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Highlighted Article

Assessing the toxicity of contaminated soils using the nematode *Caenorhabditis elegans* as test organism

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ABSTRACT

In this study, nine uncontaminated reference soils and 22 contaminated soils with different physico-chemical properties and contamination patterns were tested with a standardized toxicity test, using the nematode, *Caenorhabditis elegans*, as test organism. Fertility, growth and reproduction of *C. elegans* in the soils were compared with the exposure in standard soil Lufa St.2.2. *C. elegans* showed 100% fertility and a very low variability of growth in the reference soils. Although, reproduction varied considerably between the various reference soils, validity criteria (>30 offspring per test organism) were met in all reference soils. Moreover, Lufa St. 2.2 turned out to be a suitable and representative control soil. In order to clearly classify the effects of the polluted soils on *C. elegans*, toxicity thresholds were derived for nematode fertility (20% inhibition), growth (10% inhibition) and reproduction (40% inhibition) on the basis of the test inherent variability (MDD = minimal detectable difference), as well as their variability between the uncontaminated reference soils (MTI = maximal tolerable inhibition). The contaminated soils showed clear toxic effects on the nematodes, whereas the toxicity was better correlated to organic than to heavy metal contamination in bulk soil. Interestingly, the results of the nematode toxicity test were not well correlated with those of tests with oligochaetes, collembolans and plants, performed with the same soils, showing that the results are not redundant. The toxicity test using *C. elegans* turned out to be suitable for testing the toxicity of field collected soils and might by a valuable addition to soil test batteries.

1. Introduction

The quality of soils and soil materials is usually assessed by chemical criteria (i.e. the concentration of selected contaminants), such as in the German Soil Protection Law (BBodSchG 1998). However, the functioning of soil ecosystems is dependent on biological processes that can be disrupted by soil contamination. Therefore, it is necessary to assess the toxicity of contaminated soils on soil dwelling organisms, considering also non-measured contaminants and combinatory effects of chemical mixtures that are usually present. Moreover, using bulk soil toxicity tests, the bioavailability of contaminants can be taken into account, as the test organisms are exposed in a realistic scenario (Keddy et al., 1995). In a recent research project, a battery of several standardized chronic laboratory tests using bacteria, plants, earthworms and collembolans were validated as a suitable tool in soil toxicity assessment (ERNTE-project; Römbke et al., 2006).

However, in the test battery used so far, an important group of soil invertebrates was missing: nematodes. Free-living, non-parasitic nematodes have an important role for the functions of soils, being the most abundant and species-richest metazoans (Andrassy, 1992; Yeates, 1981). By evolving various feeding types, these invertebrates have been able to occupy key positions in terrestrial food webs (Yeates et al., 1993), thus influencing nutrient cycling in soils (Beare, 1997; Ingham et al., 1985; Yeates et al., 1982). The presence of nematodes and the structure of nematode communities are, therefore, important to agricultural production and sustainability (Fiscus and Neher, 2002). Accordingly, nematodes are suitable ecological indicators for monitoring and assessing agricultural areas (Neher, 2001). Nematodes are an emerging organism group in the field of environmental sciences (Wilson and Kakouli-Duarte, 2009), offering a variety of molecular, ecotoxicological and ecological tools for an integrated risk assessment of soils. In environmental studies, the soil-dwelling bacterivorous nematode, *Caenorhabditis elegans*, has been successfully used as a test organism for investigating complex matrices, such as soils (Donkin and Dusenbery, 1993; Freeman et al., 2000; Höss et al., 2008; Kobeticova et al., 2008; Sochova et al., 2007) and freshwater sediments (Comber et al., 2006, 2008; Höss et al.,

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2001a; Traunspurger et al., 1997). In addition, standard test guidelines are available (ASTM, 2001; ISO, 2009).

The test organisms used are exposed to potentially hazardous soil under standardized experimental conditions and the response is compared with an uncontaminated control soil (negative control), which is defined as a soil exhibiting no effect on the test organism. However, for bulk soil tests using sublethal toxicity parameters, the definition of control (reference) conditions is not a trivial task, as toxicity parameters, such as growth and reproduction, are often influenced not only by anthropogenic contaminants, but also by the natural soil properties, such as particle size distribution, organic matter or water content (Jänsch et al., 2005). For *C. elegans*, it is known that clay content and humic substances influence growth and reproduction (Höss et al., 1999, 2001b; Steinberg et al., 2002). These interactions with soil properties lead to a certain variability of the test parameters when testing field soils. Standardizing the test method can reduce part of this variability. The remaining variability, however, has to be considered when interpreting the results of a toxicity test with natural soils. A threshold for an inhibition of a toxicity parameter, which separates the natural variability (due to the influence of soil properties) from a toxic effect (due to the influence of pollution), can be derived from the variability that occurs between lowly polluted reference soils.

Table 1
Physico-chemical properties of reference soils with low anthropogenic contamination.

Soil	Use	pH	% OC	% WHC	C/ N	% Sand	% Silt	% Clay
Lufa St.2.2	Grassland	6.1	2.7	500	14	77	16	7
SHA	Field	7.4	2.2	474	14	8	70	23
GGI	Field (ploughed land)	5.5	0.9	232	16	81	16	4
HAG	Grassland	5.2	2.6	611	9	13	62	25
BWZ	Forrest	3.8	1.5	307	31	81	14	5
SOE	Field	6.6	1.6	483	10	2	83	15
SBG	Grassland (ploughed land)	5.8	3.4	653	10	27	47	26
BRG	Grassland	4.9	2.3	601	8	14	57	30
ESO5	Forest	3.1	5.1	468	28	84	12	5

OC: organic carbon; WHC: water holding capacity; C/N: carbon/nitrogen.

