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ORIGINAL PAPER

An exploratory study of peat and sawdust as enhancers in the (bio)degradation of *n*-dodecane

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Abstract Current practice for dealing with oil spills involves the use of adsorbent materials to contain the pollution prior to bioremediation of the contaminated soil and adsorbent. This work presents a study of the effects of bioavailable carbon sources in the adsorbents peat and sawdust as organic nutrients for microorganisms specialized in degrading *n*-dodecane in soil and sawdust contaminated with hydrocarbon mixtures. An experimental bioremediation system was developed using *n*-dodecane, biomass adapted to *n*-dodecane, inorganic nutrients and the two adsorbents (sterilized). Bioreactors containing peat enhanced cell growth the most and also evolved more CO_2. An advantage of peat is that its soluble carbon sources can sustain higher cell densities compared to sawdust, and this may prove decisive when cultivating endogenous microorganisms for the aerobic bioremediation of soils contaminated with hydrocarbons. However, at the end of the 68-day experiment slightly higher *n*-dodecane removal was identified in the system containing sawdust-*n*-dodecane (99.6%) than in that with peat-*n*-dodecane (98.5%), evidencing the higher hydrocarbon retention capacity of peat. Based on this study, the use of sawdust instead of peat is recommended when an adapted inoculum is available for aerobic bioremediation of organic contaminants, whereas the use of peat is advisable to boost cell densities in order to improve the probability of sustaining a viable biomass in unfavorable conditions.

Keywords Adsorbent · Bioreactor · Dodecane · Mineralization · Bioremediation

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Introduction

Current practice when dealing with petroleum spills first involves containing the pollution prior to later treatment. Aside from the polluted soil itself, many materials are used for containment purposes, including adsorbent, natural organic materials, such as sawdust (Shukla et al. 2002), straw and peat (Coleman 1994), vegetable fibers (Lim and Huang 2007; Annunciado et al. 2005); inorganic matter, such as sand (Carmody et al. 2007), volcanic ash and clay (Carmody et al. 2007); or even synthetic materials (Teas et al. 2001). Materials of choice should have a high adsorption capacity, be easily biodegradable and be markedly hydrophobic. Peat and sawdust, the focus of this study, afford excellent adsorption (from three to fifteen times their weight). In addition, they are readily available and cheap.

Peat is formed through the partial fossilization of mostly plant matter in marshlands. It essentially contains lignin and cellulose. However, the chemistry of peat is complex, as these two compounds (especially lignin) contain numerous functional groups

such as alcohols, aldehydes, ketones, organic acids, phenolic hydroxides and ethers (Viraraghavan and Ayyaswami 1987). The composition of peat varies according to its origin. The high overall polarity of peat and its complexes of chemical constituents offer a range of potential nutrients for the processes of bioremediation (Martin 1991).

Sawdust is a common byproduct of timber processing whose high lignin and lignocellulose contents make it relatively resistant to microbial decomposition. When suitably prepared and finely ground, sawdust affords good properties for containing oil spills.

Sawdust and peat are both currently used as substrates in the composting process (Godoy-Faúndez et al. 2007), since they improve aeration and increase the moisture retention capacity of the medium. Because of these properties, the adsorbent materials have been the focus of numerous studies to assess the potential for controlling episodes of hydrocarbon pollution (Teas et al. 2001). Some studies have investigated the materials as substrates in trials of growth and viability of inoculated bacteria colonies under controlled conditions (Lochhead and Thexton 1947). An adsorbent's ability to sustain microorganisms, availability and low cost are certainly advantageous, and should dictate which solid matrix to use in bioremediation processes.

The purpose of this study was to assess the capacity of peat and sawdust to sustain a microbial consortium specialized in the biodegradation of *n*-dodecane extracted from soil and sawdust contaminated with complex mixtures of petroleum hydrocarbons. *n*-Dodecane was used as a representative hydrocarbon of the liquid alkanes present in various mixtures of hydrocarbons (Geerdink et al. 1996), crude oil included, of very low solubility in water (0.0034 mg/l, at 30°C), log(K_{ow}) of 6.23, extremely low volatility (0.05 KPa, at 30°C, 71.4 times smaller than the vapor pressure of water at that temperature) and recognized biodegradability at half lives on the order of days to weeks (Margesin and Schinner 2001; Mohn and Stewart 2000).

