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Responses of earthworms to repeated exposure to three biocides applied singly and as a mixture in an agricultural field

Lisbeth Schnug [a,*], Torbjørn Ergon [b], Lena Jakob [a,1], Janeck J. Scott-Fordsmand [c], Erik J. Joner [a], Hans Petter Leinaas [d]

[a] Bioforsk – Norwegian Institute for Agricultural and Environmental Research, Frederik A. Dahls Vei 20, 1430 Ås, Norway
[b] University of Oslo, Centre for Ecological and Evolutionary Synthesis, Blindernveien 31, 0316 Oslo, Norway
[c] University of Aarhus, Department of Bioscience – Soil Fauna Ecology and Ecotoxicology, Vejlsøvej 25, 8600 Silkeborg, Denmark
[d] University of Oslo, Department of Biosciences, Blindernveien 31, 0316 Oslo, Norway

HIGHLIGHTS

- Earthworm abundance and juvenile proportions were negatively affected by the biocides.
- Species and chemical-specific differences in toxic responses were observed.
- Indication for higher sensitivity of field worms than *E. fetida*.
- Species-specific mixture toxicity patterns were observed.

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ABSTRACT

The study aimed at investigating effects of three differently acting biocides; the insecticide esfenvalerate, the fungicide picoxystrobin and the bactericide triclosan, applied individually and as a mixture, on an earthworm community in the field. A concentration–response design was chosen and results were analyzed using univariate and multivariate approaches. Effects on juvenile proportions were less pronounced and more variable than effects on abundance, but effects in general were species- and chemical-specific, and temporal variations distinct. Esfenvalerate and picoxystrobin appeared to elicit stronger effects than triclosan at laboratory-based ECx values, which is in accordance with our previous laboratory study on *Eisenia fetida*. The mixture affected abundance and juvenile proportions, but the latter only at high mixture concentrations. Esfenvalerate and picoxystrobin appeared to be the main drivers for the mixture's toxicity. Species-specific toxicity patterns question the reliability of mixture toxicity predictions derived on *E. fetida* for field earthworms. Biocide concentrations equaling EC50s (reproduction) for *E. fetida* provoked effects on the field earthworms mainly exceeding 50%, indicating effect intensification from the laboratory to field as well as the influence of indirect effects produced by species interactions. The differing results of the present field study and the previous laboratory study that imply that lower- and higher-tier studies may not be mutually exclusive, but to be used in complementary.

1. Introduction

The main group of biocides reaching arable soils is pesticides which are commonly applied in combination on a single crop in order to combat several pests simultaneously (Zhou et al., 2011). In addition, industrial biocides may reach the soil through soil amendments such as biosolids from wastewater treatment plants (Pannu et al., 2012). Consequently, a large variety of biocides enters the soil, and soil organisms are commonly exposed to chemical mixtures. Prior to market release, ecotoxicological tests are required in order to assess the risk biocides pose to the environment. Earthworms are standard test organisms for the testing of biocides due to their key role in soil fertility as well as their importance in the terrestrial food web (EPPO, 2003; Lee et al., 2008). Having no protective cuticle, and ingesting soil regularly, earthworms are more vulnerable to biocides in soil than other soil invertebrates (Jager et al., 2003; Zhou et al., 2011). Several guidelines deal with the risk assessment of biocides and their effects on earthworms, both in the laboratory and in the field (EPPO, 2003; ISO, 1999; Lee et al., 2008; OECD, 2004). Biocides are commonly tested in a

* Corresponding author. Tel.: +47 45861750.
E-mail addresses: Lisbeth.Schnug@bioforsk.no (L. Schnug), t.h.ergon@ibv.uio.no (T. Ergon), Lena.Jakob@awi.de (L. Jakob), jsf@dmu.dk (J.J. Scott-Fordsmand), Erik.Joner@bioforsk.no (E.J. Joner), h.p.leinaas@ibv.uio.no (H.P. Leinaas).
[1] Present address: Alfred Wegener Institute, Am Handelshafen 12, 27570 Bremerhaven, Germany.

