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Exposure of *Eisenia andrei* (Oligochaeta; Lumbricidea) to Cadmium Polluted Soil Inhibits Earthworm Maturation and Reproduction but not Restoration of Experimentally Depleted Coelomocytes or Regeneration of Amputated Segments*

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Lumbricid earthworms are often exposed to polluted soil. They are also commonly subjected to various stimuli and attacks by predators that induce extrusion of coelomocyte-containing coelomic fluid and/or the loss of body segments followed by the renewal of immune-competent cells and regeneration of tissues/organs. The aim of our investigations was to test the effects of exposure of the earthworm *Eisenia andrei* to cadmium-polluted soil, combined with electrostimulation-induced depletion of coelomocytes (i.e. amoebocytes and chloragocyte-derived eleocytes) or the surgical amputation of posterior segments, on earthworm maturation, reproductive output, and regenerative processes. Experimental worms were maintained up to 7 weeks either in unpolluted soil or in soil spiked with cadmium chloride (500 mg/kg air-dried soil). In juvenile worms, sexual maturation (measured by clitellum formation) was delayed and cocoon production was inhibited in Cd-exposed worms. Coelomocytes were significantly depleted by electrostimulation and the kinetics of their recovery was similar in worms kept in clean and cadmium polluted soils, in both exposure conditions amoebocyte recovery was faster than recovery of riboflavin-storing eleocytes. In adult worms, soil cadmium exposure inhibited reproduction but, at macro-anatomical level, had a negligible effect on regeneration of amputated posterior segments, visible only on histological cross-sections.

Key words: Cadmium soil pollution; earthworm maturation and reproduction; coelomocyte restoration; amoebocytes; eleocytes; riboflavin; regeneration.

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Lumbricid worms are commonly exposed to polluted soil. They are also commonly subjected to various stressors including predator attacks that induce extrusion of coelomic fluid containing coelomocytes and soluble factors involved in immune reactions (BILEJ *et al*. 2011) and/or the loss of body segments; in both cases a restorative period ensues involving the renewal of immune-competent cells (KLIMEK *et al*. 2012; SANTOCKI *et al*. 2016a, b) and regeneration of lost tissues/organs (GALUSZKA *et al*. 2015; KOCINSKI *et al*. 2016; PLYTYCZ *et al.* 2016).

The Organisation for Economic Co-operation and Development (OECD) has recommended earthworms from *Eisenia* sp. for soil contaminant testing since 1984 (OECD 1984; e.g. BRUNS *et al*. 2001; PANZARINO *et al*. 2016; OTOMO *et al*. 2016). Until recently *E. andrei* and *E. fetida* have been considered as subspecies of *Eisenia foetida* (sic) species according to the characteristic banded pigmentation pattern of *E. fetida* and the uniform reddish colour of *E. andrei*. Until the advent of "omics" technologies, there was a tendency to deploy them

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fairly indiscriminately in ecotoxicological studies. DNA barcoding has revealed that they are distinct species, and each possesses several species-specific molecular markers (ALBANI *et al*. 2003; PÉREZ-LOSADA *et al*. 2005; RORAT *et al*. 2014); thus is imperative to reexamine environmental effects on robustly defined representatives of these species.

The aim of the present investigations was to follow effects of exposure to cadmium polluted soil on regenerative processes of the composting earthworm species *Eisenia andrei* identified by methods developed previously (RORAT *et al*. 2014). In the first part of the study the focus was on juvenile worms subjected to electric shock-induced coelomocyte depletion; in the second part the adult worms were subjected to surgical amputation of posterior segments. Subsequently worms were maintained either in clean or cadmium spiked soil, and cadmium accumulation in worms' bodies, their maturation, cocoon production, restoration of experimentally depleted coelomocytes or regenerative blastema formations were monitored.

Material and Methods

Soil samples

Earthworms were maintained in commercial soil (PPUH BIOVITA, Tenczynek) of the organic type, with 51.7% organic matter, pH=6.1, with low Cd concentration (0.5±0.3), as established previously (PLYTYCZ *et al*. 2011). Air-dried soil was sieved and soaked either with cadmium chloride (CHEMPUR, Piekary Slaskie, Polska) dissolved in distilled water to concentrations of cadmium 500 mg/kg air-dried soil (polluted soil, "p") or with an equivalent volume of distilled water (control clean soil, "c"). This cadmium concentration was well tolerated by composting earthworms in a former study (KWADRANS *et al*. 2008), and was previously used for juvenile earthworms *Eisenia fetida* (ŽALTAUSKAITĖ & SODIENĖ 2014).

Cadmium concentration measurements

Cadmium concentrations in soil and in whole earthworm bodies were measured by atomic absorption spectrometry (AAS) described in a previous study (KWADRANS *et al*. 2008).

Earthworms and experimental scheme

Experiments were performed on the composting earthworms *E. andrei* (Ea) from the same laboratory colony as those used previously (SANTOCKI *et al*. 2016b). In short, worms kindly given by professor Franck Vandenbulcke from Lille University (France) were reared in the laboratory of the Department of Evolutionary Immunology, Institute of Zoology, Krakow, Poland, in commercial soil, and fed *ad libitum* a mixed diet comprised of dried/boiled tea, nettle (*Urtica dioica*) and dandelion (*Taraxacum officinale*) leaves. Experiments were performed at ambient temperature 20-23°C, during October-February.

At selected intervals (marked on X axis of Fig. 1) body weights and the occurrence of clitella were recorded, cocoons were manually collected by hand-sorting the soil and counted.

Effects of cadmium soil pollution on the maturation of the worms and restoration of coelomocytes was investigated in juvenile worms (Part A), while effects on cadmium on regeneration of amputated segments was investigated in adult worms (Part B) (Fig. 1).

