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Effect of TiO_2 nanoparticles in the earthworm reproduction test

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Abstract

Background: The increasing use of nanotechnology means that nanomaterials will enter the environment. Ecotoxicological data are therefore required so that adequate risk assessments can be carried out. In this study, we used a standardized earthworm reproduction test with *Eisenia andrei* to evaluate three types of TiO_2 nanoparticles (NM-101, NM-102, NM-103). The test was performed in natural sandy soil (RefeSol 01A) following Organisation for Economic Co-operation and Development Test Guideline No. 222. The nanoparticles differed in several aspects, such as crystalline structure, size, and the presence or absence of a coating.

Results: Uncoated nanoparticles stimulated earthworm reproduction in a concentration-dependent manner during winter testing, increasing the number of offspring by up to 50% compared to the control. However, there was no stimulation when the same test was performed in the summer. This reflected an underlying circannual rhythm observed in the control soil, characterized by the production of a significantly larger number of juveniles in summer compared with that in winter. The effect of the uncoated TiO_2 nanoparticles was to reduce or eliminate the circannual differences by increasing the reproductive rate in winter. Coated TiO_2 nanoparticles did not influence earthworm reproduction.

Conclusion: TiO_2 appears to affect earthworm reproductive activity by abolishing the circannual rhythm that depresses reproduction in the winter. Further experiments will be necessary to determine (1) the mode of action of the nanoparticles, (2) the important parameters causing the effect (e.g., relevant soil parameters), and (3) the environmental relevance of continuous earthworm reproduction we observed under laboratory conditions.

Keywords: TiO_2 nanoparticles, ecotoxicity, earthworm reproduction

Background

The increasing use of nanotechnology means that nanomaterials will inevitably enter the environment. Ecotoxicological data are therefore required so that adequate risk assessments can be carried out. Risk assessments are currently governed by European Government and Council regulations concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Nanomaterials are not mentioned explicitly, but they are covered by the substance definition [1]. In 2006, the Chemicals Committee of the Organisation for Economic Co-operation and Development [OECD] established the Working Party on Manufactured Nanomaterials [WPMN] to investigate the potential impact of nanomaterials on human health and the environment, focusing particularly on testing and assessment methods. In 2007, the WPMN launched the Sponsorship Programme on the Testing on Manufactured Nanomaterials and agreed on a priority list of nanomaterials and a list of endpoints relevant for environmental safety testing. Each material has lead sponsors that organize the testing and the preparation of a Dossier containing the results, which describe the fate and effect of nanomaterials and the preparation of guidance documents for testing and evaluation. A preliminary review on the application of OECD guidelines to manufactured nanomaterials [2] stated that the basic practices recommended by these test guidelines are suitable for the testing of nanomaterials. However, guidance for the delivery of substances to test systems, the quantification of exposure, and dose metrics needed to be adapted for the testing of nanomaterials. Preliminary guidance for sample preparation and

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dosimetry for the safety testing of manufactured nanomaterials is now under revision. The first version did not provide detailed guidance on the application of nanomaterials in aqueous or nonaqueous media, but principal procedures are listed [3].

One of the nanomaterials included in the OECD WPMN priority list is titanium dioxide, a chemically stable mineral which exists in several crystalline forms. The two tetragonal forms (anatase and rutile) are used most often in a technical context, and each crystalline structure has specific properties that determine its applications. Examples include the use of TiO_2 in white pigments and in sunscreens, the latter because of the high refractive index (n = 2.7) which allows rapid absorption of ultraviolet radiation. The photocatalytic activity of TiO_2 means that it can be used to produce easy-to-clean surfaces, for air/water purification, deodorization, and sterilization [4-8]. These multiple applications mean that environmental distribution is inevitable. Model calculations suggest that typical TiO_2 concentrations in Europe are 1.28 μg/kg in soil, 89.2 μg/kg in sludge-treated soil, and 0.015 μg/L in surface water, whereas values in the USA are approximately half of those mentioned above [9]. Current information concerning the manufacture, processing, use, and end-of-life of nanoscale TiO_2 has recently been summarized [10]. One of the standardized terrestrial test systems included in the endpoint list of the WPMN is the earthworm reproduction test [11]. This test can be performed not only in artificial soil, but also in natural soils to increase the environmental relevance of the results. Several methods have been described for the application of nanomaterials to soil, including (1) the dispersion of dry powder directly in the soil [12,13], (2) the application of a nanoparticle directly to the soil [13,14], and (3) the application of a suspension in food which is added to the soil [15]. We used TiO_2 and silver nanoparticles and various test organisms (soil microflora, plants, earthworms) to investigate five different application methods: (1) spiking the soil with powder using soil as the carrier, (2) spiking the soil with powder using silica sand as the carrier, (3) spiking the soil with an aqueous dispersion, (4) spiking the earthworm food with powder, and (5) spiking the food with an aqueous dispersion. Chemical analysis of the spiked soil showed that powders and aqueous dispersions achieved comparable homogeneity (in both cases, the standard deviation for six samples taken at each spiking concentration was < 5%). The application method did influence bioavailability, but the principal effect of the nanomaterials (i.e., toxic vs. nontoxic, stimulation or inhibition of reproduction) was similar for all methods. There was no difference in the effect from nanomaterials added directly to the soil or added to the feed, but direct application to the soil was preferred because this approach is described in the OECD guideline [11]. The application of powder produced better dose-response curves than dispersions, perhaps because the latter resulted in large nanoparticle agglomerates in the soil, thus reducing bioavailability. Experiments describing the application methods will be published separately.

