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ASSESSMENT OF BIOSOLIDS IN EARTHWORM CHOICE TESTS WITH DIFFERENT SPECIES AND SOILS

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ABSTRACT
Earthworm avoidance response is a new tool for rapid and efficient screening of potentially toxic substances added to soil environments. This technique was used to determine if five common, ecologically different earthworm species (*Allolobophora chlorotica*, *Aporrectodea longa*, *Aporrectodea caliginosa, Eisenia fetida* and *Lumbricus terrestris*) avoid soils amended with six biosolids (treated sewage sludge) applied at rates equivalent to realistic field rates of 0, 2, 5, 10 and 20 t ha^{-1}. The results showed that *A. chlorotica*, *E. fetida* and *L. terrestris* were attracted by low concentrations of biosolids (2 t ha^{-1}), whereas they avoided the highest concentration (20 t ha^{-1}). The other species did not show any preferences. An additional treatment comparing the behaviour of *E. fetida* in natural and artificial soil suggested that the type of soil can alter the preference of earthworms. Comparisons of behavioural and actual toxicity data for the same six biosolids suggest that avoidance responses by earthworms are sensitive enough to reflect different toxicities of biosolids. It is concluded that earthworm avoidance behaviour offers an ecologically relevant tool for screening the deleterious rate-effect of biosolid amended soils.

KEYWORDS: avoidance-test, earthworms, biosolids, *E. fetida,* treated sewage sludges.

1. INTRODUCTION

Sewage sludge generation and waste management is one of the most important environmental issues society has to tackle. Agricultural land application is the most commonly used method for sludge disposal (Gray, 2005). However, the disposal of sludge on land as fertilizer is associated with environmental problems due to the presence of nutrients and various toxicants such as heavy metals and pathogens that can contaminate soils, ground and surface water (Ozores-Hampton *et al.*, 2005).

European and Irish law (Council Directive 86/278/EEC; S.I. 183/1991; S.I. 148/1998; S.I. 267/2001; S.I. 378/2006) aim to promote the recycling of municipal sludge in agriculture and to set standards to protect the environment and food quality. European legislation which regulates sewage sludge amendments in agriculture land (86/271/ECC) or influence indirectly their use (91/676/ECC) imposes heavy metal limits values and nitrate/phosphorus regulation.

In recent years, different technologies have been developed for the treatment and decontamination of sludge prior to the use on land (Lo and Chen, 1990; Metcalf and Eddy, 2003; Strasser *et al.,* 1995). Biosolids, also known as treated or stabilized sewage sludge, are the final products of urban wastewater treatments employing standardised technologies. The amount of Biosolids applied to an agricultural land is dictated by their heavy metals and nutrient content (nitrogen and phosphorus) and by the crop demands.

However, it is also paramount to know what the ecological impacts of biosolids are likely to be on soil organisms, their diversity and their functions. Various ecotoxicologial methods have been used

to test such ecological impacts of sewage sludges on soil using soil invertebrates (Butt, 1999; Domene *et al.*, 2008; Pandard *et al.*, 2006; Conteras-Ramos *et al.*, 2009; Carbonell *et al.*, 2009) and in particular avoidance tests were performed with earthworms (Moreira *et al.*, 2008; Natal-da-Luz *et al.*, 2009; Kobetičová *et al.*, 2010). Based on feeding habits, earthworms are dived in two main ecological categories, detritivores and geophages (Curry and Schmidt, 2007). Detritivores are divided into two groups, the epigeics which are restricted to organic-rich horizons (litter or surface dwellers and compost worms), such as *Eisenia fetida,* and anecics which live in vertical burrows in the soil profile but feed preferentially on surface litter (deep burrowers), including *Aporrectodea longa* and *Lumbricus terrestris* (Bouché, 1977). Geophages (*endogeics* sensu Bouché, 1977) feed on the mineral soil and they produce mostly horizontal galleries near the soil surface, such as *Aporrectodea caliginosa* and *Allolobophora chlorotica* (Curry, 1994).
Acute chemical sensitivity chemoreceptors and dispersion ability give earthworms capabilities for avoiding unfavourable micro-habitats (Stephenson *et al.*, 1998). Recently, different avoidance responses of earthworm species to selected chemicals have been reported (Lukkari *et al.*, 2005; Garcia *et al*., 2008), suggesting that tests based on avoidance behaviour have great potential to give quick and ecologically relevant information in risk assessment procedures. Research is required to develop, validate and standardise these methods.
The overall objective of the present study was to develop earthworm avoidance tests for biosolid toxicity screening. The specific aims were as follows: 1) to evaluate the potential of using different earthworm species (*E. fetida*, *A. caliginosa, A. chlorotica, A. longa* and *L. terrestris*) to assess the toxicity of five biosolids; 2) to compare the efficiency of new avoidance tests and established reproduction tests; 3) to investigate the avoidance behaviour of *E. fetida* to biosolid amendments in two different substrates: natural and artificial soils.

