BEHAVIORAL AVOIDANCE TESTS TO EVALUATE EFFECTS OF CATTLE SLURRY AND DAIRY SLUDGE APPLICATION TO SOIL[1]

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SUMMARY

The application of organic wastes to agricultural soils is not risk-free and can affect soil invertebrates. Ecotoxicological tests based on the behavioral avoidance of earthworms and springtails were performed to evaluate effects of different fertilization strategies on soil quality and habitat function for soil organisms. These tests were performed in soils treated with: i) slurry and chemical fertilizers, according to the conventional fertilization management of the region, ii) conventional fertilization + sludge and iii) unfertilized reference soil. Both fertilization strategies contributed to soil acidity mitigation and caused no increase in soil heavy metal content. Avoidance test results showed no negative effects of these strategies on soil organisms, compared with the reference soil. However, results of the two fertilization managements differed: Springtails did not avoid soils fertilized with dairy sludge in any of the tested combinations. Earthworms avoided soils treated with sludge as of May 2004 (DS1), when compared with conventional fertilization. Possibly, the behavioral avoidance of earthworms is more sensitive to soil properties (other than texture, organic matter and heavy metal content) than springtails

Index terms: organic waste management, ecotoxicity tests, *Eisenia andrei*, *Folsomia candida*.

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RESUMO: *TESTES DE COMPORTAMENTO DE FUGA PARA AVALIAR OS EFEITOS DA APLICAÇÃO DE CHORUME DE BOVINO E LODO DE INDÚSTRIA LÁCTEA NO SOLO*

A aplicação de resíduos orgânicos em solos agrícolas não está isenta de riscos e pode afetar os invertebrados do solo. Foram realizados testes ecotoxicológicos de comportamento de fuga usando minhocas e colêmbolos para avaliar os efeitos de diferentes estratégias de fertilização na qualidade do solo e na sua função de habitat. Esses testes foram realizados em solos que receberam: chorume e fertilizantes químicos – a estratégia de fertilização convencional na região; lodo adicionado à estratégia convencional; e solo-referência, não fertilizado. Ambas as estratégias de fertilização contribuíram para mitigar a acidez do solo e não contribuíram para aumento do teor em metais pesados do solo. Os resultados dos testes de fuga não mostraram efeitos negativos dessas estratégias nos organismos do solo, quando se comparam com os do solo-referência. Contudo, quando as duas estratégias de fertilização foram comparadas entre si, obtiveram-se resultados diferentes. Os colêmbolos não evitaram os solos fertilizados com lodo em nenhuma das combinações testadas. No entanto, as minhocas evitaram os solos que receberam lodo desde maio de 2004 (DS1). Possivelmente, o comportamento de fuga das minhocas é mais sensível às características do solo (que não a textura, a matéria orgânica e o teor de metais pesados) do que o dos colêmbolos.

Termos de indexação: manejo de resíduos orgânicos, ensaios de ecotoxicidade, Eisenia andrei, Folsomia candida.

INTRODUCTION

Organic waste accumulation occurs over years due to the intensification of agricultural and livestock activity, industrial production and population increase in urban areas. On the other hand, agricultural intensification is gradually reducing the organic matter content in soils, thus affecting crop productivity. This is mainly a concern in Mediterranean countries, where humid winters and high summer temperatures lead to high organic matter mineralization rates (García et al., 2000). In this context, organic waste recycling in agricultural soils is one of the recommended ways to dispose of organic waste, which enriches soils with organic matter and nutrients (Petersen et al., 2003; Antolín et al., 2005). However, this practice is not risk-free, because the wastes may contain pollutants and pathogens that pose a risk to plants, animals and human health (Westerman & Bicudo, 2005).

For this reason, legislative and normative documents, rules and codes of good agricultural practice are established and frequently revised to ensure soil and water quality as well as to protect animal and human health. Moreover, the permitted limits of organic waste disposal in soils, established based on chemical analyses and ecotoxicological biological tests are often neglected at the regulatory level.