The aim of this study was to evaluate the suitability of *C. elegans* as a test organism for assessing the toxicity of contaminated soils. For this purpose, *C. elegans* was exposed to well characterized soils with varying quality and quantity of contamination, as well as to various types of reference soils, that were studied in the joint research project ERNTE (Römbke et al., 2006). The response of the nematodes to the contaminated soils was then compared with the response of other test organisms, namely the springtail *Folsomia candida* (ISO, 1999), the earthworm *Eisenia andrei* (ISO, 1998) and the plant *Brassica rapa* (ISO, 2005).

2. Materials and methods

2.1. *Soil sampling and analysis*

Nine uncontaminated field soils (including LUFA 2.2 standard soil, serving as control soil) covering a wide range of soil properties (pH, texture, organic carbon content, WHC and C/N ratio) and land use forms were selected to study the influence of soil properties on the fertility, growth and reproduction of nematodes (Jessen-Hesse et al., 2005). Samples of these reference soils were air-dried, sieved (5 mm mesh size) and stored in 25 l plastic buckets at room temperature for not longer than 3 months. Their main properties were determined using ISO standards (Table 1).

In order to determine the effect of contaminated field soils on nematodes three sets of soils were sampled. All of them were treated and characterized as the reference soils (Table 2). Firstly, six sites were chosen (BKG, BUR, CAR, DCU1, SCH and WTTNT), representing very different "old" contaminations, including heavy metals, PAHs and mineral oil (Table 2).

More in detail, two sites typical for industrial and urban areas located in the North German city of Hamburg were studied. The first case study was a former gas works site (Grasbrook) with a long period of operation: 1844–1976. The soil is heavily contaminated with organic pollutants like creosote, benzene and PAH. As remediation about 370,000 m^3 of soil were excavated at the site. Depending on its PAH content and physical properties, the soil material was classified on site to different stockpiles. In total, 12 samples were taken from these stockpiles, each one consisting of several subsamples which were mixed and homogenized (Table 2). The second case study, called Schlachthofstraße, was a former landfill site for

Table 2
Physico-chemical properties and contaminant concentrations in investigated polluted soils.

Soil	Use	pH	% OC	Cd	Cr	Cu	Hg	Ni	Pb	Zn	PAH	MKW
BKG	Garden plot	7.2	1.4	0.6	14.3	65.4	0.4	13.5	82.9	145	70	370
BUR	Former sewage field	6.3	5.1	24.8	413.0	197.0	2.0	50.0	194	706	2.3	1293
CAR	Coking plant site	7.3	1.7	0.1	7.9	3.9	0.2	6.5	9.37	26.8	88	214
DCU1	Grassland (ploughed land)	6.1	2.6	0.2	12.0	192.5	0.1	8.6	26	34.2	0.2	158
SCH	Former tank farm	6.0	1.2	0.1	6.2	1.9	0.1	4.4	26.6	18.1	1.3	4645
WTTNT	Explosives industry	7.2	5.0	28.6	20.1	256.0	10.2	29.3	2098	5674	16	981
GBK	*Case study: gas plant site*											
GBK1	GBK-1A-0412	7.8	1.0	0.0	7.7	13.0	0.1	18.0	37	41	10	n.d.
GBK2	GBK-1B-0412	7.8	2.9	0.3	17.0	145.0	1.2	17.0	195	189	237	n.d.
GBK3	GBK-2A-0412	7.7	2.9	0.3	12.0	49.0	1.0	12.0	263	264	110	n.d.
GBK4	GBK-3A-0412	7.5	5.1	0.5	16.0	86.0	2.0	18.0	400	160	210	n.d.
GBK5	GBK-3B-0412	7.5	5.5	0.0	20.0	96.0	2.0	20.0	706	150	215	n.d.
GBK6	GBK-1B-0503	7.4	1.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	50	n.d.
GBK7	GBK-1C-0503	8.7	1.9	0.0	7.0	21.0	0.2	9.0	61	114	16	n.d.
GBK8	GBK-1D-0503	7.6	1.0	0.0	6.0	24.0	0.7	8.0	72	70	16	n.d.
GBK9	GBK-1E-0503	7.7	1.2	0.0	7.0	27.0	0.5	9.0	92	107	22	n.d.
GBK10	GBK-2B-0503	7.4	3.8	0.0	7.0	42.0	1.0	15.0	142	98	220	n.d.
GBK11	GBK-3B-0503	7.3	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	68	n.d.
GBK12	GBK-3C-0503	7.3	5.4	0.0	17.0	67.0	1.5	32.0	448	128	1100	n.d.
HSH	*Case study: dumping ground*											
HSH1	HSH-OB1-0504	6.6	5.5[a]	50.0	25.0	210.0	21.0	30.0	3200	6600	53	n.d.
HSH2	HSH-OB2-0504	5.8	7.0[a]	39.0	21.0	400.0	18.0	36.0	4200	2800	118	n.d.
HSH3	HSH-OB3-0504	6.4	3.9[a]	63.0	18.0	1600.0	18.0	31.0	11000	6100	117	n.d.
HSH4	HSH-OB4-0504	7.3	5.0[a]	28.0	19.0	190.0	6.4	40.0	1500	2400	79	n.d.

