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FOREWORD

This document presents a Guidance Document on the Organic Matter Breakdown in Litter Bags. In April 2004, Germany submitted a project proposal via a Standard Project Submission Form for the development of a Test Guideline on the breakdown of organic matter in litter bags. The Working Group of National Co-ordinators of the Test Guidelines Programme approved the inclusion of this new project on its workplan at its 16th Meeting.

In September 2005, the proposed draft Test Guideline was circulated to National Co-ordinators for comments. Due to the limited information available on the validation status, including the sensitivity and performance of the proposed test method, it was suggested that the project could be re-focused on the development of a Guidance Document. This suggestion was supported by several commenters, and Germany revised the document in light of comments received. Comments were submitted from six countries: Denmark, Finland, Germany, the Netherlands, Sweden, and the United States.

The Guidance Document was approved at the 18th meeting of the Working Group of the Test Guidelines Programme. It was recognised that when experience is gained in using the test method, a retrospective review of study results might be undertaken to evaluate the sensitivity of the proposed method, and possibly propose the development of an OECD Test Guideline.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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PART A: BACKGROUND INFORMATION ON THE METHOD AND IDENTIFICATION OF THE MOST APPROPRIATE METHOD

INTRODUCTION

1. The information provided in this part of the Guidance Document is mainly based on the report prepared during the Workshop on Effects of Plant Protection Products on Functional Endpoints in Soil (EPFES Workshop in Lisbon in the year 2002 (Römbke et al. 2003)). The purpose of this Guidance Document is to identify possible and suitable approaches to the evaluation of chemicals impact, and in particular plant protection products' impact, on soil organic matter breakdown. The test method and procedures describes in this document are mainly based on previous studies conducted in Europe to address the European regulation; other OECD member countries may not currently have similar data requirements for agricultural chemicals. However, methods identified in this document, and specifically the litter bags test method, add important tools to a battery of existing standardized protocols for assessing chemical impacts on the soil biota communities. Procedures outlined in this Guidance Document are primarily intended for the evaluation of agricultural chemicals but they can also be applied to ecological risk assessment activities at contaminated sites, as well as to laboratory chemical toxicity testing.

BACKGROUND CONSIDERATIONS

2. To assess the environmental risk posed by the use of agricultural chemicals on soil organic matter breakdown, a European Directive (EC Directive 91/414/EEC (EC 1991)) and its subsequent amendments (Directive 96/12/EC; EC 1996) stipulate that plant protection products should be evaluated for their possible effects on the organic matter (OM) breakdown. This function is considered to be one of the most important properties in soil, mainly provided by the community of soil micro- and macro-organisms (Swift et al., 1979; Cadisch and Giller, 1997; Lavelle et al., 1997). Experience with the litter bag test is increasing and draft test protocols have been developed. However, questions remain. For example, is the litter bag test the most appropriate method for assessing effects of plant protection products on OM breakdown? If so, what are appropriate triggers for actions based on the test results? To support the UK Pesticides Safety Directorate (PSD), the Department for Environment, Food and Rural Affairs (DEFRA) commissioned a study to review the state of knowledge regarding test methods for assessing the breakdown of OM and their relevance to risk assessment for plant protection products. It also investigated the role of soil micro-organisms and soil fauna in plant litter decomposition (Knacker et al., 2003).

3. According to the EC Directive 91/414/EEC, a test assessing the effects of a pesticide on OM breakdown is required under certain conditions (mainly related to persistence and toxic effects on soil biota). Until recently, tests with earthworms were the only soil fauna test used routinely in the risk assessment scheme (OECD, 1984, 2005). Now, standardised test methods using Collembola (ISO, 1999), Enchytraeidae (OECD, 2005) and test proposals for Gamasida (Bakker et al., 2003) are also available. The relevance of these single-species tests for the assessment of the effects on functional endpoints is presently unclear, and for other functionally important groups of soil fauna (e.g., Diplopoda, Isopoda, Mollusca, Nematoda and Protozoa), similar standard test methods are lacking (Van Straalen and Van Gestel, 1998). Furthermore, these tests do not take into account the inter-specific relations that can modify pollutant effects.

4. Tests on microorganisms and microbial processes, standardised by OECD (e.g., OECD 2000a, 2000b), are difficult to relate to OM breakdown for 4 principal reasons:

1. The degree of functional redundancy among microflora is unknown;

2. Some tests are too general (e.g., soil respiration);

3. Some microbial tests are too specific (e.g., nitrification, enzymes);

4. The relative contribution of single enzymes to the decomposition process can hardly be quantified.

5. Multiple enzyme activities might have a predictive value for assessing the risk to OM breakdown if studied simultaneously using the 'Biolog' method (Dighton 1997), since this method does not depend on the identification of particular microbial species. It can be automated as a high-throughput screening tool and results can be obtained in 70 hours. However, this method also has drawbacks, in particular it is suitable only for extractable and cultivable microorganisms, which make up a small proportion of the soil microflora (e.g.< 0.01%; Barr et al. 2002).

6. An alternative (or additional) approach is the catabolic response profile (CRP) (Degens and Harris 1997). This approach directly assesses the catabolic diversity of microbial communities by adding a range of simple organic substrates directly to soil and measuring the short-term catabolic activity as CO_2 output. It is not limited to cultivable microorganisms, but problems of functional redundancy might occur if substrates are utilised by a wide range of microflora. Clarification is also required on which aspects of catabolic diversity are most relevant to OM breakdown. The 'Biolog' and CRP methods offer flexibility in the choice of substrates that can be used and can be developed further as predictors of OM breakdown. One must be aware, however, that all purely microbiological methods cannot reflect the interactions with other soil organisms. In addition, these methods cannot be used for registration purposes in the immediate future because no standard guideline is available.

