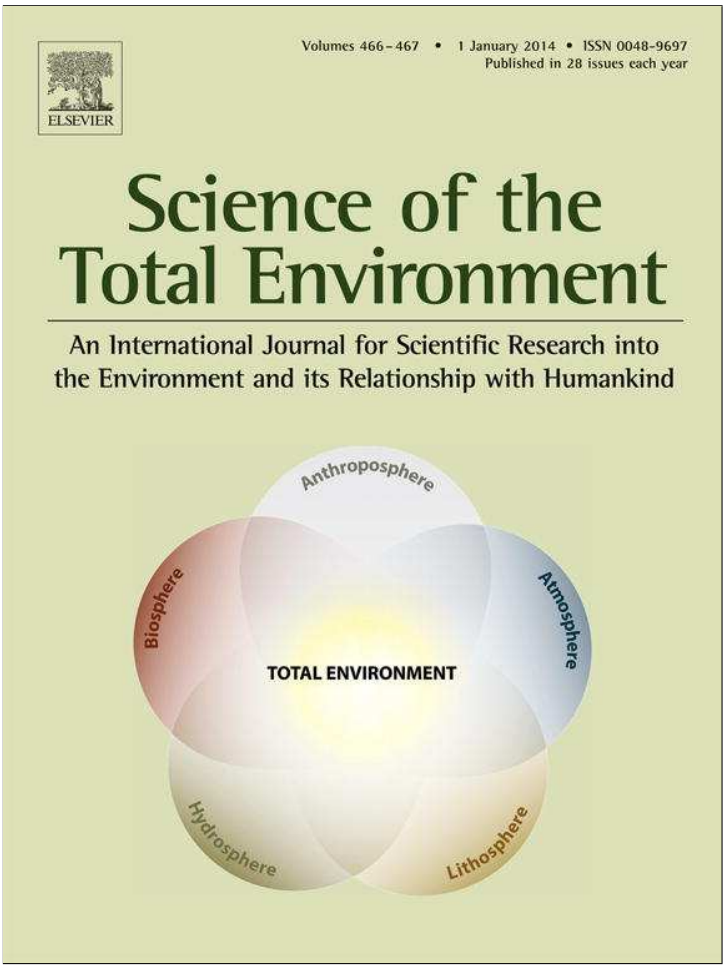

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Toxicity of tributyltin (TBT) to terrestrial organisms and its species sensitivity distribution

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HIGHLIGHTS

- New data for TBT toxicity in soils is presented for invertebrates and plants.
- SSDs were derived for different soil types and TBT forms.
- Soil type and TBT forms did not play a role on the derived HC_5 values.
- Species sensitivity distributions (SSDs) derived a HC_5 = 2.06 mg TBT/kg.

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ABSTRACT

The contamination of the terrestrial environment by disposal of tributyltin (TBT) by contaminated harbour sediments, sewage sludge and/or biocide products has been raising concerns and it may pose a risk to soil invertebrates and plants.

This study aimed to improve the amount and quality of data for TBT toxicity in soils in order to assess the ecological risk of TBT to the terrestrial ecosystems. For this, bioassays were performed with the species *Porcellionides pruinosus*, *Folsomia candida*, *Brassica rapa* and *Triticum aestivum* to evaluate the toxic effects of TBT (as chloride) on these species. Additionally, this study contributed to increase the amount of data concerning TBT toxicity on soil dwelling organisms. The results showed a dose–response relationship between TBT concentration and the increase of toxicity in all species tested. These results were collated with results from literature to construct species sensitivity distributions (SSDs) and to calculate the hazardous concentration at 5% (HC_5) for all data, for each type of soil and TBT formulation used.

The HC_5 value for TBT in soil was 2.06 mg TBT/kg soil dw. Little information is available concerning the concentrations of TBT in soils. In addition the predicted no-effect concentration (PNEC) value was determined to be 30 μg/kg soil. Only one study was found referring to TBT contaminated soils, and where TBT concentrations were lower than 0.024 μg TBT/kg for the wetland soil. Therefore it can be concluded that the real TBT concentrations determined represent low risk for environmental effects. In conclusion, the construction of SSDs and the calculation of HC_5 using all the data available showed to be a more suitable method rather than the construction of several SSDs for each soil and TBT types. Further investigations concerning TBT concentrations and toxicity on soil organisms need to be performed to increase data and improve risk calculations.

1. Introduction

Tributyltin (TBT) compounds have been widely used as antifouling agents on ships and aquaculture facilities since 1960s (Murai et al., 2005). Other uses include pesticides, fungicides, bactericides, wood preservatives, PVC stabilizers (Cruz et al., 2010) and slime control in paper mills (Corsini et al., 1997).

Tributyltin is recognized worldwide as an endocrine disruptor (Lintelmann et al., 2003), being associated with the imposex phenomenon on female dogwhelks and shell thickening on oysters during the 1980s (de Mora and Pelletier, 1997; Evans and Nicholson, 2000; Morcillo and Porte, 2000). In vitro studies suggest that TBT may cause immunotoxicity, teratotoxicity and neurotoxicity in mammals, including humans (Cooke, 2002; Girard et al., 2000; Snoeij et al., 1986; Tsunoda et al., 2006; Whalen et al., 1999).

Data compiled since the 1980s reveal that TBT compounds are present in all media of the coastal environment: water, sediments and living organisms, including large mammals (Alzieu, 1998). Attention has mainly been given to TBT pollution in water and sediments because

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of its highly toxic effect to aquatic life even at low concentrations (Hoch, 2001). However there is a lack of research regarding TBT pollution in soils (Cornelis et al., 2006). Soil contamination by TBT is possible via dredging of contaminated sediments and disposal on land, application of pesticide products (Marcic et al., 2006), atmosphere deposition (Huang et al., 2004), contaminated municipal wastewater and sewage sludge (Fent, 1996). TBT-based compounds are very toxic, bioaccumulative and persistent (Maguire, 2000), with reported half-life values in soil ranging from 15 weeks to several years (Lespes et al., 2009); therefore TBT can still be found in sediments/soils even after being banned (Undap et al., 2013). Moreover several products containing TBT are still commercialized, and antifouling paints containing TBT are illegally used in developing countries, constituting a continuous contamination source to marine waters, and consequently to sediments, and soils (Takahashi et al., 1999). Hence, their presence in the soil compartment is of great concern and the risk of TBT reaching the soil fauna and flora has to be considered. Thus it is important to assess its ecological risk to the terrestrial ecosystem.

One approach to assessing ecological risk is to characterize interspecific variation in TBT sensitivity by constructing species sensitivity distributions (SSDs). This model has been used in six European countries, USA, and Canada, among others, to set soil quality standards (SQSs) for soil and groundwater. The SSD assumes that different species, for example micro-organisms, invertebrates or plants have different responses to the same compound (Swartjes et al., 2012). SSDs are used to estimate the hazardous concentration (HC) affecting a certain proportion (p%) of species (HCp) (Maltby et al., 2005, 2009). Usually it is calculated as the HC_5 value (hazardous concentration for 5% of species) or the 95% protection level (van Straalen and van Rijn, 1998; Wheeler et al., 2002). SSD enables the calculation of the predicted no-effect concentration (PNEC) from the 5th percentile of SSD but it is only recommended when sufficient ecotoxicological data is available (MERAG, 2007). Through SSD it is also possible to calculate the maximum permissible concentration (MPC), an environmental risk limit that results from the ratio between HC_5 and an assessment factor between 1 and 5 (van Vlaardingen and Verbruggen, 2007).

