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Biodegradation of di(2-ethylhexyl)phthalate in a typical tropical soil

Silvia Marta Castelo de Moura Carrara, Dione Mari Morita, Maria Eugenia Gimenez Boscov*

Polytechnic School, University of São Paulo, Brazil

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ABSTRACT

The aim of this research was to evaluate the possibility of biodegradation of di(2-ethylhexyl)phthalate (DEHP), widely used as an industrial plasticizer and considered an endocrine-disrupting chemical included in the U.S. Environmental Protection Agency priority list, in a Brazilian tropical soil, which has not been previously reported in the literature, despite the geographic importance of tropical soils. Preliminary laboratory testing comprised respirometric, air and water permeability, and pilot scale infiltration tests. Standard respirometric tests were found inadequate for studying biodegradation in tropical contaminated soils, due to the effect of the addition of significant amounts of calcium carbonate, necessary to adjust soil pH. Pilot scale infiltration tests performed for 5 months indicated that DEHP was retained in the superficial layer of the soil, barely migrating downwards, whereas air and water permeability tests discarded in situ bioremediation. However, ex situ bioremediation was possible, using a slurry-phase reactor with acclimated microorganisms, in pilot scale tests conducted to remediate a total mass of 150 kg of contaminated soil with 100 mg DEHP/kg. The removal of DEHP in the slurry-phase reactor achieved the percentage of 99% in 49 days, with biodegradation following a first-order kinetic model with a biodegradation coefficient of 0.127 day^{-1}.

1. Introduction

Phthalates or phthalic esters, largely used as additives and plasticizers, belong to the class of refractory organic compounds. Among the phthalates, di(2-ethylhexyl)phthalate (DEHP) distinguishes as a plasticizer because of its stability, fluidity, and low volatility and is one of the most frequently employed additives in the manufacture of flexible polyvinyl chloride. As a result of widespread use of this product, phthalates are commonly detected in air, water, sediment/soil, biota, and even in human tissue [1–3].

Some contaminants, including DEHP, are known as endocrine-disrupting chemicals (EDCs) because of their capacity to affect endocrine functions, such as reproduction, in intact organisms. The EDCs adversely interfere with hormones responsible for the maintenance of homeostasis, behavior, reproduction, and development of regulation processes [4,5]. Attention has been called to "phthalate syndrome," studied in rodents, which comprises reproductive abnormalities in males characterized by malformations of some organs as a consequence of phthalate exposure [6]. Swan [7] presents a study in which evidence of phthalate syndrome in humans was first identified.

Phthalates are potentially released to the environment through production, manufacturing, use, and inadequate disposal [8]. Degradation of phthalates can occur through hydrolysis, photolysis, and biodegradation [9], but microbial degradation is considered to be the main removal mechanism. Several studies show that phthalic esters are degraded under aerobic conditions by a wide range of bacteria [10–14]. Additional tests have been performed to evaluate their degradation using sludge-amended soil [15–17]. Concentrations of phthalates were detected in depth profiles in eight field sites used for agriculture, some of which had been amended with sewage sludge, and in a 50-year preserved natural soil [18].

Recently, bioremediation bench experiments were conducted to remediate DEHP-contaminated soils (initial concentration of 5.51 mg/g of dry soil); 89% of phthalate removal was observed in 76 days because of the addition of nutrients and inoculum, reaching a final concentration of 0.63 mg/g of dry soil [19]. Laboratory studies with DEHP-contaminated soils were also performed using a sequencing batch reactor [11,20,21].

However, no information is available on laboratory or pilot plant studies to evaluate slurry-phase bioremediation performance in tropical soils, and, in some cases, results from bench scale studies may not be extrapolated to field studies, which incorporate large-scale heterogeneities, because of mass transfer limitations, soil heterogeneity, and access of bacteria to the nutrients [22,23]. According to Semple et al. [24], the bioavailability of hydrophobic organic compounds such as phthalates depends on their adsorption on minerals and soil organic matter. Thus, the degree of biodegradation of these compounds will be different in tropical soils, relative to that exhibited for temperate soils.

* Corresponding author. Tel.: +55 11 9137 9203; fax: +55 11 3091 5538.
E-mail address: meboscov@usp.br (M.E.G. Boscov).

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In situ and intrinsic bioremediation are the first options to be analyzed, but some factors such as low temperature, anaerobic conditions, low levels or absence of nutrients and co-substrates, bioavailability, and degradation potential, can limit the efficiency of in situ bioremediation [25]. Soil moisture and permeability to liquid and gas are also determining factors for the success of this method [26,27]. Screening tests such as microbial plate counts and enzyme assessment may be used to determine whether existing conditions are favorable for microbial growth, whereas respirometric tests may confirm that the microbial population is metabolically active [28].

The accelerated urbanization and industrialization processes in Brazil for the last decades have resulted in environmental problems, particularly soil pollution. In 2010, the Environmental Protection Agency of São Paulo State registered 3675 contaminated areas by various pollutants, including 17 contaminated sites with phthalates [29]. In the USA, phthalates have been found in 448 of the 1264 current National Priority List sites [30]. The Environmental Protection Agency of São Paulo State has established prevention values, which correspond to the concentration that may cause harmful alterations in soil and groundwater, and intervention values, which represent the concentration above which potential risks to human health are present. DEHP prevention value is 0.6 mg/kg of dry soil, whereas intervention values are 10 mg/kg of dry soil at industrial sites, 4 mg/kg of dry soil at residential areas, and 1.2 mg/kg of dry soil in agricultural land [31].