2. METHODS

2.1. Test substrate

Biosolids from five treatment plants (sources) throughout Ireland and one plant in Pueblo, Colorado, USA, were investigated for their effects on five different earthworm species. The sources of Biosolids were; Biosolid 1 Dublin (Ringsend), Biosolid 2 Waterford (Dungarvan), Biosolid 3 Cork (Little Island), Biosolid 4 Limerick (Dunlickey), Biosolid 5 Kildare (Osberstown) and Biosolid 6 USA (Colorado). All Irish Biosolids, 160 litres each, were collected during July 2007 and stored in sealed plastic drums. The USA Biosolid was obtained in 2008 and was stored at room temperature. Drying temperatures and dryer type used in the production of each Biosolid are given in Table 1. Chemical analysis of each Biosolid was obtained by means of Inductively Coupled Plasma Mass Spectrometry (ICP–MS), detailed results are reported by Artuso *et al.* (2010).

Table 1. Biosolid production temperatures (^{o}C), dry matter content and dryer type

	Biosolid 1	Biosolid 2	Biosolid 3	Biosolid 4	Biosolid 5	Biosolid 6
Dryer temp.	350-450	350-450	118-175	275-325	120-130	Air temp
Targeted dry matter (%)	>94	>94	>94	>94	>94	-
Measured dry matter (%)	95.4	97.5	89.6	94	95.1	82.1
Dryer type	Rotating drum	Rotating drum	Thin-film evaporator	Rotating drum	3-stage Belt	Filter-press

2.2. Test organisms

The test organisms were adults of earthworm species collected at Teagasc Oak Park, Research Centre, (Carlow, Ireland): *Allolobophora chlorotica* (Savigny, 1826), *Aporrectodea longa* (Ude, 1885), *Aporrectodea caliginosa* (Savigny, 1826) and *Lumbricus terrestris* (L.) (juveniles and adult) were extracted from a unpolluted minimum tillage field (details below) using the mustard oil method (a mix of 2 ml allyl isothiocyanate and 40 mL isopropanol [2-propanol], added to 20 l of water just before application in the field) and rinsed thoroughly in water immediately after the extraction. *Eisenia fetida* (Savigny, 1826) was produced in culture as recommended by ISO 11268-2 (1998).

2.3. Avoidance test

The avoidance test was conducted as described by ISO protocol (ISO/FDIS 17512-1).
Two different types of soil were used for the experiment: artificial and natural soil. The artificial soil used in tests comprised *Sphagnum* peat 10% (sieved through 5 mm mesh), 20% kaolinite and 70% quartz sand (80% particle size 0.2 to 2 mm) (ISO *1998* 11268-2, ISO *1999* 11267). The water holding capacity (WHC) of the soil was adjusted to 68% using distilled water and the pH to 6.0, using calcium carbonate. The natural soil was obtained from the same field from which earthworms were collected. Soil from the surface to a depth of 20 cm was used. Soil was air dried in a glasshouse and sieved through 3 mm mesh. The soil type is described as Baggotstown–Carlow Complex; this is a moderately deep, free-draining Brown Earth derived from limestone gravels with sandy loam texture. The preceding crops on the site were five winter wheat crops preceded by three winter barley crops, three spring barleys, prior to which was winter barley which was preceded by a grass ley. Soil analysis showed: pH 6.62, phosphorous 13.5 mg l^{-1}, potassium 102.7 mg l^{-1}, magnesium 131.8 mg l^{-1}, copper 2.1 mg l^{-1}, manganese 209.5 mg l^{-1} and zinc 1.5 mg l^{-1}. Soil nitrogen was very low with a requirement of 190 kg ha^{-1} for winter wheat.

