OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Breakdown of organic matter in litterbags

INTRODUCTION

1. This test guideline is designed to assess the effects of chemicals in general, but is in particular valuable for the assessment of plant protection products (PPPs) of the breakdown of organic matter in soil. The study can be used to address concerns regarding the breakdown of litter material, particularly when exposed to persistent compounds in agricultural and horticultural soils. After modifying the method accordingly, it 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.

2. The litter bag method is considered to be the most appropriate of the current methods available (Römbke et al., in press). This conclusion is primarily based on a comparison of various 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 system (under EU Directive 91/414/EEC, specifically Annex III, point 10.6.2; EC 1991). Background information from the literature is available (Kula and Römbke 1998; Knacker et al. 2003; Frampton et al. 2002). A whole range of soil organisms are considered to be involved in the breakdown of organic matter. It is one of the most important functions of organisms in the soil, because it is integral to nutrient cycling. Background information on this process and its importance for agriculture is available (Swift et al. 1979; Cadisch and Giller 1997). The focus of this method is solely on assessing risks to the process itself and not to the separate organism groups that might be involved in the process.

PRINCIPLE OF THE TEST

3. 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 from the soil after certain time periods. As an endpoint, the mass loss of the organic material in control and treatment groups is determined for each sampling date.

**INFORMATION ON THE TEST SUBSTANCE**

4. The test should be performed with a formulated plant protection product. Where appropriate the lead formulation should be used.

5. If a metabolite is of concern there are 2 ways of addressing the issue. Either the metabolite is applied and investigated in a study on its own or a study is performed using a formulation containing the active substance. In both cases, the concentration of the metabolite in soil should be measured analytically.

6. The following information relating to the test substance is required for the design of appropriate test procedures: proposed crop species, recommended concentration of plant protection product (PPP) and the timing of application(s) according to good agricultural practice (GAP), water solubility, K<sub>oc</sub>, vapour pressure and information useful for assessing the fate and behaviour of the substance in soil (e.g., mobility and routes and rates of dissipation).

**VALIDITY OF THE TEST**

7. 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**

8. Since arable land is most relevant for the majority of the proposed field uses of plant protection products, the use of arable land under cultivation as a worst-case scenario is recommended. Grassland could be used in special cases if applicable to proposed non-arable uses of a test substance.

9. The study site should be characterised by the following soil properties: particle size distribution, pH, water holding capacity and organic matter content. The soil moisture should be
measured at each plot at the start of the test and on each sampling date. Cation exchange capacity (CEC) 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 the data available concerning the fate of the product.

10. The study site should be characterised by the following biological properties: vegetation type and vegetation cover. Optionally other soil biological parameters like microbial activity or earthworm abundance may be determined. The prior history of crop cultivation and pesticide applications within the last 3 years at the test site also needs to be identified and reported.

**Study design**

11. A replicated plot design for the litter bag test is recommended. A size of 25m$^2$ (i.e. 5 x 5m) per plot is considered to be the minimum area. The PPP 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)
12. The total number of buried bags depends on the number of sampling events. As a minimum, for both treatment and control, 6 replicated plots 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
13. 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
14. The appropriate season for testing should be selected according to the intended use pattern of the test substance. If a product is applied in spring and in autumn according to GAP, it has to be decided on a case-by-case basis which represents a realistic worst-case scenario.

EXPOSURE

Plateau concentration
15. The plateau concentration expected to be present in soil after long-term use of the active substance under consideration should be achieved by soil incorporation into a depth of 10cm. The concentration 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). Calculation of the plateau concentration should be based on a soil depth of 20 cm; this takes account of mechanical tillage operations (such as ploughing). Note that for the purposes of the litter bag test, this 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.

16. For PPPs 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.

**Annual cumulative application rate**

17. 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 PPP 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**

18. 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. It is important to note that for ploughed arable soils the plateau concentration is calculated for the top 20 cm of the soil. However, a depth of 10 cm is chosen for actual soil incorporation to avoid excessive disturbance of the soil and to cause as little impact on soil organisms as possible. Therefore, the plateau concentration (mg a.s./kg dry wt. soil), which has been calculated for the top 20 cm of soil, is incorporated into the top 10 cm of soil only. Soil organisms and litter bags are exposed to the plateau concentration anyway since the bags are buried to a depth of 5 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. The control plots should be treated in the same way.

**Annual application**

19. One to 2 weeks after incorporation, the litter bags should be buried in the soil to a depth of about 5 cm horizontally. After burying the bags, the soil should slightly be compressed over the bags 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.

20. 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 Good Agricultural Practice (GAP). 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**

21. If no or little rainfall occurs within 3 days of the annual cumulative application, irrigation of the site is considered necessary to achieve optimal conditions for exposure. The amount used should be realistic according to regional and climatic conditions. A total of 10 mm of precipitation (rainfall plus irrigation) within 3 days after the spray application is desirable.

