Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2017.21392

86 (2017) 320–326
August

Sewage sludge toxicity: comparison of plants and soil invertebrates response

J. Žaltauskaitė*, R. Vaisiūnaitė, G. Sujetovienė, R. Dagiliūtė

Department of Environmental Sciences, Vytautas Magnus University, Vileikos Street 8-223, LT-44404 Kaunas, Lithuania, Tel. +370 37327904; Fax: +370 37327916; email: j.zaltauskaite@gmf.vdu.lt (J. Žaltauskaitė)

Received 30 January 2017; Accepted 29 August 2017

ABSTRACT

Sewage sludge disposal is of major concern for the quality of the receiving soils. Sewage sludge application can cause negative ecological impact to terrestrial ecosystems and pose a human health risk. Potential hazard of sewage sludge application assessment based on the sewage sludge physicochemical analysis do not evaluate possible toxic effects to soil biota. In this study, sewage sludge ecotoxicological assessment was based on the bioassays with earthworm (*Eisenia fetida*) and proso millet (*Panicum miliaceum* L.). The aim of this study was to compare the toxicity of untreated and treated (kept for 1 year in sludge storage site) sewage sludge to proso millet (*P. miliaceum* L.) and earthworm (*E. fetida*). Untreated sewage sludge was severely toxic and resulted in 100% earthworms and millet seeds mortality. Treated sewage sludge did not reduce the survival of earthworms, though negatively affected the earthworms' biomass, cocoon production rate and induced lipid peroxidation. The response of *P. miliaceum* to sewage sludge application was less pronounced. Untreated sewage sludge slightly reduced the millet growth and concentrations of photosynthetic pigments. Sewage sludge exhibited lower toxicity to plants than to soil dwelling organisms and low sewage sludge application levels had the stimulatory effect to the growth of *P. miliaceum*.

Keywords: Earthworm; Millet; Sewage sludge; Toxicity

1. Introduction

Sewage sludge production in the world is increasing and its disposal is of growing concern. Disposal pathways include landfill storage, incineration and land application. Land application of sewage sludge is a cheap and preferred sewage sludge disposal (reuse) route in European Union (EU). The Sewage Sludge Directive 86/278/EEC [1] encourages the use of sewage sludge in agriculture. In EU nearly 40% of sewage sludge is reused in agriculture or forestry and in some countries (Spain, France) the sewage sludge reuse in agriculture reaches up to 70% [2].

Reuse of sewage sludge in agriculture may be beneficial because it reduces the waste deposition in the landfills and herewith greenhouse gas emissions from landfills and may contribute to the implementation of end-of-waste policy [3]. As sewage sludge is rich in organic matter and nutrients (N and P), contains trace elements (Ca, Mg, S, etc.), its application in agricultural soils allows to maintain or improve soil physical, chemical and biological properties and to reduce the use of fertilizers. However, sewage sludge may contain pathogenic microorganisms, heavy metals and persistent organic pollutants. Heavy metals and persistent organic pollutants (PAHs, PCBs, PCDD/F, etc.) are present in sewage sludge at different concentrations [4–6] and they may accumulate in the soil, be transferred from soil to plants and be incorporated into the food chain and pose a risk to human health [7,8]. Therefore, the sewage sludge use in agriculture is regulated in such a way so as to prevent harmful effects on soil, vegetation, animals and humans.

The major problem in controlling sewage sludge application in agriculture and forestry is related to its environmental toxicity. Traditionally, the control of the quality of sewage sludge is based on chemical analysis of the sewage sludge. However, chemical data alone do not allow evaluation

---

* Corresponding author.

Presented at the 13th International Conference on Protection and Restoration of the Environment (PRE-XIII), 3–8 July 2016, Mykonos, Greece.

of possible toxic effects to biota living in receiving soils. Chemical analysis of sewage sludge is usually insufficient to provide the information on sewage sludge quality and toxicity as a high number of chemical compounds are present, some of them are in concentrations lower than detection limits and moreover various interaction effects of chemicals in mixtures can occur.

The ecotoxicity of sewage sludge previously was assessed with luminescent bacteria [9,10], soil invertebrates [11] and plants. Phytotoxicity of sewage sludge usually is evaluated using germination test [12,13] and therefore the impact on plant physiology is not assessed. The majority of studies analyzing the sewage sludge impact on plants focus on the sewage sludge application effects on the fast growing tree species (willows (*Salix* sp.), poplars (*Populus* sp.)) used in short rotation coppice and heavy metal accumulation by these tree species from sewage sludge amended soils [14–16]. However, these species may have different sensitivity to sewage sludge than other plants (herbaceous).

Earthworms are soil keystone species constituting the dominant biomass of the soil fauna. The pollutants induced adverse effects on keystone species may impair community stability and could have serious adverse ecological effects on the entire terrestrial ecosystem. As sewage sludge usually is very sticky and compact, earthworms incorporation into the sewage sludge amended soil could lead to the increase porosity of the soil and may improve soil quality. Consequently, the majority of works are dedicated to the use of vermicompost in order to improve sewage sludge quality [17,18] or remove heavy metals from the sewage sludge [19,20].

