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Abstract

Biosolids spread onto agricultural soils are potential sources of steroidal hormones able to adversely affect the soil ecosystem. Here we studied the fate of the [4-^{14}C]-17-β-estradiol hormone in laboratory microcosms experiments. Our results show first that only 2.9% of the hormone were mineralized in the soil from a French vineyard. By contrast, the mineralization increased to 9.5% when the hormone was provided in composted biosolids. Second, we found that only a minor part of the estradiol-derived ^{14}C was mobile and partly transferred to soil leachates. Indeed, the hormone was mainly stabilized in the soil as non-extractable residues. Overall, our findings show that estradiol undergoes two main processes, complete degradation and stabilisation. We conclude therefore that the environmental risk of hormones provided to the soil through composted biosolids is negligible under the conditions of our experiments.

Keywords

Composted biosolids, 17 beta-estradiol, soil, transformation, transfer

1. Introduction

In France, the increasing treatment of wastewater has resulted in the production of large amounts of biosolids and sewage sludge that are mainly recycled in farmlands as amendments, fertilizers and soil conditioners. One of the harmlessness criteria in spreading biosolids is their content in natural and man-made chemicals that can interfere with human and wildlife, alter reproduction and development functions (Birkett 2003). The contamination of sludge in persistent organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) has been studied for a long time (Webber and Lesage, 1989; Hall 1995; Blanchard et al. 2004). By contrast, little is known about less conventional contaminants and priority models have emerged among endocrine disrupters (Abad et al. 2005). Unfortunately, estrogens excreted by human and animals were not considered, although their occurrence as potential environmental contaminants has been established (Hanselman et al. 2003). In France, data regarding the elimination of hormones and estrogenic activity in wastewater treatment plants (WWTP), as well as their consecutive impact on the environment after their release from treated waters and biosolids are scarce. For that reason, we developed an integrated approach to provide information to these points. In that context, it has been shown that dehydrated biosolids from an urban WWTP in southern France contained 17β-estradiol in the range of 1-10 $\mu g.kg^{-1}$ dry weight, as well as estrone and 17α-ethinylestradiol (Muller et al. 2008). After composting, these biosolids are applied onto vineyards soils and could adversely affect the soil ecosystem. Here, our specific objective is to study the fate of 17β-estradiol entering an agricultural soil through composted biosolids, in providing the first data related to the French context.

2. Materials and methods

2.1 Chemicals and Reagents

Chemicals and solvents were of analytical grade. All chemicals, including 17β-estradiol, estrone and estriol were purchased from Sigma-Aldrich (France). [4-^{14}C]-17β-estradiol (2035 $MBq.mmol^{-1}$) has been obtained from Dislab'S System (France). Solvents were purchased from Carlo-Erba (France).

2.2 Soil characteristics

The soil was a loam collected in the 10-20 cm layer of an experimental vineyard in Gruissan (France). It comprised 40.4% sand, 43.6% silt and 16.0% clay. Its contents in organic carbon, total nitrogen and $CaCO_3$ were 1.07, 0.08 and 40.9% respectively. Soil pH_{wat} was 8.4 and its cationic exchange capacity (Metson) was 6.3 $cmol^{+}.kg^{-1}$. The soil was roughly homogenized and used without storage for the preparation of the microcosms.

2.3 Biosolid characteristics

The composted sludge was obtained from a composting platform in southern France that treats 15,000 t/year. The secondary sludge treated on the platform came from an advanced WWTP of 120,000 people-equivalent treating mainly domestic wastewater. For composting, mechanical dehydrated secondary sludge is mixed with green waste and sifting refuse (dry matter ratio 20:25:55) and is submitted to ventilated fermentation during 4 weeks. The compost is then sieved (15 mm) and kept until maturity for 8 weeks. After maturing, the compost is stored for 3 months. That final product, applied onto agricultural soils, is used in the present study. Its characteristics are reported in Table 1.

