



# Variation in stable carbon isotope fractionation during aerobic degradation of phenol and benzoate by contaminant degrading bacteria

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## Abstract

Variation in the natural abundance stable carbon isotope composition of respired CO<sub>2</sub> and biomass has been measured for two types of aerobic bacteria found in contaminated land sites. *Pseudomonas putida* strain NCIMB 10015 was cultured on phenol and benzoate and *Rhodococcus* sp. I<sub>1</sub> was cultured on phenol. Results indicate that aerobic isotope fractionations of differing magnitudes occur during aerobic biodegradation of these substrates with an isotopic depletion in the CO<sub>2</sub> ( $\Delta^{13}\text{C}_{\text{phenol-CO}_2}$ ) as much as 3.7‰ and 5.6‰ for *Pseudomonas putida* and *Rhodococcus* sp. I<sub>1</sub> respectively. This observation has significant implications for the use of a stable isotope mass balance approach in monitoring degradation processes that rely on indigenous bacterial populations. The effects of the metabolic pathway utilised in degradation and inter-species variation on the magnitude of isotope fractionation are discussed. Possible explanations for the observed isotope fractionation include differences in the metabolic pathways utilised by the organisms and differences in specific growth rates and physiology. © 1999 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

Monitoring natural attenuation (intrinsic bioremediation) and enhanced in situ bioremediation are two options for managing risk while reducing contaminants in soils and groundwater (Eganhouse et al. 1994; Aggarwal et al., 1997). These risk management techniques are potentially cost-effective, can show efficient natural mineralisation of the target contaminant, and indeed may be the only realistic option in cases when low levels of contamination occur or very large areas are involved (Jackson et al., 1996). To assess the efficiency of natural attenuation a monitoring program is

essential to ensure that; (1) changes in concentration of contaminant are due to biotic degradation reactions and not abiotic processes such as adsorption, dilution or volatilisation (Chang et al., 1987; Lovley et al., 1989; Bennett et al., 1992; Cozzarelli et al., 1994); (2) there is an accurate mass balance of contaminant flux (Baedecker and Cozzarelli 1991; Baedecker et al., 1993); (3) the remediation will occur within an effective time-scale (Bragg et al., 1994). It is important to understand biotransformation processes that take place during mineralisation of contaminants (Lovley and Lonergan, 1990; Landmeyer et al., 1996) as the design and monitoring of engineered in situ bioremediation schemes may then be optimised (Whittaker et al., 1995).

A promising technique for the monitoring of natural attenuation is to study variations in naturally occur-

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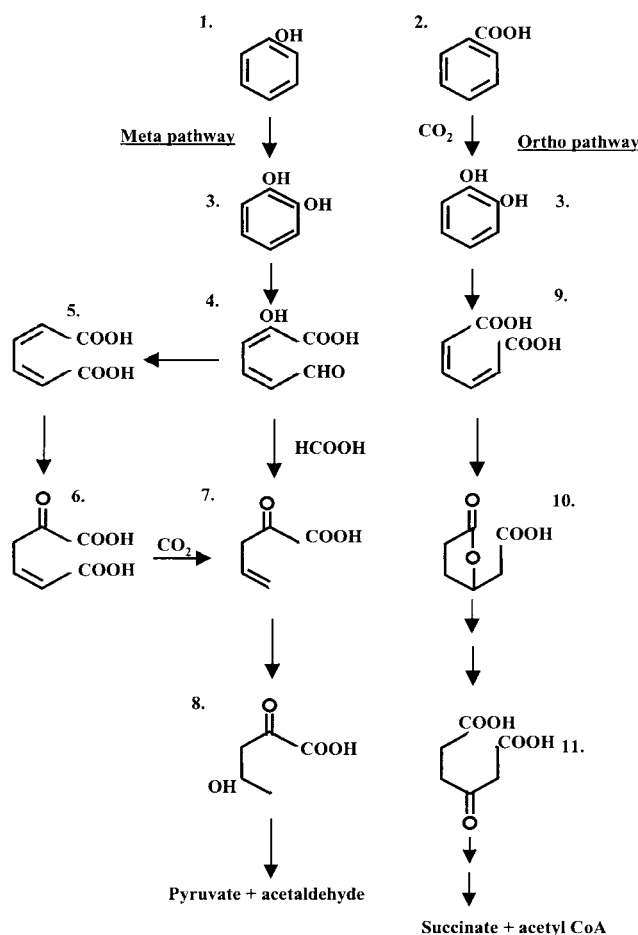


Fig. 1. The metabolism of phenol and benzoate by *Pseudomonas putida* NC1B 10015 (after Murray and Williams, 1974).

ring stable isotopic ratios of elements involved in the bioremediation process (Baedecker et al., 1988; Suchomel et al., 1990; Revesz et al., 1995). This technique can potentially be used to show not only if biological degradation is occurring but also the metabolic pathways involved and the rate of biodegradation (Aggarwal and Hinchee, 1991). As a result of the differences in mass of the isotopes (e.g.  $^{13}\text{C}$  and  $^{12}\text{C}$ ) biological and kinetic processes (e.g. enzyme catalysed reactions, diffusion, evaporation) can lead to isotopic fractionation. The result is almost a 100‰ (‰-per mil or parts per thousand) natural range in the stable isotopic ratio of carbon ( $\delta^{13}\text{C}$ ) (Hoefs, 1987). Thus, determination of the  $\delta^{13}\text{C}$  for organic compounds at a contaminated land or groundwater site may be indicative of the source of carbon (i.e. natural or anthropogenic contaminant) and also of the biogeochemical processes taking place.

