Species composition of a soil invertebrate multi-species test system determines the level of ecotoxicity

Valentina Sechi,1, Alessandra D‘Annibale, Kristine Maraldo, Anders Johansen, Rossana Bossi, John Jensen, Paul Henning Krogh

Abstract

A soil multi-species, SMS, experimental test system consisting of the natural microbial community, five collembolan species and a predatory mite along with either Enchytraeus crypticus or the earthworm Eisenia fetida were exposed to α-cypermethrin. A comparison of the performance of these two types of SMSs is given to aid the development of a standard test system. E. fetida had a positive effect on the majority of the species, reducing the negative insecticide effect. E. fetida affected the species sensitivity and decreased the degradation of the insecticide due to the organic matter incorporation of earthworm food. After 8 weeks, the EC50 was 0.76 mg kg⁻¹ for enchytraeids and ranged between 2.7 and 18.9 mg kg⁻¹ for collembolans, more sensitive than previously observed with single species. Changes observed in the community structure and function illustrates the strength of a multi-species test system as an ecotoxicological tool compared to single species tests.

1. Introduction

Soil organisms interact intimately through their contribution to the bio-geochemical cycling of carbon and mineral nutrients (Coleman et al., 2004). General types of ecological interactions are found in soil ecosystems such as predator–prey relationships, competition for resources, mutualism and commensalism. Besides having direct effects on the individual organisms, insecticides also affect these crucial species interactions ultimately having repercussions on the food-web. However, only a few laboratory experimental studies have been done on the effects of pollution on the structure and functioning of food webs in terrestrial ecosystems, especially in soils (Salminen et al., 2002; Cortet et al., 2006).

The decomposition of organic matter is a relevant functional endpoint for toxicity testing of insecticides due to its pivotal role in soil nutrient cycling. It involves a wide range of soil organism and it is employed as the litterbag method (OECD, 2006). However, the litterbag method is relatively laborious and insensitive, and gives no insight into potential impacts on the soil fauna or microbial community structure, as it does not include direct measurements related to diversity. Moreover, the choice of straw of low food quality makes the direct role of soil fauna less important in the initial decomposition phases. Given this, there is a need for new and more sensitive test systems, which incorporate community structural and functional endpoints. Where higher tier methods testing the aquatic toxicity of insecticides are relatively advanced (e.g. Boxall et al., 2002), we advocate for a development of a similar approach for soil environments as the function of individual soil organisms is most meaningful when seen as part of the entire food web (Cortet et al., 2006; Jensen et al., 2009; Knacker et al., 2004).

To evaluate the effect of faunal species interactions under controlled conditions, semi-field systems have been used to simulate field situations (Gyltenkaerne et al., 2000) in various...
degrees, either at indoor or outdoor conditions. Terrestrial Model Ecosystems (TME) are model ecosystems based on intact field soil cores containing an indigenous pool of organisms which are exposed to a toxicant by spiking (Knacker et al., 2004). However, methods closely resembling realistic field situations often have a high variability and a low stability and are difficult to reproduce. Alternatively, the indigenous animals could be extracted from field soil-cores and then added to soil cores containing the contaminant. Still the natural variation inherited from the individual sample cores may result in a relatively high variability between the replicates reducing the statistical power and the repeatability of the test (Scott-Fordsmand et al., 2008) and make it difficult to detect changes at relevant low concentrations of contaminants. The design therefore has to balance gaining realism without losing reproducibility. An attempt to improve the statistical properties is possible by a testing approach with a constructed invertebrate decomposer food web at the expense of some of the natural soil biodiversity (Cortet et al., 2003, 2006; Filsen and Krogh, 2002; Permin et al., 2006; Jensen and Scott-Fordsmand, 2012; Scott-Fordsmand et al., 2008). Compared to the TME approach, such constructed systems represent a specific but reduced faunal food web; nevertheless, they provide the basic characteristics of the soil ecosystem. In this manner, a more homogenous system is obtained with less initial variation and it can be adapted to represent different types of soil habitats. Furthermore, it gives the opportunity to study special ecological topics, such as the importance of functional redundancy and species diversity. Such a more robust higher tier test system still enables quantification of direct and indirect effects of chemicals in terrestrial food webs, and the study of possible links between functional and structural endpoints. Endpoints could include the classical endpoints survival, reproduction, growth and various microbial mediated processes, but also community structure and population dynamics. Indirect effects of pollutants via competition or predation are crucial in most ecosystems and, although community structure is complex and therefore difficult to describe, it should be taken into account (Cortet et al., 2006). Potentially, these indirect effects of a toxicant may play an important role when causality between the observed effects of pollution on the structure and functioning of stressed food webs is evaluated (Salminen et al., 2002).