Materials and methods

Reactor operation

The experiments were carried out in four glass bioreactors of 1 l capacity, continuously aerated with humid, sterile, CO_2-free atmospheric air (see Fig. 1). The air supply was provided by an arrangement of a BAR brand, UB-15 model, oil-free air compressor; an activated carbon filter; a UV sterilization system; and an NaOH trap, connected in series and located upstream from the bioreactor inlet. The air flows were distributed to the four bioreactors by means of a system of valves, such as to have the combined gas–liquid mass transfer coefficient for oxygen to each bioreactor, K_La, amount to 0.175 min^{-1}, thereby assuring excess oxygen in the air supply. The air flows amounted to 1.3 l min^{-1} for the sawdust bioreactors (R_1 and R_3) and 0.8 l min^{-1} for the peat bioreactors (R_2 and R_4).

The effectiveness of the atmospheric CO_2 retention of the system was assured through the use of two NaOH traps (T_5 and T_6 in Fig. 1). The CO_2 output of each bioreactor was monitored through the use of four 300-ml, 0.1-M NaOH traps, with one installed at the outlet of each bioreactor (T_1, T_2, T_3 and T_4). Each trap contained 2 l of 3.5 M NaOH, which was replaced before the pH fell to below 11.

The bioreactors R_1 and R_2 were loaded with 0.5% w/v (4% v/v) of previously sifted (0.15–0.18 mm) and sterilized adsorbent material. R_1 was loaded with radiata pine sawdust, and R_2 with PeatSorb^{TM} peat, the latter a product used for the containment of hydrocarbon spills. The equivalent of 38 g/l of *n*-dodecane, analytical grade, was added to bioreactors R_1 and R_2 and adsorbed on the sawdust and peat, respectively. The adsorption process for alkane was performed previously in a sterile, enclosed environment for 3 days to assure total retention of the alkane and to prevent subsequent formation of oleic phases in the bioreactors during the experiments.

The bioreactors with the adsorbent material and the adsorbed *n*-dodecane were filled to 750 ml with inorganic salt culture medium (20% v/v), vitamins (0.5% v/v) and a specialized inoculum for the biodegradation of *n*-dodecane (0.7% v/v) to an initial concentration in the aqueous phase of 10^7 cells/ml.

Bioreactors R_3 and R_4 were loaded in the same manner as bioreactors R_1 and R_2, respectively, but without *n*-dodecane.

The composition of the inorganic salt medium was as follows: 1.00 g of KH_2PO_4; 0.86 g of Na_2HPO_4; 1.00 g of NH_4Cl; 0.12 g of $MgSO_4$; 0.06 g of $CaCl_2$ and 2 ml of a solution of trace metals comprised of 3.3 mg of $MgSO_4$; 6.2 mg of $CuSO_4$; 7.6 mg of