Although the assessment of ecological risk protects the communities and ecosystems, it is usually made using single-species test data. The use of SSD allows the combination of all single-species data (Kooijman, 1987) (Forbes et al., 2008). Although this methodology requires several species datasets, their toxicity is evaluated individually and no interactions between species are attained (Forbes and Forbes, 1993; Forbes and Calow, 2002; Smith and Cairns, 1993). SSD model has also been criticized because it does not take into account the functioning of the ecosystem and for instance the bioavailability of the toxicant (Aldenberg and Jaworska, 2000). Meanwhile, the SSD approach has the advantage of providing a statement of probability of toxicity to the selected species because the exposure concentrations are combined with frequency distribution of effects (Forbes and Calow, 2002).

The main goal of this study was to improve the amount and quality of the data on TBT toxicity in soils with the purpose of assessing the ecological risk of TBT to terrestrial ecosystems. To that end, the toxicity of TBT was studied in the soil invertebrates *Porcellionides pruinosus* and *Folsomia candida* and in the plant species *Brassica rapa* and *Triticum aestivum* in order to achieve also full dose response curves. Then, the results obtained in these tests were combined with the data collected from the literature and SSDs were constructed and the HC_5 value was calculated. In order to accurately derive EC_{50}, NOEC and LOEC values, full dose response curves are needed. Therefore, and in order to achieve it, relatively high concentrations of TBT were used to calculate the EC_{50} values and then use them to construct the SSDs and derive HC_5 values. Considering the heterogeneity of data available, and also regarding the soil type and the TBT formulation used, several SSD approaches were used to improve the HC_5 output.

2. Material and methods

2.1. Test chemical and test soil

Tributyltin chloride ($[CH_3(CH_2)_3)]_3Sn$ Cl); 97% purity) with a molar mass of 325.49 g/mol was acquired from Sigma Aldrich.

An agricultural soil from the central region of Portugal with the following pedological characteristics was used: pH = 7.48, organic matter content = 2.4%, clay = 4.2%, silt = 7.0%, sand = 88.7%, density (g/cm^3) = 2.4 and water holding capacity = 26% was used. Before testing, soil samples were air dried and sieved (5 mm mesh size). The agricultural field was not treated with pesticides in the last five years (Santos et al., 2011).

Soil was spiked with a stock solution of TBT dissolved in ethanol (absolute ethanol obtained from VWR). Aliquots of this stock solution were mixed with distilled water and then mixed with the soil. Soil moisture was adjusted to 40% of maximum water holding capacity (WHC_{max}) in the isopod tests, 60% and 70% in the collembolan and the plants tests, respectively.

2.2. Test organisms

The isopods (*P. pruinosus*) used in these experiments were previously collected from an horse manure pile (Agriculture School of Coimbra, Portugal) and maintained in a laboratory culture at 25 ± 2 °C with a 16:8 h (light: dark) photoperiod. For all tests only adult males and non-gravid females (15–25 mg wet weight) with antenna were selected. The collembolans (*F. candida*) were collected from a synchronized in-house laboratory culture maintained at 19 ± 2 °C with a 16:8 (light: dark) photoperiod. Only juvenile springtails 10 to 12 days old were used in the experiment.

The two plant species used were the monocotyledonous *T. aestivum* and the dicotyledonous *B. rapa*, a rapid-cycling variety of turnip rape, based on the species list disclosed in the ISO guideline 11269-2 (ISO, 1995). Wheat seeds were acquired from an agricultural store in Esmoriz, Portugal, whereas turnip seeds were obtained from Carolina Biological Supply Company.

2.3. Experimental procedure

2.3.1. Tests with isopods

Two feeding inhibition experiments with *P. pruinosus* were run using as route of exposure: 1) contaminated food (Loureiro et al., 2006a), and 2) contaminated soil. For the food exposure route two plastic boxes overlapping, where the top box had a net bottom and the lower box a plaster bottom, were used. The net bottom allows faeces to deposit in the plaster of the lower box for later collection, avoiding coprophagy. Isopods were placed individually in the upper box with contaminated alder leaves during the test period (14 days) and food was not provided to animals one day before and after the test to allow them to empty their gut. Leaves were contaminated topically with an aqueous solution of the test chemical (TBT dissolved in ethanol and water). Five concentrations were tested (1, 2, 4, 8 and 16 μg TBT/mg leaf dw) plus water control, and ten replicates per treatment and control were used.

For the contaminated soil exposure, a feeding inhibition experiment was run with six concentrations (5.4, 17.3, 54.3, 173, 547 and 1732 mg TBT/kg soil dw), a solvent control and a water control, with ten replicates per treatment and controls.

The contaminated soil was placed into plastic boxes (Ø 80 mm; 45 mm high) and isopods were kept individually in the test containers for 14 days. Isopods and leaves were weighted at the beginning and end of the test. Leaves were dried at 50 °C before and after the experiment to obtain their dry weight. Isopod mortality was measured during the test.

Several parameters were calculated using the following the equations:

$C_R = (W_{Li} - W_{Lf})/W_{isop}$, for both exposure routes, and
$A_R = [(W_{Li} - W_{Lf}) - F]/W_{isop}$, and
$E_R = F/W_{isop}$, for the contaminated food exposure route

where, C_R — consumption ratio (mg leaf/mg isopod); A_R — assimilation ratio (mg leaf/mg isopod); E_R — egestion ratio (mg faeces/mg isopod); F — faeces produced (mg); W_{Li} — initial leaf weight (mg dw); W_{Lf} — final leaf weight (mg dw); W_{isop} — initial isopod weight (mg dw) and dw — dry weight (Loureiro et al., 2006b).

The avoidance behaviour test was also carried out according to the procedure proposed by Loureiro et al. (2005). Isopods were kept for 48 h in rectangular plastic containers divided in two sections: one section of the test box was filled up with control soil (water control) and the other with the test soil. At the end of the test the number of isopods in each soil compartment was counted and mortality was registered. Four concentrations were selected (0.2, 2, 20 and 200 mg TBT/kg soil dw) and tested against the control (water control soil), plus a control (water control soil vs water control soil) and a solvent control (water control soil vs solvent control soil), with three replicates each. The percentage of avoidance was calculated by the equation $A = [(C - T) / N] * 100$, where A is the avoidance (%), C is the number of isopods in control soil, T is the number of isopods in test soil and N is the total number of organisms (ISO, 2007). To calculate avoidance between controls with water one was labelled as C and the other as T, as in the formula.

2.3.2. Test with collembolan

The collembolan reproduction test was performed according to the ISO 11267 protocol (ISO, 1999). Four concentrations of TBT (6, 12, 24 and 48 mg TBT/kg soil dw), a solvent control and a water control were tested. Ten springtails (10 to 12 days old) were exposed to TBT in individual glass jars for 28 days. At the end, the content of each jar was transferred to larger glass vessels and filled up with water. The soil was stirred and dark ink was added to improve contrast between animals and media. The content of vessels was photographed to allow automatic counting of juveniles and adults by the image analysis software provided by Sigma Scan.

2.3.3. Tests with plants

The methodology of *T. aestivum* and *B. rapa* bioassays was adapted from the standard protocol from ISO 11269-2: 7 (ISO, 1995).