It is accepted that toxicity bioassay, using species representing the different trophic levels, is the best approach to evaluate the whole toxicity of selected matrix. Therefore, the selection of test organisms, representing different trophic levels is crucial. The aim of this study was to investigate the effects of fresh and treated sewage sludge on forage crop proso millet (*Panicum miliaceum* L.) and earthworms *Eisenia fetida*. Sewage sludge toxicity evaluation using plants and soil dwelling organisms could give more comprehensive estimation of possible environmental threats related with sewage sludge use in agriculture.

2. Materials and methods

2.1. Sewage sludge

The sewage sludge was collected from the municipal wastewater treatment plant in Kaunas, Lithuania. The Kaunas wastewater treatment plant serves a city of about 309,000 inhabitants and treats approximately 22 million m^3 of wastewater per year. The sewage sludge was collected at the end point of sewage sludge mesophilic anaerobic digestion process and was labelled as fresh sewage sludge. The second sewage sludge was collected from sewage sludge storage landfill, where it was stored for 1 year and was labelled as treated sewage sludge.

The moisture content in fresh sewage sludge was 73% ± 3%, ash content 45% ± 5%, nitrogen concentration 20 ± 2 g kg^{-1} and phosphorous concentration 30 ± 2.5 g kg^{-1}.

For heavy metal chemical analysis sewage sludge samples were dried at 105°C for 48 h and digested in HNO_3. Concentrations of heavy metals (Cu, Cd, Zn and Pb) were determined by atomic absorption spectrometry (Shimadzu AA-6800). All the reagents were of analytical reagent grade.

2.2. Toxicity evaluation

The phytotoxicity test with proso millet (*P. miliaceum* L.) was carried out according to modified organisation for economic co-operation and development (OECD) guidelines for the testing of chemicals 208 [21]. Test of seedling emergence and early seedling growth was conducted.

Fifteen seeds of *P. miliaceum* were sown in pots containing peat substrate mixed with different concentrations or fresh and treated sewage sludge. Tested sewage sludge concentrations were 25%, 50%, 75% and 100%. Pots were kept in the regime of 21°C ± 2°C temperature under 16:8 h light:dark. The following endpoints were measured to evaluate phytotoxicity: shoot height (cm), root lengths (cm), dry shoot and dry biomass and concentrations of photosynthetic pigments (chlorophyll a, b and carotenoids). The assays were performed in three replicates. Concentrations of the photosynthetic pigments (chlorophyll a, b and carotenoids) were measured spectrophotometrically in 100% acetone extract of plant tissue [22].

The toxicity test with earthworm *E. fetida* was carried out according to the OECD guidelines for the testing of chemicals [23]. The test earthworms were taken from a breeding culture kept in laboratory of Vytautas Magnus University. All selected worms were adult and fully scintillated. Earthworm's toxicity bioassay was conducted only with treated sewage sludge as fresh sewage sludge was extremely toxic for earthworms. The artificial soil was prepared with the following composition (by dry weight): 70% quartz sand, 20% clay and 10% sphagnum peat. The soil pH_{KCl} was adjusted to 6.0 ± 0.5 with powdered calcium carbonate ($CaCO_3$). The constituents of artificial soil were air-dried, mixed thoroughly and weighted (300 g) into plastic boxes. The dry soil was moistened with distilled water to obtain approximately half of the final required water content. Treated sewage sludge was mixed with artificial soil to obtain final sewage sludge content in the treatment: 25%, 50%, 75% and 100%. Three replicates were used for each of the test concentration and control. Five earthworms were added to each covered box and after 3 h they were checked to ensure that all the worms had burrowed into the soil. To ensure earthworms growth the earthworms were supplied with approximately 2.5 g of oatmeal per box weekly. The experiment was run for 7 weeks at 20°C under continuous illumination (600 lux). Mortality and growth were measured on a weekly basis (every 7 d) by counting and weighing the surviving earthworms in each box. The earthworms were considered alive if they were able to respond to mechanical stimulus. Cocoons were collected by sorting through the soil after the experiment. Any cocoons produced during the experiment were collected.

Concentration of malondialdehyde (MDA), the end product of lipid peroxidation, was used as biomarker of membrane oxidative damage. MDA content in the tissues of earthworms was determined by reaction with thiobarbituric acid (TBA) [24]. Earthworm's tissues were homogenized in Tris–NaCl buffer (pH 7.5) and after centrifugation the tissue extracts were treated with TBA and heated at 95°C for

30 min. After cooling the reaction mixture was centrifuged at 10,000×*g* for 15 min. The absorbance of the coloured supernatant was measured at 532 nm.

Earthworms growth rate during the study period was determined by linear regression and the slope of the curve (b) was used as a prediction of growth rate (g week^{-1}). Significance of difference between the linear regression slopes for different sewage sludge concentrations was assessed using Z test [25]:

$$Z = \frac{b_1 - b_2}{\sqrt{\mathrm{SE}b_1^2 + \mathrm{SE}b_2^2}} \tag{1}$$

where b_1 and b_2 are the slopes of the linear regression for the different sewage sludge concentrations, SEb is the standard error of b coefficient. The differences between the slopes were considered to be statistically significant ($p < 0.05$) when Z test exceeded the critical value of 1.96.