7. The direct determination of enzyme activity pattern of soil samples is now possible. By suspending the soil sample to buffer and by distributing it to multiwells containing fluorogenic substrates, it is possible to measure several enzyme activities within a short incubation time using a fluorometer. *E.g.* by using the ZymProfiler ® test kit, it is possible to measure form 10 to 12 different enzyme activities important in the mineralisation of P, N, S and C (Vepsäläinen et al. 2001 and 2005, Niemi and Vepsäläinen 2005). The method has been proposed to be internationally standardised at ISO TC 190 Soil Quality working group.

Are litter bags appropriate for the functional endpoint test?

Comparison of available methods and choice of the best available method for the time being

8. Five methods that could have relevance both to the functional process of OM breakdown and to the risk assessment of pesticides are reported in the literature. These are the litter bag test (Kula and Guske, 2001); the minicontainer test (Eisenbeis et al., 1999); the cotton-strip assay (Harrison et al. 1988), stable C and N isotopes (Nagel et al. 1995), and the bait-lamina assay (von Törne, 1990). The main features of these methods are summarised in Table 1 (Knacker et al., 2003). Useful comparative studies are rare (Paulus et al., 1999).

Table 1. Comparison of integrative functional methods used under field conditions to measure the process of organic matter breakdown (adapted from Knacker et al., 2003)

Method	Litter bag	Mini-container	Cotton strip	^{15}N, ^{14}C,^{13}C isotopes	Bait lamina
Principle of the method	Decomposition of OM enclosed in gauze bags or various box types	Decomposition of OM enclosed in small containers	Measurement of cellulose decomposition	Detection of isotopes from ^{15}N or ^{14}C or ^{13}C -labelled organic matter	Feeding of soil organisms on bait material
Organic material used	Straw, hay, leaf litter, cellulose or equivalent	Chopped leaves, straw, cellulose or equivalent	Standardised cotton cloth material	^{15}N or ^{14}C or ^{13}C –labelled plant material	Bait material, e.g. dried, pulverised leaves mixed with agar and charcoal
Exposure of organic matter	Bags or containers on the soil surface or buried	Mini-container on the soil surface or buried	Strips buried horizontally or vertically in soil	Material directly mixed into the soil	Perforated PVC strips inserted into the soil
Duration	1-12 months	2-6 months	2-26 months	variable	1-4 weeks
Endpoints	Mass loss; chemical, microbial and faunal parameters	Mass loss, chemical and microbial parameters	Loss of tensile strength of cotton fabric	Amount of isotopes in various soil fractions	Number of empty holes corresponds to bait material eaten
Data assessment	Comparison of mass loss with control and reference substance	Comparison of mass loss with control	Comparison of loss of tensile strength between treatments	Not defined since the method has not been used in ecotoxicology	% of empty holes; vertical distribution of empty holes
Remarks	Guidance available (Kula and Guske 2001)	Experience is limited since method has not often been applied	Tensile strength may increase due to the growth of certain fungi	Mainly applied in laboratory studies	Experience is limited since method has not often been applied
References	Bocock and Gilbert 1957, Paulus et al. 1999, Siedentop 1995	Eisenbeis 1994	Kuzniar 1948, Harrison et al. 1988	Nagel et al. 1995 (^{15}N), Rochette et al. 1999 (^{13}C)	von Törne 1990, Kratz 1998, Irmler 1998

9. Therefore, these methods were assessed on the basis of 14 suitability criteria (Table 2; see Knacker et al. 2003). Only the litter bag test was found to be sufficiently well developed and relevant to be suitable as a technique for assessing pesticide effects on OM breakdown in the field. Long-term experience in soil ecology and ecotoxicology, high practicability, and most importantly, ecological relevance were among the decisive criteria. For example, the proposed mesh size of 5 – 10 mm is seen as a compromise between ecological relevance (i.e. access of macrofauna possible) and practicability (i.e. negligible loss of OM). When comparing the potential measurement endpoints, mass loss is considered the best option; primarily because of its integrative nature. Because organic matter breakdown is operationally defined here as litter mass loss or breakdown rate, it refers to the disappearance of OM from the litter bag rather than to pure mineralisation. Effects on mineralisation in later phases (i.e. > 1 year for straw are not assessed with the litter bag test).

Table 2. Evaluation of integrative functional methods used under semi-field or field conditions to measure the process of organic matter breakdown (Knacker et al. 2003; see also for full explanation of criteria). Methods are classified as compliant with the criterion (+) or as non-compliant (-). Question marks denote that sufficient information to determine compliance is lacking. ERA = environmental risk assessment.

Criteria for the selection of test methods	Litter bag	Mini-container	Cotton-strip	Isotopes	Bait-lamina
Relevance for existing ERA schemes	+	+	+	+	-
Ecological relevance					
Quality of resource (OM)	+	+	-	+	-
Access of soil organisms to OM	+	-	+	+	+
Experience	+	-	-	?	+
Flexibility					
Use in various terrestrial ecosystems	+	+	+	+	+
Use of different resources	+	+	-	+	-
Robustness	?	?	?	+	?
Practicability	+	+	?	-	+
Sensitivity	+	?	?	?	+
Data assessment	+	+	+	+	?
Reproducibility and repeatability	+	?	?	?	?
Standardisation and validation	+	-	-	-	-

10. Pending clarification of further research, the methodology presented in Part B of this Guidance Document is considered to be sufficiently well developed for application in regulatory ecotoxicology. The integration of stable isotope methods with the litter bag test may improve information on C- and N-transformation in the field to allow the risk to be determined without requiring the full time course of the

litter bag test to be completed. Perhaps the most difficult task resulting from inclusion of the litter bag test in current risk assessment schemes will be to gather more information on the relation between single species tests and litter bag studies.