The bioassays had four replicates per treatment and controls. Ten seeds (without fungicidal treatment) were placed in a plastic pot. The plastic pots were placed on top of a plastic bowl filled with water, and soil moisture maintained by capillarity through a fibreglass wick. Four concentrations were selected based on literature (Hund-Rinke and Simon, 2005) and on previous tests: 12.5, 25, 50 and 75 mg TBT/kg, plus a control and a solvent control (ethanol). The total number of germinated seeds was recorded daily and observations were done regularly to check for any change in plant colour, other symptoms or death. At the end of the test, all plants were harvested (cut above the soil surface), and growth (shoot length) and biomass (fresh and dry weight) were recorded. In *B. rapa* the number of flower buds was also recorded. For both species the water content (*WC*) was calculated using the equation $WC = [(FW - DW) / FW] * 100$, where, *FW* is the plant fresh weight and *DW* is the plant dry weight.

During all the experiments the following conditions were observed and maintained: temperature, 20 ± 2 °C, 16:8 h (light:dark) photoperiod. At the beginning and end of each experiment, test soil pH was measured according to the ISO standard procedure (ISO, 1994).

2.4. Statistical analysis

Differences between the control soil and the solvent control exposures were checked using a t-test (Systat Software Inc., 2008). When differences were observed between controls, all TBT related effects were compared with the solvent control situation.

The comparison between the control and TBT concentrations was made using a one-way ANOVA. If data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis One Way Analysis of Variance on Ranks was performed. Whenever significant differences occurred the Dunnett's Method, Dunn's Method or the Holm–Sidak Method were carried out to discriminate statistical differences between treatments (Systat Software Inc., 2008).

The 50% effective concentration (EC_{50}) values were calculated using a nonlinear regression with a sigmoidal function, using always the best adjustment function. The AC_{50} value for the avoidance behaviour and LC_{50} values for isopods and adult collembolan survival were calculated with the Probit Analysis, using the statistical package Minitab (Minitab, 2003).

2.5. Species sensitivity distributions (SSDs)

2.5.1. Data selection

Toxicity data consists of information gathered from the literature (Amorim et al., 2008; Hund-Rinke et al., 2005; Hund-Rinke and Simon, 2005; Römbke et al., 2007) together with the results obtained in the present study. Retrieved data regarded terrestrial invertebrates *Eisenia andrei*, *Eisenia fetida*, *F. candida*, *P. pruinosus*, and *Enchytraeus albidus*, the plants *B. rapa*, *T. aestivum* and *Avena sativa* and micro-organisms. The endpoints used were EC_{50} values for reproduction (*F. candida*, *E. andrei* and *E. fetida*), feeding inhibition (*P. pruinosus*), avoidance behaviour (*P. pruinosus*, *E. albidus* and *E. fetida*), potential ammonium oxidation (PAO) (micro-organisms), biomass (*B. rapa*, *A. sativa* and *T. aestivum*), growth and water content (*B. rapa* and *T. aestivum*).

2.5.2. SSDs and HC_5 calculation

Species sensitivity distributions (SSDs) and the hazardous concentration at 5% (HC_5 value) were estimated using a log-normal distribution through the software ET_X 2.0 (Wintersen et al., 2004). Only EC_{50} values were used to calculate SSDs and HC_5 values, since this was the most frequently reported endpoint. SSDs were generated using the geometric mean of EC_{50} values for each species. Several SSDs were constructed using all the data available and only the data related to each formulation of TBT – TBTCl and TBTO – and to each type of soil: sandy (all sandy soils including the Coimbra soil from the present study), silt and loamy soils. The derivation of MPC was calculated by the ratio between HC_5 and an assessment factor of 5 (most conservative factor), following the technical guidance document (TGD, 2003). The PNEC values were determined by dividing the lowest NOEC value (from literature or this study data) by an assessment factor of 10 since NOEC values were available for long-term toxicity tests for three species of three trophic levels (TGD, 2003).

3. Results and discussion

The main aim of the present study was to improve data available on the toxicity of TBT to soil organisms. Usually it is advised that to perform an accurate risk assessment procedure, both high quantity and quality datasets are available, covering different organisms, with different functions in soil and belonging to different trophic levels. To cover more organisms with different functions and categories in the soil ecosystem, *T. aestivum* was chosen as a monocotyledon plant and the isopod *P. pruinosus* adds extra data on detritivore species.

Regarding exposure, TBT concentrations used in this study are nominal concentrations. The toxicity endpoints derived were also calculated based on the nominal concentrations and the results were reproducible

(experiments were repeated once, with consistency of the results) and are also comparable with other results available in the literature.

Regarding pH measurements, no significant changes in pH values were observed in any test, ranging from 6.26–7.06, 6.46–6.71, 6.26–6.88, 7.69–8.24 to 7.83–8.22 for feeding inhibition (soil as exposure route), avoidance behaviour, reproduction test, *B. rapa* and *T. aestivum*, respectively.

3.1. Invertebrate species

3.1.1. Isopods' feeding inhibition experiment — food as exposure route

A significant decrease with the increase of TBT concentration was observed for the three ratios calculated: consumption ratio (one-way ANOVA, $F_{5,25} = 5849$, $p < 0.05$), assimilation ratio (one-way ANOVA, $F_{5,25} = 3.180$, $p < 0.05$) and egestion ratio (one-way ANOVA, $F_{5,25} = 3.625$, $p < 0.05$) (Fig. 1a), with a NOEC of 1 μg TBT/mg leaf dw and a LOEC of 2 μg TBT/mg leaf dw.

The decrease of food consumption observed in the present study has been also reported for high metal concentrations which may indicate an avoidance response. Accordingly to Drobne and Hopkin (1995) and Joose et al. (1981), isopods can regulate the intake of contaminated food by avoiding the food. However, in the study of Ribeiro et al. (2001) isopods in the presence of organic compounds showed high elimination rates to remove the toxicants from the organism. Furthermore, isopods have also demonstrated to avoid soil contaminated by organic chemicals (Loureiro et al., 2005). Since TBT is an organometallic compound there are some uncertainties on how isopods deal with TBT contaminated food.

Several authors infer that isopods have the ability to discriminate between metal contaminated and uncontaminated food, and that they reject the contaminated food (Zidar et al., 2004). In another study with *Porcellio scaber*, it is suggested that isopods might detect metal-contaminated food by contact-chemoreception (Weißenburg and Zimmer, 2003). Other study with *P. scaber* suggests that different copper treatments cause changes in microbial populations in food, which enable the detection by differences in taste (Hassall and Rushton, 1982). Zidar et al. (2004, 2005) proposed that the rejection of copper and cadmium by *P. scaber* can be due to adverse metabolic effects of the ingested substances.

For the egestion ratio, a significant decrease occurred at 2 and 8 μg TBT/mg (Dunnett's method, $p < 0.05$), decreasing from 0.10 ± 0.08 in the control to 0.01 ± 0.00 (mean ± st. error) at 8 μg TBT/mg and reaching a null excretion at 16 μg TBT/mg (Fig. 1a). The decrease of consumption ratios can explain the decrease in the egestion ratios, because lower consumption of leaves results in less faecal production (Loureiro et al., 2006b). At the higher concentration isopods seemed to have stopped eating at a certain moment, which may be a consequence of the adverse metabolic effects caused by TBT.

Results showed a significant decrease on the assimilation ratios, mainly for animals exposed to the two highest concentrations (Dunnett's method, $p < 0.05$), and therefore meaning that food might have stayed for short periods of time in the gut, resulting in low nutrient assimilation. In all ratios, an increase occurred at 4 μg TBT/mg, followed again by a reduction. No significant differences were observed at the lower concentration (1 μg TBT/mg) (Dunnett's method, $p > 0.05$). Mortality was observed in all treatments (one death in the control) during the 14 days of test period. A mortality of 50% was calculated for the highest concentration (data not shown).

