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Enchytraeus bigeminus (Enchytraeidae, Oligochaeta) as a new candidate for ecotoxicological laboratory tests

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Abstract

In enchytraeid reproduction tests lambda-cyhalothrin and pyrimethanil were examined under three different soil moisture levels (30, 50 and 70% of the soil water holding capacity). The tests were performed with *Enchytraeus bigeminus* Nielsen & Christensen, 1963, a species that differs from other enchytraeid test species by its asexual mode of reproduction (fragmentation). The effect of chemical stressors depended on the soil moisture content. A higher toxicity was observed in soil with lower moisture levels. For lambda-cyhalothrin, the 21-day EC_{50} values for reproduction at the three levels of soil moisture were calculated to be 1.33, 3.79 and 4.75 mg active substance/kg dry weight soil, respectively. For pyrimethanil the values were 435, 499 and 829 mg active substance/kg dry weight soil. Apart from the evaluation of the combined effects of chemical stress and soil moisture, the appropriateness of the fragmenting test species *Enchytraeus bigeminus* was assessed. *E. bigeminus* tolerated temperature and pH variations, allowed obtaining reliable concentration-response relationships and was easy to handle and to culture in the laboratory. Hence this fragmenting species is considered to be suitable as an additional test species in ecotoxicological standard tests.

Keywords pyrimethanil | lambda-cyhalothrin | Clitellata | potworms | reproduction test | OECD

1. Introduction

Enchytraeids (Oligochaeta) are characteristic geobionts, involved in fertility and formation processes of many soils. In addition to their large relatives, the earthworms, they participate in the degradation of organic matter and in enhancing soil aeration and structure (Amorim 2005). Because of their importance in soil ecosystems, some representatives of this family have been chosen for standardized ecotoxicological laboratory test systems (ISO 16387 2003, OECD 220 2004). Test species are selected based on handling in the laboratory, cultivability, data availability and ecological relevance. The first choice and most common in chemical testing is *Enchytraeus albidus* Henle, 1837. Although it fulfils the first three criteria perfectly, its ecological relevance for ecotoxicological tests might be doubtful. It occurs mainly in decaying organic material, such as in the marine littoral or in compost (Schmelz & Collado 2010) and hence colonizes habitats that might not be representative for pesticide application. Both ISO and OECD guidelines recommend additional enchytraeid species, and the ones considered so far (*E. buchholzi* Vejdovský, 1879; *E. bulbosus* Nielsen & Christensen, 1963; *E. crypticus* Westheide & Graefe, 1992; *E. luxuriosus* Schmelz & Collado, 1999) have sexual reproduction in common. In this study, we explored the suitability of *Enchytraeus bigeminus* Nielsen & Christensen, 1963 as another possible species for ecotoxicological standard testing. *E. bigeminus* may reproduce sexually, but reproduces mostly by fragmentation. This might be advantageous because – due to its short generation cycle – test duration

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could be shortened and mass cultures can be initiated within a few days. Furthermore fragmenting species may offer a lower response variation due to genetic uniformity of the test population (Weltje 2003). However, effects of chemicals on sexual reproduction cannot be determined. In contrast to *E. albidus*, *E. bigeminus* colonizes habitats that are more representative for a possible pesticide usage. It belongs to the 'open landscape species' (Heck et al. 1999) and is found in lawns, grasslands and fields (Heck et al. 1999, Römbke et al. 2002).

To evaluate the advantages and limitations of *E. bigeminus* for chemical testing, two model substances were selected: lambda-cyhalothrin, an insecticide, and pyrimethanil, a fungicide. With these chemicals, reproduction tests according to OECD 220 (2004) were performed. The tests were conducted to examine the toxicity of the chemicals on the test species, but also to assess the effects of environmental factors. IPCC (Intergovernmental Panel on Climate Change) forecasts predict changes in temperature as well as in precipitation. The worst-case scenario forecasts a temperature rise up to 6.4°C by 2100 (Meehl et al. 2007). Furthermore a decline in summer precipitation (30–45 %) and an increase in winter precipitation (15–30 %) in central Europe is prognosed (Alcamo et al. 2007). Due to precipitation changes, a change in soil moisture may be expected. Therefore, we conducted the experimental tests at a wide temperature range of 19 to 27°C and at three defined levels of soil moisture: 30, 50 and 70 % of the maximum water holding capacity (WHC). Besides the effects of soil moisture on the toxicity of the pesticides, the tolerance of *E. bigeminus* against changing environmental factors is discussed.