The aim of this research was to evaluate the possibility of DEHP biodegradation in a tropical saprolitic soil that occurs widely in the metropolitan area of São Paulo and in other industrialized and highly populated areas in Brazil.

Natural soil samples spiked with DEHP were used in this investigation to guarantee higher control of boundary conditions. To determine the mobility of DEHP in the subsoil, DEHP infiltration tests were conducted in pilot scale. Evaluation of DEHP degradation was divided into two stages. In the first stage, laboratory respirometric and geotechnical tests were conducted to investigate the feasibility of in situ DEHP biodegradation by the natural microbiota of a non-contaminated ("clean") soil and by exogenous acclimated microorganisms. In situ bioremediation was discarded considering the geotechnical characteristics, particularly permeability of soil to water and air. In the second stage, the performance of an ex situ bioremediation process consisting of a slurry-phase biological treatment was verified by means of pilot tests. Only aerobic conditions were researched, inasmuch as tropical soils are generally non-saturated, constituting thick layers of fine soils associated with low water table.

2. Tropical soils

Tropical soils comprise one-third of the superficial soils in the world, with 75% of the world's population living on these soils [32]. Tropical soils present properties and behavior peculiarities when compared to non-tropical (sedimentary and temperate climate) soils due to geological and pedological processes typical of humid tropical regions [33]. From a geotechnical standpoint, two large families are easily identified in the broad group of tropical soils: lateritic soils and saprolites or saprolitic soils. Lateritic soils are generally found in Earth's inter-tropical latitudes, in climatic conditions favorable to intense and fast weathering of rocks, corresponding to high temperatures, humid environment, abundant rainfall and water seepage. Lateritic soils cover 1% of the surface of the planet and 21% of the inter-tropical latitudes. In Brazil, lateritic soils are present in almost every region, in the superficial layers of the pedological horizon, with thicknesses varying from centimeters to tens of meters. In humid tropical regions, chemical weathering of soils is more intense, resulting in great loss of bases and silica and relative accumulation of iron and aluminum oxides, a process called laterization. The intensity of action of chemical weathering is directly proportional to the temperature increase. The hotter and more humid the weather, the greater is the depth of the soil subjected to physical and chemical changes [34,35]. Another factor is the interference of the temperature on the amount of organic matter in the soil. Under high temperature and good aeration, mineralization of organic matter occurs rapidly, releasing nutrients for plants. Thus, in warm climates, the conditions are favorable for increased microbial activity, generally resulting in soil with low organic matter [35,36].

Saprolitic soils are formed by rapid and intensive weathering of rocks, during which process mechanical characteristics are modified, but visual appearance, discontinuities, schistosity, faults, foliations and intrusions are preserved. Rock weathering is initially characterized by alterations in color and texture and hardness loss despite little volumetric change; development of micro-fissures is followed by element dissolution from soil grains and partial or total transformation of primary minerals. As weathering advances through increasing fissures, inherited features gradually disappear because of the transformation and reorganization of the original phases. Weathering tends to decrease along the profile depth, which presents finer material composed of more expressive fraction of secondary minerals near the surface, and rock fragments and coarse grains composed of primary minerals in deeper layers; these soils are essentially heterogeneous. Saprolitic soils occur in five continents, i.e., Africa, America, Asia, Australia and Europe. Large areas of Brazil contain saprolitic soils, which may be exposed or covered by lateritic soils. Saprolitic soils derived from acidic rocks occur over Brazilian and Guianas Pre-Cambrian Shields, which constitute the base of the South American continent [37]. Granite and other acidic rocks are widely distributed throughout the continental crust and are the most abundant type of basement rock underlying the sedimentary cover of the continents.

3. Materials and methods

3.1. Soil

The investigated soil was a saprolitic silty clay from the C horizon of a residual profile of migmatite, collected from a cut slope in the campus of the University of São Paulo, Brazil, 4.4 m below the ground surface. The natural soil was non-contaminated, i.e., "clean soil", and was contaminated in the laboratory, an option to guarantee higher control of boundary conditions.

The soil was composed of 5% medium sand, 36% fine sand, 35% silt, and 24% clay. The specific gravity of the solids, G_s, was measured as 2.7. In situ unit weight, water content and saturation degree were, respectively, 1.81 t/m^3, 23.5% and 75% [38].

Organic matter content, cation exchange capacity, pH and concentrations of metals and semi-metals were determined according to [39]. Buffer capacity, bulk density, residual water moisture and field capacity were estimated following [40]. Organic carbon content was measured by means of elemental analysis (Perkin Elmer Analyser). Total Kjeldahl nitrogen and total phosphorous were determined by procedures in [41]. Heterotrophic bacteria were counted according to [42].