$\text{CO}_2$  is produced in the soil by respiration of microorganisms and plant roots, and by abiotic chemical

oxidation of soil organic matter or organic contaminants. The concentration and isotopic composition of the  $\text{CO}_2$  and total dissolved inorganic carbon (TDIC) have been used as an indicator of contaminant degradation in soil and groundwater (Suchomel et al., 1990; Hinchee et al., 1991; Revesz et al., 1996). A stable isotope mass-balance approach can be employed to estimate the proportion of carbon in a system for soil or groundwater (Eq. 1).

$$[\text{CO}_2]_{\text{HC}} \times (\delta^{13}\text{C}_{\text{HC}}) + (([\text{CO}_2]_{\text{total}} - [\text{CO}_2]_{\text{HC}}) \times (\delta^{13}\text{C}_{\text{initial}})) = \delta^{13}\text{C}_{\text{total}} \times [\text{CO}_2]_{\text{total}} \quad (1)$$

Where  $[\text{CO}_2]_{\text{HC}}$  is the amount of  $\text{CO}_2$  arising from hydrocarbon degradation,  $[\text{CO}_2]_{\text{total}}$  is the total amount of  $\text{CO}_2$  in the system,  $\delta^{13}\text{C}_{\text{initial}}$  is the carbon isotope composition of background carbon dioxide in the system,  $\delta^{13}\text{C}_{\text{total}}$  is the carbon isotope composition of the  $\text{CO}_2$  at each measurement point; and  $\delta^{13}\text{C}_{\text{HC}}$  is the

carbon isotope composition of the hydrocarbon contaminant (note: substitution of TDIC for CO<sub>2</sub> when describing aqueous phase carbon balance; geochemical modelling of carbon sources is also highly recommended) (Baehr and Hult, 1987; Chang et al., 1990; Kalin, 1995; Essaid et al., 1995; Kalin et al., 1996).

This can be rearranged to calculate the quantity of CO<sub>2</sub> derived from hydrocarbon contaminant (HC) degradation (Eq. 2):

$$[\text{CO}_2]_{\text{HC}} = [\text{CO}_2]_{\text{total}} \times \frac{\delta^{13}\text{C}_{\text{total}} - \delta^{13}\text{C}_{\text{initial}}}{\delta^{13}\text{C}_{\text{HC}} - \delta^{13}\text{C}_{\text{initial}}} \quad (2)$$

However, the underlying assumption for applying Eq. (1) is the conservative nature of  $\delta^{13}\text{C}$  in the system (i.e. no isotope fractionation) during biodegradation (Stahl, 1980).

There has been increasing evidence that the carbon isotope signature is not conserved during biogeochemical degradation of contaminants (Dempster et al., 1997). The objective of this study was to determine isotopic fractionation during aerobic microbial production of CO<sub>2</sub> in order to correct the  $\delta^{13}\text{C}_{\text{HC}}$  value in Eq. 1 and allow a more accurate determination of the concentration of carbon dioxide in the soil zone resulting from biodegradation of a hydrocarbon contaminant. Once the total mass of CO<sub>2</sub> produced by natural and contaminant degradation processes is known, a mass-balance estimate of the flux of CO<sub>2</sub> derived from contaminant degradation in the system can be made. Finally, during biogeochemical modelling, this stable isotope data can be used to give accurate estimate of the rate of degradation and a prediction made of the time necessary to achieve a given degree of bioremediation (Kalin et al., 1996). In this paper we present results of laboratory studies that show significant carbon isotope fractionation during aerobic degradation of organic contaminants. The extent of the fractionation appears to vary depending on the bacterial species involved and the degradation pathway utilised.

The main micro-organism employed in this work, *Pseudomonas putida*, was first described in detail by Dagley and Gibson (1965). *P. putida* NCIB strain 10015 metabolises phenol by the *meta* (or  $\alpha$ -keto-acid) pathway (Dagley and Gibson, 1965; Sala-Trepat et al., 1972; Bayley and Dagley, 1969) and metabolises benzoate by the *ortho* (or  $\beta$ -ketodipate) pathway (Feist and Hegeman, 1969) (Fig. 1). The *ortho* and *meta* degradation pathways are quite distinct in that the former includes an initial decarboxylation reaction prior to the formation of catechol. The *meta* pathway has a decarboxylation step further 'downstream' in the degradation pathway. In this reaction decarboxylase enzyme, 4-oxalocrotonate decarboxylase, converts 4-oxalocrotonate to 2-oxo-4-pentanoate. During the utilisation of phenol by *P. putida*, all the metabolic inter-

mediates enter the TCA cycle as acetyl CoA after decarboxylation of pyruvate. In contrast, during the utilisation of benzoate by *P. putida*, the metabolic intermediates enter the TCA cycle as a 4-carbon intermediate (succinate) and a 2-carbon fragment (acetyl-CoA). Stable isotope effects occurring during metabolism may therefore vary depending on the substrate. In this work we studied the carbon isotope fractionation between CO<sub>2</sub> and the substrate, and CO<sub>2</sub> and the biomass produced by the organism, when cultured on phenol and benzoate. A carbon mass balance was calculated for *P. putida* cultured on phenol. We also report CO<sub>2</sub> production, O<sub>2</sub> and substrate utilisation during growth. In further work a bacterial species isolated from a phenol and cresol contaminated land site in Ireland, *Rhodococcus* Sp. I<sub>1</sub> (Kulakov et al., 1998) was cultured on phenol, and the isotope fractionation between CO<sub>2</sub> and the substrate, and CO<sub>2</sub> and the biomass produced was compared with results for *P. putida*.

## 2. Experimental methods

### 2.1. Culture of organisms

*Pseudomonas putida* strain NCIMB 10015 (Dagley et al., 1960) was obtained from the National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, United Kingdom. *Rhodococcus* Sp. I<sub>1</sub> (Kulakov et al., 1998) was obtained from the QUESTOR Centre at the Queen's University of Belfast, Belfast, N. Ireland. *Rhodococcus* and *Pseudomonad* species are known to degrade a variety of hydrocarbons that can have a negative effect on the environment. Therefore, we cultured these species on a contaminant of environmental concern, phenol, and on an analogous compound, benzoate. Bacteria were cultured under sterile conditions in sealed 500 ml conical flasks at 30°C in 100 ml of a standard minimal salts growth media buffered at pH 7.0 containing the following constituents (gl<sup>-1</sup>): Na<sub>2</sub>HPO<sub>4</sub>, 6.0; KH<sub>2</sub>PO<sub>4</sub>, 3.0; NaCl, 0.5; NH<sub>4</sub>Cl, 1.0; MgSO<sub>4</sub>, 0.06; and CaCl<sub>2</sub>, 0.003. The cultures were inoculated with a bacterial suspension (5 ml) from a culture grown on the medium and phenol or benzoate, and sampled midway through the exponential phase. Growth was monitored by measuring absorbance at 540 nm using a Beckman Du640B UV-VIS spectrometer. Cells were harvested by centrifugation at 10,000 g, washed in 0.05 M phosphate buffer (pH 7.5), re-suspended in Pyrex combustion tubes and dried at 80°C to a constant weight. For each experiment, a fresh bacterial sample was taken from the same culture that was stored at -70°C and mixed with glycerol to assure no changes in the bacterial inoculum during the duration of the experiment. The concentrations of the