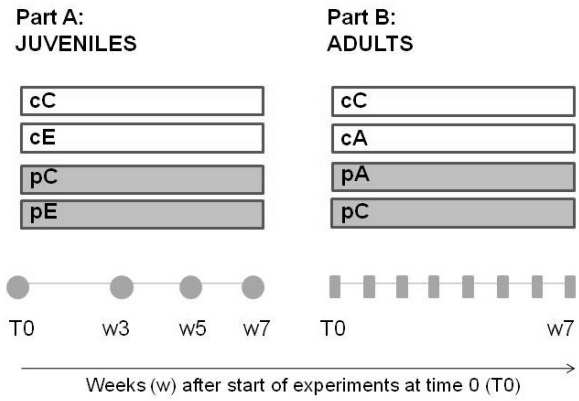

Fig. 1. Scheme of experiments on effects of cadmium-polluted soil on restoration of coelomocytes after experimental depletion in juvenile worms (Part A) and on regeneration of amputated posterior segments in adult worms *Eisenia andrei* (Part B). Empty boxes – clean control soil (c); grey boxes – Cd-polluted soil (p); C – control worms; E – experimental worms with extruded coelomocytes; A – worms with amputated posterior segments. Descriptions in the text.

Part A. Juvenile worms

In total 78 juvenile non-clitellated individuals of *Eisenia andrei* of similar body weights (0.20±0.04 g) were either untreated (control, C) or subjected to coelomocyte extrusion by electric shock (experimental – E) and put in boxes with unpolluted soil (control – c) or soil soaked with cadmium chloride (polluted soil – p), forming groups cC, cE, pC, pE, each of them in 3 boxes, 6 worms per box. On the 3rd, 5th and 7th week after the start of experiments, cocoons were removed and counted, clitellum development was recorded in all

weighed worms, and 2 worms from each box (i.e. 6 worms per each group: cC, cE, pC, pE) were electrostimulated for the first time (cC, pC) or the second time (cE, pE) for analysis of extruded coelomocyte-containing coelomic fluid while their bodies were used for measurements of cadmium accumulation. An additional group of 6 worms was subjected to analysis 2 hours after the first coelomocyte expulsion.

Coelomocytes (i.e. amoebocytes and eleocytes) from electrostimulated worms were counted and coelomocyte lysates were used for spectrofluorimetric measurements of riboflavin stored in granules of eleocytes. Analyses were performed at time 0 and then 3, 5, and 7 weeks later in 6 worms per group.

Analyses of coelomocytes and fluorophores

Coelomocyte extrusion by electrostimulation (30s with 4.5V electric current), differential cell counting in a haemocytometer, and spectrofluorimetric analysis of riboflavin in coelomocyte lysates were performed by the same methods as described previously (SANTOCKI *et al*. 2016b).

Part B. Adult worms

In total 24 adult worms of similar body weights (0.53+0.08g) were subjected to amputation of 40 tail segments under CO_2-water anesthesia and put to either clean or cadmium-polluted soil samples forming cA and pA groups, 6 worm each. Untreated worms kept in clean or polluted soil comprised the control groups, cC and pC, 6-worms each. They were inspected at weekly intervals for cocoon production and tail segment regeneration expressed as numbers of blastema segments. Photographs were taken with a SONY SLTA 58K camera.

Histology of regenerating posterior segments

On the 7th week after amputation of several segments of earthworms from cA and pA groups, 6 worms each, were fixed for histological observations in a freshly prepared mixture of 6 ml formaldehyde, 1 ml acetic acid and 18 ml distilled water for 3 days at room temperature. After thorough washing in distilled water, tissue samples were dehydrated in a graded ethyl alcohol series, immersed with benzene and embedded in paraffin wax. Serial sections (10 µm) were made with a Micron rotary microtome, mounted on gelatin coated slides and stained with Mayer's hematoxylin and eosin as described earlier (MOLNAR *et al*. 2015). Stained sections were observed and photographed with a Nicon-Eclipse80 photomicroscope.

Statistics

Recorded parameter values for earthworms and their coelomocytes were expressed as mean values and standard deviations (X±SD) and analysed by MANOVA and post hoc Tukey's test; $P<0.05$ was established as the level of significance.

Results

Worm viability and cadmium concentration in earthworm bodies

No earthworm mortality in juveniles or adults was recorded throughout the experiments, independent of the conditions.

Cadmium recovery from reference material (animal tissue) was 78%. In bodies of worms from the clean soil, cadmium concentrations were very low (2.5 mg/kg). After transfer to Cd-polluted soil, cadmium concentrations significantly increased to 541 and 982-933 mg/kg after 3 and 5-7 weeks, respectively (Table 1).

Table 1

Cadmium concentration in the whole *E. andrei* bodies. Means not sharing letters are statistically significantly different

Time of exposure to Cd-soaked soil (weeks)	Cd concentration in earthworm bodies (mg*kg^{-1} dw); X±SD; n=6
0w	2.5±2.2^{a}
3w	541±154^{b}
5w	982±367^{c}
7w	933±276^{c}

Body weight

Body weight gain was appr. 0.20 g at the start of experiments and during the first 3 weeks increased to 0.30 g in cC worms and to 0.26-0.27 g in remaining groups, and then increased rapidly until the 5th week in cC and cE worms (to 0.43-0.42 g, respectively), but at a significantly slower rate in pC and pE worms (to 0.33 g), and stayed at similar levels until the 7th week (Fig. 2A).