2.4 Terrestrial Model Ecosystem experiments

Here we used the Terrestrial Model Ecosystems (TMEs) successfully developed by our team (Ghanem et al. 2006). Three experimental conditions have been retained for incubations. In the first TME used as a reference (soil), labelled 17β-estradiol (555 kBq) and unlabelled chemical were sprayed onto the soil (2 kg, dw) to ensure the final estradiol concentration of 75 $\mu g.kg^{-1}$ dry soil. In the second TME (soil + low sludge dose), the soil was amended with 2.9 g spiked dry composted sludge (equivalent to 3 T $dw.ha^{-1}$) as an agronomic reality. In that case, the final concentration of hormon was also 75 $\mu g.kg^{-1}$ dry soil/sludge mixture. In the third TME (soil + high sludge dose), the "worst case" hypothesis of contamination, the soil was supplemented with 29 g spiked dry composted sludge (equivalent to 30 T $dw.ha^{-1}$). The concentration of estradiol remained steady in the composted sludge, but its final amount was 750 $\mu g.kg^{-1}$ dry soil/sludge mixture. Composted sludge samples were spiked using an acetonic solution of labelled and unlabelled hormon. Then, the solvent was allowed to evaporate and the sludge samples were "aged" for one day at 4°C to avoid any transformation of the chemicals. The spiked samples were then applied onto the fresh soil, mixed, and the soil was moistened to 80% of its moisture holding capacity. Finally, the TMEs were closed.

2.5 Incubations in TMEs and analytical procedures

The TMEs were incubated for 91 days under 16 h light at 25°C and 8 h darkness at 18°C. A stream (0.5 $L.min^{-1}$) of wet air was continuously flushed through the TMEs to allow $^{14}CO_2$ trapping in NaOH solutions. Moisture was also checked every week and adjusted. After 28 and 91 days of incubation, water was sprayed onto the soils to mimic 20-mm rainfalls and to allow recovery of leachates.

NaOH solutions were changed every 7 days and $^{14}C0_2$ was determined. 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/composted sludge mixtures after 14, 28, 63, and 91 days of incubation. Radioactivity was extracted by shaking with 45 mL acetone in presence of 800 mg hyflosupercel (VWR, France), using a soil/liquid ratio of 1/3. Each extraction (1 h) was performed in duplicate and followed by filtration on 8 g hyflosupercel.

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

After on-line concentration of aqueous solutions on a MCH 10 column (C_{18}, 4 cm x 4.6 mm i.d.; Mougin et al., 1994) or direct injection of 100 µl aliquots of organic extracts, HPLC analysis was performed using an analytical column TSK ODS-80TM (25 cm x 4.6 mm i.d.) set at 30°C. The mobile phase consisted of a mixture of acetonitrile/water (15/85; v/v) at a flow rate of 1 mL.min-1. After one min, it was increased to 100% acetonitrile in 20 min and maintained during the following 4 min. Radioactivity eluted from the column was monitored.

2.6 Bioavailability of 17-β-estradiol in the soil solution

The amounts of 17-β-estradiol in the soil solution were determined using the protocol of Gaillardon and Dur (1995) adapted by Kollmann et al. (2003). Soil and soil/composted sludge samples (10 g equivalent dry matter) were placed in 5-cm diameter Petri dishes to give a 3-4 mm thick layer. Composted sludge samples were freeze-dried and ground to ensure the homogeneity of the mixture with soils. Composted sludge aliquots were then spiked with labelled (6 kBq) and unlabelled estradiol to allow 75 $µg.kg^{-1}$ (soil + low sludge dose) and 750 $µg.kg^{-1}$ dry soil/sludge mixtures (soil +high sludge dose). After adding the chemical, the solvent (ethanol) was left to evaporate for 30 min. The chemicals were sorbed onto the sludge for 24 hours at 4°C before the sludge was mixed with the soil. At this time, an ethanolic

solution of labelled (6.0 kBq) 17β-estradiol was applied to the surface of the soil alone. All the dishes were placed in the dark at 4°C to avoid biotransformation.

Concentrations of estradiol in the soil solution were determined just after soil treatment and after 24 hours of sorption. Two superposed 42.5 mm diameter glass micro fibber filters GF/A (Whatman) were laid on the soil surface and a slight pressure was applied for 10 s to favour wetting of the filters. The upper filter was then recovered. The volume of soil solution and the dissolved radioactivity retained in the filter were determined by weighing and liquid scintillation counting.