Loureiro et al. (2006a) states that egestion is a parameter of ecological relevance, because faecal production occurs in the primary phase of leaf decomposition and it is straightly related to isopod function. Although no differences were observed on the EC_{50} values calculated, the egestion ratio was a sensitive parameter, showing the lowest EC_{50} value in this test (Table 1).

Novak and Trapp (2005) performed a study to investigate the feasibility of using land deposited harbour sludge for plant production. TBT

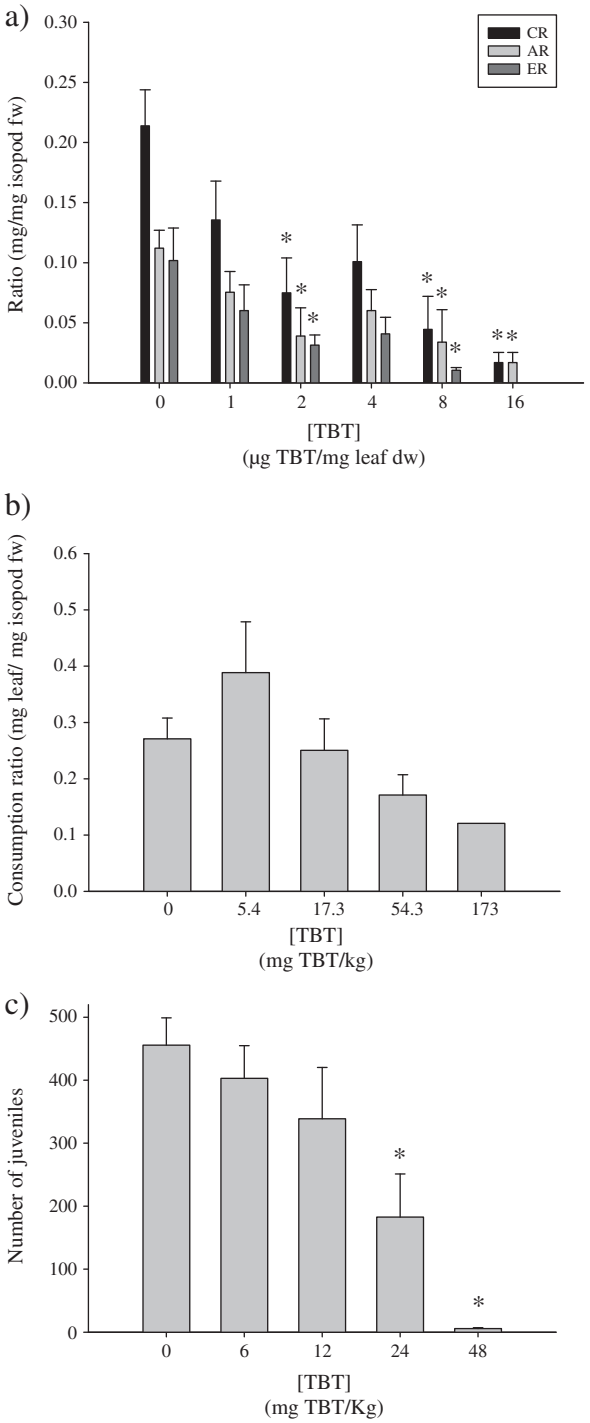

Fig. 1. a) Consumption (CR), assimilation (AR) and egestion (ER) ratios (mg/mg isopod fw) of *Porcellionides pruinosus* exposed to TBT via food; b) Consumption ratio (mg leaf/mg isopod fw) of *Porcellionides pruinosus* exposed to TBT via soil. On the 173 mg TBT/kg treatment, data shown refers only to one isopod; c) Reproduction rate (number of juveniles) of *Folsomia candida* exposed to TBT. All data is expressed as mean values and standard error (*$p < 0.05$, Dunnett's method).

Table 1
EC_{50} values (μg TBT/mg leaf dw) (with SE (standard error) values between brackets) of feeding parameters for *Porcellionides pruinosus* exposed to TBT via food (alder leaves) for 14 days.

Feeding parameters	EC_{50}	r^2
Consumption ratio	1.58 (0.86)	0.51
Assimilation ratio	1.90 (1.53)	0.35
Egestion ratio	1.29 (0.83)	0.40

concentrations were measured in several plants, and in barley corn TBT was <5 μg/kg dw, in reed (whole plant) it was 105 μg/kg dw, and in clover/grass (whole plant) it was 50 μg/kg dw. The TBT uptake into stem and leaves was very low and not related to concentrations in soil (Novak and Trapp, 2005). In the study of Ciucani et al. (2003) willow trees were able to uptake TBT although, none or a very low amount of TBT was translocated to the upper stem and leaves. Plants growing outside, on dumped sediments, with TBT concentrations ranging from 170 to 590 μg/kg (mean value of 490 μg/kg) had taken up maximally 15 μg/kg TBT into the above-ground parts (Ciucani et al., 2003). The concentrations measured in plants are far below the concentrations used in this study therefore TBT contamination in plants may not constitute a risk for isopods in terms of consumption of contaminated leaf litter, for short term exposures.

3.1.2. Isopod's feeding inhibition experiment — soil as exposure route

Student's t test results found no significant differences between the control and the solvent control ($p > 0.05$). No statistical differences were found between concentrations and the control (one-way ANOVA, $F_{3,32} = 2.824$, $p > 0.05$) (Fig. 1b). Food consumption decreased from 0.27 ± 0.12 (control) and 0.40 ± 0.24 (5.4 mg TBT/kg) to 0.12 ± 0.00 at the 173 mg TBT/kg concentration. At this concentration only one isopod survived, with low consumption ratio. Possibly isopods also fed on soil particles (Shachak et al., 1976) thus the ingested TBT contaminated particles may induce stress and/or adverse metabolic effects on animals, causing the decrease of leaf consumption. In addition, TBT in soil pore water can have been also uptaken by uropods, as already reported in other studies (Drobne and Fajgekj, 1993). Since the mortality reached 100% for the two highest concentrations, it was possible to calculate a LC_{50} value of 99.23 mg TBT/kg of soil (Table 2). No mortality was observed in the control. A mortality of 90% was observed at 173 mg TBT/kg, 20% at 5.4 mg TBT/kg and 10% at both 17.3 and 54.3 mg TBT/kg. As high mortality occurred in the two highest concentrations, results on the consumption ratio from both highest exposures were not used.

In a study on earthworms (*E. fetida*) exposed to TBT contaminated soil (132 μg/kg soil dw) Schaefer (2005) reported 42% mortality. Although the mortality rate was different, the TBT concentration used was much lower than the one used in this study, and in that study isopods were fed during the experiment. Römbke et al. (2007) performed a study where the earthworm *E. andrei*, the plant *B. rapa* and the collembolan *F. candida* were exposed to different soil types contaminated with TBTO. For the exposure to three sandy soils, the LC_{50} values were of 8.5 and 15.3 mg TBTO/kg (the LC_{50} was not determined for one soil) for *E. andrei*; and 20.7, 91.9 and 127.1 mg TBTO/kg for *F. candida*. The LC_{50} value of 91.9 mg TBTO/kg registered for the collembolan was similar to the LC_{50} obtained in this test. Again, earthworms revealed lower LC_{50} values, and are considered to be the most sensitive species (Römbke et al., 2007).