Table 1

Mass balance carbon during the culture of *Pseudomonas putida* on phenol based on carbon isotopes and on measured carbon (note: low values after 11 h reflect an increase in dissolved metabolites evident by heavy coloration of liquid)

Time (h)	mmole C (phenol)	mmole C (biomass)	mmole C (CO <sub>2</sub> )	mmole total C	Iso. mass balance (%)	Carbon mass balance (%)
1.5	3.00	0.15	Trace	3.15	100.1	100.0
3.0	2.94	0.17	Trace	3.11	98.8	98.7
4.5	2.89	0.23	0.03	3.15	100.3	100.0
6.0	2.73	0.30	0.06	3.09	98.8	98.1
7.5	2.63	0.49	0.13	3.25	104.3	103.2
9.0	2.04	0.68	0.27	2.99	97.3	94.9
11.5	1.03	1.09	0.64	2.76	92.8	87.6
13.5	0.06	1.76	0.87	2.69	09.9	85.4

growth substrates, phenol or benzoate (as sodium salt), were 5 mM. Each experiment consisted of 27 × 500-ml sterile flasks each sealed with gas impermeable Mini-inert valves and containing 100 ml of standard growth media, substrate and microbial inoculum. Triplicate flasks were removed from the shaker at intervals and analysed for CO<sub>2</sub>, O<sub>2</sub> and N<sub>2</sub> headspace concentrations, total substrate concentrations, and biomass weight. The  $\delta^{13}\text{C}$  isotopic ratios of CO<sub>2</sub> and of the biomass were also determined.

### 2.2. Substrate and oxygen utilisation and measurement of metabolic by-products

For determination of O<sub>2</sub> utilisation and CO<sub>2</sub> production, a sample (1 ml) was withdrawn from the flask headspace using a gas tight syringe and injected into a Perkin Elmer Sigma 3 gas chromatograph (GC) fitted with a CTR-I (Alltech) packed column (6 ft ×  $\frac{1}{4}$  in OD, SS) and equipped with a thermal conductivity detector (TCD). The temperature was maintained at 56°C for 5 min. Helium (30 ml min<sup>-1</sup>) was used as a carrier gas. Ambient air (20.9% O<sub>2</sub>, 79.9% N<sub>2</sub> and 0.04% CO<sub>2</sub>) and a synthetic gas mixture (varying O<sub>2</sub>, N<sub>2</sub>, CO<sub>2</sub> concentrations) were used as known standards for calibration.

In substrate utilisation studies, a sample (1 ml) of culture was obtained by inverting the flask and aseptically withdrawing the medium through the opened mini-inert valve with a disposable sterile syringe. Culture medium (150  $\mu\text{l}$ ) was added to methanol (1.35 ml) in a 1.5 ml Eppendorf tube and then ultra centrifuged (10,000 *g*) for 5 min. An aliquot (1 ml) of supernatant was withdrawn and phenol or benzoate concentration determined using a Beckman Gold HPLC system equipped with a UV detector. The HPLC program was as follows; 100% water for 5 min then a 20-min gradient to 100% CH<sub>3</sub>OH and a cleaning phase of 5 min 100% CH<sub>3</sub>OH. For determination of benzoate, the aqueous phase was acidified to pH 3

using conc. H<sub>2</sub>SO<sub>4</sub>. The substrate eluting from the column was quantified by measuring absorbance at 272 nm using standard solutions of sodium benzoate and phenol for calibration.

### 2.3. Stable carbon isotope analysis

Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS) has been used in contaminant degradation studies (Dempster et al., 1997) for analysis of trace amounts of contaminants. However, for this research it was decided to use the more accurate dual-inlet IRMS method so that any small isotope effects could be determined with greater confidence. The controlled nature of the experiments allowed each batch experiment to be undertaken in volumes sufficient for the method. Isotopic analysis of CO<sub>2</sub> in the headspace was conducted after standard vacuum line separation of CO<sub>2</sub>. The CO<sub>2</sub> was sampled through the septum of the valve on each flask. Biomass samples were dried to a constant weight in Pyrex combustion tubes, flame sealed after adding CuO (2 g) and silver wire (50 mg) and then combusted for 16 h at 550°C. The sealed tubes were opened on the vacuum line and the CO<sub>2</sub> cryogenically isolated. The phenol and sodium benzoate used as growth substrates were also combusted to CO<sub>2</sub> using this technique. The  $\delta^{13}\text{C}$  of the CO<sub>2</sub> samples was measured using a Micromass Prism III IRMS and reported with respect to PDB (Craig, 1957). The CO<sub>2</sub> from internal laboratory standards, combusted C<sub>3</sub> cellulose (Rozanski et al., 1992), was analysed every six samples and show a long-term precision during this study of better than  $\pm 0.1\%$ . It should be noted that because each flask contains a separate inoculum, the variation between replicates for  $\delta^{13}\text{C}$  analysis of CO<sub>2</sub> and biomass at each time step is due to biological variability and not the internal precision of the instrument. Results reported in this paper are the mean of, at least, triplicates of individual flasks, and the standard deviations have been reported.