On the basis of these results, we designed experiments to determine the potential effects of TiO_2 on earthworm reproduction in natural sandy soil with low sorption capacity, using three different nanomaterials included in the OECD Sponsorship Programme (NM 101, 103, 105) and spiking the soil with powder using soil as a carrier.

Results

The reproduction test results are presented in Table 1 (NM-101), Table 2 (NM-103), and Table 3 (NM-105). Each of the nanomaterials was tested at least twice, and all tests fulfilled test guideline validity criteria, i.e., (1) ≥30 juveniles must be produced in each of the replicate control vessels by the end of the test; (2) the reproductive coefficient of variation in the control vessels must be ≤30%; and (3) adult mortality in the control vessels over the initial 4 weeks of the test must be ≤10%.

We observed no mortality at all. The guideline also states that earthworm biomass must be monitored during the test. The biomass increased during the incubation period because food was added to the containers. The biomass change differed between the control and test soils, but the differences were not statistically different for any of the nanoparticles (Tables 1, 2, 3).

Two of the tests with NM-105 (tests 1 and 3) revealed a concentration-dependent stimulation of reproductive activity (Table 3), which ranged from 39% to 49% in test 1 (TiO_2 concentrations 50, 100, and 200 mg/kg dry matter) and from 9% to 38% in test 3 (TiO_2 concentrations 50, 200, 500, 750, and 1,000 mg/kg dry matter). All test concentrations resulted in a statistically significant increase in reproductive activity compared to the controls. Both tests started in January. In contrast, test 2 was started in spring, and there was no concentration-dependent stimulation of reproduction.

Reproduction was also stimulated by the two highest test concentrations of NM-101 (test 1, Table 1), and this test also commenced in February. In contrast, neither of the tests with NM-103 (Table 2) affected reproductive activity. One of these tests started in January, and the other, in April.

Figure 1 shows the mean number of juveniles in the control vessels (containing the natural soil RefeSol 01A) in tests starting at different times of the year. There is a clear circannual rhythm, with fewer juveniles in the tests starting in winter (January and February) but more in those starting in spring and summer (April to

Table 1 Effects of NM-101 in the earthworm reproduction test

Test and test start	Test concentration (mg/kg sdm)	Mortality (%)	Biomass per vessel at test start (g) ± SD	Biomass per vessel at test end (g) ± SD	Increase in biomass (%)	Number of juveniles per test vessel ± SD	SD (%)	Effect on reproduction (%)
Test 1 - February 2010	0 (control)	0	3.65 ± 0.21	6.06 ± 0.26	66	303 ± 25	8.3	-
	50	0	3.40 ± 0.18	6.22 ± 0.29	83	322 ± 20	6.2	-6.3[a]
	100	0	3.33 ± 0.32	6.31 ± 0.13	91	353 ± 11	3.1	-16.5[a]*
	200	0	3.27 ± 0.14	6.24 ± 0.38	91	373 ± 41	11.0	-23.8[a]**
Test 2 - January 2011	0 (control)	0	3.65 ± 0.22	5.65 ± 0.26	55	223 ± 15	6.7	-
	50	0	3.56 ± 0.28	5.83 ± 0.38	64	213 ± 22	10.3	4.5
	100	0	3.57 ± 0.22	5.94 ± 0.30	67	210 ± 16	7.6	5.8
	200	0	3.60 ± 0.24	5.77 ± 0.28	60	213 ± 47	22.1	4.5
	400	0	3.44 ± 0.15	5.88 ± 0.39	71	234 ± 20	8.6	-4.9[a]

[a]Negative values indicate stimulation. sdm, soil dry matter; SD, standard deviation. Asterisks indicate a statistically significant difference to controls (*$0.05 \geq p \geq 0.01$; **$0.01 \geq p \geq 0.001$).

