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Effects of calcium cyanamide on Collembola in a standardized field study. Part 2: lessons learned for chemical risk assessment in field studies with soil invertebrates

J. Römbke[1*], A. van der Veen[1], P. Ebke[2] and P. Stegger[2]

Abstract

Background: Data from a one-year field study on the effect of a fertilizer (calcium cyanamide, trade name: Perlka®) on Collembola served as the basis for a broader discussion on performance and evaluation of these data for terrestrial risk assessment according to the REACH regulations) of the European Union.

Performance: Details of the technical performance of this study have been described in Part 1 of this article. In this part, the experiences made in this very large field study with a non-pesticide (i.e., a fertilizer) is discussed in a wider context, in particular by asking: Is the existing information suitable for preparing a field test guideline for Collembola – and, secondly, would such a guideline be suitable for improving current REACH and/or EFSA guidelines for testing very different chemicals in the soil compartment?

Results: By discussing individually the most important properties of such studies we could show that by combining experiences from earthworm field studies, literature, and our field study higher-tier testing with Collembola is not only technically possible, but also suitable for the evaluation of chemicals in soil (i.e., similar to existing OECD approaches for earthworms).

Conclusion: Due to our experience as well as information from literature, we could show that such Collembola field studies are suitable for improving risk assessment procedures in the soil compartment. This is in particular true, when realizing that the long-term protection of soil biodiversity is of high value both from an ecological as well as an eco-toxicological point of view. Thus, a specific Collembolan field test guideline is urgently needed.

Keywords: Springtails, Terrestrial risk assessment, Perlka®, Field test guideline, Soil biodiversity, REACH

Introduction

In the first part of this mini-series, the background and the technical performance as well as the most important results of a field study on the effects of the fertilizer calcium cyanamide (Perlka®) on springtails (Collembola) were described [1]. In the following (i.e., the second part of this mini-series), the performance and the evaluation of that Collembola field study is discussed in a wider context, meaning that all major aspects of such ecotoxicological field studies with soil invertebrates are addressed. In fact, the idea of testing and evaluating the effects of chemicals on soil organisms, mainly earthworms, in the field (i.e., under natural conditions) is already quite old, at least in the context of assessing the effects of Plant Protection Products (PPPs), also known as pesticides (e.g., [2]). It is not clear, however, how often they were performed

*Correspondence: j-roembke@ect.de
[1] ECT Oekotoxikologie GmbH, Böttgerstraße 2-14, 65439 Flörsheim/Main, Germany
Full list of author information is available at the end of the article

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in the 1980s, but since the mid-1990s such field studies were used as highest tier test for pesticides in some European countries such as Germany [3]. Actually, field testing became more regular when the European Union published their ideas for a tiered (i.e. lab to field) test strategy using earthworms as representative test organisms for the soil compartment as part of the registration process of pesticides [4]. Basically, this testing strategy is still in place, meaning that within the last 30 years dozens of similar field tests have been performed, most of them according to the (frequently updated) ISO guideline 11268-3 [5] and under GLP (Good Laboratory Practice) rules. In the nineties, partly long-lasting, i.e. up to six years, field studies addressing the effects of pesticides on Collembola were performed (e.g., [6]). According to this author, pitfall sampling is suitable for detecting long-term changes in springtail abundance and diversity at an arable field under rotation of grass and winter wheat. Within the last years, an elaborated version of the existing earthworm field test guideline has been prepared, which is based on experiences made in a huge field study performed in Germany [7]. It is not yet clear whether these improvements, mainly regarding study design and statistical data assessment [8] can be transferred directly to other field tests with soil invertebrates, in particular with Collembola.

In the first part of this mini-series, the performance and the effects of the fertilizer calcium cyanamide on Collembola in a standardized field study are described [1, 9]. Springtails were selected as study organisms because the collembolan species *Folsomia candida* reacted more sensitively than other test species such as earthworms in several OECD laboratory tests under GLP conditions when exposed to the first metabolite cyanamide of calcium cyanamide [10]. Springtails (Collembola) belong to the soil arthropod mesofauna and play an important role within the soil organism community in many soils of the temperate regions of Europe (including agricultural lands; e.g., [11–13]). Therefore, and in line with general ECHA recommendations [14], the performance of a Collembola field study is considered to be the most appropriate approach to reduce uncertainties regarding the effects of this chemical on soil organisms under field conditions.

In this second part of the mini-series, we want to discuss whether the experiences made (both regarding the study performance as well as concerning data assessment) are relevant for ecotoxicological field studies with springtails in general, independently which chemicals are tested. In addition, we want to support hazard and risk assessors in both industry and agencies in the interpretation of large and complex data sets, which are typical for such field studies with chemicals. Thus, we will discuss our experiences made in the calcium cyanamide study in a wider context, i.e. by looking at soil invertebrate field studies in general. In fact, we assume that the need for these higher-tier studies will increase in the foreseeable future, due to increasing legal requirements (as listed, for example, in a recent EFSA document [15]) as well as increasing scientific insights in the structure and functions of soil invertebrate communities (e.g., [16, 17]). In parallel, the loss of (soil) biodiversity is getting more and more public attention, even on a global scale (e.g., [18]).

Finally, we will discuss the most important parts of the technical performance of such studies, but also those issues addressing their interpretation and consequences within the last decades. In this context, it has to be highlighted that our starting point, a Collembolan field study with a fertilizer, is an exception, since most of these studies focused on PPPs used earthworm abundance and diversity as biological endpoints. The experiences made within the last 40 years with such field studies are a good starting point for their further development as well as their improved implementation in chemical risk assessment procedures for soil ecosystems.

So far, no guideline for a field study with Collembola exists. In addition, within the REACH regulation, which covers the assessment of calcium cyanamide, no detailed guidance for the evaluation of agricultural chemicals in ecotoxicological field studies is available [14]. However, the experiences gained in earthworm field studies which have been performed regularly for the last 30 years in the context of the registration of PPPs (e.g., [5, 7, 19, 20]) can be used for field studies with other organisms as well. The rules for their evaluation are mainly based on documents published by legal bodies and subordinate authorities within the European Union [4, 15]. In addition, two reports addressing the evaluation of field studies published by the Dutch government were used for an external quality control, since they formulate minimum requirements for earthworm as well as non-target arthropod field studies [21, 22].

Finally, based on the experience gained in earthworm field studies for the assessment of the effects of chemicals on Collembola as well as the specific problems of assessing a granulated test item, i.e., calcium cyanamide (Perlka®; in contrast to the usually tested liquid formulations of PPPs) will be discussed in the following. They provide a sound basis both for the technical performance as well as a robust assessment of the results of these higher tier testing approaches for chemical risk assessment [7].

