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Christian Mougin[1], Frederic Dappozze[1], Agathe Brault[1], Christian Malosse[1], Jens Ejbye Schmidt[2], Najat Amellal-Nassr[3], Dominique Patureau[4]

[1]Unité de Phytopharmacie et Médiateurs Chimiques, Route de, Saint-Cyr, 78026 Versailles Cedex, France
Present address: Physicochimie et Ecotoxicologie des Sols d'Agrosystèmes Contaminés, Versailles Cedex, France
[2]Environment & Resources, Technical University of Denmark, Lyngby, Denmark
[3]RITTMO, 24, rue du Moulin, 68740 Nambsheim, France
[4]INRA, Laboratoire Biotechnologie de l'Environnement, Avenue des Etangs, 11100 Narbonne, France

Abstract
We studied the fate of ^{14}C-labelled phthalic acid and benzo[a]pyrene applied to the soil by the way of contaminated sewage sludge in model ecosystems allowing the simultaneous assessment of physicochemical and biological descriptors. Here we show that the mineralisation of phthalic acid is higher than 30% after 90 days in the situation of direct soil contamination, amendment with contaminated digested or composted sludge. It is reduced to 10% in the presence of the raw sludge. In that case, the values of phospholipidic fatty acids and dehydrogenase activity are the highest. By contrast, benzo[a]pyrene is recalcitrant to biodegradation whatever the type of soil contamination. We show also that the chemicals present in the sludge are poorly transferred to soil leachates and plant seedlings.

Keywords Sewage sludge; PAHs; Phthalates; Transfer; PLFAs; Enzymes

Introduction

The intensive development of wastewater treatment in Europe has resulted in the production of increasing amounts of sewage sludge. Actually, landfilling of sludge on soils, mainly from agricultural use, becomes the most important mean of disposal in the European Union, accounting in France for more than 60% of the produced sewage sludge. In a context of modern and sustainable agriculture, the recycling of nutrients and organic matter from organic wastes is thus a cheap way reducing artificial fertilization, and maintaining the biological activity of the soil.

Nevertheless, sludge contains numerous toxic heavy metals, and also organic pollutants that can exert adverse effects on biological life inside and outside the soil ecosystem. That point has received increasing attention from the European Union, because some of the chemicals can be toxic for micro-organisms, and carcinogenic, mutagenic and endocrine disrupters for animals and humans. For that reason, a review concerning priority organic pollutants has been published by European Union in 2001. Furthermore, a draft for future European directive is intended to improve sewage sludge management with respect to human, animal and plant health, quality of ground and surface waters, and long-term quality of the soil (Abad et al. 2005).

Unfortunately, data concerning the fate of pollutants in soils, their transfer to other environmental compartments and their impacts on living beings are mostly obtained from distinct experiments, so that their interpretation remains difficult. Terrestrial model ecosystems have been designed to allow the simultaneous monitoring of physicochemical and biological descriptors, and to overcome that problem. We used such an approach successfully in the case of sludge spiked with pesticides (Ghanem et al. 2006).

An integrated approach has been provided by the Biowaste project, including the treatment of sewage sludge for removal of priority pollutants and the safe application on agricultural land. A long-term field study reported previously that a part of sludges PAHs was preserved in the soils for a long period after sludge addition (Lichtfouse et al. 2005a). In the present laboratory experiment, our ob- jectives are to study in more details the fate of chemicals entering the soil through different types of contaminated sludge, in relation with expression of the soil biological activity. Phthalic acid, the main metabolite formed during the breakdown of numerous phthalic esters in the environment, and benzo[a]pyrene, a chemical representing polycyclic aromatic hydrocarbons (Lichtfouse et al. 2005b), were selected as model pollutants. These xenobiotics exhibit opposite physicochemical properties governing their transformation and mobility in soil-plant-water systems.

Soil and sludge spiking

Experiments have been conducted in terrestrial model ecosystems. The reference one comprised soil only. In that case, 0.7 kg of soil was spiked either with a mixture of labelled and unlabelled phthalic acid (300 kBq), or benzo[a]pyrene (BaP, 370 kBq) to ensure final amounts of 1000 and 80 µg chemical per system.