2. Materials ans methods

2.1. Test species

The test species used in the present study was *E. bigeminus*. This species is not proposed as a possible candidate for ecotoxicological tests in the ISO and OECD guidelines and has thus far only rarely been used in laboratory studies (e.g. Christensen & Jensen 1995). It is one of six enchytraeid species known to reproduce mostly by fragmentation (Niva et al. 2012). Living individuals have a body length of between 0.5 and 15 mm (Schmelz et al. 2000). The species is probably common in Southern Europe and South America (i.e. Brazil), where it colonizes soils rich in organic matter (Schmelz & Collado 2010, Niva et al. 2012). It was also found in Iran (Dózsa-Farkas 1995). This might be an indication for a preference for warmer climatic zones. A list of records of *E. bigeminus* on a global scale is presented in Collado et al. (2012).

Christensen (1973) stated that its reproduction strategy depends on the density of individuals. At low densities it reproduces sexually, while at high densities only few mature individuals occur and reproduction by fragmenting strongly dominates. However, in the cultures used by us, clitellate individuals and cocoons were never observed. During fragmentation *E. bigeminus* divides into up to seven fragments. These need six days to regenerate a new anterior end and a further seven days for intermediary and posterior fragments at 20–22°C (Christensen 1964, Christensen 1973). The generation time of fragmenting *E. bigeminus* is comparatively short compared to the sexually reproducing test species proposed by OECD 220 (2004): 33 days (18°C) for *E. albidus* (Römbke & Moser 2002), 17.4 days (21°C) for *E. crypticus* (Westheide & Graefe 1992) and about 25 days (20°C) for *E. buchholzi* (Learner 1972). For *E. luxuriosus* and *E. bulbosus*, no data concerning life cycle are available. Therefore, the test duration was reduced to 3 weeks instead of the guideline's recommendation of 4 and 6 weeks, respectively.

The *E. bigeminus* culture was established without temperature control at 19–27°C. The animals were kept in plastic petri dishes containing a ca 5 mm layer of agar in constant darkness.

2.2. Model substances lambda-cyhalothrin and pyrimethanil

The reproduction tests were performed with two substances. They were selected according to the following criteria: for both of them ecotoxicological data are available, including Oligochaeta, but none for enchytraeids. Furthermore physical-chemical characteristics such as the mode of action and degradability are known and their half-life is longer than the applied test duration. Hence, a continuous chemical exposure can be assumed. Last but not least these are commercially available pesticides used globally in agriculture. The first was lambda-cyhalothrin, a pyrethroid insecticide with sodium-channel modulator as mode of action (CAS 91465-08-6). Its mean half life in soil is estimated to be 56 days under standard laboratory conditions (20°C and soil moisture of 40–60 % WHC) (European Commission 2001). Lambda-cyhalothrin has a high octanol water partition coefficient (Log K_{OW} = 7.0) (European Commission 2001) and, hence, tends to adsorb to organic matter, either to biota but certainly also to organic compounds in soils. Since water solubility is low (0.005 $mg/l_{21°C,\ pH\ 6.5}$) (European Commission 2001) and to achieve a more direct link to agricultural reality, lambda-cyhalothrin was applied as the formulation 'Karate® Zeon™', distributed by Syngenta Crop Protection AG, Switzerland. For agricultural usage the maximum application rate for 'Karate® Zeon™' is 75 ml/ha. This rate corresponds to 7.73 g lambda-cyhalothrin per hectare. Applying the assumptions of EU regulatory procedures, namely a soil density of 1.5 g/cm³ and a mean incorporation depth of 5 cm (European Economic Community 2007), the maximum application rate would theoretically result in a soil concentration of 10.3 µg lambda-cyhalothrin/kg dw soil. This calculation provides the necessary comparison with data generated in laboratory experiments. The concentrations of the reproduction test were chosen based on previous range finding tests (data not shown) and included 10 concentrations with lambda-cyhalothrin plus one negative control in untreated soil. The concentrations were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 mg a.s./kg dw soil (nominal values).

The second model substance was pyrimethanil (CAS 53112-28-0), a broad spectrum fungicide, with methionin biosynthesis inhibition as mode of action. Under standardized laboratory conditions, its mean half life in soil has been determined to be 56 days. Its lipophilicity is moderate (log K_{OW} = 2.84) as well as its water solubility (121 $mg/l_{25°C,\ pH\ 6.1}$) (European Commission 2005). Similar to lambda-cyhalothrin, pyrimethanil was applied as a commercial formulation, i.e. 'SCALA', distributed by BASF SE, Germany. The maximum application rate for Scala is 2.5 l/ha, equivalent to 1 kg pyrimethanil per hectare. Applying the same calculation as mentioned above, the maximum application rate would lead to 1.33 mg pyrimethanil/kg dw soil. The concentrations of the reproduction tests performed with pyrimethanil were 44.2, 62.5, 88.4, 125, 177, 250, 354, 500, 707 and 1000 mg a.s./kg dw soil (nominal values). Besides the chemical exposure, a negative control with untreated soil was also used. The concentration range was chosen based on prior range finding tests (data not shown).

