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Effect of the exposure to metal lead on the regenerative ability of *Lumbriculus variegatus* (Oligochaeta)

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ABSTRACT

Lumbriculus variegatus is a recommended species for use in sediment toxicity tests and is known to have a remarkable power of segmental regeneration. Here, we tested the effects of a chemical stressor on the regenerative ability of *L. variegatus* and investigated the potential of regenerative ability as an additional new parameter in standard toxicity tests. The worms were cut into two equal segments, and exposed to various concentrations of lead. Two assays were performed: one with sediment spiked with lead and the other with water spiked with lead. The endpoints were segmental regeneration, survival and behaviour. Regenerative ability was clearly affected by exposure to lead-contaminated sediment and lead-contaminated water. Organisms exposed to lead grew more slowly than those not exposed; worms exposed to contaminated water showed higher mortalities than those exposed to contaminated sediment. Results showed that *L. variegatus*' regenerative ability, as a developmental test parameter, is more sensitive than mortality.

1. Introduction

The evaluation of the effects on reproduction may offer an important contribution to the risk assessment of metals in sediments and becomes even more interesting when the organism reproduces by a peculiar and uncommon process. One form of asexual reproduction, transverse fission, has evolved in a number of linear, limbless animals, such as the platyhelminths, nemerteans and annelids (Bely, 1999). In annelids, fission is found in four (out of fewer than 14) Oligochaeta families (Brinkhurst and Jamieson, 1971; Christensen, 1984). Reproduction by fission must involve two processes: (a) physical separation of an individual into two (or more) pieces, and (b) reconstitution of a whole individual from each piece or at least two pieces (Bely, 1999). Much of the current understanding of the developmental mechanisms underlying regeneration has emerged from studies using invertebrate model systems (Sanchez-Alvarado, 2000). Invertebrate animals are champions of regeneration, often rapidly regenerating much of their body from small isolated fragments (Martinez et al., 2005).

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Lumbriculus variegatus (Müller 1774) is recommended for use in toxicity tests with sediments. This recommendation is based on its ease of culture and handling, known chemical exposure history, adequate tissue mass for chemical analysis, tolerance of a wide range of physico-chemical sediment characteristics, low sensitivity to contaminants associated with sediment, and amenability to long-term exposures without feeding (Ingersoll et al., 2003). Judging by the number of internationally-published articles, the most common oligochaeta species used in evaluations of freshwater toxicity has been *L. variegatus* (Leppänen, 1999). *L. variegatus*, a freshwater oligochaete known to have remarkable powers of segmental regeneration (Hyman, 1916), has been proposed by the American Society of Testing and Materials (ASTM, 1995) as a standard organism for tests of sediment bioaccumulation and is listed by the Organization for Economic Co-operation and Development (OECD, 1992) as a good organism for bioaccumulation studies. Reproduction of *L. variegatus* under laboratory conditions is always by asexual fragmentation, in which a worm spontaneously divides into two or more body fragments. Each surviving fragment then undergoes rapid regeneration of body segments to form a new head, tail, or both. Eventually, each fragment grows into a normal-sized worm, comprising a combination of older and newer segments representing two or more "generations" of development (Lesiuk and Drewes, 1999). The regeneration is achieved after seven days at 20 °C and goes through eight characteristic stages, even if the sectioning is artificially induced (Veltz-Balatre, 2000). This worm does not have a chitinous exoskeleton and can only accumulate metal in soft tissue, simplifying the interpretation of the relationship between survival and body concentrations of metals (Meyer et al., 2002).

Lead is a metal well known as a toxic material (IPCS, 1995) that occurs naturally in the environment. The introduction of unleaded gasoline has contributed enormously to the decrease of lead emissions into the environment. However, it is still one of the most commonly-used elements, in many industries (Goyer, 1991). On a global scale, there is abundant evidence of lead pollution, but it is often difficult to determine its effects on the environment (Depledge et al., 1994). Lead was chosen as the model substance in this test because of its affinity to sediment particles and its persistence in the environment.

Some authors contend there is a need for further research into the toxicity of metals in freshwater invertebrates (Bat et al., 2000). Here, we test the effects of a chemical stressor on the regenerative ability of *L. variegatus* and investigate the potential of using regenerative ability as a new parameter in standard toxicity tests with this species.