Plastic containers, 20 x 12 x 5 cm, were used for each trial replicate. Containers had a removable aluminium flat sheet which divided the container transversely into two equal volumes. Two hundred and fifty grammes of dried field soil was introduced into the control section of the container (marked left side) while a similar weight of field soil-biosolid mixture was added to the test section (marked right side).
Three rates of biosolids equivalent to 2, 10 and 20 t ha^{-1} were mixed with the soil substrate. Every species was tested singularly and *L. terrestris* was tested for adults and juveniles separately. Ten clitellate worms of *A. chlorotica*, *A. caliginosa* and *E. fetida* were placed per box, five adults for *A. caliginosa*, four for *L. terrestris* and two for *A. longa*. Replication was 5-fold except for *E. fetida* tests which was 6-fold. Worm species and number as well as trial replication, biosolid source and rate and soil type are given in Table 2. The substrate-biosolid mix was moistened, using distilled water, so that no free water was visible when the soil was compressed (ISO *17512-1* 2005). Worms were placed on the line dividing the two sections following removal of the aluminium divider. Perforated snap-on plastic lids were placed on containers. Containers were placed in a controlled environment cabined (CEC) for 2 days at 20°C in a 16:8 h light: dark regime. After this period, the control and test soils were separated and the number of earthworms in each section counted as described in ISO (2005).

Table 2. Earthworm species and number investigated in 'avoidance' tests using soils with and without biosolids. Trial replication, biosolid number (source), soil source and equivalent field rates of application investigated are also shown

Species	Worms per container	Replication	Biosolids	Rates (t ha^{-1})	Soil
E. fetida	10	6	1,2,3,4,5,6	2, 10, 20	Natural
E. fetida	10	6	1,2,3,4,5,6	2, 10, 20	Artificial
A. chlorotica	10	5	1,2,3,4,5	2, 10, 20	Natural
L. terrestris	4	5	1,2,3,4,5	2, 10	Natural
L. terrestris	5	5	1,2,3,4,5	20	Natural
*L. terrestris**	5	5	1, 3	2, 10	Natural
A. caliginosa	5	5	1, 3	2, 10	Natural
A. longa	2	5	1, 3	2, 10	Natural

*juvenile worms

Table 3. Eisenia fetida preference between soil (control) and soil amended with biosolids from six locations and applied at three rates, laboratory 'avoidance test'. Ten adult worms for each replicate which was 6-fold.

Biosolid	*Eisenia fetida,* natural soil						*Eisenia fetida,* artificial soil					
	2 t ha^{-1}		10 t ha^{-1}		20 t ha^{-1}		2 t ha^{-1}		10 t ha^{-1}		20 t ha^{-1}	
	Control	Biosolid	Control	Biosolid	Control	Biosolid	Control	Biosolid	Control	Biosolid	Control	Biosolid
1	1.67	8.33	3.83	6.17	3.67	6.33	3.67	6.33	6.50	3.50	4.50	5.50
2	1.5	8.5	4.0	6.0	7.83	2.17	3.50	6.50	6.83	3.17	5.33	4.67
3	1.33	8.67	3.17	6.83	7.67	2.33	4.83	5.17	8.17	1.83	6.17	3.83
4	1.67	8.33	3.33	6.67	5.50	4.50	5.33	4.67	6.17	3.83	4.33	5.67
5	0.67	9.33	2.67	7.33	6.33	3.67	3.50	6.50	7.50	2.50	4.33	5.67
6	3.67	6.33	6.50	3.50	6.67	3.33	4.33	5.67	6.33	3.67	8.83	1.17
[1]Pr. significance	0.8 ***		0.6 *		0.4 **		0.6 **		0.3 ***		0.4 ns	
[2]O.R. significance			2.8 ***		2.1 ***				3.1 ***		0.5 **	
[2]O.R. significance				5.8 ***						1.7 **		

[1]Probability of worm being in biosolid amended soil. [2]Odds Ratio *i.e.* The number of times a worm is more likely to be found in biosolid relative to control.
* = P< 0.05, ** = P< 0.01, *** = P< 0.001.

Table 4. The number of *Eisenia fetida* recovered from each biosolid amended soil and untreated control soils for combined rates of biosolids (2, 10 and 20 t ha^{-1}) and combined controls. The probability and significance of finding worms in biosolid amended natural and artificial soil is presented.