The aim of the study was to test the performance and validity of a soil multi-species (SMS) test systems, to underpin future adoption for ecotoxicity testing, by measuring the effects of an insecticide, α-cypermethrin, on soil invertebrate populations and microbial community composition. In addition, we wanted to assess the suitability and sensitivity of two alternative worm species in the SMS system and to gain knowledge regarding the performance of two different soil communities as insecticides in agriculture (Hartnik et al., 2008a) and have been used during the past 25 years. For practical reasons replicates of the experiment were allocated into two blocks with twenty four soil mesocosms per block set up at two dates. The second block was run two weeks later than the first block. The exposure time covered a period of eight weeks. At each sampling date, after four and eight weeks twelve mesocosms were harvested destructively for analyses.

2. Materials and methods

A simplified invertebrate food-web was constructed in the laboratory based on species composition found in a Danish agro-ecosystem (Fjellberg, 2007). Seven culturable species (all cultured at the Dept. of Bioscience, Aarhus University) were selected from different functional groups (Table 1). Mutualism, competition and predation were introduced by addition of five collemboles, one enchytraeid and earthworm, and a predatory mite species. A total of forty-eight soil mesocosms were set-up in a design which included zero (control) and three concentrations (1, 5, 25 mg kg⁻¹) of α-cypermethrin (α-CYP), two different soil fauna communities and two sampling dates.

We chose the pyrethroid insecticide α-cypermethrin (α-CYP) as it has known effects on invertebrates. The recommended application level in Danish agriculture is 100 g ha⁻¹, or approximately 0.07 mg kg⁻¹ soil in the upper 10 cm of the ploughing layer. The pyrethroids are a dominant group of plant-protection products widely used as insecticides in agriculture (Hartnik et al., 2008a) and have been used during the past 25 years. For practical reasons replicates of the experiment were allocated into two blocks with twenty four soil mesocosms per block set up at two dates. The second block was run two weeks later than the first block. The exposure time covered a period of eight weeks. At each sampling date, after four and eight weeks twelve mesocosms were harvested destructively for analyses.

2.1. Communities

Two different faunal communities (abbreviated COM), were constructed, including three trophic levels and different functional groups. All animals employed were obtained from laboratory cultures. Both communities included five species of springtails and the predator mite 

**Table 1**

<table>
<thead>
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<th>Functional group</th>
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<th>Life-form</th>
<th>No. indiv.</th>
<th>No. indiv. m⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predator mite</td>
<td>Hypoaspis aculeifer</td>
<td>Hemi-eudaphic</td>
<td>10</td>
<td>1270</td>
</tr>
<tr>
<td>Collembolans d retivore</td>
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<td>20</td>
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6 ml of water was added to the soil surface. Each mesocosm was covered by a number of individuals was estimated to be minimum numbers necessary to cultures. Twenty-four groups of ranging between 30 and 70 mg f.w. per individual were selected from our laboratory fresh biomass, and then randomly allocated to the 24 mesocosms.

At day one, the half of the cylinders received each

\[ \text{PLFA} \times 10^{17:0} \] (Kroppenstedt, 1985). The total amount of PLFAs was used as an indicator of the quantity of microbial biomass.

2.6. PFLA analysis

The extraction of phospholipid fatty acids (PLFA) was performed according to a modified procedure of Frostegård et al. (1993) (Johansen and Olson, 2005; Johansen et al., 2003). The main task of the microbial community was identified in samples of 5 g DW of soil: Gram-positive bacteria (PLFAs 11:0, 15:0, 11:0, 11:0, 17:0) (O’Leary and Wilkinson, 1988), Gram-negative bacteria (PLFAs 18:1ω7c, 17:0, cy19:0) (Wilkinson, 1988), fungi (PLFA 18:2ω6:0) (Federle, 1986) and actinomycetes (PLFAs 10me17:0, 10me18:0) (Kroppenstedt, 1985). The total amount of PLFAs was used as an indicator of the quantity of microbial biomass.

2.7. α-Cypermethrin analysis

Twenty representative soil sub-samples (3 g) were used to determine the concentration of α-CYP in the soil after eight weeks in both communities. Soil sub-samples were freeze-dried and extracted by sonication for 16 h with a mixture of dichloromethane and acetone as solvent (50:50, v/v). Before extraction, the samples were spiked with 13C–cis-permethrin. The solvent phase was separated by centrifugation; the extract was evaporated to nearly dryness and reconstituted in isooctane. An Agilent 7890 GC (Agilent, CA, USA) coupled to an Agilent 5975C mass spectrometer was used for the analysis. The sample was injected in splitless mode and the analyses were separated on an HP-5MS column (30 m, 0.25 mm i.d., 0.5 μm film thickness). The GC–MS was operated in selected ion monitoring (SIM) with Negative Chemical Ionization (NCI). Quantification of α-CYP in samples was performed by external calibration.

2.8. Statistical analysis

The effect of each experimental main factor, i.e. insecticide community and time and their interactions on earthworms, Collembola, mites and enchytraeids were determined using a mixed model specified with PROC MIXED (SAS Institute Inc., 2011). The blocks were included as random factors in the model while insecticide treatment, community and time effect were all considered fixed factors. Data were tested for normal distribution and homogeneity of variance and log10 (x + 1) transformed if necessary. Data are presented as untransformed means in graphs and tables.