In the assays with sludge, we considered a "worst case hypothesis" of soil contamination, corresponding to a spreading of 30 T dry sludge ha^{-1}, the maximal value permitted by the French regulation. Each sludge (RS, AS, CS; 28.5 dry sample) was mixed with 0.7 kg soil. Sludge has been previously spiked with the same amounts of chemicals (phthalic acid or BaP) than the soil alone. Finally, total phthalate and hydrocarbon concentrations were 50 and 4 mg kg^{-1} dry sludge.

Experimental

Chemicals

High purity reagents were purchased from Sigma–Aldrich and VWR. Ring-^{14}C-U-phthalic acid (740 MBq $mmol^{-1}$) and 7-^{14}C-benzo[a]pyrene (984 MBq $mmol^{-1}$) have been obtained from American Radiolabeled Chemicals and Sigma-Aldrich. High purity grade solvents were from Carlo Erba.

Soil characteristics

The loamy clayey soil was collected in the 10-20 cm layer of a field in Franche-Comté (east of France). It comprised 23.5% sand, 47.8% silt and 28.5% clay. Its content in organic carbon, total nitrogen and $CaCO_3$ were 1.56, 0.20 and 3.2%, respectively. Soil pH_{wat} was 7.8 and its cationic exchange capacity was 13.3 cmol kg^{-1}. The soil was roughly homogenized and immediately used.

Sludge characteristics

Three types of sludge have been collected in an urban wastewater treatment plant from Franche-

Comté. The plant was located in an urban area and was sized for 50,000 equivalent inhabitants. The types of sludge comprised the non- transformed raw sludge (RS), the anaerobic digested sludge (AS) and the anaerobic digested and composed sludge (CS), whose characteristics are reported in Table 1.

Incubations in terrestrial model ecosystems

Terrestrial model ecosystems consisted in glass cylinders (10 cm i.d. and 50 cm depth with inlets and outlets), filled with 1.8 kg soil at 80% of their moisture holding capacity, and incubated during 3 days. After that period, the upper layer comprising spiked soil alone or soil/spiked sludge mixtures was added and the cylinders were closed. A stream (0.5 l min^{-1}) of wet air was continuously flushed to allow $^{14}CO_2$ trapping in two vials containing 1N NaOH. Incubations were performed during 90 days at 23◦C under 16 h light and 8 h darkness. Leachates were collected after 60 days of incubation by watering the soil with 190 ml water (equivalent to 20 mm rainwater). Wheat seedlings (obtained by the sowing of two seeds after 15 days of incubation) were harvested after a further 45-day period of growth, and dried.

Analytical procedures

NaOH solutions were changed every 7 days. Soil cores (1.5 cm i.d. and 20 cm depth) were performed to determine extractable and bound ^{14}C in the soil and soil/sludge mixtures after 14, 28, 56 and 90 days of incubation. In experiments with phthalic acid, 10 g soil samples were supplemented with 20 ml hot water (60°C) and 5 ml ethanol, and radioactivity was then extracted by shaking (1 min) and sonication (15 min). Samples were then centrifuged at 3000 g for 5 min. Finally, pellets were suspended in 20 ml methanol, briefly shaken and filtered. The efficiency of the extraction protocol was higher than 90%.

Extractable radioactivity associated with benzo[a]pyrene was extracted two times from soil samples (10 g) with acetonitrile (50 ml) by shaking for 1 h. Extracts were then pooled and filtered. The efficiency of the extraction protocol was higher than 95%.

The radioactivity was measured in all liquid fractions (extracts, leachates) by liquid scintillation counting. Non-extractable radioactivity (soil, soil/sludge samples, seedlings) was determined by combustion, followed by liquid scintillation counting.

HPLC analysis was performed by injecting 100 µl of the organic extracts onto an analytical column TSK ODS- 80TM (25 cm 4.6 mm i.d.) set at 30°C. The mobile phase consisted of a mixture of acetonitrile/water (30/70; v/v) at a flow rate of 1 ml min^{-1}. After 1 min, it was increased to 100% acetonitrile in 20 min and maintained during the following 5 min. Radioactivity eluted from the column was monitored.