September). No data are available for March and October to December. The maximum difference in reproductive activity seen between April 2010 (365 offspring) and January 2011 (208 offspring) was 157, which is 75% of the number of winter juveniles. The maximum standard deviation of 22% is significantly lower than the variation in the number of offspring during the year, indicating that the fluctuation in the absolute number of juveniles cannot be explained by biological variability.

The circannual difference is less obvious in test soils containing NM-105. Figure 2 shows some of the results as an example, comparing the control vessel (control from the tests with NM-105) with test soils containing 200 mg/kg NM-105 or NM-103. For the controls, the difference in reproductive activity between January 2010 (212 juveniles) and May 2010 (340 juveniles) was 128, which is 60% of the number of winter juveniles (January 2010). The difference in reproductive activity between January 2011 (208 juveniles) and May 2010 (340 juveniles) was 132, which is 63% of the winter juveniles (January 2011). Therefore, the difference in reproductive activity between summer and winter is comparable. The differences between summer and winter are statistically significant ($p \leq 0.05$). For NM-105 (200 mg/kg), no statistically significant differences were detected ($p \leq 0.05$). The difference in reproductive activity between May 2010 (290 offspring) and January 2011 (265 offspring) was 25, which is 9% of the number of winter juveniles (January 2011). The difference in reproductive activity between January 2010 (315 offspring) and January 2011 (265 offspring) was 50, which is 19% of the number of winter juveniles (January 2011) for NM-105. For NM-103, the difference between the spring juvenile numbers (April 2010, 343 juveniles) and the winter juvenile numbers (January 2011, 233 juveniles) is 110, which amounts to 47% of the winter juvenile numbers. This

Table 2 Effects of NM-103 in the earthworm reproduction test

Test and test start	Test concentration (mg/kg sdm)	Mortality (%)	Biomass per vessel at test start (g) ± SD	Biomass per vessel at test end (g) ± SD	Increase in biomass (%)	Number of juveniles per test vessel ± SD	SD (%)	Effect on reproduction (%)
Test 1 - April 2010	0 (control)	0	3.86 ± 0.22	5.55 ± 0.27	44	365 ± 43	11.8	-
	50	0	3.86 ± 0.22	5.51 ± 0.50	44	338 ± 20	5.9	7.4
	100	0	3.83 ± 0.27	5.73 ± 0.58	52	372 ± 57	15.3	-1.9[a]
	200	0	3.78 ± 0.38	5.70 ± 0.27	56	343 ± 34	9.9	6.0
Test 2 - January 2011	0 (control)	0	3.65 ± 0.22	5.65 ± 0.26	55	223 ± 15	6.7	-
	50	0	3.67 ± 0.33	5.68 ± 0.15	55	240 ± 31	12.9	-7.6[a]
	100	0	3.47 ± 0.27	5.77 ± 0.30	67	252 ± 42	16.7	-13.0[a]
	200	0	3.45 ± 0.13	5.71 ± 0.48	66	233 ± 40	17.2	-4.5[a]
	400	0	3.61 ± 0.22	5.98 ± 0.40	66	237 ± 38	16.0	-6.3[a]

[a]Negative values indicate stimulation. sdm, soil dry matter; SD, standard deviation.

Table 3 Effects of NM-105 in the earthworm reproduction test

Test and test start	Test concentration (mg/kg sdm)	Mortality (%)	Biomass per vessel at test start (g) ± SD	Biomass per vessel at test end (g) ± SD	Increase in biomass (%)	Number of juveniles per test vessel ± SD	SD (%)	Effect on reproduction (%)
Test 1 - January 2010	0 (control)	0	3.29 ± 0.24	5.47 ± 0.36	67	212 ± 46	21.7	-
	50	0	3.37 ± 0.43	5.80 ± 0.15	74	295 ± 44	14.9	-39.2[a]**
	100	0	3.49 ± 0.13	5.47 ± 0.10	57	299 ± 74	14.7	-41.0[a]**
	200	0	3.45 ± 0.14	5.47 ± 0.11	59	315 ± 42	13.3	-48.6[a]**
Test 2 - May 2010	0 (control)	0	3.81 ± 0.30	5.37 ± 0.34	41	340 ± 39	11.5	-
	50	0	3.62 ± 0.10	5.09 ± 0.20	41	341 ± 33	9.7	-0.3[a]
	100	0	3.66 ± 0.11	5.66 ± 0.33	55	343 ± 28	8.2	-0.9[a]
	200	0	3.58 ± 0.11	5.17 ± 0.40	44	290 ± 24	8.3	14.7
	500	0	3.54 ± 0.08	5.24 ± 0.40	48	253 ± 62	24.5	25.5
	1,000	0	3.63 ± 0.23	5.57 ± 0.26	54	319 ± 43	13.5	6.5
Test 3 - January 2011	0 (control)	0	3.70 ± 0.26	5.26 ± 0.40	42	208 ± 15	7.2	-
	50	0	3.68 ± 0.16	5.36 ± 0.08	46	239 ± 22	9.2	-8.8[a]*
	100	0	3.57 ± 0.20	5.39 ± 0.19	52	252 ± 15	6.0	-14.9[a]**
	200	0	3.46 ± 0.09	5.58 ± 0.24	61**	265 ± 31	11.7	-27.4[a]**
	500	0	3.59 ± 0.22	5.37 ± 0.46	50	238 ± 11	4.6	-14.4[a]**
	750	0	3.58 ± 0.12	5.43 ± 0.36	52	279 ± 27	9.8	-34.1[a]**
	1,000	0	3.43 ± 0.18	5.29 ± 0.53	54	286 ± 21	7.3	-37.5[a]**