3.2. Air and water permeability tests

The hydraulic conductivity of the soil was determined by constant-head flexible-wall permeability tests performed using a TriFlex-2 permeameter from ELE International Inc., with a confining pressure of 30 kPa and a hydraulic gradient of 60 m/m. Soil specimens were compacted at a dry-unit weight similar to

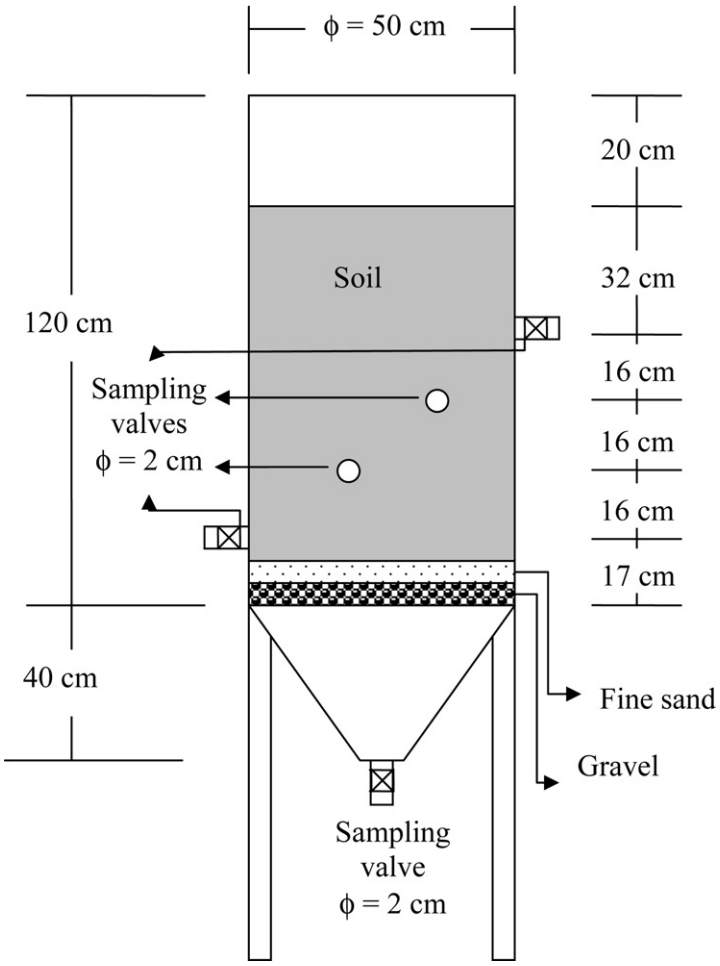


Fig. 1. Pilot scale reactor for infiltration test.

in situ conditions to verify the feasibility of bioventing. The air permeability test was executed according to ASTM-D4525 [43] in a constant-head flexible wall permeameter consisting of an adapted triaxial test cell, with application of air pressures from 10 to 90 kPa to the specimen base while maintaining atmospheric pressure at the top of the specimen.

3.3. Infiltration tests

A DEHP emulsion was applied to the upper surface of a soil specimen placed in an acrylic cylindrical reactor with diameter of 50 cm and height of 120 cm (Fig. 1). The reactor had 5 points for pore water collection uniformly distributed along its height. Bottles were connected to each point to store samples until enough volume for chemical analysis was collected.

The soil was compacted inside the reactor in 9 lifts, each with 10 cm of thickness, resulting in 0.9 m of total height, at the in situ value of dry unit weight, 1.47 t/m^3. Molding water content was 20%, which for this soil is the most favorable situation for in situ bioremediation, resulting in an initial saturation degree of 64%.

Infiltration of water through the top surface proceeded during the 2 months before DEHP application. To contaminate the soil with 100 mg DEHP/kg of dry soil, 26 mL of DEHP were mixed with 2 L of water, resulting in a solution with a concentration of approximately 0.4 mg/L and a free phase of DEHP, which is a common situation in spillages of this compound.

After the complete infiltration of the liquid phase, 10 L of water were applied weekly at the top surface to enhance DEHP migration through the specimen. Water samples were collected at five collection points and DEHP concentration was determined by gas chromatography. After 5 months, soil samples were extracted from each point of collection, and DEHP content in the soil was also determined by gas chromatography by means of EPA Methods 3540C and 8061A [44].

3.4. Respirometric studies

Bartha and Pramer [45] first described a microcosm method to measure aerobic soil biodegradability, an approach on which most laboratory methods are based. Microcosm tests have proven to be reliable for studying biodegradation of many organic chemicals [28,46,47]. Additional studies have used respirometric tests to evaluate biodegradation of organic compounds by measuring oxygen uptake [28,48] or carbon dioxide production [49]. Currently, automated respirometers that continuously measure O_2 consumption and CO_2 production provide useful tools for monitoring ex situ bioremediation projects [48].

DEHP biodegradability in soil was measured by Bartha biometer flasks [45], standardized in Brazil by the Environmental Protection Agency of São Paulo State [40]. This technique consists of exposing the chemical to the soil, with or without addition of microorganisms and nutrients, and measuring carbon dioxide production over time.

The microcosms consisted of 250 mL borosilicate glass vials fit with valves that allowed carbon dioxide samples to be absorbed into 10 mL of a KOH solution 0.2 M in a sealed system. The mass of

carbon dioxide absorbed into the KOH solution was calculated by Eq. (1):

$$m_{CO_2} = V_{HCl} \cdot 50 \cdot f_{HCl} \quad (1)$$

m_{CO_2}, mass of CO_2 absorbed into KOH solution (μmol); V, volume of HCl solution 0.1 M (used as titrant) (mL); 50, factor that transforms equivalents in μmol of CO_2; f_{HCl}, factor of HCl concentration 0.1 M.