Phospholipidic fatty acids measurements

First, 2 g soil samples were freeze-dried and ground whereas higher plant materials and gravels are withdrawn. Phospholipidic fatty acids (PLFAs) were extracted using a modification of the Bligh and Dyer (1959) method. Briefly, fatty acids are extracted by shaking in a chloroform/methanol/citrate buffer. The organic phase is then submitted to solid phase extraction on silica gel. Apolar lipids, glycolipids and PLFAs are eluted by chloroform, acetone and methanol, respectively. Then, PLFAs are trans- methylated under mild alkaline condition to yield fatty acid methyl esters. Finally, fatty acid methyl ester C19:0 is added to the mixtures as internal standard and samples are analysed by GC-MS. The method has been validated in several case studies including grassland or cropped soils, and then used in microcosm experiments.
Samples ware then analysed by GC-MS with a mass spectrometer (ion trap Saturn II, Varian) equipped with a VF5-MS column (50 m, 0.20 mm i.d., FT = 0.33, Varian), and helium as a carrier gas (20 psi). The temperature program was 120°C (1 min) to 310◦C at 4°C min^{-1}. Bacterial PLFAs were 15:0, i15:0, a15:0, i16:0, 16:1ω9, i17:0, a17:0, 17:0, cy17, cy19. Fungal PLFA was 18:2ω6,9 (linoleic acid).

Enzyme activities

Measurement of dehydrogenase activity was adapted from Casida et al. (1964). Twenty-four millilitres of distilled water and 1 ml aqueous solution of 5% 1,3,5- triphenyltetrazolium chloride were mixed with 5 g fresh soil in 30 ml centrifuge tubes. Controls were also prepared without the substrate. Incubation was performed at 25°C during 16 h. After that period, 20 ml acetone was added to the mixture which was shaken for 5 min in the dark. A shaking was repeated every hour. Finally, 1,3,5- triphenyltetrazolium chloride was added to the control and all the mixtures were centrifuged 5 min at 6000 g. The absorbance of the supernatant (red colour in case of formazan

formed) was measured at 485 nm.

Urease activity (EC 3.5.1.5) was measured according to Kandeler and Gerber (1988) with slight modification. Fifty millilitres of distilled water was added to 1 g fresh soil and shaken for 10 min. Aliquots of the soil solution (500 µl) were supplemented with 100 µl of 0.4 M urea, which was omitted in the controls. The mixtures were shaken vigorously and incubated at 25◦C for 4 h. After that, 100 µl of a salicylate solution (Hach reagent no. 23952-66, 1 bag dissolved in 1 ml water) was added. The mixtures were shaken and the reaction allowed proceeding for 3 min. Hundred microlitres of a cyanurate solution (Hach reagent no. 23954-66, 1 bag dissolved in 1 ml water) was then added. Controls were supplemented with urea. After mixing, the reaction proceeded a further 30 min. Finally, the mixtures were centrifuged for 2 min at 10,000 g. The absorbance of the supernatant (blue colour in case of NH_4^+-N released) was measured at 610 nm.

Results and discussion

Fate of phthalic acid in terrestrial model ecosystems

Phthalic acid amounted in all soils and mixtures to 50 mg kg^{-1} dry sludge, a value often measured in real samples, because phthalic acid is a common transformation product of numerous phthalic esters. Evolution of phthalic-acid was calculated from mass-balance analysis of the ecosystems incubated with soil alone and soil supplemented with each of the three types of spiked sludge (Fig. 1).

Quite similar amounts of phthalic acid (25–31%) were mineralised during the first 2 weeks of incubation in soil alone, soil with anaerobic digested sludge and soil with composted sludge. By contrast, its mineralisation in soil with raw sludge was lower, representing only 12% of initial radioactivity during the same period. In all cases, the mineralisation of phthalic acid proceeded very slowly until the end of the experiment.