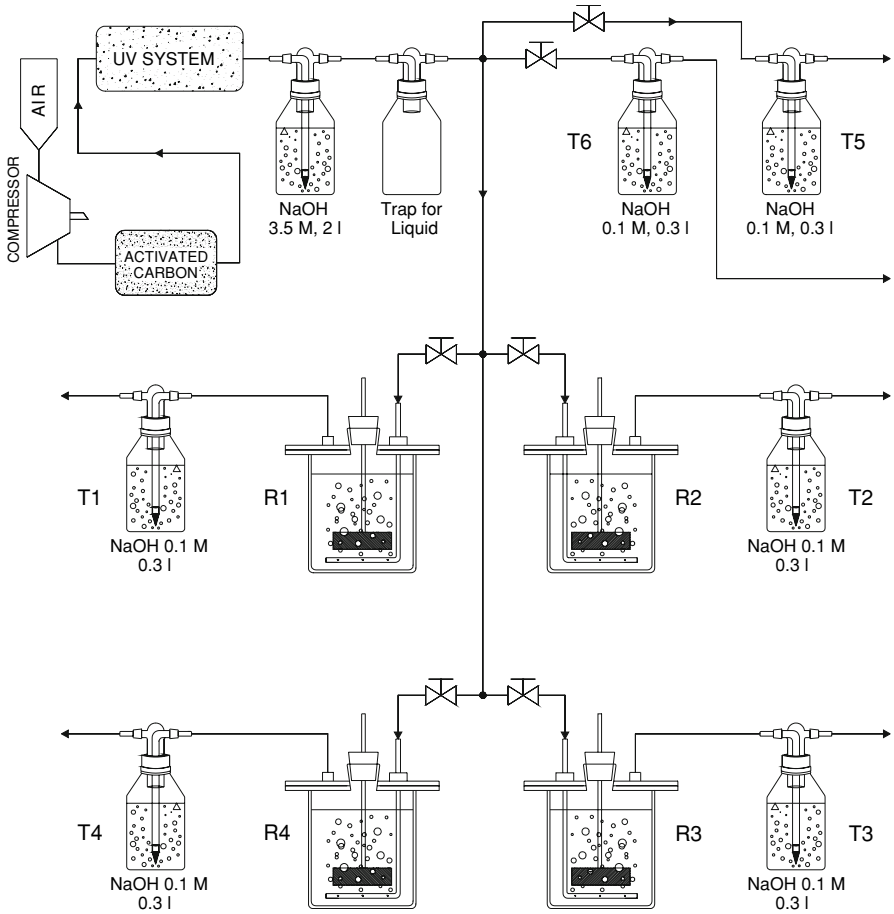

Fig. 1 Experimental bioremediation system. R_1 contains sawdust and *n*-dodecane; R_2, peat and *n*-dodecane; R_3, sawdust only; and R_4, peat only

$ZnSO_4$; and 11.7 mg of Na_2MoO_4 in 1 l of HCl 0.1 N, all dissolved in 1 l of deionized water.

The vitamin solution used was made up of 20 mg of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), 20 mg of nicotinic acid ($C_6H_5NO_2$), 1 mg of biotin ($C_{10}H_{16}N_2O_3S$), 1 mg of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), 1 mg of folic acid ($C_{19}H_{19}N_7O_6$), 20 mg pyridoxine hydrochloride ($C_8H_{11}NO_3{\cdot}HCl$), 20 mg of p-aminobenzoic acid ($H_2NC_6H_4COOH$), 20 mg of inositol ($C_6H_{12}O_6$), 20 mg of thiamin hydrochloride ($C_{12}H_{17}ClN_4OS{\cdot}HCl$), and 20 mg of riboflavin ($C_{17}H_2ON_4O_6$) in a 1 l solution.

The bioreactors were kept at 30°C during the entire period of experimentation by immersion in a Memmert brand thermostat regulated bath, continuously agitated by means of a 4-blade VELP Scientific multiple shaker at 100 rpm^{-1} in the dark. The bioreactors were operated on a batch basis, with the exception of the gaseous phase. Stationary biomass growth and CO_2 production were used to determine the cutoff point for the bioremediation experiment.

All flasks, air lines, bioreactors and their connectors were sterilized chemically prior to the experiments.

Microorganism extraction and adaptation to *n*-dodecane

The biomass specialized in the degradation of *n*-dodecane used in these experiments was extracted from a soil-sawdust mixture (50:50% w/w) contaminated with complex mixtures of petroleum hydrocarbons that included *n*-dodecane (fuels, solvents and lubricating oils). The soil and sawdust were obtained from a secure landfill of a mining company in northern Chile where they had been retained for

2 years, showing evidence of microbial activity (accumulations of gases). Prior to the extraction of the biomass, the soil-sawdust mixture used was segregated and passed through a sieve to obtain particles sizing less than 850 μm.

At this stage, the biomass in the fine contaminated soil and adsorbent was extracted and adapted to *n*-dodecane ($nC_{12}H_{26}$) over a period of 8 days (Fig. 2, series AB). Soil (1% w/v), analytical grade anhydrous $nC_{12}H_{26}$ (1% v/w) (Sigma Aldrich), inorganic salt culture medium (20% v/v) and deionized water was added to complete to 200 ml in two 500 ml graduated Erlenmeyer flasks (M_1 and M_2). The procedure was repeated using two additional graduated flasks (M_3 and M_4), but with twice the amount of soil (2% w/v). The four Erlenmeyer flasks were incubated in a shaker (Lab-Line 3527 Orbit-Environ Shaker) at 150 rev min^{-1} and kept at 30°C for 8 days.