	Natural soil				Artificial soil			
Biosolid	Control	Biosolid	**[1]Pr.**	**Signif.**	Control	Biosolid	**Pr.**	**Signif.**
1	9.2	20.8	**0.71**	*******	14.7	15.3	**0.51**	**n.s.**
2	13.3	16.7	**0.56**	**n.s.**	15.7	14.3	**0.47**	**n.s.**
3	12.2	17.8	**0.60**	*****	19.2	10.8	**0.35**	**
4	10.5	19.5	**0.66**	*******	15.8	14.2	**0.47**	**n.s.**
5	9.7	20.3	**0.64**	******	15.3	14.7	**0.48**	**n.s.**
6	16.8	13.2	**0.42**	**n.s.**	19.5	10.5	**0.34**	***

[1]Probability of worm being in biosolid amended soil. * = P< 0.05, ** = P< 0.01, *** = P< 0.001.

2.4. Data analysis

The binary outcomes of the avoidance tests were analysed using a generalized linear mixed model. Random effects were used to model the correlations within each test, *i.e.* the binary outcomes for each worm were not taken as independent within tests. Proc Glimmix (Statistical Analysis Systems Institute SAS 9.1 2004) was used to fit the analysis models. Analysis was carried out for each species separately and biosolid source was a blocking factor. Comparisons between levels of the blocking factor were not supported by randomisation and therefore the p-values are used only as a guide to exploring these differences. Multiple comparison procedures were used to control error rates in pairwise comparisons. Odds ratios were used to summarise the differences in the binary outcomes where an odds ratio of one means no difference, e.g. a worm is one times as likely to be found in the biosolid-amended mixture compared to unamended soil, that is, equally likely to be found in either.

3. RESULTS

3.1. Eisenia fetida

Comparing the numbers of *E. fetida* recovered from natural soil and natural soil amended with biosolids at the 2 t ha^{-1} rate showed that worms had a significant ($p< 0.0001$) preference for biosolid amended soil compared to untreated control soil (Table 3). The probability of finding a worm in the amended soil was 80%. Worms also had a significant ($p=0.02$) preference for the amended soil at the 10 t ha^{-1} rate with a 60% probability of worms being found in the amended soil. At the 20 t ha^{-1} rate, however, worms had a significant ($p=0.006$) preference for the untreated soil with only a 40% probability of finding worms in the amended soil. In the case of biosolid amended natural soil, increasing the rate of biosolid application resulted in significantly ($p<0.0001$) fewer worms in the amended soils. At the 2 t ha^{-1} rate, amended soil had significantly ($p< 0.0001$) more worms than either the 10 or 20 t ha^{-1} rate; similarly, the 10 t ha^{-1} rate had significantly ($p=0.0005$) more worms than the 20 t ha^{-1} rate (Table 3).

Comparisons of overall *E. fetida* preferences for amended and control soils for individual biosolids are presented in Table 4. The p-values are the result of a test of the measured probability versus 0.5, i.e, equally likely to be found in either material. The Worms had a significant preference for amended natural soil for biosolid 1 (71%), biosolid 3 (60%), biosolid 4 (66%) and biosolid 5 (64%). Worm numbers did not differ significantly between amended and control soils for either biosolid 2 and biosolid 6 (Table 4). Data analysis of worm numbers in natural soil amended with one of the six biosolids (blocking factor comparisons) showed biosolid 6 had significantly fewer worms than either biosolid 1 or biosolid 4. Differences between the remaining biosolids were not significant.

In the case of artificial soil amended with biosolids, there were significantly ($p=0.01$) more *E. fetida* in the amended soil at the 2 t ha^{-1} rate relative to the controls (Table 3). However, at the 10 t ha^{-1} rate there were significantly ($p< 0.0001$) more worms in the controls than in the amended soil. There were also more worms in the controls relative to the amended soil at the 20 t ha^{-1} rate, however, the latter difference was not significant. Comparing worm preferences for amended artificial soil at the different rates showed worm numbers were significantly ($p<0.0001$) greater for the 2 t ha^{-1} rate than the 10 t ha^{-1} rate and 20 t ha^{-1} rate ($p=0.006$). Unexpectedly, artificial soil amended with biosolid at the 20 t ha^{-1} rate had significantly ($p=0.007$) more worms than that for the 10 t ha^{-1} rate.