In addition, two further aims were considered when compiling this article:

- To provide the basis for the preparation of a Collembola field study guideline, using the experiences of this study as well as those from the literature, in order to address the specific problems of testing chemicals such as fertilizers in the field.
- To use the experiences made in this field test for the improvement of the current REACH and EFSA requirements. In particular, specific guidance is needed to allow registrants to perform a field study on the effects of chemical stressors on soil organism communities under field conditions in cases where chemicals are intentionally applied to (arable) soil.

Test item, study site, and application regime

Performance

The Collembola field study with calcium cyanamide (formulation: Perlka®) was performed on a hay meadow near Homberg (Ohm), Hesse, Germany (soil texture: silt loam [23]. Since 2010, this meadow was managed without the use of mineral fertilizers and pesticides. Granular calcium cyanamide (Perlka®) was applied twice at two rates (200 and 400 kg Perlka®/ha) with an interval of six months. Agrichlor (a.i. chlorpyrifos) was sprayed once as a reference item. This chemical was selected since it is known to cause toxic effects on springtails—and this was the main reason for using it. The fact that it is a granular product did not play a role in this decision.

Control plots were left untreated. Additional fertilizer control plots were treated with two applications of the urea fertilizer Piagran®46 with 172.9 kg/ha (i.e., about the same total nitrogen rate as in the high dosed test item, i.e., 400 kg Perlka®/ha), using a commercial spreader for granules and the reference item with a movable field plot sprayer. Almost 100% of the test item was applied and target quantity as well as distribution homogeneity was determined according to DIN 13739-1 [24]. The results were evaluated with nominal application rates, which enclosed the average application rate of 300 kg/ha used once per growing season as recommended rate for different crops [25]. The study area was irrigated by approx. 10 mm water within 3 days after the first application [5].

Evaluation

The identification and selection of an appropriate study site as well as the application of the study substance and its irrigation followed both recommendations covering good agricultural practice [26] as well as specifications given in existing earthworm field test guidelines (e.g., [5, 7]) as much as possible. Special attendance had to be given to the specific properties of this granular study item. In this case specific equipment was needed in order to evenly distribute the correct amount of fertilizer granules per area on the study plots (including specific methods to check the respective application rates). The results presented in Part 1 [1] of this mini-series prove that such an application can be reliably performed when using appropriate equipment, taking into account relevant guidelines for specific applications (DIN 13739-1 [24]).

General comment

Actually, and despite the fact that similar experiences have already been made in standard earthworm field studies with pesticides (e.g., regarding applications of pesticides with adjuvants) it is recommended to include information on the handling of test chemicals with "special" properties (e.g., low solubility, granular appearance, etc.) in existing or new field test guidelines (i.e. those addressing earthworm and Collembola tests).

Study design

Performance

A randomized block design with five treatments (untreated control, fertilizer control, reference item, and two application rates of the test item with four replicates each (20 plots in total) was used. Each plot had a size of 10 × 12 m (120 m^2) separated by a strip of untreated land (width: 3 m). The study area was surrounded by an unused margin of at least 10 m width.

Evaluation

This design is identical with the one which has been used for decades in standard earthworm field studies (ISO 11268-3 [5]). Within the last 20 years some field studies on the effects of PPPs on Collembola have been performed (often under GLP), either with springtails alone or, more often, in combination with earthworms, but very few of them have been published (as an exception see, e.g., [27]). With regard to the design itself as well as background information (e.g., environmental data such as precipitation, temperature, etc.), the technical requirements of earthworm and springtail field studies are similar. One potential difference is the size of the study plots, since the area required for Collembola tests could be smaller than that for earthworms due to their smaller body size, higher abundance, and lower dispersal ability [28]. However, in order to be on the safe side, in the present study almost the same plot size was chosen as recommended for earthworm field studies (10 × 12 m). The selection of the most appropriate field-study design has recently been intensively discussed ([7, 8]), but it is not yet clear how the outcome of this exercise will be implemented in international guidelines.

General comment

The selection of an "optimized" study design, again using earthworms as an example, has already been started and has been submitted to OECD [7]. Regarding the statistical evaluation, an elaborated proposal has currently been made by Daniels et al. [8]. These authors developed the CPCAT approach (Closure Principle Computational Approach Test), which is particularly suitable for large data sets. It has been proposed as a complementary method for deriving NOECs, and a dose–response design was recommended to deduce ECx values, which are always preferable for the in-depth understanding of the ecotoxicological profile of a substance.

Sampling of the Collembola

Performance

Springtails were sampled in two ways: (1) pitfall traps (4 traps per plot), which were exposed for 4 days; and (2) soil cores (6 cores per plot) at 11 dates, respectively. Collembola were extracted via MacFadyen heat extraction from the soil cores. Both methods were performed according to ISO guideline 23611-2 [29], without deviations. The use of these methods proves that this Collembola field study was performed according to current regulatory quality requirements.

Evaluation

Both methods have been used for about 50–100 years [30] in soil ecology as well as in soil monitoring studies. They were standardized by ISO more than 20 years ago, and have been evaluated every 5 years (newest versions: ISO 11268-3:2014 [5]) and 23611-2:2006, confirmed in 2015 [29]). Thus, the sampling of springtails was performed by adapting existing earthworm guidelines for monitoring springtail communities (e.g., [31, 32]). The preparation of a new ecotoxicological field study guideline with Collembola analogous to the current earthworm field study (ISO 11268-3 [5]) would be important from a regulatory point-of-view.

General comment

From a scientific point-of-view this issue has been widely clarified. As a step forward the existing information on Collembolan seasonality, small-scaled distribution as well as should be formalized and agreed-on by the respective bodies within ISO and OECD. This process will take a couple of years, but contributions such as this paper will be helpful in order to get a common understanding within the scientific and regulatory communities.

Statistical evaluation of the study results

Performance

Abundance values for sum of Collembola as well as for individual species were tested for normality (Shapiro–Wilk's test) and homogeneity of variance (Levene test), using ToxRat Professional (Version 3.3.0 ToxRat® Solutions GmbH, 52477 Alsdorf, Germany). Depending on these results a Student's *t*-test, Welch's *t*-test or Mann–Whitney *U* test were selected to compare control and reference item. The tests were calculated one-sided smaller ($\alpha = 0.05$). The difference between control and fertilizer control was calculated in the same way. These tests were performed two-sided ($\alpha = 0.05$).