[a] % OC estimated from loss on ignition (OC = 0.5 × LOI).

construction waste, slag and sludge (about 40,000 t), which were deposited between 1924 and 1945. The site was planned to be remediated by soil removal of the landfill mound. Four samples (HSH1–4) were taken from the first 20 cm depth at different sites of the mound, again consisting of four subsamples each. According to the chemical assessment of these soil and waste mixtures, the main pollutants were heavy metals such as arsenic, cadmium, lead, mercury and zinc, while PAH concentrations were in a medium range (Table 2).

2.2. Nematode toxicity test

C. elegans var. Bristol, strain N2, was maintained as stocks of dauer larvae (an alternative juvenile stage that occurs with a lack of food) on nematode growth medium (NGM) agar (17 g bacto agar, 2.5 g bacto peptone and 3 g NaCl l^{-1}, after autoclaving, add 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KH_2PO_4 and 1 ml of 5 mg ml^{-1} cholesterol solution in ethanol; Brenner, 1974) according to standard procedures (Lewis and Fleming, 1995; Sulston and Hodgkin, 1988). The nematode bioassay with *C. elegans* was carried out, with few modifications (five test organisms instead of ten), according to standard methods (ISO, 2009). For the soil test, 0.3 g of soil (air-dry weight) was moistened with 0.2 ml of M9-medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl and 0.25 g $MgSO_4 \cdot 7H_2O$ l^{-1}) in test wells (12-well polystyrene multidishes; Nunc, Wiesbaden, Germany) and then mixed with 0.5 ml of *Escherichia coli* (OP50, approximately 10^{10} cells ml^{-1}) suspended in M9-medium as the food supply (ASTM, 2001; ISO, 2009). At the start of the test, five first-stage (J1) juvenile worms were transferred to each test well. The mean initial body length of the test organisms was 258 μm (±33 μm, SD). Four replicates were set up for each treatment. After 96 h of incubation at 20 °C, the test was stopped by heat-killing the worms at approximately 50 °C. The samples were then mixed with 0.5 ml of an aqueous solution of Rose Bengal (0.5 g l^{-1}) to stain the worms for easier counting and stored at 4 °C until further use.

For recovery of the test organisms, nematodes were isolated from the soil according to standard procedures (ISO, 2009), using a mixture of a suspension of colloidal silica (Ludox TM50; Sigma–Aldrich, Munich, Germany) and deionized water (density: 1.13 g cm^{-3}). Soil and nematodes were removed from the test wells with a Pasteur pipette by washing with approximately 5 ml of Ludox. This suspension was transferred to a centrifuge tube (15 ml), thoroughly mixed, and centrifuged for 10 min at 800*g*. The supernatant, which contained the nematodes, was poured into a Petri dish, and the pellet, containing soil particles, was resuspended with diluted Ludox and again centrifuged to extract any remaining nematodes. On average, 77% (±20% SD; $n = 129$) of the test organisms were recovered by this procedure. Nematode reproduction was quantified by counting the juvenile offspring under a dissecting microscope at 25-fold magnification. Nematode growth was determined by measuring the body length at 100-fold magnification using a light microscope. Growth was calculated by subtracting the mean initial body length of the test organisms from the mean body length after incubation. Nematode fertility was quantified by calculating the percentage of gravid test organisms (≥ 1 egg inside the body).

2.3. Statistical analysis

The following coefficients of variation were defined to describe the variability of the test parameters.

The test inherent coefficient of variation, CV_i, considers variability of a test parameter regardless of any environmental factor and is calculated from the variance of a test parameter within each of the investigated reference soils:

$$CV_x = SD_x / Mean_x \times 100, \tag{1}$$

where $Mean_x$ and SD_x are mean and standard deviation of a test parameter calculated from the replicates of soil *x*. As a measure of the test inherent variability the mean CV_i over all CV_{ix} was calculated.

The soil coefficient of variation, CV_s, considers the influence of sediment characteristics (besides pollution) and is calculated from the variance of a test parameter between various investigated sediments with low anthropogenic contamination:

$$CV_s = SD_{rs} / Mean_{rs} \times 100, \tag{2}$$

where $Mean_{rs}$ and SD_{rs} are mean and standard deviation of the test parameter over all reference soils.

Two measures were calculated to estimate the suitable toxicity threshold for the toxicity parameters.

The minimal detectable difference (MDD) based on the test inherent variability of a test parameter is calculated for each reference soil compared with the control soil Lufa St. 2.2:

$$\%MDD_x = \frac{100t\sqrt{\frac{(SD_c)^2}{n_c} + \frac{(SD_x)^2}{n_x}}}{Mean_x}, \tag{3}$$

where *t* is the tabulated value of Student's *t* distribution (alpha = 0.05, one-sided, df = n_C+n_t−2), SD_C and SD_x is the standard deviation of the test parameter in the control soil and the reference soil *x*, and n_C and n_x are the number of replicates in the control soil and the reference soil *x* sediment, and $Mean_x$ is the mean of the test parameter in soil *x*. For estimation of the toxicity threshold, the mean MDD of all investigated reference soils was calculated.

The maximal tolerable inhibition (MTI) is based on the variance of a test parameter between the various reference soils, referring to a certain control (in this case: Lufa St.2.2):

$$\%MTI = 100 - \frac{P_C}{P_C - (Mean\ P_S - SD\ P_S)}, \tag{4}$$

where P_c is control value of parameter *P* and Mean P_s and SD P_s are mean and standard deviation of parameter *P* over all reference soils.

Bivariate correlations were analyzed with non-parametric Spearman correlations, and, if indicated, with the Pearson correlation (in case of normal distribution of data; $p > 0.05$, Kolmogorov–Smirnov) using SPSS software.

