b
28	1.32E+08^b	1.63E+08^b	1.42E+08^b	3.54E+08^b

C	Control		
W	Windrow	B	Biopile
W+N	Windrow + Nutrient	B+N	Biopile + Nutrient
W+N+I	Windrow + Nutrient + Inoculum	B+N+I	Biopile + Nutrient + Inoculum

Table 3: Quantification of TPH and sub-ranges of hydrocarbons in the windrow and biopile treatments during the time course of the remediation study (with standard error of the mean)

Hydrocarbon Fraction		Start	SE	Week 14 C	SE	W	SE	W+N	SE	W+N+I	SE	Week 28 C	SE	W	SE	W+N	SE	W+N+I	SE
	Total	13,009	152	11844	982	3691	381	2208	147	1474	129	10639	1034	539	47	419	30	361	38
Al	C10-12	71	21	64	8	21	3	12	2	10	1	43	4	10	1	0	0	10	0
Al	C12-16	1432	161	1123	98	453	18	421	28	350	20	1311	155	21	1	23	1	10	1
Al	C16-21	3124	209	2314	87	521	12	411	12	434	21	2671	31	110	21	24	10	31	3
Al	C21-35	2100	981	1876	121	629	31	349	21	219	12	1932	63	43	2	49	8	38	4
Ar	C10-12	21	11	34	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ar	C12-16	671	54	576	34	130	14	54	2	10	2	422	23	21	1	12	3	17	1
Ar	C16-21	2376	329	3021	176	631	32	480	17	210	16	2398	101	103	13	67	5	71	5
Ar	C21-35	3214	210	2876	109	1306	121	481	21	241	21	1862	12	231	19	244	10	184	21

		Start	SE	C	SE	B	SE	B+N	SE	B+N+I	SE	C	SE	B	SE	B+N	SE	B+N+I	SE
	Total	13,009	152	11844	982	8561	522	9649	637	8884	1091	10639	1034	2655	320	2989	308	2604	319
Al	C10-12	71	21	64	8	55	4	70	4	23	1	43	4	25	3	32	4	42	3
Al	C12-16	1432	161	1123	98	981	74	1129	76	870	108	1311	155	421	54	548	72	461	62
Al	C16-21	3124	209	2314	87	1850	109	2131	101	2231	39	2671	43	254	12	162	41	238	18
Al	C21-35	2100	981	1876	121	1293	87	1082	24	879	31	1932	61	549	21	487	30	504	42
Ar	C10-12	21	11	34	2	10	1	0	0	0	0	0	0	0	0	0	0	0	0
Ar	C12-16	671	54	576	34	290	3	310	13	281	19	422	23	132	16	213	17	376	28
Ar	C16-21	2376	329	3021	176	2101	76	2876	18	2845	101	2398	59	421	31	545	105	419	34
Ar	C21-35	3214	210	2876	109	1981	81	2051	23	1755	65	1862	102	853	42	911	53	564	31

Al   Aliphatic   Ar   Aromatic

C   Control   SE   Standard Error
W   Windrow   B   Biopile
W+N   Windrow + Nutrient   B+N   Biopile + Nutrient
W+N+I   Windrow + Nutrient + Inoculum   B+N+I   Biopile + Nutrient + Inoculum

Table 4a Bioluminescence as expressed as a percentage of control (non-hydrocarbon impacted soil) obtained for the constitutive biosensors used in the experiment (wd ith standard error of the mean)

Escherichia coli HB101

Week	C	SE	W	SE	WN	SE	WNI	SE	B	SE	BN	SE	BNI	SE
0	44.20	2.1	43.20	1.2	40.20	2.1	38.90	3.2	39.40	1.7	41.30	1.8	40.20	2.0
2	32.40	3.2	68.70	2.0	70.20	2.7	66.80	2.0	33.40	2.3	37.60	1.2	36.50	2.3
6	45.10	1.7	71.20	1.6	73.80	1.3	69.10	1.8	49.50	3.0	51.30	1.4	40.40	3.4
12	52.50	1.2	68.90	2.4	75.20	1.1	76.40	1.7	56.70	2.9	58.70	2.0	61.20	2.9
18	60.32	1.5	86.20	3.0	93.20	1.9	88.10	1.5	62.70	2.5	60.30	2.1	63.80	1.8
26	57.80	2.7	91.10	1.0	94.60	1.3	90.12	2.1	66.80	2.2	65.70	2.0	66.90	1.8
28	62.30	2.9	88.20	2.1	91.30	2.1	94.70	1.9	69.10	2.0	71.60	1.0	74.30	2.2