All concentrations mentioned in this paper refer to the corresponding concentrations of the active substance (a.s.) in dry weight soil (kg dw soil), either lambda-cyhalothrin or pyrimethanil, as nominal values.

2.3. Experimental testing

All reproduction tests were conducted on the basis of OECD guideline 220 (2004), but with some deviations. The tests ran under a 16/8 h light/dark regime without climatic control at a temperature range of 19 to 27°C according to a minimum-maximum thermometer. The exposure was conducted in spiked OECD artificial soil, to which the chemical was added. OECD soil contains 10% peat, 20% kaolin, approximately 70% quartz sand and circa 0.3 to 1% calcium carbonate to regulate the pH-value. In order to evaluate a possible interaction of environmental and chemical stress, all chemical treatments (either with lambda-cyhalothrin or pyrimethanil) were combined with three different soil moisture levels (30, 50 and 70% w/w of WHC).

The water holding capacity (WHC), measured as described in Annex 2 of OECD 220 (2004), of the soil used in the tests with lambda-cyhalothrin had a WHC of 60.4%, while that of the soil in the tests with pyrimethanil was determined to be 58.1%. Hence, water was added in a way that the actual water content in the three moisture levels corresponded to approximately 18, 30 and 41% w/w. To achieve the desired soil moisture (in % w/w of WHC), the soil was pre-moistened before the start of the test with the respective volume of deionized water less the application volume of 10 ml. This was added at day 0 and mixed thoroughly in the soil. Each test vessel was filled with the respective amount of soil, corresponding to 20 g dry weight soil and covered with Parafilm afterwards. All vessels used in the experiments were made of glass with a volume of approximately 250 ml. Besides the test vessels, two extra vessels per treatment were established to assess the soil moisture and pH at the beginning and the end of the test. The soil moisture was measured gravimetrically (ca. 10 g fresh weight in tests with lambda-cyhalothrin, ca. 1 g fresh weight in case of pyrimethanil). The pH values were determined in calcium chloride for the tests with lambda-cyhalothrin and in 3 repetitions with a pH insertion electrode for the tests with pyrimethanil. The test design with 10 chemical concentrations plus negative controls combined with the three moisture levels led to 132 test vessels including four replicates per treatment plus 66 extra vessels for soil moisture and pH determination (two replicates per treatment) for each test substance.

At the beginning of an experiment, 10 full-grown enchytraeids of about 8–12 mm length were randomly inserted in the test vessels and fed with 50 mg autoclaved oat flakes. Thereafter, feeding (25 mg oat flakes) as well as remoistening to initial weights, was conducted once per week. Because of the short generation time of this fragmenting species, the test duration recommended in the ISO and OECD guidelines (4 weeks) was reduced to 3 weeks. After completion, the soil of the test vessels was saturated with 70% (v/v) ethanol to kill the enchytraeids. After a couple minutes, each vessel was filled with deionized water and the suspension floated into a lab tray. Additionally 0.3 ml of diluted Rose Bengal (3 g Rose Bengal/l ethanol) were added as described in annex 6 of OECD 220 (2004). In order to receive a homogenous distribution of the stain and a flat layer of the soil, the lab tray was carefully slued. After at least 12 hours under the fume hood, the water was evaporated and the enchytraeids coloured deep violet. The individual enchytraeids laid above the soil particles and could be easily counted under a stereo microscope.

For *E. bigeminus* the validity criteria were established for small *Enchytraeus*-species applying OECD 220 (2004): at least 50 juveniles on average in the control. Furthermore the adult mortality should not exceed 20% at the end of the test. Since *E. bigeminus* may reproduce by fragmentation, the evaluation of adult mortality was omitted. Finally the coefficient of variation of the mean number of juveniles of the controls should not be higher than 50%. The endpoint statistically assessed in this study was the total number of individuals, referring to fragments and regenerated worms.