To calculate the differences between means in controls and treatments for each taxon and sampling date the multiple *t*-test by Williams [33, 34] was used providing also the NOER (no observed effect rate) on the population level. The minimal detectable difference (MDD) at the NOER was determined in accordance to Brock et al. [35] using the software Community Analysis (CA) 4.3.14 [36].

Evaluation

The statistical evaluation of the study results followed accepted international guidelines (e.g., ISO 11268-3 [5]), i.e., the same statistical test procedures as in ISO earthworm studies were used (e.g., Student's *t*-test, Welch's *t*-test or Mann–Whitney *U* test) as well as the multiple *t*-test [33, 34]. Thus, the statistical approach used here is fully reliable. However, in the future the CPCAT approach has to be considered as an alternative for such data sets [8].

In the absence of a guideline for conducting Collembola field studies, there is also no corresponding guidance for evaluating the results. For the evaluation of effects in field studies, ECHA [14] refers to the risk assessment for plant protection products with non-target arthropods [37]. Therefore, the effect classes according to De Jong et al. [22] were used as a good approximation, but they do not provide a scaling classification for the magnitude of effects.

More recently, EFSA [15] suggested a scaling of the magnitude of effects on populations or functional groups of microarthropods like Collembola for the definition of specific protection goals. Thus, detailed threshold values are available in case of detecting significant effects. In case no effects have been observed, this could also be caused by poor quality of the data. Therefore, a measure for the magnitude of detectable effects is also needed. EFSA [15] suggested to use the MDD concept according to Brock et al. [35], which originally was developed for the aquatic risk assessment of PPPs. Recently, the mandatory recommendation of MDD calculations for higher-tier studies with NTAs and other soil organisms was considered to be premature, because criteria for the interpretation of MDD values are currently lacking [38]. Nevertheless, MDD values are considered as a measure for the robustness of the derived ecotoxicological thresholds in order to achieve a defined degree of certainty for the assessment and the resulting regulatory decisions [39].

The MDD values according to Brock et al. [35] have been included in the evaluation in order to provide a measure for data quality [40]. It was found that the MDD values for total abundance and the most frequent species *Lepidocyrtus violaceus* allow to detect effects defined as "small" (i.e., ≤ 34% difference to the control value) by EFSA [15]. For three other species the MDD values were in a range of medium effects (i.e., 35–65% difference to the control value) at six to seven sampling dates [15]. For further three species at five to all sampling dates MDDs were at least in a range to detect large effects (i.e., > 65% difference to the control value). MDDs for another two species at one to two sampling dates were in the range to detect possible large effects. Therefore, at least 7 species could be classified as "MDD-category 1 species" according to Brock et al. [35]. As these species include representatives of all three ecological life-form types (epedaphic, hemidaphic, euedaphic) this data set is suitable for a reliable risk assessment of Collembola as representatives of soil mesofauna.

General comment

Scientifically, the statistical evaluation of complex data sets such as the diversity and abundance of soil invertebrates in the field is currently intensively discussed, but so far new approaches (e.g., CPCAT) [8] have not (yet) been transformed into international guidelines. This will take some years, but it seems that the main issues of such a guideline are in general agreed-on, mainly due to the experiences and improvements made in earthworm field studies [7]. Some formal issues such as the question whether the respective guideline or guidance documents will cover all environmentally relevant chemicals or will focus on one specific chemical group (such as PPPs) may delay the finalization of this process.

Validity of the study results

Performance

First, on each of the three dates after the first application, a statistically significant reduction of at least 50% was observed for the reference item compared to the untreated control for total abundance of Collembola. The same is true for six of the eight species (75%) in pitfall traps and soil cores for which a statistical evaluation was possible. Second, a "representative" community is expected to exist at the study site. In the pitfall traps and soil cores in total 16 Collembola species were found. For seven of them, a reliable statistical evaluation was possible during the study.

Evaluation

According to the earthworm field study guideline (ISO 11268-3 [5]) a 50% reduction has to be achieved with a single application of the reference substance in terms of total abundance or biomass. For the current study, the stricter requirements of the guidance for NTAs were used defining the validity criteria with at least 50% effect of the reference item on at least one sample date for at least 10% of the analysed taxa [22]. In the current study, this criterion was fulfilled on the first three sampling dates after the first application for pitfall and soil core samples, thus proving the sensitivity of the study system, too.

General comment

This issue may need some more discussion in order to get a commonly agreed approach. In addition, more practical experience with Collembolan field studies would be helpful for the preparation of a specific guidance document for Collembolan field studies (e.g., from different regions, various stressors, etc.). However, due to the fact that the basic approach has been taken over from existing guidelines it is very likely that it is a very practical field method.

Summary of study results I: diversity analysis

In the following, the effects of the test item on the number of species, Shannon index and evenness are presented in order to get an overview on the results of this study. The means of both treatments for pitfall traps compared to the untreated control for the three parameters are shown separately (Fig. 1). Figure 2 shows the values of both treatments for the three parameters for soil core samples compared to the untreated control. For both treatment levels of pitfall trap samples and soil core samples, no clearly treatment-related differences compared to the untreated control could be observed for the three parameters. Therefore, differences observed were assigned to effect 2.