2.3. Statistical analysis

A one-way analysis of variance (ANOVA) was used to assess the sewage sludge concentration effect on estimated endpoints. Significant differences between control and treatment samples were determined by the Dunnett's test and $p < 0.05$ were considered to be significant. Significant differences between treatments were determined by Student's test and $p < 0.05$ were considered to be significant. All the statistical analysis was carried out using Statistica software.

3. Results

3.1. Heavy metal content

None of the measured metals concentrations exceeded the limit values for heavy metal concentrations in sewage sludge for use in agriculture [1,26] (Table 1).

The concentrations of heavy metals, with the exception of Ni, in the sewage sludge deposited for 1 year were lower than in the untreated sludge (t test, $p < 0.05$). During 1 year of sewage sludge storage the concentrations of Cu decreased by 15.32%, Cd by 62.18% and Cr by 78.38%. Concentrations of Zn and Ni changed very slightly.

3.2. Sewage sludge phytotoxicity

Fresh sewage sludge had a significant effect on the proso millet seedling emergence (ANOVA, $F = 91.04$, $p < 0.01$). Not even one seedling has emerged in the treatment of 100% fresh sewage sludge; however, in other samples amended with different amount of fresh sewage sludge, the seedling emergence was higher than 62.22%. The seedling emergence was not adversely affected by treated sewage sludge (ANOVA, $F = 1.85$, $p = 0.20$) and in all treatments the emergence exceeded 64.44%.

Fresh sewage sludge application had a significant effect on the root length of proso millets (ANOVA, $F = 271.46$, $p < 0.01$; Fig. 1(A)). The root length of plants exposed to fresh sewage sludge was significantly lower than that of control plants (Dunnett's test, $p < 0.001$). Root length decreases along with fresh sewage sludge concentration in

Table 1
Sewage sludge characteristics (mg kg^{-1})

	Untreated sewage sludge	Treated sewage sludge	Limit values, Directive 86/278/EEC [1]	Limit values, working document on sludge [26]
Cu	171.59 ± 2.90	145.31 ± 5.94	1,000–1,750	1,000
Cd	3.49 ± 0.05	1.32 ± 0.04	20–40	10
Zn	83.36 ± 0.12	81.27 ± 0.11	2,500–4,000	2,500
Ni	14.50 ± 0.12	15.19 ± 0.18	300–400	300
Cr	218.86 ± 3.21	47.31 ± 1.17	–	1,000

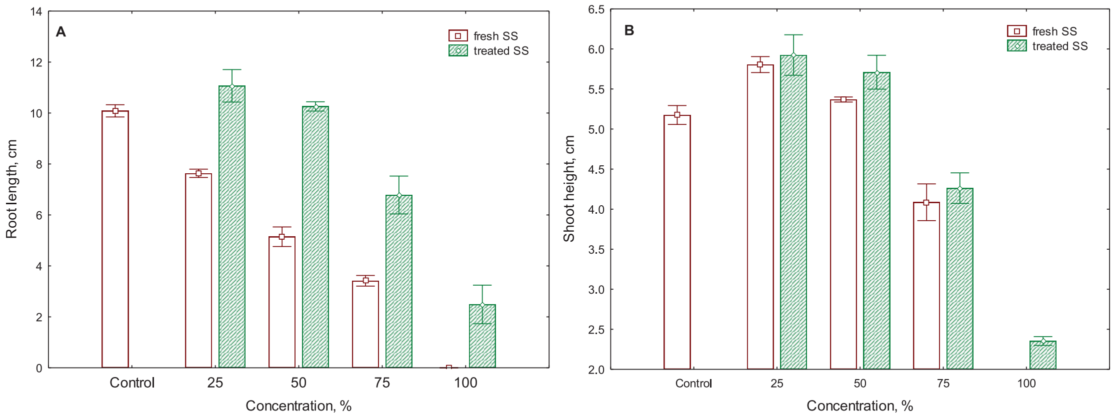

Fig. 1. Root length (A) and shoot height (B) of proso millet (*Panicum miliaceum* L.) exposed to different concentrations of fresh and treated sewage sludge.

the soil ($R^2 = 0.98$, $p < 0.01$). Low application of treated sewage sludge (25%) slightly ameliorated the growth of roots, though high concentrations of treated sewage sludge in the soil led to a decrease of millet root length ($p < 0.01$). The better growth of *P. miliaceum* after treated sewage sludge application could be explained by higher amount of available nutrients in soil comparing with the control soil.

Low and moderate concentrations of both, fresh and treated, sewage sludge concentrations in the soil slightly stimulated the growth of shoots of proso millet (Fig. 1(B)). However, high concentrations of sewage sludge adversely affected the growth of shoots and the proso millet shoots were up to 54.51% lower than the shoots of control plants (Dunnett's test, $p < 0.005$).

Low concentrations of fresh sewage sludge had no effect on the dry weight of millet roots. However, roots of plants exposed to 50%–75% of fresh sewage sludge in the soil presented two times lower dry weight than that of control plants (Dunnett's test, $p < 0.005$; Fig. 2(A)). Soil application with treated sewage sludge resulted in higher roots dry weight compared with control plants with exception of the treatment with 100% of treated sewage sludge. Both fresh and treated SS had no adverse effect on the shoots dry weight (Fig. 2(B)). The shoots dry weight of plants exposed to 25%–50% of sewage sludge concentrations in the soil was up to two times higher than the dry weight of control plants.