Special considerations for the bait-lamina method

11. During the last years, experience with the bait-lamina method increased. The suitability and practicability of this additional functional method could be shown in studies assessing the effects of the fungicide carbendazim in Terrestrial Model Ecosystems from four European grassland and crop sites (Förster et al., 2004) as well as evaluating the impact of metals in the vicinity of an English smelter plant (Filzek et al., 2004). Referring to these studies and experiences the International Organisation for Standardisation (ISO) is currently considering the standardisation of this method. However, the investigated endpoint of the bait-lamina-test is not OM breakdown but the feeding activity of soil invertebrates (mainly macro- and mesofauna). For example, in a study comparing OM breakdown and feeding activity in four forest types, no correlation between these two data sets could be found (Römbke et al., 2006). Therefore, because of its functional endpoint and simplicity, the bait-lamina test may be a valuable addition to the battery of soil tests but not an alternative to the litter bag test.

SPECIFIC ISSUES

12. In the following, some specific issues concerning the litter bag test are discussed:

Selection of the most appropriate test substrate

13. In the past, various natural materials (all types of plant residues, predominantly tree leaves and crop residues) as well as more standardised organic material (e.g. cellulose) have been used in litter bag tests (an overview is given in Knacker et al., 2003). When assessing the effects of pesticides in a higher tier test and thus under - as far as possible - field relevant conditions, the most appropriate land use type is arable land and the most relevant crops are cereals. According to literature, out of 34 studies investigating the effects of pesticides on OM breakdown, wheat straw was used in 10 cases (in the other studies on arable land barley and maize residues were used), meaning that experience with this material is good. In addition, the decomposition of straw is slow and the decomposition of wheat straw is even the slowest, which fits to the overall aim of these litter bag tests (i.e. assessing persistent compounds). Therefore, ecological and field relevance as well as practicability support the choice of wheat straw as test substrate in litter bag tests.

Influence of soil type on OM breakdown

14. Information on the influence of soil type is limited. Breakdown of OM can be affected indirectly by soil pH, which can be explained by the presence of different soil organism communities: for example, earthworms are often missing in acid soils (e.g. Roper and Smith, 1991). However, in arable soils with usually similar (slightly acid to neutral) pH-values, such an effect is not expected. Soil texture did not affect mineralisation rates in studies by Sharkov and Lodko (1997) and Thomsen et al. (2001). Climatic and biological factors (which may differ between seasons) are considered to have greater influence on OM breakdown than soil properties. The current text of the Guidance Document takes this issue into account by requiring a comprehensive characterisation of the soil at the study site. In addition, it is recognised that any field study reflects a unique situation in space and time. However, by selecting an arable site under cultivation considered as the most relevant case and by following the application requirements for the test compound in a litter bag test, the results of such test can be considered representative.

Justification of the proposed standard exposure scenario

15. The standard scenario proposed in this method is based on the interpretation of the requirements for the registration of pesticides as outlined described in EC Directive 91/414/EEC, i.e. the test is focussing on persistent pesticides. If the test is used for other purposes (e.g. for soil quality assessment), the design may vary. In addition, the test performance and in particular the application of the test substance aims to be as close as possible to realistic Good Agricultural Practices This means that the persistent test substance is applied in a 2-step process:

Step 1: The long-term plateau concentration is incorporated into the uppermost 10 cm of the soil.

Step 2: The accumulated annual application rate considering crop interception is applied on the soil surface after burying of litter bags. Seed treatments or granules should be applied according to the relevant conditions of use of the product.

16. The main principles of the calculation of the plateau concentration are the long-term use of a product for several years according to the label (until a steady state plateau has been reached), mechanical incorporation of the product into the soil layer according to Good Agricultural Practice (20 cm deep ploughing considered as a relevant case for arable soils, 10 cm for permanent crops), and natural fate of the substance in the soil layer (preferably based on data from field studies). The plateau concentration in soil also must be determined in line with the requirements of EC Directive 91/414/EEC and using the relevant FOCUS (2000) guidance.

Further exposure scenarios

17. As mentioned above, the performance of the litter bag test should reflect the use pattern of the test substance as close as possible. Therefore, the EPFES workshop identified a combination of the plateau concentration followed by the application of the accumulated annual application rate (i.e. the organic material wheat straw was not directly treated) as the most relevant exposure scenario for persistent pesticides. Thus, the main aim of the current test method is the investigation of effects of long-term residues of persistent pesticides in soil on organic matter breakdown.

18. However, a scenario in which treated crop residues are used as test substrate was considered to be appropriate under certain circumstances. During the development of the current method, several tests in which the test substance was applied on top of the litter bags before burying them have been performed (i.e. with a different exposure scenario as compared to the standard scenario). Thus, the test method itself is exactly the same except the application procedure. As already indicated, care must be taken when using such results in assessing the risk of a pesticide under normal agricultural conditions.

The use of functional tests in soil quality assessment

19. The EPFES workshop concluded that protocols for measuring effects on OM breakdown for contaminated site soil assessments should be a higher-tier component in an accepted tiered framework (such as that initiated by CLARINET (Schelwald, 2001)). This conclusion is also supported by experience on the use of such methods in soil quality assessment, in particular when studying pollution gradients (e.g. Phillipsen et al., 1999; Filzek et al.; 2004; Kools, 2006).

Sensitivity of the litter bag test

20. Both enhancement and delay of the decomposition process are considered to be an ecologically significant effect as a change in each direction may indicate a disturbance of the process.