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stepwise (tiered) approach, from simple laboratory tests (lower-tier) to field or semi-field tests (higher-tier) (Jensen and Mesman, 2006). For high-risk biocides, field studies are mandatory (EPPO, 2003; Pannu et al., 2012). Yet, the first evaluation of the ecotoxicity of a biocide is derived from single-species laboratory tests, mainly using *Eisenia fetida* (Savigny) as a standard test species (Jänsch et al., 2006). From these tests, effect concentrations are derived, commonly the median effective concentration (EC50), which is a statistically robust toxicity parameter (Warne and van Dam, 2008). However, extrapolating such results to natural earthworm communities bears a considerable degree of uncertainty (Baveco and De Roos, 1996). Laboratory tests indicate toxicity for individual endpoints, e.g. cocoon production, while effects inflicted on field populations will reflect a combination of both, direct and indirect effects (Baveco and De Roos, 1996; van Gestel, 1992). Earthworms in natural habitats also have greater possibilities to choose microsites providing favorable conditions, and escape from unwanted conditions, which may influence exposure (Holmstrup, 2000; Schaefer, 2003). Field studies account for these factors, and may give a better understanding of the true impact of a biocide on natural earthworm communities if combined with lower-tier studies. However, field studies are costly and often only conducted for high-risk biocides (Jensen and Mesman, 2006; van Gestel, 1992), which in turn reduces the opportunities to validate lower-tier test predictions with field data. In order to account for the uncertainty in extrapolation among species, from acute to chronic effects and from ideal laboratory conditions to realistic field conditions, arbitrary assessment factors are applied to lower-tier effect concentrations (Jänsch et al., 2006). The significance of laboratory-based risk assessments to natural ecosystems has been investigated, comparing laboratory- and field-derived toxicity of different biocides on earthworms and other soil organisms (e.g. Heimbach, 1998; Holmstrup, 2000; Jänsch et al., 2006; van Gestel, 1992), but data are still sparse. While mixture effects of biocides on soil-organisms have been studied in laboratory multi-species experiments (Santos et al., 2010, 2011), to our knowledge no studies have yet compared laboratory and field data of biocide mixtures. Further, higher-tier toxicity data are dominated by older biocides, such as organophosphorous and organochlorine insecticides, while less data is available on synthetic pyrethroid insecticides and strobilurin fungicides, widely used in Europe. Field studies are also mainly conducted with only few biocide concentrations, commonly based on the recommended application rate (Jänsch et al., 2006). These low concentrations may be more realistic, but effects may also remain undetectable due to increasing variation in the data with decreasing concentrations. Hence, field studies are often difficult to interpret. Increasing the number of concentration levels may be more beneficial than having replicates, since this enables the use of regression analysis such as generalized linear models, a powerful tool for detecting toxic effects (Warne and van Dam, 2008). The usefulness of field studies for investigating the potential risk of biocides may consequently be higher if the study follows a concentration–response design (Römbke et al., 2009).

In the present study, a concentration-response based field experiment was conducted, using three differently acting biocides, individually and as a mixture: Esfenvalerate is a pyrethroid insecticide and acts as a sodium channel antagonist disrupting the nervous system of arthropods (Tomlin, 1994) and possibly also Lumbricidae due to similarities in the nervous system (Scholtz, 2002). The strobilurin fungicide picoxystrobin disrupts respiration in fungi and probably also in soil invertebrates by inhibiting the complex III cytochrome bc1 at the Qo site (Krieger, 2001). Consequently, picoxystrobin may affect earthworms both directly and indirectly by reducing soil fungi and hence food (Bonkowski et al., 2000). Triclosan is a bactericide used in many consumer products and reaches the soil mainly through amendments with sewage sludge from waste water treatment plants (Amorim et al., 2010). Triclosan inhibits fatty acid synthesis in bacteria and may alter gene transcription (Jang et al., 2008). It has also been shown that triclosan may be genotoxic to *E. fetida* (Lin et al., 2010). All three biocides are likely to be found in arable soils due to spraying with plant protection products and application of sewage sludge, and appear to be toxic to earthworms (esfenvalerate: e.g. EFSA, 2013; Schnug et al., 2013; Schnug et al., 2014a; picoxystrobin: e.g. Ctgb, 2005; Schnug et al.. 2013; Schnug et al., 2014a; Wang et al., 2012; triclosan: e.g. Amorim et al., 2010; Lin et al., 2010; Pannu et al., 2012; Schnug et al., 2013; Schnug et al., 2014a; mixture: Schnug et al., 2013).