3. Results and discussion

3.1 Mineralization of 17β-estradiol in soils with respect to the type of contamination

The mineralization of 17β-estradiol was monitored with respect to the type of soil contamination (Figure 1). When the hormone was spread directly onto the soil, its mineralization proceeded slowly and only 2.9% of the initial compound was transformed to labelled carbon dioxide after 91 days of incubation. That low mineralization of the hormone has been reported in a similar type of soil by Colucci et al. (2001) and in a sandy soil by Fan et al. (2007). The mineralization rate increased when estradiol was contained in the composted biosolid applied onto the soil. In that case, labelled carbon dioxide amounted to 4.9% and 9.5% for respectively, the soil + low sludge dose and soil + high sludge dose situations after 20 days of incubation. At the end of the experiment, these values represented 7.1% for the soil + low sludge dose and 14.6% for the soil + high sludge dose. These percents corresponded to 0.19, 0.32 and 6.27 µg of the initial ^{14}C converted to carbon dioxide. Avoiding the lag-phase for calculations, the first-order mineralization rate constants (*k*) for estradiol were 0.0002 h^{-1} in the soil alone, 0.0018 h^{-1} in the soil + low sludge dose and 0.0017 h^{-1} the soil + high sludge dose. The differences of *k* values clearly demonstrate the importance of the two types of soil contamination on estradiol transformation: direct spreading or amendment with composted biosolids. That stimulation of contaminant mineralization was unusual, as other studies often reported a decrease of the transformation of any contaminant in the presence of biosolids (Dubroca et al. 2005; Ghanem et al. 2006). One first hypothesis should be that the autochthonous microflora of the soil from the vineyard is unable to efficiently degrade estrogenic compounds, and that the biosolids provide efficient microbial inocula that are adapted for the degradation of the hormone. One second hypothesis could

concern a low availability of nutrients for micro-organisms in the soil, where biosolids provide high amounts or organic matter. One last hypothesis may be related to the different sorption of the chemical in the soil and the biosolids that impacts the bioavailability of contaminants for degrading organisms. Nevertheless, in biosolid/soil mixtures, a decrease in the transformation/mineralization of a contaminant is often attributed to a stronger sorption mediated by the organic matter constituting the biosolids (Kollmann et al, 2003).

An experiment was performed to clarify that last point (Figure 2). Just after the soil spreading, estradiol concentration measured in the soil solution was 16.80 ± 3.14 $\mu g.L^{-1}$. It was only 2.21 ± 0.26 $\mu g.L^{-1}$ when the same amount of hormone was provided by the low sludge dose. By contrast, it was increased to 53.87 ± 3.04 $\mu g.L^{-1}$ in the soil amended with the high sludge dose, where the initial amount of hormone was ten-fold higher. After a sorption period of 24 hours, estradiol concentrations in the soil solution were generally reduced, amounting to 7.37 ± 0.73 $\mu g.L^{-1}$ for the soil alone, 2.63 ± 0.42 $\mu g.L^{-1}$ for the soil + low sludge dose, 42.18 ± 0.66 $\mu g.L^{-1}$ for the soil + high sludge dose. These results demonstrated the low bioavailability of estradiol for soil organisms, especially in the situation of good agricultural practice. When expressed in molarity, mean hormone concentrations in the soil solutions ranged from 0.01 to 0.2 µM. These values can be used to design further experiments for the assessment of hormone toxicity on soil (micro)-organisms.

3.2 Distribution of labelled carbon among extractable and non-extractable fractions

The mass-balance analysis of the labelled carbon in the TMEs is shown in Figure 3. Whatever the soil treatment, the radioactivity extracted by the acetonic solution was higher to 95% at the beginning of the experiment. But it was strongly decreased to 17.5-25.0% of the initial labelled carbon after 14 days of incubation, and amounted only to 2.0-8.1% at the end of the experiment. The highest values were always noticed in the non amended soil, where HPLC analysis showed the presence of estradiol and estrone in the acetonic extract between 14 and 28 days of incubation (not shown). In all cases, the remaining part of the radioactivity was mainly stabilized in the soils as non-extractable residues. Our results agree with those reported by Fan et al. (2007) who reported that humic substances of the soils immobilized the majority of estrogenic hormones. Taken together, mass-balance and HPLC analyses suggested two distinct pathways of estradiol biological transformation according to the microbial potentialities under aerobic conditions. One involves the transformation of estradiol to estrone, and can be transitional. The second could lead to a more efficient transformation of

the steroids by ring breakdown and carbon stabilization within the soil constituents (Colucci et al, 2001; Fan et al, 2007).