Comparison of overall *E. fetida* preference for amended and control artificial soil for individual biosolids are given in Table 4. There was no significant difference in the number of worms recorded between the control and amended soil for biosolid 1, 2, 4 and 5, while significantly fewer worms were found in soil amended with biosolid 3 ($p=0.002$) and biosolid 6 ($p=0.001$). Data analysis of worm numbers in artificial soil amended with any one of the six biosolids showed that worms did not have a significant preference between biosolids. The comparison between the artificial and the natural soils showed the number of worms was significantly higher ($p<0.0001$) in the natural soil than in the artificial at the 2 and 10 t ha^{-1} rates, whereas at 20 t ha^{-1} there was no significant difference between the two soils.

3.2. Allolobophora chlorotica

Data analysis incorporating results for all five Irish biosolids showed there were significantly ($p<0.0001$) more *A. chlorotica* in natural soil amended with biosolids relative to control soil at 2 and 10 t ha^{-1} rate (Table 5). At the 20 t ha^{-1} rate, however, the amended soil had significantly fewer worms than the control soil. In the case of biosolid amended natural soil, increasing the rate of biosolid application resulted in significantly (p<0.0001) fewer worms in the amended soils. At the 2 t ha^{-1} rate, amended soil had significantly (p=0.0001) more *A. chlorotica* than either the 10 or 20 t ha^{-1} rates (Table 5). Similarly, the 10 t ha^{-1} rate had significantly more worms than the 20 t ha^{-1} rate. The odds ratio showed worms were 16 times more likely to be found in amended soil at 10 rather than 20 t ha^{-1} rate. Comparing the number of worms recovered from amended soil for each biosolid with that for untreated controls showed only biosolid 3 had significantly (p=0.008) fewer worms than that for the control. Comparisons between biosolids showed biosolid 3 had significantly fewer worms than the remaining four.

Table 5. Allolobophora chlorotica preference between soil (control) and soil amended with biosolids from five Irish locations and applied at three rates, laboratory 'avoidance test'. Ten adult worms for each of the six replicates

Biosolid	*Allolobophora chlorotica,* natural soil					
	2 t ha^{-1}		10 t ha^{-1}		20 t ha^{-1}	
	Control	Biosolid	Control	Biosolid	Control	Biosolid
1	0.2	9.8	0.6	9.4	8.4	1.6
2	0.8	9.2	2.8	7.2	8.2	1.8
3	1.6	8.4	6.6	3.4	9.6	0.4
4	0.8	9.2	1.0	9.0	4.8	5.2
5	0.4	9.6	2.6	7.4	8.8	1.2
[1]Pr. significance	0.94 ***		0.76 ***		0.16 ***	
[2]O.R. significance			5.6 ***		16.2 ***	
[2]O.R. significance				90.9 ***		

[1]Probability of worm being in biosolid.
[2]Odds Ratio *i.e.* The number of times a worm is more likely to be found in biosolid relative to control.
* = P< 0.05, ** = P< 0.01, *** = P< 0.001.

3.3. Lumbricus terrestris

As with other worm species, adult *L .terrestris* worms had a significant (p=0.006) overall preference for biosolid amended soils relative to untreated control soil at 2 t ha^{-1} application rate (Table 6). At the 10 t ha^{-1} rate, *L. terrestris* did not show a preference for amended soil relative to control soil.However, at the 20 t ha^{-1} rate, worms had a significant (p<0.0001) preference for untreated relative to amended soil. Comparing worm preference for amended soil at 2, 10 and 20 t ha^{-1} rates showed the 2 t ha^{-1} rate had non-significant (p<0.0001) more than for the 20 t ha^{-1} rate. Amended soil at the 10 t ha^{-1} rate had significantly (p=0.01) more worms than the 20 t ha^{-1} rate. Data analysis across all rates for the five biosolids showed soils amended with biosolid 2 and biosolid 3 had significantly fewer *L. terrestris* when compared with untreated soil. Comparisons between biosolids showed soil amended with biosolid 1 had significantly more worms than soil amended with either biosolid 2 (p=0.007) and biosolid 3 (p=0.008).

In the case of juvenile *L. terrestris,* amended soils included only the 2 and 10 t ha^{-1} rates. Juvenile *L. terrestris* had a significant (p=0.004) preference for biosolid (aggregate data for biosolids 1 and 3) amended soil when compared with control soil. Juvenile worms were 83% more likely to be found in the amended soil (Table 6) Worms did not show a preference between amended soil and control soil when biosolids were applied at 10 t ha^{-1} rate. Juveniles had a significant (p=0.01) preference for amended soil at the 2 t ha^{-1} rate relative to that for the 10 t ha^{-1} rate. Comparisons between soil amended with biosolid 1 and 3 showed worms had a significant preference for the biosolid 1 mixture.