Nevertheless, ecotoxicological tests are being used to obtain information about the effects of organic waste on soil quality and habitat of soil organisms (Crouau et al., 2002; Domene et al., 2008; Moreira et al., 2008; Natal-da-Luz et al., 2009a,b). In short-term ecotoxicological tests, behavioral avoidance tests are highly sensitive to evaluate soil contamination (Yeardley et al., 1996; Natal-da-Luz et al., 2004; Aldaya et al., 2006; Niemeyer et al., 2006). These tests have a great potential for evaluation of the soil as habitat (Hund-Rinke & Wiechering, 2001; Hund-Rinke et al., 2003) and as an early screening tool in assessments of lower levels of ecological risk at contaminated sites (Loureiro et al., 2005; Natal-da-Luz et al., 2004). These tests are based on the principle that organisms respond to unfavourable conditions, leaving or avoiding a contaminated soil. They constitute a quick and cost-effective means to detect low concentrations of organic contaminants (Yeardley et al., 1996).

Results of different studies using biological assays show that the suitability of a bioassay to characterize wastes depends on their origin, treatment and pollution impacts (Domene et al., 2008, Natal-da-Luz et al., 2009a,b).

The aim of this study was to perform a preliminary ecotoxicological evaluation of the soil quality degradation after slurry and dairy sludge application to agricultural soils. Behavioral avoidance tests with *Eisenia andrei* (Oligochaeta) and *Folsomia candida* (Springtails) were performed to complement soil chemical analyses.

MATERIALS AND METHODS

The experiment was conducted in fields in Vilalba (Lugo, NW Spain), with mainly humic, haplic and gleyic Umbrisols (FAO, 1998). In this region, the main agricultural activities are beef and dairy cattle and grassland management. Conventionally, chemical fertilizers and cattle slurry are widely applied after every forage cut. Application rates of chemical fertilisers and cattle slurry are based on the annual nutrient requirements of mixed pasture, not exceeding 170 kg ha^{-1} yr^{-1} N for organic materials.

There is a dairy processing and packaging plant in the region. Dairy industry effluents undergo a biological treatment that turn them into a semi-liquid sludge. Nowadays, this sludge is reapplied as a fertilizer to complement the conventional fertilization management. A single annual application of 80 m^3 ha^{-1} of dairy sludge (DS) to pastures at the beginning of the growing season is currently recommended to achieve best yields, aside from while favouring clover in competition with grass (López-Mosquera et al., 2001). The annual quantities of heavy metal applied in this sludge should never exceed the maximum levels determined by the Sludge European Directive (European Commission, 1986).

In March 2007, soil was collected from the top 15 cm soil from two close fields under two fertilization managements (1 and 2): one fertilized conventionally (C) (i.e., cattle slurry and chemical fertilizers) and the other including DS. At this time, a total volume of 104.18 m^3 ha^{-1} dairy sludge had been applied to field DS1 (distributed as 69.79 m^3 ha^{-1} in 2004 and 34.39 m^3 ha^{-1} in 2006) and 149.86 m^3 ha^{-1} to field DS2 (distributed as 107.32 m^3 ha^{-1} in 2005 and 42.54 m^3 ha^{-1} in 2006). The tested soils treated with cattle slurry (C) and dairy sludge (DS) had in common: a low dry matter content (< 20 g L^{-1}), basic pH (near 7) and heavy metal concentration below legal limits. The organic wastes differed mainly regarding potassium (higher in cattle slurry) and for calcium, chromium and lead (higher in DS). "Reference Soil" (R) samples were also collected from a shrub area, free of pesticide and fertilizer inputs, which represents the original landscape of the region.

In the laboratory, soils were air-dried and sieved through a 2 mm mesh for chemical characterisation and through a 5 mm mesh for the avoidance tests.

Three replicates of every soil sample were analysed for pH in water and in KCl (in a w/v suspension of 1:1 and 1:2,5, respectively), total C and N by combustion, extractable P using $NaHCO_3$ (Olsen & Dean, 1965), exchange cations (Ca^{2+}, Mg^{2+}, Na^+, K^+, and Al^{3+}) by atomic absorption spectrophotometry (Peech et al., 1947), texture by a discontinuous sedimentation method (Guitián & Carballas, 1976), water holding capacity (ISO, 1998) and heavy metals (Cr, Cd, Pb, Zn and Ni) quantified by atomic absorption after HNO_3 digestion (USEPA, 1995).