Soil treatment with fresh and treated sewage sludge had significant effect on the concentrations of photosynthetic pigments in the plants (ANOVA, $F_{fresh} > 9.86$, $F_{treated} > 6.27$, $p < 0.01$; Figs. 3(A) and 3(B)). Concentrations of photosynthetic pigments decreased along with the fresh sewage sludge concentration in the soil ($R^2 > 0.38$, $p < 0.05$) and carotenoids were the most sensitive to the treatment with fresh sewage sludge.

Treatment with 25% of treated sewage sludge in the soil had no negative impact of the concentration of chlorophyll a and b, though higher treated sewage sludge concentrations in the soil led to reduced concentrations of both chlorophyll a and b. Carotenoids concentrations in the leaves of proso millet grown in the soil with different concentrations of treated sludge were significantly lower than in the control plants tissues (Dunnett's test, $p < 0.05$) and decreased along with treated sewage sludge concentration in the soil ($R^2 > 0.26$, $p < 0.05$).

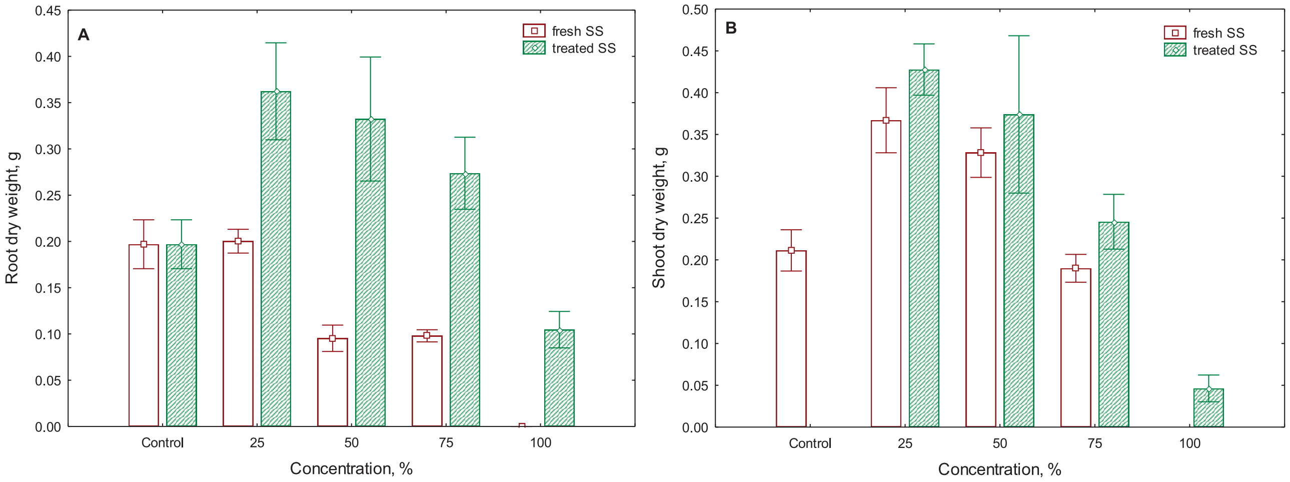

Fig. 2. Root dry weight (A) and shoot dry weight (B) of proso millet (*Panicum miliaceum* L.) exposed to different concentrations of fresh and treated sewage sludge.

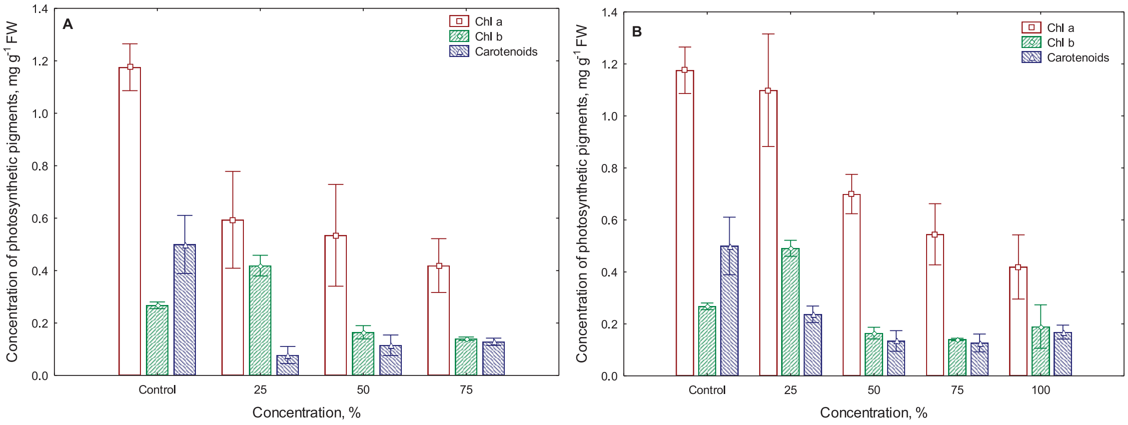

Fig. 3. Concentrations of chlorophylls a (Chl a), chlorophylls b (Chl b) and carotenoids in the leaves of proso millet (*Panicum miliaceum* L.) exposed to different concentrations of fresh (A) and treated (B) sewage sludge.