Radioactivity in all the extracts from soil samples represented only a low fraction (less than 6%) of initial radioactivity all along the experiment. For that reason, it was not possible to identify labelled chemicals in the extracts by the mean of HPLC analysis. By contrast, radioactivity was mainly stabilised in the soil as non-extractable residues. The highest amounts of bound residues (65–86%) were found in the soil with raw sludge situation, where mineralisation was the lowest.

Our results suggest than in all situations, excepted in the soil with raw sludge one, phthalic acid

transformation proceeds extensively under an aerobic pathway, leading to both mineralisation and bound residue formation. In addition, total radioactivity counted in these three situations was lesser than 80% of initial radioactivity. So, we cannot exclude a transformation of phthalic acid to volatile compounds (CH_4) that are not trapped according to our experimental setup. In counter part, addition of raw sludge in the soil, thus leading to a high organic load, results mainly in the formation of bound residues due to the high adsorption capacity of this type of sludge.
Radioactivity measured in leachates collected at day 56 was very low when expressed in percentage. However, leachates from soil with raw sludge contained the highest radioactivity (0.03%) and the highest calculated concentration (1.7 µg l^{-1}). In the presence of raw sludge, phthalic acid seems less adsorbed than in the other situations, and not extensively degraded. Thus, it is assumed that the chemical remains more mobile. Only traces of radioactivity have been detected in wheat seedlings.

Fate of benzo[a]pyrene in terrestrial model ecosystems

The second set of experiment concerned BaP. Its amount in all soils and mixtures corresponded to 4 mg kg^{-1} dry sludge, which was two-fold higher than the amount permitted by the French regulation. Mineralisation of BaP in microcosms was very low when compared to that of phthalic acid (Fig. 2). Amounts of trapped $^{14}CO_2$ were the highest and identical in soil and soil with composted sludge mixture (1% after 90 days). By contrast, the amounts evolved in the two others conditions, soil with raw sludge and soil with anaerobic digested sludge, were slower (0.5%). In these two last situations, the mineralisation started significantly after 56 days of incubation (not shown). Finally, in the four situations, a 7-day lag phase was noticed where no mineralisation occurred.
The greatest part of the radioactivity present in the soil has been recovered in the organic extracts, whatever the incubation condition. It amounted to 84-89% of initial radioactivity after 2 weeks. Then, the extractable radioactivity decreased after 2 weeks of incubation, whereas the amount of non-extractable residues increased accordingly. There were no clear differences between the four types of incubation conditions. HPLC analysis of the extracts revealed BaP as the main radioactive compound. According to a previous work (Rama et al. 1998), a more polar and minor peak was attributed to the mixture of BaP quinones.

Taken together, these results suggest a poor microbial activity regarding the transformation of the

hydrocarbon, resulting in a very weak final mineralisation and a moderate stabilisation of the chemical as bound residues.

Previous results obtained in a 25-year field experiment showed that the total PAHs levels increased in the soil during successive sludge additions, and slowly decreased with time after the last treatment (Lichtfouse et al. 2005a). Our present results, showing that BaP mainly adsorbs onto soil particles and may accumulate, confirm the field study.

Only traces of radioactivity (less than 0.01% of initial amount) have been detected in leachates collected from all soils and mixtures containing labelled BaP. As a consequence, calculated concentrations of chemical in the leachates were below the ng l^{-1} level. There was also no transfer of labelled material to wheat plants. These results confirm also the very low mobility of the hydrocarbon within the soil matrix.

PLFA measurements in terrestrial model ecosystems containing phthalic acid

At the beginning of the incubations in the presence of phthalic acid, soil and composted sludge samples contained low levels of bacterial PLFAs, whereas the two other types of sludge were enriched in microbial lipids (Fig. 3). Their levels decreased after mixing with the soil during the first 2 weeks, and remain steady all along the experiment. By contrast, in the presence of raw sludge, high levels of PLFAs have measured at 56 days. Identical profiles have been obtained for fungal PLFAs, and for both types of micro-organisms in the presence of BaP (not shown). It is noteworthy that raw sludge may introduce high levels of micro-organisms in the soil, and also supply nutrients for their development and activity.

