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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Soil Microorganisms: Carbon Transformation Test

INTRODUCTION

1. This Test Guideline describes a laboratory test method designed to investigate long term potential effects of a single exposure of crop protection products and possibly other chemicals on carbon transformation activity of soil microorganisms. The test is principally based on the recommendations of the European and Mediterranean Plant Protection Organisation (1). However, other guidelines, including those of the German Biologische Bundesanstalt (2), the US Environmental Protection Agency (3) and SETAC (4) were taken into account. An OECD Workshop on Soil/Sediment Selection, held at Belgirate, Italy, in 1995 (5) agreed, in particular, on the number and type of soils for use in this test. Recommendations for collection, handling and storage of soil samples are based on an ISO Guidance Document (6) and recommendations from the Belgirate Workshop.

INITIAL CONSIDERATIONS

2. In the assessment and evaluation of toxic characteristics of test substances, determination of effects on soil microbial activity may be required, e.g. when data on the potential side effects of crop protection products on soil microflora are required or when exposure of soil microorganisms to chemicals other than crop protection products is expected. The carbon transformation test is carried out to determine the effects of such chemicals on soil microflora. If agrochemicals (e.g. crop protection products, fertilisers, forestry chemicals) are tested, both carbon transformation and nitrogen transformation tests are conducted. If non-agrochemicals are tested, the nitrogen transformation test is sufficient. However, if EC_{50} values of the nitrogen transformation test for such chemicals fall within the range found for commercially available nitrification inhibitors (e.g. nitrapyrin), a carbon transformation test can be conducted to gain further information.

3. Soils consist of living and non-living components which exist in complex and heterogeneous mixtures. Microorganisms play an important role in breakdown and transformation of organic matter in fertile soils with many species contributing to different aspects of soil fertility. Any long-term interference with these biochemical processes could potentially interfere with nutrient cycling and this could alter the soil fertility. Transformation of carbon and nitrogen occurs in all fertile soils. Although the microbial communities responsible for these processes differ from soil to soil, the pathways of transformation are essentially the same.

4. The test described in this Guideline is designed to detect long-term adverse effects of a substance on the process of carbon transformation in aerobic surface soils. The test is sensitive to changes in size and activity of microbial communities responsible for carbon transformation since it subjects these communities to both chemical stress and carbon starvation. A sandy soil low in organic matter is used. This soil is treated with the test substance and incubated under conditions that allow rapid microbial metabolism. Under these conditions, sources of readily available carbon in the soil are rapidly depleted. This causes carbon starvation which both kills microbial cells and induces dormancy and/or sporulation. If the test runs for more than 28 days, the sum of these reactions can be measured in (untreated soil) controls as a progressive loss of metabolically active microbial biomass (7). If the biomass in carbon-stressed soil, under the conditions of the test, is affected by the presence of a chemical, it may not return to the same

level as the control. Hence, disturbances caused by the test substance at any time during the test will often last until the end of the test.

5. The tests from which this Guideline was developed were primarily designed for substances for which the amount reaching the soil can be anticipated. This is the case, for example, for crop protection products for which the application rate in the field is known. For agrochemicals, testing of two doses relevant to the anticipated or predicted application rate is sufficient. Agrochemicals can be tested as active ingredients (a.i.) or as formulated products. However, the test is not limited to chemicals with predictable environmental concentrations. By changing both the amounts of test substance applied to the soil, and the way in which the data are evaluated, the test can also be used for chemicals for which the amount expected to reach the soil is not known. Thus, with non-agrochemicals, the effects of a series of concentrations on carbon transformation are determined. The data from these tests are used to prepare a dose-response curve and calculate EC_x values, where x is defined % effect.

PRINCIPLE OF THE TEST

6. Sieved soil is either treated with the test substance or left untreated (control). If agrochemicals are tested, a minimum of two test concentrations are recommended and these should be chosen in relation to the highest concentration anticipated in the field. After 0, 7, 14 and 28 days incubation, samples of treated and control soils are mixed with glucose, and glucose-induced respiration rates are measured for 12 consecutive hours. Respiration rates are expressed as carbon dioxide released (mg carbon dioxide/kg dry soil/h) or oxygen consumed (mg oxygen/kg soil/h). The mean respiration rate in the treated soil samples is compared with that in control and the percent deviation of the treated from the control is calculated. All tests run for at least 28 days. If, on the 28th day, differences between treated and untreated soils are equal to or greater than 25 % measurements are continued in 14 day intervals for a maximum of 100 days. If chemicals other than agrochemicals are tested, a series of concentrations of the test substance are added to samples of the soil, and glucose induced respiration rates (i.e. the mean of the quantities of carbon dioxide formed or oxygen consumed) are measured after 28 days. Results from tests with a series of concentrations are analysed using a regression model, and the EC_x values are calculated (i.e. EC_{50}, EC_{25} and/or EC_{10}). See annex for definitions.