Microcosm studies were conducted after DEHP addition to soil samples collected in a non-contaminated site. Two conditions were investigated: biodegradation by the natural microbiota present in non-contaminated ("clean") soil and biodegradation by exogenous acclimated microorganisms. The first condition was considered as a reference to analyze the efficiency of DEHP biodegradation by acclimated microorganisms present in the aeration tank of an industrial wastewater activated sludge process.

The first series of experiments was immediately performed after the collection of the soil in the field and pH adjustment to approximately 7.0 with a $CaCO_3$ solution. The soil was contaminated with a solution of DEHP mixed with acetone HPLC-grade to obtain a homogeneous contamination. If only DEHP was mixed with the soil, mud clods were produced. Because of the high solubility of DEHP in acetone, total dissolution was achieved, without formation of a DEHP free phase, for the three tested concentrations: 1 mg/kg, 10 mg/kg and 100 mg/kg. Acetone evaporation, controlled by means of soil water content, was complete in 3 days. Acetone introduction did not affect the soil's natural microbiology, according to a parallel research [50]. Nutrients were then applied to the contaminated soil; a carbon:nitrogen:phosphorous ratio of 300:5:1 was used in all tests, according to [40]. Soil water content was adjusted to 30%, which corresponded to 70% of the field capacity of the soil. Respirometers were incubated at a constant temperature of (20 ± 2) °C. Evolved CO_2 was measured 2 days after the incubation and, subsequently, once a week for 98 days.

Control experiments were conducted with natural uncontaminated soil, acetone and nutrients. Triplicate experiments were performed for each DEHP concentration and for the control, resulting in the preparation of 12 respirometers (9 tests and 3 controls).

The procedure for the second series was similar to that of the first, with the addition of exogenous microorganisms to the soil to obtain 10^7 CFU/g of dry soil. The sludge containing microorganisms adapted to phthalates was collected in an aeration tank from an activated sludge process of a wastewater treatment plant from an industry that produces plasticizers. Because the plant received other organic compounds, e.g., alcohols and organic acids that could be easily biodegraded, the sludge was submitted to a sedimentation process to separate the microorganisms from the liquid portion. The sludge was collected into a 50 L recipient, and the supernatant was removed after 30 min of sedimentation. Dechlorinated drinking water was introduced until the original volume was reached and the water and sludge were thoroughly mixed; the supernatant was removed after 30 min of sedimentation. This process was repeated three times. The following characteristics of the sludge were determined: temperature, pH, total Kjeldahl nitrogen, phosphorus, DEHP concentration, and bacterial counting, which protocols followed the Standard Methods for the Examination of Water and Wastewater [41]. Table 3 shows chemical characteristics of the sludge used to provide exogenous acclimated microorganisms to the slurry-phase experiments.

3.5. Ex situ bioremediation test

3.5.1. Experiment

A cement mixer was chosen as a reactor because mixing intensity is a critical factor in slurry-phase bioreactor design and performance. The mixer was installed under a wood shed in the campus of the University of São Paulo. A timer was connected to the equipment and programmed to operate the cement mixer for 2 min each 12 min, 24 h a day for 49 days of treatment.

Initial water content was adopted as 67%, which corresponds to a saturation degree within the range of 40–85% recommended for slurry-phase bioremediation, according to [51], and guaranteed a slurry consistency.

Inoculum for soil contamination has already been described in Section 3.4. A dry mass of 150 kg of soil was used in the pilot test. First, soil pH was adjusted with $CaCO_3$ addition and measured after one week, when the equilibrium of carbonates was expected to be reached. Subsequently, the soil was introduced into the cement mixer. To form slurry and reach the previously defined water content, 105 L of liquids were added in the following sequence: 10 L of DEPH emulsion, 80 L of water and 15 L of sludge with microorganisms. DEHP emulsion was prepared by mixing 15 g of DEHP with 10 L of water, providing an initial DEHP concentration of 100 mg/kg of dry soil. After the introduction of the emulsion, the cement mixer was turned on for 2 h to obtain a homogenous mixture between soil and pollutant.

The treatment was initiated after the introduction of 15 L of sludge to the slurry. Twenty liters of water were added weekly to the slurry. Treatment was monitored weekly by measurement of pH, water content, and DEHP concentration. Applied nutrients were $(NH_4)_2SO_4$ and K_2HPO_4, monitored every week to maintain the specified ratio C:N:P of 300:5:1. Total heterotrophic bacteria were monitored weekly and counted by means of a pour-plate count. Nitrogen and phosphorus quantification protocols were performed following the Standard Methods for the Examination of Water and Wastewater [41].

3.5.2. DEHP quantification

Gas chromatographic analyses of DEHP were performed on a Hewlett-Packard 6890 Series Gas Chromatograph (Palo Alto, CA, USA) equipped with a split–splitless injector. A Hewlett-Packard HP-5 Porapack capillary column (cross-linked methylsilicone) of 30 m × 0.32 mm and with a phase thickness of 0.5 μm was selected to separate the analytes. Nitrogen was used as a carrier gas at a flow rate of 6.0 mL/min. Samples were injected by the auto sampler in the splitless mode at 270 °C. Nitrogen flow rate was maintained at 68.6 mL/min.