3.3. Sewage sludge toxicity to E. fetida

No mortality of earthworms *E. fetida* was observed during the 7 weeks exposure to different treated sewage sludge concentrations. None of the tested treated sewage sludge concentration resulted in earthworms weight decrease during the 7-week period (Fig. 4(A)). The pattern of growth of earthworms exposed to treated sewage sludge was very similar to that in the control group; however, the earthworms exposed to different sewage sludge concentrations grew slower than those of control and resulted in the final lower fresh weight than that of control earthworms. Earthworm's weight growth rate (g week^{-1}) was determined by linear regression b coefficient value (Table 2).

The growth rate of earthworms exposed to 25%–75% treated sludge concentrations in the soil was very similar and no significant differences between the treatments and control were observed (Z test). The slowest growth was recorded in the treatment with 100% treated sewage sludge and was by 20.57% lower than the growth rate of control earthworms.

Reproduction of earthworms was estimated as number of cocoons produced by adult earthworm. As the first cocoons were observed only at the sixth week from the beginning of the experiment, the number of cocoons is provided only after 6 and 7 weeks of exposure (Fig. 4(B)). ANOVA (one-way) indicated that treated sewage sludge had a significant effect on the cocoons production ($F = 4.95$, $p < 0.05$) and cocoons productions in all the treatments with treated sewage sludge was lower than in the control (Dunnett's test, $p < 0.05$). Cocoons production rate (after 7 weeks) decreased along with treated sewage sludge concentration in the soil ($R^2 = 0.41$, $p < 0.05$).

A significant effect of treated sewage sludge (ANOVA, $F = 4.31$, $p < 0.05$) on the level of lipid peroxidation, measured as the concentration of MDA, in the tissue of *E. fetida* was found (Fig. 5). MDA concentrations in the tissue of earthworms exposed to 25%–100% of treated sewage sludge in the soil were by 13.67%–73.38% higher than in the control organisms. Concentration of MDA in the tissue of *E. fetida* increased along with treated sludge concentration in soil ($R^2 = 0.51$, $p < 0.05$).

4. Discussion

Both proso millet (*P. miliaceum* L.) and earthworms (*E. fetida* L.) showed a strong response after the treatment with fresh and treated sewage sludge. Chemical analysis of fresh and treated sewage sludge showed that the level of heavy metals in the sewage sludge met the requirements for sewage

Table 2
Estimated growth rate of *Eisenia fetida* exposed to treated sewage sludge in soil for 7 weeks

Treated SS concentration, %	Growth rate, g week^{-1}
0	0.067a
25	0.064a
50	0.069ab
75	0.064a
100	0.055ac

Note: Values followed by the same letter are not significantly different as determined by Z test.

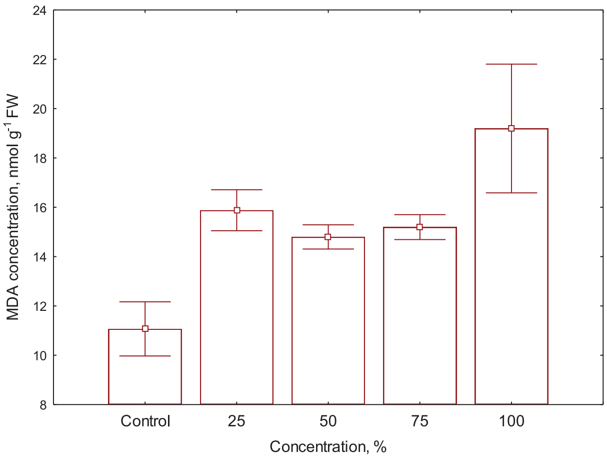

Fig. 5. MDA content in the tissue of *Eisenia fetida* exposed to different treated sewage sludge concentrations in soil for 7 weeks.

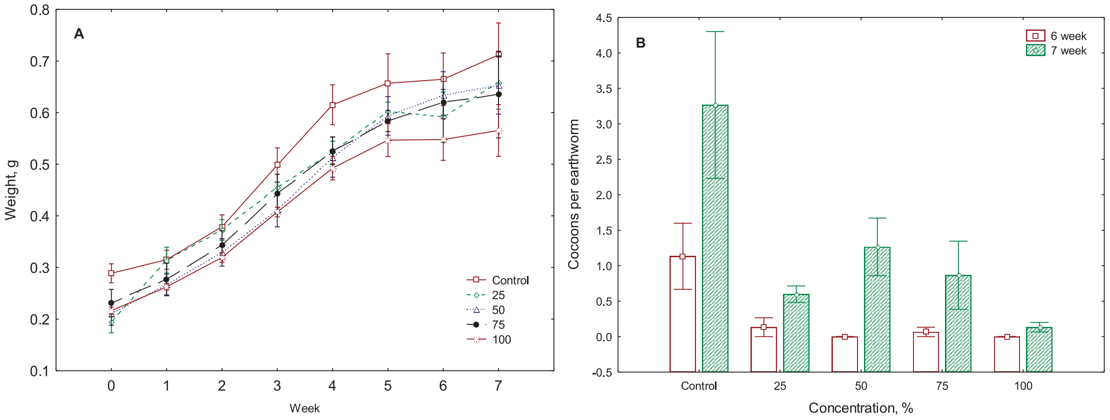

Fig. 4. Fresh weight (A) and reproduction (measured as cocoons production) (B) of earthworms *Eisenia fetida* exposed to different concentrations of treated sewage sludge in soil for 7 weeks.