Following the incubation period, two samples were extracted from each flask. The first sample was transferred to four new graduated Erlenmeyer flasks containing the enriched culture medium specified above, while the other was used for direct counting of microorganisms by means of a Leite Biomed optical microscope. The newly inoculated Erlenmeyer flasks were agitated and incubated under the same conditions as above. The process was repeated a further three times (see Fig. 2).

Once the cultures had been adapted to *n*-dodecane, the solution from the final sample (series PI4) that contained the most microorganisms' concentration (M_1) was used to inoculate each bioreactor (R_1, R_2, R_3 and R_4).

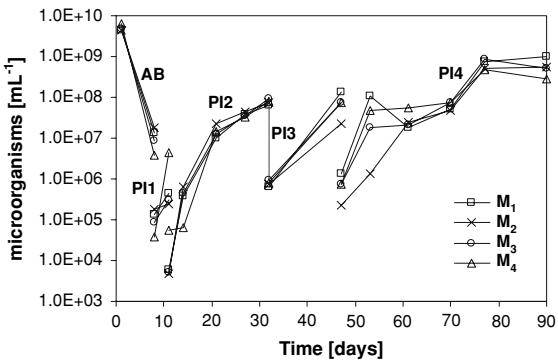

Fig. 2 Microbial adaptation and growth in *n*-dodecane. Each curve represents a new inoculation

Quantification of microbial activity

Biomass growth was monitored in each bioreactor through direct cell counts. In parallel, CO_2 production was measured indirectly. The pH of each CO_2 retention trap was determined on a daily basis, and titrations were performed of the NaOH content in the solutions each time the traps were changed. The CO_2 traps contained 300 ml of 0.1 M standardized NaOH and were replaced periodically to ensure that pH did not fall below 11.

Direct microorganism counts were carried out every six days for each bioreactor, using a Neubauer chamber and a Leite Biomed optical microscope.

Each time the traps were changed (every 2–5 days), two 25 ml samples were extracted from the weakened NaOH for titration. The first sample was used to determine total alkalinity, titrating with normalized HCl 0.1 M against a methyl orange indicator. The second sample was treated with an excess of barium chloride ($BaCl_2$ 10% w/v) to precipitate a carbonate ion in the form of $BaCO_3$ (Eq. 1), leaving a solution containing only the NaOH that did not react with CO_2. A sample was extracted from the supernatant solution and was titrated with HCl 0.1 M using phenolphthalein as the indicator. The difference between the HCl used in the two titrations determined the acid needed to completely neutralize the Na_2CO_3 formed (Ayres 1968; Kolthoff and Stenger 1947), as shown in Eq. 2 (Rieman et al. 1942).

$$Na_2CO_3 + BaCl_2 \rightarrow BaCO_3 \downarrow + 2NaCl \quad (1)$$

$$Na_2CO_3 + 2HCl \rightarrow 2Na^+ + 2Cl^- + H_2CO_3 \quad (2)$$

Since Na_2CO_3 is stoichiometrically equivalent to CO_2 and two moles of HCl are required to neutralize one mole of Na_2CO_3, the CO_2 that the microorganisms produce in mineralizing the *n*-dodecane as well as other biodegradable organic matter in the adsorbent materials can thus be determined.

Residual *n*-dodecane concentrations

The multi-phase nature of the bioreactor mixtures made it impossible to obtain consistently representative samples, so we could not determine *n*-dodecane concentrations during the course of the experiment.