2.4. Statistical analysis

In order to assess an influence of soil moisture on reproduction in the absence of a chemical stressor, a two-way ANOVA was conducted with Statistica 10 (StatSoft, Inc. 2011) with number of individuals as the dependent variable and soil moisture and the two tests as fixed factors (n = 4). To achieve variance homogeneity of the dependent variable, a square root transformation was applied. The ANOVA was followed by a Bonferroni post hoc test with $\alpha = 5\%$.

The statistical evaluation of the concentration-response curves was conducted with R 2.13.1 (R Development Core Team 2011) and the drc package (Ritz & Streibig 2005). A three parametric log-logistic function (LL2.3) was used to fit the response (number of individuals at test end) to the concentration of the pesticide:

$f_{(x)} = d / (1 + \exp^{(b(\log(x)-e)})$ with x = nominal concentration, b = slope at inflection point, d = upper limit and e = ln inflection point. We assumed Poisson error distribution as the numbers of individuals are count data. From the fitted curves, EC_x values relating to 50%, 20% and 10% effect, respectively, were derived together with their respective 95% confidence intervals using the function ‘fls’ in the drc package.

3. Results

3.1. Validity of the tests

The pH in the tests with lambda-cyhalothrin was on average 6.21 (range: 6.11 – 6.34; n = 33) at the beginning of the experiment and 5.98 (range: 5.84–6.26; n = 66) at the end. Hence, the pH recommended by OECD 220 (2004) (= 6.0 ± 0.5) was achieved throughout the experiment. The pH values in the pyrimethanil test ranged from 5.25 to 7.20 with an average of 6.44 (n = 33) at the beginning of the experiment. After 21 days the average pH was 5.87 (range: 5.14–6.72; n = 66). The validity range for the pH according to OECD guideline 220 (2004), i.e. 6.0 ± 0.5, was not maintained for all additional vessels in this experiment. Potential consequences on the test outcome will be discussed below.

The means and standard deviations for each soil moisture treatment of the test with lambda-cyhalothrin were 33.9 ± 0.8, 53.7 ± 1.4 and 73.1 ± 1.7% (n = 33) at the beginning and 33.4 ± 1.7, 52.8 ± 1.6 and 73.4 ± 1.5% (n = 66) at end of the experiment. This clear variation allowed a separate analysis for further endpoints and discussion. According to OECD 220 (2004), the soil moisture should be adjusted to 50 ± 10% of WHC. The values in this experiment ranged between 29.2 and 36.6% WHC for the treatment with dry soil (n = 99) and between 48.2 and 55.9% WHC for the treatment aiming at 50% WHC (n = 99). In the soil with a WHC of 70%, the water content varied between 71.0 and 76.8% WHC (n = 99). Applying the same recommendations for maximum deviation, the actual soil moisture of this experiment fulfilled that criterion.

In the experiment with pyrimethanil, the following means and standard deviations for soil moisture were calculated for the respective treatment: 30.3 ± 1.3, 48.3 ± 1.3 and 68.7 ± 5.4% WHC (n = 33) at test start and 26.0 ± 3.1, 46.1 ± 3.1 and 61.2 ± 7.1% WHC (n = 66; for the treatment with 70% nominal soil moisture n = 65) at the end of the test. For technical reasons (amount of soil was too small), the gravimetric measurement of soil moisture in the test with pyrimethanil was performed with a smaller amount of soil. Hence, the deviations were larger in comparison to the study with lambda-cyhalothrin. For the nominal soil moisture of 30% of the WHC, the values ranged between 18.4 and 32.9% WHC (n = 99) during the experiment. For the treatment aiming 50% of the WHC, they ranged from 37.2 to 50.9% WHC (n = 99). Finally the treatment with a nominal value of 70% of the WHC, the values ranged between 47.0 and 76% WHC (n = 98). Even though the deviation of the nominal values were larger than recommended by the guideline (pH as well as soil moisture), it should be mentioned that these values result from additional vessels and need not necessarily reflect the conditions in the test vessels. Actually, the question whether an ecotoxicological test is valid or not depends on the fact whether specific validity criteria (most importantly the number of juveniles in the control vessels being larger than 50 individuals) are fulfilled or not.

In none of the vessels were cocoons observed, indicating that under test conditions fragmentation strongly dominates. Since 50% of WHC is the soil moisture required by OECD 220 (2004), only this moisture treatment was considered when determining the validity of the tests. In the experiment with lambda-cyhalothrin, between 144 and 425 enchytraeids (including fragments) were counted per control vessel (mean: 299; n = 4), while in the pyrimethanil study between 310 and 388 worms were found in the control vessels (mean: 355; n = 4). The coefficients of variation around the means were calculated as 40.5% in the study examining lambda-cyhalothrin and 9.71% in those of pyrimethanil. Hence, the validity criterion of both reproduction tests as defined for small species in the guideline (OECD 2004) was fulfilled.