[a]Negative values indicate stimulation. sdm, soil dry matter; SD, standard deviation. Asterisks indicate a statistically significant difference to controls (*$0.05 \geq p \geq 0.01$; **$0.01 \geq p \geq 0.001$).

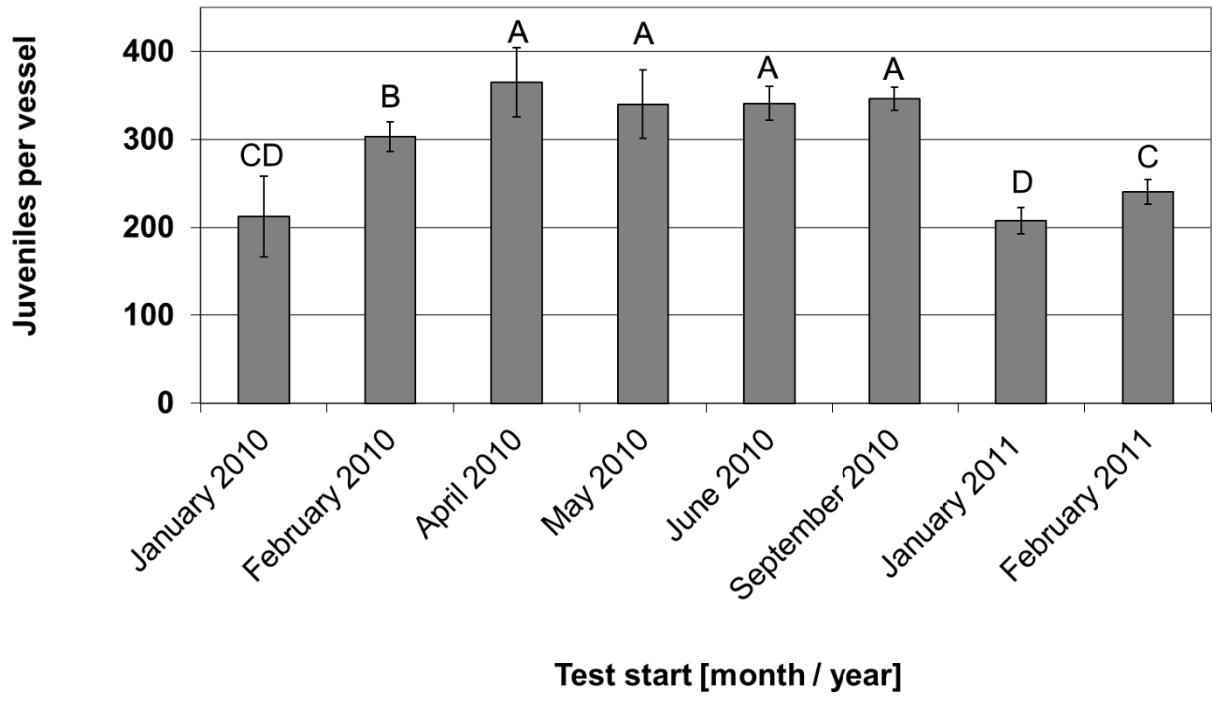

Figure 1 Number of offspring per control vessel in the reproduction test with *E. andrei*. All tests were performed according to OECD Test Guideline No. 222 [2] in natural soil. The figure shows the mean number of juveniles in the control vessels (containing the natural soil RefeSol 01A) and the standard deviation in tests starting at different times of the year. Results with the same letters are not statistically different.