The temperature program was set at an initial temperature of 180 °C, which was maintained for 2 min and then increased to 300 °C, remaining at this value for 5 min and resulting in a total time run of 13 min. The detector was FID at a temperature of 300 °C with compressed air and hydrogen at flow rates of, respectively, 350 mL/min and 30 mL/min. The make-up gas was nitrogen at a flow rate of 30 mL/min.

Reagents at the highest analytical purity (HPLC grade) were used for extractions, chromatography calibration curves and injections.

Sub-products of DEHP biodegradation were determined with gas chromatography and mass spectrometry. The process of extraction of DEHP from soil was the same for DEHP analysis and sub-product identification, in which 10–30 g of soil were placed in an extraction cartridge with the same amount of sodium sulfate pa (anhydrous), previously dried at 400 °C for 4 h. The cartridge was inserted in a Soxhlet extractor, connected to a condenser, and a flask of 500 mL containing 150 mL of acetone and 150 mL of hexane. The extraction was performed for 24 h. The sample was cooled to room temperature and preserved under refrigeration.

Sub-products of degradation were identified by gas chromatograph Shimadzu GC-17A's, coupled to a mass spectrometer Shimadzu Massa. The GCMS-QP5050 operated with a ZB5 (5% phenyl–95% dimethylpolysiloxane), internal diameter of 0.25 mm, length of 30 m, and film thickness of 0.25 μm was selected to separate the analytes. Helium was used as a carrier gas at a flow rate of 4.9 mL/min, with a constant pressure of 250 kPa, at a linear

Table 1
Soil characterization.

Characterization	Parameters	Value
Chemical	Organic matter content	0.3%
	Cation exchange capacity	21 mmolc/kg
	pH	4.1 4.7
	Buffer capacity	20 mg $CaCO_3$/10 g dry soil
	P	1 g/kg
	K	0.3 mmolc/kg
	Ca	3 mmolc/kg
	Mg	1 mmolc/kg
	Al	9 mmolc/kg
	S	15 g/kg
	B	0.1 g/kg
	Cu	0.1 g/kg
	Fe	2 g/kg
	Mn	0.5 g/kg
	Zn	1.7 g/kg
	Cd	<0.01 g/kg
	Cr	<0.01 g/kg
	Ni	<0.01 g/kg
	Pb	1.17 g/kg
Geotechnical	Bulk density	1.16 g/cm^3
	Residual water moisture	78%
	Field capacity	43%

speed of 81 cm/s. Samples were injected in the split mode at 280 °C. The temperature program was set with an initial temperature of 60 °C, which was maintained for 1 min and then increased to 280 °C, resulting in a total time run of 65 min.

4. Results and discussion

4.1. Soil characterization

Results of chemical, geotechnical and biological characterization tests are shown in Table 1. Obtained values of organic matter content, cation exchange capacity (CEC) and pH are within the range of typical values for tropical soils [52–54]. According to Embrapa [55], this soil presents low CEC (<270 mmolc/kg), within the range for Brazilian soils, 79% of which present CEC lower than 100 mmolc/kg [56]. A buffering capacity test showed that 20 mg of $CaCO_3$ for 10 g of dry soil were necessary to maintain a soil pH of 7.5 at 25 °C. The association of low CEC, low organic matter content, low pH, and high aluminum content is typical for tropical soils in general, and particularly for saprolitic soils derived from acidic rocks.

Hydraulic conductivity of the soil and intrinsic air permeability determined in triplicate specimens molded at in situ density were, respectively, 6×10^{-8} m/s and 7×10^{-15} m^2. The low hydraulic conductivity may impair bacteria access to DEHP and migration of nutrients in the soil. Intrinsic air permeability is below the recommended value of 10^{-12} m^2 for bioventing [57]. According to the National Research Council In Situ Bioremediation [58], among the most important site characteristics for in situ bioremediation is the transmissivity of the subsurface to fluids: minimum limits for hydraulic conductivity and intrinsic permeability are, respectively, 10^{-6} m/s and 10^{-13} m^2 to guarantee circulation of water and air.

Results indicated that in situ treatment was not feasible for the soil under investigation; therefore, it was decided to study soil decontamination by slurry-phase biological treatment, using a cement mixer as a reactor.

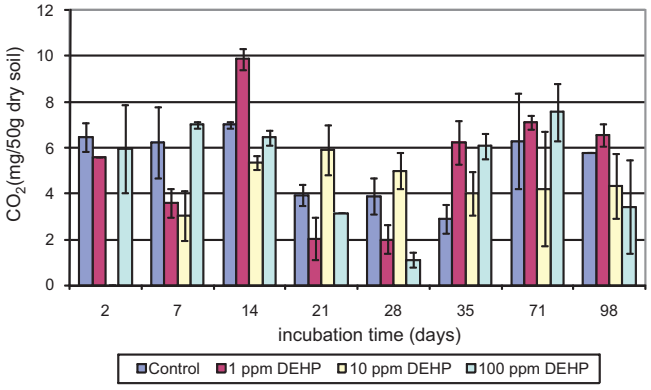


Fig. 2. CO_2 production as a function of incubation time for respirometric tests with indigenous microorganisms.