Redundancy analysis (RDA) was performed using Canoco 4.55 (Microcomputer Power). Analogous to a principal component analysis (PCA), RDA maps information from a large number of variables onto a smaller number of linear combinations, thereby simplifying the data interpretation. However, in RDA ordination axes are not only a linear combination of primary variables, but also a linear combination of further external variables. By an additional regression step within the algorithm only the amount of variance, which can be attributed to the external variables is mapped on the ordination axes. The eigenvalues of the resulting ordination axes are a measure for the information content, the ecological relevance and the amount of variance explained by the axes. Significances of environmental variables for explaining the biological data in RDA were calculated by a Monte-Carlo permutation test (499 permutations). Variable selection in RDA was done by an automatic forward selection by means of the significance (Ter Braak and Šmilauer, 2002). Biological data were expressed as % inhibition to the control soil, Lufa St. 2.2. Negative values for inhibition (means better performance than in the control) were set to zero, assuming no toxicity in these samples. All data that were used for the RDA analysis were normalized in relation to the maximum value.

3. Results

3.1. Response of nematodes to reference soils

The response of *C. elegans* to the soils from the reference sites was very homogeneous in terms of fertility and growth. Nematode fertility was found to be 100% in all reference soils (Fig. 1a), growth ranged from 1232 to 1383 μm, with a mean growth of 1306 μm (SD: 52 μm; Fig. 1b). Reproduction showed a higher variance between the reference soils, with mean values ranging from 44 to 144 offspring per test organism and a mean of 108 (SD: 33; Fig. 1c). In all reference soils, the validity criteria for the control of 30 offspring and 80% fertility were met (ISO, 2009). A large part of the variance in reproduction was caused by one soil, ESo5, where the nematodes only had 44 offspring per test organism. Omitting this soil from calculations would decrease the variance considerably (range: 80–144; mean: 115±24 offspring per test organism). The test inherent variability of growth and reproduction, measured as test inherent variance coefficient (CV_i), was 4.8±2.1 and 19.3±12.6, respectively. The minimal detectable difference (MDD) for these two parameters was found to be 7.2±1.6% and 24.7±13.0%, respectively. As there was no variability in nematode fertility, CV_i and MDD were not calculated. With 1279 μm, 100% fertility and 113 offspring per test organism, the response of *C. elegans* to the control soil Lufa St. 2.2 was found to be very close to the mean values, calculated over all reference soils.

The variability of growth could not be related to any of the measured soil properties ($p > 0.05$; Pearson correlation). Nematode reproduction, however, was positively correlated to the pH measured in the reference soils ($r^2 = 0.57$, $p = 0.019$; Pearson correlation).

3.2. Toxicity thresholds

For growth and reproduction, the minimal detectable difference (MDD) was found to be 7.2±1.6% and 24.7±13.0%, respectively. The maximal tolerable inhibition (MTI) to the control soil Lufa St. 2.2 was calculated to 2% and 34% for growth and

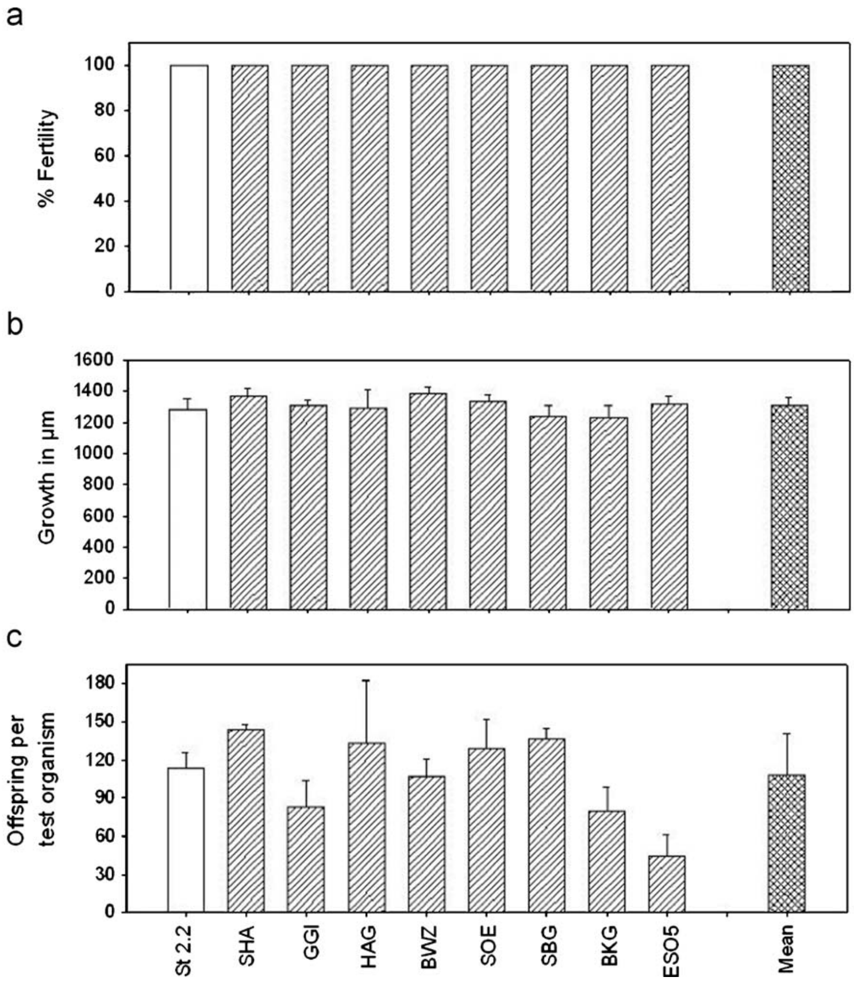

Fig. 1. Fertility (a; %), growth (b; µm), and reproduction (c; offspring per test organisms) of *C. elegans* after 96 h exposure in reference soils; bars = mean, error bars = standard deviation ($n = 4$); Mean = mean and standard deviation over all single mean values ($n = 9$).

reproduction, respectively. Based on the observed variability, the toxicity thresholds for growth and reproduction were set to 10% and 40%, respectively. The test parameter fertility did not show any variation within the unpolluted reference soils. Thus any significant deviation from 100% can be regarded as toxic effect. Therefore, the toxicity threshold was set to 20%, analogous to the validity criterion.