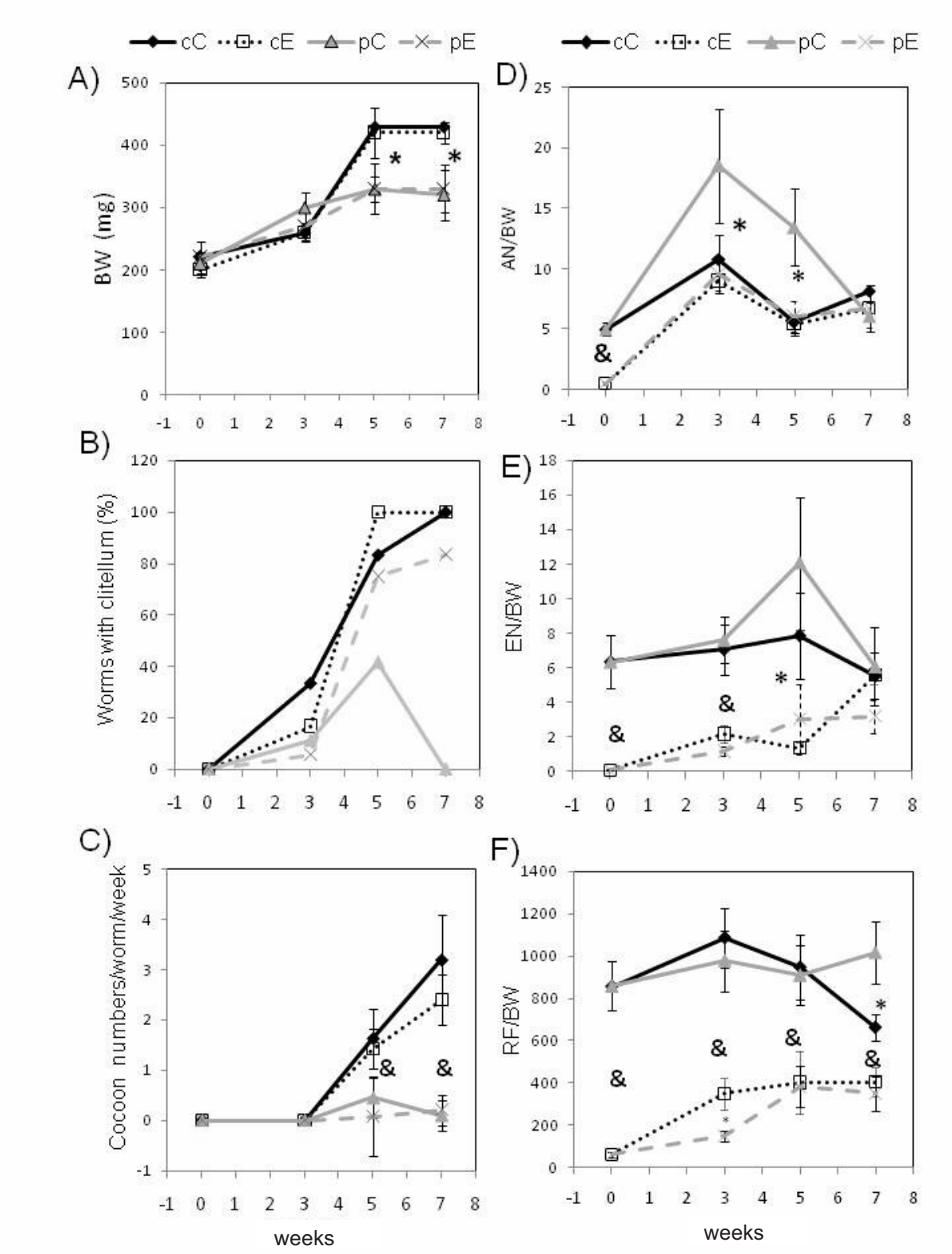

Fig. 2. Characteristics of growth, maturation, reproduction, and coelomocyte-based immune system of earthworms *E. andrei* at the start of experiments either untreated (C) or subjected to electrostimulation-induced coelomocyte depletion (E), and transferred to either clean soil (c) or cadmium-polluted soil (p), forming cC (black solid lines), cE (black dotted lines), pC (grey soild lines), pG (grey dashed lines) groups, and monitored at time 0 and then after 2 hours, and 3, 5 and 7 weeks. The results are expressed as a time-course of changes in: A – body weights (BW); B – acquisition of maturation reflected by clitellum formation; C – cocoon production per worm, per week; components of coelomic fluid expressed per gram body weights (BW); D – numbers of amoebocytes (AN/BW); E – numbers of eleocytes (EN/BW); F – riboflavin content in coelomocyte lysates (RF/BW); X±SD, 6 worms per group. Statistically significant differences between groups from c/p soil*, and C/E worms$^{\&}$.

Clitellum development

At the start of experiments all worms had no signs of clitellum, while 3 weeks later clitella were partly or fully developed in some specimens from all groups of earthworms, either untreated (C) or electrostimulated at the start of experiments (E); more clitella were counted in worms living in clean soil (cC, cE) than in those from cadmium-polluted soil (pC, pE). The percentages of partly or fully clitellated worms were 33, 17, 11, and 6% of worms from cC, cE, pC, pE groups, respectively. In the untreated worms kept in the clean soil, partly or fully developed clitella were recorded in 83 and 100% worms investigated on the 5^{th} or 7^{th} week, respectively. All electrostimulated worms in clean soil (cE) were clitellate already from week 5. In contrast, in the untreated worms kept in polluted soil (pE), 42% of worms had partly or fully developed clitella in week 5, but clitella atrophied later on, as two weeks later clitella were hardly visible. However, most animals from the coelomocyte-depleted group kept in polluted soil (pE) had developed clitella both on the week 5 and 7 (75 and 83%, respectively) (Fig. 2B).

Cocoon production

In worms exposed to either clean or Cd-polluted soil as juveniles (appr. 0.2 g b.w.), no cocoons were deposited during the first 3 weeks of experiments. Then the numbers of cocoons increased in boxes with cC and cE worms reaching on week 7 more than 3 and more than 2 cocoons per week per worm in cC and cE worms, respectively, while numbers of cocoons were close to zero in pC and pE worms from boxes with cadmium-polluted soil (Fig. 2C).

In worms exposed to either clean or Cd-polluted soil as adults (appr. 0.5 g b.w.), the numbers of cocoons produced during the 7-week experimental period was more than two times higher in the clean than cadmium-polluted soil, in both conditions being slightly higher in worms regenerating amputated tail segments (Table 2).