Avoidance tests were performed following the guidelines established by ISO 17512-1 for earthworms (ISO, 2008) and by ISO/CD 17512-2 for springtails (ISO, 2007). Earthworms *Eisenia andrei* (Oligochaeta: Lumbricidae) with well-developed *clitellum* and 10–12 day old springtails *Folsomia candida* (Collembola: Isotomidae) were selected for the test. The earthworms and springtails were maintained in laboratory cultures under a photoperiod of 16 h light and 8 h dark at 20 ± 2 °C. Rectangular plastic containers (length 20 cm; width 12 cm; height 5 cm) and cylindrical plastic containers (∅: 7 cm, height 6 cm) were used, respectively, for *E. andrei* and *F. candida.* The moisture content of each soil was adjusted to 50 % of the corresponding water holding capacity. Soil pH and moisture were quantified at the beginning and end of the tests. In both tests, containers were divided with cardboard in two sections that were filled with equal amounts of dry matter of the test soil and reference/control soil (200 g for tests with *E. andrei* and 30 g in tests with *F. candida*). The following combinations were tested: i) all pasture fields (C1, C2, DS1 and DS2) against reference soil and ii) the two fields of each set, i.e., sludge-treated fields (DS1, DS2) against conventionally fertilized fields (C1, C2) as "control soil". Five replicates per combination were performed. After soil addition the cardboard division was removed and 10 earthworms, previously washed and dried with absorbent paper, or 20 springtails, were carefully placed on the midline of each container. To keep individuals from escaping and to reduce water loss, the test containers were covered with a lid. For test containers with earthworms, a few holes were drilled in the lids to allow aeration. The tests were run for 48 h at 20 ± 2 °C with a photoperiod of 16 h light and 8 h dark. At the end of the exposure time, the cardboard division was reinserted on the middle line of each container and individuals of both sections were counted. Earthworms found on the midline of the test container were counted as 0.5 individuals. For tests with springtails, the soil of one section was emptied into another container and both containers were flooded with water. Afterwards, a few drops of pen ink were added and springtails floating on the water surface were counted.

One-way Analysis of Variance (ANOVA) was used to compare the effects of different fertilization strategies on soil parameters. To identify the effects of fertilization strategies on individual sites, planned (orthogonal) comparisons were performed. The effects of the following site treatments were analyzed in three replications: within fertilized fields and between fertilized and reference soil. Avoidance test data were analysed using Fisher's exact test (Zar, 1996), which compares the observed distribution of the individuals in a specific tested combination with the theoretical distribution where a non-avoidance response is assumed as null hypothesis. For avoidance tests, a one-tailed hypothesis was applied, since only an avoidance response regarding the tested soil can be considered (Natal-da-Luz et al., 2004). In this case, the null hypothesis, rejected for $p \leq 0.05$, considers that at least half of the organisms stay in the tested soil, indicating an absence of avoidance response for that soil.

RESULTS AND DISCUSSION

The differences between the tested soils were statistically significant for the analysed physical-chemical properties. Values for pH H_2O, pH KCl, extractable P as well as for exchangeable Ca and Mg were higher in pasture soils (C and DS) than the reference soil (R) and they had a loamy-sand or a loamy-clay-sand texture. On the other hand, reference soil contained the highest organic matter (OM) content, C/N rate, exchangeable Al content, Al saturation, heavy metal concentrations (significance depending on the metal) and had a loamy texture (Table 1).

Table 1. Physical-chemical soil properties and mean comparisons between fertilization managements (mean ± standard deviation)