The quantification of the total amount of *n*-dodecane remaining in all phases of each of the four bioreactors was determined by using FID gas chromatography and sacrificing the complete content of each bioreactor. Alkane was extracted with dichloromethane, by following EPA 3510C (Separatory Funnel Liquid–Liquid Extraction). The sample was then analyzed by gas chromatography coupled with a flame ionization detector (FID) to determine the residual concentrations of *n*-dodecane in each bioreactor (EPA 8015b method for non-halogenated organics using GC/FID). A 1-µl sample was injected into a Hewlett Packard 5890 Series II gas chromatograph with an FID. An HP–1 column was used (30 m × 0.32 mm i.d. × 0.25 µm film thickness). The oven was set at 50°C, held for 2 min, and the temperature was then increased 10°C min^{-1} to 315°C, which was sustained for 15.5 min. High purity helium was used as the transport gas at a rate of 1.0 ml min^{-1}. The standard used was Supelco *n*-dodecane.

Statistical analysis

The results obtained for cell density in each of the four bioreactors were compared statistically by using a two-way ANOVA analysis in Minitab^{TM} version 13.1.

Results

Microorganisms' adaptation to *n*-dodecane

In the contaminated soil-sawdust mixture, microbial colonies use a mixture of hydrocarbons as their source of carbon and energy. Once extracted from the soil, microbial colonies were placed in a medium where *n*-dodecane was the only source of carbon available, and so a process of microbial selection occurred (Atlas and Bartha 1998) in which only the microorganisms capable of degrading *n*-dodecane and withstanding the alkane concentrations in the medium survived (Fig. 2). The number of microorganisms diminished by three orders of magnitude during this adaptation period (AB), as was observed in each Erlenmeyer flask (M_1, M_2, M_3 and M_4).

Once the microorganisms had adapted to *n*-dodecane (Fig. 2, series AB), the preparation of inoculum was repeated four times consecutively (Fig. 2, series PI), lasting 3, 24, 15 and 43 days, respectively.

Quantification of microbial activity

At this point, degradation of *n*-dodecane and soluble material from the adsorbents was tested using the previously adapted *n*-dodecane degrader microorganisms. Biomass growth and CO_2 production were monitored continuously.

Biomass growth

Every 6 days throughout the 68-day period of experimentation, samples of the aqueous phase of the bioreactors were taken in quadruplicate to determine microbial growth by means of direct counting.

Biomass growth resulting from degradation of *n*-dodecane and other bioavailable organic compounds in the adsorbent materials is shown in Fig. 3. Slightly higher growth was observed in the two bioreactors containing peat (R_2 and R_4) than in the other two containing sawdust (R_1 and R_3). The two-way ANOVA showed that curves R1-R3 and R2-R4 in Fig. 3 are different, with a P-value < 0.001.

CO_2 production

The results obtained through the quantification of the CO_2 produced in each bioreactor corroborated our findings from the direct counting of microorganisms as per the above. Higher CO_2 production occurred in the bioreactors containing peat (R_2 and R_4) than in the sawdust-rich media (R_1 and R_3). CO_2 production was determined from daily assays of the pH in the retention traps (Fig. 4). NaOH titrations were performed from samples each time the traps were replaced, which corroborated the findings from the pH measurements (data not shown).

When considering that all of the CO_2 produced in the bioreactors with *n*-dodecane was due to its mineralization, alkane removal in the bioreactor with sawdust and *n*-dodecane can be estimated at 12.9%. In the case of the bioreactor with peat and *n*-dodecane,