A direct comparison of EC_{50} values between the two feeding inhibition tests (via food and via soil exposure) cannot be made due to the different routes of exposure, with the data now available, although the different modes of TBT ingestion have to be considered. In this study the possible TBT uptake could be made by ingestion of soil particles and/or soil pore water. According to what is described in the literature, isopods can intake water from soil through uropods (Drobne and Fajgekj, 1993), ingesting soil and absorption through the cuticle (Loureiro et al., 2005). Given the tendency of TBT to adsorb onto particles, TBT ingestion by water intake may not have been significant.

Table 2
EC_{50} values (mg TBT/Kg soil dw) (with SE (standard error) values between brackets), LC_{50} (95% confidence intervals (CI)), NOEC and LOEC for the test species *Porcellionides pruinosus* (feeding inhibition test — soil as exposure route) and *Folsomia candida* exposed to TBT.

Test species	EC_{50}	r^2	LC_{50}	NOEC	LOEC
Porcellionides pruinosus	59.85 (29.85)	0.14	99.23 (60.91–137.55)	n.d.	n.d.
Folsomia candida	19.31 (3.75)	0.63	22.82 (20.09–25.54)	12	24

n.d. = data not determined.

3.1.3. Isopod's avoidance behaviour response test

No mortality was observed in any of the treatments used, showing that the organisms could escape from the TBT contaminated soils. Isopod avoidance behaviour was observed for 0.2, 2 and 20 mg TBT/kg, with $A = 60\%$. No avoidance was observed in the control ($A = 6.67\%$). At the highest concentration (200 mg TBT/kg) it was observed that 100% of the isopods avoided the contaminated soil, which is an indication of loss in habitat function; this soil function loss is usually considered when more than 80% of the isopods are found in the control soil (Hund-Rinke et al., 2003; ISO, 2007).

The AC_{50} value determined was lower than the lowest concentration used in the experiment ($AC_{50} < 0.2$ mg TBT/kg). The results suggest that isopods are able to detect TBT in soil and avoid or escape at very low concentrations. No significant differences were found between controls by Student's t test ($p > 0.05$), so it is very unlikely that the solvent influenced avoidance behaviour at 0.2, 2 and 20 mg TBT/kg, because the solvent concentrations used were much lower. The fact that isopods were able to avoid contaminated soil indicates that animals could orient normally at those TBT concentrations, for 48 h, which is the usual length of time used on avoidance behaviour tests to test compounds that affect the central nervous system. In addition, this avoidance behaviour can also support what was discussed above regarding the avoidance of contaminated food.

3.1.4. Collembolan reproduction test

No significant effects of ethanol on reproduction were observed (control vs solvent control, Student's t test $p > 0.05$). The number of juveniles decreased with the increase of TBT concentrations (one-way ANOVA, $F_{4,25} = 11.166$, $p < 0.05$). Significant differences were found in the two highest concentrations (24 and 48 mg TBT/kg) when compared with the control (Dunnett's method) (Fig. 1c), decreasing from 455.60 ± 137.17 (control) to 5.60 ± 3.51 (48 mg TBT/kg). A mortality of 50% and 100% was observed for the 24 mg TBT/kg and 48 mg TBT/kg concentrations, respectively.

An EC_{50} value for the production of juveniles of 19.31 mg TBT/kg soil dw was calculated (Table 2), but regarding the high mortality observed, we cannot state clearly if the decrease on juvenile production was related to a direct effect on springtail reproduction or if it was due to the high mortality of juveniles. In the study performed by Römbke et al. (2007), EC_{50} values of the collembolan reproduction test ranged from 23.4 to 177.8 mg TBTO/kg soil dw, according to the soil type.

For the three sandy soils used by Römbke et al. (2007) – BWZ, ESo5, GGI (with a similar sand content to the one used in this study) – the EC_{50} values were of 76.4 (ESo5), 26.0 (BWZ) and 23.4 mg TBTO/kg soil dw (GGI). The differences observed in the EC_{50} can be attributed to differences in other soil properties (pH, organic C content and cation exchange capacity (CEC)), since they can influence the availability of contaminants (Römbke et al., 2007). Another explanation could be attributed to TBT type, since in this study TBTCl was used instead of TBTO that was used in the study by Römbke et al. (2007). This author attributes the organic content of the soil as a property that primarily influenced the toxicity of TBTO to the three species tested.

Hund-Rinke and Simon (2005) also tested the effects of TBTCl upon exposure on sandy, silty and loamy soils in *E. fetida*, *F. candida*, in the plants *B. rapa* and *A. sativa* and in micro-organisms. The EC_{50} values obtained for collembolan reproduction were of 22, 11 and 66 mg TBT/kg, for sandy, silty and loamy soils, respectively. Again, the EC_{50} value for the sandy soil was similar to the value obtained in this test. In both studies, earthworms showed to be a highly sensitive species when compared to collembolan and plants, with EC_{50} values of 2.0 (BWZ) and 0.5 (GGI) (Römbke et al., 2007), 1.3 (sandy soil), 3.0 (silty soil) and 2.7 mg TBT/kg (loamy soil) (Hund-Rinke and Simon, 2005).

The determined LC_{50} value was of 22.82 (Table 2), similar to that obtained by Römbke et al. (2007) for the BWZ soil (20.7). The LC_{50} values of that study ranged from 20.7 to 806.5 mg TBTO/kg between all soil types. The sandy soils GGI and ESo5 showed LC_{50} values of 91.9 and 127.1, respectively. Again, earthworms were more sensitive to TBT than the other test-species, exhibiting LC_{50} values between 8.5 and 15.3 mg TBT/kg, depending on the soil type (Römbke et al., 2007). In the study of Römbke et al. (2007) the artificial OECD soil showed to induce the highest TBT toxicity to earthworms (LC_{50} of 56.2 and EC_{50} of 13.4 mg TBT/kg dw soil) and plants (EC_{50} of 535.5 mg TBT/kg dw soil). For collembolan, the soil HAG induced the highest TBT toxicity with LC_{50} of 806.5 and EC_{50} of 177.8 mg TBT/kg dw soil.

Plagellat et al. (2004) determined the TBT concentrations in sewage sludge of 18.6–648.5 μg/kg dw and Fent and Müller (1991) determined values of 0.28–1.51 mg/kg dw. Fent (1996) indicates TBT concentrations in sewage sludge from different urban and suburban areas in several countries ranging between 0.04 and 3.4 mg/kg dw. Those concentrations are far below the concentrations used and the EC_{50} values calculated in this study; however the disposal of sewage and their effects on soil fauna will be dependent on the mixing processes used. When sludge is mixed only on the first top cm of the soil surface, the isopod *P. pruinosus* might be able to detect it. Furthermore, due to the high persistence of TBT in soils (Lespes et al., 2009) the continuous dump of sewage sludge in crops for soil amendment cannot be ignored.

3.2. Plant species

3.2.1. Seed emergence and growth parameters in B. rapa

In the control replicates seed emergence was observed for *B. rapa* after three days. Apparently, TBT concentrations higher than 50 mg TBT/kg not only caused a delay on germination of the turnip seeds but also completely inhibited seed germination in some replicates. There was a dose response pattern for germination of plants exposed to TBT (data not shown).