3.3 Transfer of the radioactive materials to soil leachates

We studied the mobility of labelled carbon to leachates after 28 and 91 days of incubation (Table 2). At both times, labelled carbon was transferred to leachates in very low amounts when expressed in percentage of the applied dose. When expressed as calculated concentrations of equivalent estradiol, the values were lower than those measured in the soil solution after 24 hours. However, they are in many cases significant and exceed the $\mu g.L^{-1}$ threshold for water contamination (2.06 and 5.71 $\mu g.L^{-1}$ equivalent hormone). All the values were increased in the second period of watering, suggesting that the radioactive materials belongs to fragments of the initial hormone resulting from degradation, or is associated to soluble organic matter provided by the biosolids. Unfortunately, we were unable to identify that radioactivity using HPLC.

4. Conclusion

On the basis of the present study concerning a French case study, it is concluded that 17β-estradiol introduced in the soil through spiked composted biosolids undergoes under aerobic conditions both mineralization and stabilization as non extractable residues. As a consequence, the fractions of the chemical either mobile through the soil or available for soil organisms remain very low. In addition, and because of its low specific activity, we used a great amount of labelled 17β-estradiol in our experiments. By contrast, the amounts naturally remaining in the biosolids after composting were lower, and below the limit of detection of the analytical method (3 $\mu g.kg^{-1}$ dry weight, D. Patureau, personnel communication). Taken together, our results suggest that the environmental risk of estradiol provided to the soil through composted biosolids is negligible.

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Table 1. Characteristics of the composted sludge used in the study.

Organic carbon, $g.kg^{-1}$ dry weight	309
Nitrogen (N) total, $g.kg^{-1}$ dry weight	25.8
C /N ratio	12
pH_{Water}	7.6
Dry weight %	57.5

Table 2. Transfer of the radioactive materials to soil leachates (LS: low sludge dose; HS: high sludge dose).

Rainfall	28 days event			91 days event		
Condition	Soil	Soil + LS	Soil + HS	Soil	Soil + LS	Soil + HS
Leachate	% of the initial radioactivity					
	0.02	0.10	0.03	0.04	0.05	0.01
	Equivalent concentration of estradiol ($\mu g.L^{-1}$)					
	0.41	0.62	2.60	0.73	0.71	5.71

Fig. 1. Mineralization of [4-^{14}C]-17β-estradiol in soil spread with the hormone, soil amended with the low amount of spiked biosolids and soil amended with the high amount of spiked biosolids. The results are expressed as means of triplicate. The standard deviation was less than 5 % of the mean.