The aims of the present study were (1) to measure responses of an earthworm community in the field under exposure to different biocides, including effects observed after two consecutive biocide applications, and (2) to evaluate the significance of laboratory-derived effect concentrations for higher-tier earthworm community parameters. The study aimed further at (3) investigating whether responses upon mixture exposure divert from the individual biocide exposures. The aims were addressed by measuring earthworm abundance and juvenile proportions at concentrations known to inhibit cocoon production in *E. fetida* (hereafter referred to as *Eisenia*-ECx, e.g. *Eisenia*-EC50 is the concentration provoking 50% inhibition of cocoon production in *E. fetida*) (Schnug et al., 2013). These responses capture important elements of the three main processes determining earthworm population dynamics, maturation, reproduction and mortality (Baveco and De Roos, 1996). Responses were assessed in spring and autumn of both study years (2010 and 2011) in order to investigate temporal variations of effects.

2. Material and methods

The field study was carried out in 2010 and 2011, with identical experimental set-ups in both years; i.e. each experimental plot receiving the same treatment two years in a row.

2.1. Experimental site

The experimental site (49 × 42 m) was located in south-east Norway on a field at Ås (N 59° 65′ E 10° 75′), which was divided into 30 plots measuring 3 × 5 m. Buffer belts (3–4.5 m wide) between plots and along the field boundaries separated the plots (Fig. 1). The topsoil was a loam with 40% sand, 43% silt, 17% clay, 1.49% organic carbon, and a pH of 5.8 (further details in Schnug et al, 2014b). The field had been used as experimental site for many years, and was drilled with turnips (*Brassica rapa* L.), rape (*Brassica napus* L.) and barley (*Hordeum vulgare L.*) during the last three years before our study. The last pesticide applications were in 2009 with the herbicides clopyralid and glyphosate. Since these were applied on the entire field site, including control plots, and are dissipated to 90% within <160 days (DT90) (http://sitem.herts.ac.uk/aeru/) they should not affect the interpretation of our results. Soil moisture and temperature were recorded continuously during the growth season using a data logger placed in the center of the field. The field was plowed each spring, sown with barley on April 27th 2010 and April 30th 2011, and threshed on August 30th 2010 and August 13th 2011.

2.2. Biocides and concentration levels

The biocides used were the insecticide Sumi Alpha® (DuPont®, 50 g esfenvalerate l^{-1} Sumi Alpha, CAS 66230-04-4), the fungicide Acanto® 250 SC (DuPont®, 250 g picoxystrobin l^{-1} Acanto, CAS 117428-22-5), and the bactericide triclosan (Irgasan®, CAS 3380-34-5, purity ≥97.0%). The two former were purchased from the local supplier of agricultural equipment (Felleskjøpet), Norway, and Triclosan from Sigma-Aldrich, Steinheim, Germany. Besides the active ingredients, the formulations contained inert ingredients and 15% phenylxylylethane (Sumi Alpha) and <10% propylene glycol (Acanto). The latter two substances are likely less toxic and are no known synergists with other substances (Addison et al., 1982; Madsen et al., 2001).

Biocides were applied individually and as a ternary mixture. Priority was given to multiple concentrations of each biocide and the mixture