Table 2

Cocoon production by adult worms either untreated (C) or regenerating amputated posterior segments (A) kept for 7 weeks in boxes either in clean (c) or Cd-polluted (p) soil, 6 worms per box

Experimental groups	cC	cA	pC	pA
Cumulative numbers of cocoons	46	53	17	20

Coelomocytes

Among worms untreated at the start of experiments, the numbers of amoebocytes per body weight were significantly higher after 3 and 5 week maintenance in polluted soil (pC), being significantly higher than the number of coelomocytes in worms living in clean soil (cC), and then dropped to the level characteristic for the cC group. During electrostimulation at the start of experiments (w0), worms lost about 90% of amoebocytes but amoebocyte numbers reached levels similar to those in cC group already 3 weeks later and stayed at this level both in the cE and pE groups maintained in both the clean and polluted soil samples (Fig. 2D).

Numbers of eleocytes per body weights were relatively stable during the whole experimental period in the untreated control worms living in clean soil (cC group), but were temporarily increased on week 5 in their untreated counterparts kept in polluted soil (pC group), but this difference was statistically insignificant. Eleocytes were almost completely depleted by electrostimulation performed at time zero, as evidenced by their low numbers in the group of worms investigated 2 hours later. In contrast to amoebocytes, the numbers of eleocytes increased very slowly both in the cE and pE groups of worms being close to the values characteristic for the untreated animals only in week 7 (Fig. 2E).

Riboflavin

Spectrofluorimetric measurements revealed that riboflavin content in coelomocyte lysates from the untreated worms was similar in groups kept in both clean and polluted soil (cC and pC), 3 and 5 weeks after the start of experiments, but on week 7 riboflavin content in cC worms dropped and was significantly lower than that of pC worms at this time point, putatively due to its involvement in reproduction. In worms subjected to electrostimulation at the start of experiments, riboflavin content dropped to 7% of its initial value. Then the amount of riboflavin increased faster in cE worms kept in the clean soil than in pE worms from polluted soil during the first 3 initial weeks, but 5 and 7 weeks after the start of experiments riboflavin content was very similar in cE and pE groups, being always significantly lower than in the untreated cC and pC worms (Fig. 2F).

Regeneration of posterior segments

At the macroanatomical level, soil cadmium had a negligible effect on regeneration of amputated posterior segments, as kinetics of formation of regeneration blastema and a gradual increase of numbers of newly formed segments were even

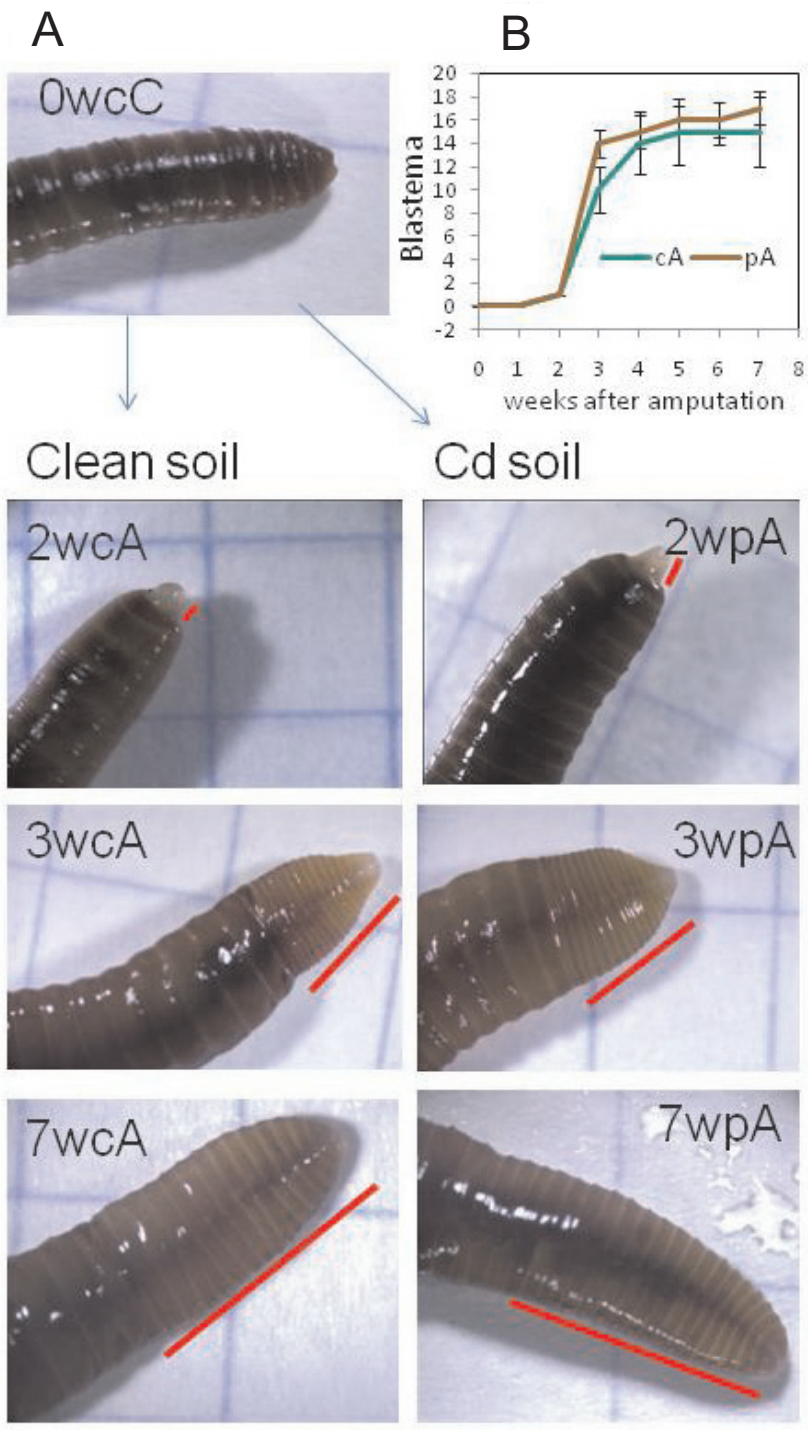

Fig. 3. Regeneration of amputated posterior segments of adult specimens of *E. andrei* transferred after surgery either to clean soil or cadmium-polluted soil. A – photos of worms before surgery and 2, 3 or 7 weeks later during regeneration blastema (red lines) formation; B – time-course of new segment formation in worms kept in the clean (cA) or polluted soil (pA). Means ±SD; 6 worms per group.

slightly faster in cadmium-exposed worms than in their counterparts kept in clean soil (Fig. 3A,B).