sludge use in agriculture [1,26] (Table 1). Heavy metals concentrations in treated sewage sludge were slightly lower than in fresh one and this phenomenon could be explained by leaching of metals [27] and was also reported by other researchers [28]. However, the levels of single chemicals could not reflect the whole toxicity of the complex matrix. The sewage sludge is a mixture of different inorganic and organic chemicals which may interact and this in turn may lead to higher or lower toxicity (due to synergistic or antagonistic interactions). This was shown in our study, despite the fact that fresh sewage sludge chemical composition met the legislation; it has executed severe toxicity to plants and earthworms. Consequently, the bioassays with the organisms from the different trophic chain levels could provide more realistic evaluation of the potential sewage sludge impact on the environment.

Fresh sewage sludge was toxic for plants early growth and induced lethal effect to earthworms. Fresh sewage sludge application reduced the plants' growth and the concentrations of photosynthetic pigments in the plant tissues. The main factors determining phytotoxicity of sewage sludge are sewage sludge properties, application dose, plant species, soil type, endpoint and duration of exposure. Application of unstabilized and stabilized (aerobically and anaerobically) sewage sludge reduced germination index of *Lepidium sativum* and *Hordeum vulgare* [29]. Sewage sludge application on the different type of soils (sandy, loamy and OECD soil) resulted in different *L. sativum*, *Sorghum saccharatum* and *Synapsis alba* response. The lowest EC_{50} for root growth was determined in OECD soil and *L. sativum* was the most sensitive to sewage sludge application [30]. Generally, low sewage sludge dose application leads to the stimulation of plant growth and high doses may result in a lowered growth [28]. As the majority of sewage sludge phytotoxicity studies are dedicated to seed germination and root growth, the data of sewage sludge impact on the content of photosynthetic pigments are very scarce. Seleiman et al. [31] recorded reduced net photosynthesis in maize (*Zea mays* L.) and hemp (*Cannabis sativa* L.) grown in sewage sludge amended soil, though the plants grown in soil amended with sewage sludge–peat mix showed higher biomass and more intense photosynthesis. In the present study, the concentrations of photosynthetic pigments were adversely affected by fresh and treated sewage sludge and this endpoint was more sensitive than the growth of shoots and roots. It suggests that this endpoint could be more widely used for sewage sludge phytotoxicity determination prior to the use in agriculture.

The toxicity of treated sewage sludge to plants and earthworms was less pronounced and our results are consistent with the data of other research. Kocik et al. [17] investigated the suitability of willows (*Salix viminalis*) and earthworms (*E. fetida*) for soils treated with sewage sludge and found out that willows grew only in the sewage sludge which was stored for 12 years. Ramírez et al. [28] compared fresh, composted and thermally dried sewage sludge phytotoxicty with *Brassica rapa*, *Lolium perenne* and *Trifolium perenne* and found that fresh sludge had the highest inhibitory effect on the germination and shoot length. Moreover, application of treated sewage sludge had a stimulatory effect on proso millet growth. Low and moderate doses of treated sewage sludge stimulated the growth and biomass increment of plants roots and shoots; however, the impact on the concentrations of photosynthetic pigments was negative and no stimulation was recorded. Wang et al. [32] investigated the effects of sewage sludge application on the growth of grasses (*Zoysia japonica* and *Poa annua*) and observed that the plants biomass growth was well promoted in sewage sludge amended soil though significant increase in heavy metals content in soil and grasses was also observed. The reduction of the sludge toxicity during the composting is explained by volatilization, degradation and pollutants binding to the organic matrix [33]. Fresh sewage sludge was highly toxic and had high cytotoxic, genotoxic and mutagenic potential for *Allium cepa*, though the toxicity decreased with the duration of attenuation period and it was shown that a period of 12 months of natural attenuation should be sufficient to decrease the toxicity of the sewage sludge [34].