4.2. Respirometric studies

Results of the first series of respirometric tests performed with the natural microorganisms of non-contaminated soil are presented in Fig. 2, with CO_2 production as a function of incubation time for the concentrations of 1, 10 and 100 mg DEHP/kg for the control test. Corresponding results of the second series of respirometric tests performed with exogenous microorganisms are shown in Fig. 3.

The difference between CO_2 production in a contaminated respirometric test and in the control test indicates the amount of occurred DEHP biodegradation. Mean values from the sets of triplicates were used to test for significant differences at each time interval using a Tukey's test ($p < 0.05$), but the majority of differences were not statistically significant.

The remarkable variability of test results may be attributable to the high amount of $CaCO_3$ necessary to neutralize the acidic soil. Figs. 2 and 3 show high production of CO_2 in the control tests, a consequence of the acidity of the investigated soil, which required the addition of a significant mass of calcium carbonate to adjust soil pH, releasing CO_2 according to the following reaction [59]:

$$\text{Soil}]^{H^+}_{Al^{3+}} + 2CaCO_3 + H_2O \rightarrow \text{Soil}]^{Ca}_{Ca} + Al(OH)_3 + 2CO_2 \uparrow \quad (2)$$

It should be emphasized that, although respirometric tests are recommended by many authors [28,45–48], including the EPA [26], for the assessment of biodegradation occurrence, they may not be adequate for tropical soils because of their typical acidity [52–54]. Addition of a significant mass of calcium carbonate to adjust soil pH has a deleterious effect in the performance of respirometric tests. An interesting alternative may be the measurement of O_2 uptake

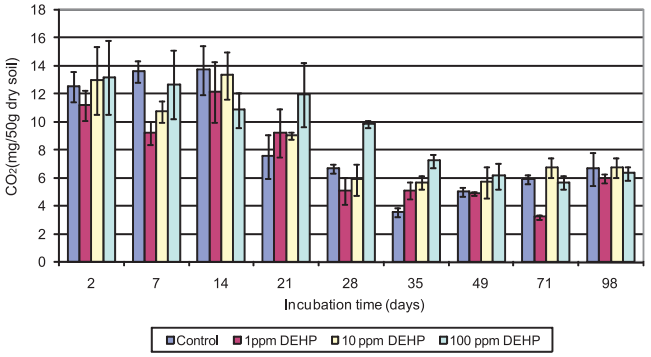


Fig. 3. CO_2 production as a function of incubation time for respirometric tests with exogenous microorganisms.

Table 2
DEHP concentration in the soil pore water during the infiltration test.

Point of collection	Depth (cm)	Time (days)	pH	Water temperature (°C)	DEHP concentration in water (μg/L)	DEHP concentration in soil (mg/kg)
Top	0–2					3885.2
1	16.6	23	6.3	26.4	nd	1.4
		70	6.4	26.0	nd	
		133	6.8	26.0	nd	
2	33.2	50	5.9	26.4	1.7	0.1
		120	7.2	26.0	2.8	
		133	6.7	26.0	nd	
3	49.8	38	4.8	28.8	1.3	0.1
		89	6.3	26.0	1.4	
		133	7.1	26.0	–	
4	66.4	70	3.8	26.4	nd	0.2
		133	4.3	26.0	–	
Bottom	83.0					0.2

nd, not detected.

by microorganisms instead of CO_2 production and the adaptation of test procedures relative to pH adjustment.

4.3. Infiltration test

Table 2 presents DEHP concentration in the soil pore water and soil samples as a function of depth and time after the application of the DEHP emulsion to the upper surface of the soil specimen. Measured pH values between 6.0 and 7.0 may be explained by the effect of DEHP migration, as they are slightly higher than the pH of the water applied to the upper surface of the soil specimen. The low concentrations of DEHP in the soil pore water, on the other hand, indicate that the pollutant was retained by the soil particles. Despite the continuous introduction of water, DEHP presents low solubility in water; therefore, the low measured concentrations may also be related to the low mobility of the pollutant. Staples et al. [60] present some values of DEHP solubility in water obtained by various researchers, the majority of which are lower than 0.4 mg/L.

Almost all DEHP applied to the soil was retained in the upper 2 cm of the soil specimen. The expected retention of DEHP is important information to consider when selecting feasible remediation techniques. Spillages of DEHP do not tend to infiltrate deeply into the subsoil, unless it presents preferential flow paths, e.g., features that are much more permeable than the soil mass. Tropical residual soils generally constitute thick layers of unsaturated soils composed of an expressive fines fraction (silt and clay fractions). In the case of an oil spillage (such as phthalate), infiltration through the soil is very limited because of the retention by capillarity in the fine pores, resulting from the interfacial tension between oil and air, as well as oil and water, and by the wettability of the oil on the surface of soil grains, forming an adhered film. Furthermore, air and water permeability coefficients are too low to indicate in situ bioremediation. A feasible technique for these soils is ex situ bioremediation of the contaminated material excavated from the superficial layer of the soil.

Table 3
Sludge characterization.