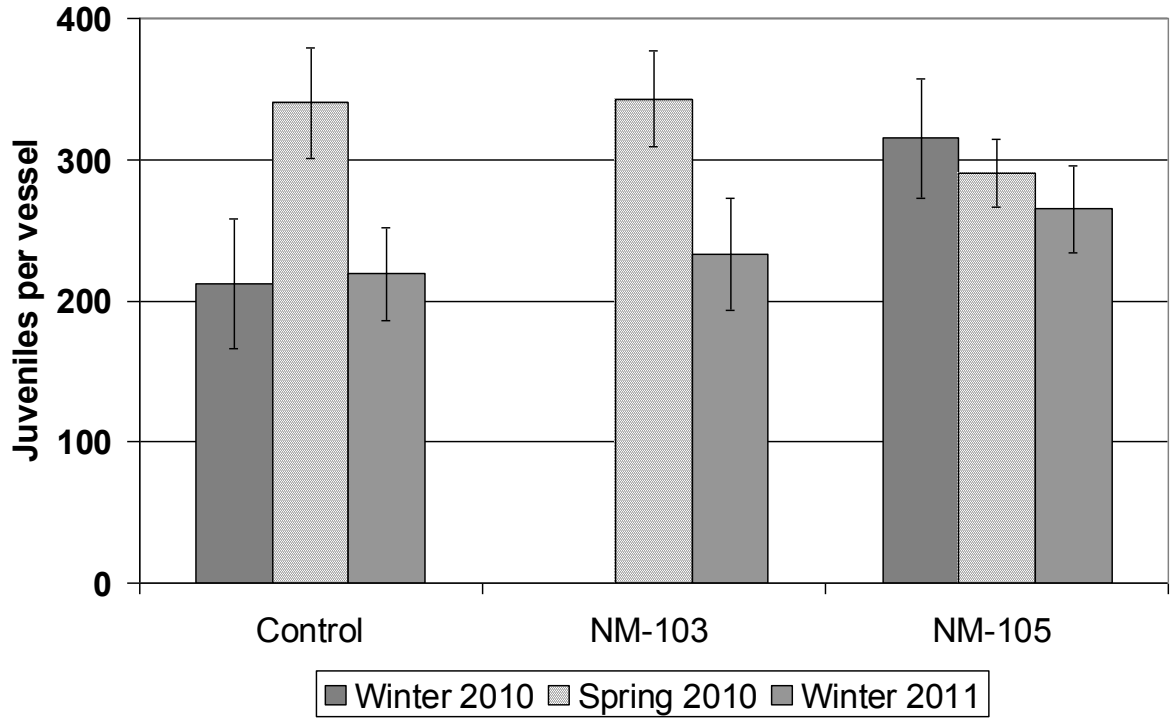

Figure 2 Difference in reproductive activity of Eisenia between summer and winter and influence of TiO_2. All tests were performed according to OECD Test Guideline No. 222 [2] in natural soil. The figure shows some of the results as an example comparing the control vessel (control from the tests with NM-105) with test soils containing 200 mg/kg NM-105 or NM-103. The mean number of offspring per vessel and the standard deviation are presented.

difference is statistically significant and comparable to the values obtained for the controls presented in this figure (Figure 2).

Discussion

Test performance

Our experiments showed that the OECD Test Guideline No. 222 (Earthworm Reproduction Test) is technically suitable for the testing of solid nanomaterials in natural soils. We did not encounter any handling difficulties, and the application of TiO_2 did not result in remarkably high standard deviations for the number of juveniles compared to the untreated controls, indicating that the test material was distributed homogeneously. The range of the standard deviations for all treated replicates presented in Tables 1, 2, 3 (3.1% to 24.5%) is comparable to the range for all control samples (6.7% to 21.7%).

Stimulation of reproductive activity

The observed stimulation of earthworm reproductive activity reflected the existence of an underlying circannual rhythm in the control vessels which was diminished or eliminated in the presence of NM-105. Circannual biological rhythms have been described for both vertebrates and invertebrates, but the underlying mechanisms are not yet understood [16]. Rozen [17,18] collected earthworms (*Dendrobaena octaedra*) in the field and cultured them in the laboratory under constant conditions, but even so, the reproductive rate was higher in the spring and summer than in the winter, indicating that reproductive activity was internally regulated. Neurosecretory hormones regulate cyclical functions such as reproductive behavior and secondary sex characteristics in earthworms [18,19], but whether TiO_2 influences the production of these hormones or the transduction of hormone-dependent signals remains to be determined.

