Journal of Thermal Analysis and Calorimetry, Vol. 57 (1999) 303-312

CALORIMETRIC STUDY OF THE BIOREMEDIATION OF A POLLUTED SOIL

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Abstract

The calorimetric cells of a Setaram BT 2.15 flux calorimeter have been modified, in order to measure the heat production associated with microbial growth, with a continuous flow of gas and liquid through the sample. Good conditions for the growth of the microorganisms present in a polluted soil were determined and the possibility of the bioremediation examined. It was shown that the biodegradation of hydrocarbons adsorbed for a long time in the soil is a very slow process difficult to study with calorimetry. On the contrary, sodium succinate and different C_{14} hydrocarbons were easily biodegradated, producing a large quantity of heat.

Keywords: bioremediation, calorimetry, hydrocarbons biodegradation, microbial growth

Introduction

Bioremediation is the use of microorganisms to degrade environmental pollution; the extent of bioremediation is critically dependent upon the presence of degrading microorganisms, the environmental conditions, the types of contaminants and the bioavailability of the contaminants to biodegradative bacteria.

Bacteria capable of utilizing hydrocarbons as sources for energy are well documented [1–3], and *in situ* bioremediation is a very promising concept emerging as one of several technologies for restoring contaminated sites [4–6].

Decontamination can be performed either with indigenous microorganisms present on the site or with inoculated microbial consortia. In both cases the technique is based on the addition of electron acceptor (O_2, NO_3^-, etc.) and nutrients (NH_4^+, PO_4^{3-}, etc.) to increase the activity of the microorganisms.

Our laboratory has been involved in the evaluation of the possibility of the bioremediation of a polluted site; it consists of 40 000 m^3 of soil contaminated with hydrocarbons (aliphatics and polyaromatics) and heavy metals. This evaluation was made on columns containing 10 kg of soil and feeded with air and a nutrients solution [7]. The efficiency of the process was followed by chemical analysis of the soil and of the lixiviate. In parallel with this 'macro-scale' test, we have developed a calorimetric 'mini-scale' test on 5 g of soil which gives much faster response after the modifications of the experimental conditions.

The high complexity of biological systems, which are open systems and exchange energy and matter with their surrounding and are far from equilibrium, re-

1418–2874/99/ $ 5.00

Akadémiai Kiadó, Budapest
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quires the application of classical and irreversible thermodynamics [8–10]. However measurements by calorimetry of the sum of the net heat change of all the reactions that occur during cellular growth is a widely used technique; it has the advantage to be independent of the complexity of the reaction pathway, taking into account only the initial and final energy state of the system and supply an energy value proportional to the microbial growth [11] .

Among others, the following phenomena have been studied with calorimetry: regulation of the metabolism with energy reserves [12], influence of the temperature [13] or the pH [14–15] on bacterial growth, limitation of bacterial growth by nutrients or electron acceptor [16], toxicity of different chemicals (heavy metals, antibiotics [17], organic compounds [18]). Modifications of the calorimetric cells allowed us to reproduce in a BT 2.15 Setaram calorimeter the experimental conditions used in the 'macro-scale' experiments on columns and to test the influence of several parameters on the bioremediation.

Experimental

Soil

A sample of 2 kg of soil was collected on the polluted site, sieved (≤2 mm) and stored in a closed vessel at 4°C. The chemical and microbiological analysis of the sample were performed as described in details elsewhere [7]; heavy metals were extracted with nitric acid and analysed by ICP-MS.

Hydrocarbons were extracted with dichloroethane and analysed qualitatively with GC-MS and quantitatively with FTIR; it consist of a small quantity of aliphatics (C_{14}–C_{18}), and mainly phthalic esters and polycyclics (triterpenes). Microorganisms were isolated, developed on different culture media after successive dilutions, counted and the following were identified: *Pseudomonas* (*fluorescens, Stutzeri, vesicularis, aureofasciens* and *aeruginosa*), *Corynebacterium aquaticum, Oerskovia sp., Bacillus* (*Iacterosporus* and *subtilis*) *Clostridium* (*perfringens* and *butyricum*), *Rhodotorula* (*rubra* and *minuta*), *Candida (famata* and *guillermondii*), *Cephalosporium sp., Aureobasidium pullulans.*

Table 1 shows the main results of these analysis.