2. Material and methods

2.1. Culture

The laboratory cultures of *L. variegatus*, used throughout these tests, originated from the University of Joensuu, Finland. The animals were reared in polyethylene aquariums (8.5 cm × 17.5 cm × 12 cm), covered with lids, containing ASTM (ASTM, 1980) medium (pH 7.6 ± 0.3), at 20 °C, in a temperature-controlled room (16:8 h light:dark cycle and 50% humidity). A commercially available sand-pebble mixture (grain sizes: 0–8 mm) was acid washed (pH 2), ashified (4 h, 450 °C) and used as sediment. The aquaria contained a 2 cm layer of sediment with continuous and moderated aeration. The worms were fed with Tetraphyll, applied two or three times a week, at approximately 5 mg/30 worms.

2.2. Spiking

Two separate experiments were conducted to evaluate the effect of contaminated sediment and the effect of contaminated water on the regeneration and survival of *L. variegatus*.

The whole sediment (sediments and associated pore water that have had minimal manipulation, US EPA, 2000) used in both experiments was formulated sediment and had the following characteristics: 4.9% sand, 74.4% clay and 20.7% silt; pH of pore water 6.77; ammonia of pore water 3.04 mg/kg and total carbon content 0.54%. Metal-spike solutions were prepared by dissolving the appropriate amount of metal salt ($(CH_3COO)_2Pb{\cdot}3H_2O$; supplied by Merck) in distilled water. These solutions were immediately added to the sediment, which was then capped and rapidly shaken for 1 min. The spiked sediments equilibrated for a minimum of 48 h, to allow the lead to adsorb to the sediment particles. During this time, the sediments were shaken every day for approximately 2 min. The contaminated water was replaced by ASTM water before adding the worms. For the experiment with contaminated water, lead solutions were prepared with de-chlorinated tap water (due to the interaction between Pb^{2+} and Cl^{2-} ions, from ASTM, which leads to precipitation). Target lead concentrations were 2.0, 4.0, 8.0, 20.0, 70.0 and 85.0 mg/kg (dry weight) and mg/l – for sediment (dry sediment) and water; a control with uncontaminated ASTM/tap water was used. The sediment was sampled for metal analysis at the start and termination of each test; the overlying water (ASTM medium) was sampled at the beginning and end of both tests. Water samples were read in the Atomic Absorption Spectrophotometer (AAS). For sediment and tissue samples, the Inductively Coupled Plasma (ICP) method was used, and the complete digestion was performed according to SMEWW 3120B (Standard Methods for Examination of Water and Wastewater) (limit of quantitation: 0.015 mg/l).

2.3. Exposure design

Short-term (10-day) tests were performed and exposures were conducted at ±20 °C, in a temperature-controlled room (16:8 h light:dark cycle and 50% humidity), in 100 ml plastic beakers, containing 20 ml of whole sediment and 20 ml of ASTM, (for the contaminated sediment) or tap water (for the contaminated water), in a static system. Three replicates per concentration were used, each with six adult worms (about 3 cm each). Each adult was cut into two equally long pieces (totalling 12 fragments per beaker, 36 fragments per concentration); there was no bleeding from the amputation process. Each treatment received the same number of head and tail fragments, minimizing eventual differences in the comparative regenerative abilities of tail and head between treatments. The worms were exposed immediately after amputation and

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carefully introduced into the beakers with the help of a plastic Pasteur pipette. Worms were fed once a week with 2.4 mg of Tetraphyll per pot. Mortality/survival, size class/growth (size class 1: worm < 2 cm; size class 2: 2 cm < worm < 2.5 cm; size class 3: worm > 2.5 cm), colour, activity and presence in sediment or water were monitored every 48 h. For this task, the worms were removed from the sediment and carefully observed and measured. The surviving worms were collected and dried at 40 °C, for about 24 h. Before drying, worms were rinsed rapidly in distilled water and gently dried with filter paper. For water, sediment and whole-body samples the Inductively Coupled Plasma (ICP-OES) method was used (ICP-OES acc. to DIN EN ISO 11885; limit of quantitation (LOQ): 0.010 mg/l for water and 0.1 mg/kg for sediment and whole-body). Bioaccumulation factors (BAFs) were calculated for each metal according to the formula [BAF = metal concentration (mg/kg dry wt.) in tissue/metal concentration (mg/kg dry wt.) in sediment], according to Barron (1995).