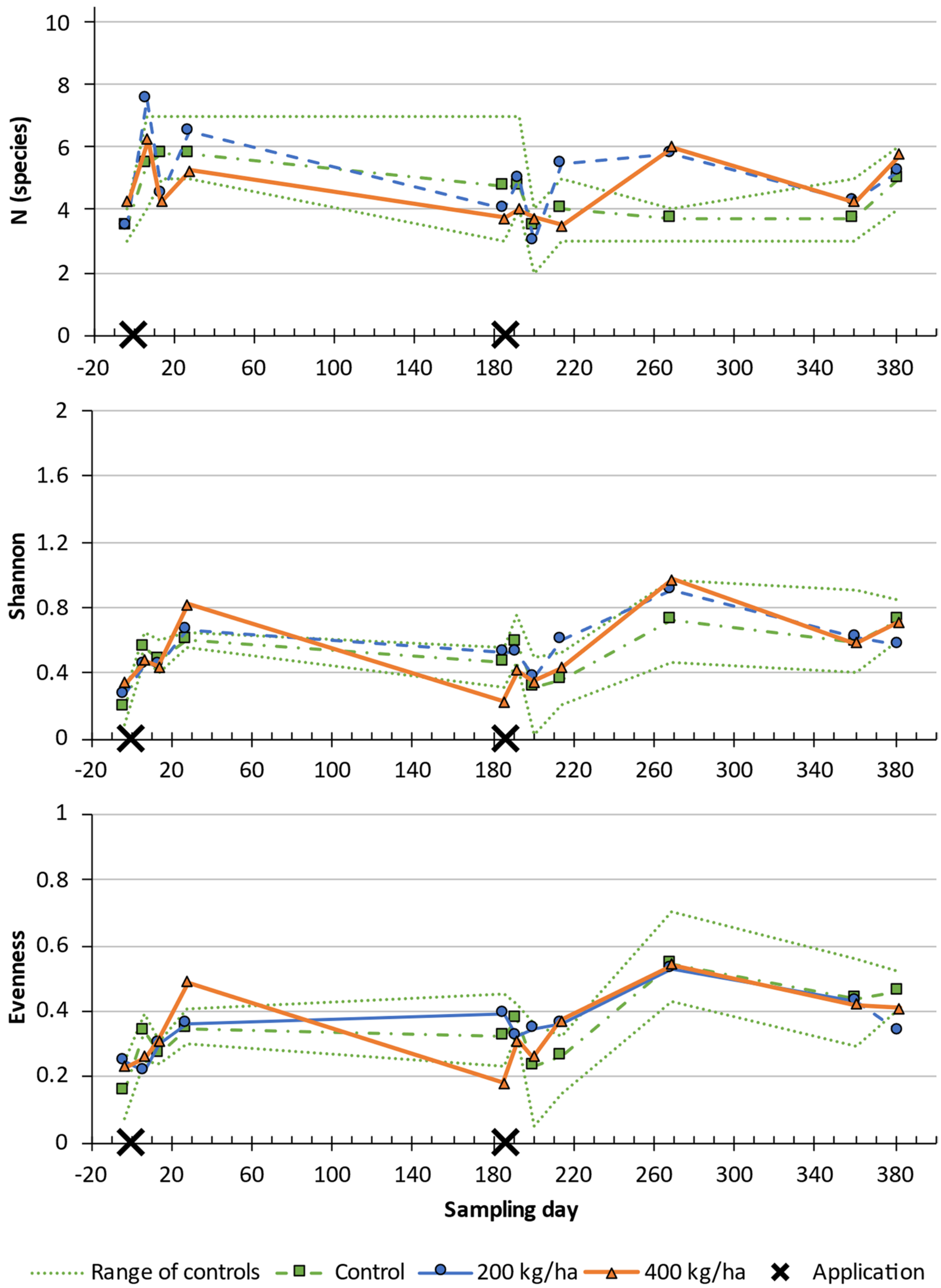

Fig. 1 Diversity analysis of pitfall trap in comparison to the untreated control. Means per treatment level and range of controls

Summary of study results II: effect class analysis

Performance

A classification of effects according to De Jong et al. [22] was performed, leading to an assessment of effect class 1, 2 and 5 (effect classes 3 and 4 did not occur in our study):

- Effect class 1 (effects could not be demonstrated; NOER): no (statistically significant) effects were observed as a result of the treatment and/or observed differences between treatment and control showed no clear causal relationship.

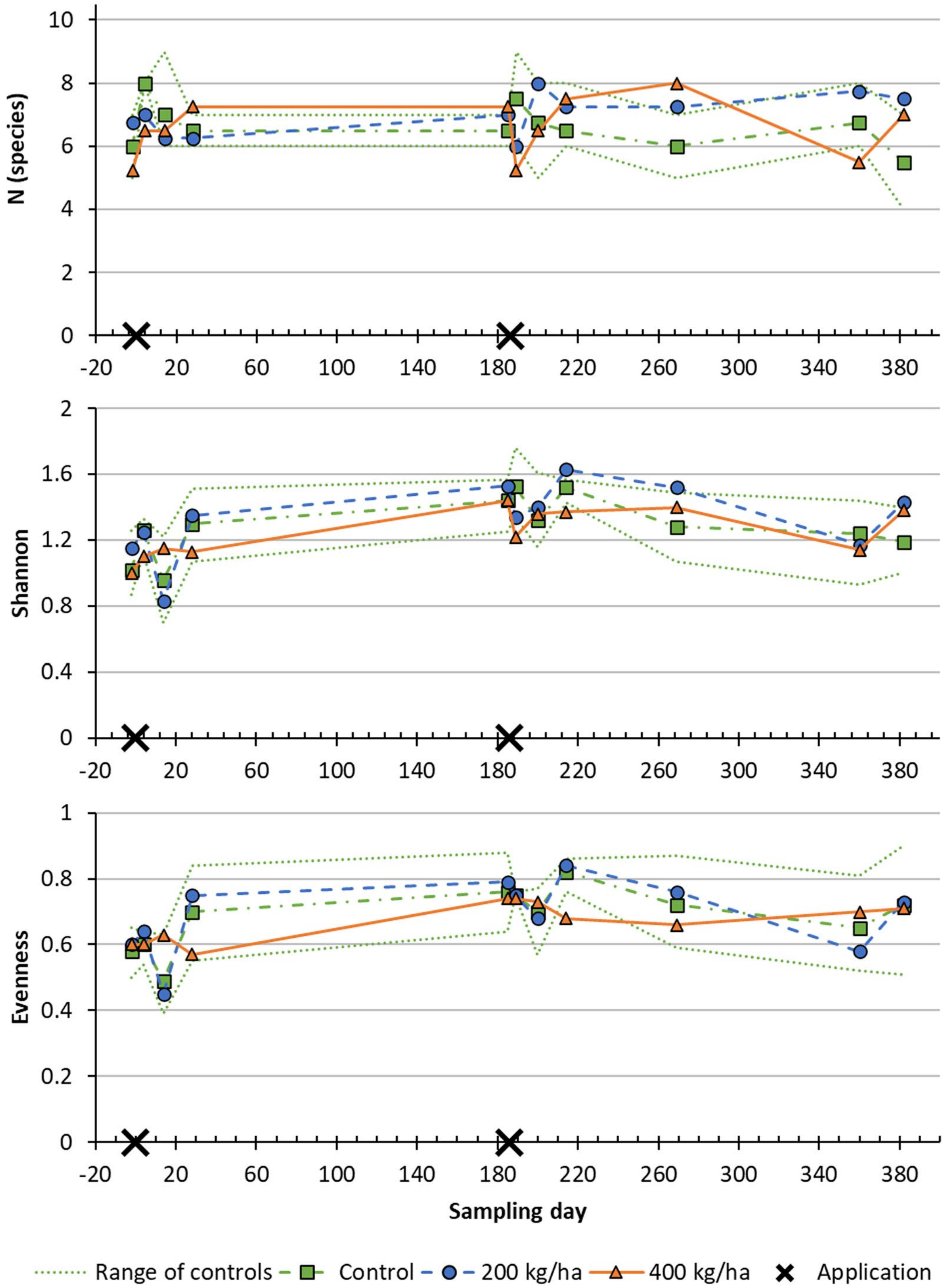

Fig. 2 Diversity analysis of soil core samples in comparison to the untreated control. Means per treatment level and range of controls

- Effect class 2 (slight and transient effects): quantitatively restricted response of one or a few taxa and only observed on one sampling occasion. Any isolated effect (whether an increase or a decrease) is assessed as class 2, for both treatment-related and not-treatment-related effects.
- Effect class 5 (pronounced effects; recovery within 8 months after first application): clear response of taxa, effects last longer than 4 months, but full

recovery happens within 8 months after the first application; effects were observed at two or more sampling instances.