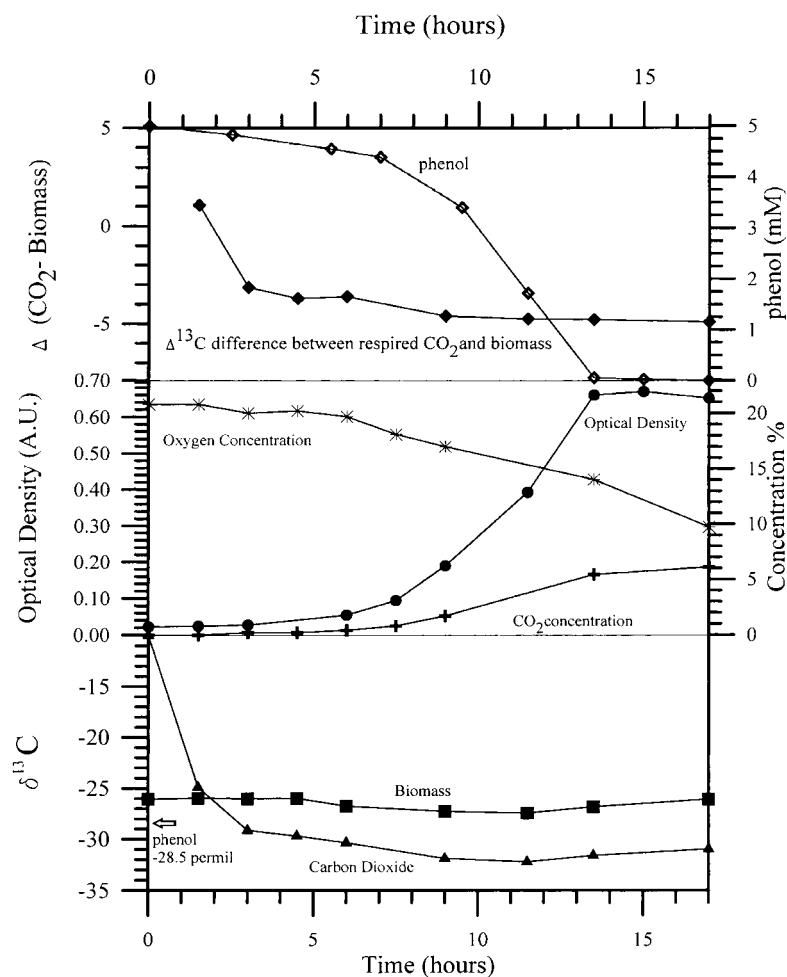


Fig. 2. The carbon isotope composition of CO<sub>2</sub> and biomass during degradation of phenol by *Pseudomonas putida*.

#### 2.4. Mass balance calculations

The results for mass balance calculations of carbon and carbon isotopes for *Pseudomonas putida* are shown in Table 1. The experiments presented were conducted under closed conditions thereby limiting the carbon sources to the substrate and trace amounts of residual CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> and inoculum biomass. The minimal salts solution has sufficient salinity to greatly minimise dissolved CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (<2% total carbon). The initial mass of the microbes, substrate and intermediates added in the inoculum is approximately 5% of the total carbon.

The mole fraction of total carbon represented by the cell biomass was calculated by using CHN ratios of *Pseudomonas* biomass reported in the literature (Goldberg et al., 1976) and determining the mass of carbon at the end of the exponential growth phase.

The mole fraction of carbon represented as CO<sub>2</sub> in the headspace was calculated by using the ideal gas equation (Eq. 3),

$$PV = nRT \quad (3)$$

where  $P$  is the pressure in the headspace, which for these experiments has been approximated as atmospheric pressure,  $V$  is the volume % CO<sub>2</sub> in the microcosm headspace,  $n$  is the total moles of carbon,  $R$  is the ideal gas constant and  $T$  is the temperature of the system. This equation can obviously be rearranged to solve for the molar quantity of carbon in the headspace,  $n$ .

The mole fraction of carbon remaining in solution as phenol was calculated from concentrations data used for determining substrate utilisation. The stable carbon isotopic balance was calculated for the bio-

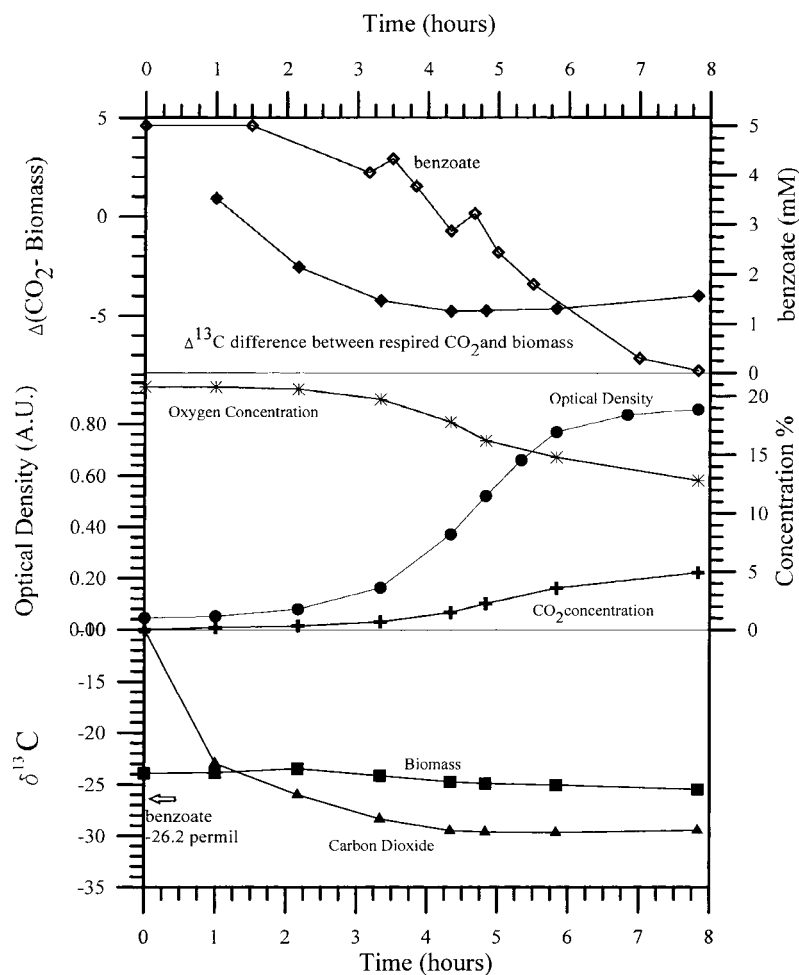


Fig. 3. The carbon isotope composition of CO<sub>2</sub> and biomass during degradation of benzoate by *Pseudomonas putida*.

mass, CO<sub>2</sub> and phenol. These calculations show that 85–100% of carbon is accounted for as CO<sub>2</sub>, phenol or biomass and the amount of carbon dissolved as CO<sub>2</sub> in the media and as dissolved metabolic intermediates is insignificant.