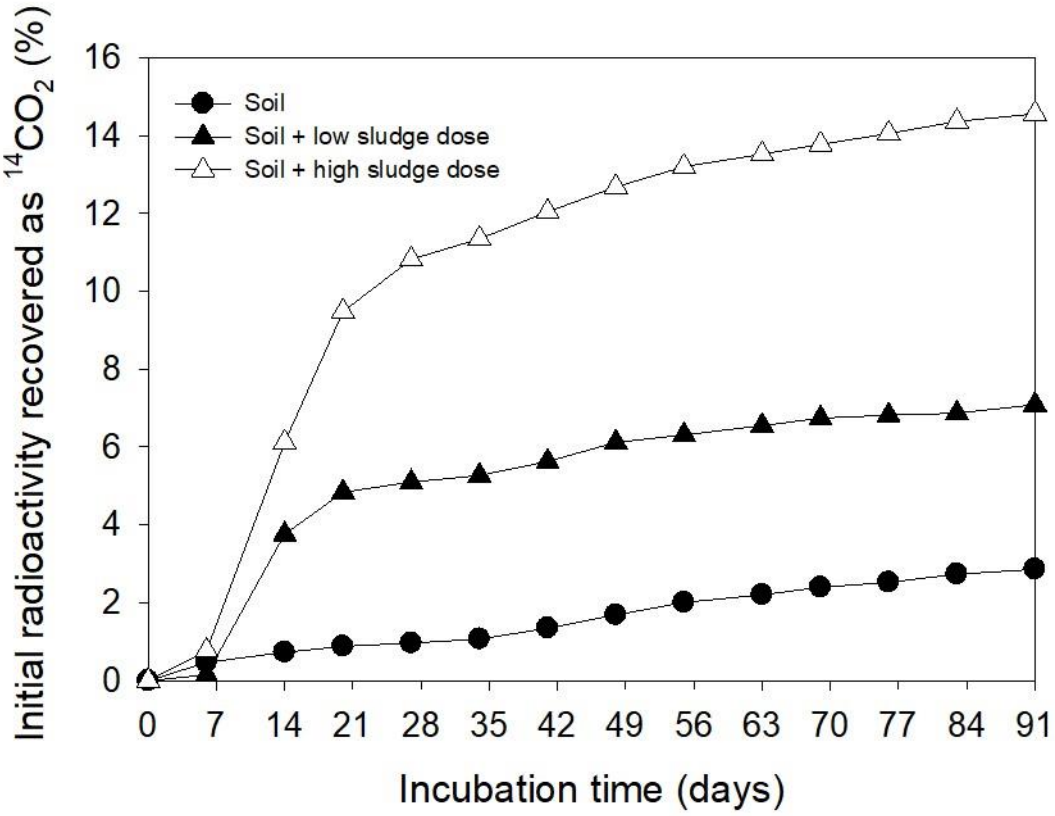

Fig. 2. Concentrations of 17β-estradiol in the soil solutions of soil spread with the hormone, soil amended with the low amount of spiked biosolids and soil amended with the high amount of spiked biosolids. The results are expressed as means ± standard deviation of triplicate.

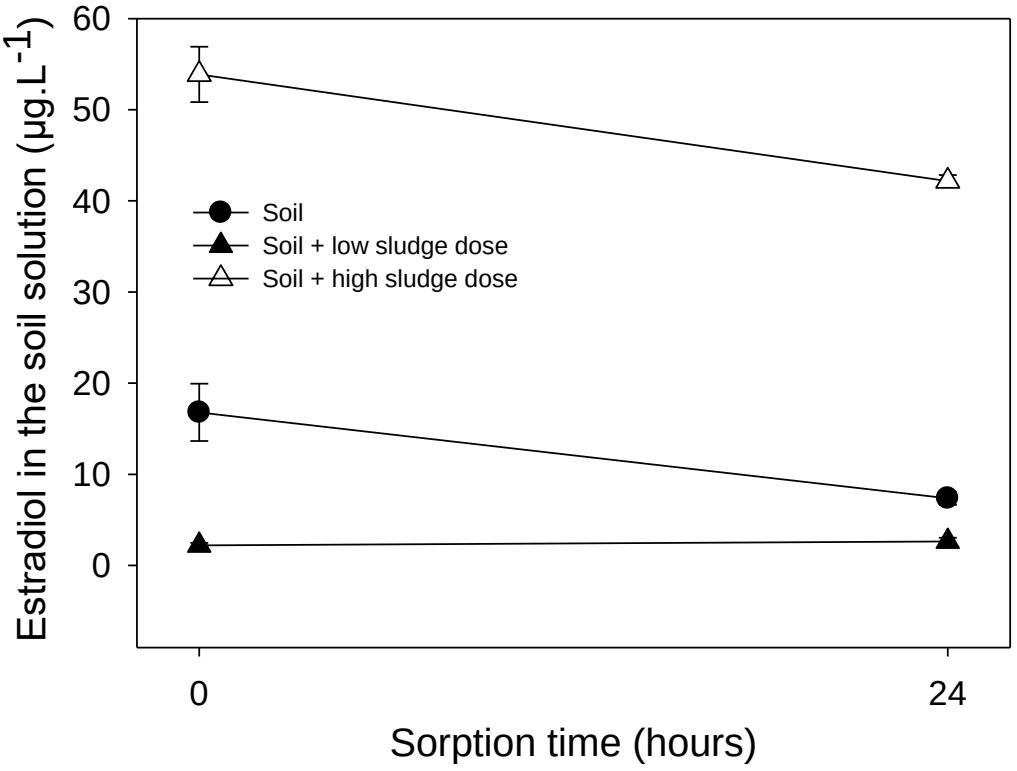

Fig. 3. Mass-balance analysis of the labelled carbon in soil spread with 17β-estradiol, soil amended with the low amount of spiked biosolids and soil amended with the high amount of spiked biosolids. The results are expressed as means of triplicate. The standard deviation was less than 15 % of the mean.