VALIDITY OF THE TEST

7. Evaluations of test results with agrochemicals are based on relatively small differences (i.e. average value ± 25 %) between the carbon dioxide released or the oxygen consumed in (or by) control and treated soil samples, so large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15 %.

DESCRIPTION OF THE METHOD

Apparatus

8. Test containers made of chemically inert material are used. They should be of a suitable capacity in compliance with the procedure used for incubation of soils, i.e. incubation in bulk or as a series of individual soil samples (see paragraph 23). Care should be taken both to minimise water loss and to allow gas exchange during the test (e.g. the test containers may be covered with perforated polyethylene foil). When volatile substances are tested, sealable and gas-tight containers should be used. These should be of a size such that approximately one quarter of their volume is filled with the soil sample.

9. For determination of glucose-induced respiration, incubation systems and instruments for measurement of carbon dioxide production or oxygen consumption are required. Examples of such systems and instruments are found in the literature (8)(9)(10)(11).

Selection and number of soils

10. One single soil is used. The recommended soil characteristics are as follows:

- sand content: not less than 50% and not greater than 75%;
- pH: 5.5 - 7.5;
- organic carbon content: 0.5 - 1.5 %;
- the microbial biomass should be measured (12)(13) and its carbon content should be at least 1 % of the total soil organic carbon.

11. In most cases, a soil with these characteristics represents a worst case situation, since adsorption of the test chemical is minimised and its availability to the microflora is maximum. Consequently, tests with other soils are generally unnecessary. However, in certain circumstances, e.g. where the anticipated major use of the test substance is in particular soils such as acidic forest soils, or for electrostatically charged chemicals, it may be necessary to substitute an additional soil.

Collection and storage of soil samples

Collection

12. Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, dates of treatments with crop protection products, treatments with organic and inorganic fertilisers, additions of biological materials or accidental contaminations. The site chosen for soil collection should be one which allows long-term use. Permanent pastures, fields with annual cereal crops (except maize) or densely sown green manures are suitable. The selected sampling site should not have been treated with crop protection products for a minimum of one year before sampling. Also, no organic fertiliser should have been applied for at least six months. The use of mineral fertiliser is only acceptable when in accordance with the requirements of the crop and soil samples should not be taken until at least three months after fertiliser application. The use of soil treated with fertilisers with known biocidal effects (e.g. calcium cyanamide) should be avoided.

13. Sampling should be avoided during or immediately following long periods (greater than 30 days) of drought or water logging. For ploughed soils, samples should be taken from a depth of 0 down to 20 cm. For grassland (pasture) or other soils where ploughing does not occur over longer periods (at least one growing season), the maximum depth of sampling may be slightly more than 20 cm (e.g. to 25 cm).

14. Soil samples should be transported using containers and under temperature conditions which guarantee that the initial soil properties are not significantly altered.

Storage

15. The use of soils freshly collected from the field is preferred. If storage in the laboratory cannot be avoided, soils may be stored in the dark at 4 ± 2 °C for a maximum of three months. During the storage of soils, aerobic conditions must be ensured. If soils are collected from areas where they are frozen for at least three months per year, storage for six months at minus 18 °C can be considered. The microbial biomass of stored soils is measured prior to each experiment and the carbon in the biomass should be at least 1 % of the total soil organic carbon content (see paragraph 10).

Handling and preparation of soil for the test

Pre-incubation

16. If the soil was stored (see paragraph 15), pre-incubation is recommended for a period between 2 and 28 days. The temperature and moisture content of the soil during pre-incubation should be similar to that used in the test (see paragraphs 17 and 24).

Physical-chemical characteristics

17. The soil is manually cleared of large objects (e.g. stones, parts of plants, etc.) and then moist sieved without excess drying to a particle size less than or equal to 2 mm. The moisture content of the soil sample should be adjusted with distilled or deionised water to a value between 40 % and 60 % of the maximum water holding capacity.

Preparation of the test substance for the application to soil

18. The test substance is normally applied using a carrier. The carrier can be water (for water soluble substances) or an inert solid such as fine quartz sand (particle size: 0.1-0.5 mm). Liquid carriers other than water (e.g. organic solvents such as acetone, chloroform) should be avoided since they can damage the microflora. If sand is used as a carrier, it can be coated with the test substance dissolved or suspended in an appropriate solvent. In such cases, the solvent should be removed by evaporation before mixing with the soil. For an optimum distribution of the test substance in soil, a ratio of 10 g of sand per kilogram of soil (dry weight) is recommended. Control samples are treated with the equivalent amount of water and/or quartz sand only.