3.2. Impact of soil moisture on maximum reproduction

The concentration-response curves for the experiment lambda-cyhalothrin effects on enchytraeids are shown in Fig. 1. In the controls (as well as in the chemical treatments), a clear dependency of reproduction on soil moisture was visible in addition to the concentration-dependent reduction in reproduction (see below). Especially in vessels with soil moistened to 30% of WHC, the number of individuals was lower in comparison to the treatment with 50 and 70% WHC, respectively. In the latter two moisture regimes, comparable numbers of enchytraeids were counted, but with slightly greater values in 70% WHC.

In the reproduction tests with pyrimethanil (Fig. 2), the number of individuals in the control vessels was reduced by a simulated drought stress as well. In comparison to the first experiment with lambda-cyhalothrin, the enchytraeids seemed to be even more affected by drought, with 36 worms on average in contrast to an average of 126 in the first test. The treatment with a soil moisture of 70% WHC led to a marginally lower number of individuals in the control vessels compared to the 50% WHC treatment without a chemical stressor.

The two-way ANOVA of both experiments (Table 1) detected no difference between the numbers of worms in the control vessels of the two experiments. But as Figs 1 and 2 already indicate, a significant dependence between number of enchytraeids and the main factor 'soil moisture' was observed. Furthermore the Bonferroni post hoc test indicated a significant difference between the nominal groups of 30 and 50% as well as between 30 and 70% WHC, but none for the comparison of the groups 50 and 70% WHC. Hence, the simulated drought stress led to a considerable reduction of reproduction in comparison to the treatments with higher soil moisture.

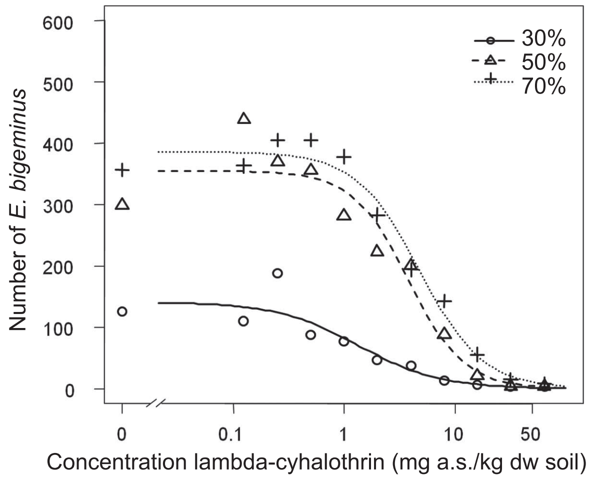

Figure 1. Effects of lambda-cyhalothrin [mg a.s./kg dw soil] on *E. bigeminus* in soils hydrated to different moisture levels expressed as percent of water holding capacity (WHC) (values are means; n = 4).

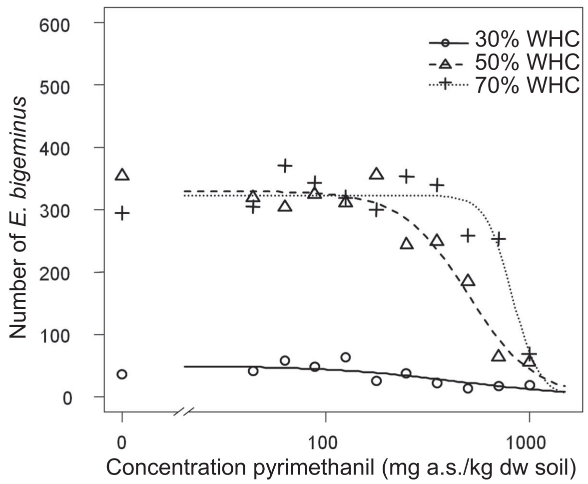

Figure 2. Effect of pyrimethanil [mg a.s./kg dw soil] on *E. bigeminus* in soils hydrated to different moisture levels expressed as percent of water holding capacity (WHC) (values are means; n = 4).

Table 1. Results of the two-way ANOVA of maximum reproduction in the controls.