Physical-chemical property	Sampled field				
	C1	DS1	C2	DS2	R
pH H_2O	6.18 ± 0.01	5.73 ± 0.01	5.77 ± 0.03	5.86 ± 0.01	5.08 ± 0.02
pH KCl	5.20 ± 0.01	4.50 ± 0.01	4.66 ± 0.02	4.78 ± 0.01	4.08 ± 0.01
OM (%)	12.20 ± 0.66	10.16 ± 0.36	11.25 ± 0.26	11.23 ± 0.17	13.39 ± 0.17
N (%)	0.56 ± 0.03	0.49 ± 0.02	0.53 ± 0.02	0.57 ± 0.01	0.51 ± 0.00
C/N	12.52 ± 0.01	11.99 ± 0.01	12.37 ± 0.09	11.51 ± 0.04	15.24 ± 0.13
P (mg kg^{-1})	22.19 ± 0.20	8.55 ±0.46	21.47 ± 0.52	23.76 ± 3.48	4.88 ± 0.20
Ca^{2+} ($cmol_c$ kg^{-1})	7.01 ± 0.05	3.02 ± 0.25	3.29 ± 0.19	3.59 ± 0.04	0.68 ± 0.05
Mg^{2+} ($cmol_c$ kg^{-1})	0.87 ± 0.00	0.65 ± 0.05	0.74 ± 0.07	0.91 ± 0.04	0.50 ± 0.04
Na^+ ($cmol_c$ kg^{-1})	0.29 ± 0.01	0.30 ± 0.03	0.30 ± 0.03	0.33 ± 0.00	0.30 ± 0.01
K^+ ($cmol_c$ kg^{-1})	0.54 ± 0.02	0.26 ± 0.02	0.59 ± 0.18	0.27 ± 0.00	0.50 ± 0.04
Al^{3+} ($cmol_c$ kg^{-1})	0.18 ± 0.00	0.72 ± 0.03	0.93 ± 0.14	0.70 ± 0.01	1.65 ± 0.06
Al sat. (%)	2.06 ± 0.00	14.54 ± 0.97	15.92 ± 0.78	12.04 ± 0.02	45.53 ± 1.32
CEC	8.89 ± 0.07	4.95 ± 0.32	5.85 ± 0.59	5.80 ± 0.09	3.62 ± 0.16
Cr (mg kg^{-1})	8.43 ± 0.18	16.40 ± 1.10	13.58 ± 0.08	12.53 ± 0.03	19.30 ± 0.55
Cu (mg kg^{-1})	8.45 ± 0.10	14.98 ± 1.03	11.45 ± 0.00	11.48 ± 0.08	21.63 ± 0.27
Pb (mg kg^{-1})	0.50 ± 0.50	2.75 ± 0.25	6.25 ± 0.25	5.50 ± 1.00	5.00 ± 0.50
Zn (mg kg^{-1})	23.81 ± 0.14	31.84 ± 1.00	30.70 ± 0.28	29.00 ± 0.62	33.09 ± 0.45
Ni (mg kg^{-1})	0.00 ± 0.00	3.50 ± 0.40	2.00 ± 0.05	0.78 ± 0.78	9.05 ± 1.15
Sand (%)	68.75	60.57	57.60	57.65	38.77
Silt (%)	16.95	20.85	22.75	22.30	37.77
Clay (%)	14.30	18.58	19.64	20.05	23.46
Texture	Loam-sandy	Loam-sandy	Loam-sandy	Loam-clay sandy	Loamy

	Significance of Mean Planned Comparisons (a)										
	C-R	DS-R	C-DS	C1-DS1	C2-DS2	C1-C2	DS1-DS2	C1-R	C2-R	DS1-R	DS2-R
pH H_2O	***	***	ns	***	***	***	***	***	***	***	***
pH KCl	***	**	*	***	***	***	***	***	***	***	***
OM (%)	**	***	*	***	ns	**	**	**	***	***	***
N (%)	ns	ns	ns	***	*	*	***	**	ns	ns	**
C/N	***	***	***	***	***	*	***	***	***	***	***
P (mg kg^{-1})	***	*	ns	***	ns	ns	***	***	***	*	***
Ca^{2+}	***	*	*	***	*	***	***	***	***	***	***
Mg^{2+}	**	**	ns	***	***	**	***	***	***	**	***
Na^+ ($cmol_c$ kg^{-1})	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
K^+ ($cmol_c$ kg^{-1})	ns	***	***	**	***	ns	ns	ns	ns	**	**
Al^{3+} ($cmol_c$ kg^{-1})	***	***	ns	***	**	***	ns	***	***	***	***
Al sat. (%)	***	***	ns	***	***	***	**	***	***	***	***
CEC	***	ns	*	***	ns	***	**	***	***	***	***
Cr (mg kg^{-1})	***	*	*	***	*	***	***	***	***	***	***
Cu (mg kg^{-1})	***	***	**	***	ns	***	***	***	***	***	***
Pb (mg kg^{-1})	ns	ns	ns	***	ns	***	***	***	*	***	ns
Zn (mg kg^{-1})	**	ns	ns	***	**	***	***	***	***	*	***
Ni (mg kg^{-1})	***	***	ns	***	*	**	***	***	***	***	***

C1 and C2: fields of each set treated with conventional fertilization. DS1and DS2: Fields of each set treated with sludge as of May 2004 (DS1) or May 2005 (DS2). R: reference soil. (a) Planned contrasts (orthogonal): 1) compare means recorded in each fertilization management and with reference soil (first three columns), 2).compare means in each field, strategies and soil reference. Significance level: *: p≤ 0.05, **: p≤ 0.010, ***: p≤ 0.001.

