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DOSSIER ON TITANIUM DIOXIDE - PART 1 - NM 105 ANNEX 18

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1 Test with Microorganisms - Carbon Transformation Test

1.1 Test principle

The effects of the test item on carbon transformation were determined in a field soil. After mixing the test item into the soil the soil was incubated at 20 ± 2 °C for 28 days in the dark. Samples were taken at test start and after 28 days of incubation. The test item was applied once. For measurement of carbon transformation a short-term respiration test (glucose-induced respiration rates) in soil was performed. The test was conducted according to the "Soil Microorganisms: Carbon Transformation Test" Guideline OECD 217.

1.2 Materials and methods

1.2.1 Test guideline

The test was performed according to:

OECD Guidelines for the Testing of Chemicals Test No. 217: "Soil Microorganisms: Carbon Transformation Test" (2000).

1.2.2 GLP

The test was performed following the principles of GLP. In deviation to GLP no archiving of the raw data was performed and the quality assurance unit was not involved with respect to the inspection of the test, of the raw data and of the report. All laboratory equipment (e.g. balances, thermometers, pH-meters) were controlled and documented according to GLP.

1.2.3 Test material

P25 - distributed by Evonik for the OECD Sponsorship Programme; the test substance corresponds to the sub-sampled nanomaterial NM-105. The nanoparticles were stored in the dark at room temperature until use.

1.2.4 Test type

Static

1.2.5 Analytical monitoring

Due to the high natural concentration of TiO_2 in the test soil no specific chemical analyses were performed in the soil.

The zeta potential was measured in the test dispersions using a Malvern Zetasizer Nano ZS. The particle size distribution in the dispersion was not determined. It would give no information on the size distribution in soil or food. A measurement of the Zeta-potential or the particle size distribution in soil is not yet possible.

1.2.6 Test item – preparation protocol

We tested two different modes of application: spiking via powder and spiking via dispersion.

The nominal concentrations of the test item in the test containers were 9.3, 21, 45, and 100 mg P25/kg soil, dry mass (application via powder) and 9.3 and 21 mg/kg (application via dispersion). Three replicates per concentration were conducted.

Spiking of soil with TiO_2 powder

For the first application the TiO_2 powder was mixed directly into the soil, whereby air-dried test soil (1% of the total amount) was used as a carrier for the powder. Amounts of TiO_2 powder that are suitable to achieve the desired final soil content were mixed homogenously with the dry soil. Care was taken to avoid a modification of the TiO_2 crystalline structure. Uncontaminated test soil (between 20 and 30% of WHC_{max}) was spread on a plate, the carrier material with the TiO_2 powder was distributed on the test soil, and all was mixed carefully. In the same way, 5 g/kg TM grinded lucerne was mixed into the soil. For the test with contaminated soil, the soil was adjusted to a water-holding capacity of 55% of the maximum water-holding capacity (WHC_{max}).
Test concentrations were: 9.3, 21, 45 and 100 mg/kg soil dry matter (d.m.).

Spiking of soil with aqueous TiO_2 dispersion

The second application trial was to spray a TiO_2 dispersion that had been prepared with a magnetic flea (900 rpm; 1 min) and ultrasonication (3 min) in a bath sonicator. Test soil was dried to about 10% of WHC_{max} and spread on a plate. 5 g/kg TM of grinded lucerne were mixed into the soil. Immediately after preparation a predetermined amount of the highly concentrated TiO_2 dispersion was sprayed on the soil by means of a syringe coupled with a cannula, and thoroughly mixed. Finally, the test soil was adjusted to a water-holding capacity of 55% of WHCmax. A maximum concentration of 202 mg/L application dispersion of TiO_2 nanoparticles was considered as adequate for the tests. Higher concentrations would have sedimented rapidly preventing a homogenous distribution of the nanomaterial in the soil. The maximum water content in the test soil should be about 55% of the maximum water-holding capacity. Due to these limitations only soil contents of 9.3 and 21 mg/kg were tested.

Test concentrations were: dispersion with 92 and 202 mg/L deionized water; application of 202 ml test dispersion to 2.0 kg test soil (d.m.) corresponding to 9.3 ml and application of 208 ml test dispersion to 2.0 kg test soil (d.m.) corresponding to 21 mg/kg soil (d.m.)

1.2.7 Test organism

A sandy soil with the individual soil microflora was investigated.

1.3 Study design

1.3.1 Total exposure period

The exposure period was 28 days:
June 22, 2010 - July 20, 2010.