2.4. *Histology*

After the exposure to contaminated water, a standard histology technique was performed using worms from the control and from each tested concentration. The procedure consisted of: fixation in Bouin solution for 24 h, 70% ethanol for 24 h, dehydration, paraffin embedding, sectioning (8 μm), staining using Haematoxyline–Eosin and mounting (with Eukitt™). The slides obtained after this process were observed under a light microscope to search for damage caused by lead exposure.

2.5. *Statistical analysis*

The statistical relationship between lead in the sediment and mortality/growth was determined by bivariate correlation using a Pearson's coefficient in a two-tailed test ($p < 0.05$), using Minitab software (version 14.1). The overall effect of lead concentrations on mortality and growth was investigated using a Kruskal–Wallis one-way ANOVA on ranks ($p < 0.001$), followed by a post hoc Dunn's test ($p < 0.05$) to check for significant differences. The software used was *SigmaStat for Windows*, version 3.10.

Table 1 – Total mortalities for worms exposed to contaminated sediment and worms exposed to contaminated water.

Pb concentrations	Total mortality % (contaminated sediment)	Total mortality % (contaminated water)
CTR	0.0	5.6
2.0	0.0	13.9
4.0	13.9	13.9
8.0	19.4	72.2
20.0	19.4	69.4
70.0	22.2	100.0
85.0	27.8	100.0

3. Results

3.1. *Spiked sediment (contaminated sediment/clean water)*

Some study parameters, such as colour, activity after a small prod, and presence in sediment or in water were monitored during the experiment. No changes in colour occurred in organisms exposed to any lead concentration. Both fragments (head and tail) were always equally active and no avoidance was observed: despite the contaminated sediment, worms never escaped to clean water.

No mortality was observed for control worms, demonstrating that the holding facilities and handling techniques were acceptable for conducting such tests, as required by the viability criteria (mean survival for control should be 90%) (ASTM,

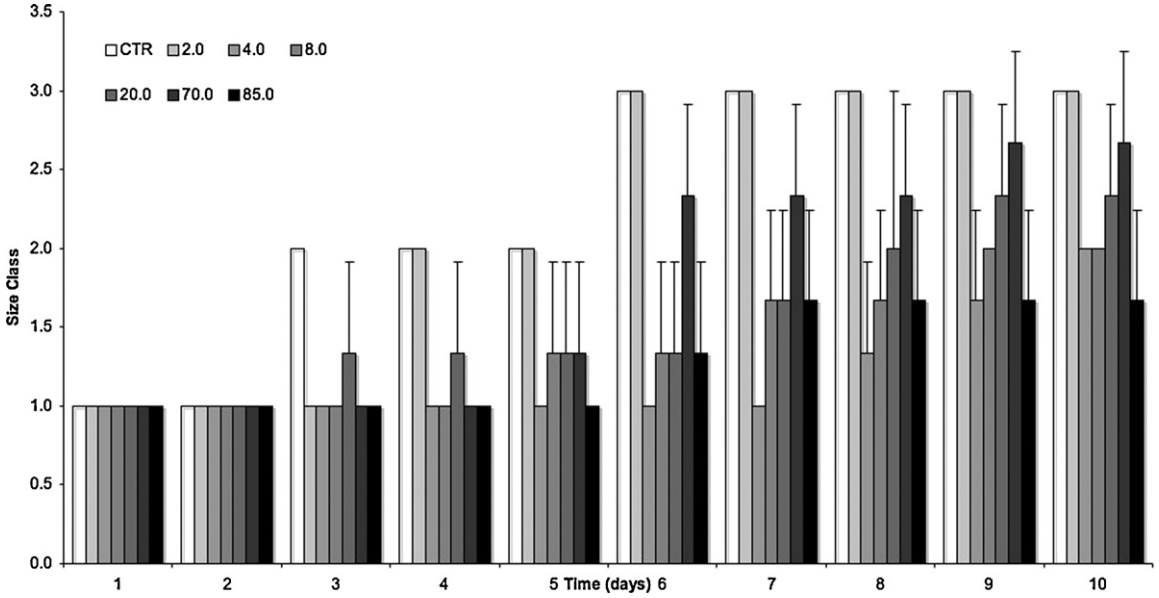

Fig. 1 – Growth (size class) of worms (mean + STDEV) exposed to contaminated sediment.

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Table 2 – Lead concentration in sediment (mean ± STDEV) (mg/kg), water (mean ± STDEV) (mg/l) and whole-body (mg/kg), for the contaminated sediment/clean water experiment.