Factors in the soil can also influence the circannual rhythm of earthworm reproduction observed in the control vessels because soil collected in winter but used for tests performed in summer also reduced reproductive activity. This effect was ameliorated by the addition of NM-105, stimulating reproduction by 17% at 200 mg/kg and by 27% at 500 mg/kg. There was no reproductive stimulation in a simultaneous test using freshly collected soil (data not shown). No obvious circannual rhythm in the controls was observed when earthworms were tested in artificial soil (14 tests were performed within a period of 4 years, with individual tests starting in nearly all months).

Any risk assessment for TiO_2 needs to consider the environmental relevance of any observations, and in this context, it is unknown whether earthworms in the field will be affected in the same manner as those under test conditions in natural soil. In many locations, the winter temperature falls below the 20°C we maintained in the laboratory, which will affect earthworm activity generally and may also suppress any impact of TiO_2.

In contrast to our results, a TiO_2 nanomaterial comparable to NM-105 resulted in a statistically significant 50% reduction in earthworm reproductive activity at a concentration of 1 g/kg [20]. The test was performed in a natural soil (sandy loam) with a slightly higher carbon content than the soil used in this study, and the nanoparticles were applied as a stock suspension. One significant difference between the experiments was the treatment of the soil. Whereas our treatment followed the relevant ISO guidelines for soil preparation and storage [21], the comparable study [20] used soil that was dried at 80°C, ground, sieved, and stored at room temperature until required, which could affect the bioavailability of nanoparticles and the effect of soil components significantly. Furthermore, because the study was designed as a limit test, the missing information on the test timing and the absolute number of juveniles reduces its comparability with our data.

Ecotoxicity of TiO_2 and influence of substance properties

Previous studies have shown that TiO_2 nanoparticles have a low toxicity, with effects on *Eisenia fetida* reproduction, metabolism, and DNA becoming evident at concentrations > 1,000 mg/kg [12]. Furthermore, several endpoints for *E. fetida* have been studied using artificial and field soils supplemented with TiO_2 nanoparticles by aqueous dispersion or dry powder mixing, and no significant effect has been observed on survival, cocoon production, cocoon viability, or total number of juveniles hatched from the cocoons up to a concentration of 10 g/kg. However, earthworms avoided certain artificial soils supplemented with 1 to 5 g/kg TiO_2 nanoparticles depending on the nature of the particles and could distinguish between particles in the nanometer and micrometer ranges. A TiO_2 nanomaterial comparable to NM-105 resulted in 45% avoidance at 1 g/kg and of 37% avoidance at 10 g/kg, whereas a micrometer-range nanomaterial was not avoided [22].

We found that the uncoated nanomaterial NM-105 had a clear effect on earthworm reproduction at the lowest test concentration (50 mg/kg). The nanoparticles selected for testing within the framework of the OECD Sponsorship Programme differed in several aspects, including crystalline structure, size, Brunauer-Emmett-Teller [BET] surface, and the presence or absence of a coating, all of which could potentially influence earthworm reproduction. However, because the nanoparticles differed in more than one parameter, it may be difficult to identify the most relevant properties affecting earthworm reproduction. This could be determined by testing panels of nanoparticles differing in single parameters.

The presence or absence of a coating is important because coatings can be worn away or degraded, modifying the particle structure and therefore its potential toxicity over time. In our experiments, NM-103 had no effect on earthworm reproduction, but we cannot exclude the possibility of a delayed impact after the coating is modified or lost. For example, in tests against *Vibrio fisheri*, the toxicity of soil eluates containing Fe/Co nanoparticles with a capping agent increased over time, suggesting that aging may have contributed to the degradation of the capping agent and a release of Co [23]. The comprehensive risk assessment of coated nanomaterials must therefore include the potential for aging and structural modifications after prolonged exposure to the soil. In a study dealing with aged TiO_2 composites, the apoptotic frequency appears to be more sensitive to TiO_2 nanoparticles than a conventional endpoint (mortality). Aged TiO_2 composites did not induce mortality in the earthworm *Lumbricus terrestris* up to the highest test concentration (100 mg/kg), but the apoptotic frequency increased [24]. Fresh (non-aged) material was not studied at the same time, so the influence of aging cannot be determined. However, the data indicate that TiO_2 can affect soil organisms beyond conventional effects such as increased mortality and reduced reproduction. The natural circannual rhythm in earthworm reproduction was abolished in soils spiked with NM-105, but was maintained in soils spiked with NM-103. The two materials have a similar primary particle size (20 vs. 21 nm), the same BET surface (60 m^2/g), but differ in their crystal structure and coating. NM-105 is uncoated and its crystal structure is a mixture of rutile and anatase, whereas NM-103 has a hydrophobic coating and a purely rutile crystal structure. The coating is likely to be responsible for the differential effects of the particles by preventing contact between TiO_2 and the environment. In the soil spiked with NM-105, earthworms are directly exposed to TiO_2, whereas this is not the case with NM-103.