21. At the EPFES workshop it was discussed that a difference of > 25 % in mass loss compared to control at test termination indicates an unacceptable risk. If a difference of > 10 % in mass loss occurs at test termination, concern should be raised and at least a higher tier risk assessment is needed. The method therefore should be able to detect differences in the magnitudes given.

22. For the time being no studies are known which exceeded this triggers at the end of the study indicating an unacceptable risk of a substance. Several of these studies showed an effect > 10 % compared to control at one or more sampling dates during the course of the study. From these data it is concluded that the method is sufficiently sensitive to detect effects of a test substance on breakdown of organic matter.

Validity of the litter bag test

23. Due to a lack of time and money, no validation exercise (ring-test) was performed up to now. The need for validation was considered an important research need as an outcome of the EPFES workshop (Römbke et al, 2003). The litter bag test has already been used within pesticide registration in EU-member states. A check of about 15 of these data sets, conducted according to the method described in part B, showed that the given validity criterion (60 % mass loss in the control plots at the end of the study) have been fulfilled in the studies.

PART B: DESCRIPTION OF THE TEST METHOD

INTRODUCTION

24. This guidance document is designed to assess the effects of chemicals in general, and pesticides in particular on the breakdown of organic matter in soil. The test can be used to address concerns regarding the breakdown of plant litter material, particularly when exposed to persistent compounds in agricultural and horticultural soils. After appropriate modifications, this method can be used to assess the effects of other chemicals (e.g. biocides) as well as for the assessment of soil quality at contaminated sites.

25. The litter bag method is considered to be the most appropriate one for assessing the effects of chemicals on soil function among the currently available methods (Kula and Römbke 1998; Römbke et al., 2003). This conclusion is primarily based on a comparison of five methods using criteria such as practicability and the amount of experience with the different methods (Knacker et al., 2003). Experience with litter bag tests has been collected within the EU pesticide registration (see EU Directive 91/414/EEC, specifically Annex III, point 10.6.2; EC 1991). The focus of this method is solely on assessing risks to the process itself and not to the specific organism groups that might be involved in the process.

PRINCIPLE OF THE TEST

26. Litter bags containing dried organic material are buried in the soil of a field site which is treated with the test substance in an amount representative of realistic worst-case agricultural use. The litter bags are removed chronosequentially from the soil after specified time periods. The measurement endpoint, mass loss of the organic material, is quantified in control and treatment groups for each sampling date.

INFORMATION ON THE TEST SUBSTANCE

27. If a plant protection product is to be tested, the test should be performed with a formulated product. The relevant formulation should be used where appropriate.

28. For metabolites, there are 2 ways of addressing the issue. The preferred way of testing is to apply the metabolite and investigate it in a study on its own. As a second choice, e.g. if synthesis of the metabolite is a problem, a study may be performed using a formulation containing the active substance. In both cases, the concentration of the metabolite in soil should be measured analytically.

29. The following information relating to the test substance and its agricultural use is required for the design of appropriate test procedures: proposed crop species, recommended concentration of the pesticide and mode and timing of application(s) according to relevant use conditions,water solubility, K_{oc}, vapour pressure, and information pertinent to the fate and transport of the substance in soil (e.g., mobility, residence time and routes and rates of dissipation), available data on toxicity to soil organisms.

VALIDITY OF THE TEST

30. The test is considered valid if at least 60% mass loss has occurred at the end of the study in the control plots. For the time being, a maximum coefficient of variation of 40% for mass loss in the control plots ($n = 6$) is recommended for those data generated within the first 6 months of a test.

DESCRIPTION OF THE TEST

Site selection and characterisation

31. Because arable land is most relevant for the majority of the proposed field uses of plant protection products, the use of arable land under cultivation is considered the most relevant scenario for testing. Grassland could be used in special cases if applicable to proposed non-arable uses of a test substance.

32. The study site soil should be characterised and the following soil properties should be reported: particle size distribution (texture), pH, water holding capacity, and organic matter content before starting the test. When the distribution of these parameters in the site is not homogenous, this information has to be considered for the lay-out of the plots (in extreme cases, the site may not be suitable for testing purposes). The soil moisture content should be measured at each plot at the depth of the litterbags (i.e. 5 cm), at the start of the test and on each sampling date. Determination of the Cation Exchange Capacity (CEC) is recommended because it may provide additional information concerning the fate of the pesticide in the soil. Information on the sorption of the test chemical to the soil of the site (K_{om} or K_{oc}) may be obtained from available data on the fate of the product.

33. The following biological properties should be characterised at the study site: vegetation (crop) type and cover. Optionally, other soil biological parameters, including microbial activity or earthworm abundance may be determined. The history of crop cultivation and pesticide applications within the last three years at the test site also need to be identified and reported.

Study design

34. The replicates (= plots) of both control and treatment should be randomly distributed at the study site. A size of $25m^2$ (i.e. 5 x 5m) per plot is considered to be the minimum area. The pesticide should be applied homogeneously across the entire test plot including right up to the border of each plot; however, no bags should be placed within 1 m of the plot border. In addition, plots have to be separated by untreated, ca. 3m wide strips in order to avoid cross-contamination (see Fig. 1). Bags should be distributed evenly within each plot. Random sampling of the bags must be performed.