Pseudomonas putida F1 Tn5

Week	C	SE	W	SE	WN	SE	WNI	SE	B	SE	BN	SE	BNI	SE
0	37.10	1.2	38.20	1.1	35.40	1.9	33.20	2.2	33.60	1.8	32.60	1.9	32.50	1.2
2	23.40	1.6	63.20	1.8	64.60	2.1	62.60	1.9	22.90	2.0	34.50	0.3	32.87	2.1
6	40.60	1.6	66.20	1.6	70.60	1.2	65.80	1.4	44.70	1.4	44.70	2.3	35.70	2.7
12	56.30	1.4	63.90	2.1	73.80	1.1	71.90	1.4	49.90	2.1	50.60	1.6	55.80	3.0
18	58.80	1.1	82.70	1.9	90.30	2.1	83.70	1.3	56.30	1.3	57.10	1.8	60.30	1.1
26	60.20	1.2	87.40	1.2	92.70	1.2	85.60	1.1	61.90	1.9	61.00	1.6	63.80	1.6
28	59.70	2.0	91.30	2.1	88.50	2.2	92.80	1.2	63.70	2.1	67.30	1.5	71.70	2.2

Table 4b Bioluminescence as expressed as a percentage of a positive control sample (containing 2.8 μ/ 100ml^{-1}) obtained for *Escherichia coli* HMS174 (with standard error of the mean)

Escherichia coli HMS174

Week	C	SE	W	SE	WN	SE	WNI	SE	B	SE	BN	SE	BNI	SE
0	16.40	0.2	18.31	0.5	17.66	0.3	16.43	0.5	15.72	0.7	14.99	0.9	15.12	1.0
2	12.34	1.0	4.23	0.4	2.13	0.7	3.54	0.4	11.54	0.6	9.32	1.0	8.76	0.8

6	8.31	0.2	1.29	0.7	2.22	0.3	3.02	0.4	6.76	0.9	7.49	1.0	6.81	1.0
12	6.22	0.4	0.23	0.1	0.03	0.0	0.14	0.0	2.17	0.1	2.01	0.1	3.01	0.1
18	3.21	0.4	0.43	0.0	0.12	0.0	0.11	0.0	1.32	0.4	1.29	0.3	1.04	0.1
26	1.29	0.2	0.59	0.0	0.19	0.0	0.09	0.0	0.41	0.0	0.54	0.1	0.38	0.0
28	1.39	0.1	0.39	0.1	0.13	0.1	0.18	0.0	0.12	0.1	0.19	0.1	0.11	0.1

C	Control	SE	Standard Error
W	Windrow	B	Biopile
WN	Windrow + Nutrients	BN	Biopile + Nutrients
WNI	Windrow + Nutrients + Inoculum	BNI	Biopile + Nutrients + Inoculum

1

Table 5 Percentage of survival of mustard seeds (with standard error of the mean)

Week	C	SE	W	SE	WN	SE	WNI	SE	B	SE	BN	SE	BNI	SE
0	**22**	**0.4**	**24**	**0.6**	**28**	**0.5**	**18**	**0.4**	**21**	**0.6**	**17**	**0.6**	**19**	**0.5**
12	36	0.4	32	0.4	38	0.4	22	0.3	38	0.4	44	0.5	30	0.5
28	92	0.2	74	0.4	94	0.2	76	0.3	88	0.3	90	0.2	84	0.5

Table 6 Percentage of earthworm survival after the 14 day duration assay (with standard error of the mean)

Week	C	SE	W	SE	WN	SE	WNI	SE	B	SE	BN	SE	BNI	SE
0	10	0	0	0	10	10	20	10	10	0	0	0	0	0
12	40	0	50	20	50	10	100	0	20	20	10	10	80	0
28	100	0	80	20	80	0	90	10	70	10	90	10	90	10