Enzyme activities in terrestrial model ecosystems containing phthalic acid

Dehydrogenase activity was low at the beginning of the experiment performed in the presence of phthalic acid (Fig. 4). Then, it remained quite steady until 90 days in three of the incubations conditions: soil alone, soil with anaerobic digested sludge and soil with composted sludge. By contrast, a strong increase has been noticed in the soils with raw sludge along the experiment, with maximal values measured at day 26. Dehydrogenase activity remained always the highest in soil with raw sludge condition, whereas it was the lowest in the soil alone. A quite similar enzyme profile occurred in incubations in the presence of BaP. The highest dehydrogenase activity, a

marker of overall microbial activity, was the highest in the soil supplemented with raw sludge, where PLFA amounts showed also their maxi- mal values, and where phthalic acid transformation was the lowest. That result is probably due to the great availability of nutrients and substrates provided by the sludge, which behave as competitors for phthalic acid or BaP.

In the experiment performed in the presence of phthalic acid, there was no clear difference of urease activity ac- cording to the incubation condition (not shown). Enzyme activity was the highest (around 30 nmol h^{-1} g^{-1} dry soil) between 14 and 56 days of incubation. No or low urease activity has been noticed in the 90-day samples. A similar situation has been noticed in the presence of BaP, with maximal activities amounting only to 11 nmol h^{-1} g^{-1} dry soil. Urease appeared a poor indicator in order to distinguish our incubation conditions and the impacts of different types of sludge. The low activities measured for both enzymes after 90 days could be due to anoxic conditions caused by the addition of water (at 60 days) in order to obtain leachates.

Conclusion

Using experimental ecosystems allowing the simultaneous assessment of physicochemical and biological descriptors, we showed that the mineralisation and stabilisation as bound residues of phthalic acid was extensive in the soil alone, and in the soil amended with digested or com- posted sludge. The chemical was less transformed in the presence of the raw sludge. By contrast, BaP was quite not transformed under similar incubations conditions and ac- cumulated in the soil as sorbed chemical. That last result strengthened a previous study performed at a long-term field scale. The highest phthalic acid transformation occurred in soil/sludge mixtures where PLFA and dehydrogenase activity provided generally their lowest values.

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Table 1. Characteristics sludge samples used in the study

	RS	AS	CS
Dry matter (%)	4	13	48
Organic carbon (C) (%)	1	3	9
Total nitrogen (N) (%)	0.2	0.6	0.6
C/N ratio	5	5	13
P_2O_5 (%)	0.2	1	1
pH_{wat}	6.5	8.3	8.5

RS, raw sludge; AS, anaerobic digested sludge; CS, anaerobic digested and composed sludge

Fig 1. Mass-balance analysis of soil and soil/sludge mixtures (RS, raw sludge; AS, anaerobic digested sludge; CS, anaerobic digested and composed sludge) after treatment with ^{14}C phthalic acid. Colours refer, from the bottom of the figure to the top, to: non-extractable ^{14}C, white; ^{14}C extracted by water/ethanol mixture, black; $^{14}CO_2$, white