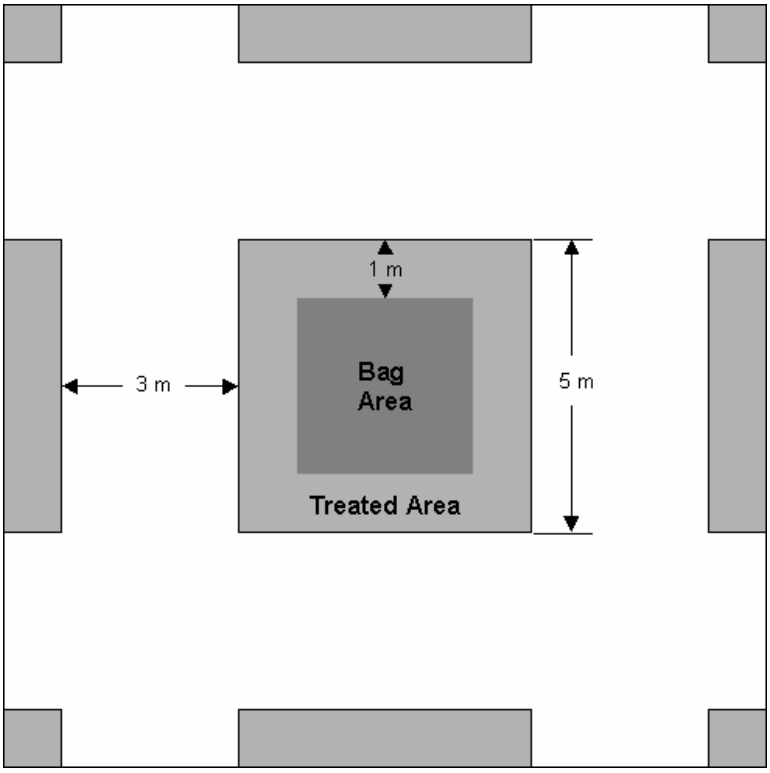

Fig. 1: Scheme of the recommended plot design (grey area)

35. The total number of buried bags depends on the number of sampling events. As a minimum, six replicated plots for treatment and six replicated plots for control, each with 8 litter bags per sampling date, are recommended. This results in a total of 96 litter bags for both treatment and control per sampling date. The test should include at least 1 treatment rate and a control.

Litter bags

36. Litter bags should be constructed using a non-degradable material (e.g., synthetic mesh material) with a mesh size of 5 to 10 mm. The size of the bags should be ca. 10 x 20 cm. Each bag should be filled with 4 g dry mass of wheat straw (i.e. stalks, not leaves) only, to ensure close contact between the test substance in soil and the wheat straw in the litter bags. Straw should be dried for at least 4 hours at 30 to 35 °C before filling bags. In litter bags that are not individually marked before burying the amount of litter should be 4 g +/– 0.1 g; if marked bags are used, the individual weight of 4 g +/– 10% must be recorded per bag. The straw ash-free weight is determined by combusting a representative straw sample (4 g each) with 10 replicates. Combustion conditions (duration and temperature) may differ between laboratories, but must be identical to those used during processing of the sampled litter bags. The litter material (wheat straw) should be placed into the litter bag in a thin and even layer.

Season for testing

37. The appropriate season for testing should be selected according to the intended use pattern of the test substance. If a product may be applied in spring and in autumn according to the label, it has to be decided on a case-by-case basis which represents a realistic worst-case scenario.

EXPOSURE

Plateau concentration

38. The plateau concentration expected to be present in soil after long-term use of the active substance under consideration (in mg active substance/kg dry weight soil) should be calculated using guidance from the FOrum for the Coordination of pesticide fate models and their USe (FOCUS) soil and groundwater groups (FOCUS 1996, 2000). Note that for the purposes of the litter bag test, the minimum or baseline steady-state plateau concentration does not include the final annual cumulative dose for the year of the study, i.e. it is not the peak plateau concentration. This annual cumulative dose is applied subsequently. The calculation of the plateau concentration, as per the guidance from FOCUS, is based on a soil depth of 20 cm; this takes account of mechanical tillage operations (such as ploughing).

39. For certain cases, e.g. pesticides applied to less cultivated sites like orchards or minimum tillage systems, there may be special considerations required when calculating the long-term soil PEC (Predicted Environmental Concentration) and achieving this concentration in soil. In such cases it will be more precautionary to assume that the PEC soil may be determined over a depth of 5 cm.

40. However, in the litter bag test performed on arable land, only a soil depth of 10 cm has been chosen for achieving the plateau concentration. This soil depth of 10 cm has been chosen to avoid excessive disturbance deeper in the soil and to cause as little impact on soil organisms as possible (for details of the application see § 18). As the litter bags are buried in 5 cm depth (see paragraph 19) they are fully exposed to the plateau concentration achieved.

Annual cumulative application rate

41. The annual cumulative application should be made in 1 dose on bare soil or on soil with only little plant cover. “Annual cumulative application” refers to the sum of all applications of the pesticide within a year. This should make no allowance for degradation of the test substance in soil. The crop interception levels for the applications at different growth stages should however be taken into account (see FOCUS 2000).

PROCEDURE

Application

Plateau concentration

42. The test substance should be applied at the amount required to achieve the steady-state plateau concentration within the top 10 cm of the soil. To that end, 50% of the amount necessary for reaching the plateau concentration in 20 cm soil depth (see guidance from FOCUS) is used. Finally, this amount is mechanically incorporated into the top 10 cm. It is not appropriate at this stage to water the test substance into the soil because of the resulting unpredictable distribution of the test substance in soil. Therefore, a careful mechanical incorporation with a grub or harrow is recommended to yield an even distribution of the test substance within the uppermost 10 cm soil layer. If a study is undertaken in permanent crops a special design may be needed as no mechanical incorporation into the soil is possible. The control plots should be treated exactly in the same way except for the addition of the test substance. For the timing of the incorporation of the plateau concentration during the study see ANNEX III.

Annual application

43. One to 2 weeks after incorporation of the test substance, the litter bags should be buried horizontally in the soil to a depth of about 5 cm. After burying the bags, the soil above the bags should be slightly compressed to ensure good soil contact with the wheat straw. The total annual application rate should then be applied within 1 week of the litter bags being buried. The application should be made over bare soil or on soil with a low level of plant cover (e.g., turf should be closely mown). If plant cover is present, this has to be considered in relation to the interception rates and dose applied. For the timing of the annual application during the study see ANNEX III.