C   Control   SE   Standard Error
W   Windrow   B   Biopile
W+N   Windrow + Nutrient   B+N   Biopile + Nutrient
W+N+I Windrow + Nutrient + Inoculum   B+N+I Biopile + Nutrient + Inoculum

Table 7 Treatments that <u>pass</u> the remedial target criteria

Three Months	GAC		SSAC		SCTL	
Fractions	Residential without plant	Industrial / Commercial	Residential without plant	Industrial / Commercial	Residential without plant	Industrial / Commercial
EC10-C12	None Pass	C, W, WN, WNI, B, BN, & BNI	WN & WNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC12-C16	None Pass	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC16-C35	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC35-C44	C, W ,WN ,WNI, B, BN,& BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC10-C12 Aro	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC12-C16 Aro	WN & WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC16-C21 Aro	WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI
EC21-C35 Aro	WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
RA Mark	Fail	Pass	Fail	Pass	Fail	Pass

Six Months	GAC		SSAC		SCTL	
Fractions	Residential without plant	Industrial / Commercial	Residential without plant	Industrial / Commercial	Residential without plant	Industrial / Commercial
EC10-C12	WN	C, W, WN, WNI, B, BN, & BNI	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC12-C16	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC16-C35	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	-	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC35-C44	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	-	-	-
EC10-C12 Aro	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC12-C16 Aro	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, & B	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC16-C21 Aro	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
EC21-C35 Aro	W, WN, & WNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI	C, W, WN, WNI, B, BN, & BNI
RA Mark	Fail	Pass	Fail	Pass	Pass*	Pass

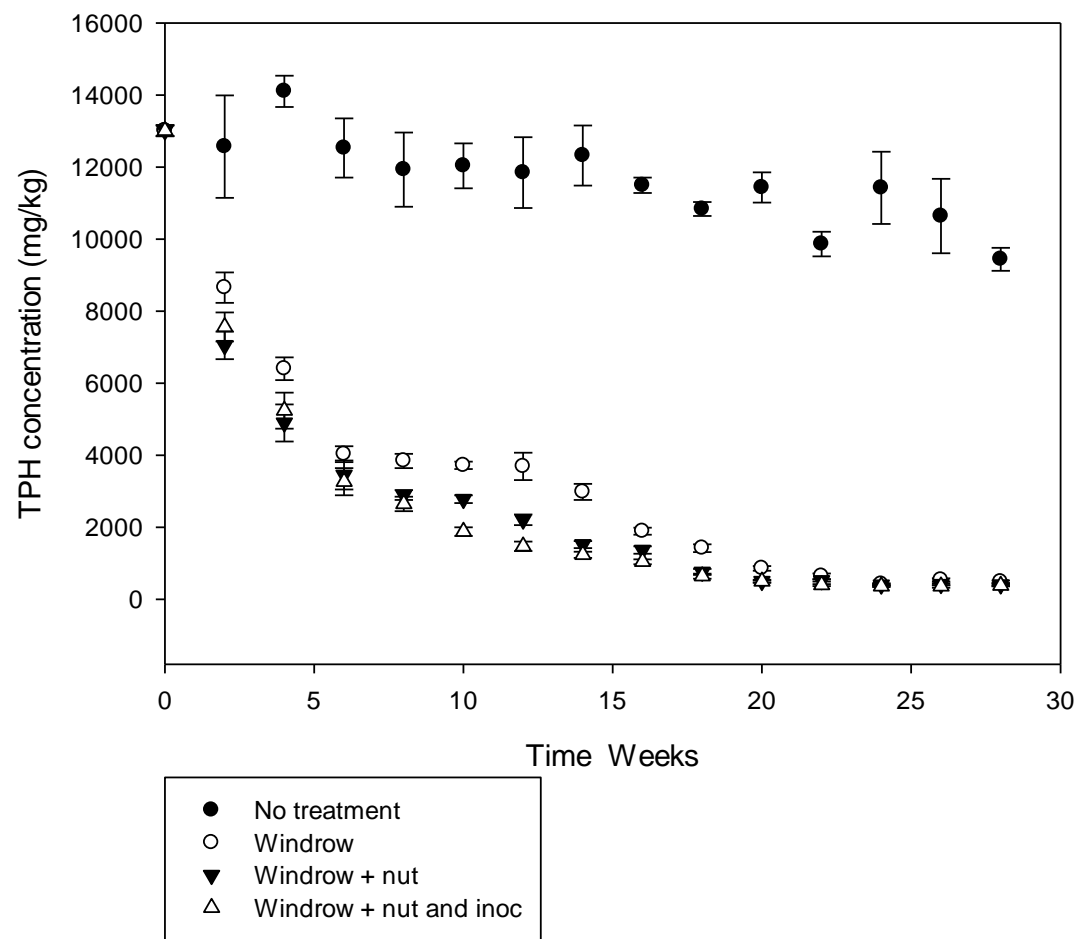|				
RA	Risk Assessment			
GAC	General Action Concentration		SSAC	Site Specific Action Concentration
SCTL	Soil Clean-up Target Level		Ali	Aliphatic Fraction
C	Control		Aro	Aromatic Fraction
W	Windrow		B	Biopile
WN	Windrow + Nutrient		BN	Biopile + Nutrient
WNI	Windrow + Nutrient + Inoculum		BNI	Biopile + Nutrient + Inoculum