1.4 Test conditions

1.4.1 Environmental conditions

Physico-chemical data

The incubation temperature was measured continuously with a thermograph. With 20 - 21 °C the permitted range of 20 ± 2 °C was kept. Incubation occurred in the dark. During the whole test the soil dry mass was maintained at 88.7% (controls), 88.3% (powder application: 9.3 mg/kg), 89.4% (powder application: 21 mg/kg), 88.8% (powder application: 45 mg/kg), 88.8% (powder application: 100 mg/kg), 89.3% (dispersion application: 9.3 mg/kg) and 89.1% (dispersion application: 21 mg/kg).

1.4.2 Test soil

The test soil was a natural sandy soil (Certified RefeSol 01-A, batch IME-01: sand 71%, silt: 24%, clay: 5%, org C: 0.93%, pH 5.7, clay: 5%). The soil was sieved to 2 mm. It was not sterilized and stored outdoors on the grounds of the test facility in high-grade stainless steel basins with drainage and ground contact.

For at least one year prior to soil sampling in the field, no plant protection products had been applied to the sampling site. Neither organic nor mineral fertilizers had been applied to the soil for six and three months, respectively, prior to soil sampling.

Table 1: Test soil for microbial tests: Soil parameters

Soil name	RefeSol 01-A
Soil batch	IME-01
Soil texture	Loamy sand
Clay (%)	5
Silt (%)	24
Sand (%)	71
WHC (g H_2O/kg soil dry weight)	264
CECeff (mmol/kg)	37.9
pH	5.7
Total org. C (%)	0.93
Microbial biomass (mg C/kg dry mass soil), calculated from respiration activity	92
Microbial biomass (% of total org. C)	1.0
Total nitrogen (%)	0.09
NO_3^- (mg/kg dry weight)	81.7

WHC = water holding capacity

CECeff = effective cation exchange capacity

Table 2: Test soil for microbial tests: Storage information

Soil name	RefeSol 01-A
Soil batch	IME-01
Date of field sampling	11.06.2010
Start of indoor storage at room temperature to reduce the water content and to allow sieving; the soil was distributed in a thin layer; surface drying was prevented by periodically turning the soil.	11.-13.09.2008
Date of sieving for the study	13.06.2010
Start of soil conditioning **	13.06.2010
Date of application	22.06.2010

** Soil conditioning was performed at room temperature in the dark.

1.4.3 Concentration levels

For the application via powder the nominal contents in the test containers with TiO_2 were 9.3, 21.0, 45.0 and 100.0 mg/kg soil dry matter.

For the application via dispersion the nominal contents in the test containers with TiO_2 were 9.3 and 21.0 mg/kg soil dry matter.

1.4.4 Any other information on materials and methods

Frequency of treatment

Treatment was performed once at test start.

Control group and treatment

The control consists of soil. Three replicates were conducted per control.

Statistical method

Data evaluation:

In this report numerical values were frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with rounded values in comparison to results obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and of no practical concern.

Statistical calculations:

For each concentration the quantity of consumed oxygen was determined. According to the guidelines for non-agrochemicals, the glucose-induced respiration rates found in the treated samples after 28 days were compared to the respiration rates found in the controls. Furthermore, the percent inhibition value for the test concentrations was calculated. The percent deviation of the respiration rates were calculated in comparison to the control. ECx, LOEC and NOEC calculations were performed with the computer software ToxRat Professional version 2.10 (release 19.02.2009) by ToxRat® Solutions GmbH.

Test procedure

Three incubation containers per treatment were filled up with 733 g moist spiked soil. Further three incubation containers were filled with 733 g of control soil.

The test was carried out in the dark at 20 ± 2 °C for 28 days. During the test the moisture content of the soil was maintained at 40 - 60% of WHC_{max} with a range of not more than of 5%. The mass in the test vessels was measured weekly. Evaporated water was supplemented by adding deionised water.

Samples of each treated and control replicate were analysed for glucose-induced respiration at the beginning (day 0) and at the end of the exposure period (28 days).

The soil samples (100 g dry mass) were mixed with 4000 mg glucose per kg dry weight. The glucose concentration was based on a range finding test for the soil to achieve maximum activity. The glucose-amended soil samples were continuously incubated in an apparatus for the measurement of respiration rates (day 0: Sapromat® Voith Inc.; day 28: Sensomat, Aqualytik) at 20 ± 2 °C. The oxygen consumed was measured consecutively for at least 12 hours. Measurements started as soon as possible after glucose supplement. For evaluation the linear phase of oxygen consumption was used.

1.5 Results

1.5.1 Zeta potential

The Zeta potential is presented in Table 3.