	Df[a]	Mean Sq[b]	Fvalue	Pr (>F)[c]
experiment	1	24.7	2.41	0.138
moisture	2	232	22.6	< 0.001
experiment * moisture	2	27.5	2.67	0.096
residuals	18	10.3		

[a] – Degrees of freedom, [b] – Mean Squares, [c] – p-value

3.3. Toxicity of the model substances and their dependency on soil moisture

Besides the influence of soil moisture on the maximum reproduction in the controls, the concentration-response curves indicate a different reaction towards lambda-cyhalothrin for each moisture treatment (Fig. 1). The estimated EC_{50} values clearly increased with increasing moisture for both pesticides (Fig. 3). The EC_{50} values for each moisture treatment were calculated to be 1.34 (30% WHC), 3.79 (50% WHC) and 4.77 mg a.s./kg dw soil (70% WHC), respectively. These three EC_{50} values can be regarded as being clearly different since their confidence intervals do not overlap, indicating an increasing toxicity with decreasing soil moisture. EC_{10} and EC_{20} values as surrogate endpoints for NOECs (No Observed Effect Concentration) are presented with their confidence intervals in Table 2. Their confidence intervals of the upper two soil moisture levels overlap, indicating no influence at lower effect levels. A possible influence through dry soil was also observable at these effect levels.

The progressions of the three different concentration-response curves of the experiment with pyrimethanil are rather dissimilar as well (Fig. 2). That of the treatment with 30% WHC is very flat due to low numbers of enchytraeids throughout all chemical treatments. On the other hand, enchytraeids exposed to pyrimethanil at 70% WHC reacted less sensitively towards this substance and, hence, the decrease occurred in higher concentrations in comparison to the treatment at 50% WHC. The EC_{50} values (mg a.s./kg dw soil) were calculated to be 437, 499 and 829 in the treatments with 30, 50 and 70% of WHC, respectively. Due to the flat concentration-response curve of the treatment with 30%, the EC_{50} calculation was associated with uncertainties leading to relatively large confidence intervals. Therefore the EC_{50} determined in the two treatments at 30% and 50% WHC were not considered to be different since their confidence intervals overlapped. Only the EC_{50} resulting from the treatment at 70% WHC was clearly different from those determined at lower moisture levels. The EC_{10} and EC_{20} may be considered different among moisture levels, because their confidence intervals differed (Table 2). In both experiments a positive correlation between EC_{50} and soil moisture was observed: low soil moisture led to enhanced toxicity while higher soil moisture in general led to lower toxicity.

4. Discussion

4.1. Maximum reproduction

The validity criterion concerning the number of juveniles in the control vessels was fulfilled in both experiments in the treatments with medium soil moistures. In a ring test with *E. albidus*, a high coefficient of variation of the number of juveniles of the control vessels (= 43–48%) was already reported (Römbke & Moser 2002) and hence the range of variability that was found in our study can be regarded to be a normal deviation.

The statistical analysis confirmed an influence of moisture on the maximum reproduction, namely a decrease in offspring under simulated drought stress. Such an impairment of reproduction by low soil moistures has already been demonstrated for a number of enchytraeids. Beylich & Achazi (1999) found a decrease

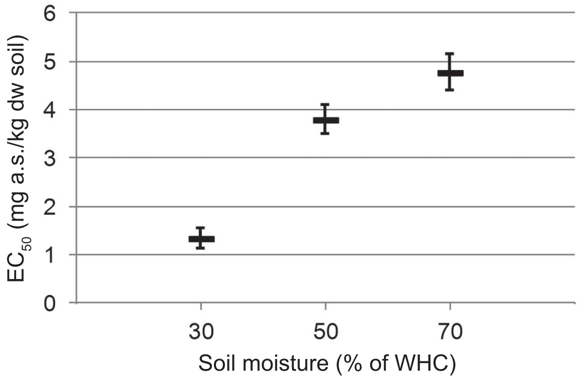

Figure 3. Relationship between the EC_{50} values for lambda-cyhalothrin [mg a.s./kg dw soil] with their 95% confidence intervals of the reproduction test with *E. bigeminus* and soil moisture [% of water holding capacity (WHC)]. n = 4.

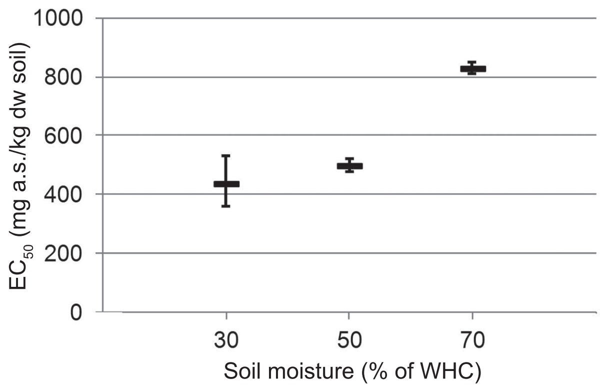

Figure 4. Relationship between the EC_{50} values for pyrimethanil [mg a.s./kg dw soil] with their 95% confidence intervals of the reproduction test with *E. bigeminus* and soil moisture [% of water holding capacity (WHC)]. n = 4.