44. Special use patterns such as seed treatment or granule applications should, as far as possible be assessed in accordance with the proposed agricultural use. In the case of treated seeds or granules, these should be sown/applied according to the relevant use conditions. If an active substance is used for both a seed dressing and spray application in the same crop and season, it may be appropriate to incorporate seeds dressed at the normal rate and then after burying the litter bags, apply the additional annual spray application rate onto the soil surface.

Irrigation

45. If no or little rainfall occurs within three days of the annual cumulative application, irrigation of the site with water is considered necessary to achieve optimal conditions for exposure. The amount used should be realistic according to regional and climatic conditions. A total of at least 10 mm (i.e. 10 L per square meter) of precipitation (rainfall plus irrigation) within three days after the spray application is desirable.

Soil analysis for test substance

46. The pesticide concentration in soil must be measured by soil residue analysis to verify the exposure concentration in soil and to ensure that the litter bags are exposed to the test substance. Sub-samples of soil should be collected and analysed immediately after incorporation of the plateau concentration into the soil. Three days after the spray application of the annual cumulative dose, a second set of soil sub-samples should be collected (if irrigation is undertaken, soil samples for residue analysis should be collected after irrigation), see Annex III. Collection of soil samples for residue analyses should be performed according to standardised protocols and standardised analytical methods to measure the pesticide should also be used where possible. In light of the wide variability in field studies it is recommended that a range of 50% to 150% of the nominal concentration should be reached.

Maintenance during the test

Plant cover on the study site

47. Ideally, the soil of cultivated sites should be free of vegetation during the pesticide application period; however, it is appropriate to allow crop plants to grow during the remaining test period. Sowing of plants (e.g. crop species such as clover) must be done after the plateau concentration has been incorporated into the soil but should be done before the litter bags are buried and the annual cumulative application is made. No harvesting of the crop should be performed at the test site in order to avoid disturbance of the soil.

48. When testing herbicides, the use pattern of the product and/or the timing of application must not impact growth of the crop species. Depending on the use pattern of the product, it is important to choose a suitable crop plant and sowing period. Differences in plant cover between control and treatment plots can be manipulated, for example, by applying an already registered herbicide that is known not to affect

organic matter breakdown or by hand weeding. Any additional influence must be kept to a minimum, and control and treatment plots must be treated in the same way.

Other treatments

49. Apart from the circumstances mentioned above, the use of fertilisers or other pesticides should be avoided as far as possible during the test. If treatments are necessary to ensure plant growth and homogeneity of the study site, control and treatment should be treated in the same way. The number, timing, and rates of application(s) of pesticides and/or fertilisers to the study site during the study and the previous three years should be reported.

Recording climatic data

50. Precipitation and air temperature data should preferably be recorded directly at the study site in order to characterise the climatic conditions during the test period. Otherwise, such data should be obtained from a weather station located as close as possible to the study site.

Sampling and test duration

51. The test should include at least three sampling dates within the first six months, with the first sampling after about 1 month. The test duration is at least 6 months with a maximum in the standard test design of 12 months. If 60% mass loss in the control is not reached within 6 months, then the study should be continued for up to 12 months.

52. If statistically significant differences in organic matter mass loss or breakdown rate between control and treatment bags are observed after 6 months (rate calculation based on the samplings between 0 and 6 months), then continuation of the test for a maximum of 12 months from the start is recommended. Also, any indication that litter breakdown rates between the control plots and treatment plots are diverging should also lead to continuation of the study. It is recommended to consider an additional sampling (e.g., after 9 months) in case the study has to be prolonged after 6 months. The potential need to continue the study (including additional samplings beyond 6 months) must be accounted for when determining the number of litter bags to bury at the start of the study.

53. Usually the study is terminated after 12 months, but if it is still not clear at this time whether there is difference between control and treated plots, then several options are available (e.g., performing a litter bag test following a dose-response design or recommended use pattern).

Collection and processing of litter bags

Collection of litter bags

54. A randomisation procedure has to be used to identify those litter bags which are taken at each individual sampling date. They are taken from the plots manually, placed individually into plastic bags (if unmarked litter bags were used, the plastic bags must be marked) and promptly transported to the laboratory. Care must be taken that material lost from the litter bags and found in the plastic bags will be included in further processing. The litter bags should be processed as soon as possible after collection. Collected bags (or their content alone) are placed into open plastic trays. Processing of the litter bags in the laboratory depends on the method used to separate straw from soil material input. If this separation is not conducted immediately by wet sieving, the bags must be air-dried, for example, in open plastic boxes to interrupt biological activity.

Separation of straw and soil material

55. After air-drying the litter bags, any visible extraneous plant material (e.g., roots), soil organisms (e.g., earthworms), and debris must be removed by physically separating them from the remaining litter. High amounts of soil material will also disturb ignition of straw and influence combustion results by releasing humidity and organic matter. Therefore, soil within the bags must be separated as far as possible from the remaining litter material. Separation can be done by dry or wet sieving (see below), and whichever method is used, a mesh size of 0.5 - 0.63 mm is recommended so that only straw and coarse sand particles remain on the sieve.

Dry sieving

56. The dried contents of the litter bag are lightly mortared and sieved. Dry sieving does not require an additional, time-consuming drying step and it is most appropriate for the evaluation of litter bags removed from sandy soils. Subsequently, the samples can be stored at 4 °C in airtight containers for up to 2 weeks.