In order to analyse dose–response relationships, EC10 and EC50 were determined for the population abundance after four and eight weeks for both communities using the linear regression function:

\[
\text{Abundance} = a \cdot \text{conc} + c, \quad \text{EC10} = (c + 0.5 - c) / a, \quad \text{EC50} = (c + 0.5 - c) / a
\]

In one case, an exponential decay function was applied:

\[
\text{Abundance} = c \cdot \exp[\text{conc}], \quad \text{EC10} = -\log_{10}(0.9) / a, \quad \text{EC50} = -\log_{10}(0.5) / a
\]

Where a is the slope or decay rate and c is the control abundance level. We used a significant decline with increasing concentration as revealed by linear regression of log-transformed abundances of a species to trigger the calculation of EC values. The EC10 and EC50 were point estimates using the estimate function of SAS/PROC NLMIXED (SAS Institute Inc., 2011).

Statistical analysis of quantitative PLFA (nmol g−1) data was performed by using SYSTAT software v. 9.0, employing a GLM procedure to test for main effects and interactions, and Bonferroni post-test to compare effect of individual insecticide concentrations. Principal component analysis was performed on the data sets from the PLFA analysis (log10 transformed molar % data) using the Unscrambler software v. 7.6 (CAMO ASA, NO). The resulting values from each individual principal component were subject to statistical treatments as described above.

The relationship between the community with two levels, and the nominal concentration was introduced into this model, to asses if the concentration of α-CYP was influenced by the community factor. The x is the nominal concentration of α-CYP and the i and j subscripts indicate the treatment levels of the continuous α-CYP factor, acting as a regressor, and the categorical community factor, respectively. The concentration mean were obtained by invoking the PREDOM option of the PROC MIXED procedure (SAS Institute Inc., 2011).

3. Results

3.1. α-cypermethrin fate

Measured soil concentrations of α-CYP revealed degradation to a minimum of 19% and a maximum of 74% of the nominal concentration. The largest reduction in the concentration was observed in COM-C at an initial concentration of 5 mg kg−1 and the lowest level of degradation was noticed at 25 mg kg−1 in COM-F. Modelling the relationship between the initial concentration as approximated by the nominal concentration and the measured concentration after eight weeks established the significant influence of the type of community on the fate of α-CYP (P < 0.05) where the soil with enchytraeids had half the content of α-CYP compared with the earthworm community (Table 2 and Fig. 1).
3.2. α-cypermethrin effect

The effects of α-CYP on the population abundance varied according to species and insecticide concentrations (Fig. 2). The EC10 and EC50 were estimated only where soil organisms showed a significant effect of α-CYP. The EC10s and EC50s for microarthropods ranged between 2 and 19 mg kg⁻¹ with the exception of P. minuta in COM-F and H. aculeifer in COM-C where both EC10 and EC50 were about five times lower than in the alternate community. E. crypticus was more sensitive than E. fetida, i.e., after eight weeks the EC10 was 0.12 mg kg⁻¹ for E. crypticus and 11.5 mg kg⁻¹ for E. fetida. In the same way, after eight weeks the estimated EC50 was 0.76 mg kg⁻¹ for E. crypticus and >25 mg kg⁻¹ for E. fetida (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Nominal conc. mg kg⁻¹</th>
<th>Community</th>
<th>Mean conc. 8 week mg kg⁻¹</th>
<th>95% C.L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>COM-F</td>
<td>0.5</td>
<td>[0.5–1.4]</td>
</tr>
<tr>
<td>5.0</td>
<td>COM-F</td>
<td>2.9</td>
<td>[2.0–3.7]</td>
</tr>
<tr>
<td>25</td>
<td>COM-F</td>
<td>18.5</td>
<td>[17–20]</td>
</tr>
<tr>
<td>1.0</td>
<td>COM-C</td>
<td>0.1</td>
<td>[0.8–1.1]</td>
</tr>
<tr>
<td>5.0</td>
<td>COM-C</td>
<td>1.1</td>
<td>[0.3–2.0]</td>
</tr>
<tr>
<td>25</td>
<td>COM-C</td>
<td>9.7</td>
<td>[8.6–11]</td>
</tr>
</tbody>
</table>

3.3. Microarthropods

Most of the microarthropods were sensitive to the insecticide. F. fimetaria was the most abundant species and its abundance was negatively affected by α-CYP (F-test, p < 0.05). Effects of α-CYP were highly significant for P. fimata (F-test, p < 0.01), a significant decrease of P. fimata abundance with the increasing of insecticide concentration. The M. macrochaeta population decreased with time (F-test, p < 0.01) and α-CYP (F-test, p < 0.05). The predatory mite H. aculeifer was significantly affected by the insecticide (p < 0.001) while the insecticide had no significant effect on H. nitidus.