Concentrations	Initial sediment concentrations	Final sediment concentrations	Final water concentrations	Final whole-body concentrations
CTR	<0.005	<0.005	<0.005	<0.005
2.0	2.0 ± 0.63	1.9 ± 0.44	<0.005	–
4.0	4.1 ± 1.98	3.9 ± 3.12	<0.005	–
8.0	7.1 ± 1.21	8.6 ± 1.27	<0.005	94.1
20.0	19.4 ± 1.16	18.8 ± 8.30	0.1 ± 0.02	410.0
70.0	68.7 ± 2.80	67.9 ± 7.40	0.1 ± 0.01	2071.0
85.0	83.1 ± 1.58	80.4 ± 6.82	0.1 ± 0.01	2541.0

1990). Mortalities (Table 1) for exposed worms ranged from 0.0% to 27.8%, and increased with increasing lead concentrations. However, mortalities were never high: the highest tested concentrations had survivorship of 83.2%. Statistical analysis showed that there was no significant difference ($p = 0.059$) between the tested concentrations and the control, indicating that exposure to contaminated sediment did not produce significant toxic effects that caused mortality.

The regenerative ability and growth (Fig. 1) of the artificially cut fragments were affected by exposure to sediments contaminated with lead: the higher the lead concentration in the sediment, the less the exposed worm grew. However, the results were not significantly different between tested concentrations. Only worms exposed to the lower concentration (2.0 mg/kg) were not affected, growing as the control organisms.

The initial concentrations of lead in the sediment (Table 2) presented expected values, confirming that the spiking method was correct, well-performed and, in general, kept constant until the end of the test. Water samples (Table 2) showed that lead concentrations decreased from the beginning to the end of the test, due to an increased binding of lead to the sediment. Correlations were calculated between the concentration of lead in the sediment and the concentration of lead in the whole-body, mortality and growth (Table 3). The strongest correlation was found between lead in the whole-body and lead in the sediment ($r = 0.999$), which means that when the concentration of lead increased in the sediment it also increased in the whole-body: worms were therefore accumulating lead from the sediment. A strong correlation was also found between the concentration of lead in the sediment and mortality ($r = 0.733$): mortalities were higher when the concentration of lead in the sediment was higher.

Table 3 – Correlations between concentration of lead in the sediment/water and mortality, growth and concentration of lead in the whole-body.

	r	P-value
Pb sediment/Pb whole-body	0.999	0.000
Pb sediment/mortality	0.733	0.097
Pb sediment/growth	−0.244	0.642
Pb water/Pb whole-body	0.982	0.018
Pb water/mortality	0.831	0.040
Pb water/growth	−0.840	0.036

3.2. *Spiked water (clean sediment/contaminated water)*

As in the previous test, parameters such as colour, activity and avoidance were not influenced by the exposure to contaminated water. Very high mortalities (Table 1) were observed for organisms exposed to 8.0, 20.0 and 70.0 mg/l. Mortality ranged from 13.9% to 100% and increased when the lead concentration was higher. 100% mortality was recorded for worms exposed to the highest tested concentration, 85.0 mg/l, and was reached very quickly, within 3–4 days. Statistically significant differences ($p < 0.05$) were observed for concentrations 8.0, 20.0, 70.0 and 85.0 mg/l, indicating that exposure to concentrations equal or above 8.0 mg/l had a significant toxic effect on *L. variegatus*.

The regenerative ability and growth of the fragments were also affected (Fig. 2). Only worms exposed to 2.0 mg/kg regenerated and grew like the controls; all the other tested concentrations inhibited the regenerative ability of *L. variegatus*. Exposure to water contaminated with levels of lead reduced the growth of the study species, as these organisms grew less than unexposed worms. Nevertheless, the differences between concentrations were not statistically significant.

Histology was performed using worms exposed to contaminated water and clean sediment. Cuticle, epithelium, muscle, digestive system, tiflosol and reproductive organs were examined for differences and potential damages caused by the exposure to lead, but no changes were observed in any vital structures of *L. variegatus*.