Table 1 Heavy metals, hydrocarbons (HC) and microorganisms (MO) content of the soil

Cu	Cd	Pb	Ni	Cr	Zn	HC	MO
ppm	ppm	ppm	ppm	ppm	ppm	ppm	ufc* g^{-1}
124	15	1140	53	32	3700	5800	$2 \cdot 10^6$

*ufc: units forming colonies

Calorimetric cell

We have developed a flow cell usable in a Setaram BT 2.15 flux calorimeter (Fig. l). The hermetically closed cell made of stainless steel (diam.: 1.5 cm; height:

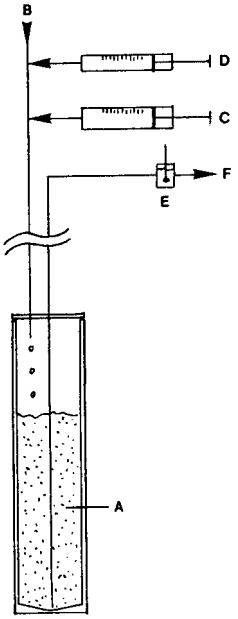

Fig. 1 Schematic representation of the calorimetric cell. A – sample (5 g of soil); B – inlet oxygen (0.5 ml min^{-1}); C – nutrients solution (1 ml day^{-1}); D – carbon source; E – pH measurement; F – outlet

8 cm) is fitted with an inlet and an outlet stainless steel tube (diam. 2.0/1.8 mm). Oxygen, nutrients solution and eventually a carbon source enter the cells, percolate through the soil sample and leave the cells continuously.

Experimental conditions

Five grams of soil and 1.5 ml of water were introduced in the reference and sample cells for all experiments; the water insoluble carbon sources were mixed with the soil of the sample cell, when the water soluble carbon sources were either introduced continuously with a syringe, or mixed with the soil. The two cells were closed, introduced in the calorimeter stabilised at 30°C, and the oxygen and the different liquids immediately percolated through both cells.

In all experiments an oxygen flow rate of 0.5 ml min^{-1} (Hi-Tek flow meter) was supplied to both cells; oxygen was humidified by bubbling through water at 33°C before entering the cells. Nutrients solution (1 g l^{-1} NH_4NO_3 and 0.5 g l^{-1} K_2HPO_4 in water), or sometimes pure water, was introduced in the cells by means of motor driven syringes at a flow rate of 1 ml day^{-1}. The pH of the solutions leaving the cells was measured with a micro electrode (Schott 10162).

The sensitivity of the calorimeter was 200 mV mW^{-1} throughout the experiments. Because of the necessary thermal equilibration of the calorimeter, the measurements were started 30 min after the introduction of the cells; the heat values were stored every 30 min (Wavetek 50 A) and analysed with Excel software.

Results and discussion

Preliminary experiments

Sodium succinate ($C_4H_4O_4Na_2$), an intermediate of the cycle of Krebs, is a specific carbon source for the aerobic bacteria in general. Preliminary experiments were performed in order to determine good conditions for observing the growth of the bacteria present in the soil with sodium succinate as carbon source. The following parameters were tested: the composition and flow rate of the nutrients solution, the nature and flow rate of the electron acceptor, the flow rate of the succinate. The heat production observed when the reference cell is feeded with pure water (1 ml day^{-1}) and the sample cell with different solutions is schematically represented on Fig. 2; oxygen was supplied to both cells at a flow rate of 0.5 ml min^{-1}. This figure shows that 40 mg day^{-1} of sodium succinate alone (curve a) produce only a small heat evolution due to the lack of nutrients in the medium.