1
2 **<u>Figure legends</u>:**
3
4
5 **Fig. 1** TPH degradation from Windrows field time course of remediation.

6

7 **Fig. 2** TPH degradation from Biopile field time course of remediation.

8

9 **Fig. 3** Respiration values from the Windrow field time course remediation study

10

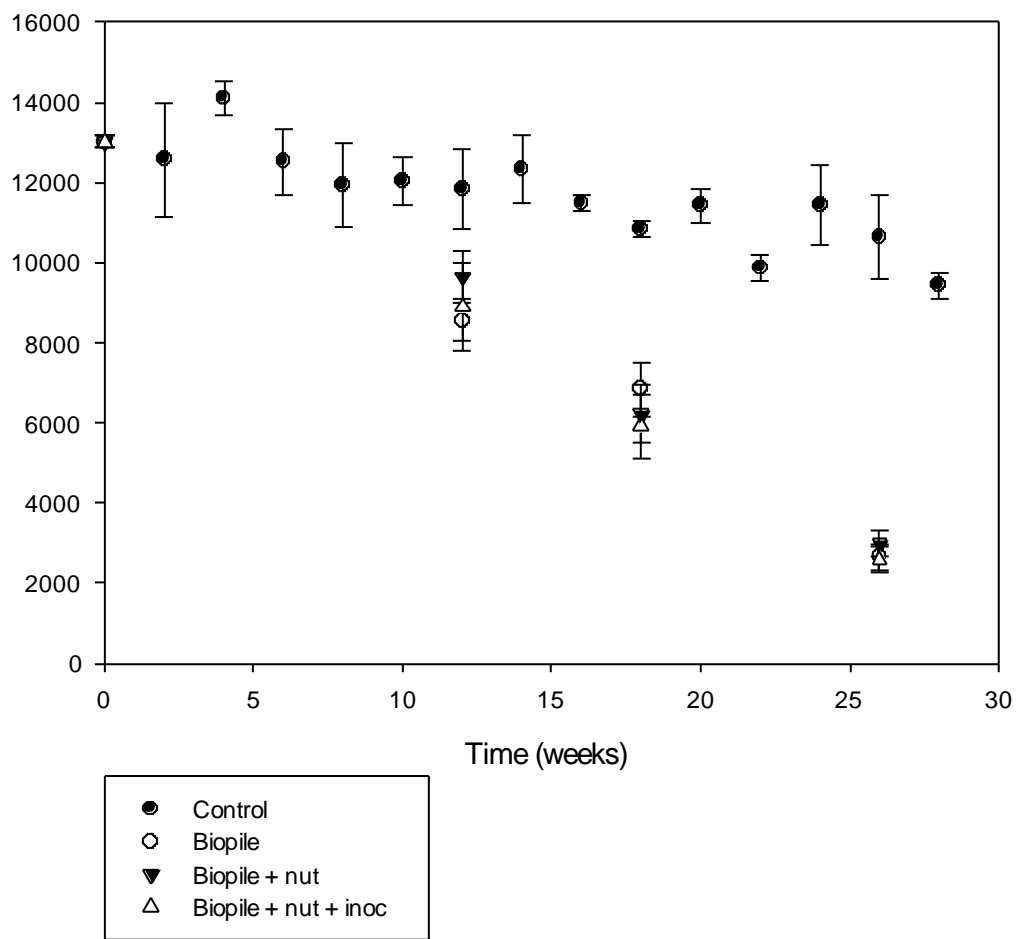11 **Fig. 4** Respiration values from the Biopile field time course remediation study