Our results indicate that soil dwelling invertebrates may be more vulnerable to the soil amendments with sewage sludge than plants. Different plants and soil invertebrates sensitivity to sewage sludge and various compost treatments were shown by several researchers [30,35,36]. Fresh sewage sludge was severely toxic to earthworms and even 25% sewage sludge concentration in the soil was lethal. Treated sewage sludge application had no lethal consequences, but it reduced earthworms' growth rate, reproduction and evoked oxidative stress (measured as MDA concentration). Previously sewage sludge toxicity to earthworms was based on the survival, biomass and reproduction and no data are available about growth rate and oxidative stress. Furthermore, earthworms' response to sewage sludge application may be different depending on the duration of exposure. Earthworms *Eisenia andrei* survival was unaffected by the sewage sludge compost dosage and its degree of composting during 28 d of exposure [35]. Though the impact on the fresh weight of *E. andrei* was different depending on the duration of exposure to composts with sewage sludge. And the 14 d exposure resulted in lowered *E. andrei* fresh weight, though 28 d exposure had no significant inhibition on the fresh weight [36]. In the present study, sewage sludge inhibitory effect on the fresh weight of *E. fetida* was more pronounced after 7 weeks of exposure in comparison with that after 2 or 4 weeks of exposure. It may suggest that chronic, more prolonged exposure to sewage sludge may have more detrimental effects on the earthworm biomass. Earthworm's growth rate was shown to be sensitive parameter of soil pollution by heavy metals and various organic compounds [37–39]. Sewage sludge application induced membranes lipid peroxidation in the tissues of *E. fetida*, measured as the concentrations of MDA. It indicates that pollutants present in the sewage sludge participate in the production of reactive oxygen species in the cells and evoked oxidative stress. The level of MDA is a good indicator of oxidative stress induced by various contaminants and high level of MDA could be linked to slower growth ($r_s = -0.87$, $p = 0.05$) and results in smaller weight ($r = -0.59$, $p = 0.03$). Moreover, the life-cycle parameters such as growth rate and reproduction play an important role in the population viability and size. Earthworm's growth rate and reproduction decreased with sewage sludge concentration in the soil indicating that high application rates or long-term exposure could have detrimental consequences to this soil keystone species population viability and size.

5. Conclusions

Results indicate that sewage sludge application meeting legislative limits may exhibit plant and soil invertebrates toxicity. Toxicity of sewage sludge was shown to be sewage sludge treatment dependent and fresh sewage sludge was more toxic to plant and earthworms. The forage crop *P. miliaceum* and earthworms *E. fetida* responded differently to sewage sludge application and earthworms were more sensitive than plants. It suggests that bioassays with plants could underestimate the potential hazard of sewage sludge application in agriculture and could not reflect the whole sewage sludge toxicity. The results clearly demonstrate that prior using sewage sludge in agriculture, ecotoxicity should be detected using bioassays with several species representing different trophic levels.

References

[1] Council Directive 86/278/EEC on the Protection of the Environment, and in Particular of the Soil, When Sewage Sludge is Used in Agriculture, 1986.

[2] N. Roig, J. Sierra, E. Marti, M. Nadal, M. Schuhmacher, J.L. Domingo, Long-term amendment of Spanish soils with sewage sludge: effects on soil functioning, Agric. Ecosyst. Environ., 158 (2012) 41–48.

[3] Council Directive 2008/98/EC, of 19 November 2008, on waste and repealing certain Directives. OJ L 312, 22.11.2008, p. 3–30.

[4] S.R. Smith, A critical review of the bioavailability and impacts of heavy metals in municipal solid waste composts compared to sewage sludge, Environ. Int., 35 (2009) 142–156.

[5] C.E. Gökçe, S. Güneysu, S. Arayici, Investigation of the environmental distribution of endocrine disrupting materials in sewage sludge, Desal. Wat. Treat., 57 (2016) 2564–2569.

[6] N.A. Suciu, L. Lamastra, M. Trevisan, PAHs content of sewage sludge in Europe and its use as soil fertilizer, Waste Manage., 41 (2015) 119–127.

[7] N.S. Chary, C.T. Kamala, D.S. Suman Raj, Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer, Ecotoxicol. Environ. Saf., 69 (2008) 513–524.

[8] A. Passuello, M. Mari, M. Nadal, M. Schuhmacher, J.L. Domingo, POP accumulation in the food chain: Integrated risk model for sewage sludge application in agricultural soils, Environ. Int., 36 (2010) 577–583.

[9] N. Roig, J. Sierra, M. Nadal, E. Marti, P. Navalón-Madrigal, M. Schuhmacher, J.L. Domingo, Relationship between pollutant content and ecotoxicity of sewage sludges from Spanish wastewater treatment plants, Sci. Total Environ., 425 (2012) 99–109.

[10] A. Kapanen, M. Vikman, J. Rajasärkkä, M. Vitta, M. Itävaara, Biotest for environmental quality assessment of composted sewage sludge, Waste Manage., 33 (2013) 1451–1460.

[11] T. Natal-da-Luz, S. Tidona, B. Jesus, P.V. Morais, J.P. Sousa, The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation, J. Soils Sediments, 9 (2009) 246–260.

[12] P. Oleszczuk, A. Malara, I. Jośko, A. Lesiuk, The phytotoxicity changes of sewage sludge-amended soils, Water Air Soil Pollut., 223 (2012) 4937–4948.

[13] P. Alvarenga, C. Mourinha, M. Farto, T. Santos, P. Palma, J. Sengo, M.-C. Morais, C. Cunha-Queda, Sewage sludge, compost and other representative organic wastes as agricultural soil amendments: benefits versus limiting factors, Waste Manage., 40 (2015) 44–52.

[14] I. Dimitriou, J. Eriksson, A. Adler, P. Aronsson, T. Verwijst, fate of heavy metals after application of sewage sludge and wood–ash mixtures to short-rotation willow coppice, Environ. Pollut., 142 (2006) 160–169.

[15] A.P. Maxted, C.R. Black, H.M. West, N.M.J. Crout, S.P. Mcgrath, S.D. Young, Phytoextraction of cadmium and zinc by *Salix* from soil historically amended with sewage sludge, Plant Soil, 290 (2007) 157–172.