Seven weeks after amputation, the damaging effects of cadmium were visible on the cross sections through regenerating segments filled with coelomocytes (Fig. 4A and 4B). In the Cd-intoxicated worms, the body wall muscles were incompletely differentiated (Fig. 4C'), nuclei of midgut epithelium and coelomocytes were swollen (Fig. 4D'), and the ventral nerve cord (VNC) ganglia had irregular structures (Fig. 4E'), different from those of respective structures in regenerating segments of earthworms maintained in unpolluted soil (compare with Figs 4C, 4D, 4E).

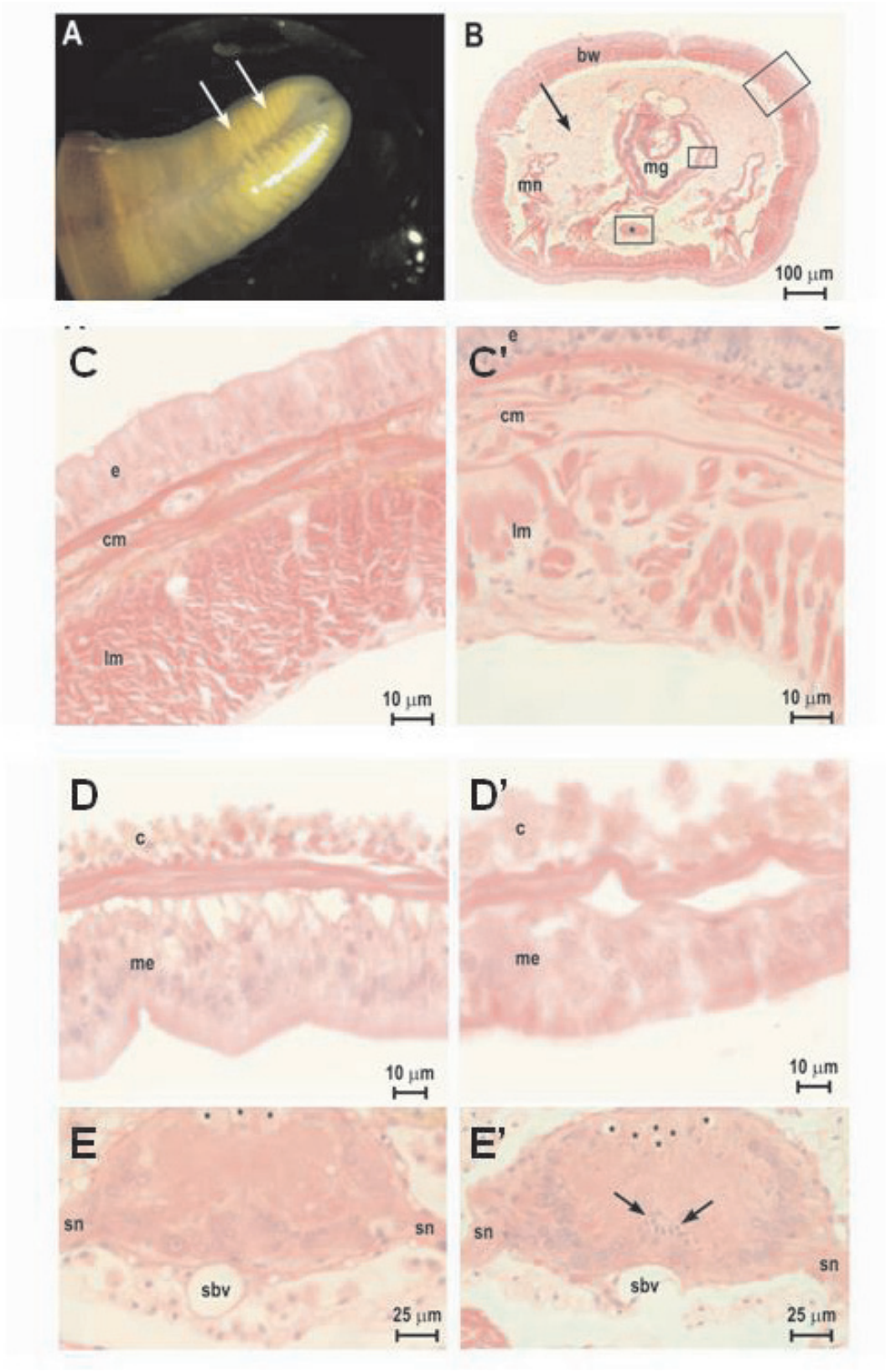

Fig. 4. Morphology of regenerating posterior segments during week 7 after amputation in *E. andrei* kept in clean soil (control) or cadmium polluted soil. A – Accumulation of coelomocytes (arrows) in regenerating posterior segments; B – coelomocytes in a cross section of a segment. bw: body wall; metanephridium; mg; midgut; asterisk: VNC ganglion. Rectangles show histologically observed body parts of the control (left panel below) and Cd-poisoned earthworms (right panel below). CC') body wall. e: body wall epithelium; cm: circular muscles; lm: longitudinal muscles. DD') midgut. In D' note Cd-stimulated nuclear swelling of both chloragocytes (c) and midgut epithelium (e). EE') ventral nerve cord ganglia (VNC). In E' the VNC ganglia have an irregular structure, i.e. the pattern of neurons is not identical with that of normal ganglia; undifferentiated cells (arrows) are located close to neural somata and extranumerary dorsal giant axon (asterisks) occurred. sn; segmental nerve; sbv: subneural blood vessel.