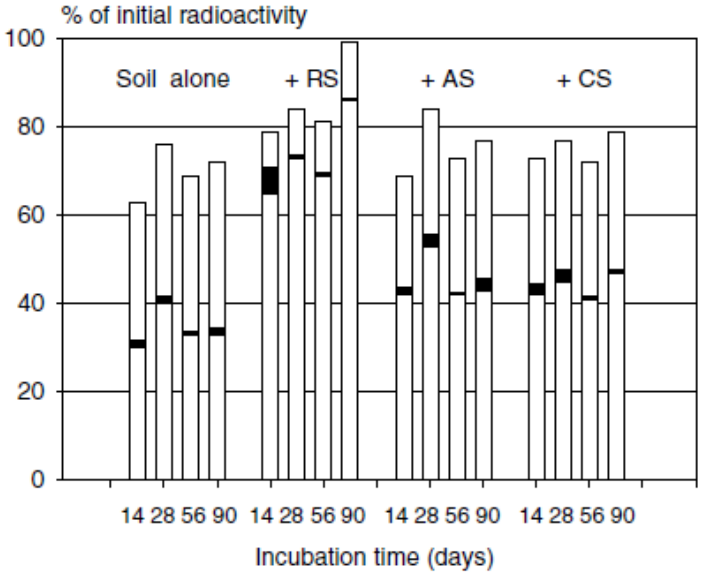

Fig. 2. Mass-balance analysis of soil and soil/sludge mixtures (RS, raw sludge; AS, anaerobic digested sludge; CS, anaerobic digested and composed sludge) after treatment with ^{14}C benzo[a]pyrene. Colours refer, from the bottom of the figure to the top, to: non- extractable ^{14}C, white; ^{14}C extracted by acetonitrile, black; $^{14}CO_2$, white

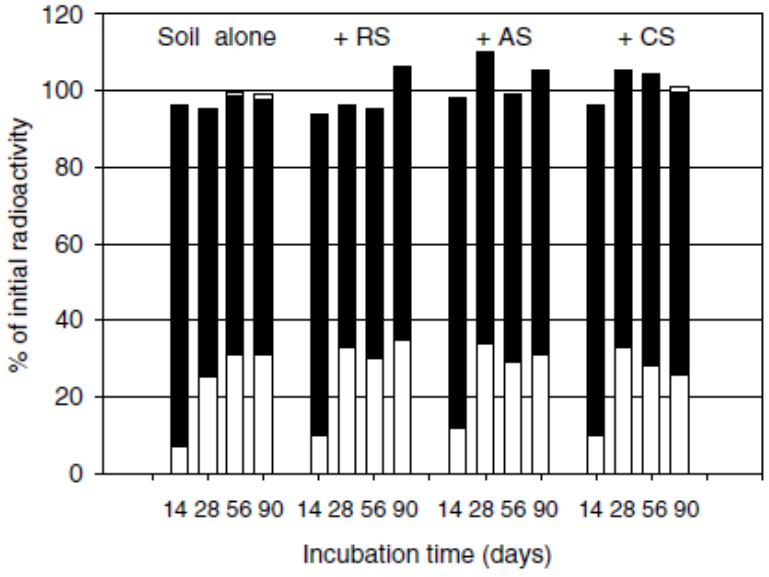

Fig. 3. Bacterial PLFAs in soil samples from terrestrial model ecosystems containing phthalic acid. Symbols refer to: (O) soil alone; (●) soil with raw sludge; (■) soil with anaerobic digested sludge; (▲) soil with anaerobic digested and composed sludge

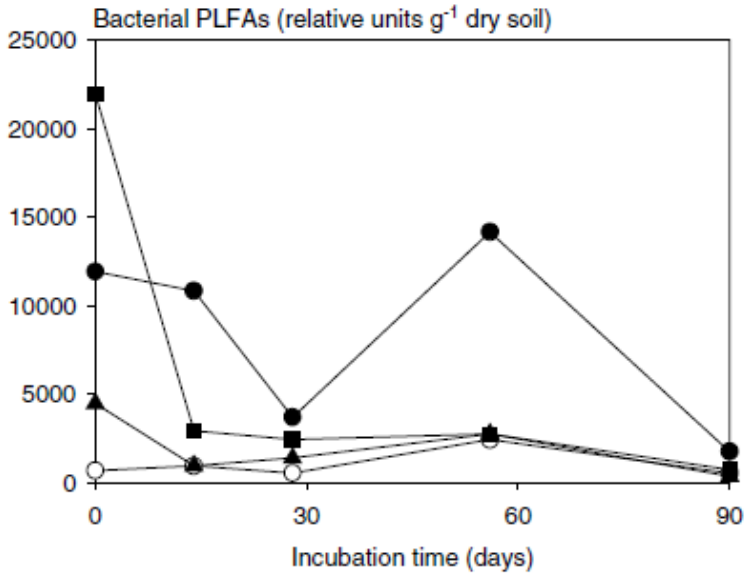

Fig. 4. Dehydrogenase activity in soil samples from terrestrial model ecosystems containing phthalic acid. Symbols refer to: (O) soil alone; (●), soil with raw sludge; (■), soil with anaerobic digested sludge; (▲) soil with anaerobic digested and composed sludge