The acidic properties of the reference soil are representative of the soils of this region. Conventional fertilization and/or sludge disposal has contributed to soil acidity mitigation, increasing pH and reducing Al saturation (Table 1). Many studies showed that organic residues contribute to reduce problems with soil acidification, since they reduce Al toxicity in addition to contributing to OM and nutrient enrichment (Mokolobate & Haynes, 2002). In addition, the low concentrations of heavy metals found in the DS used in this study did not cause an increase in soil concentration, compared with reference soil (Table 1). Nowadays, the production of sludge and other organic wastes from varied sources is on the rise. Sludge from dairy industries is considered to contain lower levels of heavy metals and organic pollutants than sewage sludge from other sources (Bertsch, 2000; López-Mosquera et al., 2005). For this reason, some national programmes encourage a different political approach for this sludge (Issa et al., 2007).

When the pasture soils under different fertilization managements were compared, the values of pH KCl, OM, C/N rate and exchangeable Ca and K were highest in conventionally fertilized (C) fields. On the contrary, values of Cr and Cu were highest in fields fertilized with DS (mean comparisons in Table 1). Moreover, differences were detected among studied soils, even when the same OM source was used (C1-C2 and DS1-DS2) (Table 1). Dairy sludge was applied to Field DS2 later than to DS1 (see Materials and Methods), but total amounts applied to DS2 were higher, contributing to better soil properties in this soil (Table 1).

Results of the avoidance tests are shown in figure 1. No mortality was observed in any test container.

When the sown pasture soils (C and DS) were compared with reference soil (R), no behavioral avoidance response by either earthworms or springtails was found (Figure 1). This could be expected due to the low heavy metal contents in the tested soils (Table 1). Moreira et al. (2008), evaluating the loss of habitat function after organic waste disposal, also observed no avoidance response of these organisms in any treatment applied. In that study, earthworms preferred soils fertilized with sludge or compost to the artificial OECD's soil (Organization for Economic Co-operation and Development). The more favourable properties to organism development observed in the tested soils, e.g., the higher pH values and nutrient concentrations (mainly P and Ca) (Edwards & Bohlen, 1996), could also explain the no-avoidance response of these organisms. However, despite the no-avoidance response of earthworms and springtails to the pasture soils, chronic effects (eg. on reproduction, bioaccumulation) after longer exposure periods cannot be ruled out.

In a comparison of the different fertilization strategies, different results were observed in the tests with earthworms, depending on the tested combination (Figure 1). Comparing C2 (with conventional fertilization) and DS2 fields (with sludge application as of May 2005), earthworms did not avoid soils where