Plant growth showed a significant decrease upon TBT exposure when compared to the solvent control (Kruskal–Wallis one-way ANOVA, $H = 67.703$, $df = 4$, $p < 0.001$, Dunn's method, $p < 0.05$). Different concentrations of TBT in *B. rapa* produced significant effects on biomass production (fresh weight) when compared to the control replicates (Fig. 2a), with a NOEC lower than 12.5 mg TBT/kg and a LOEC of 12.5 mg TBT/kg (Kruskal–Wallis one-way ANOVA, $H = 63.045$, $df = 4$, $p < 0.001$, Dunn's method, $p < 0.05$). Effects were also observed for dry weight (Kruskal–Wallis one-way ANOVA, $H = 50.152$, $df = 4$, $p < 0.001$, Dunn's method, $p < 0.05$) and also for the water content (Kruskal–Wallis one-way ANOVA, $H = 10.671$, $df = 4$, $p = 0.031$, Dunn's method, $p < 0.05$). For these parameters it was possible to observe a dose–response relationship and, therefore, EC_{50} values were calculated (Table 3).

In the *B. rapa* replicates exposed to 25 mg TBT/kg smaller plants were observed when compared to the control, but with more flower buds. At the end of the bioassay, there was a significant difference in the number of flower buds between treatments (Kruskal–Wallis one-Way ANOVA, $H = 22.993$, $df = 4$, $p < 0.001$), whereas at concentrations higher than 25 mg TBT/kg there was a decrease in their number.

At the end of the 14 days, some plants of the highest concentration were very small, showed chlorosis (yellow leaves) and some revealed signs of necrosis (brown/dark colour leaves). It can be assumed that TBT is toxic to these species, with consequences on seed germination, growth parameters and viability.

3.2.2. Seed emergence and growth parameters in T. aestivum

Seed emergence for *T. aestivum* was observed in control replicates after five days. TBT concentrations higher than 50 mg TBT/kg caused a delay in wheat germination. In some plants and at the highest concentration (75 mg TBT/kg) abnormal germination was observed on wheat caryopsis. In some plants the caryopsis coat did not release from the shoot, compromising the plant's growth.

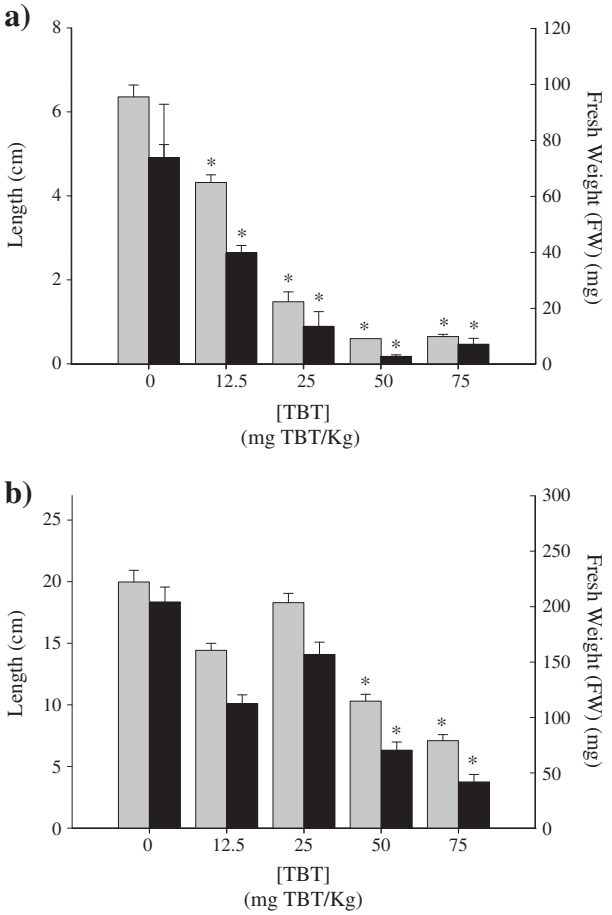

Fig. 2. a) Length and biomass production (fresh weight) of *Brassica rapa* exposed to different concentrations of TBT. b) Length and biomass production (fresh weight) of *Triticum aestivum* exposed to different concentrations of TBT. All data is expressed as mean values and standard error (*$p < 0.05$, Dunn's test). Grey bars are for data on plant's length and black bars refer to fresh weight data.

A significant decrease on the plant's growth (expressed as length) exposed to TBT was observed when compared to the control (Kruskal–Wallis one-way ANOVA, $H = 67.898$, $df = 4$, $p < 0.001$, Dunn's method, $p < 0.05$). Thus, biomass production (fresh weight) was also affected, showing a significant decrease too (Fig. 2b) (Kruskal–Wallis one-way ANOVA, $H = 62.004$, $df = 4$, $p < 0.001$, Dunn's method, $p < 0.05$). Significant differences were observed also for the water content (Kruskal–Wallis one-way ANOVA, $H = 10.671$, $df = 4$, $p = 0.031$, Dunn's method, $p < 0.05$).

The results from the exposure to 25 mg TBT/kg in *T. aestivum* (Fig. 2b) might be explained by the fact that in some concentrations TBT may be favourable for plants, as TBT has a biocidal action, being

Table 3
EC_{50} (as mg TBT/kg) (with SE (standard error) values between brackets), NOEC (as mg TBT/kg) and LOEC (as mg TBT/kg) values for length, fresh and dry weight obtained from the exposure of *Brassica rapa* and *Triticum aestivum* to TBT.

Test species	Parameter	EC_{50} value	r^2	NOEC	LOEC
Brassica rapa	Length	14.81 (1.66)	0.66	<12.5	12.5
	Fresh weight	11.83 (1.64)	0.49	<12.5	12.5
	Dry weight	12.33 (2.63)	0.40	<12.5	12.5
Triticum aestivum	Length	33.82 (35.12)	0.48	25	50
	Fresh weight	28.40 (6.41)	0.45	25	50
	Dry weight	52.80 (6.90)	0.36	n.d.	n.d.

n.d. = data not determined.

toxic, for instance to some insects, soil invertebrates and fungi that act against plants (Novak and Trapp, 2005). In our assay, it was observed that fungi were present on the top soil and might have been beneficial to some plants (replicates) exposed to lower TBT concentrations.

As noted, TBT showed toxic effects to both plant species. It seems that *B. rapa* is slightly more sensitive when exposed to TBT compared with *T. aestivum*, having an EC_{50} value (for length) less than half the EC_{50} value for *T. aestivum* (Table 3).

Other signs of toxicity of TBT in plants are the observation of chlorosis. This suggests that TBT may act in the photosynthetic apparatus and, probably, the protein rubisco (ribulose biphosphate carboxylase oxygenase) is also affected (Desimone et al., 1996). Biomass production also depends on proteins. If these are affected, some biochemical processes are also affected, compromising growth (Desimone et al., 1996; Loureiro et al., 2006b). Therefore, in future studies photosynthetic efficiency evaluation may also be useful to clarify the mode of action of TBT in plants.

TBT sorption is pH dependent and the persistence of TBT is higher when the pH is high (Marcic et al., 2006). Organotins are also more bioavailable in sandy soils, than in loamy soils (Novak and Trapp, 2005) and our studied soil had a high percentage of sand, 88.7%. In our results, the EC_{50} value for biomass production of 11.83 mg TBT/kg to *B. rapa* was similar to the Hund-Rinke and Simon (2005) study (25 mg TBT/kg). This may be explained by the pedological characteristics of the soil, with a similar percentage of sand, around 70–80%, and organic matter content around 2%.

In the natural soil SBG an EC_{50} value of 189.2 mg TBTO/kg was determined for *B. rapa* (Römbke et al., 2007). These differences, as previously referred and comparing to the EC_{50} values obtained in the present work, are probably due to differences in characteristics of soil but may also be related to the TBT type used (TBTCl vs TBTO). In the artificial soil OECD the EC_{50} value was 535.5 mg TBTO/kg (Römbke et al., 2007), suggesting that the use of natural soils shows more realistic scenarios than the use of artificial ones.