19. When testing volatile chemicals, losses during treatment should be avoided and an attempt should be made to ensure homogeneous distribution in the soil (e.g. the test substance should be injected into the soil at several places).

Test concentrations

20. If crop protection products or other chemicals with predictable environmental concentrations are tested, at least two concentrations should be used. The lower concentration should reflect at least the maximum amount expected to reach the soil under practical conditions whereas the higher concentration should be a multiple of the lower concentration. The concentrations of test substance added to soil are calculated assuming uniform incorporation to a depth of 5 cm and a soil bulk density of 1.5. For agrochemicals that are applied directly to soil, or for chemicals for which the quantity reaching the soil can be predicted, the test concentrations recommended are the Predictable Environmental Concentration (PEC) and five times that concentration. Substances that are expected to be applied to soils several times in one

season should be tested at concentrations derived from multiplying the PEC by the maximum anticipated number of applications. The upper concentration tested, however, should not exceed ten times the maximum single application rate.

21. If non-agrochemicals are tested, a geometric series of at least five concentrations is used. The concentrations tested should cover the range needed to determine the EC_x values.

PERFORMANCE OF THE TEST

Conditions of exposure

Treatment and control

22. If agrochemicals are tested, the soil is divided into three portions of equal weight. Two portions are mixed with the carrier containing the product, and the other is mixed with the carrier without the product (control). A minimum of three replicates for both treated and untreated soils is recommended. If agrochemicals are tested, the soil is divided into six portions of equal weight. Five of the samples are mixed with the carrier containing the test substance, and the sixth sample is mixed with the carrier without the chemical. Three replicates for both treatments and control are recommended. Care should be taken to ensure homogeneous distribution of the test substance in the treated soil samples. During mixing, compacting or balling of the soil should be avoided.

Incubation of soil samples

23. Incubation of soil samples can be performed in two ways: as bulk samples of each treated and untreated soil or as a series of individual and equally sized subsamples of each treated and untreated soil. However, when volatile substances are tested, the test should only be performed with a series of individual subsamples. When soils are incubated in bulk, large quantities of each treated and untreated soils are prepared and subsamples to be analysed are taken as needed during the test. The amount initially prepared for each treatment and control depends on the size of the subsamples, the number of replicates used for analysis and the anticipated maximum number of sampling times. Soils incubated in bulk should be thoroughly mixed before subsampling. When soils are incubated as a series of individual soil samples, each treated and untreated bulk soil is divided into the required number of subsamples, and these are utilised as needed. In the experiments where more than two sampling times can be anticipated, enough subsamples should be prepared to account for all replicates and all sampling times. At least three replicate samples of the test soil should be incubated under aerobic conditions (see paragraph 22). During all tests, appropriate containers with sufficient headspace should be used to avoid development of anaerobic conditions. When volatile substances are tested, the test should only be performed with a series of individual subsamples.

Test conditions and duration

24. The test is carried out in the dark at room temperature of 20 ± 2 °C. The moisture content of soil samples should be maintained during the test between 40 % and 60 % of the maximum water holding capacity of the soil (see paragraph 17) with a range of ± 5 %. Distilled, deionised water can be added as needed.

25. The minimum duration of tests is 28 days. If agrochemicals are tested, the quantities of carbon dioxide released or oxygen consumed in treated and control samples are compared. If these differ by more than 25 % on day 28, the test is continued until a difference equal to or less than 25 % is obtained, or for a maximum of 100 days, whichever is shorter. If non-agrochemicals are tested, the test is terminated after 28 days. On day 28, the quantities of carbon dioxide released or oxygen consumed in treated and control soil samples are determined and the EC_x values are calculated.

<u>Sampling and analysis of soils</u>

Soil sampling schedule

26. If agrochemicals are tested, soil samples are analysed for glucose-induced respiration rates on days 0, 7**,** 14 and 28. If a prolonged test is required, further measurements should be made at 14 days intervals after day 28.

27. If non-agrochemicals are tested, at least five test concentrations are used and soil samples are analysed for glucose-induced respiration at the beginning (day 0) and at the end of the exposure period (28 days). An intermediate measurement, e.g. at day 7, may be added if deemed necessary**.** The data obtained on day 28 are used to determine EC_x value for the chemical. If desired, data from day 0 control samples can be used to estimate the initial quantities of metabolically active microbial biomass in the soil (12).