Table 3: P25 (NM-105) - C-transformation: Zeta potential of the stock dispersion for application via dispersion

Sample	Zeta potential [mV]
100 mg/L	18 mV

1.5.2 Carbon transformation

Effect concentrations

For both application forms, inhibitory effects were not observed; no EC-values were calculated. There is no statistically significant difference between the treatments and the control. The NOEC is higher than the highest test concentration (≥ 100 mg/kg).

Respiration measurement

For each treatment three replicate vessels were incubated. From each vessel one soil sample was taken for measurement. The results showed a large variation between the replicates. This was especially true for the measurement at day 28, where another measuring device than applied for day 0 had to be used due to technical reasons (day 0: Sapromat with continuous oxygen supply depending on respiration activity; the amount of oxygen supplied is the measure for microbial respiration activity; day 28: OxiTop (= respirometer without oxygen supply; a decrease in pressure is the measure for microbial respiration activity; 500 mL incubation vessels). In previous projects the comparability of both measuring devices was proven (joint project sponsored by BMBF: FKZ 0330303; Project: Biologische Testverfahren in der Vor-Ort-Analytik zur Beurteilung der Qualität von Böden und Bodenmaterialien; Teilvorhaben 2: Mikrobielle Atmungsaktivität).

Looking at the replicates in several cases two values were identical or very similar, whereas one value differed obviously. In these cases a further assessment was performed after eliminating the "extreme" values. The results of the short-term respiration measurement are presented as mean values in Table 4 and for better visualisation in Figure 1. The evaluation based on all measured values and the evaluation based on the reduced number of values is listed. Table 5 shows the percental deviation compared to the control. For single values of the replicates see Table 6.

Table 4: P25 (NM-105) - C-transformation: Mean short-term respiration rate [mg O_2/(kg*h)]

			Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9,3	21
Consideration of all values								
Day 0	Mean	3.3	3.8	3.6	3.5	3.8	3.8	3.7
	Std.Dev.	0.7	0.5	0.1	0.2	0.5	0.2	0.2
	CV	20.8	12.1	1.6	4.4	12.1	5.7	4.7
Day 28	Mean	2.6	2.3	2.8	3.9	3.6	2.3	2.1
	Std.Dev.	0.5	0.8	0.9	0.8	0.9	0.8	0.5
	CV	18.0	34.8	32.6	19.4	24.1	34.8	22.7
Elimination of extreme values								
Day 0	Mean	3.7	3.8	3.6	3.5	3.8	3.8	3.7
	Std.Dev.	0.2	0.5	0.1	0.2	0.5	0.2	0.2
	CV	5.4	12.1	1.6	4.4	12.1	5.7	4.7
Day 28	Mean	2.3	2.3	2.3	3.9	3.1	2.3	2.3
	Std.Dev.	0.0	0.8	0.0	0.8	0.0	0.8	0.0
	CV	0.0	34.8	0.0	19.4	0.0	34.8	21.7

Table 5: P25 (NM-105) - C-transformation: Mean short-term respiration rate; deviation from control [%]

	Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
	9.3	21	45	100	9.3	21
Consideration of all values						
Day 0	-15,2	-9,1	-6.1	9.1	-15.2	-15.2
Day 28	11.5	-7.7	-50.0	-38.5	11.5	19.2
Elimination of extreme values						
Day 0	-2.7	2.7	6.3	-1.4	-1.4	0
Day 28	0	0	-69.6	-34.8	0	0

Table 6: P25 (NM-105) - C-transformation: Short-term respiration rate (SIR) [mg O_2/(kg*h)]

Single values of the replicates; values eliminated for the evaluation ("extreme" values are marked bold)

Date of sampling	Replicate		Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Test start	1	**2.5**	3.9	3.5	3.5	3.3	-	3.6
	2	3.8	4.2	3.5	3.3	4.2	3.9	3.9
	3	3.5	3.3	3.6	3.6	-	3.6	3.6
Day 28	1 (500)	2.3	3.1	2.3	3.1	3.1	3.1	2.3
	2 (500)	**3.1**	1.5	2.3	3.9	3.1	2.3	**1.5**
	3 (500)	2.3	2.3	**3.9**	4.6	**4.6**	1.5	2.3