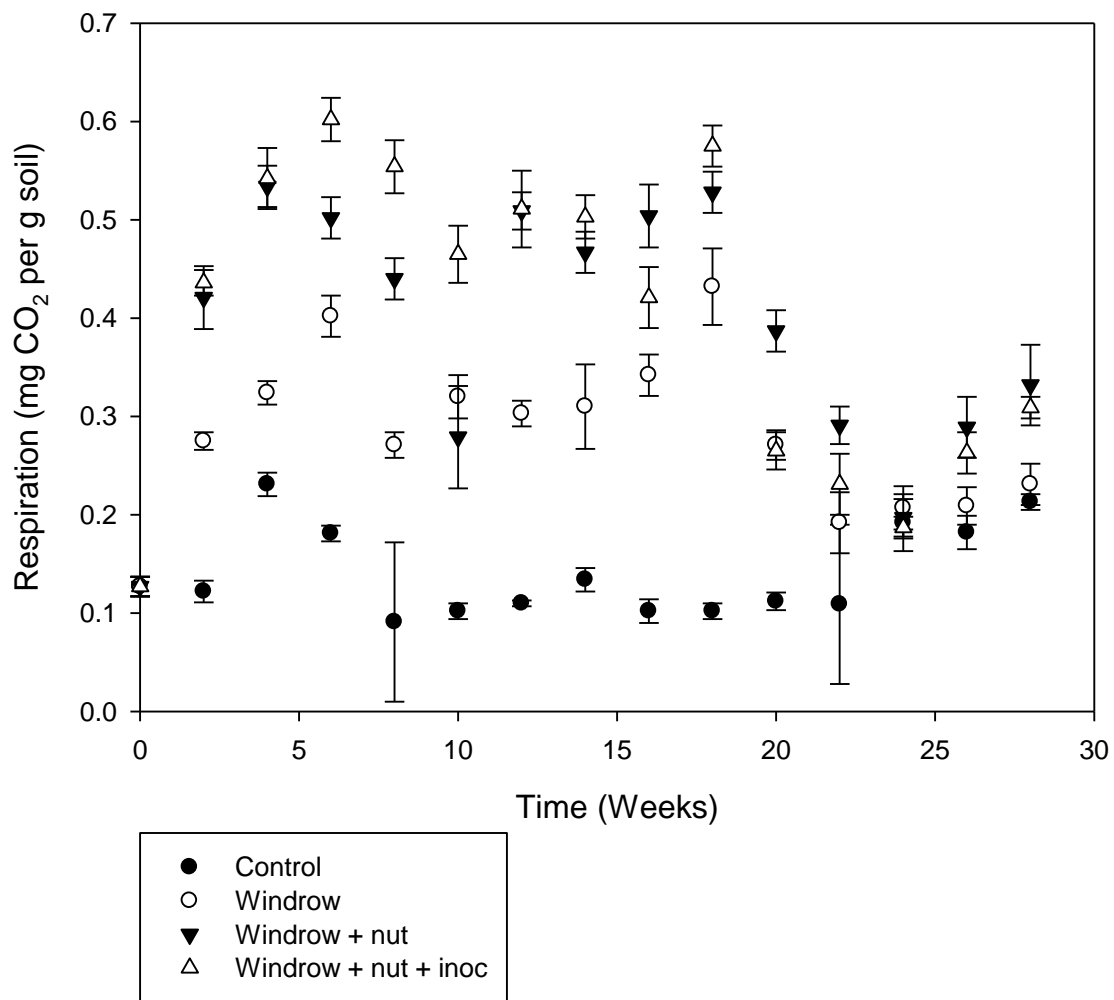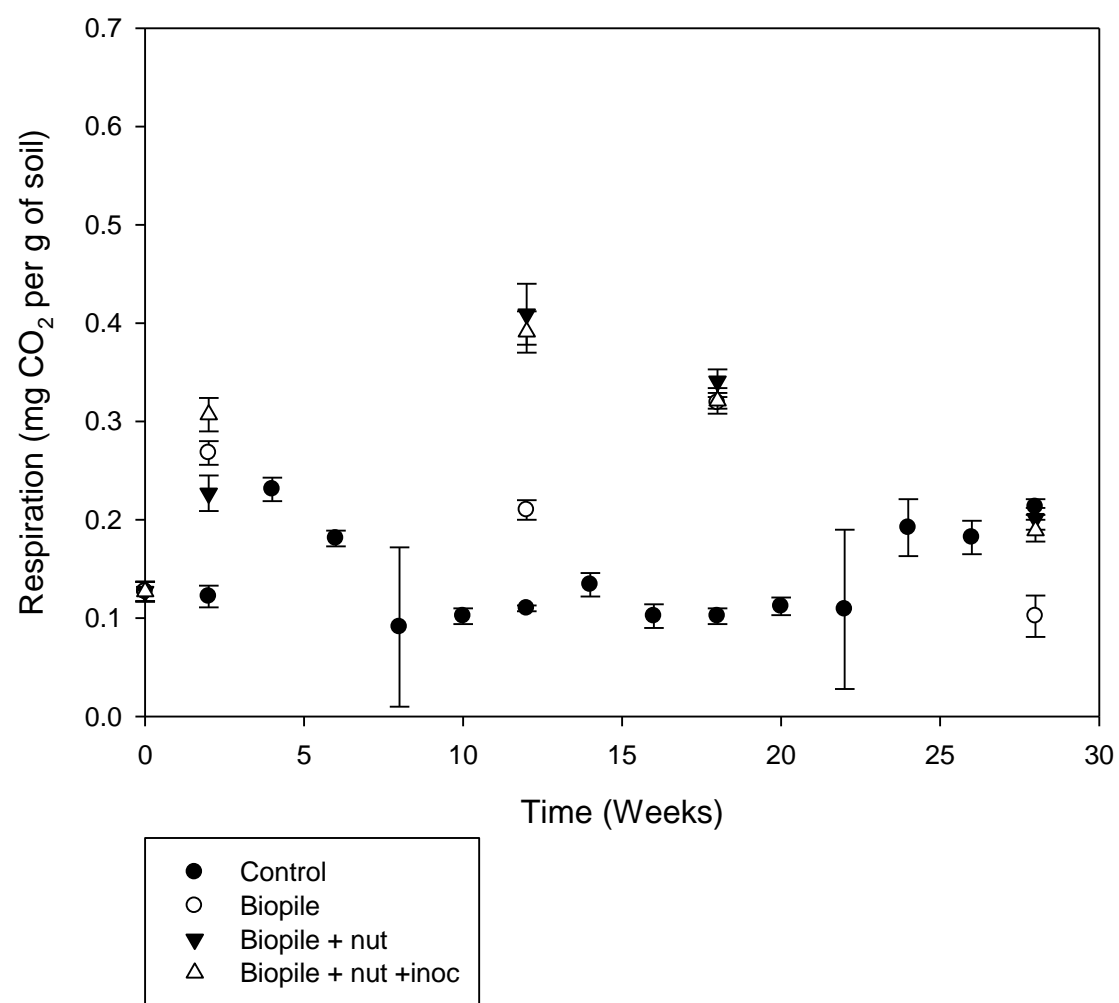12

13

1
2
3
4 **Figure 1**

1
2
3
4 **Figure 2**
5
6

Figure 3

1
2
3
4
5
6

1
2
3
4　**Figure 4**