3.3. *Response of nematodes to contaminated soils*

Nematode reproduction indicated 17 of 22 soils as toxic (inhibition of >40% compared with the control soil, Lufa St. 2.2), and thus turned out to be the most sensitive toxicity parameter (Fig. 2c). Regarding the parameter growth, still 14 soils turned out to be toxic (>10% inhibition; Fig. 2b), while the inhibition of fertility exceeded the toxicity threshold of 20% only in 8 of 22 soils (Fig. 2a). The most toxic soils were found at the former gas plant site (GBK2-4, GBK6-9) and at one site of the dumping ground (HSH2), where reproduction, growth and fertility were strongly inhibited. However, also in soils of BKG, CAR, and WTTNT, and of all other GBK sites (GBK1, 5 and 10–12), the reproduction of *C. elegans* was strongly inhibited. In soils of BUR, DCU1, SCH, HSH3 and HSH4, no toxicity occurred.

Within the contaminated soils, the inhibition of nematode growth correlated negatively with the organic carbon normalized PAH concentrations (mg PAH g^{-1} OC; $r^2 = 0.22$, $p < 0.05$). When regarding the whole data set (reference and contaminated soils), the correlation of toxicity with PAH concentrations was even more pronounced. Growth, fertility and reproduction were negatively correlated with bulk soil PAH concentrations ($r^2 = 0.45, 0.20, 0.31$, respectively, $p < 0.05$) and even stronger with organic carbon normalized PAH concentrations ($r^2 = 0.48, 0.26, 0.43$, respectively, $p < 0.01$). For the whole data set, inhibition of nematode growth was also negatively correlated with Cu and Hg concentrations in bulk soil ($r^2 = 0.21, 0.22$, respectively, $p < 0.05$), and with organic carbon normalized concentrations of Cu, Hg, Pb and Zn ($r^2 = 0.24$, 0.31, 0.32, 0.34, respectively, $p < 0.01$). Also inhibition of reproduction was negatively correlated with organic carbon normalized Pb concentrations ($r^2 = 0.16$, $p < 0.05$).

The toxicity parameters were negatively correlated with the pH of the soil samples, either when considering only the contaminated soils (growth: $r^2 = 0.20$, $p < 0.05$; fertility: $r^2 = 0.24$, $p < 0.05$; reproduction: $r^2 = 0.77$, $p < 0.001$), or the whole data set (growth: r^2 0.34, $p < 0.01$; fertility: $r^2 = 0.31$, $p < 0.01$; reproduction: $r^2 = 0.54$, $p < 0.001$).

3.4. *Comparison of the different soil toxicity test*

To compare the complex data set of four different toxicity tests, 22 soils, and nine environmental variables, a redundancy analysis (RDA) was performed. The first two axes of the RDA plot explained 82.0% of the variance (eigenvalues: axis 1: 0.366; axis 2: 0.191;