### 3. Results and discussion

#### 3.1. Degradation of phenol by *Pseudomonas putida*

The carbon isotopic composition of CO<sub>2</sub> and biomass at various stages during degradation of phenol by *P. putida* are shown in Fig. 2 together with the growth curve, O<sub>2</sub> and phenol utilisation and CO<sub>2</sub> production. The phenol substrate initially possessed a  $\delta^{13}\text{C}$  of  $-28.5\text{‰} \pm 0.1\text{‰}$ . At time = 0 the  $\delta^{13}\text{C}$  of CO<sub>2</sub> in the culture flask was  $-10\text{‰}$  (atmospheric back-

ground CO<sub>2</sub> value) but then falls to its most depleted value at 11.5 h of  $-32.2\text{‰} \pm 0.2\text{‰}$ . This represents a depletion of 3.7‰ relative to the initial  $\delta^{13}\text{C}$  of phenol substrate. (This was shown to be independent of the  $\delta^{13}\text{C}$  of the original phenol, as duplicate experiments demonstrated a similar  $\delta^{13}\text{C}$  CO<sub>2</sub> depletion of 3.7‰ at 11.5 h for *P. putida* grown on phenol with a  $\delta^{13}\text{C}$  of  $-29.9\text{‰}$ ). Near the end of the growth cycle, the  $\delta^{13}\text{C}$  of CO<sub>2</sub> stabilised to a value of  $-31\text{‰}$ , showing an average depletion of 2.5‰ relative to the growth substrate of  $-28.5\text{‰} \pm 0.1\text{‰}$ . After 9 h, the delta value ( $\Delta^{13}\text{C}$ ) between the biomass and respired CO<sub>2</sub> remains relatively constant. The  $\delta^{13}\text{C}$  of biomass is initially  $-26.0\text{‰} \pm 0.1\text{‰}$ , enriched by 2.5‰ with respect to the phenol, and became slightly more depleted at 11.5 h by 1.2‰ before stabilising at  $-26.0\text{‰} \pm 0.1\text{‰}$ . A mass balance calculation was performed (Table 1) to study the partitioning of carbon from the substrate into CO<sub>2</sub>

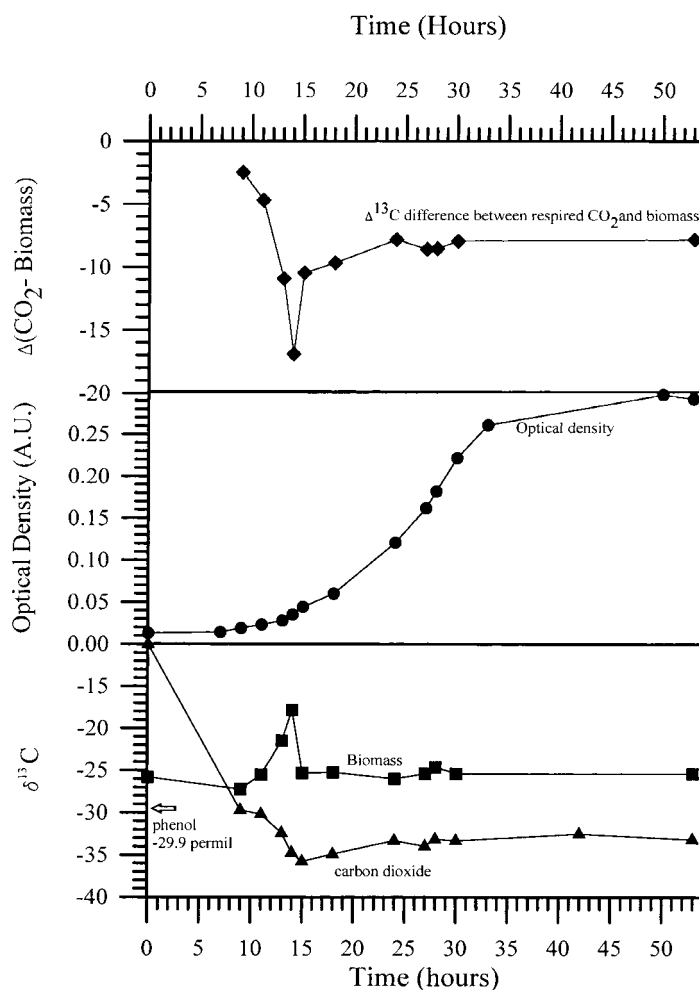


Fig. 4. The carbon isotope composition of  $\text{CO}_2$  and biomass during degradation of phenol by *Rhodococcus* Sp. I<sub>1</sub>.

and biomass. The results indicate that in the initial stages of growth (until  $t = 6$  h) the mass balance accounts for 99% of the carbon. As the growth continues the mass balance accounts for a minimum of 91% of the substrate carbon. The remaining 9% of carbon unaccounted for is likely to be intermediate compounds and perhaps some dissolved  $\text{CO}_2$  although the high ionic concentration of the media would limit bicarbonate formation.

### 3.2. Degradation of benzoate by *Pseudomonas putida*

The carbon isotope composition of  $\text{CO}_2$  and biomass at various stages during degradation of benzoate by *P. putida* are shown in Fig. 3 together with the growth curve,  $\text{O}_2$  and benzoate utilisation and  $\text{CO}_2$  production. The benzoate substrate initially possessed

a  $\delta^{13}\text{C}$  of  $-26.2\text{‰} \pm 0.1\text{‰}$ . At time=0 the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  in the culture flask was  $-10\text{‰}$  (atmospheric background  $\text{CO}_2$  value) but rapidly falls attaining a minimum value at 4.5 h,  $-29.6\text{‰} \pm 0.1\text{‰}$ . This represents a depletion of 3.4‰ relative to the initial  $\delta^{13}\text{C}$  of the benzoate substrate. Subsequently, the  $\delta^{13}\text{C}$  of  $\text{CO}_2$  produced does not significantly differ from this depletion. The biomass possesses an initial  $\delta^{13}\text{C}$  of  $-23.9\text{‰} \pm 0.2\text{‰}$  which decreases steadily becomes more depleted relative to the benzoate until the biomass production ceases when the  $\delta^{13}\text{C}$  averages  $-25.5\text{‰} \pm 0.2\text{‰}$ , an enrichment of only 0.7‰ relative to the benzoate. The variation in  $\Delta^{13}\text{C}$  between  $\text{CO}_2$  and *P. putida* biomass during growth are also shown in Fig. 3. It is interesting to note that *P. putida* utilises 20% more oxygen and produces 38% more  $\text{CO}_2$  when metabolising phenol (Fig. 2) than benzoate (Fig. 3).