Parameters	Values
pH	6.4
T (°C)	34
NTK (mg/L)	135
P (mg/L)	41.8
Total heterotrophic bacteria concentration (CFU/mL)	4.30×10^7
DEHP (mg/L)	0.61

4.4. Slurry-phase experiments

Table 3 shows chemical characteristics of the sludge used to provide exogenous acclimated microorganisms to the slurry-phase experiments. Nutrient concentrations in the soil over time are presented in Table 4. Guidelines of the Environmental Protection Agency of the State of São Paulo, Brazil, for respirometric tests [40] recommend a ratio C:N:P of 300:5:1, but the literature generally suggests a ratio of 100:10:1 for remediation of soils contaminated with organic pollutants. Respirometric tests and the slurry-phase test were performed following Brazilian procedures; the first sample in Table 4 indicated a nutrient ratio of 300:6:4.5, higher than that recommended by the adopted procedure; hence, nitrogen and phosphorous were not introduced. However, a lag phase in the slurry-phase test was observed, and nitrogen content did not decrease; therefore, on the 35th day, 28 g of $(NH_4)_2SO_4$ were introduced to achieve a nutrient ratio of approximately 100:10:1. DEHP degradation was enhanced with the increase of the nitrogen content because of assimilation of the ammonium nitrogen by the microorganisms and its conversion to organic nitrogen in the cells' bodies.

Table 5 presents the water content, pH measured in H_2O, temperature, and total heterotrophic bacteria over the time of the experiment. $CaCO_3$ introduced prior to the treatment increased soil pH from 4.9 to approximately 7.0. Temperature and pH did not vary

Table 4
Nutrients concentrations in the soil during the bioremediation process.

Time (days)	Organic nitrogen (mg/kg of dry soil)	Total ammonium nitrogen (mg/kg of dry soil)	Total phosphorous (mg/kg of dry soil)	Ratio C:N:P
0	13.0	2.55	1.90	100:2:1.5 300:6:4.5
21	33.9	2.69	1.55	100:2:1 300:6:3
35	34.1	15.7[a]	0.71	100:11:0.5 300:33:1.5
49	46.7	4.76	0.72	100:3.5:0.5 300:10.5:1.5

[a] Introduction of 28 g of $(NH_4)_2SO_4$.

Table 5
Water content, pH, temperature and total bacteria along experimental time.

Time (days)	Water content (%)	pH	Temperature (°C)	Total bacteria (CFU/gdw)
0	69.95	7.45	24.0	3.6×10^6
7	55.85	7.45	24.0	7.2×10^5
14	66.87	7.49	26.5	1.2×10^5
21	71.33	7.20	26.0	1.2×10^6
28	71.19	7.31	24.0	7.9×10^5
35	82.40	7.96	24.0	3.7×10^6
43	83.16	7.97	24.0	2.0×10^6
49	74.93	7.80	27.0	2.1×10^6

gdw, g of dry weight of soil.

significantly during the period of the experiment. This is different than expected according to the literature [60,61] and is probably attributable to the low concentrations of acidic organic compounds produced during biodegradation compared to the mass of $CaCO_3$ introduced to adjust the soil pH. Water content stayed close to the range recommended for the slurry-phase treatment (40–85%).

During the lag phase, the number of total heterotrophic bacteria decreased almost tenfold, but after 20 days, the microorganisms recovered and growth was observed. A greater growth occurred at the 35th day because of the introduction of nitrogen. The microorganisms' previous acclimation to DEPH in the wastewater treatment process from a phthalate industry resulted in good performance, showing that the introduction of the sludge containing DEHP degraders was important for the fast removal of the chemical compound and the success of the bioremediation process. Although the scope of this research was not the isolation and identification of DEHP degraders, the importance of this subject must be emphasized.

Although the soil had been contaminated with 100 mg DEHP/kg of dry soil, part of the DEHP was lost in the contamination process, resulting in a lower concentration being applied to the soil, i.e., 68.2 mg DEHP/kg of dry soil. The water added to the soil may have caused ester hydrolysis [62].

DEHP biodegradation by exogenous microorganisms determined by chromatography is presented in Fig. 4. A first-order kinetic equation was fit to the experimental points, resulting in a biodegradation coefficient of 0.127 day^{-1} and a correlation coefficient of 0.94. Similar biodegradation coefficients were obtained by [11,13,17,61,63,64].

After 49 days of treatment, DEHP concentration decreased from 68.2 mg/kg of dry soil to 0.8 mg/kg of dry soil, corresponding to 98.8% of degradation. The final DEHP concentration reached in 49 days was lower than the intervention value for agricultural land (1.2 mg/kg of dry soil) established by the Environmental Protection Agency of the State of São Paulo. Thirty-four days would be necessary to reach the reference value of 10 mg/kg of dry soil.