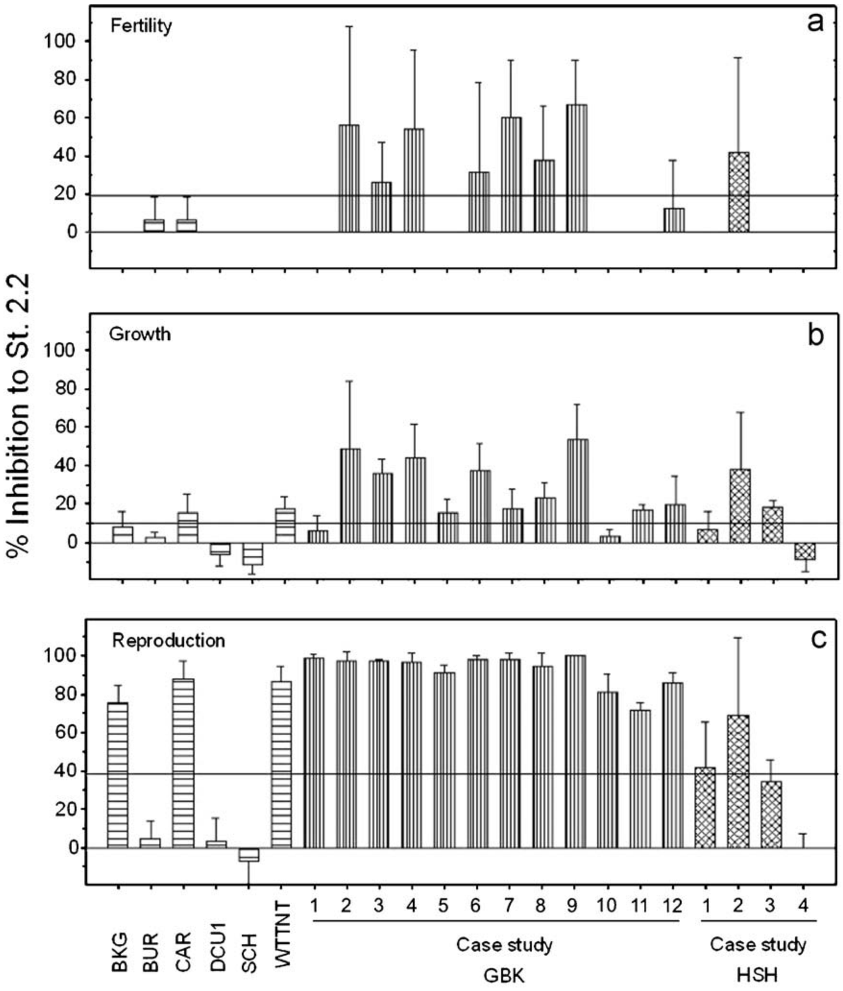

Fig. 2. % Inhibition (to the control soil Lufa St. 2.2) of fertility (a), growth (b) and reproduction (c) of *C. elegans* after 96 h exposure in various contaminated soils; bars = mean, error bars = standard deviation ($n = 4$); horizontal lines indicate toxicity threshold for the respective parameter: fertility = 20%; growth = 10%; reproduction = 40%; for sample codes see Table 2.

sum of eigenvalues of all axes: 0.683). All environmental variables (soil properties) that were introduced as co-variables, explained 68% of the variance. However, only three variables, pH, Zn and Hg, contributed significantly ($p<0.05$, Monte-Carlo permutation test), explaining 22%, 12% and 10% of the variance, respectively. The RDA shows that the responses of *F. candida*, *E. andrei* and *B. rapa* follow the first axis together with the heavy metal and organic matter contents in the soils (Fig. 3). The response of *C. elegans* to the various contaminated soils pointed at a completely different direction, following the pH and PAH concentrations (Fig. 3). When trying to relate the responses of the different test systems in bivariate correlations, effects on *E. andrei* were significantly correlated with those on *B. rapa* ($r^2 = 0.39$, $p<0.01$) and *F. candida* ($r^2 = 0.23$, $p<0.05$). The responses of the toxicity parameters of *C. elegans* were significantly correlated to each other (growth—fertility: $r^2 = 0.63$, $p<0.001$; growth—reproduction: $r^2 = 0.43$, $p<0.01$; fertility—reproduction: $r^2 = 0.38$, $p<0.01$), but not to any other test system ($p>0.05$). Neither were effects on *F. candida* correlated to the effects on *B. rapa* ($p>0.05$).

Table 3 shows that in 12 soils at least two test systems indicated a significant toxicity, meaning effects above the respective toxicity threshold as given in ISO guideline 17616 (ISO, 2006); see also (Römbke et al., 2006). However, only one soil (WTTNT) showed a toxic effect in all four tests. Only in two soils (DCU1, HSH4), none of the test organisms were affected.

4. Discussion

The nematode *C. elegans* turned out to be a suitable test organism for testing various types of natural soils. In reference soils of varying physico-chemical properties with low anthropogenic contamination, that are representative for a wide range of agricultural and forest soils of temperate regions (Römbke et al., 2006), all validity criteria (>80% fertility; >30 offspring; ISO, 2009) were met. An important indicator for the reliability of a test system is the test inherent variability, meaning the variability of a test parameter between the various replicates within each reference soil sample. The test inherent variability of the toxicity parameters was found to be quite small and similar to values that were found in studies with freshwater sediments. Höss et al. (1999) investigated growth of *C. elegans* in 26 unpolluted sediments and found a comparable test inherent variability ($CV_i = 6.8 \pm 3.7\%$) to the value calculated in this study. Also, a more recent study on the response of *C. elegans* to freshwater sediments with low contamination shows a similar test inherent variability of growth and reproduction ($CV_i = 6.5\%$ and 23.3%, respectively; unpublished data).

When testing the toxicity of field collected soil samples, it is important to compare to a reliable and representative control. In this study, Lufa St. 2.2, widely used in soil ecotoxicology (Løkke and Van Gestel, 1998), was chosen as control soil. The results show

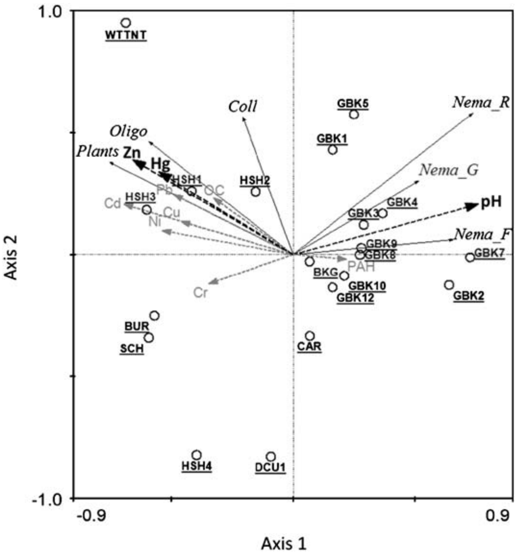

Fig. 3. Redundancy analysis (RDA) of soil samples on the basis of the response of all four different soil toxicity tests expressed as % inhibition of the respective toxicity parameter: Nema_G = *C. elegans* growth, Nema_F = *C. elegans* fertility, Nema_R = *C. elegans* reproduction, Coll = *F. candida* reproduction, Oligo = *E. andrei* reproduction, plants = *B. rapa* shoot length; with soil properties as explaining environmental variables; gray variables do not contribute significantly (Monte-Carlo permutation test; $p > 0.05$); black and bold variables contribute significantly (Monte-Carlo permutation test; $p < 0.05$); eigenvalues: Axis 1: 0.366, Axis 2: 0.119; for sample codes see Tables 1 and 2.