Table 6. Lumbricus terrestris preference between soil (control) and soil amended with biosolids from five Irish locations and applied at three rates, laboratory 'avoidance test'. Four adult worms and five juvenile worms were used for each of six replicates

	Adult *Lumbricus terrestris*, natural soil					
	2 t ha^{-1}		10 t ha^{-1}		20 t ha^{-1}	
Biosolid	Control	Biosolid	Control	Biosolid	Control	Biosolid
1	1.2	2.8	1.0	3.0	2.6	2.4
2	1.6	2.4	2.6	1.4	4.8	0.2
3	1.6	2.4	3.4	0.6	4.4	0.6
4	1.6	2.4	0.8	3.2	4.0	1.0
5	2.0	2.0	2.8	1.2	2.8	2.2
[1]Pr. significance	0.60 **		0.46 ns		0.24 ***	
[2]O.R. significance (2 vs 10 t ha^{-1}; 10 vs 20 t ha^{-1})		1.7 ns			2.7 **	
[2]O.R. significance (2 vs 20 t ha^{-1})				4.7 ***		

	Juvenile *Lumbricus terrestris*, natural soil			
	2 t ha^{-1}		10 t ha^{-1}	
Biosolid	Control	Biosolid	Control	Biosolid
1	0.8	4.2	1.6	3.4
3	1.0	4.0	3.6	1.4
[1]Pr. significance	0.83 **		0.47 ns	
[2]O.R. significance			5.5 **	

[1]Probability of worm being in biosolid.
[2]Odds Ratio *i.e.* The number of times a worm is more likely to be found in biosolid relative to control.
* = P< 0.05, ** = P< 0.01, *** = P< 0.001.

3.4. Apporectodea caliginosa and A. longa

Investigations involving *Aporrectodea longa* and *A. caliginosa* (details in Table 2) did not indicate differences in worm preferences between amended and control soils with either biosolid 1 or biosolid 3, for the two application rates of 2 and 10 t ha^{-1} (data not shown).

4. DISCUSSION

4.1. Earthworm preference and biosolids toxicity

Results of avoidance test with *A. chlorotica*, *E. fetida* and *L. terrestris* clearly showed that earthworms make a choice when offered unamended control soil and soil amended with biosolids. This confirms that the principle of these tests is suited to determining the preference by soil invertebrates for or avoidance of soils containing exogenous materials (Hund-Rinke and Wiechering, 2001; Yeardley *et al.*, 1996). Results showed that the species reacted similarly to biosolids amendments: they all were attracted by low concentrations of biosolids (2 t ha^{-1}), whereas they avoided the highest concentration (20 t ha^{-1}). As suggested by Hamilton *et al*. (1988), low rates of biosolids probably attracted earthworms as a food source because of its higher organic matter content compared with the untreated controls. The same behaviour was observed by Natal-da-Luz *et al.* (2009) and Moreira *et al.* (2008) when *E. andrei* earthworms were added to artificial soil mixed with 6 t ha^{-1} urban sewage sludge. However, Natal da Luz *et al.* (2009) found that worms were still attracted by sewage sludge when applied at 25 and 45 t ha^{-1}, whereas, in this study at 20 t ha^{-1} rate all the three species avoided the amended soil.

The repulsive effect observed when biosolids were applied at 20 t ha^{-1} has to be interpreted carefully since it may be related not just to the toxicity of the material tested but also to the artificial conditions of the experiment such as release of ammonia and decrease in pH (Crouau *et al.,* 2002). These other conditions that could act as stimuli affecting earthworm behaviour should be monitored in future studies.

The results from comparisons of 6 biosolids indicate that avoidance responses by earthworms are sensitive enough to reflect different toxicities of different biosolids. The avoidance behaviour observed at 20 t ha^{-1} may be attributed to the high levels of zinc and copper measured in the five Irish biosolids (between 202 and 530.7 µg g^{-1} for copper and between 83.4 and 681 µg g^{-1} for zinc). A pronounced avoiding behaviour was observed in treatments with biosolids 2 and 3 where the concentration of zinc was particularly high (547 and 681 µg g^{-1}). Considering the results obtained by Lukkari *et al.* (2005) for the effects of Cu/Zn concentration pairing on *Aporrectodea tuberculata* avoidance behaviour, the level of Cu/Zn present at the higher concentrations of soil-biosolids mixtures were high enough to affect the avoidance behaviour of the earthworms.

