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1. Introduction and Aim
The aim of this guideline is to describe the application of the nanomaterial-suspension to soil which is subsequently used in following tests: a) Determination of the mortality of the nanomaterial towards *Eisenia fetida* in soil after 7 and 14 days. B) Investigation of the effect of the nanomaterial on the reproduction of *E. fetida* after 56 days.

1.1 Guidelines and Recommendations
This guideline is constructed to meet the requirements of following guidelines:

2 Scope of the Guideline
This SOP is valid for the application of a nanomaterial-suspension to soil, which is subsequently tested in an acute toxicity or reproduction test with the earthworm *Eisenia fetida* (Savigny 1826) in soil.

3 Materials and Instruments
- Soil (e.g. natural, ReFeSol 01-A)
- Ultrasonic probe (e.g. Sonoplus 200 W, BANDELIN electronic GmbH & Co. KG, Berlin, Germany)
- 250 ml beakers for the suspensions
- Demineralized water for the suspensions
- General labware

4 Procedures and Descriptions

4.1 Determination of the Maximum Water Holding Capacity (WHK$_{\text{max}}$) of the Soil
The determination of the maximum water holding capacity of the soil is conducted according to Annex C of ISO 11268-2.

4.2 Test Beakers
Nanomaterial suspensions are prepared in commercially available 250 ml beakers.
4.3 Calculations

It is necessary to know the maximum water holding capacity (WHK_{max}) of each soil lot for calculating the test parameters.

4.3.1 Calculation of the Water Amount to be added to the Soil

A) Total water amount

\[
\text{Target WHK} \times \frac{\text{WHK}_{\text{max}}}{100} = \text{water amount [g] per 100 g}
\]

WHK = water holding capacity in %
WHK_{max} = maximum water holding capacity of the soil lot in %

\[
\text{Water amount [g] per 100 g} \times \frac{\text{Soil dry weight}}{100} = \text{total water amount [g]}
\]

Example:

Soil dry weight: 2050 g; WHK_{max} = 50.00 %; target % WHK = 55 %

\[
\frac{55.0}{100} \times 50.0 = 27.5 \text{ g (water amount per 100 g)}
\]

\[
\frac{2050}{100} \times 27.5 = 563.75 \text{ g (total water amount)}
\]

B) Water Amount for the Suspension

The nanomaterial is suspended in the total water amount (see calculations above) that has to be added to the soil. But it has to be considered that the suspension beaker has to be rinsed with water after the suspension has been added to the soil. For this, an aliquot of the total water amount is used. In preliminary experiments a water amount of 3 ml per 250 ml beaker was tested as useful for rinsing the beaker. Consequently the amount of water which is used for the suspensions is calculated by subtracting the water amount necessary for rinsing the beaker from the total water amount. Furthermore, it should be noted that the concentration of the suspension should only be so high that it can be measured with dynamic light scattering, e.g., by using a specially constructed cell for high concentrations. Moreover the volume of the suspension should not be too high so that the energy input is still high enough to suspend the particles (experiences showed that a volume of 50-250 ml was useful).

Total water amount - water amount for rinsing the beaker = water amount for suspension
4.4 Preparation of the Nanomaterial-Suspension

The nanomaterial is weighed and then stirred into the water amount for suspension with a spatula. Next, the ultrasonic probe is immersed 1.5-2 cm into the suspension. A pulsed ultrasound is used (0.2 s). To prevent heating of the suspension the beaker is placed into an ice bath. The duration of ultra-sonication depends on the nanomaterial (experience shows that 5-30 min are sufficient for suspending the material). If necessary the pH of the suspension is adjusted with NaOH or HCl after the ultra-sonication. For determining the optimal pH value of the suspension and duration of ultra-sonication preliminary tests can be done. Therefore, suspensions are characterized with regard to their hydrodynamic diameter and zetapotential by using dynamic light scattering (zetasizer; see SOP – Characterization of a nanomaterial-suspension).

4.5 Preparation of Control and Treatment Substrates

Treatment substrates (TS) are prepared directly before test initiation. A treatment substrate consists of soil, nanomaterial-suspension and amount of rinsing water. After addition of the total water amount to the soil the water content of the soil has to lie between 40 and 60% of the WHK$_{\text{max}}$ of the soil. The soil should be moist but after pressing the soil no water should escape (OECD 207).

First the soil is weighed into a bowl, then the nanomaterial-suspension is poured on the soil by gently mixing all components. Next the beaker of the suspension is rinsed with the specific amount of water that is subsequently added to the soil. Thereafter, the TS is mixed for 5 min.

Finally, the specific wet weight of the TS corresponding to a soil dry weight of 500 g is weighed into the test beakers which are then loosely closed with a lid.

5 Collection of Raw Data

Following raw data have to be collected:

- Soil constitution and parameters (e.g. WHK$_{\text{max}}$)
- Initial weights of the soil
- Preparation of the nanomaterial-suspension
- Application of the total water amount to the soil

6 Literature

ISO-Guideline 11268-1: 1993 "Soil quality - Effects of pollutants on earthworms (Eisenia fetida) - Part 1: Determination of acute toxicity using artificial soil substrate"


ISO/DIS 11465 (1996): Soil Quality - Determination of dry matter and water content on a mass basis - Gravimetric method

OECD (4. April 1984): Guideline for the testing of chemicals Nr. 207 "Earthworm, Acute Toxicity Test"

OECD (13. April 2004): Guideline for the testing of chemicals Nr. 222 "Earthworm Reproduction Test"

7 Safety Recommendations

The safety recommendations for handling the test substance are listed in the specific safety data sheets.