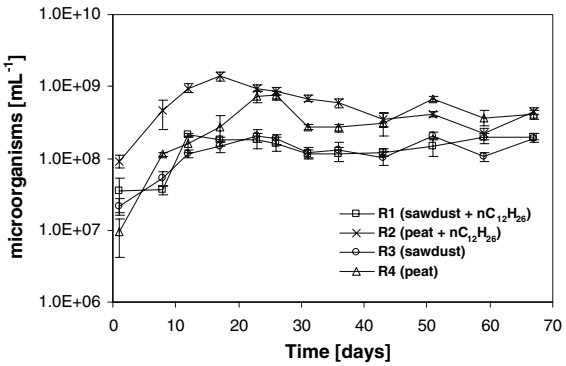

Fig. 3 Microorganisms growth in the aqueous phase of each bioreactor

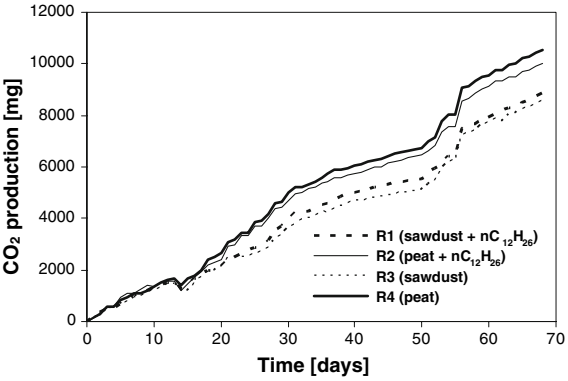

Fig. 4 Accumulated CO_2 evolved from bioreactors R_1 (sawdust + *n*-dodecane), R_2 (peat + *n*-dodecane), R_3 (sawdust) and R_4 (peat), measured via pH

the CO_2 produced corresponded to an alkane removal of 14.5%.

Residual *n*-dodecane concentrations

At the end of the experimental period, all of the contents of each bioreactor were subjected to analysis by chromatography to determine residual alkane concentrations in all phases. The results obtained are shown on Table 1.

A significant, although small, difference was observed between the residual concentration of *n*-dodecane in the bioreactor containing sawdust and *n*-dodecane (99.6% removal) and the bioreactor containing peat and alkane (98.5% removal), evidencing the superior hydrocarbon retaining capacity of peat in comparison with sawdust.

This observation regarding the removal rate estimated on the basis of CO_2 production in bioreactors R_1 and R_2, together with the observation referred to in the preceding paragraph, makes it possible to establish the existence of a biodegradation mechanism that reduces the *n*-dodecane concentrations in the bioreactors that contain *n*-dodecane. Although mineralization (CO_2 production) does not allow it to be established that complete biodegradation of alkane occurred, alkane's very low solubility and vapor pressure reinforce the fact that metabolism of alkane occurs rather than its loss due to volatilization from the system.

Discussion

Bioremediation analysis

The mineralization pathway for all alkanes, and *n*-dodecane in particular, is: alkanes, alcohols, aldehydes, carboxylic acids and CO_2 (Eq. 3). CO_2 is produced from the carboxylic acid (acetic acid) at the end of the pathway, while the longer-chained carboxylic acid needs to be degraded again. Yet complete mineralization of *n*-dodecane is unlikely to occur due to the volatility of the lighter intermediary products. In this connection, the very low removal of *n*-dodecane estimated on the basis of CO_2 production in the system and the high alkane removal found by chromatography testing at the end of the experimental period indicate the loss of these intermediate degradation compounds through volatilization. In fact, the vapor pressure of acetic acid as a degradation product of *n*-dodecane is 53 times higher than that of alkane.

$$C_{12}H_{26} + \frac{1}{2}O_2 \rightarrow C_{10}H_{21} - CH_2CH_2OH \rightarrow C_{10}H_{21} - CH_2CHO \rightarrow C_{10}H_{21} - CH_2COOH \rightarrow C_8H_{17} - CH_2COOH + CH_3COOH \quad (3)$$

The same underlying principle also holds when considering either peat or sawdust. However, rather than starting the degradation process with a single compound, *n*-dodecane, peat and sawdust contain many organic compounds. Although the degrader biomass could also contain lignin degrading biomass

Table 1 Concentration of residual *n*-dodecane in each bioreactor

Bioreactor	R_1 (sawdust + $nC_{12}H_{26}$)	R_2 (peat + $nC_{12}H_{26}$)	R_3 (sawdust)	R_4 (peat)
$nC_{12}H_{26\ INITIAL}$ (mg l^{-1})	38000	38000	0	0
$nC_{12}H_{26\ FINAL}$ (mg l^{-1})	151	574	n.d.	n.d.

n.d. not detected. The gas chromatography results were significantly different as the margin of error was 20%

on account of its origin, peat, in addition to having these compounds, is of a complexity that is much greater than that of sawdust. In particular, peat contains as part of its organic fraction (which is often greater than 90%) humic substances in varying degrees of polymerization and solubility. The most soluble fraction of the humic substances is made up of fulvic acids, which are of smaller molecular weight and have greater contents of carboxyl and hydroxyl groups in their structure. The humic acids, another group of humic substances in peat, also have particular functional characteristics including a great number of carboxyl radicals in their structure and fractions that are soluble at neutral pH. The most relevant group of these substances are the phenol, alcohol, quinone and ketone groups, although the presence of nitrogenated groups has also been demonstrated. This great variety of compounds that are part of peat and that can become partially soluble in an aqueous system represent a metabolic advantage for the degrader microorganisms employed, and particularly when compared against sawdust, which has compounds with fewer structural functional groups, most of them insoluble.