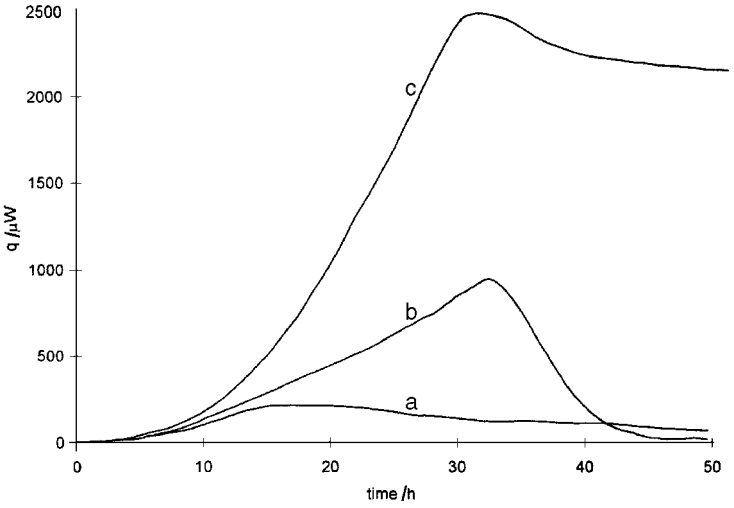

Fig. 2 Heat production as a function of time. Reference cell: 1 ml day^{-1} of pure water. Sample cell: (curve a) 40 mg day^{-1} of sodium succinate; (curve b) 1 ml day^{-1} of nutrients solution; (curve c) 1 ml day^{-1} of nutrients solution and 40 mg day^{-1} of sodium succinate

Nutrients alone (1 mg day^{-1} NH_4NO_3; 0.5 mg day^{-1} K_2HPO_4; curve b) produce a heat evolution during a short time, due to the presence in the soil of a small quantity of a carbon source easily available for the microbial growth.

The supply of nutrients and succinate (curve c) produces a heat evolution of more than 2000 μW which continue as long as the sample cell is supplied with oxygen, nutrients and succinate.

Figure 3 shows the influence of the quantity of succinate on the heat production. An increase from 40 to 80 mg day^{-1} (point b) induces an increase of the heat, when 160 mg day^{-1} (point c) reduces the heat production; this phenomena is probably due

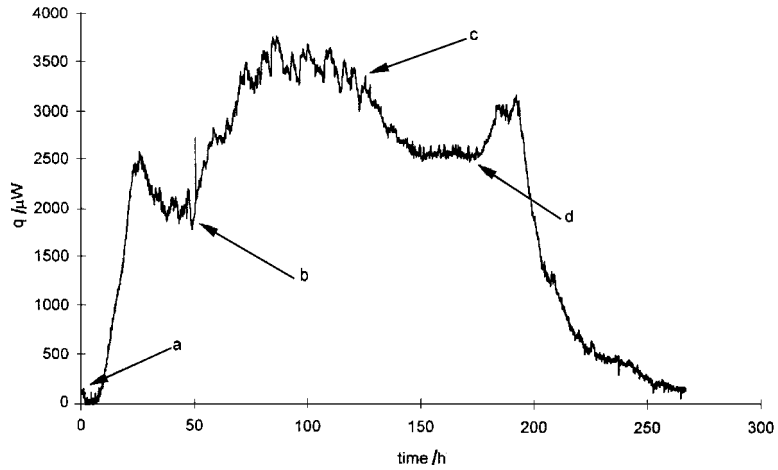

Fig. 3 Heat production as a function of time; influence of the quantity of sodium succinate. Reference cell: 1 ml day^{-1} of nutrients solution. Sample cell: 1 ml day^{-1} of nutrients solution and a – 40 mg day^{-1}; b – 80 mg day^{-1}; c – 160 mg day^{-1}; d – 0 mg day^{-1} of sodium succinate

to a toxic effect of the succinate on the microbial metabolism. At the point d the nutrients solution alone is supplied to the sample cell; after an increase of the heat due to the decrease of the toxicity, the heat production slowly declines with the diminution of the carbon source.