NM-101 is also uncoated, and at the highest test concentration (200 mg/kg), this material was able to stimulate earthworm reproduction by 24% in an experiment initiated in winter 2010, but there was no observed effect in a similar experiment initiated in winter 2011. Similarly, the impact of NM-105 was less pronounced in the winter 2011 test compared to the winter 2010 test, suggesting that these differences (NM-101: a small effect in winter 2010, no effect in winter 2011) are likely to

Table 4 Nanomaterial properties (data from the Joint Research Centre, European Commission)

Nanoparticles	NM-101	NM-103	NM-105
Producer	Sachtleben	Sachtleben	Evonik
Trade name	Hombikat UV 100	UV-Titan M262	AEROXIDE® TiO_2 P25
Crystal structure	Anatase	Rutile	Rutile-anatase
Purpose	Active component for photocatalytic reactions	UV screening agent in sunscreen	Active component for photocatalytic reactions
Primary particle size (according to Scherrer, nm)	8	20	21
Composition (%)	TiO_2 = 91.7	TiO_2 = 89; Al_2O_3 = 6.2	TiO_2 > 99
BET surface (m^2/g)	> 250	60	60
Coating	None	Hydrophobic	None

BET, Brunauer-Emmett-Teller.

reflect biological variability. NM-101 and NM-105 both lack a coating, but they differ in several other aspects such as crystal structure, size, and BET surface (Table 4). Any of these parameters could be responsible for the qualitatively different effects of the two materials, with NM-105 appearing generally more potent, but this needs to be addressed in further investigations. It is also unclear whether the effect is triggered primarily by the chemical properties of TiO_2 or by the nanoparticle size (no bulk material with a primary particle size above the nanoscale range was tested).

Test soil

We carried out our experiments in accordance with OECD Test Guideline No. 222, which allows the use of natural soils. Our results indicate that the outcome of the test depends both on the time of the year the test soil is collected and the time of the year the test is carried out. This is the first time to our knowledge that the timing of a terrestrial test has been shown to influence the results. The influence of TiO_2 on circadian or circannual rhythm has already been reported in aquatic organisms. In zebra fish embryos, exposure to TiO_2 affected the regulation on genes controlling the circadian rhythm [25]. In the mysid *Praunus flexuosus*, seasonal differences in sensitivity to copper was observed, with no mortality in winter but a 96-h LC_{50} of 30.8 μg/L in summer [26]. Further investigations will be necessary to determine the conditions that need to be considered when tests are performed using natural soils. Standardized test guidelines must guarantee that results obtained in accordance with the guidelines are comparable and can be used for regulatory purposes. Therefore, the test medium and test conditions must be carefully specified in guidelines relating to the risk assessment of chemical substances.

Conclusions

Our experiments showed that OECD Test Guideline No. 222 (Earthworm Reproduction Test) can be used to test solid nanomaterials and that the preparation of test materials using 1% dry soil as a carrier is a suitable application method. We conclude that TiO_2 nanomaterials can affect earthworm reproduction if the test is carried out according to OECD Test Guideline No. 222 using natural sandy soil. The circannual biological rhythm of earthworm reproductive activity is affected, but the following issues remain to be clarified in further experiments:

1. We need to determine the specific properties of nanomaterials that are responsible for disturbing the circannual rhythm in earthworm reproductive activity. We found that NM-105 was more potent than NM-101, but we do not know whether the primary particle size, BET surface, crystalline structure, or impurities are relevant parameters. We also do not know whether the effect is caused by the chemical properties of TiO_2 or the size of particles (i.e., the nanoparticle size as opposed to its bulk form) or a combination of the above.
2. We need to determine whether nanomaterials can be modified to prevent them from disturbing the circannual rhythm of earthworms.
3. We need to determine why the circannual biological rhythm is more pronounced in natural soil than artificial soil and which properties of the soil are responsible for the effect. We need to test a range of soils to determine whether the effect is widespread. Most importantly, we need to consider how OECD Test Guideline No. 222 must be modified to ensure its general applicability (i.e., whether certain soil types should be excluded).
4. Finally, we need to understand the environmental relevance of the disturbance of the circannual biological rhythm caused by TiO_2 and exclude the possibility that the effect is limited to earthworms under test conditions. More data concerning the mode of action of TiO_2 nanoparticles would be useful in this regard.