The organisms showed to be sensitive also to the community effect. A significant difference was detected between the two communities (p < 0.01) H. nitidus with the highest abundance in COM-F (Fig. 2). After eight weeks the population abundance was higher in the COM-F and lower in the COM-C (p < 0.05). In COM-C we observed also a significant decrease in the abundance from week four to week eight (p < 0.05). The F. fimetaria abundance was significantly higher (p < 0.05) in COM-F where the population increased significantly (p < 0.01) with time. After eight weeks a significant difference was detected between 5 and 25 mg kg⁻¹ (p < 0.05).

Significant effects of communities (COM), insecticide and time were detected also for P. minuta. After eight weeks a larger abundance was detected in COM-F compared to COM-C (p < 0.01). In the COM-F a significant increase in abundance were observed between 0 and 1 mg kg⁻¹ (F-test, p < 0.05). P. fimata has a significantly larger abundance in COM-F after eight weeks (p < 0.05) compare to COM-C, while the M. macrochaeta population did not differ between the communities. The predator mite H. aculeifer was instead significantly more abundant in the COM-C. The effect of the factors Time, COM and α-CYP on the species abundance is summarized in Table 3.

3.4. Oligochaeta

E. crypticus collapsed when exposed to 25 mg kg⁻¹ as no individuals were found in this treatment (Fig. 3). The abundance was negatively affected by the insecticide (p < 0.001). After eight weeks the population was reduced by 66% and by 94% at an exposure of 1 and 5 mg kg⁻¹, respectively, compared to the control (Fig. 3). No mortality of earthworms was observed in any of the test containers. The biomass of E. fetida increased with time at all α-CYP concentrations (Fig. 3). A negative effect at 5 and 25 mg kg⁻¹ was observed (p < 0.05) (Table 3). After eight weeks, the biomass was reduced by 16% at 25 mg α-CYP kg⁻¹.

The soil carbon content in COM-C was 1.4% [1.2–1.7] and 1.6% [1.4–1.9] in COM-F at week 4 which did not differ significantly. At 8 weeks there was a significantly higher carbon content in COM-F than in COM-C (Tukey’s test, P < 0.01), resulting in 1.4% [1.2–1.7] in COM-C and 2.4% [2.1–2.6] in COM-F.

3.5. Soil microbial community

At week 4, the total PLFA content in soil was lower (varying from 10 to 100%) in the COM-C compared to COM-F (Fig. 4b and a, respectively; main effect p = 0.03) tending to decrease (not significantly) with increasing insecticide concentration, while at week 8 it was similar in the two communities and unaffected by the insecticide concentration. This pattern was also observed with main taxonomic groups in general (fungi, Gram-negatives, Gram-positives, actinomycetes), however, significant only for Gram-negative bacteria (Fig. 4e and f, main effect p = 0.001; indicated with an asterisk). The effect of the insecticide was also most pronounced with Gram-negative bacteria in the COM-C (main effect p = 0.002). Fungal PLFA differed somewhat by increasing with insecticide concentration at week 4, although only in COM-C (Fig. 4d; interaction effect p = 0.013). Relative changes (control = 100%) in the soil PLFA concentrations are presented in Fig. suppl. 1 a–f.

Principal component analysis of the PLFA profiles revealed that the 4-week samples from the COM-C at 25 mg kg⁻¹ α-CYP were indeed very different from any of the other treatments, separating them from the other samples along the PC1 axis which explained...
Fig. 2. Mean abundance ± S.E. per mesocosm unit of Hypoaspis aculeifer, Heteromurus nitidus, Folsomia fimetaria, Proisotoma minuta, Protaphorura fimata and Mesaphorura macrochaeta after 4 and 8 weeks in the two different communities COM-C: Enchytraeus crypticus and COM-F: Eisenia fetida spiked with α-cypermethrin (concentrations 0 mg kg⁻¹, 1 mg kg⁻¹, 5 mg kg⁻¹ 25 mg kg⁻¹). Different lower case letters above groups of bars indicate significant differences between 4 and 8 weeks, (P < 0.05). Different capital letters above groups of bars indicate significant differences between communities (COM-F and COM-C), (P < 0.05) (Tukey–Kramer, Adj P < 0.05).
98% of the variation (Fig. suppl. 2). These samples were omitted in an additional PCA to better reveal the relationship between the remaining samples in the score plot (Fig. 5a). In general, the variation was quite high and, accordingly, the separation between α-CYP treatments and communities not very good. However, a COM-F cluster along PC1 (explaining 59% of variation; main effect of community \(p = 0.02\)), except the 4-week samples at 25 mg kg\(^{-1}\) α-CYP which ordinated to the left. The samples from the COM-C were more scattered in the score plot. There were no systematic trends in relation to insecticide treatment (no significant main effect of insecticide concentration). The time of incubation affected the ordination (main effect \(p = 0.002\)) as samples from week 4 and 8 were mostly located to the left and right, respectively. The loading plot (Fig. 5b) showed that fatty acids like 15:0, a15:0, 15:0, i16:0 and a16:0 (often found in Gram-positive bacteria) dominated the right side of the plot, while methylated fatty acids and 18:2\(u_6,9\) (indicative of actinomycetes and fungi, respectively) were found in the left side of the plot.