Wet sieving

57. The litter bag content is carefully washed in the sieve using tap water to remove any remaining soil particles. Wet sieving may have advantages when litter bags are removed from heavier soils. The washing of litter can be done immediately after the litter bags have been collected. Once sieved, the remaining straw must be dried for at least 12 h at 30 to 35°C (see paragraph no. 13) to avoid further microbial degradation.

Drying and grinding of straw

58. Because all results should be based on ash-free dry weight (AFDW), it must be assured that the litter material (whether wet or dry sieved) has been dried for at least 12 hours at 30 to 35 °C to adjust its moisture content and achieve conditions comparable to those during preparation of litter bags. Depending on the size of porcelain dishes used for combustion, the straw may be chopped and ground in order to homogenise the sample and promote combustion.

Ignition of straw

59. An empty, dry porcelain crucible should be weighed, filled with the oven-dried straw remnants from the litter bag and weighed again before combustion. Combustion efficacy is influenced by temperature, duration, surface area of the crucible, amount of straw included, and the amount of soil material remaining. Because the combustion results during evaluation are compared to those obtained by combustion of pure straw under standardised conditions in each laboratory, no general instructions with respect to temperature and duration are given. Experience has shown that a minimum temperature of 600°C and duration of 30 minutes is usually required. After ignition, the crucible should be cooled down under defined conditions (e.g., in a desiccator) until it can be handled and then re-weighed.

Calculation of loss on ignition and decomposition

60. Ash-free dry weight is calculated by subtraction of resulting ignition residue from the straw remnants (dry weight). Breakdown (organic matter mass loss) is calculated by subtracting the ash-free dry weight of the remaining litter from the ash-free dry weight of the initial input. Because separation of straw and soil material will not always be sufficient, a soil and a litter correction factor should be used to account for the release of organic matter from soil particles (= Soil correction factor [SCF]) or the mineral content,

including incomplete ignition, of the wheat straw material (STraw Correction Factor (StCF)). All ignition results are then corrected by these factors.

61. Before starting the test, both correction factors are calculated by the loss of ignition, under standardised conditions, of different amounts of either soil material from the study site or wheat straw used in the test (10 replicates each). They are defined as follows:

Burning of soil samples: SCF = (soil input – ash residue) / soil input

Burning of straw samples: StCF = ash residue / straw input

Procedure for obtaining the results

62. A stepwise calculation should be followed for evaluation of the results:

- Loss on ignition (LOI) = MAT – ASH

  (MAT = g input material from a litter bag; ASH = ash residue after burning)

- Corrected loss on ignition (CLOI) = LOI - (SCF x (ASH/1-SCF))

- Non degraded straw (NDS) = CLOI + (StCF x CLOI x SCF/1-SCF)

63. In Annex II an example is calculated in order to clarify the use of the correction factors.

Summary and timetable of the litter bag test

64. The individual steps of the test are summarised in Annex III (please note that the days after starting the tests have been approximated and will depend on the actual weather conditions and the cultivation measures that are necessary at various dates between the sampling events).

REPORTING

Description of results

65. Weight of the remaining wheat straw (ash-free dry weight) should be assessed for each sampling date after including the correction factors described above. The results should be expressed as the mean percent mass loss of the wheat straw.

Comparison of start weight and end weight per litter bag

66. The following items should be reported:

- Sampling date
- Start weight of the wheat straw in each litter bag (g)
- End weight after ignition of the wheat straw in each litter bag (g)
- Loss in g (start weight – end weight)
- % loss

Formula:

% mass loss = ((start weight – end weight) / start weight) x 100

or

% mass loss = (1 – (end weight / start weight)) x 100)

67. Per sampling date and plot of each treatment the weight loss (mean and standard deviation of the 6 plots) has to be calculated.

Comparison between control and treatment given as mean of the 6 plots

68. The following items should be reported:

- Sampling date
- Mean mass loss in control in % (including standard deviation)
- Mean mass loss in treatment in % (including standard deviation)
- % Effect (mass loss) in comparison to control
- Negative numbers indicate an enhancement of mass loss compared to control

Formula:

% effect (mass loss) = ((mean mass loss control – mean mass loss treatment) / mean mass loss control) x 100

or

% effect (mass loss) = (1– (mean mass loss treatment / mean mass loss control)) x 100

69. Additionally the breakdown (mass loss) rate between each individual sampling date and between the start of the study and the last sampling date should be reported for the control and the treatment. It is calculated as the quotient of mass loss over time. Daily (or annual) decay rate constants of litter residues can be calculated by using the single negative exponential decay model $m_t/m_o = e^{-kt}$, where m_t/m_o = proportion of mass remaining at time *t, t* = time elapsed in days (years), and *k* = the derived daily (annual) decay constant. This approach will allow to integrate mass loss data over the entire test duration instead of relying on data comparisons for individual sampling dates.

Treatment of results

70. The mean value of 8 litter bags per plot and sampling date will be used for statistical analysis. These data should be checked for normality and homoscedasticity (i.e., the distribution of 2 random variables). If the data are skewed when plotted or the variances are unequal across treatments, it might be necessary to transform the data. The question of whether to transform raw data shall be decided on a case-by-case basis. The assumptions of normality and equal variances must be re-examined after the data have been transformed. If transformation has conferred normality and homoscedasticity, then a student *t*-test (2-sided) should be applied to the data for maximum power. Otherwise, the non-parametric Wilcoxon-Mann-Whitney test (2-sided) should be applied to the data for each sampling period. For experimental designs involving multiple exposure concentrations, ANOVA procedures can be applied to the data, if the assumptions of the model (normality and equal variances among treatment plots) have been met. If the data

cannot be transformed to satisfy the parametric assumptions, for example the non-parametric Kruskal-Wallis rank analyses for multiple comparisons can be applied.