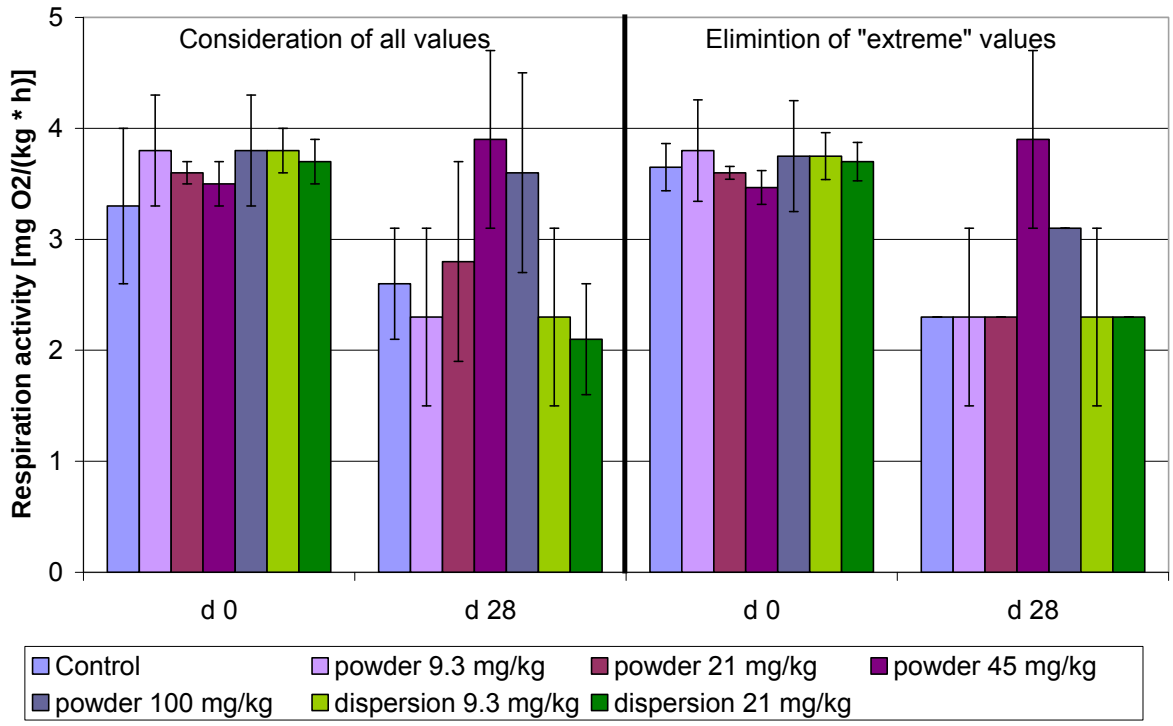

Figure 1: P25 (NM-105) – C-Tranformation: Mean short-term respiration rate [(mg O_2/(kg*h)]

1.5.3 Further information

To confirm the results the test was repeated. The results are presented in Table 7, Table 8 and Figure 2.

Effect concentrations:

For both application forms, inhibitory effects were not observed and no EC-values were calculated.

There is no statistically significant difference between the treatments and the control after an incubation period of 28 d. The NOEC increases the highest test concentration (≥ 100 mg/kg).

Respiration measurement:

Deviating from the first test, there was a small concentration-dependent inhibition at day 0; at day 28 a concentration-dependent stimulation was not measured. A statistical difference to the control was not observed. The results obtained at day 28 were independent of the application form (application of powder / application via dispersion). Therefore the conclusion drawn from the results from both tests (first test and repeated test) is the same: P25 does not affect the microbial respiration activity.

Table 7: P25 (NM-105) - C-transformation: Mean short-term respiration rate [mg O_2/(kg*h)]

			Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
		Control	9.3	21	45	100	9.3	21
Day 0	Mean	5.87	5.33	5.07	4.80	4.80	4.80	5.07
	Std.Dev.	0.46	0.92	0.46	0.00	0.00	0.00	0.46
	CV	7.9	17.3	9.1	0.0	0.0	0.0	9.1
	Statistical significance	---	n.s.	n.s.	* [1]	* [1]	* [1]	n.s.
Day 28	Mean	3.20	2.93	3.20	3.20	3.47	2.93	3.73
	Std.Dev.	0.00	0.46	0.00	0.00	1.22	0.46	0.46
	CV	0.0	15.7	0.0	0.0	35.3	15.7	12.4
	Statistical significance	---	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Statistical significance: p > 0.05

Table 8: P25 (NM-105) - C-transformation: Mean short-term respiration rate, deviation from control [%]

	Application via powder [mg TiO_2/kg]				Application via dispersion [mg TiO_2/kg]	
	9.3	21	45	100	9.3	21
Day 0	9.2	13.6	18.2	18.2	18.2	13.6
Day 28	8.4	0	0	-8.4	8.4	-16.6