### Table 3

The effect on individual species abundances in the mesocosms by the factors (Time, COM and α-CYP) and interactions of those. COM: indicates community effect, COM-F vs. COM-C effect; Time: indicates different effects after 4 and 8 weeks; α-CYP: Pesticide effect (Conc. 0, 1, 5, 25 mg kg\(^{-1}\)). Significance is indicated by:*: \(P < 0.05\), **: \(P < 0.01\). ↑ and ↓ indicate positive (↑) or negative effects (↓) driven by the factors on the population abundance.

<table>
<thead>
<tr>
<th>Species</th>
<th>Time</th>
<th>COM</th>
<th>α-CYP</th>
<th>Time*Conc.</th>
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<tr>
<td>Hypoaspis aculeifer</td>
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<td>Mesaphorura macrochaeta</td>
<td>**↑</td>
<td></td>
<td></td>
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<tr>
<td>Eisenia fetida</td>
<td>*↑</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Enchytraeus crypticus</td>
<td>*↑</td>
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</tbody>
</table>

**Fig. 3.** (a) Fresh body weight in g of the earthworm *Eisenia fetida* (mean ± S.E) in the mesocosm after α-cypermethrin exposure (concentration 0 mg kg\(^{-1}\) (control), 1 mg kg\(^{-1}\), 5 mg kg\(^{-1}\) and 25 mg kg\(^{-1}\)) at week 4 and 8. Different capital letters above groups of bars indicate significant differences between week 4 and 8 (\(P < 0.05\)). (b) Number of the enchytraeids *Enchytraeus crypticus* (mean ± S.E) in the mesocosm after α-cypermethrin exposure at week 4 and 8. Different letters above the bars indicate significant pesticide effect (Tukey–Kramer, Adj \(P < 0.05\)). *E. crypticus* was absent at 25 mg α-cypermethrin kg\(^{-1}\) soil.
4. Discussion

4.1. a-Cypermethrin fate

The soil concentration of a-CYP at 8 weeks reveals a degradation of the insecticide, probably by the soil microbial community, but with no apparent impact on the microbial community composition as indicated by the results of the PLFA measurements. This suggests that the observed differential degradation pattern in the soil is caused by the contrasting behaviour of the two oligochaete species as the degradation was significantly increased in the COM-C. The observed biodegradation was quantitatively highest at 25 mg kg\(^{-1}\) in COM-C, maybe indicating a higher bioactivity in this treatment, where *E. crypticus* was eradicated within 4 weeks. The lowest level of degradation was observed in the COM-F at all pesticide levels and was similar to degradation levels previously observed for a range of single species in a sandy loam (Hartnik et al., 2008a). Due to the epigeic feeding habits of *E. fetida*, it was feeding selectively on the added manure (not contaminated), increasing the organic matter present within the soil as also confirmed by the higher content of soil organic carbon found in COM-F. Hence, the higher level of organic carbon of 1% originating from *E. fetida* casts has reduced the bioavailability of the a-CYP compared to COM-C, as a-CYP has a soil organic carbon/water partitioning coefficient, log \(K_{OC}\), of 6.14 which indicates a high affinity for binding to organic matter (Hartnik et al., 2008b).

4.2. a-cypermethrin effects

a-CYP had a general negative effect on the soil fauna significantly decreasing the abundance of collembolans, mites and enchytraeids and the fresh body weight of *E. fetida*. The two different communities responded in a different manner to the insecticide. We observed a positive effect due to the presence of the earthworms in the COM-F where generally a larger abundance of collembolan populations was detected. Oppositely, the predator mite was more abundant in the COM-C. Enchytraeids were much more sensitive to a-CYP compared to earthworms at all concentration levels.

![Fig. 4. The effect of the community treatments and a-cypermethrin observed for indicators of the microbial biomass.](image)

![Fig. 5. Overview of treatment effects in PCA (a) score plot of all samples based on microbial membrane fatty acids (PLFA) variables. The time of incubation and a-cypermethrin concentration are indicated inside the symbols (first and second digit, respectively). The PLFA variables are shown in the loading plot (b).](image)

**Fig. 4.** The effect of the community treatments and a-cypermethrin observed for indicators of the microbial biomass. The soil content of membrane fatty acids, PLFA, nmol g\(^{-1}\) dry soil, was harvested after 4 and 8 weeks. The PLFA originated from the total microbial community, and fungal and Gram-negative bacterial biomass in the two communities with *Eisenia fetida* (COM-F) or *Enchytraeus crypticus* (COM-C) spiked with a-cypermethrin. Bars indicate S.E. (n = 2–4).