3.3. Species sensitivity distributions

To our knowledge, no SSDs have been constructed for TBT in soils and no studies have been performed on the effects of TBT on the species *P. pruinosus* and *T. aestivum*. Leung et al. (2007) estimated SSDs for freshwater species for posterior comparison with SSDs of saltwater species. A HC_5 value of 30.13 ng TBT/L was calculated for freshwater species suggesting that those species are much less susceptible to TBT than saltwater species (HC_5 of 3.55 ng TBT/L).

The HC_5 values calculated in the present study for TBTCl and TBTO were 1.49 and 4.28 mg TBT/kg soil dw, respectively (Table 4). Regarding the soil type, the lowest HC_5 value was observed for silty soils (1.36 mg TBT/kg soil dw), followed by loamy soils (1.55 mg TBT/kg soil dw) and sandy soils (1.56 mg TBT/kg soil dw). The HC_5 calculated with all available data was 2.06 mg/kg soil dw. Regarding the soil type it would be expected that TBT toxicity would derive different HC_5 values, although this has not been confirmed. Soil properties influence the behaviour of compounds and therefore their bioavailability and toxicity to organisms. In addition, the main exposure routes and bioavailable fractions are different among organisms: toxicity to collembolans is mainly driven by soil pore water; soil particles and leaf contamination are the main route of exposure to isopods; soil and dermal uptake are the main routes for earthworms. An overall analysis of the SSD graphs revealed that invertebrates are more affected by TBT than plants. The analysis demonstrated that earthworms and the isopod *P. pruinosus* were the most sensitive species to TBT whereas *A. sativa* was the less sensitive. For the potworm *E. albidus* only one data point was available, and this species was considered the least sensitive to TBT, followed by *F. candida* (Fig. 3). According to the data retrieved from the SSD graphs *E. fetida* seems to be the most sensitive species, followed by *E. andrei* and *P. pruinosus*.

Table 4
Hazardous concentrations 5% (HC_5: mg/kg soil dw) obtained from species sensitivity distributions and maximum permissible concentration (MPC: mg/kg soil dw) calculated for all available data, TBT type and soil type (values were calculated using the geometric mean of EC_{50} values for each species). n is the sample size and tests for normality included: the Anderson–Darling test, the Kolmogorov–Smirnov test and the Cramer von Mises test. PNEC values (mg/kg soil dw) were determined with the lowest NOEC value divided by the respective assessment factor according to the TGD.

		n	HC_5	95% interval	Normality tests	MPC	PNEC
TBT type	TBTCl	7	1.49	0.09–5.77	Accepted	0.30	1.2
	TBTO	4	4.28	0.06–17.19	Accepted[b]	0.86	0.03
Soil type	Sandy soils	9	1.56	0.18–5.05	Accepted	0.31	0.03
	Silty soils	6	1.36	0.03–7.10	Accepted	0.27	0.03
	Loamy soils	5	1.55[a]	0.01–11.10	Accepted	0.31[a]	–
All data		9	2.06	0.24–6.58	Accepted	0.41	0.03

[a] Only one EC_{50} value available for each species (except for *E. fetida*, with two EC_{50} values available), thus the calculation of the geometric mean was not possible.
[b] Except for the Anderson–Darling test and the Kolmogorov–Smirnov test for normality, where significance level was 0.1 and 0.05; and for the Cramer von Mises test where significance level was 0.1.

As expected, the available data is dominated by test results from standard species (*E. albidus*, *E. fetida*, *E. andrei*, *F. candida*, *B. rapa* and *T. aestivum*). However, some standard test species are selected because they are easy to handle in laboratory tests rather than for their ecological relevance, which is another criticism to the use of SSDs in risk assessment; species in datasets are often not representative of the ecosystem (Forbes and Forbes, 1993; Forbes and Calow, 2002). Frampton et al. (2006) encourage the use of soil invertebrate species with more ecological relevance in order to improve the ecological realism of distribution-based risk assessments. To improve the quality and amount of data related to TBT toxicity in soil dwelling species the isopod *P. pruinosus*, a non-standard test species, was tested because of its ecological relevance, mainly in agricultural soils. Isopods are crucial in the decomposition and nutrient cycling processes in soil ecosystems (Loureiro et al., 2002; Zimmer, 2002) and this particular synanthropic species plays an important role in the decomposition of agriculture and cattle wastes (Loureiro et al., 2002). Besides their role in soil maintenance this species presents important ecological, structural and functional traits since they are exposed to chemical compounds by different possible pathways: ingestion of soil particles and/or soil pore water, uptake of contaminated leaves/plants, intake of contaminated water from soil through uropods (Drobne and Fajgekj, 1993) and absorption through the cuticle (Loureiro et al., 2005). The common wheat, *T. aestivum*, was also an important species to test because it is the most widely grown crop in the world, playing an important role in the beginning of agriculture and as food source, mainly used in making bread.

As mentioned above, Römbke et al. (2007) and Hund-Rinke and Simon (2005) studies showed that different soil types influence the toxicity of TBT. The different charged anions/anionic groups of TBT (e.g. hydroxide — TBTH, chloride — TBTCl or oxide — TBTO) can also influence the toxicity of this compound (Antizar-Ladislao, 2008; Fromme et al., 2005; Hoch, 2001). With that knowledge and data, SSDs and HC_5 values derived from different soils and TBT types were estimated in order to evaluate the influence of each soil type and different charged anions (O^- and Cl^-) on the toxicity of the test chemical (Fig. 3 and Table 4). According to the technical guidance document (TGD, 2003) for SSDs, the geometric mean should be the input value when there is equivalent data on the same endpoint and species. This means that data are collected from tests conducted under similar conditions (physical and geochemical) (MERAG, 2007; TGD, 2003) thus, the SSDs of this study were constructed using the geometric mean.

The use of the most sensitive endpoint is another approach to determine SSDs and is considered to be a very conservative one. However, this method selects only the lowest values and therefore does not take into account all the available data. Thus, it is more likely to be biased by outliers, mainly when using larger datasets, where the probability