Both substrates were completely mineralised during the course of the experiments, with the benzoate being utilised in approximately half the period in which phenol was utilised. It is also interesting to note that the CO<sub>2</sub> production continues at the end of the experiment. This could be due to possible utilisation of an intermediate compound in the culture medium or metabolic recycling of biomass. The unusual substrate concentration fluctuation during growth is likely to be an analytical artefact associated with intermediate metabolites in solution that adsorb UV radiation at or near 272 nm.

### 3.3. Degradation of phenol by *Rhodococcus Sp. I<sub>1</sub>*

The carbon isotopic value of CO<sub>2</sub> and biomass at various stages during phenol degradation by *R. Sp. I<sub>1</sub>* are displayed in Fig. 4 together with the growth curve. The δ<sup>13</sup>C of phenol used as growth substrate was -29.9‰ ± 0.1‰. At time = 0 the δ<sup>13</sup>C of the CO<sub>2</sub> in the culture flask was -10‰ (atmospheric background CO<sub>2</sub> value) and shows increasing depletion during initial growth attaining a maximum depletion of -35.5‰ ± 0.4‰ after 14 h before rising slightly to -33.5‰ ± 0.3‰ after 24 h. A maximum Δ<sup>13</sup>C of 5.6‰ for respired CO<sub>2</sub> relative to the phenol was recorded after 14 h and decreased to 3.6‰ after 24 h. The biomass δ<sup>13</sup>C value displayed an initial rapid enrichment to -17.5‰ at 14 h. As biomass accumulated the δ<sup>13</sup>C decreased to -25.5‰ ± 0.1‰ after 15 h. Change in the Δ<sup>13</sup>C between CO<sub>2</sub> and *R. Sp. I<sub>1</sub>* biomass with time is also shown in Fig. 4.

The results of these experiments have significance as regards the use of isotope mass-balance (Eq. 1) for monitoring biodegradation rates during natural attenuation or enhanced bioremediation schemes. As indicated in the introduction, one of the underlying assumptions for Eq. (1) is that stable carbon isotope ratios are maintained, i.e. the process is conservative, during the aerobic degradation process. The δ<sup>13</sup>C of CO<sub>2</sub> observed during aerobic degradation of aromatic organic compounds in this study with maximum depletions of 5.6, 3.7 and 3.4‰ with regard to the substrate, signifies isotopic discrimination during biodegradation (Figs. 2, 3 and 4) i.e. a non-conservative process. These results were obtained in controlled experiments (single carbon source) that were closed systems using single strains of bacteria. The results indicate that study of isotope fractionation during substrate utilisation using site-specific mixtures of microbes is needed to provide an indication of the correction (Δ or the fractionation factor α) required for application of Eq. (2) to natural attenuation processes. This produces Eq. (4).

$$[\text{CO}_2]_{\text{HC}} = [\text{CO}_2]_{\text{total}} \times \frac{\delta^{13}\text{C}_{\text{total}} - \delta^{13}\text{C}_{\text{initial}}}{\delta^{13}\text{C}_{\text{HC}^*} - \delta^{13}\text{C}_{\text{initial}}} \quad (4)$$

where: δ<sup>13</sup>C<sub>HC\*</sub> = δ<sup>13</sup>C<sub>HC</sub> + Δ<sub>HC-CO<sub>2</sub></sub> (Δ<sub>HC-CO<sub>2</sub></sub> ≈ 1000 lnα<sub>HC-CO<sub>2</sub></sub> of microbe), α is the fractionation factor between the original hydrocarbon and microbiologically respired CO<sub>2</sub> (determined experimentally). If Eq. (2) were not adjusted for the Δ<sub>HC-CO<sub>2</sub></sub>, the calculated mass of CO<sub>2</sub> from degradation contaminants given the results found here (and a δ<sup>13</sup>C<sub>initial</sub> = -24‰) would be overestimated by as much as 40%.

The magnitude of the isotope fractionation observed during aerobic degradation appears to be dependent on both the species and the phase of growth. This study demonstrates that there is a significant difference of 2‰ between *Pseudomonas putida* and *Rhodococcus Sp. I<sub>1</sub>* in the δ<sup>13</sup>C of the CO<sub>2</sub> observed during growth. However, toward the end of the growth phase for each species this difference was only 0.9‰. Therefore, Δ<sub>HC-CO<sub>2</sub></sub> (Eq. 4) may depend not only on the species but on the phase of growth suggesting that interpretation of δ<sup>13</sup>C<sub>CO<sub>2</sub></sub> during aerobic degradation should initially be considered site-specific and temporally variable until field-based study of different strains of bacteria can provide common, time averaged or modelled values Δ<sub>HC-CO<sub>2</sub></sub>.

*Pseudomonas putida* degrades benzoate with an initial decarboxylation step. Benzoate is metabolised to catechol. We therefore thought it probable that the initial magnitude of isotope fractionation (Δ<sub>HC-CO<sub>2</sub></sub>) would be dependent on the metabolic pathway of degradation as there is no such initial step with phenol as the substrate (Fig. 1). To test this hypothesis we compared the isotope effects during degradation of phenol and benzoate by *P. putida*, which, as stated previously, metabolises phenol and benzoate via the separate *meta* and *ortho* pathways respectively. Unexpectedly, the maximum isotope fractionation observed (Δ<sub>HC-CO<sub>2</sub></sub> substrate to CO<sub>2</sub>) for both pathways is similar, depletions of 3.7‰ for phenol and 3.4‰ for benzoate being recorded. However, at the completion of substrate utilisation there is a significant difference between the *meta* (phenol utilising) pathway, depletion of 2.5‰, and the *ortho* (benzoate utilising) pathway, depletion of 3.7‰. It is also interesting to note there is little change in the isotope fractionation during benzoate utilisation via the *ortho* metabolic pathway (Fig. 3). Variation in the isotope fractionation of CO<sub>2</sub> is observed at different time intervals when the *Rhodococcus* and *Pseudomonad* species are grown on phenol (Figs. 2 and 4). If a different isotope effect was observed early in the growth phase due to the decarboxylation reaction (Fig. 1, steps 6–7), this would imply that cleavage of the bond by the decarboxylase enzyme 4-oxalocrotonate decarboxylase is rate limiting (Cook, 1991). This would suggest any difference in



stable isotope effects due to metabolic pathway are related to secondary metabolic processes or effects related to entry of the metabolites into the TCA cycle, and not to those which control the initial uptake of phenol or benzoate. More research is continuing in this area to better define isotope effects resulting from different metabolic pathways.