4.2. Method development and comparisons

Comparing the behaviour of *E. fetida* in artificial and natural soils to which biosolids at various rates were added showed similar results for the two soils at 2 t ha^{-1} rate, whereas a dissimilar responses were recorded at 10 and 20 t ha^{-1}. At 10 t ha^{-1} in natural soil earthworms chose the biosolid amended section, while in the artificial soil they preferred the controls. The movement of the test organisms toward the amended soil when biosolids where added at 2 t ha^{-1} is probably related to the absence of repulsive effect of the contaminants in the biosolids. However, soil properties such as pH and organic matter content can affect the avoidance behaviour of soil organisms (Amorim *et al.*, 2005; 2008; Natal da Luz *et al.*, 2004). In this study, the pH levels of the soils were similar (6.5 in the artificial soil, 6.0-7.0 in the field soil), while the artificial soil had a higher organic matter content than the field soil used (10% in the artificial soil, 6.4% field soil). We can hypothesize that in artificial soil, *E.fetida* was finding enough food in the control section whereas in natural soil, the low content of organic matter encouraged them to seek out food in the biosolid amended section. The central issues in using field soil – standardisation and inter-test comparability – have been addressed by other authors (Römbke and Amorim, 2004).

The ecological differences of the earthworms species employed in this study were expected to influence the outcome of the avoidance tests (Doube *et al.*, 1997; Lukkari and Haimi, 2005). Tomlin (1992), in fact, suggested that ecological characteristics of earthworm species (epigeic, anecic and endogeic) may affect their susceptibility to soil contaminants. *E. fetida* is considered to be less sensitive to soil factors than other earthworm species (Spurgeon *et al.*, 2000; Lukkari *et al.*, 2005; Frampton *et al.*, 2006; Owojori and Reinecke, 2009). However, in this study there was no evidence of different behavioural patterns between earthworm species. These results indicate the suitability of *E. fetida* as standard species for ecotoxicological tests (Kobetičová *et al.*, 2010), since it is easily produced in laboratory conditions and it had similar responses to biosolids applications as the other soil-dwelling species that occur in agricultural soils such as *L. terrestris* and *A. caliginosa*. This assumption is supported by Langdon *et al*. (2005) who determined the effect of lead on survival and avoidance response of *E. andrei*, *L. rubellus* and *A. caliginosa*, in a standard artificial soil. Little or no variations among the three ecologically different earthworms species were observed in the avoidance test results.

The main trends observed for *E. fetida* in the avoidance tests, were also detected in ISO Mortality and Reproduction tests performed with the same biosolids (Artuso *et al*., 2010). Lukkari *et al.* (2005) observed that *A. tuberculata* avoided contaminated soil at lower metal concentrations than those level that induced significant negative responses in the acute and reproduction test. The results of this study showed the high sensitivity of this simple and rapid preference test, including at lower application rate (2 t ha^{-1}) of biosolids which are realistic for field situation. Several studies indicated a similar or higher sensitivity of avoidance test compared with sub-lethal measures (Hund-Rinke *et* *al.*, 2005; Hund-Rinke and Wiechering, 2001; Schaefer, 2003; Owojori and Reinecke, 2009). However, in risk assessment studies where the toxicity of contaminated soils is monitored, the avoidance test should be considered as screening test and carried out in conjunction with toxicity tests (Hund-Rinke *et al.*, 2003).

Based on our results, it is concluded that earthworm avoidance tests, which are simple and rapid, offer an efficient screening tool to determine the impact of adding biosolids to the soil. The tests can detect differences between different biosolids, but stimuli not related to toxicity that could alter earthworm behaviours need to be monitored in future studies. The use of natural field soil is recommended because it creates more realistic conditions, but standardisation is reduced. Regarding the choice of earthworm species, the results of this study confirm the suitability of *E. fetida* for biological screening of biosolids. For assessing the toxicity of biosolids, *E. fetida*, *L. terrestris* and *A. caliginosa* are ecologically relevant species and they are sensitive to biosolid applications. However, when a rapid and efficient screening is required, cultured *E. fetida* are much more convenient.

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