[16] J. Žaltauskaitė, S. Judeikytė, G. Sujetovienė, R. Dagiliūtė, Sewage sludge application effects to first year willows (*Salix Viminalis* L.) growth, Waste Biomass Valorization, 8 (2017) 1813–1818.

[17] A. Kocik, M. Truchan, A. Rozen, Application of willows (*Salix viminalis*) and earthworms (*Eisenia fetida*) in sewage sludge treatment, Eur. J. Soil Biol., 43 (2007) S327–S331.

[18] G. Masciandaro, V. Bianchi, C. Macci, S. Doni, B. Ceccanti, R. Iannelli, Potential of on-site vermicomposting of sewage sludge in soil quality improvement, Desal. Wat. Treat., 23 (2010) 123–128.

[19] M.A. Domínguez-Crespo, Z.E. Sánchez-Hernández, A.M. Torres-Huerta, M. Negrete-Rodríguez, E. Conde-Barajas, A. Flores-Vela, Effect of the heavy metals Cu, Ni, Cd and Zn on the growth and reproduction of epigeic earthworms (*E. fetida*) during the vermistabilization of municipal sewage sludge, Water Air Soil Pollut., 223 (2012) 915–931.

[20] A.B. Azizi, M.P.M. Lim, Z.M. Noor, N. Abdullah, Vermiremoval of heavy metal in sewage sludge by utilising *Lumbricus rubellus*, Ecotoxicol. Environ. Saf., 90 (2013) 13–20.

[21] OECD Guideline for the Testing of Chemicals 208, Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, 2006.

[22] D. von Wettstein, Chlorophyll-letale und der submikroskopische Formwechsel der Plastiden, Exp. Cell Res., 12 (1957) 427–506.

[23] OECD Guideline for the Testing of Chemicals 222, Earthworm Reproduction Test, 2004.

[24] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Methods Enzymol., 52 (1978) 302–310.

[25] C.C. Clogg, E. Petkova, A. Haritou, Statistical methods for comparing regression coefficients between models, Am. J. Sociol., 100 (1995) 1261–1293.

[26] European Commission, DG Environment, Working Document – Sludge and Biowaste, Brussels, 2010.

[27] V. Paulauskas, N. Sabienė, E. Zaleckas, Wastewater sewage sludge leaching and alkaline stabilization, Ekologija, 4 (2006) 76–82.

[28] W.A. Ramírez, X. Domene, O. Ortiz, J.M. Alcañiz, Toxic effects of digested, composted and thermally dried sewage sludge on three plants, Bioresour. Technol., 99 (2008) 7168–7175.

[29] A. Fuentes, M. Lloréns, J. Sáez, M.I. Aguilar, A.B. Peréz-Marín, J.F. Ortuño, V.F. Meseguer, Ecotoxicity, phytotoxicity and extractability of heavy metals from different stabilized sewage sludges, Environ. Pollut., 143 (2006) 355–360.

[30] P. Oleszczuk, H. Hollert, Comparison of sewage sludge toxicity to plants and invertebrates in three different soils, Chemosphere, 83 (2011) 502–509.

[31] M.F. Seleiman, A. Santanen, F.L. Stoddard, P. Mäkelä, Feedstock quality and growth of bioenergy crops fertilized with sewage sludge, Chemosphere, 89 (2012) 1211–1217.

[32] X. Wang, T. Chen, Y. Ge, Y. Jis, Studies on land application of sewage sludge and its limiting factors, J. Hazard. Mater., 160 (2008) 554–558.

[33] A. Kapanen, M. Itävaara, Ecotoxicity tests for compost applications, Ecotoxicol. Environ. Saf., 49 (2001) 1–16.

[34] D.E.C. Mazzeo, T.C.C. Fernandes, C.E. Levy, C.S. Fontanetti, M.A. Marin-Morales, Monitoring the natural attenuation of a sewage sludge toxicity using the *Allium cepa* test, Ecol. Indic., 56 (2015) 60–69.

[35] X. Domene, L. Solà, W. Ramírez, J.M. Alcañiz, P. Andrés, Soil bioassays as tools for sludge compost quality assessment, Waste Manage., 31 (2011) 512–522.

[36] X. Domene, W. Ramírez, S. Mattana, J.M. Alcañiz, P. Andrés, Ecological risk assessment of organic waste amendments using the species sensitivity distribution from a soil organisms test battery, Environ. Pollut., 1255 (2008) 227–236.

[37] D.J. Spurgeon, C. Svendsen, P. Kille, A.J. Morgan, J.M. Weeks, Responses of earthworms (*Lumbricus rubellus*) to copper and cadmium as determined by measurement of juvenile traits in a specifically designed test system, Ecotoxicol. Environ. Saf., 57 (2004) 54–64.

[38] J. Žaltauskaitė, D. Miškelytė, Antimicrobial personal care products effects in the terrestrial environment, Fresenius Environ. Bull., 23 (2014) 2859–2864.

[39] J. Žaltauskaitė, I. Sodienė, Effects of cadmium and lead on the life-cycle parameters of juvenile earthworm *Eisenia fetida,* Ecotoxicol. Environ. Saf., 103 (2014) 9–16.