In this study, at one date a pronounced effect was found for one species, but recovery did occur within 8 months. This observation was noted as effect class 5.

Pitfall traps

For the low treatment level compared to the untreated control, a statistically significant lower abundance in the range of a small effect was observed for the total abundance of Collembola and for one species (*Sminthurinus aureus*) at one sampling date. Therefore, this treatment level was assigned to effect class 2 (Table 1, lower part). For the high treatment level compared to the untreated control, some effects were observed at individual dates, both on total abundance and on two individual species. Therefore, they were assigned to effect class 2. For *Sminthurinus aureus*, a response on two consecutive samplings dates could be observed after the first application in autumn. For the sampling in the following spring, a meanwhile recovery could be assumed. Thus, the statistically significant lower abundance in the high treatment level on the last sampling date was not considered to be treatment-related and the effect was assigned to effect class 5. For pitfall trap samples compared to fertilizer control, the differences observed showed no clear dose–response relationship or consistent trend and were therefore assigned to effect class 2 at the highest.

Concerning the effect class assessment of the endpoint diversity in the same samples (Table 1, upper part), single differences with opposing trends were observed at the low treatment level in comparison to the untreated control for the parameter "Number of Species" and "Evenness", but not for the Shannon index. This results in an effect class 2 for the former two parameters and an effect class 1 for the latter index. At the high treatment level, opposing trends were found for each parameter (in the case of the Shannon index without a dose–response relationship).

Table 1 Effect classification according to De Jong et al. [22] for the endpoints "diversity" and "abundance" in soil core and pitfall samples compared to the untreated control (Williams test, one-sided, $\alpha = 0.05$)

Treatment	200 kg/ha		400 kg/ha	
Sample type	Pitfall	Soil core	Pitfall	Soil core
Diversity				
Number of species	2↑	2↑	2↓↑[a]	2↓↑[a]
Shannon index	1	2↑	2↓↑[a,b]	1
Evenness	2↓	1	2↓↑[a]	2↓↑[a]
Abundance				
Sum Collembola	2↓	2↓↑[c]	2↓[d]	2↓↑[c]
Isotoma viridis	1	2↑[c]	2↓	2↑[c]
Lepidocyrtus violaceus	1	2↓↑[c]	2↓↑[d]	2↓↑[c]
Lepidocyrtus lignorum	1	(1)	1	(1)
Heteromurus nitidus	(1)	–	(1)	–
Sminthurinus aureus	(2↓)	(1)	(5↓)[e]	(2↓)[f]
Parisotoma notabilis	–	1	–	2↓[c]
Folsomia manolachi	–	2↑[c]	–	2↑[c]
Protaphorura armata	–	(1)	–	(1)
Tullbergia simplex	–	1	–	1

Classes in brackets: numbers too low for a reliable evaluation

[a] Single differences with opposing trends

[b] Difference on day 185 without dose–response relationship

[c] No dose–response relationship

[d] Isolated decreases on day 28 after 1 application, after 2nd application no clear dose–response relationship

[e] Effect on two consecutive samplings in autumn, next sampling five months later (following spring), for difference on last sampling no treatment relation considered

[f] Questionable due to low abundance

Soil cores

For the low as well as the high treatment level compared to the untreated control, no ambiguous effects were observed. Comparing the same results with those of the fertilizer control did not change this outcome. Due to statistically significant differences observed on single sampling dates (but without a clear dose–response relationship), effect class 2 was assigned to total abundance of Collembola and three species in the low treatment rate and total abundance of Collembola and five species in the high treatment rate compared to the untreated control. For the low treatment level compared to the fertilizer control, an effect class 2 was assigned to *Lepidocyrtus violaceus* due to a statistically significant higher abundance on a single sampling date not seen to be treatment-related. For the high treatment level compared to the fertilizer control, effect class 2 was assigned to total abundance of Collembola and two species. Thus, for all Collembola species a full recovery within one year after the first application was demonstrated. Concerning the endpoint diversity for comparing the treatments to the untreated control and fertilizer control, only single statistically significant differences with opposing trends were observed resulting in an effect class 1–2 for the different diversity measures.

Evaluation

The ISO guideline 11268-3 [5] gives no recommendation for the assessment of the study results. However, the guidance document for the evaluation of NTA studies

[22] provides a suggestion for an effect classification that was used for the current study to assign effects classes to observed statistically significant differences.

The results of the application of the reference item clearly proof the sensitivity of the springtail species to a chemical stressor. Therefore, these results are well-suited for hazard and risk assessment purposes of the test item calcium cyanamide (for details see Part 1 of this mini-series [1]).

In addition, our results prove that the study substance caused differentiated effects on the Collembola community and on individual species. Thus, the main expectation regarding the results of such a field study is fulfilled. Any community of soil invertebrates, such as Collembola consists of many species which are, due to ecological, partly site-specific reasons occurring in different numbers. In addition, species are not evenly distributed in the (soil) environment, due to "local" hotspots, caused, e.g., by food accumulation or differences in soil properties. Therefore, it is necessary that various species are evaluated in such studies—a criterion which has also been fulfilled here. Finally, not only the sheer occurrence of the individual species is an important endpoint, but also their ability to recover after being stressed by an external factor such as the applied test item.

Standardized performance

Performance

So far, no specific guideline for the ecotoxicological field studying of Collembola is available. However, such studies have been performed as GLP or non-GLP studies quite regularly in the context of the risk assessment of PPPs, either with Collembola alone or in combination with earthworms. Very few of these studies were published [27]. In order to be on the "safe side" we considered as much as possible existing guidelines, in particular from earthworm field studies (e.g., ISO 11268-3 [5]). Actually, these guidelines are regularly checked regarding their content and relevance. For example, based on content of the existing ISO guideline 11268-3 [5] OECD is going to publish soon an improved earthworm field study guideline, focusing mainly on new statistical evaluation methods. Such a change will have also clear effects on the study design and, thus, performance (e.g., [7, 8]).