The main limiting factor of the *in situ* bioremediation is generally due to the difficulty to ensure a sufficient availability of oxygen as electron acceptor everywhere

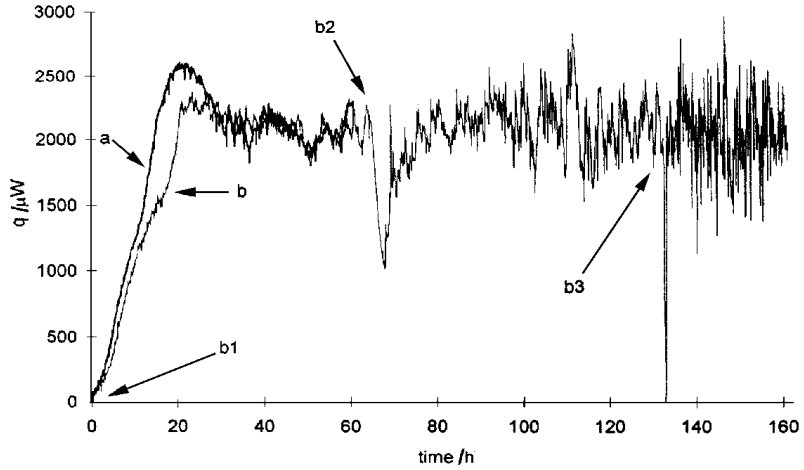

Fig. 4 Heat production as a function of time; influence of the addition of hydrogen peroxide. Reference cell: 1 ml day^{-1} of nutrients solution. Sample cell: 1 ml day^{-1} of nutrients solution and curve a – 0 mg day^{-1} of H_2O_2; curve b1: bl – 0.12 mg day^{-1}; b2 – 0.5 mg day^{-1}; b3 – 1.2 mg day^{-1} of H_2O_2

in the polluted site. Hydrogen peroxide, which is soluble in water and decomposes spontaneously according to the Eq. (1)

$$2H_2O_2 \rightarrow O_2+2H_2O \quad (1)$$

has been proposed as a source of oxygen for in situ bioremediation treatment [19–21].

Figure 4 shows the effect of the supply of an increasing quantity of H_2O_2 (0.5, 1.9 and 4.8 mg ml^{-1}) in the sample cell at a flow rate of 0.24 ml day^{-1}. The same heat production is obtained with and without addition of H_2O_2 to 0.5 ml min^{-1} O_2 (curves a and b between 0 and 60 h). An increase of the quantity of H_2O_2 (points b2 and b3) is not favorable; on the contrary the heat production associated with the microbial growth is perturbated by the decomposition of H_2O_2. No toxicity of H_2O_2 [22] was observed within the concentrations used.

From these preliminary experiments we have selected the experimental conditions indicated in the previous section. (O_2: 0.5 ml min^{-1}; nutrients (1 g l^{-1} NH_4NO_3 + 0.5 g l^{-1} K_2HPO_4): 1 ml day^{-1}).

Hydrocarbon degradation

The biodegradation of polycyclic hydrocarbons and aliphatic hydrocarbons present for a long time in a polluted soil is a very slow process, even with the supply of oxygen and nutrients [7]. During 47 days we have supplied the sample and the reference cell with oxygen and a nutrients solution; the reference soil was sterilizided with the addition of 2% of formol to the nutrients solution; a very low heat production was observed after 22 days (<200 μW) and the GC and FTIR analysis of the