**SOIL ANALYSIS FOR TEST SUBSTANCE**

22. The PPP concentration in soil must be measured by residue analysis to verify the exposure concentration in soil and to ensure that the litter bags are exposed to the test substance. Subsamples of soil should be collected and analysed immediately after incorporation of the plateau concentration into the soil. A second set of soil subsamples should be collected after the spray application of the annual cumulative dose (if irrigation is undertaken soil samples for residue analysis should be collected after irrigation). Collection of soil samples for residue analyses should be performed according to standardised protocols and standardised analytical methods to measure the PPP 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**

23. 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.

24. 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 a herbicide that is known not to affect organic matter breakdown or by hand weeding. Every additional influence must be kept to a minimum, and control and treatment plots must be treated identically. This means that in case a herbicide or a substance toxic to plants is tested, the plants growing incidentally in control plots should be carefully removed.

Other treatments
25. 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 identically. The number, timing, and rates of application(s) of pesticides and fertilisers to the study site during the study and the previous 3 years should be reported.

Recording climatic data
26. Precipitation and air temperature data from a weather station located nearby to the study site should be recorded in order to characterise the climatic conditions during the test period.

SAMPLING AND TEST DURATION
27. The test should include at least 3 sampling dates within the first 6 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 within 6 months 60% mass loss in the control is not reached, then the study should be continued for up to 12 months.

28. If statistically significant differences in litter mass loss or breakdown rate between control and treatment bags are observed after 6 months (rate calculation based on the samplings at 0 and 6 months), then continuation of the test for a maximum of 6 months 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.

29. Usually the study is terminated after 12 months, but if there is still a concern at this time regarding the 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**

30. Litter bags are taken randomly from the plots manually, put into individually plastic bags (if unmarked litter bags were used, the plastic bags must be marked) and promptly transported to the laboratory. 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**

31. 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 out 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**

32. 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
33. 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 to avoid further microbial degradation. Dried samples must be cooled down in desiccators before weighing.

Drying and grinding of straw
34. 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
35. 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 dessicator) until it can be handled and then reweighed.

Calculation of loss on ignition and decomposition
36. Ash-free dry weight is calculated by subtraction of resulting ignition residue from the straw remnants (dry weight). Breakdown (litter 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 may 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.
37. 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:** \( \text{SCF} = \frac{\text{soil input} - \text{ash residue}}{\text{soil input}} \)
- **Burning of straw samples:** \( \text{StCF} = \frac{\text{ash residue}}{\text{straw input}} \)

**Procedure for obtaining the results**

38. 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)

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**

39. 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**

40. Weight of the remaining plant material (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 % mass loss of the wheat straw.

**Comparison of start weight and end weight per litter bag**

41. The following items should be indicated:

- 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:
\[
\% \text{ mass loss} = \frac{(\text{start weight} - \text{end weight})}{\text{start weight}} \times 100
\]
or
\[
\% \text{ mass loss} = 100 - \left(\frac{\text{end weight}}{\text{start weight}}\right) \times 100
\]

Per sampling date and plot of each treatment the mean % loss has to be calculated \((n = 6)\).

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

42. The following items should be indicated:
   - Sampling date
   - Mean mass loss in control in %
   - Mean mass loss in treatment in %
   - % Effect (mass loss) in comparison to control

Negative numbers indicate an enhancement of mass loss compared to control

Formula:
\[
\% \text{ effect (loss)} = \left(\frac{\text{mean mass loss control} - \text{mean mass loss treatment}}{\text{mean mass loss control}}\right) \times 100
\]
or
\[
\% \text{ Effect (loss)} = (1 - \left(\frac{\% \text{ mass loss treatment}}{\text{mass loss control}}\right) \times 100
\]

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.

**Treatment of results**

43. The mean values of 8 litterbags per plot 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**

44. 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
   - History of the test site (pesticide use within the last 3 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
   - Residue analysis (including method description)

5) Test results:
   - Mass of remaining wheat straw in % of the starting weight per litter bag, plot and treatment
   - % 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 = effect (reduction of OM breakdown)

• 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 guideline and any unusual occurrences during the test

REFERENCES


### ANNEX I

**Definitions and Units**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AFDW</td>
<td>Ash-Free Dry Weight</td>
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<tr>
<td>CEC</td>
<td>Cation Exchange Capacity</td>
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<tr>
<td>CLOI</td>
<td>Corrected Loss On Ignition</td>
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<tr>
<td>FOCUS</td>
<td>FOrum for the Coordination of pesticide fate models and their Use</td>
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<tr>
<td>GAP</td>
<td>Good Agricultural Practice</td>
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<td>LOI</td>
<td>Loss On Ignition</td>
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<tr>
<td>NDS</td>
<td>Non Degraded Straw</td>
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<tr>
<td>PEC</td>
<td>Predicted Environmental Concentration</td>
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<tr>
<td>PPP</td>
<td>Plant Protection Product</td>
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<tr>
<td>SCF</td>
<td>Soil Correction Factor</td>
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<tr>
<td>StCF</td>
<td>Straw Correction Factor</td>
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</table>
ANNEX II