Discussion

A relatively high nominal cadmium concentration, i.e. 500 mg*kg^{-1} soil, did not induce *E. andrei* mortality during the present experiments, while the same concentration was lethal for some juveniles of *E. fetida* (ŽALTAUSKAITĖ & SODIENĖ 2014). However, *E. andrei* worms for the present experiments were kept in organic soil from commercial supplier containing 51% of organic matter, while *E. fetida* were maintained in artificial soil consisting of 70% quartz, 20% clay and 10% powdered calcium carbonate (ŽALTAUSKAITĖ & SODIENĖ (2014). It has been shown that low organic matter in soil increased Cd toxicity causing mortality and reproduction impairment of *E. fetida*, probably due to a lack of energy to maintain protection mechanisms (IRIZAR *et al*. 2015). Cadmium concentration in bodies of *E. andrei* exposed to Cd-spiked soil increased in a time-dependent manner, as recorded previously in another composting species, *Dendrobaena veneta*, tested at similar experimental conditions (KWADRANS *et al*. 2008).

Cadmium inhibited *E. andrei* maturation and reproduction

The results of the present experiments on juveniles of *E. and*rei indicated that cadmium exposure slowed down body weight gain. Stabilization of body weight from the 5th week in worms kept in both clean and polluted soil may be connected with accumulation of waste metabolic products in soil and/or a trade-off mechanism between body weight gain and reproduction.

Sexual maturation and reproduction measured by cocoon deposition were evidently inhibited in *E. andrei* exposed to cadmium-spiked soil. A similar response to cadmium exposure, i.e. absent or delayed maturation and reduced cocoon production, was observed in juveniles of *E. fetida* (ŽALTAUSKAITĖ & SODIENĖ 2014). In the present experiments, the clitellum developed faster in worms living in unpolluted soil than in their counterparts kept in polluted soil. Interestingly, in the polluted soil there was a rapid atrophy of developing clitella in the untreated worms while clitella development was rescued in Cd-exposed worms subjected to coelomocyte expulsion followed by restoration of depleted coelomocytes. Maybe factors derived from regenerating cells/tissues can delay degenerative processes. This phenomenon is worthy of further exploration.

The disrupted structure of ovaries (SIEKIERSKA & URBAŃSKA-JASIK 2002) and suprapharyngeal and subpharyngeal ganglia (SIEKIERSKA 2003) were described in cadmium-exposed *D. veneta.* Morphologically similar changes in the ovary cells were induced after amputation of anterior segments in the earthworm *D. veneta* and these changes were fully reversible (SIEKIERSKA 2007). Cocoon deposition by *D. veneta* was reversibly inhibited by amputation of anterior segments or surgical removal of suprapharyngeal ganglia (brains) (OKRZESIK *et al*. 2013; MOLNAR *et al*. 2015; PLYTYCZ & MORGAN 2015). This indicates the great plasticity of earthworm gonads. The damaging effects of cadmium on gonads was described in other model organisms, such as the invertebrate *Bombyx mori* (YAN *et al*. 2016; YUAN *et al*. 2016), and fish, tilapia *Oreochromis niloticus* (LUO *et al*. 2015). In tilapia, Cd exposures affected gonad development by altering steroid hormone levels and sex-related gene expressions (LUO *et al*. 2015). Adverse effects of cadmium on testes of avian and mammalian species are reviewed by MARETTOVÁ *et al*. (2015).

Immune system and regenerative processes are resistant to cadmium exposure

The significant increase of amoebocyte numbers in untreated worms exposed for 3 or 5 weeks to Cd-polluted soil may be connected with their proliferation in response to modified bacterial biota of cadmium-polluted soil. A drop of amoebocyte numbers between the 5th and 7th week may reflect an exhaustion of proliferation and/or formation of multicellular bodies encapsulating invaders, being too large to be expelled during electrostimulation for analyses.

A fast restoration of immunocompetent amoebocytes after experimental expulsion was recorded in previous studies (KLIMEK *et al*. 2012; SANTOCKI *et al*. 2016 a, b); the results of present investigation evidenced that the kinetics of restoration was undisturbed by cadmium exposure.

In juvenile worms restoring the coelomocyte system after experimental depletion, the numbers of riboflavin-storing eleocytes reached a level characteristic for intact worms faster than the amount of riboflavin in coelomocyte lysates. Perhaps extensive growth and maturation of juvenile specimens is energetically highly demanding, thus riboflavin is continuously metabolized to its metabolically active forms FMN and FAD. Involvement of riboflavin in developmental processes was described in larvae of the polychaete *Capitella teleta* (BURNS *et al*. 2014). The opposite situation was recorded earlier in adult specimens of *D. veneta* (KLIMEK *et al.* 2012) and *E. andrei* (SANTOCKI *et al*. 2016b) restoring coelomocyte system, as eleocytes freshly detached from chloragogenous tissue possessed higher amounts of riboflavin than the old eleocytes.

The main protective mechanism of the earthworm immune system against cadmium pollution seems to be expression of cadmium metallothionein, MT-2 (e.g. BRULLE *et al*. 2007; HOMA *et al*. 2007, 2015; CALISI *et al*. 2014; KOWALD *et al*. 2016). Studies on metallothioneins in worms regenerating coelomocyte systems or lost body segments are in progress.

Abundance of riboflavin in eleocytes from *Eisenia* sp. may be responsible for very efficient regeneration blastema formation after autotomy or amputation of posterior segments, while formation of blastema was consistently absent in another composting species, *D. veneta*, with a moderate amount of riboflavin in eleocytes (PLYTYCZ *et al*. 2016). Involvement of riboflavin in efficient formation of regeneration blastema was evidenced in the earthworm *Eudrilus eugeniae* (JOHNSON *et al*. 2012). Putatively, abundance of riboflavin can also be involved in resistance of regeneration blastema of *E. andrei* to cadmium soil pollution.