The stationary phase observed in Fig. 3 shows the above effect. The bioreactors with peat reached cell concentrations in the aqueous phase that they were considerably higher than the bioreactors with sawdust. Likewise, secondary species from the incomplete mineralization of *n*-dodecane, peat and sawdust provided sufficient organic nutrients to maintain cell density almost constant until the end of the experiment. Biomass viability should only tail off once the pool of bioavailable nutrients is depleted.

At the maximum cell density (18 days), the bioavailable compounds in the peat became depleted, and the biomass descended gently as it readapted to *n*-dodecane, to new organic peat compounds and to any byproducts. Biomass concentration stabilized at 42 days when consumption began of those compounds in peat that are more difficult to degrade and the secondary products of earlier degradation.

The biomass in the control systems containing peat or sawdust as carbon sources both had to readapt to the adsorbent materials' soluble organic compounds. As in the hydrocarbon polluted systems, the peat system continuously sustained a higher biomass than the sawdust system.

In mixtures of peat and *n*-dodecane, alkane may form a film around the peat particles and reduce the solubilization of soluble compounds and hence their bioavailability for the degrader biomass suspended in the aqueous phase. This situation is observed in Fig. 4, where the bioreactor with peat and without *n*-dodecane had a greater CO_2 output than the bioreactor with peat and *n*-dodecane. In the case of the bioreactors with sawdust, the small presence only of soluble material in the sawdust is reflected in a smaller CO_2 production in the bioreactor that had sawdust only, although mineralization of certain compounds was observed nevertheless, possibly due to lignin-degrading microorganisms present in the microbial consortium selected.

The differences between the sawdust and sawdust-*n*-dodecane systems (Fig. 3, curves R_1 and R_3) were much less apparent. The two-way ANOVA analysis showed that there is no statistical significant difference (P-value = 0.10) between both growth curves. The biomass profiles of the two systems were similar. Sawdust contains few easily biodegradable soluble compounds. The remaining compounds and secondary products are very difficult to degrade. An *n*-dodecane film around a sawdust particle would tend to increase the bioavailability of alkane. So, the only explanation for the higher CO_2 production in the sawdust-*n*-dodecane system is that, on the overall balance, the alkane and its byproducts are easier to degrade than the sawdust-related carbon sources.

Processes involving *n*-dodecane and the soluble organic compounds from the peat and sawdust ought

to be considered when evaluating *n*-dodecane degradation, CO_2 production and biomass growth.

Conclusions

The soluble carbon sources of peat can sustain higher cell densities compared to sawdust. This may prove decisive when cultivating endogenous microorganisms for the aerobic bioremediation of soils contaminated with hydrocarbons, although in these experiments, the content of lignin degrading biomass is evident in the specialized biomass selected for the degradation of *n*-dodecane.

In a bioremediation process, peat can provide the degrading biomass a large variety of metabolizable soluble compounds that are easier to degrade than *n*-dodecane. Alkane is considered to be rapidly biodegradable, and therefore can be used as a transitory sustainer of biomass in processes of this kind, and in particular during an adaptive process by degrader microorganisms. Moreover, peat's high hydrocarbon retention capacity (one of the main reasons for its use on spills) could increase the residual concentrations reached in bioremediation systems, although in the case studied the final concentration of *n*-dodecane obtained in the bioreactor with peat was nevertheless small (574 ppm). According to this study, the use of sawdust rather than peat could be recommended in situations in which a specialized inoculum and a suitable balance of nutrients are available and the attainment of small residual concentrations is expected.

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