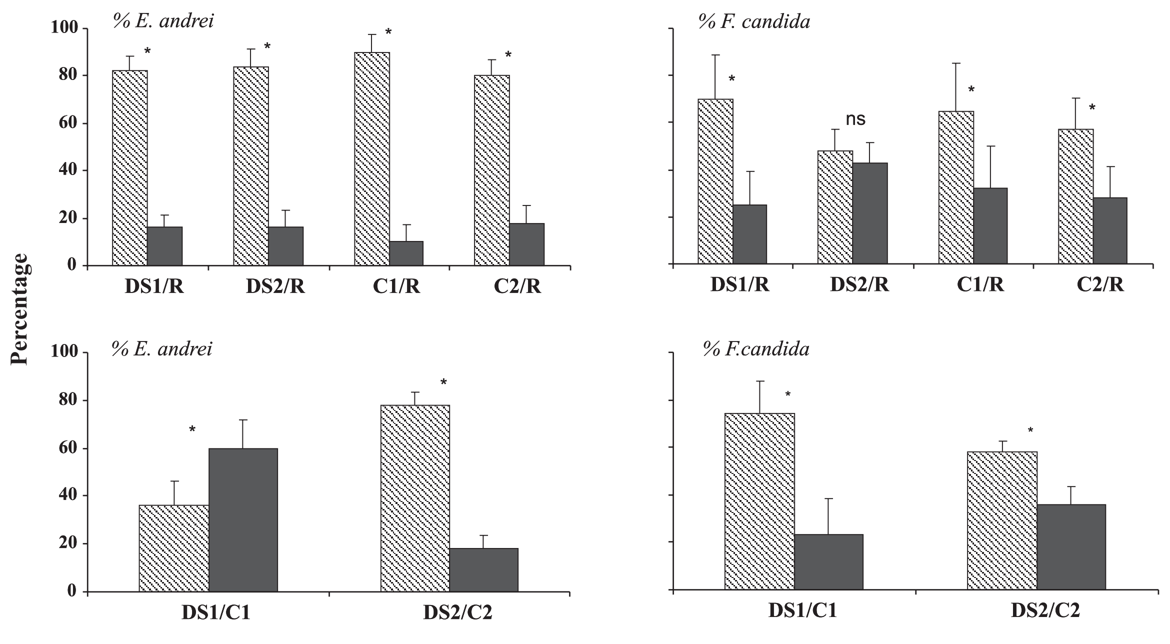

Figure 1. Avoidance response of earthworms (*Eisenia andrei*) and springtails (*Folsomia candida*) in different tested soils. C1 and C2: fields treated with conventional fertilization. DS1, DS2: Fields treated with sludge as of May 2004 (DS1), May 2005 (DS2). R: reference soil. Values are the average percentage of organisms found on control or tested sides. Vertical lines represent standard deviation. *: significantly higher percentage of organisms on the control side than on the tested side ($p < 0.05$).

sludge had been applied (Figure 1). On the other hand, earthworms avoided sludge -treated soils, when C1 (with conventional fertilization) and DS1 (with sludge application as of May 2004) fields were compared (Figure 1). These different responses could be related with differences in soil properties. Natal-da-Luz et al. (2008), testing combinations of artificial soils with different OM content and texture, showed that the avoidance response of soil organisms could be influenced by these properties. According to these authors, earthworms preferred soils with higher OM content and coarse texture; this response is stronger if the difference between soils is high, regardless of the soil contamination level. In this case, both soils (C1 and DS1) had the same texture type, but all chemical properties analysed (except Na) were significantly different (Table 1). Favourable properties of soil C1 could have influenced the earthworm behaviour. The positive effect of suitable Ca and pH values for earthworms is well-known (Edwards & Bohlen, 1996). However, little information is available in literature concerning other soil chemical parameters. Some authors described toxic effects of Al to these organisms (Philips & Bolger 1998; van Gestel & Hoogerwerf, 2001), but to date this soil property is not taken into account in behavioural avoidance tests. In this study, comparing both soils (DS1 and C1) high significant differences ($p \leq 0.001$) were observed for Al saturation (Table 3) and earthworms preferred soils with the lowest values (DS1) (Figure 1). Also, some soil propoerties not analysed in this study may be involved.

On the other hand, springtails did not avoid soils fertilized with DS in any of the tested combinations (Figure 1). Hence, soils fertilized with complementary DS did not affect the soil habitat function of these invertebrates. The results of other studies involving the ecotoxicological assessment of organic wastes, including sludge from agro-industries, using *F. candida,* were similar (Natal-da-Luz et al., 2009a). Springtails were less influenced by soil chemical parameters than earthworms. This is in agreement with Natal-da-Luz et al. (2008), who considered that springtails were better suited for avoidance tests performed in natural soils with different chemical properties.

CONCLUSIONS

1. Both fertilization strategies, conventional or with dairy sludge, contributed to soil acidity mitigation and caused no increase in soil heavy metal content.

2. Results of avoidance tests showed no negative effects of conventional or dairy sludge fertilization on soil organisms, compared with the reference soil.

3. Different results were obtained for earthworm behaviour in response to fertilization strategies: soils where sludge had been applied as of May 2005 (DS2) were not avoided, but avoidance was observed in sludge-treated soils as of May 2004 (DS1), compared to conventional fertilization. A possible explanation involves soil properties, other than texture, organic matter and heavy metal content.

4. Springtails were less influenced by soil chemical properties than earthworms and did not avoid soils fertilized with dairy sludge in any of the tested combinations.

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