Table 2. EC_{10} and EC_{20} values [mg a.s./kg d.w. soil] with their 95% confidence interval of the reproduction test with *E. bigeminus* exposed to lambda-cyhalothrin or pyrimethanil under different soil moisture levels [% of water holding capacity (WHC)]. n = 4.

	Moisture	Lambda-cyhalothrin	Pyrimethanil
EC_{10}	30	0.21 (0.16–0.28)	85.6 (56.4–130)
	50	1.06 (0.90–1.24)	220 (199–244)
	70	1.12 (0.97–1.30)	598 (560–639)
EC_{20}	30	0.41 (0.33–0.52)	156 (114–215)
	50	1.70 (1.49–1.93)	298 (276–322)
	70	1.92 (1.70–2.15)	675 (642–709)

in reproduction for *Enchytraeus buchholzi* in Lufa soil 2.2 containing 15 % or less water content, comparable to 28.2 % WHC in that soil. Dirven-van Breemen et al. (1994) examined the reactions of *E. albidus* and *Enchytraeus crypticus* to different soil moisture levels. They used OECD standard soil with 10 % peat with a total water content of 15, 35, 55, 65 and 90 % w/w. *E. albidus* reproduced best at 55 %, *E. crypticus* at 35 %, measured as the number of juveniles per adult per week. In treatments with the lowest soil moisture no juveniles were found, neither with *E. albidus* nor *E. crypticus*. The soil moisture of the study of Dirven-van Breemen et al. (1994) was reported in % w/w total water content. Our three soil moisture levels refer to approximately 18, 30 and 41 % w/w total water content, respectively. For *E. bigeminus* no specific data are available in the literature, but our results are in agreement with published studies indicating that drought stress may reduce the reproduction success of enchytraeids.

4.2. Toxicity of model substances

The EC_x values followed a positive trend, i.e. higher toxicity (decreasing EC_x values), with decreasing soil moisture. The EC_{50} of lambda-cyhalothrin was reduced by a factor of 2.8 due to exposure at 30 % WHC in contrast to the EC_{50} under standard conditions. An enhanced sensitivity due to drought stress may be of great relevance if global climate change worsens and aridity occurs in higher frequencies. At high soil moisture (70 % WHC), the toxicity of lambda-cyhalothrin was slightly reduced in comparison to medium soil moisture by a factor of 1.3 for the EC_{50} value.

The toxicity of pyrimethanil showed a similar pattern regarding soil moisture. When exposed to pyrimethanil under simultaneous drought stress, the EC_x values were lower up to a factor of 2.6 for the EC_{10}, although the confidence intervals of the EC_{50} did not indicate a difference between the treatments at 30 and 50 % of the WHC. The enchytraeids were less sensitive to the chemical stressor in the high soil moisture treatment (70 % WHC), based on an increase of a factor of up to 2.7 in the EC_x values. Nevertheless it should be mentioned that there are several possible reasons for this dependency of toxicity on soil moisture: bioavailability and/or degradation may depend on soil moisture and thereby influence toxicity by influencing (internal and/or external) exposure levels. Toxicity may also be influenced by internal processes within the test organisms, i.e., lower metabolic activity or reduced fitness at lower soil moistures. The aspect concerning degradation might be of lower importance, since both substances have a mean half life in soil of 56 days under standard conditions, which is more than twice as long as the test duration.

Since no ecotoxicological data for enchytraeids are available for the test substances, a comparison with data of earthworm tests may be appropriate. Garcia et al. (2011) examined the toxicity of lambda-cyhalothrin in an earthworm reproduction test with *Eisenia fetida*. In OECD standard soil they detected an EC_{50} of 37.4 and a NOEC (No Observed Effect Concentration) of 10.0 mg a.s./kg dw soil. The earthworm EC_{50} value is about ten times greater in comparison to the EC_{50} generated under standard conditions with *E. bigeminus*. In accordance with the European Commission (2003), we compared our EC_{10} data that can be considered as NOECs. The EC_{10} of *E. bigeminus* (50 % WHC) was calculated to be 1.06 mg a.s./kg dw soil (Table 2) and, hence, is also about a factor of 10 lower than the NOEC reported by Garcia et al. (2011). This difference is probably due to the use of different taxonomic families. It indicates a higher sensitivity of *E. bigeminus* towards lambda-cyhalothrin in comparison to the earthworm *E. fetida*. Nevertheless a risk for enchytraeids due to lambda-cyhalothrin even under additional drought stress appears unlikely, since the predicted environmental concentration in soil is about 20-fold lower than the lowest EC_{10} value.