Evaluation

With regard to the general study design and activities such as the collection of environmental background data, e.g., precipitation, temperature, etc., the requirements for earthworms and springtail field studies are similar. In addition, in terms of chemical application as well as plot size we used these documents as a template. Due to the smaller body size and lower dispersal ability of (especially) endogeic Collembola in comparison to earthworms the size of the study plots could have probably been smaller [28]. However, and in order to be on the safe side, our plot size corresponded to that used for earthworm testing.

Representativity of the springtail community at the study site

Performance

According to the approach for earthworm field monitoring studies (ISO 23611-1 [41]), the study organism group found at the study site has to be representative for the respective organism group at comparable sites. Usually, abundance data for individual species (or the whole community and even age groups, respectively) as well as information regarding community composition (i.e., percentage of each species within a community) are needed. In order to collect this information, we checked the central German database Edaphobase, located at the Senckenberg Museum Görlitz [42] for Collembola data at comparable sites; i.e., sites which are as much as possible similar to our study site in terms of location, climate, soil properties (e.g., pH), vegetation and land use (here: grassland).

Based on this database, a list of 25 species was extracted which should occur at grasslands similar to the Homberg site with a probability of at least 50% (Table 2). In addition, we have had a closer look at two additional studies focusing on Collembolan communities at grassland sites comparable to the Homberg site: a succession experiment covering 86 sites (each one sampled once) performed near Giessen in Upper Hesse (Germany) [43], i.e., in the same region as our study site, and a monitoring meadow site located in a hilly area of Eastern Bavaria [44]. In Table 2, the main information regarding the Collembolan community at the study site as well as the two comparable sites mentioned above is summarized.

Evaluation

When evaluating the representativeness of the results of this field study the following issues have to be discussed:

(1) The high dominance of one species (here e.g.,: *L. violaceus*) at the study site has no impact on the study reliability per se, since the dominance structure was comparable at the different treatments. In addition, this high dominance percentage applied only to surface-active Collembola (i.e., those caught in pitfall traps). In contrast, among soil-dwelling

Table 2 Comparison of Collembolan species expected to occur with a probability of ≥ 50% at "Grassland with slightly acid soils" in southern Germany (Edaphobase; Senckenberg-Museum, Görlitz) with those found in three field studies (differing in sampling methods)

Expected species according to edaphobase (25)	Species found at the three comparable sites			Ecological type
	Homberg Study	Chauvat et al. 2007	Toschki et al. 2020	
Brachystomella parvula				Hemiedaphic
Dicyrtomina violacea				Epedaphic
Entomobrya lanuginose			●	Epedaphic
Folsomia manolachei	●	●	●	Hemiedaphic
Folsomia quadrioculata				Hemiedaphic
Friesea mirabilis	●	●	●	Hemiedaphic
Isotoma viridis	●	●	●	Epedaphic
Isotomiella minor		●	●	Euedaphic
Lepidocyrtus cyaneus		●	●	Epedaphic
Lepidocyrtus lanuginosus		●	●	Epedaphic
Lepidocyrtus lignorum	●		●	Epedaphic
Lepidocyrtus violaceus	●	●		Epedaphic
Megalothorax minimus		●		Euedaphic
Mesaphorura macrochaeta			●	Euedaphic
Metaphorura affinis				Euedaphic
Neanura muscorum	●			Hemiedaphic
Parisotoma notabilis	●	●	●	Hemiedaphic
Protaphorura armata	●	●	●	Euedaphic
Protaphorura campata		●		Euedaphic
Pseudosinella alba	●	●	●	Hemiedaphic
Sminthurinus aureus	●			Hemiedaphic
Sphaeridia pumilis		●	●	Hemiedaphic
Stenaphorura denisi				Euedaphic
Stenaphorura quadrispina				Euedaphic
Supraphorura furcifera				Euedaphic
	10	13	13	
Species not expected according to edaphobase	**Homberg study**	**Chauvat et al. [43]**	**Toschki et al. 2020**	**Ecological type**
Ceratophysella sp.	●			Not known
Heteromurus nitidus	●		●	Euedaphic
Orchesella flavescens	●	●	●	Hemiedaphic
Pogonognathellus flavescens	●	●	●	Epedaphic
Sminthurus viridis	●			Euedaphic
Tullbergia simplex	●			Euedaphic
	6	2	3	
Total number found (in brackets: species not listed)	16	15 (+ 16)	16 (+ 12)	

Additional species found at Homberg are listed but not for the two other studies (16 and 12)

Collembola, the most dominant species accounted for approximately 50% of the respective community. In the Bavarian monitoring study, performed at 36 sites at four German federal states [44], the dominance spectrum of Collembola was checked, with particular attention to the species *L. violaceus*. According to this source, it is a wide-spread species in grasslands, especially in soils with a high organic matter content. High differences between individual samples and/or sampling dates indicate that the species reacts strongly to the occurrence of local "hot spots" of organic matter. In fact, the abundance (and thus also the dominance) of individual springtail species can vary considerably in time and space depending on (often small-scale) differences in soil parameters. The dominance of a species does not say anything about the usefulness of the data for the risk assessment, if the abundance of the remaining species is sufficient for a reliable statistical evaluation.

(2) Usually, species number and composition are considered to be more stable indicators than parameters such as abundance which are highly dependent on—relatively quickly changing—environmental factors such as climatic conditions or food availability. According to the information provided from Edaphobase [45], sites comparable to the test site in Homberg could host about 25 Collembolan species with a probability of $\geq 50\%$. Of course, this does not mean that all of them have to occur at the same time or site (e.g., due to site-specific factors). Anyway, this information has been used as a reference for the evaluation of the springtail community at the test site.

(3) According to the information provided in Table 3, the number of species is comparable between the study site and the individual as well as the regional data sets on Collembolan communities when keeping in mind the different sampling efforts between this study and the information collected in the database. The number of springtail species found at the study site (16; one site sampled 11 times) does not differ much from the numbers found at several sites in Upper Hesse (19 species; 86 sites sampled once; [43]) nor at an arable meadow site in Bavaria (14 species, sampled twice at three sites; [44]). The higher species number found in the study of Chauvat et al. [43] is probably caused by the clearly higher sampling effort, especially in terms of sites. Seven species were found in all three studies.