Correlations were calculated between the concentration of lead in the water and the concentration of lead in the whole-body (Table 4), mortality, and growth (Table 3). The strongest correlation was found between the concentration of lead in the whole-body and the concentration of lead in the water ($r = 0.982$), followed by the correlation between lead in the water and the mortality of *L. variegatus* ($r = 0.831$). These two strong positive correlations indicate that when lead concentration increased in the water, it also increased in the whole-body and caused mortality. A negative strong correlation ($r = -0.840$) was found between the concentration of lead in the water and the worms' growth: as the concentration of lead in the water increased, the growth of the organisms decreased. Bioaccumulation factors (Table 5) increased with increasing lead concentrations, as expected. Worms exposed to higher lead concentrations presented a higher BAF, suggesting a considerable potential for bioaccumulation.

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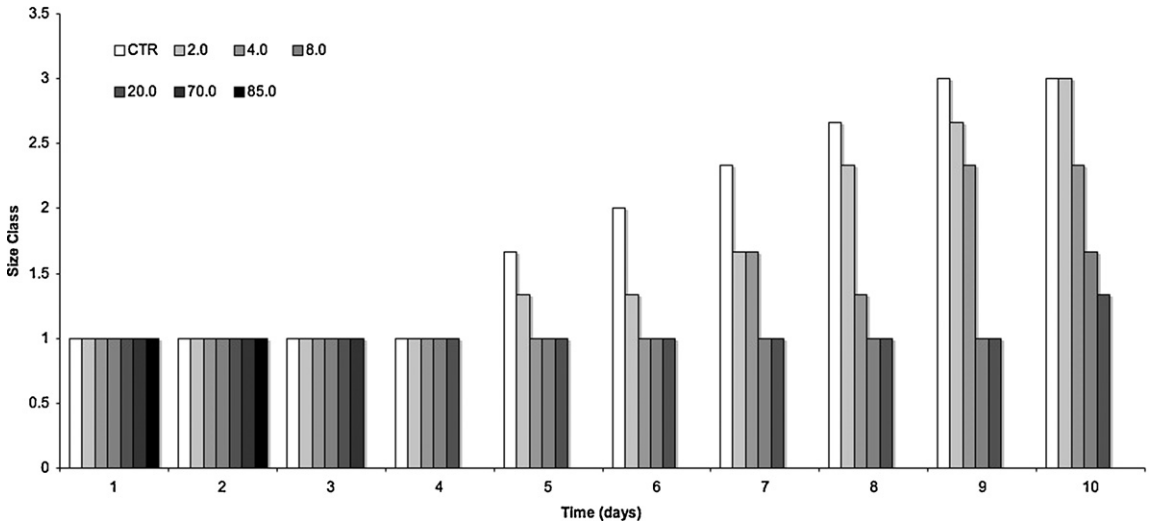

Fig. 2 – Growth (size class) of worms (mean + STDEV) exposed to contaminated water.

Table 4 – Lead concentration in sediment (mean ± STDEV) (mg/kg), water (mean ± STDEV) (mg/l) and whole-body (mg/kg) samples, for the clean sediment/contaminated water experiment.

Concentrations	Final sediment concentrations	Initial water concentrations	Final water concentrations	Final whole-body concentrations
CTR	<0.005	<0.005	<0.005	<0.005
2.0	2.0 ± 0.10	0.1 ± 0.02	<0.005	66.2
4.0	4.2 ± 0.30	2.2 ± 0.07	0.1 ± 0.02	160.0
8.0	13.1 ± 1.55	8.1 ± 0.42	0.1 ± 0.03	953.0
20.0	11.9 ± 2.52	14.1 ± 0.32	0.2 ± 0.13	1276.0
70.0	48.4 ± 6.36	63.4 ± 0.78	6.6 ± 1.20	1675.0
85.0	73.3 ± 8.01	76.1 ± 1.06	41.5 ± 5.50	–

Table 5 – Bioaccumulation factors.

Concentrations	BAFs contaminated sediment
2.0	[a]
4.0	[a]
8.0	10.9
20.0	21.8
70.0	30.5
85.0	31.6

[a] BAFs were not calculated because the value for whole sediment was below detection level.

4. Discussion

Regenerative ability was the parameter most affected by exposure to contaminated sediment and water. Worms exposed to concentrations above 2.0 mg/kg and 2.0 mg/l grew more slowly than non-exposed worms; hence, growth was inhibited by exposure to lead-contaminated sediment and contaminated water. It was evident that the presence of lead had a negative effect on the growth of *L. variegatus*. Growth was a sensitive test parameter in determining the effects of lead exposure at these concentrations; similar results were highlighted by Sardo and Soares (2010), who found that exposure to sediments in which lead was present did not support good *L. variegatus* growth. In addition, Maboeta et al. (1999) concluded that the growth of the oligochaete *Perionyx excavatus* was a sensitive endpoint for lead toxicity, while Khalil et al. (1996) found that metal salts mixed into soil had a negative, concentration-related effect on the growth of the oligochaete *Aporrectodea caliginosa*. Furthermore, exposure to lead-contaminated water at higher concentrations, in addition to inhibiting regeneration and growth, killed the worms. This suggests that direct uptake through the skin might be a more important and faster contamination route than uptake via sediment ingestion.