Questions still remain about the possible reasons for the observed differences in fractionation ( $\Delta_{\text{HC-CO}_2}$ ) between the two species. One explanation may be related to the difference in growth rate between the *Rhodococcus* and *Pseudomonas* species. Complete growth of the *R. Sp. I<sub>1</sub>* did not occur until 55 h, whereas growth and utilisation of phenol and by *P. putida* was complete at about 8 h. The specific growth rates of the *P. putida* and *R. Sp. I<sub>1</sub>* strains were 0.13 and 0.05 h<sup>-1</sup> respectively. The differences in isotope fractionation measured near the beginning of the logarithmic growth phase for *P. putida* ( $\Delta_{\text{HC-CO}_2} = 3.7\%$ ) and *R. Sp. I<sub>1</sub>* ( $\Delta_{\text{HC-CO}_2} = 5.6\%$ ) and measured near the end of the logarithmic growth phase (*P. putida*  $\Delta_{\text{HC-CO}_2} = 2.5\%$  and *R. Sp. I<sub>1</sub>*  $\Delta_{\text{HC-CO}_2} = 3.6\%$ ) likely relate to a rate limiting metabolic step (or steps) found in *R. Sp. I<sub>1</sub>* that is different for *P. putida*. Little is currently known about the metabolic pathways of phenol degradation by *R. Sp. I<sub>1</sub>* and more work is needed in this area to better define the time-dependent nature of the observed stable isotope effects.

Another possible explanation for interspecies variation relates to possible fundamental differences in the cell wall structure between the two species and the mechanism of substrate uptake into the cell. The *R. Sp. I<sub>1</sub>* strain is gram positive and the *P. putida* strain is gram negative. Any differences in kinetic isotope effects associated with the uptake of the substrate would be accentuated early on in the breakdown of the contaminant and become less important with decreasing substrate availability. The complex biological mechanisms of substrate penetration through the cell wall are not well understood for the bacteria studied.

#### 4. Conclusions

Aerobic biodegradation of aerobic contaminants has been regarded as an isotopically conservative process and thus isotope fractionation has not been considered when using naturally occurring stable isotopes to quantify rates of biodegradation in soils and groundwater. Our study suggests that aerobic degradation produces CO<sub>2</sub> that is significantly lighter ( $\delta^{13}\text{C}$ ) than the growth substrate, and that the magnitude of the isotope fractionation may vary with growth rate or substrate utilisation. This fractionation may also be dependent on species and/or on the degradative metabolic pathway employed by a particular micro-organ-

ism. This implies that site-specific and time dependent/averaged studies are necessary to determine the indigenous bacterial population present and to quantify the stable isotope fractionation occurring biologically. These results would facilitate an accurate isotope mass-balance of CO<sub>2</sub> produced by biodegradation of the target contaminant.

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#### References

- Aggarwal, P.K., Fuller, M.E., Gurgas, M.M., Manning, J.F., Dillon, M.A., 1997. Use of stable oxygen and carbon isotope analysis for monitoring the pathways and rates of intrinsic and enhanced in-situ biodegradation. *Environmental Science and Technology* 31, 590–596.
- Aggarwal, P.K., Hinchee, R.E., 1991. Monitoring in-situ biodegradation of hydrocarbons by using stable carbon isotopes. *Environmental Science and Technology* 25, 1178–1180.
- Baedecker, M.J., Cozzarelli, I.M., Siegel, D.I., Bennett, P.C., 1988. Processes related to the distribution of carbon isotopes in ground water affected by crude oil. *Geological Society of America Abstracts with Programs* 20, 365.
- Baedecker, M.J., Cozzarelli, I.M., 1991. Geochemical modeling of organic degradation reactions in an aquifer contaminated with crude oil. In: Mallard, G.E., Aronson, D.A. (Eds.), *US Geological Survey Toxic Substances Hydrology Program—Proceedings of the Technical Meeting*, Monterey, California, March 11–15, 1991. *US Geological Survey Water-Resources Investigations Report* 91-4034, pp. 627–632.
- Baedecker, M.J., Cozzarelli, I.M., Siegel, D.I., Bennett, P.C., Eganhouse, R.P., 1993. Crude oil in a shallow sand and gravel aquifer. III. Biogeochemical reactions and mass balance modeling in anoxic ground water. *Applied Geochemistry* 8, 569–586.
- Bayley, R.C., Dagley, S., 1969. Oxobenzoic acids as metabolites in the bacterial degradation of catechols. *Biochemical Journal* 111, 111–121.
- Baehr, A.L., Hult, M.F., 1987. Mathematical modeling of hydrocarbon and oxygen transport coupled with microbial degradation in the unsaturated zone. In: Franks, B.J. (Ed.), *US Geological Survey Program on Toxic Waste—Ground-Water Contamination—Proceedings of the technical meeting*, Pensacola, Florida, March 23–27, 1987. *US Geological Survey Open-File Report* 87–109, pp. C27–C28.