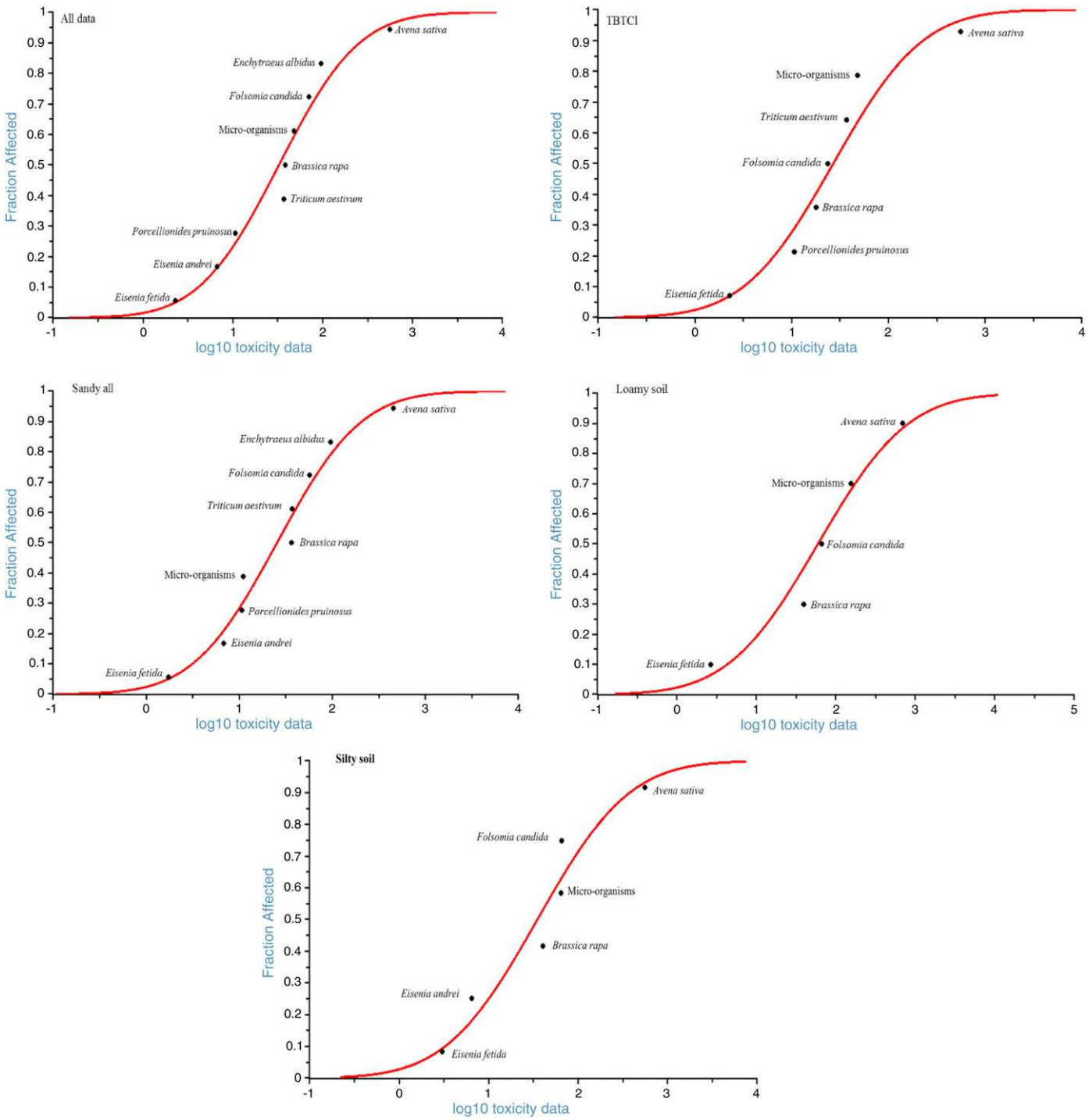

Fig. 3. Species sensitivity distributions (SSDs) for all data together, TBTCl, sandy soil, silty soil and loamy soil based on EC_{50} values data. SSD graphics were constructed using the geometric means (with the exception of loamy soil, which only present one EC_{50} value for each species). "Sandy all" includes all sandy soils tested, namely Lufa 2.2, Coimbra soil, OECD, BWZ, ESo5 and GGI soils (see Table 1SD).

to include extreme values (namely very low values) is higher (Smith and Cairns, 1993; Wheeler et al., 2002).

Differences in experimental conditions such as endpoints, strain of species and other factors, may result in intraspecific variation between test results. Using geometric means the intraspecific variation is disregarded whereas the interspecific variation is taken into account (Duboudin et al., 2004). As Duboudin et al. (2004) reported, it is not rightful to calculate geometric mean, ignoring intraspecific variation, and neither is legitimate to consider the entire amount of data as it stands without taking into account the label species, because for some species the amount of data available is higher than for others. In this case this is observed for *F. candida* and *B. rapa*, whose dataset is higher (see Table 1SD).

Overall, and considering the SSD curves obtained in the present study it seems most appropriate to construct one single SSD curve with all soil types, once the amount of data and species differs from each soil type. Therefore the liability and strength of each curve would be different regarding the amount of data points available. The same is

observed for SSDs of TBTCl and TBTO, since only four species (*B. rapa*, *F. candida*, *E. albidus* and *E. andrei*) were tested for toxicity of TBTO. Furthermore, the HC_5 for TBTCl and TBTO showed to be of the same order of magnitude suggesting that it may be also more appropriate to construct SSDs without discriminating the TBT type.

MPC calculation revealed similar values, with the highest value recorded for TBTO (0.86 mg TBT/kg soil dw) and the lowest for silty soils (0.27 mg TBT/kg soil dw). A MPC of 0.41 mg TBT/kg soil dw was estimated using the complete dataset. A similar PNEC value was obtained for all groups (0.03 mg TBT/kg soil dw) with the exception of TBTCl (1.2 mg TBT/kg soil dw) (Table 4). PNEC values were generally lower than MPC values suggesting that for this case they may be more conservative. The PNEC estimated for TBTCl is the highest value (even when compared to MPC values) because the lowest available NOEC was for *F. candida* and not for the most sensitive species. The PNECs, HC_5 values and consequently the MPC values obtained are much lower than the concentrations used and endpoints derived for all studies.

Brand et al. (2012) reported a similar $MPC_{soil.eco}$ (for ecosystems) value of 0.13 mg/kg dw, however van Herwijnen (2012) determined a much lower $MPC_{soil.eco}$ value (2.3 ng/kg dw). Brand et al. (2012) also stated that taking into account the serious risk concentration (SRC) for ecosystems and for secondary poisoning there is a risk for earthworms to accumulate TBT. Furthermore, Brand et al. (2012) stated that secondary poisoning is relevant because there is risk for predators at MPC and SRC levels.

Preliminary remediation goals (PRGs) attributed baseline values for TBT in soils at residential and industrial areas (US-EPA, Region 9) of 18 mg/kg and 180 mg/kg dm, respectively (Beuselinck and Valle, 2005). In the report of Brand et al. (2012) the proposed intervention value for TBT in soils is 20 mg/kg dw. Considering the HC_5, MPC and PNEC values obtained in this study and the reports available, the proposed PRG and intervention values may pose a risk for soil organisms and should be reconsidered. Huang et al. (2004) determined TBT concentrations lower than 0.01 ng Sn/g in wetland soils, corresponding to 0.024 μg TBT/kg. To the best of our knowledge, no more data is available for TBT concentrations for soils in the environment. Comparing the PNEC value obtained for all the data in our study (0.03 mg TBT/kg soil) with the real TBT concentrations found in wetland soils, one may conclude that it would represent low risk for environmental effects (ratio between the predicted environmental concentration (PEC) and the PNEC is smaller than 1). However, the lack of information about real TBT concentrations in soils hinders further interpretations about environmental risks of TBT to terrestrial organisms.

4. Conclusions

The data produced in the present study from springtails, isopods and plants improved the quantity and quality of data regarding the toxicity of TBT in soil.

In the SSD graphs the plant species *A. sativa* showed to be the less sensitive species to TBT and *E. albidus* was the terrestrial invertebrate less affected. The earthworms (*E. fetida* and *E. andrei*) and the isopod *P. pruinosus* were the most sensitive species to TBT.

SSDs built upon the complete dataset available showed to be a more suitable method rather than constructing several SSDs considering the soil and TBT types. The HC_5 value calculated for TBT in soil was 2.06 mg TBT/kg soil dw, estimating a MPC value of 0.41 mg TBT/kg soil dw. A PNEC value of 0.03 mg TBT/kg soil dw was also recorded. Further studies are needed to obtain a better knowledge and comprehension of TBT toxicity towards soil fauna and flora, as well as research in order to assess real TBT concentrations in soils and therefore estimate the risk in the terrestrial environment. In addition, it is advised that intervention values for TBT contamination in soils are revised as they represent concentrations that may impair soil function and structure.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.08.002.

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