**Fig. 5.** Overview of treatment effects in PCA (a) score plot of all samples based on microbial membrane fatty acids (PLFA) variables. The time of incubation and a-cypermethrin concentration are indicated inside the symbols (first and second digit, respectively). The PLFA variables are shown in the loading plot (b).
Table 4
EC50s and the EC10s, mg kg\(^{-1}\), of tested species after week 4 and 8. EC50s and EC10s have been estimated only for significant declines of species abundances with \(\alpha\)-cypermethrin concentration and similarly for the fresh body weight of Eisenia fetida. N.E., the EC was not estimated as no effects were detected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Time</th>
<th>Com</th>
<th>EC10 mg kg(^{-1})</th>
<th>95% CI</th>
<th>EC50 mg kg(^{-1})</th>
<th>95% CI</th>
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</thead>
<tbody>
<tr>
<td>Hypoaspis australis</td>
<td>Week 4</td>
<td>COM-C</td>
<td>2.7</td>
<td>1.2–4.2</td>
<td>13.4</td>
<td>5.8–21.1</td>
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<td></td>
<td>Week 8</td>
<td>COM-C</td>
<td>0.5</td>
<td>0.3–0.7</td>
<td>3.2</td>
<td>1.7–4.7</td>
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<td></td>
<td>Week 8</td>
<td>COM-F</td>
<td>2.8</td>
<td>0.1–5.5</td>
<td>14.1</td>
<td>6.0–27.6</td>
</tr>
<tr>
<td>Heteromurus nitidus</td>
<td></td>
<td>N.E.</td>
<td></td>
<td></td>
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<tr>
<td>Folosomia fimetaria</td>
<td>Week 4</td>
<td>COM-C</td>
<td>2.8</td>
<td>1.0–4.7</td>
<td>14.2</td>
<td>4.9–23.6</td>
</tr>
<tr>
<td></td>
<td>Week 8</td>
<td>COM-C</td>
<td>3.8</td>
<td>0.6–6.9</td>
<td>18.9</td>
<td>3.0–34.7</td>
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<td>Week 8</td>
<td>COM-F</td>
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<td>2.1–3.3</td>
<td>13.6</td>
<td>10.5–16.7</td>
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<td>Protarcheta minuta</td>
<td>Week 8</td>
<td>COM-C</td>
<td>2.9</td>
<td>2.1–7.9</td>
<td>14.4</td>
<td>10.5–39</td>
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<td>Week 8</td>
<td>COM-F</td>
<td>0.4</td>
<td>0.1–0.7</td>
<td>2.7</td>
<td>0.8–4.6</td>
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<tr>
<td>Protarcheta fimata</td>
<td>Week 4</td>
<td>COM-C</td>
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<td>0.4–6.8</td>
<td>15.9</td>
<td>2.1–33.9</td>
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<td>Week 4</td>
<td>COM-F</td>
<td>2.9</td>
<td>0.2–5.6</td>
<td>14.7</td>
<td>1.1–28.2</td>
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<tr>
<td>Mesaphorura macrochaeta</td>
<td>Week 4</td>
<td>COM-C</td>
<td>3.1</td>
<td>0.2–6.0</td>
<td>15.5</td>
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<td>Week 4</td>
<td>COM-F</td>
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<td>0.7–4.6</td>
<td>13.4</td>
<td>3.6–23</td>
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<td>Week 8</td>
<td>COM-F</td>
<td>2.2</td>
<td>0.1–4.4</td>
<td>10.8</td>
<td>0.5–22.0</td>
</tr>
<tr>
<td>Eisenia fetida</td>
<td>Week 4</td>
<td>COM-F</td>
<td>4.8</td>
<td>1.6–7.9</td>
<td>23.8</td>
<td>8.2–39.5</td>
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<td>Week 8</td>
<td>COM-F</td>
<td>11.5</td>
<td>2.0–21.0</td>
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<tr>
<td>Enchytraeus crypticus</td>
<td>Week 4</td>
<td>COM-C</td>
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<td>0.05–0.43</td>
<td>1.26</td>
<td>0.34–2.9</td>
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<td>Week 8</td>
<td>COM-C</td>
<td>0.12</td>
<td>0.03–0.20</td>
<td>0.76</td>
<td>0.19–1.32</td>
</tr>
</tbody>
</table>

4.3. Collembola

H. nitidus and P. minuta were less affected by \(\alpha\)-CYP probably due to their epi- and hemiedaphic behaviour. They are living on and close to the soil surface, respectively, and they may reside and feed on the uncontaminated food added weekly on the top of the mesocosm soil, so this would reduce their exposure to the insecticide. H. nitidus attained a high abundance likely to be caused by favourable feeding conditions. Contrary to our expectations, P. minuta was characterized by a low density. Larsen et al. (2009) found that P. minuta has a high metabolic rate and high fecundity at least compared with P. fimata. The same study indicated that P. minuta may have higher nutritional requirements for reproduction as the N:P ratio of its eggs is significantly higher than for P. fimata (Larsen et al., 2009). Thus, we suggest that the food quality may not be optimal for P. minuta, but we cannot exclude other influences such as competition.