- Bennett, P.C., Hiebert, F.K., Baedecker, M.J., 1992. Microbial influences on inorganic solute mobilization and transport in an oil contaminated aquifer. Geological Society of America Abstracts with Programs 24 (7), A128.
- Bragg, J.R., Prince, R.C., Harner, E.J., Atlas, R.M., 1994. Effectiveness of bioremediation for the Exxon-Valdez oil spill. *Nature* 368, 413–418.
- Chang, F.H., Hult, M.F., Noben, N.N., 1987. Quantitative studies of biodegradation of petroleum and some model hydrocarbons in ground water and sediment environments. In: Fairchild, D.M. (Ed.), National Conference on Ground-Water Quality and Agricultural Practices—Proceedings of Ground-Water Quality and Agricultural Practices, May 1–2, 1986. Lewis Publishers, pp. 295–318.
- Chang, F.H., Denzin, B., Wang, H., 1990. A kinetic model for microbial activity on crude oil in groundwater environments. In: Morello, J.A. (Ed.), 90th Annual Meeting of the American Society for Microbiology, Anaheim, California, May 13–17, American Society for Microbiology, p. 298.
- Cozzarelli, I.M., Baedecker, M.J., Eganhouse, R.P., Goerlitz, D.F., 1994. The geochemical evolution of low-molecular-weight organic acids derived from the degradation of petroleum contaminants in groundwater. *Geochimica et Cosmochimica Acta* 58 (2), 863–877.
- Cook, P.F. (Ed.), 1991. *Enzyme Mechanisms from Isotope Effects*, 1st ed. CRC Press, Boca Raton.
- Craig, H., 1957. Isotopic standards for carbon and oxygen and correction factors for mass spectrometric analysis of carbon dioxide. *Geochimica et Cosmochimica Acta* 44, 1903–1907.
- Dagley, S., Evans, W.C., Ribbons, D.W., 1960. New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature* 188, 560–566.
- Dagley, S., Gibson, D.T., 1965. The bacterial degradation of catechol. *Biochemical Journal* 95, 466–474.
- Dempster, H.S., Sherwood, Lollar B., Feenstra, S., 1997. Tracing organic contaminants in ground water: A new methodology using compound specific isotope analysis. *Environmental Science and Technology* 31, 3193–3197.
- Eganhouse, R.P., Baedecker, M.J., Cozzarelli, I.M., 1994. Biogeochemical processes in an aquifer contaminated by crude oil: An overview of studies at the Bemidji, Minnesota, research site. In: Symposium on Intrinsic Bioremediation of Ground Water, Denver, CO, August 30–September 1, 1994, Environmental Protection Agency, Washington, DC, pp. 111–120.
- Essaid, H.I., Bekins, B.A., Godsy, E.M., Warren, E., Baedecker, M.J., Cozzarelli, I.M., 1995. Simulation of aerobic and anaerobic biodegradation processes at a crude-oil spill site. *Water Resources Research* 31 (12), 3309–3327.
- Feist, C.F., Hegeman, G.D., 1969. Phenol and benzoate metabolism by *Pseudomonas putida*: Regulation of Tangential Pathways. *Journal of Bacteriology* 100, 869–877.
- Goldberg, I., Rock, J.S., Ben-Bassat, A., Mateles, R.I., 1976. Bacterial yields on methanol, methylamine, formaldehyde and formate. *Biotechnology and Bioengineering* XVIII, 1657–1668.
- Hoefs, J., (Ed.) 1987. *Stable Isotope Geochemistry*, 3rd ed. Springer-Verlag, Berlin, p. 434.
- Hinchee, R.E., Downey, D.C., Dupont, R.R., Aggarwal, P.K., Miller, R.N., 1991. Enhancing biodegradation of petroleum-hydrocarbons through soil venting. *Journal of Hazardous Materials* 27, 315.
- Jackson, A.W., Pardue, J.H., Araujo, R., 1996. Monitoring crude oil mineralization in salt marshes: use of stable isotopic ratios. *Environmental Science and Technology* 30, 1139–1144.
- Kalin, R.M., 1995. Basic concepts and formulations for isotope-geochemical process investigations, procedures and methodologies of geochemical modelling of groundwater systems. In: Yurtsever, Y. (Ed.), *Manual on Mathematical Models in Isotope Hydrology*, Chapter 4, IAEA TECHDOC 910, pp. 155–206.
- Kalin, R.M., Larkin, M., Harper, D., Sivakumar, S., 1996. Stable isotope fingerprinting of natural bioattenuation of contaminated land. In: SAGTA Seminar and Workshop 'Contaminated Land Research—The Way Forward' 12–14 February, ICI Runcorn.
- Kulakov, L.A., Delcroix, V.A., Larkin, M.J., Ksenzenko, V.N., Kulakova, A.N., 1998. Cloning of new *Rhodococcus* extradiol dioxygenase genes and study of their distribution in different *Rhodococcus* strains. *Microbiology* 144, 955–963.
- Landmeyer, J.E., Vroblesky, D.A., Chapelle, F.H., 1996. Stable carbon isotope evidence of biodegradation zonation in a shallow jet-fuel contaminated aquifer. *Environmental Science and Technology* 30, 1120–1128.
- Lovley, D.R., Baedecker, M.J., Lonergan, D.J., Cozzarelli, I.M., Phillips, E.P., Siegel, D.I., 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. *Nature* 339, 297–299.
- Lovley, D.R., Lonergan, D.J., 1990. Anaerobic oxidation of toluene, phenol, and *p*-Cresol by the dissimilatory iron-reducing organism, GS-15. *Applied and Environmental Microbiology* 56 (6), 1858.
- Murray, K., Williams, P.A., 1974. Role of catechol and the methycatechols as inducers of aromatic metabolism in *Pseudomonas putida*. *Journal of Bacteriology* 117, 1153–1157.
- Revesz, K., Coplen, T.B., Baedecker, M.J., Glynn, P.D., Hult, M.F., 1995. Methane production and consumption monitored by stable H and C isotope ratios at a crude oil spill site, Bemidji, Minnesota. *Applied Biochemistry* 10, 505–516.
- Revesz, K., Coplen, T.B., Baedecker, M.J., Hult, M.F., 1996. Use of carbon and hydrocarbon stable isotopes to investigate the production and fate of methane at a toxic waste site, Bemidji, Minnesota. In: Morganwalp, D.W., Aronson, D.A. (Eds.), *US Geological Survey Toxic Substances Hydrology Program—Proceedings of the technical meeting*, Colorado Springs, Colorado, September 20–24, 1993. US Geological Survey Water-Resources Investigations Report.
- Rozanski, K., Stichler, W., Gonfiantini, R., Scott, E.M., Beukens, R.P., Kromer, B., Van Der Plicht, J., 1992. The IAEA 14C Intercomparison Exercise 1990. *Radiocarbon* 34 (3), 506–519.
- Sala-Trepat, J.M., Murray, J.M., Williams, P.A., 1972. The

- metabolic divergence in the meta cleavage of catechols by *Pseudomonas putida*. NCIMB 10015. *European Journal of Biochemistry* 28, 347–356.
- Stahl, W.J., 1980. Compositional changes and  $^{13}\text{C}/^{12}\text{C}$  fractionations during the degradation of hydrocarbons by bacteria. *Geochimica et Cosmochimica Acta* 44, 1903–1907.
- Suchomel, K.H., Kremer, D.K., Long, A., 1990. Production and transport of carbon dioxide in a contaminated vadose zone: A stable and radioactive carbon isotope study. *Environmental Science and Technology* 24, 1824–1831.
- Whittaker, M., Pollard, S.J.T., Fallick, T.E., 1995. Characterisation of refractory wastes at heavy oil-contaminated sites: A review of conventional and novel analytical methods. *Environmental Technology* 16, 1009–1033.