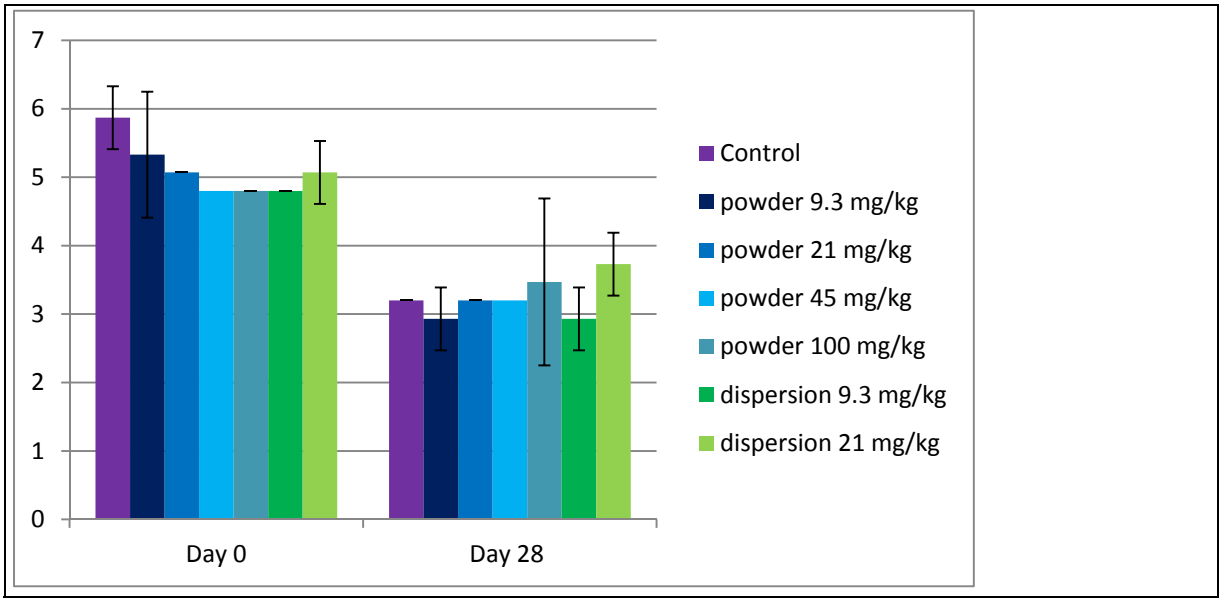

Figure 2: P25 (NM-105) – C-Tranformation: Mean short-term respiration rate ([mg O2/(kg*h)] (test repetition)

1.6 Validity

A validity criterion is only available in the guideline for the testing of agrochemicals. The evaluation of the results from the tests performed with agrochemicals are based on relatively small differences (i.e. average value ± 25%) between the carbon dioxide released or the oxygen consumed in control and treated soil samples; accordingly large variations in the controls can lead to false results. Therefore, the variation between replicate control samples should be less than ± 15%.

For non-agrochemicals concentration-effect relationships are relevant. Therefore, a variation of 15% is of minor importance. From the results it is obvious that there are no concentration-effect relationships and P25 (NM-105) does not affect microbial respiration activity. Nevertheless, the validity criteria for agrochemicals are fulfilled.

- The variation between replicate control samples should be less than 15 %. The variation is 8 % (day 0) and 0 % (day 28). Therefore, the test is considered to be valid.

1.7 Reference substance

In the guideline the investigation of a reference substance is not demanded. A reference substance was not tested.

1.8 Conclusion

The TiO_2 nanoparticles tested by means of

- Application via powder on soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil, dry matter

- Application via dispersion on soil: 9.3 and 21.0 mg/kg soil, dry matter

did not cause a reduced carbon transformation activity.

1.9 Executive Summary

TiO_2 nanoparticles (P25) were tested in the microbial carbon transformation assay (test guideline OECD 217). Soil was spiked with the test item via powder and via dispersion. As test substrate a natural sandy soil was used. Following test concentrations were investigated:

- Application via powder on soil: 9.3, 21.0, 45.0 and 100.0 mg/kg soil, dry matter
- Application via dispersion on soil: 9.3 and 21.0 mg/kg soil, dry matter.

For each treatment three replicate vessels were incubated. From each vessel one soil sample was taken for measurement.

For both application forms, no inhibitory effect was observed and no EC-values were calculated. There is no statistically significant difference between the treatments and the control. The NOEC is higher than the highest test concentration (≥ 100 mg/kg). This result was confirmed by a repetition of the test.