It is clear that no method of sample pre-treatment preserved the speciation of lead intact. A study from Davidson et al. (1999) confirms that the extraction procedures should be applied to wet, sieved sediment immediately after sampling, if environmentally-relevant information is to be obtained. Lead concentrations present in sediment, water and whole-body were as expected for both tests. For mammals, it is well known that lead is usually associated with bone structure, through the replacement of Ca^{2+} by Pb^{2+} ions (Timbrell, 2000). However worms, while lacking calcareous structures, present other specific structures. The chloragogen cells of lumbricid worms, which surround the gut and the large blood vessels, contain numerous granules, chloragosomes, which, among other abilities, bind toxic cations and organic xenobiotcs, enabling the worms to survive mild poisonings (Fischer, 1977). It has been documented that chloragosomes are involved in the accumulation and immobilization of metals (Ireland and Richards,

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1977; Morgan and Morgan, 1989). Minõ et al. (2006) found that the uptake of lead by *L. variegatus* was linearly related to the nominal concentration of the metal in the bioassays and according to Aisemberg et al. (2005), the levels of lead concentration in *L. variegatus* and the levels of metal exposure showed strong positive correlations in 48 h short-term tests, which could be described by highly significant linear regression equations. These data are consistent with our results, which showed that worms exposed to higher concentrations of lead presented a higher value of lead in the whole-body. The fact that earthworms can accumulate lead was determined in several previous studies (Ernst et al., 2008; Nahmani et al., 2009).

Some protocols (US EPA, 2000; ASTM, 2001) recommend a holding period in clean water at the end of the sediment exposure, to allow organisms to purge their gut of sediment prior to analysis. This inclusion of a purging period has been questioned by some authors because of concerns that tissue-bound chemicals will depurate from the organisms during holding in clean water, thereby under-representing the steady-state concentration in organisms residing in the sediment (Dawson et al., 2003; Sheedy et al., 1998). Here, we preferred not to use any purging period. There was no attempt to remove sediment from *L. variegatus* intestines by allowing a recovery period (putting the worms in water for 24 h), because an increase in the water content of worms could decrease or erase negative effects on weight (Dalby et al., 1996; Capowiez et al., 2005). Histology was performed using worms exposed to contaminated water and clean sediment but no changes were detected in any of the scanned structures. Short-term tests are not sufficient to test histological effects in *L. variegatus* new long-term tests should be performed to investigate histological impacts.

Comparisons of our data with other reports in the literature are somewhat difficult as (i) there are few organisms with such a remarkable ability to regenerate, and (ii) regeneration is not a common parameter in ecotoxicological tests, although some studies have been performed using growth as an endpoint. Maboeta et al. (1999) concluded that the growth of the soil oligochaete *P. excavatus* was a sensitive endpoint for lead toxicity. A study with another soil oligochaete demonstrated that metals had a negative, concentration-related effect on the growth, which decreased with increasing concentrations of the metal (Khalil et al., 1996).

One of the aims of this study was to investigate the use of the regenerative ability of *L. variegatus* as a new additional parameter for standard toxicity tests. From the results, the authors believe that the regenerative ability of the tested species was clearly affected by exposure both to lead-contaminated sediment and to lead-contaminated water. As a result, lead will affect the presence of this species because of interference in the regeneration process, the natural asexual reproductive method. It is clear that regeneration tests cannot replace classical sediment toxicity tests, but the data suggest that useful information can be obtained from the monitoring of this parameter. Therefore, the authors propose that regenerative ability, a sensitive and ecologically relevant parameter, should be used in toxicity tests whenever the test species present such abilities.

5. Conclusion

L. variegatus' regenerative ability was clearly affected by exposure to lead-contaminated sediment and to lead-contaminated water: exposed organisms grew more slowly, which suggests that lead could interfere in the natural asexual reproductive method of this species and thus affect its presence in nature.

Conflict of interest statement

Nothing declared.

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