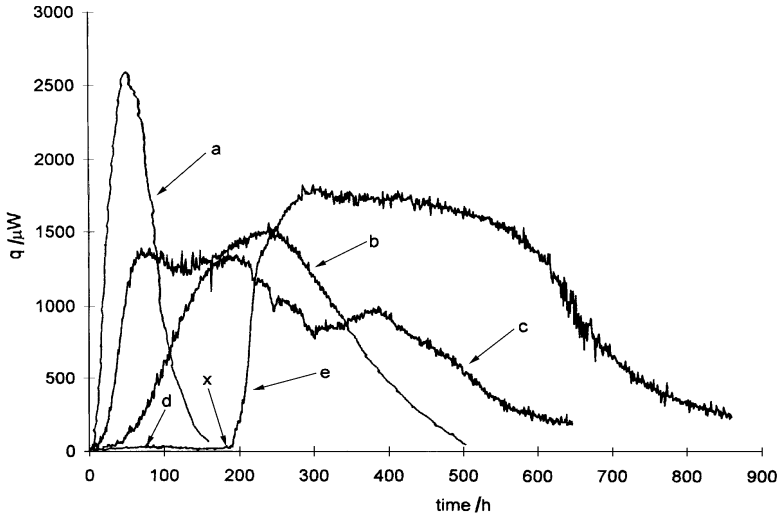

Fig. 5 Heat production as a function of time for different carbon sources. Reference cell: 1 ml day^{-1} of nutrients solution. Sample cell: 1 ml day^{-1} of nutrients solution and a – 80 mg of sodium succinate; b – 80 mg of dodecane; c – 80 mg of dodecene; d – 80 mg of 2, 2, 4, 6, 6-pentamethylheptane; e – 80 mg of dodecane added to d at the point x

sample at the end shows the disappearing of the lighter aliphatic hydrocarbons (C_{12}–C_{18}) and a decrease of ca 10% of the total hydrocarbons. These results are in good agreement with the results obtaind with the 'macro-scale' experiments [7].

We have then tested the biodegradation of three different C_{12} aliphatic hydrocarbons added to the polluted soil. Figure 5 shows the results obtained with the addition of 80 mg of dodecane, 80 mg of dodecene and 80 mg of 2, 2, 4, 6, 6-pentamethylheptane, compared with the addition of the same quantity of sodium succinate.

To compare these different curves, we have defined the following parameters:

•The latence time τ [h] is the time elapsed from the beginning of the measurement until a measurable heat production occurs.

• The maximum heat production q_{max} [μW]; this quantity is related to the microorganisms concentration N [ufc g^{-1}](see below).

• The kinetic of the microbial gropth σ [μW h^{-1}]. Generally this kinetic follows an exponential law [23–25]; in our case an exponential growth is observed during a very short period and we have chosen to express the kinetic with the slope of the linear regression of the first part of the curves.

• The total energy measured Q [J]; it corresponds to the area under the curve. This value can be compared with the energy of combustion Q_c [J] of the quantity of the carbon source supplied. α is the ratio Q/Q_c (see below).

Estimation of the number of microorganisms

The determination of the number of microorganisms is a long and tedious task. The six experimental points shown in Fig. 6 were obtained by determining the number of microorganisms at the maximum of the heat production. Assuming that the heat production measured during the microbial growth is proportional to the popula-

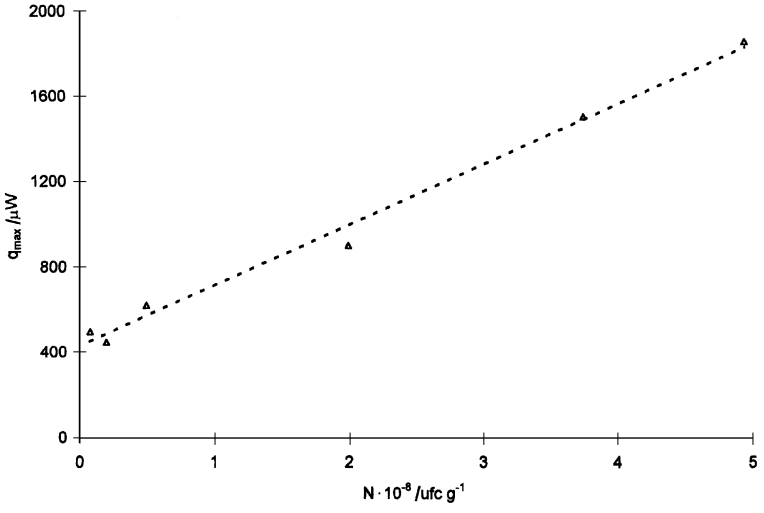

Fig. 6 Maximum heat production as a function of the number of microorganisms. Linear regression: slope=2.8 pW $cell^{-1}$; r^2=0.991

tion [23, 25], the slope of the graph q_{max}=f(N) (Fig. 6) represents the heat production for one microorganism. We obtained a value of 2.8 pW cell^{-1}, which is within the range of those determined by the calorimetry of pure culture (2.1 pW cell^{-1} [26]; 4.1 pW cell^{-1} [27]) or by the calorimetry of soil microorganisms (6.7 pW cell^{-1} [23]).