Table 3
Comparison of toxic effects (% inhibition to control soil, Lufa St. 2.2) of various soils on different test organisms.

Soil	Percent inhibition compared with St. 2.2			
	C. elegans	*E. andrei*	*F. candida*	*B. rapa*
BKG	***75.7***	***100.0***	***100.0***	35.4
BUR	4.5	***52.7***	45.3	***70.7***
CAR	***87.9***	22.6	−31.9	−3.2
DCU1	3.0	0.5	−18.0	−0.7
SCH	−6.8	***81.9***	29.9	***79.0***
WTTNT	***86.3***	***100.0***	***100.0***	***100.0***
GBK1	***99.0***	***63.9***	30.3	10.2
GBK2	***97.3***	9.1	−24.8	25.9
GBK3	***97.1***	−6.6	***62.5***	15.8
GBK4	***96.9***	37.6	***68.6***	17.8
GBK5	***91.3***	40.1	***99.6***	21.6
GBK6	***98.0***	39.9	−51.7	23.9
GBK7	***98.4***	***50.6***	−19.2	29.4
GBK8	***94.4***	11.9	−44.3	6.7
GBK9	***99.9***	43.6	−33.6	4.7
GBK10	***81.4***	29.8	−54.0	19.4
GBK11	***71.6***	25.8	−90.9	25.2
GBK12	***86.1***	39.9	−26.8	20.7
HSH1	***41.7***	***98.6***	33.4	***89.1***
HSH2	***68.7***	***99.8***	−7.5	***66.6***
HSH3	34.3	***98.8***	20.8	***100.0***
HSH4	0.1	39.1	7.1	23.8

Toxicity parameters and thresholds: reproduction for *C. elegans* (40% inhibition), *E. andrei* (50% inhibition) and *F. candida* (50% inhibition), shoot length for *B. rapa* (50% inhibition); effects above the respective toxicity threshold are printed bold and italic.

that Lufa St. 2.2 is a suitable control soil, as the response of *C. elegans* to Lufa St. 2.2 showed a very low variability between the various replicates (CVI = 5.7% and 11.5% for growth and reproduction, respectively). Moreover, nematode growth and reproduction were very similar to the mean values of those test parameters in the other reference soils (Fig. 1).

The threshold for an inhibition of a toxicity parameter, which separates the natural variability (due soil properties) from a toxic effect (due to pollution), was derived from the variability that occurred between the lowly polluted reference soils. The variability of nematode growth in the reference soils was very small, with a soil coefficient of variance (CV_s) of 4%. This is a considerably lower variability than was found for freshwater sediments (10.1%, Höss et al., 1999; 12.3%, unpublished data). Reproduction showed with 30.6% a higher CV_s than growth, is, however, still lower than the variability in freshwater sediments ($CV_s = 49.9\%$; Höss et al., submitted). The higher variability of reproduction might be explained by a generally higher sensitivity of this parameter to environmental factors, such as experimental conditions (e.g. food density, test duration, temperature), which is indicated by the higher test inherent variability. Alternatively, influences of soil properties, such as organic matter (Höss et al., 2001b), might also cause the higher variability of reproduction. However, the only soil property that was significantly correlated to nematode fertility, growth or reproduction was the pH. Regarding the reference soils, *C. elegans* favored the soils where higher pH values were measured. As the pH in the tested soil suspension is buffered with a phosphate buffer (M9-medium) to 7.2, a direct pH effect can be ruled out. Thus, a variable, closely related to pH, but not measured in this study, might have been responsible for the variability of the test parameters. The reproduction of *F. candida* that was also exposed to the reference soils investigated in this study, showed a comparable variability ($CV_s = 23.4\%$) to the nematode reproduction, while reproduction of *E. andrei* varied to a larger extent between the various reference soils ($CV_s = 43.8\%$) (Römbke et al., 2006).

In line with previous research (Anderson et al., 2001; Höss et al., 2002), reproduction of *C. elegans* showed the strongest response to the contaminated soils, being inhibited up to 100%. However, due to the high toxicity threshold of 40%, the number of soils that were detected as toxic using reproduction was only marginally higher than using growth (17 and 14 soils, respectively). However, the compensation of the background noise by raising the toxicity threshold seems appropriate to get more reliable information on the toxicity of soils. In comparison, effects on nematode growth were less severe, however, due to the low variability of this toxicity parameter, equally significant than the effects on reproduction.

The strongest effects on *C. elegans* were found in soils from the former gas plant site, Grasbrook, that mainly are characterized by a high PAH contamination, while the soils from the dumping ground with high heavy metal loads affected the nematodes to a lower extent. The significant negative correlations of nematode growth and reproduction with organic carbon normalized PAH concentrations suggest that the toxicity was at least partly caused by bioavailable PAHs. In a recent remediation study, survival of *C. elegans* was found to be significantly correlated with the aqueous soluble PAH fraction of the soils before the start of the remediation (Cofield et al., 2008). The soluble PAH concentrations, where lethal effects occurred (56–99% mortality) ranged from 2.7 to 5.2 $mg\,l^{-1}$, corresponding to total soil concentrations of 2.6–3.3 g/kg. This is 3–30 times higher than the concentrations that were measured in the present study in soils with high inhibitions of sublethal toxicity parameters. In freshwater sediments, the EC50 of the PAH fluoranthene for reproduction was determined to be 490 $mg\,kg^{-1}$ dry weight (Höss et al., 2007), which is in the same order of magnitude to the PAH contaminated soils investigated here. Moreover, it could be shown that PAHs induce a multiple response in *C. elegans*, when regarding the molecular level (Reichert and Menzel, 2005). Fluoranthene induced the expression of cytochrome P450 genes, i.e. CYP35s, that code for enzymes involved in the detoxification of xenobiotics, in a concentration dependent manner (Menzel et al., 2005).