F. fimetaria was the most abundant species probably due to its preference for organic matter, here in the form of manure that favours its proliferation (Krogh et al., 1997). It was negatively affected because of competition or just due to suboptimal conditions for the relatively high density. Larsen et al. (2009) found a high metabolic rate and high fecundity at least compared with P. fimata. The same study indicated that P. minuta may have higher nutritional requirements for reproduction as the N:P ratio of its eggs is significantly higher than for P. fimata (Larsen et al., 2009). Thus, we suggest that the food quality may not be optimal for P. minuta, but we cannot exclude other influences such as competition.

4.4. Predator mite

The predatory mite H. aculeifer was affected by \(\alpha\)-CYP, however this effect could be due to both direct exposure to the insecticide or/and to the reduction of prey availability. As its running velocity may be slowed down by \(\alpha\)-CYP as demonstrated for spiders, this may decrease its capture efficiency of prey collembolans (Baatrup and Bayley, 1993; Baatrup et al., 2006).

The population of mites decreased by the increasing insecticide concentration but did also show a higher abundance in the COM-C compared with the abundance in COM-F. H. aculeifer is known to be a generalist predator preying on both collembolans and enchytraeids (Heckmann et al., 2007) and the presence of E. crypticus in COM-C could explain the higher survival of H. aculeifer due to more food being available.

4.5. Oligochaeta

The two oligochaetes showed a significantly different sensitivity to the insecticide, as the population of E. crypticus was reduced by 94% when exposed to 5 mg kg\(^{-1}\) and collapsed when exposed to 25 mg kg\(^{-1}\) as opposed to E. fetida where the effects observed at the same test concentrations were less evident (Fig. 2). This is likely caused by the different body size of the two oligochaetes.

For the enchytraeids, predation by H. aculeifer could have been an important factor. Studies have shown that direct toxic effects of the toxicants and the presence of predators together can enhance (additively or synergistically) the total negative effects on prey populations (Salminen et al., 2002; Schoener, 1983; Shi et al., 1985). Thus, the E. crypticus population was exposed to double stress (toxicity and predation) that might result in an enhanced response to the insecticide.

Compared to Hartnik et al. (2008a), the enchytraeids were markedly more sensitive in the SMS test system than in a single species test. Hartnik et al. (2008a) reported a 4 week EC10 estimate of 0.99 [0.34–1.60] mg kg\(^{-1}\) and an EC50 of 4.91 [3.7–6.5] mg kg\(^{-1}\) for E. crypticus when exposed in a single species test, whereas the
EC10 and EC50 observed in the current SMS study after the same period, were 0.19 [−0.05−0.43] and 1.26 [−0.34−2.9] mg kg⁻¹, respectively. The effect on the earthworm in terms of the EC in the present study was almost similar to those revealed in the single species test by Hartnik et al. (2008a) (EC10 1.57 and EC50 31.0 mg kg⁻¹).

4.6. Microbial community

α-CYP had only limited impact on total microbial biomass and as this effect was observed only with the enchytraeid community it seems likely that the effect was indirect through disturbance of the faunal community and its activities (e.g. grazing behaviour). In support of this, Zhuang et al. (2011) found no effects of α-CYP on a range of microbial variables like enzyme activities and the general metabolic activity, even at insecticide concentrations three times higher than in the present study. Xie et al. (2009) observed that α-CYP at 10 mg kg⁻¹ had a negative impact on functional diversity of the soil microbial community, but only when applied in combination with heavy metals. Microbial communities on plant surfaces may have increased biomass and change their structure after exposure to α-CYP as shown by PLFA and PCR-DGGE analysis (Zhang et al., 2009); maybe due to the utilization of the insecticide as a substrate. The COM-C microbial community composition was also altered at the highest insecticide concentration in the present study to an extent which governed the outcome of the PCA analysis completely (98% of explained variation); mostly due to a decrease in Gram-negative bacteria. The collapse of the enchytraeid population in COM-C might have an impact on the microbial community, e.g. indirectly through changes in their manipulation/degradation of organic matter or directly due to grazing on bacterial and fungal populations. Waldrop et al. (2012) observed (in situ forest soils) that the number of bacteria decreased with increasing densities of Enchytraeids, indicating a predator-prey relationship, while at low enchytraeid densities the bacterial population also decreases, indicating that a certain level of faunal activity may stimulate bacterial proliferation. With collembolans, Larsen et al. (2008) demonstrated a species dependent and differential utilization of hyphae from a range of fungal species. This may support that the variations in fungal biomass may be caused by changes in collembolan populations, e.g. P. minuta which has been shown to feed on fungal material by Castaño-Meneses et al. (2004). Although the present setup represented a simplified biological system, in comparison to natural soils, the interactions between this reduced number of species were still numerous and complex. This was also apparent in the PCA plot where the week-4 samples from COM-C and COM-F were separating along the PC1 axis and where the level of pesticide contributed to this distribution (especially COM-C 25 mg kg⁻¹ soil (Fig. suppl. 2). Hence, the type of community had great influence on the microbial community composition in the first part of the incubation period. After 8 weeks, the community composition was apparently not different as the data points from both communities grouped up together at the right side of the PCA score plot, regardless of initial pesticide concentration. Although still present in substantial amounts, the pesticide exposure to animals and microorganism may have decreased due to adsorption to soil particles, thus diminishing the effect of the pesticide concentration at week 8. The distribution pattern of the PLFA variables in the PCA loading plot indicated that Gram positive bacteria were numerous late in the experiment as fatty acids typical for this group of bacteria were dominating the right side of the plot — determining the grouping of all treatments at week 8. Overall, the distribution of scores indicates that the treatments had a differential impact on the microbial community composition early in the experiment, but developed into similar communities with time. This is often observed after perturbation of microbial communities; e.g. with heavy metals (Ekelund et al., 2003) or microbial inoculants (Johansen and Olsson, 2005).