Example how to calculate the amount of non degraded (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**
1. It is assumed that we have 5g of dry material (organic matter plus soil) coming from a buried litterbag (after being dried, sieved, etc, according to the guideline = MAT)
2. After burning, the ash residue is 1.83g (= ASH)

**Theoretical Assumption:**
3. 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.
4. 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
  \[ \text{LOI} = (5g - 1.83 g) = 3.17 [g] \]
- Corrected loss on ignition (CLOI) = LOI - (SCF x (ASH / (1-SCF)))
  \[ \text{CLOI} = 3.17 - (0.1 x (1.83/(1-SCF))) = 2.96667 [g] \]
- Non degraded straw (NDS) = CLOI + (StCF x CLOI x SCF/(1-SCF))
  \[ \text{NDS} = 2.96667 + (0.01 x 2.96667 x SCF/(1-SCF)) = 2.96996 [g] \]

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)

<table>
<thead>
<tr>
<th>Time (days/ months)</th>
<th>Activity/ task</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-litter bag burial</strong></td>
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<tr>
<td>Pre-litter bag burial</td>
<td>1. Selection of the test site and characterisation of soil and site properties</td>
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<tr>
<td></td>
<td>2. Preparation of straw and litter bags</td>
</tr>
<tr>
<td></td>
<td>3. Preparation of field site and spraying equipment</td>
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<tr>
<td>Day 0</td>
<td>1. Preparation of stock solution for field plateau application</td>
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<td></td>
<td>2. First application of test substance to plateau concentration</td>
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<td></td>
<td>3. Incorporation of the test substance(s) into the soil</td>
</tr>
<tr>
<td></td>
<td>4. PPP residue sampling for chemical analysis</td>
</tr>
<tr>
<td>Day 0 to 14</td>
<td>If necessary sowing of plants prior to burying of the litter bags</td>
</tr>
<tr>
<td>Day 7 to 14</td>
<td>Burying of the litter bags</td>
</tr>
<tr>
<td><strong>Post-litter bag burial</strong></td>
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</tr>
<tr>
<td>Between Day 7 and 21 (within one week</td>
<td>4. Preparation of stock solution for annual cumulative application</td>
</tr>
<tr>
<td>after burying the bags)</td>
<td>1. Second application of the test substance (annual cumulative application rate)</td>
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<tr>
<td></td>
<td>Between Day 10 and 24 (within 3 days after the second application)</td>
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<tr>
<td></td>
<td>1. If necessary, irrigation of the treated plots</td>
</tr>
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<td></td>
<td>2. Soil sampling and PPP residue analysis</td>
</tr>
<tr>
<td>One month (after burying the bags)</td>
<td>1. First sampling period, possible need for cultivation (e.g. weeding) activity.</td>
</tr>
<tr>
<td></td>
<td>2. Determination of the ash-free dry weight of the remaining straw in the laboratory</td>
</tr>
<tr>
<td>Three months (after burying the bags)</td>
<td>1. Second sampling period, possible need for cultivation (e.g. weeding)</td>
</tr>
<tr>
<td></td>
<td>2. Determination of the ash-free dry weight of the remaining straw in the laboratory</td>
</tr>
<tr>
<td>Six months (after burying the bags)</td>
<td>1. Third sampling period, possible need for cultivation (e.g. weeding)</td>
</tr>
<tr>
<td>(after burying the bags)</td>
<td>2. Determination of the ash-free dry weight of the remaining straw in the laboratory</td>
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<td>3. Decision on whether the test can be terminated (i.e. mass loss in the control &gt; 60%); otherwise repeat this step after a further 3 or 6 months.</td>
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<tr>
<td>Nine months (after burying the bags)</td>
<td>1. Recommended additional fourth sampling period (possible need for cultivation (e.g. weeding))</td>
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<tr>
<td>recommended additional sampling and</td>
<td>2. Determination of the ash-free dry weight of the remaining straw in the laboratory</td>
</tr>
<tr>
<td>decision stage for study termination</td>
<td>3. Decision on whether the test can be terminated (i.e. mass loss in the control &gt; 60%); otherwise repeat this step after a further 3 months</td>
</tr>
<tr>
<td>Up to 12 months (after burying the bags)</td>
<td>1. Final sampling period. Termination of the standard test.</td>
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<td></td>
<td>2. Determination of the ash-free dry weight of remaining straw (if any) in the laboratory.</td>
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<td></td>
<td>3. Risk analysis</td>
</tr>
<tr>
<td></td>
<td>4. Decision to extend beyond 12 months or move to higher tier testing.</td>
</tr>
</tbody>
</table>