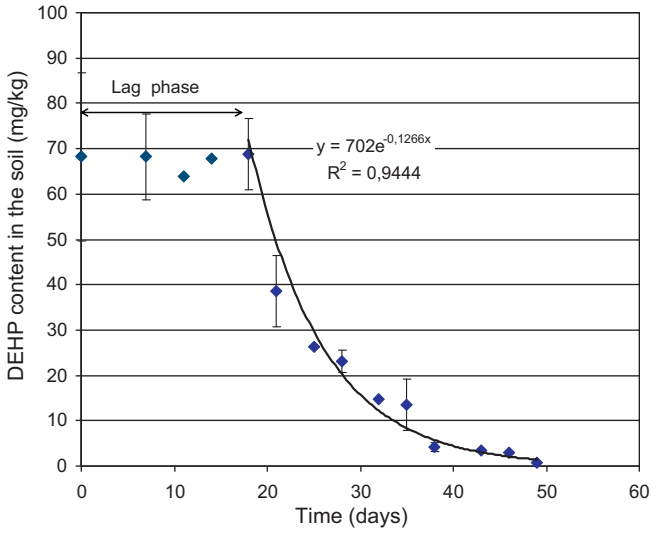


Fig. 4. Evolution of DEHP concentration in the soil in the slurry-phase experiment.

Gas chromatography and mass spectrometry analyses with soil samples along the slurry-phase test indicated the presence of subproducts of DEHP biodegradation in the soil. Until the 35th day, there was a predominance of DEHP (Fig. 5); subsequently, phthalates of small molecular weights, such as isobutyl o-phthalate and butyl octyl ester, and organic acids, such as 9-octadecenoic acid, octanoic acid, octadecanoic acid and n-hexadecanoic acid, were recorded, as shown in Fig. 6 [11]. Pyruvic acid, among the final products of DEHP biodegradation, was identified at day 49.

After the treatment, the cement mixer was turned off and the soil was left there to verify the water loss as a function of time. The water content decreased naturally from 75% to 31% in three weeks.

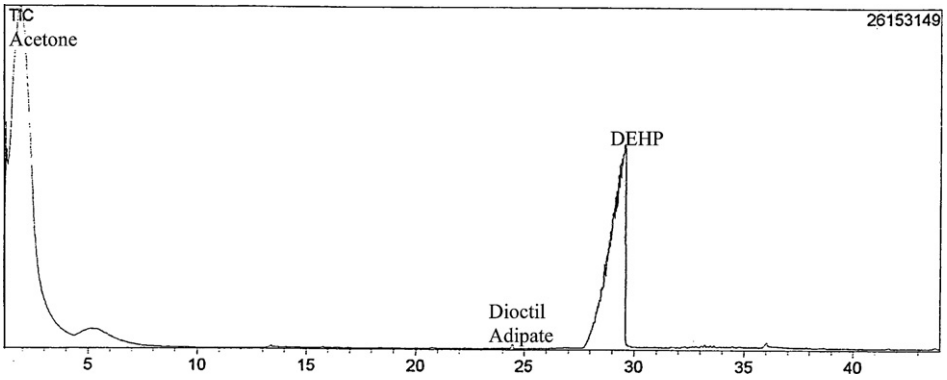
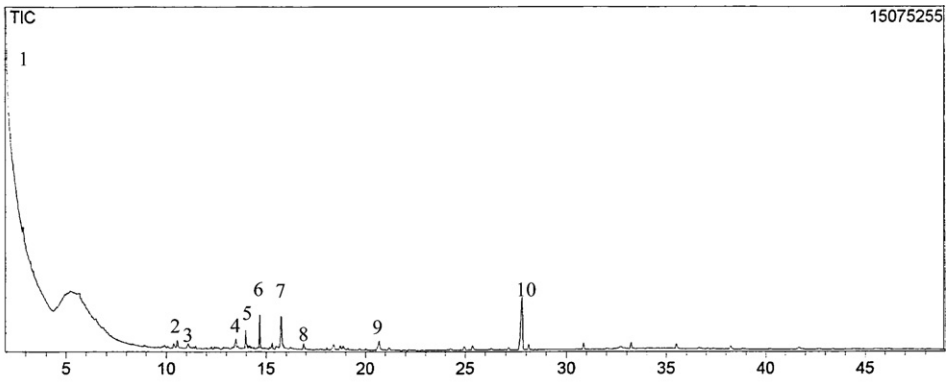


Fig. 5. Gas chromatography and mass spectrometry analyses – contaminated soil in the beginning of the slurry-phase experiment.



1 – acetone
2 - pyruvic acid
3 - octanoic acid, 7-oxo
4 - n-octadecanoic acid
5 - phosphorothioic acid, o,o-diethyl o-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester
6 - di-isobutyl phthalate
7 - di-n-buthyl phthalate
8 - 1-tetradecanol
9 - 9-octadecenoic acid (Z)
10-DEHP

Fig. 6. Gas chromatography and mass spectrometry analyses – contaminated soil in 45th day of the slurry-phase experiment.

5. Conclusions

Standard respirometric were found inadequate for investigating bioremediation in tropical soils, which are typically acidic. Infiltration tests, however, provided an indication of the retention and/or mobility of the pollutant, DEHP, in a structured tropical soil.

Ex situ bioremediation is an appropriate remediation technique for the investigated soil, considering the low soil permeability to water (5.8×10^{-8} m/s), as well as to air (7×10^{-15} m^2). The cement-mixer, used as a reactor, was capable of adequately mixing the soil, nutrients (C:N:P = 100:10:1) and water, and the treatment system achieved a 98.8% DEHP removal (initial concentration = 100 mg/kg) from the soil after 49 days using acclimated microorganisms. DEHP biodegradation could be adequately modeled using a first-order kinetic equation, with a biodegradation coefficient of 0.127 day^{-1} for the specific conditions tested in this study.

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