For pyrimethanil a NOEC of 8.24 mg a.s./kg dw soil was reported for earthworm reproduction (European Commission 2005), which is considerably lower than the EC_{10} determined in the present study under standard conditions for *E. bigeminus* (220 mg a.s./kg dw soil). In the earthworm reproduction test, the chemical was sprayed onto the surface and, hence, the exposure scenario differed strongly from the one used in the present study (i.e. homogenous mixture in soil). A risk due to exposure to pyrimethanil is not expected for enchytraeids, as the lowest EC_{10} is more than 60-fold higher than the estimated concentration of the maximum application rate in the field.

4.3. *Enchytraeus bigeminus* as test organism

So far *E. bigeminus* has rarely been used in ecotoxicological tests. One of the aims of the present study was to examine whether and how a fragmenting species could be involved in standardized test systems. Neither the OECD (2004) guideline nor that of ISO (2003) proposes such a candidate. As stated above, the usage of a fragmenting species may be regarded as advantageous because test duration can be shortened and the variance of response may be reduced (Weltje 2003). In fact, the use of non-sexually reproducing species is often considered as positive since clonal laboratory cultures are more uniform genetically than cultures of sexually reproducing species, meaning that for the selection of test species this mode of reproduction is a positive or at least a neutral factor. However, *E. bigeminus* is polyploid, and different strains of the species sensu lato are highly divergent at the DNA level (Collado et al. 2012), suggesting different sensitivities to chemicals among strains. Therefore the use of the same strain in laboratory tests is recommendable. The molecular characterization of the strain of *E. bigeminus* (barcoding) used in these tests is under way (Schmelz, pers. com.).

Apart from *E. bigeminus*, another fragmenting enchytraeid species, *Cognettia sphagnetorum* (Vejdovský, 1879), has been proposed as a candidate species for ecotoxicological studies (e.g. Sjögren et al. 1995). However, due to its strong preference for acidic soils its use as a test species is limited and not applicable to agricultural soils that have a pH of usually > 5.5. Furthermore, maintaining stable laboratory cultures and obtaining reproducible test results is not straightforward (Römbke unpublished, Schmelz pers. com.).

In the reproduction tests a high tolerance against varying abiotic factors was observed for *E. bigeminus*, which might be due to the fact that this species is adapted to the environmental conditions in warmer climatic zones (Southern Europe, Brazil, Iran). Since the experiments were conducted without climatic control, the temperature varied between 19° and 27°C. The pH values deviated slightly from the range recommended by OECD (2004), but it is unlikely that these differences had a strong effect on the test results. In the validation of the enchytraeid reproduction test (OECD 2004), conducted in a ring test with 29 participants, it was recognized that small deviations do not have a great effect on enchytraeids in general, and hence only those tests with a pH lower than 4.5 and higher than 7.5 were excluded (Römbke & Moser 2002). It seems that *E. bigeminus* was not affected by different pH or temperature values and was also tolerant towards different moisture levels (even in the treatment at 30 % WHC, the validity criterion was fulfilled in the study with lambda-cyhalothrin). This statement is backed by the fact that no outlier in abundance occurred in the experiments. In addition clear concentration-response relationships could be generated as well. Due to these results *E. bigeminus* might be a good choice for testing of chemicals and also for assessing different natural soils which possess a wide variation of environmental variables. Christensen & Jensen (1995) used *E. bigeminus* for assessing the toxicity of three pesticides (dimethoate, pirimicarb and fenpropimorph). Their test duration was even shorter (7 days). The formation of new segments in an active growth phase and mortality was evaluated. They detected clear concentration-response relationships for each chemical using *E. bigeminus* as a test organism. Although the test design differs considerably from the present study, it supports the finding that the fragmenting species *E. bigeminus* is a potential candidate for ecotoxicological tests that may offer robust results within a short time period.

5. Conclusions

With regard to the toxicity of the two model substances, clear effects on the number of descendants were detected that depended on soil moisture. Toxicity was enhanced in drier soils, while higher than standard soil moistures led to a reduced toxicity. Furthermore the fragmenting test species *E. bigeminus* can be recommended as a suitable candidate for chemical testing in standardized tests as well as in environmental monitoring, particularly for the examination of natural soils since *E. bigeminus* endures a rather broad range of abiotic factor variations.

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