(4) The species found could be classified according to their ecological group, usually given as absolute number as well as percentage of the total number in their preferred soil layer (see Table 3). First of all, it should be noted that all three ecological groups are represented at each site, but differences are visible: the number of endogeic species is relatively low at Homberg in comparison to the two other ones. All sites are similar in terms of general land use and main soil parameters (at least pH), but there are (probably) other differences, especially in terms of land use treatments (i.e., what is done when—and how often). In addition, the different sampling efforts (i.e., crop vs. meadow) have to be considered: Chauvat et al. [43] and Toschki et al. [44] used only soil cores (containing both the litter layer and the upper soil), but not pitfall traps as in the present study. Thus, species composition could reflect these differences.

(5) Looking at the distribution of the expected and found species (and their respective ecological group) in the three German field studies (Table 3) and using the available information from Edaphobase [45] as a "yardstick", it seems that:

- The total number of species found at the three sites is quite similar ($n = 14$–19), but at the same time it is lower than the total number of species which could occur at German grassland sites with comparable soil conditions ($n = 25$; according to the current version of Edaphobase). This difference is probably caused by the respective land use activities. In addition, differences in the sampling techniques may also play a role;
- Regarding the depth distribution of the species found in comparison to the expected numbers

Table 3 Comparison of numbers of Collembolan species found at the study site (Homberg) and two comparable German grassland studies (absolute and relative numbers), separated by their respective ecological group

Species found per group	Homberg study	Chauvat et al. [43]	Toschki et al. 2020	Number of species expected
Epigeic species	4	4	5	7
Hemiedaphic species	9	6	5	9
Euedaphic species	3	3	3	9
Total number found	16	13	13	25
Epigeic species (%)	25	31	38	28
Hemiedaphic species (%)	56	46	38	36
Euedaphic species (%)	19	23	31	36
Total percentage found (%)	100	100	100	100

In the last column the number (and their relative percentage of the total number) of species expected at these sites is given using the actual information on springtail findings as given in the database Edaphobase there is no big difference between the percentage of epigeic species ($n = 30$–38), but a striking difference regarding the occurrence of hemi- and euedaphic species: in Bavaria there is almost no difference between these two groups regarding their depth preference, but in upper Hesse twice as many hemiedaphic compared to euedaphic species have been found. At the Homberg site this difference was even larger—only one euedaphic species was found there compared to three in the two other studies.

Again, it should be stated that the differences listed here are (partly) caused by different sampling techniques, local soil and site conditions and/or differences in the site history. Actually, the number of sites which have been studied in such detail as those discussed and which are located at comparable sites and used comparable sampling methods and time periods is still very low. Therefore, the Collembolan springtail community at the Homberg sites is considered to be representative for springtail communities of central European grasslands.

Potential for recovery

According to recent regulatory documents, especially in the context of assessing pesticides (e.g., [38]), the recovery (in fact, the potential for recovery), has to be shown when evaluating the effects of a substance (here: Perlka®) on the community structure and abundance of an organism group (here: Collembola) within a "reasonable amount of time". A community of organisms usually consists of many species with different ecological requirements, but also with different life cycles (including generation times). For soil ecosystems in Europe, it is reasonable to assume that the biggest organisms (i.e., here: earthworms) have also the longest lifetime. Therefore, respective regulatory documents require a recovery time of (in this case) soil organisms of one year at the longest [15].

Under REACH, few details regarding this endpoint have been provided. However, in the document REACH R.7.11.4.2 [14] "Field data and model ecosystems" it is stated that: "Where significant effects are detected the duration of effects and the range of taxa affected should be taken into consideration [37]." In our field study these recommendations (which have originally been proposed for non-target-arthropods, including at least some soil organisms) were followed. Thus, under REACH data from field studies, including the investigation of soil organisms, population recovery within one year should be considered for terrestrial hazard assessment.

In detail, the recovery of a population in field studies is influenced by the dispersal potential of the organisms, plot size, species phenology, and the surrounding habitat structure of the plot experiment [46, 47]. The time to recover observed in small plots can be misleading for mobile species that move in and out of plots during the course of a study. In contrast to larger arthropods such as spiders or beetles which are often very mobile many Collembolans live in the soil (i.e., belong to the endogeic ecological group) and show a different behaviour. These soil-inhabitants are not able to move over wide distances [28]. In addition, they are by a factor of 5 to 10 smaller than the beetle and the spider species mentioned above, meaning that their range of active movement is also smaller. Thus, the approach used in this study has been accepted for registration purposes by European agencies for about three decades. Finally, in the studies analysed so far Collembola have been tested at 10×10 m plots: i.e., these plots were originally designed for PPP field studies which focus on much larger and (at least) partly more mobile organisms than springtails. Results gained in studies with such a trial design have been accepted as the highest tier in soil environmental risk assessment for about 40 years.

Conclusions of the methodological discussion

Summarizing the discussion of the last chapters, we conclude that the presented field study is valid and meaningful regarding all criteria proposed so far for ecotoxicological field studies with soil invertebrates. In detail, the performance of the study is in line with international guidelines describing ecotoxicological field work or biological soil monitoring activities. It can be concluded that this study yields robust data for the ecotoxicological assessment of calcium cyanamide fertilizer (Perlka®) under field conditions, in particular the soil compartment.

Regulatory considerations

General introduction

Without doubt higher-tier field studies focusing on the most sensitive species group are a powerful tool to achieve a robust hazard assessment under realistic conditions. When assessing the impact of pesticides on NTAs such studies have been used for more than 20 years in order to assess the risk of substances for organisms living on the soil surface (e.g., [37, 48]). Additionally, the potential for recovery of the studied organisms (e.g., earthworms or less often Collembola) determined in field studies with PPPs (e.g., Frampton [6] has been considered as an important endpoint by authorities and industry for at least the same number of years, especially in the context of the registration of PPPs (e.g., [15, 38]). Currently, OECD is preparing a new Guidance Document for the performance of earthworm field studies which is basically also applicable to the testing of other soil organisms regarding the design of such studies and the statistical evaluation of the results [7, 8].

Results of the hazard assessment of the Collembola field study in the light of REACH requirements

The REACH Regulation (Regulation (EC) 1907/2006) defines the objective of the environmental hazard assessment in Annex I Section 3.0.1 as identifying "the concentration of the substance below which adverse effects in the environmental sphere of concern are not expected to occur" [49]. Depending on the registered tonnage, short-term and/or long-term effects must be studied in order to fulfil the information requirements under the REACH Regulation which are defined in its Annexes VII to X. The appropriate test should be selected depending "on the outcome of the chemical safety assessment" [49].