The number of microorganisms N (ufc g^{-1}) was then estimated by dividing q_{max} (μW) of each curve by 2.8 pW cell^{-1} and by 5 g.

Energy of combustion of the carbon sources

The Eqs (2) and (3) represent the reactions of combustion of the succinic acid and the dodecane respectively:

$$C_4H_6O_{4(aq)}+3.5O_{2(aq)} \rightarrow 4CO_{2(aq)}+3H_2O_{(aq)} \quad (2)$$

$$C_{12}H_{26(1)}+18.5O_{2(aq)} \rightarrow 12CO_{2(aq)}+13H_2O_{(1)} \quad (3)$$

The energy of combustion was calculated from the enthalpy of formation of each species taken in the state indicated in both equations [10, 28]. The values obtained are Q_c=1560 kJ mol^{-1} and Q_c=8221 kJ mol^{-1} for the Eqs (2) and (3) respectively. The enthalpy of formation of the sodium succinate, the dodecene and the 2, 2, 4, 6, 6-pentamethylheptane were not found in the literature, so that the values calculated above were used for these compounds. Q represents approximatively 45% of Q_c; the difference between the total energy measured Q and the combustion energy Q_c is most probably due to the enthalpy changes associated with the anabolic processes [9, 29, 30].

Table 2 shows the values of the parameters described above obtained with three experiments for each different carbon sources used. The sodium succinate is the most easily metabolized carbon source, then the dodecane and the dodecene. The

Table 2 Parameters of the curves of Fig. 5

Carbon source*	τ/ h	q_{max}/ μW	MO conc./ ufc g^{-1}	σ/ μW h^{-1}	α/ %
succinate (curve a)	2–7	2300–2600	1.7–1.9·10^8	70–90	46–47
dodecane (curve b)	15–25	1400–1600	1.0–1.1·10^8	20–30	43–44
dodecene (curve c)	25–35	1200–1400	0.8–1.1·10^8	10–15	42–43
pentamethyl heptane (curve d)	>190	<20	/	/	/
pentamethyl heptane + dodecane (curve e)	1	1800	1.3·10^8	38	37

* 80 mg of each carbon source

specific capacity of bioremediation of the soil can be roughly estimated from the calorimetry and the analysis of the carbon source in the soil at the end of the experiment; we have obtained 12 mg day^{-1} g^{-1} for the sodium succinate, 1.5 mg day^{-1} g^{-1} for the dodecane and 1.0 mg day^{-1} g^{-1} for the dodecene. The 2, 2, 4, 6, 6-pentamethylheptane produces no measurable heat evolution during 190 h (Fig. 5, curve d). At the point ×80 mg of dodecane was added to the sample, and an important heat evolution was observed after 1 h (Fig. 5, curve e). If we calculate the heat of combustion of 80 mg of 2, 2, 4, 6, 6-pentamethylheptane and of 80 mg of dodecane, α=37% and σ has a value of 38 $\mu W\ h^{-1}$; this value is higher than that measured with the dodecane alone (20–30 $\mu W\ h^{-1}$). This finding can only be explained by the adaptation of the microorganisms to the degradation of the 2, 2, 4, 6, 6-pentamethylheptane which was already in progress when the dodecane has been introduced.

Conclusions

The use of calorimetry with a continuous flow of oxygen and nutrients through the sample is a good technique to study the possibility of the bioremediation of a polluted soil. The biodegradation of the hydrocarbons adsorbed for a long time in the soil is a too slow process to be measured by calorimetry, even in the presence of oxygen and nutrients. On the other hand, the biodegradation of sodium succinate, dodecane and dodecene produces a measurable heat evolution, which corresponds to the increase of the microbial population from $2 \cdot 10^6$ to more than 10^8 ufc g^{-1}.

The specific bioremediation capacity of the soil has been estimated to 12, 1.5 and 1.0 mg day^{-1} g^{-1} for the sodium succinate, the dodecane and the dodecene respectively.

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The author wishes to thank Hélène Lartigue, Jocelyne Favet, Laurent Borella and Frédéric Garret-Flaudy for their collaboration.

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