4.7. Community level effects

There was a clear difference in the effect of the insecticide on the species composition when comparing the two communities. The presence of E. fetida had a positive effect on the majority of the species, reducing the negative effect of the insecticide, compared with the number of individuals in the COM-C at the same concentration. Our results showed that earthworms did not significantly affect the amount of fungal PLFA, but they enhanced the content of soil carbon in COM-F and the bioavailability of α-CYP (see 4.1). In addition, positive effects of earthworms could have been caused by their biological activity that improved the nutrient availability for the mesofauna by providing and processing the organic matter (Monroy et al., 2011). Salmon and Ponge (2001) have also shown that collembolans may feed on earthworm urine and mucus. These epidermal excreta contain nitrogen molecules and mucus contains carbohydrates easy to assimilate for collembolans.

Studies performed by Cortet et al. (2008) and Hartnik et al. (2008a) revealed that toxicity can change depending on whether it is tested in single species — or a multispecies test system. Our results are confirmative in this respect by showing that employing enchytraeids instead of earthworms increased the sensitivity of the ecotoxicological responses. It is also clear that different species have different sensitivity to the insecticide but also different combinations of species and their interaction can dramatically change the level of toxicity. E. fetida excreta, physical disturbance and bioturbation of the soil, expectedly, resulted in mixing of the organic matter, i.e. the added earthworm food consisting of cattle manure, into the soil. So feeding substrate were available to maintain a microbial community only changing marginally due to α-CYP and during the course of the experiment – in contrast to the community with enchytraeids where we observed a decrease in bacterial PLFA and a simultaneous increase in fungal PLFA. This change in the microbial composition could have been influenced by the collapse of the enchytraeids population at the high α-CYP concentration. The enchytraeids, preyed upon by H. aculeifer, became less abundant with increasing insecticide concentration, so the mite may have shifted its predation to collembolans, which are less abundant than in the COM-F. The interaction between H. aculeifer and the collembolans could result both in alleviation of grazing on microorganisms but also increased availability of nutrients mineralized through the turnover of the faunal prey components. A summary diagram showing the biotic interactions driving the changes in the mesocosm community is presented in Fig. 6.

5. Conclusion

The two different oligochaetes gave rise to dramatically different community responses to the insecticide as well as creating different conditions resulting in different degradation dynamics of α-cypermethrin. Hence, addition of manure for feeding of E. fetida is crucial for our understanding of the resulting soil conditions and fate and effects. Our interpretation of the observed changes in the community structure of soil microorganisms and soil fauna illustrates the strength of a multi-species test system as an ecotoxicological tool compared to single species tests when it comes to bridging the effects observed in the laboratory to field conditions. Based on a simple toxicity criterion of “the more sensitive the better” we would recommend selection of E. crypticus over E. fetida.
However, as both oligochaete species led to a successful outcome with no microarthropods going extinct, E. fetida could be selected if soil ecological functions such as bioturbation is a relevant endpoint. The SMS test system could reveal both direct and indirect effects and the effect were more evident after eight weeks. The system was capable of detecting population dynamics and species interactions not present in single species tests, and included interactions between faunal and microbial communities. Although our conclusions were drawn from observations with a pyrethroid insecticide, the results have important implications for future risk assessment of chemicals, and species interaction should be taken into consideration when developing ecologically relevant methodology for assessment of ecotoxicity.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.10.008.

References


Jänsch, S., Frampton, G.K., Römbke, J., Van den Brink, P.J., Scott-Fordsmand, J.J., 2006. Effects of insecticides on soil invertebrates in model ecosystem and field Fig. 6. Schematic overview of the mesocosm biotic elements and their interactions depicted as ecosystem function arrows including microbial grazing and predation, all potentially affected by the stressor. Underlying the biotic elements is the soil matrix of chemical and physical properties depicted as an oval, in particular under the influence of Eisenia fetida functioning such as organic matter (OM) consumption and bioturbation.

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studies: a review and comparison with laboratory toxicity data. Environ. Toxicol. Chem. 25, 2490–2501.