In spite of the weaker correlation of nematode toxicity with heavy metal concentrations in soils, metals might have caused a toxic effect in some of the samples. WTTNT and HSH2, inducing highly toxic effects, as well as HSH1 and HSH3, showing toxicities close to the toxicity thresholds, were characterized by heavy metal concentrations that are expected to cause toxicity to *C. elegans* (Cu: 256–1600 $\mathrm{mg\,kg^{-1}}$ dry wt; Zn: 2800–6600 $\mathrm{mg\,kg^{-1}}$ dry wt; Table 2). Lethal toxicity of Cu and Zn in soils on *C. elegans* were found at 200–1000 and 250–600 $\mathrm{mg\,kg^{-1}}$ dry wt, respectively, depending on texture and organic content of the soils (Boyd and Williams, 2003; Donkin and Dusenbery, 1993, 1994).

The effects in the contaminated soils were also related to pH, but this time, the correlation was negative, in contrast to the reference soils. As stated above, the pH cannot have affected *C. elegans* directly, as the pH was adjusted to 7.2 in all treatments. It is known that the bioavailability of metals is strongly related to the pH (Allen, 2001). However, at a low pH metals are more soluble in the pore water phase, thus being better available for the nematodes. Therefore, higher toxicities in samples with high pHs cannot be explained by an indirect metal effect. Maybe, the pH is an indicator for the quality of humic substances, that are able to either affect nematodes directly (Höss et al., 2001b), or influence the bioavailability of organic pollutants, such as PAHs (Haitzer et al., 1998).

The fact that the nematode toxicity data did not correlate with the toxicity data of oligochaetes, collembolans and plants, determined in the same soils, might be explained by varying sensitivities towards different classes of toxicants. In this study, *C. elegans* seemed to be more sensitive to organic than to heavy metal pollution, and vice versa for oligochaetes and plants. In this respect the nematode test is a suitable addition to the currently used battery of earthworm, collembolan and plant tests.

Another reason for differences in toxicity might be different exposure routes for the different test organisms, resulting in varying bioavailabilities of the toxicants. Bezchlebova et al. (2007) compared the toxicity of the polychlorinated insecticide toxaphene on various soil invertebrates, including the nematode *C. elegans*, the collembolan *F. candida*, the oligochaetes, *Eisenia fetida*, *Enchytraeus albidus* and *Enchytraeus crypticus*. *F. candida* was most sensitive with a lowest observed effect concentration (LOEC) of 6.2 $\mathrm{mg\,kg^{-1}}$, followed by *C. elegans*, *E. fetida* and the *Enchytraeus* species (no observed effect concentration [NOEC]: 69, 496 and $>600\,\mathrm{mg\,kg^{-1}}$, respectively). For N-heterocyclic PAHs (NPAHs) a different sensitivity ranking was observed for the same organisms with springtails being most sensitive, followed by the annelids and the nematodes (Hofman et al., 2008). However, it should be noted that, with 48 h, the exposure period for *C. elegans* was much lower than for the other species (28 days). Moreover, *C. elegans* was exposed to the NPAHs in natural soil, while for the other test organisms artificial OECD soil was spiked with the chemicals (Hofman et al., 2008). Even if the organic content is lower or similar, the bioavailability of organic chemicals can be considerably higher in artificial compared with natural soils (van Gestel and Ma, 1990). In a study with aquatic sediments, linear alkylbenzene sulphonates (LAS) had a similar toxicity to *Lumbriculus variegatus* and *C. elegans* in spiked sediments, with LOECs of 82 and 100 $\mathrm{mg\,kg^{-1}}$, respectively (Comber et al., 2006). Using five metallic salts in artificial OECD soil, Peredney and Williams (2000) compared lethality data with *C. elegans* to lethality data with the earthworm *E. fetida*. The *C. elegans* exposure was for 24 h compared with earthworm acute exposure of 14 days. *C. elegans* were more sensitive than the earthworm for two of the five metals, Pb and Cd, and less sensitive compared with Cu, Ni and Zn. Overall, the two species had similar values to the 24-h nematode data compared with 14-day earthworm data.

5. Conclusions

The nematode *C. elegans* is a suitable test organism for testing the toxicity of field collected soils, representing a wide range of agricultural and forest soils of temperate regions, using sublethal toxicity parameters. For reference soils with low anthropogenic contamination, growth and reproduction of *C. elegans* showed a low variability between the replicates of a particular reference soil (test inherent variability), as well as between the various soils (soil variability), while fertility was not affected at all. In the control soil, Lufa St. 2.2, the nematodes showed fertility, growth and reproduction, which are representative for the tested reference soils. In polluted soils with varying quantities and qualities of contamination, *C. elegans* was mostly affected by soils with high PAH contamination, while other test organisms, such as earth worms, springtails and plants, were more affected by heavy metal polluted samples. Considering the importance of nematodes for soil ecosystems, the toxicity test with *C. elegans* might be a valuable addition to a soil toxicity test battery, covering complementary exposure routes and thus providing non-redundant information on the toxicity of contaminated soils.

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