Test report

71. The test report should include the following information:

1) Test substance (including the active substance):

- Test substance identification according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature, batch, lot and CAS-number, purity
- Properties of the test substance
- Source

2) Litter bags:

- Material, loading methods, and procedures

3) Soil properties and biological properties of the site:

- Soil classification
- Particle size distribution
- pH, organic matter content
- Water holding capacity
- Vegetation type and vegetation cover
- Climatic data (at least average air temperature, precipitation)
- Maintenance of the site during the study
- History of the test site (pesticide use within the last three years, cropping pattern)

4) Application:

- Date and description of the technique used to apply the test substance to the soil
- Calculations and methods to determine application rates and the amount to be applied to the plot
- Calibration details for spraying equipment if appropriate
- Soil residue analysis (including method description)

5) Test results:

- Mass of remaining wheat straw in percent of the starting weight per litter bag, plot and treatment
- Percent mass loss in each litter bag (marked bags) or group mean at each time interval per plot and per treatment and control
- Mass loss in treatment compared to control mass loss for each plot and time interval
- Breakdown (mass loss) rate between each individual sampling date and between the start of the study and the last sampling date for the control and the treatment.
- Statistics
- Graph of the time course of mass loss for the treatment and control
- Deviations from procedures described in this guidance document and any unusual occurrences during the test

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ANNEX I

Abbreviations

AFDW	Ash-Free Dry Weight
CEC	Cation Exchange Capacity
CLOI	Corrected Loss On Ignition
ERA	Environmental Risk Assessment
FOCUS	FOrum for the Coordination of pesticide fate models and their USe
LOI	Loss On Ignition
NDS	Non Degraded Straw
OM	Organic Matter
PEC	Predicted Environmental Concentration
SCF	Soil Correction Factor
StCF	Straw Correction Factor

ANNEX II

Example how to calculate the amount of non degraded (non decomposed) straw using the soil and straw correction coefficients

Assumptions (just for practical purposes):

A given soil has 10% of organic matter

A given straw type has 99% of organic matter

Soil correction factor (SCF)

If 2g of soil result in 1.8g of ash residue after burning in a muffle oven at standardised conditions, so the SCF is: SCF = (2 – 1.8) / 2 = 0.1

Straw correction factor (StCF)

If 2g of straw result in 0.02 g of ash residue after burning in a muffle at standardised conditions, so the StCF is: StCF = 0.02 / 2 = 0.01

Practical Measurement

It is assumed that we have 5g of dry material (organic matter plus soil) coming from a buried litter bag (after being dried, sieved, etc, according to the guideline = MAT)

After burning, the ash residue is 1.83g (= ASH)

Theoretical Assumption:

It is assumed that these 5g are 3g of straw plus 2g of soil (of course this is not known in a real case!). This means that the weight of the ash residue will be 90% of the soil weight plus 1% of the straw weight.

In theory, the ash-free dry weight (AFDW) of the straw would be 2.97g and the AFDW of the soil would be 0.2g

Calculations:

Loss on ignition (LOI) = MAT – ASH

LOI = (5g – 1.83 g) = 3.17 [g]

Corrected loss on ignition (CLOI) = LOI - (SCF x (ASH / (1-SCF)))

CLOI = 3.17 – (0.1 x (1.83/(1-SCF))) = 2.96667 [g]

Non degraded straw (NDS) = CLOI + (StCF x CLOI x SCF/(1-SCF))

NDS = 2.96667 + (0.01 x 2.96667 x SCF/(1-SCF)) = **2.96996 [g]**

All further calculations have to be done using this value.

ANNEX III

Summary of the key tasks required to be undertaken for a litter bag test (dependent upon the ensuing climate and cropping regime)

Time (days/ months)	Activity/ task
Pre-litter bag burial	Selection of the test site and characterisation of soil and site properties Preparation of straw and litter bags Preparation of field site and spraying equipment
Day 0	Preparation of stock solution for field plateau application First application of test substance to plateau concentration Incorporation of the test substance(s) into the soil pesticide residue sampling for chemical analysis
Day 0 to 14	If necessary sowing of plants prior to burying of the litter bags
Day 7 to 14	Burying of the litter bags
Post-litter bag burial	
Between Day 7 and 21 (within one week after burying the bags)	Preparation of stock solution for annual cumulative application Second application of the test substance (annual cumulative application rate)
Between Day 10 and 24 (within 3 days after the second application)	If necessary, irrigation of the treated plots Soil sampling and pesticide residue analysis
One month (after burying the bags)	First sampling period, possible need for cultivation (e.g. weeding) activity. Determination of the ash-free dry weight of the remaining straw in the laboratory

Three months (after burying the bags)	Second sampling period, possible need for cultivation (e.g. weeding) Determination of the ash-free dry weight of the remaining straw in the laboratory
Six months (after burying the bags) – Decision stage for study termination	Third sampling period, possible need for cultivation (e.g. weeding) Determination of the ash-free dry weight of the remaining straw in the laboratory Decision on whether the test can be terminated (i.e. mass loss in the control > 60%); otherwise repeat this step after a further 3 or 6 months.
Nine months (after burying the bags) recommended additional sampling and decision stage for study termination	Recommended additional fourth sampling period (possible need for cultivation (e.g. weeding) Determination of the ash-free dry weight of the remaining straw in the laboratory Decision on whether the test can be terminated (i.e. mass loss in the control > 60%); otherwise repeat this step after a further 3 months
Up to 12 months (after burying the bags)	Final sampling period. Termination of the standard test. Determination of the ash-free dry weight of remaining straw (if any) in the laboratory. Risk analysis Decision to extend beyond 12 months or move to higher tier testing.