Measurement of glucose-induced respiration rates

28. The glucose-induced respiration rate in each treated and control replicate is determined at each sampling time. The soil samples are mixed with a sufficient amount of glucose to elicit an immediate maximum respiratory response. The amount of glucose needed to elicit a maximum respiratory response from a given soil can be determined in a preliminary test using a series of concentrations of glucose (14). However, for sandy soils with 0.5-1.5 % organic carbon, 2000 mg to 4000 mg glucose per kg dry weight soil is usually sufficient. The glucose can be ground to a powder with clean quartz sand (10 g sand/kg dry weight soil) and homogeneously mixed with the soil.

29. The glucose amended soil samples are incubated in a suitable apparatus for measurement of respiration rates either continuously, every hour, or every two hours (see paragraph 9) at 20 ± 2 °C. The carbon dioxide released or the oxygen consumed is measured for 12 consecutive hours and measurements should start as soon as possible, i.e. within 1 to 2 hours after glucose supplement. The total quantities of carbon dioxide released or oxygen consumed during the 12 hours are measured and mean respiration rates are determined.

<u>DATA AND REPORTING</u>

<u>Data</u>

30. If agrochemicals are tested, the carbon dioxide released from, or oxygen consumed by each replicate soil sample should be recorded, and the mean values of all replicates should be provided in tabular form. Results should be evaluated by appropriate and generally acceptable statistical methods (e.g. F-test, 5% significance level). Glucose-induced respiration rates are expressed in mg carbon dioxide/kg dry weight soil/h or mg oxygen/dry weight soil/h. The mean carbon dioxide formation rate or mean oxygen consumption rate in each treatment is compared with that in control, and the percent deviation from the control is calculated.

31. If tests are conducted with non-agrochemicals, the quantities of carbon dioxide released or oxygen consumed by each replicate is determined, and a dose-response curve is prepared for estimation of the ECx values. The glucose-induced respiration rates (i.e. mg carbon dioxide/kg dry weight soil/h or mg oxygen/dry weight soil/h) found in the treated samples after 28 days are compared to that found in control. From these data, the % inhibition values for each test concentration are calculated. These percentages are plotted against concentration, and statistical procedures are used to calculate the EC_x values. Confidence limits (p = 0.95) for the calculated ECx are also determined using standard procedures (15)(16)(17).

Interpretation of Results

32. When results from tests with agrochemicals are evaluated, and the difference in respiration rates between the lower treatment (i.e. the maximum predicted concentration) and control is equal to or less than 25 % at any sampling time after day 28, the product can be evaluated as having no long-term influence on carbon transformation in soils. When results from tests with chemicals other than agrochemicals are evaluated, the EC_{50}, EC_{25} and/or EC_{10} values are used.

Test Report

33. The test report must include the following information:

Complete identification of the soil used including:

- geographical reference of the site (latitude, longitude);
- information on the history of the site (i.e. vegetation cover, treatments with crop protection products, treatments with fertilisers, accidental contamination, etc.)
- use pattern (e.g. agricultural soil, forest, etc.);
- depth of sampling (cm);
- sand/silt/clay content (% dry weight);
- pH (in water);
- organic carbon content (% dry weight);
- nitrogen content (% dry weight);
- cation exchange capacity (mmol/kg);
- initial microbial biomass in terms of percentage of the total organic carbon;
- reference of the methods used for the determination of each parameter;
- all information relating to the collection and storage of soil samples;
- details of pre-incubation of soil if any.

Test substance:

- physical nature and, where relevant, physical-chemical properties;
- chemical identification data, where relevant, including structural formula, purity (i.e. for crop protection products the percentage of active ingredient), nitrogen content.

Test conditions:

- details of the amendment of soil with organic substrate;
- number of concentrations of test chemical used and, where appropriate, justification of the selected concentrations;
- details of the application of test substance to soil;
- incubation temperature;

- soil moisture content at the beginning and during the test;
- method of soil incubation used (i.e. as bulk or as a series of individual subsamples);
- number of replicates;
- sampling times.

Results:

- method and equipment used for measurement of respiration rates;
- tabulated data including individual and mean values for quantities of carbon dioxide or oxygen;
- variation between the replicates in treated and control samples;
- explanations of corrections made in the calculations, if relevant;
- the percent variation of glucose-induced respiration rates at each sampling time or, if appropriate, the EC_{50} with 95 per cent confidence limit, other EC_x (i.e. EC_{25} or EC_{10}) with confidence intervals, and a graph of the dose-response curve;
- statistical treatment of results, where appropriate;
- all information and observations helpful for the interpretation of the results.

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ANNEX

DEFINITIONS

Carbon transformation is the degradation by microorganisms of organic matter to form inorganic end-product carbon dioxide.

EC_x (Effective Concentration) is the concentration of the test substance in soil that results in a x percent inhibition of carbon transformation in carbon dioxide.

EC_{50} (Median Effective Concentration) is the concentration of test substance in soil that results in a 50 per cent inhibition of carbon transformation in carbon dioxide.