Summary and Conclusions

Ecological studies on *Eisenia* sp. focused mainly on effects of environmental factors on earthworm viability, life-history strategies, growth rates and cocoon production, while immunological studies explored the effects of pollutants on coelomocytes and soluble factors present in worm coelomic fluids.

Prolonged exposure of juvenile specimens of *E. andrei* to cadmium polluted soil at 20-23^{o}C (1) delayed worm maturation and inhibited reproduction; (2) induced proliferation of amoebocytes in the untreated worms with slight effects on eleocytes and eleocyte-derived riboflavin; (3) had no or slight adverse effects on restoration of experimentally depleted amoebocytes and riboflavin-storing eleocytes; (4) In adult worms, cadmium accumulation inhibited reproduction but had little effect on efficiency of regeneration of amputated posterior segments**.** In general, the results of the present experiments show that in *E. andrei* cadmium accumulation inhibits reproduction but has no or few adverse effects on regeneration.

Mechanisms involved in resistance of regenerative processes to cadmium exposure are worthy of further elucidations, with special emphasis on tissue-specific expression of Cd-metallothioneins and other stress-proteins, as well as various stress-related mechanisms that play a protective role in particular organs of cadmium exposed earthworms (e.g. CHEN *et al*. 2017; BEAUMELLE *et al*. 2016).

References

ALBANI J.R., DEMUYNCK S., GRUMIAUX F., LEPRĘTRE A. 2003. Fluorescence fingerprints of *Eisenia fetida* and *Eisenia andrei*. Photochem. Photobiol. **78**: 599-602.

BEAUMELLE L., VILE D., LAMY I., VANDENBULCKE F., GIMBERT F., HEDDE M. 2016. A structural equation model of soil metal bioavailability to earthworms: confronting causal theory and observations using a laboratory exposure to field-contaminated soils. Sci. Total Environ. **569-570**: 961-972.

BILEJ M., PROCHAZKOVA P., SILEROVA M., JOSKOVA R. 2011. Earthworm immunity. Adv. Exp. Med. Biol. **708**: 66-79.

BRULLE F., MITTA G., LEROUX R., LEMIERE S., LEPRETRE A., VANDENBULCKE F. 2007. The strong induction of metallothionein gene following cadmium exposure transiently affects the expression of many genes in *Eisenia fetida*: a trade-off mechanism? *Comp. Biochem. Physiol. Toxicol. & Pharmacol. CBP* **144**: 334-341.

BRUNS E., EGELER P.H., MOSER T., ROMBKE J., STEFFCZYK A., SPORLIEN P. 2001. Standardisierung und Validierung eines Bioakkumulationstests mit terrestrischen Oligochaete. Report o the German Federal Environmental Agency (Umweltbundesamt Berlin), R&D No: 298.64.416.

BURNS R.T., PECHENIK J.A., BIGGERS W.J., SCAVO G., LEHMAN C. 2014. The B vitamins nicotinamide (B3) and riboflavin (B2) stimulate metamorphosis in larvae of the deposit-feeding polychaete *Capitella teleta*: implications for a sensory ligand-gated ion channel. PLoS One. **9**:e109535.

CALISI A., LIONETTO M.G., DE LORENZIS E., LEOMANNI A., SCHETTINO T. 2014. Metallothionein induction in the coelomic fluid of the earthworm *Lumbricus terrestris* following heavy metal exposure: a short report. Biomed. Res. Int. **2014**:109386. doi: 10.1155/2014/109386.

CHEN X., WANG X., GU X., JIANG Y., JI R. 2017. Oxidative stress responses and insights into the sensitivity of the earthworms *Metaphire guillelmi* and *Eisenia fetida* to soil cadmium. Sci. Total Environ. **574**: 300-306.

GAŁUSZKA A., KLIMEK B., BIGAJ J., PLYTYCZ B. 2015. Endogeic earthworm *Aporrectodea caliginosa* as a model species for studies of modulation of regeneration by environmental stressors. Acta Biol. Crac. Zool. **57**: 15-23

HOMA J., RORAT A., KRUK J., COCQUERELLE C., PLYTYCZ B.,VANDENBULCKE F. 2015. Dermal exposure of *Eisenia andrei* earthworms: Effects of heavy metals on metallothionein and phytochelatin synthase gene expressions in coelomocytes. Environ.Toxicol. Chem. **34**: 1397-1404.

HOMA J., STÜRZENBAUM S.R, MORGAN A.J., PLYTYCZ B. 2007. Disrupted homeostasis in coelomocytes of *Eisenia fetida* and *Allolobophora chlorotica* exposed dermally to heavy metals. Eur. J. Soil Biol. **43**: 273-280.

IRIZAR A., RODRÍGUEZ M.P., IZQUIERDO A., CANCIO I., MARIGÓMEZ I., SOTO M. 2015. Effects of soil organic matter content on cadmium toxicity in *Eisenia fetida*: implications for the use of biomarkers and standard toxicity tests. Arch. Environ. Contam. Toxicol. **68**: 181-92.

JOHNSON R.S.S.C., ELAIYA R.S., BERYL V.Y., EDITH A.J.A., AMUTHA K., DINESH S.M. *et al.* 2012. Autofluorescence in BrdU-positive cells and augmentation of regeneration kinetics by riboflavin. Stem. Cells Dev. **21**: 2071-2083.

KLIMEK M., KRUK J., PLYTYCZ B. 2012. Restoration of coelomocytes in the earthworm *Dendrobaena veneta.* Acta Biol. Cracov. Zool. **54**: 11-17.

KOCINSKI M., TAKACS V., MOLNAR L., MORGAN A.J., BIGAj J., PLYTYCZ B. 2016. Experimental induction of autotomy in two potential model lumbricid earthworms *Eisenia andrei* and *Aporrectodea caliginosa*. ISJ. **13**: 11-17.

KOWALD G.R., STÜRZENBAUM S.R., BLINDAUER C.A. 2016. Earthworm *Lumbricus rubellus* MT-2: Metal binding and

protein folding of a true cadmium-MT. Int J Mol Sci. **17**. pii: E65. doi: 10.3390/ijms17010065.