In case of calcium cyanamide, the appropriate test method was selected from a set of laboratory tests, which were performed with the metabolite cyanamide, using Collembola, earthworms [10] and spiders [50] as test species, according to international guidelines. A potential hazard for the soil compartment was identified based on the Collembolan species *Folsomia candida*, which was determined to be the most sensitive soil-inhabiting species [10]. For higher tier investigation of the potential effects on Collembola it was decided to perform a field study.

In the REACH Guidance document R.7c [14], section R.7.11.4.2 "Field data and model ecosystems", it is stated that: "Where significant effects are detected the duration of effects and range of taxa affected should be taken into consideration." This is in line with recommendations for above-ground arthropods in the context of the registration of PPPs [37]. In addition, the REACH Guidance document R.7c [14] states that population level effects are the desired endpoint.

However, under the REACH Regulation No. 1907/2006 [49], no specific and detailed guidance on the performance and/or the assessment of the hazard of a substance under field conditions has been provided. Indications on how to handle such issues can be derived from one of the REACH Guidance documents. In the REACH Guidance Document R.7c [14], section R.7.11.3.2 "(semi-) Field data", difficulties of field studies are shortly mentioned, but not further discussed. Nevertheless, ECHA concludes that "field studies are the most accurate assessment of the impact of a substance on soil function and structure under climatic conditions." This is exactly the reason why in different regulatory chemical registration documents (most prominently: PPPs) field studies are required in cases of doubt, because they are performed under realistic conditions, i.e. no extrapolation to the "real world" is necessary. However, one important difficulty is the lack of standardization of field studies. The REACH Guidance Document R.7c (R.7.11.4.2 [14]) also states that "fixed trigger values for acceptability of effects are not recommended, as the impact of treatments can be significantly different for different organisms".

In order to achieve a high level of applicability although no guideline is available for field studies with Collembola, this study with calcium cyanamide was therefore designed as similar as possible to the standardized earthworm field guideline (ISO 11268-3 [5]). This field study clearly focuses on the investigation of long-term effects (including potential recovery) of a Collembolan population. In other words, the practical approach chosen (the Collembola field study (Part 1; [1]) follows exactly the requirement of the REACH Guidance Document R.7c [14].

The results of this study show that the investigated Collembola population had recovered at the end of the study which was one year after the first application and only six months after the second application. A valid NOEC was derived from the Collembola field study enabling the derivation of a meaningful PNEC for the terrestrial compartment. Taking into consideration data from both levels (laboratory, field), it can be concluded that the results of the Collembola field study are suitable to derive protective values for the soil invertebrate community as such.

In conclusion, this field study is a milestone in the hazard assessment of calcium cyanamide as a fertilizer in the terrestrial compartment—and at the same time an example how higher-tier studies could be performed and assessed.

Finally, in regulatory soil ecotoxicology there is no agreement so far which soil organism groups should be evaluated as a minimum requirement when assessing the hazard or risk of chemicals in the field. Most often seven organism groups are listed as potential candidates (see, e.g., [15]), but this is a proposal at best. With regard to the REACH Regulation, no specifications are given for field studies. Based on our experience, earthworms are regularly required as the representative group in regulatory risk assessment for the soil compartment—and only for this group a test guideline for the performance of field studies exists as a higher-tier study. It has been standardized already back in the early eighties by a German agency [3]. Later on, this guideline document was transformed into an ISO standard [5]. Currently, OECD is working on the modification of this document in order to publish a new OECD Guidance Document within the next three years [7]. The ISO document could also be used as a template for Collembola field studies. According to recent procedures in pesticide testing, Collembola field studies are regularly asked for by EU and national regulatory authorities.

Summary and conclusions

In summary, this Collembola field study was planned and performed according to established ISO and EFSA documents referring to earthworm testing taking into consideration relevant sections of guidance documents for field studies from the open literature [21, 22]. The study was performed under the rules of GLP. No methodological limitations were identified. In addition, the requirements of the REACH Regulation were also fulfilled. Thus, the results of this field study are fully reliable and relevant for the hazard assessment of the fertilizer calcium cyanamide in the soil compartment. In addition, and referring to our three aims listed in the introduction we can conclude that:

- Firstly, the hazard of calcium cyanamide applied as a granulate to Collembola could be assessed in a standardized way. In terms of diversity and ecological functions, this group is an important representative for the soil organism community of many European soils.
- Secondly, the information collected in this field study could be used as a basis for discussions on a Collembola field study guideline, which would be suitable for a broad range of chemicals. Using this information—and in combination with experience gained in earthworm field studies, a standardized field study with Collembola could be formulated according to ISO and/or OECD rules.
- Thirdly, based on the information collected in this study it would be possible to start the process of including such a study into the canon of the REACH regulation. An inclusion of a field-relevant assessment would improve the current hazard and risk assessment under REACH. In addition, it could be helpful for other national or international assessment processes as well.

In conclusion, due to the long-term experience in agricultural and ecotoxicological field testing, the results of field studies are a relevant contribution to the overall evaluation of substances which may enter the soil compartment. Thus, such studies are an important improvement for an overall and systematic evaluation of the effects of anthropogenic stress on soil biodiversity and functions [15].

Abbreviations

a.i.: Active ingredient; BBA: Biologische Bundesanstalt für Land- und Forstwirtschaft (German Federal Institute for Agriculture and Forestry); CPCAT : Closure Principle Computational Approach Test; DIN: Deutsches Institut für Normung (German Institute for Standardization); EC: European Community; ECHA: European Chemicals Agency; EFSA: European Food Safety Authority; EU: European Union; FAO: Food and Agriculture Organization of the United Nations; GLP: Good Laboratory Practice; GSBI: Global Soil Biodiversity Initiative; ISO: International Organisation for Standardization; ITPS: Intergovernmental Technical Panel on Soils; MDD: Minimal detectable difference; NOER: No observed effect rate; NTA: Non-target arthropods; OECD: Organisation for Economic Co-operation and Development; PPPs: Plant protection products; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH regulation (EC 1907/2006)); SCBD: Secretariat of the Convention on Biological Diversity.

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Author contributions

JR: assessment of the study—writing—original draft. AV: assessment of the study (regulatory aspects)—writing—original draft, review and editing. PS: conceptualization, methodology, investigation, review and editing. PE: conceptualization, funding acquisition, resources, review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]ECT Oekotoxikologie GmbH, Böttgerstraße 2-14, 65439 Flörsheim/Main, Germany. [2]Mesocosm GmbH, Forschungszentrum Neu-Ulrichstein, Neu-Ulrichstein 5, 35315 Homberg (Ohm), Germany.

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