KWADRANS A., LITWA J., WOŁOSZCZAKIEWICZ S., KSIĘŻARCZYK E., KLIMEK M., DUCHNOWSKI M., KRUK J., PLYTYCZ B. 2008. Changes in coelomocytes of the earthworm, *Dendrobaena veneta*, exposed to cadmium, copper, lead or nickEl-contaminated soil. Acta Biol. Cracov. Zool. **49**: 57-62.

LUO Y., SHAN D., ZHONG H., ZHOU Y., CHEN W., CAO J., GUO Z., XIAO J., HE F., HUANG Y., LI J., HUANG H., XU P. 2015. Subchronic effects of cadmium on the gonads, expressions of steroid hormones and sex-related genes in tilapia *Oreochromis niloticus*. Ecotoxicology. **24**: 2213-2223.

MARETTOVÁ E., MARETTA M., LEGÁTH J. 2015. Toxic effects of cadmium on testis of birds and mammals: a review. Anim Reprod Sci. **155**: 1-10. doi: 10.1016/j.anireprosci.

MOLNAR L., POLLAK E., SKOPEK Z., GUTT E., KRUK J., MORGAN A.J., PLYTYCZ B. 2015. Immune system participates in brain regeneration and restoration of reproduction in the earthworm *Dendrobaena veneta*. Dev. Comp. Immunol. **52**: 269-279.

OKRZESIK J., KACHAMAKOWA-TROJANOWSKA N., JOZKOWICZ A., MORGAN A.J., PLYTYCZ B. 2013. Reversible inhibition of reproduction during regeneration of cerebral ganglia and celomocytes in the earthworm *Dendrobaena veneta*. ISJ **10**: 151-161.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT. 1984. Test No. 207: Earthworm, acute toxicity tests, Section 2: Effects on biotic systems, OECD Guidelines for the Testing of Chemicals. Paris, France.

OTOMO P.V., OTOMO L.V., BEZUIDENHOUT C.C., MABOETA M.S. 2016. Preliminary evidence of differences in cadmium tolerance in metal-free stocks of the standard earthworm test species *Eisenia andrei* (Oligochaeta). Ecotoxicology **25**: 1119-1125.

PANZARINO O., HYRŠL P., DOBEŠ P., VOJTEK L., VERNILE P., BARI G., TERZANO R., SPAGNUOLO M., DE LILLO E. 2016. Rank-based biomarker index to assess cadmium ecotoxicity on the earthworm *Eisenia andrei*. Chemosphere **145**: 480-486.

PÉREZ-LOSADA M., EIROA J., MATO S., DOMÍNGUEZ J. 2005. Phylogenetic species delimitation of the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences. Pedobiologia **49**: 317-324.

PLYTYCZ B., BIGAJ J., FALNIOWSKI A., MORGAN A.J. 2016. Unexpected results and open questions from experiments on regeneration in lumbricid worms. Inv. Surv. J. **13**: 315-326.

PLYTYCZ B., KLIMEK M., KLIMEK B.A., SZYMANSKI W., KRUK J., MORGAN A.J. 2011. Riboflavin content in the coelomocytes of contrasting earthworm species is differentially affected by edaphic variables including organic matter and metal content. Pedobiologia **54S**: S43-S48.

PLYTYCZ B., MORGAN A.J. 2015. Interactions between earthworm neuroendocrine and immune systems. ISJ **12**: 176-178.

RORAT A., KACHAMAKOVA-TROJANOWSKA N., JOZKOWICZ A., KRUK J., COCQUERELLE C., VANDENBULCKE F., SANTOCKI M., PLYTYCZ B. 2014. Coelomocyte-derived fluorescence and DNA markers of composting earthworm species. J. Exp. Zool. A Ecol. Genet. Physiol. **321**: 8-40.

SANTOCKI M., FALNIOWSKi A., PLYTYCZ B. 2016a. Restoration of experimentally depleted coelomocytes in juvenile and adult composting earthworms *Eisenia andrei*, *E. fetida* and *Dendrobaena veneta*. Appl. Soil Ecol. **104**: 163-173.

SANTOCKI M., MORGAN A.J., PLYTYCZ B. 2016b. Differential time course of restoration of experimentally depleted coelomocytes and fluorophores in the earthworm *Eisenia andrei*. Folia Biol. (Kraków) **64**: 121-130.

SIEKIERSKA E. 2003. Cadmium effect on the structure of supra- and subpharyngeal ganglia and the neurosecretory processes in earthworm *Dendrobaena veneta* (Rosa) 2003. Environ. Pollut. **126**: 21-28

SIEKIERSKA E., URBAŃSKA-JASIK D. 2002. Cadmium effect on the ovarian structure in earthworm *Dendrobaena veneta* (Rosa). Environ. Pollut. **120**: 289-297.

SIEKIERSKA E. 2007. Changes in the ovary cells structure in the earthworm *Dendrobaena veneta* (Rosa) in neurosecretion deficiency and after exposure to cadmium. Eur. J. Soil Biol. **43**: S311-S315.

YAN S.Q., XING R., ZHOU Y.F., LI K.L., SU Y.Y., QIU J.F., ZHANG Y.H., ZHANG K.Q., HE Y., LU X.P., XU S.Q. 2016. Reproductive toxicity and gender differences induced by cadmium telluride quantum dots in an invertebrate model organism. Sci. Rep. **6**: 34182. doi: 0.1038/srep34182.

YUAN H., QIN F., GUO W., GU H., SHAO A. 2016. Oxidative stress and spermatogenesis suppression in the testis of cadmium-treated *Bombyx mori* larvae. Environ. Sci. Pollut. Res. Int. **23**: 5763-5770. doi: 10.1007/s11356-015-5818-3.

ŽALTAUSKAITĖ J., SODIENĖ I. 2014. Effects of cadmium and lead on the life-cycle parameters of juvenile earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. **103**: 9-16.