Methods

Test soil

We carried out our experiments using the reference soil RefeSol 01A (sieved ≤2 mm) [27], a loamy, medium-acidic, and lightly humic sand, whose physicochemical properties are presented in Table 5. RefeSol soils were selected on behalf of the German Federal Environment Agency (*Umweltbundesamt*). They are suitable for testing the influence of substances on the habitat function of soils (bioavailability, effects on organisms). The soil RefeSol 01A reflects the properties mentioned in various terrestrial ecotoxicological guidelines of the OECD (e.g., tests with plants and soil microflora). The soils were sampled in the field and stored in high-grade stainless steel basins with drainage and ground contact on the open-air grounds of the institute. During the period of all the experiments performed in the study, red clover was sown on the stored soils. No pesticides were used. Appropriate amounts of soil were sampled 1 to 4 weeks before the test. If the soil was too wet for sieving, it was dried at room temperature from 20% to 30% of the maximum water-holding capacity [WHC_{max}] with period turning to avoid surface drying. If the tests did not start immediately after sieving, the soil was stored in the dark at 4°C under aerobic conditions [21]. We used RefeSol 01A as both the test and carrier soils.

Nanoparticle properties

We studied three different TiO_2 nanoparticles from the OECD Sponsorship Programme (NM 101, 103, 105) using the single batch applied by all participants. The properties of the used nanoparticles are presented in Table 4.

Six priority physicochemical characteristics have been specified as parameters to investigate in ecotoxicological studies, i.e., size, dissolution, surface area, surface charge, and surface composition/surface chemistry [28]. Surface charge and dissolution are strongly influenced by the environment [29-31], e.g., organic materials prevent nanomaterial agglomeration and result in a more homogenous distribution. It is therefore necessary to characterize the nanomaterials in the test medium in order to demonstrate a link between the chemical analysis and the effects data. Current methods are insufficient; therefore, it is necessary to develop a novel procedure including new extraction, cleanup, separation, and storage methods that minimize artifacts and increase the speed, sensitivity, and specificity of analytical techniques, as well as new techniques that can distinguish between abundant, naturally occurring particles and manufactured nanoparticles. The state of the art is included in the publications of Fareé et al. [32] and von der Kammer et al. [33]. Titanium is naturally abundant in soils, and no further characterization of the nanomaterials was attempted beyond the information presented in Table 4 because of yet unsolved problems in the characterization of manufactured TiO_2 nanoparticles in soil.

Table 5 Physicochemical properties of RefeSol 01A soil

Physicochemical properties	RefeSol 01A
pH	5.67
C_{org} (%)	0.93
N_{all} (mg/kg)	882
CEC_{eff} (mmolc/kg)	37.9
Sand (%)	71
Silt (%)	24
Clay (%)	5
WHC_{max} (ml H_2O/kg)	227

CEC, cation-exchange capacity; WHC_{max}, maximum water-holding capacity.

Application and test concentrations

The TiO_2 particles were applied by mixing the powdered test material and air-dried carrier soil which had the same physicochemical properties as the test soil (Table 5). Enough TiO_2 powder was added to the carrier so that the correct final test concentration was achieved when 1% carrier soil and 99% test soil were mixed to homogeneity (see below). The soil was mixed with a spoon rather than a pestle to avoid modifying the TiO_2 crystalline structure. Uncontaminated soil (at 20% to 30% of the WHC_{max}) was spread on a plate, and the spiked carrier soil was evenly distributed over the test soil before manually mixing. The mixed soil was adjusted to 55% WHC_{max} using deionized water. The standard test concentrations for TiO_2 were 50, 100, and 200 mg/kg soil dry matter, although we also tested higher concentrations in some experiments (400, 500, 750, and 1,000 mg/kg soil dry matter).

Ecotoxicological tests

All tests were performed as described in OECD Test Guideline No. 222: 'Earthworm Reproduction Test with *E. fetida*' [11], which allows the use of *E. fetida* and *Eisenia andrei* as test organisms. We used *E. andrei* which has been cultured in our laboratory for more than 15 years. The earthworms were acclimated to the test soil for 7 days prior to testing.

We filled polypropylene containers (Bellaplast GmbH, Alf, Germany) to a depth of approximately 5 cm with 640 g dry mass of soil (55% WHC_{max}) and then spread 40 g (wet weight) of cow dung (air-dried, ground, and moistened before application) onto the surface. The cows were kept in an ethical husbandry. The tests were performed with eight replicates for the control and four replicates for each TiO_2 concentration.

Ten earthworms weighing between 300 and 450 mg were added to each container, and the containers were incubated at 20 ± 2°C with a light/dark cycle of 16:8 h (approximately 700 lx). Once per week, the water content was checked gravimetrically and evaporated water was replaced. Every 7 days, 20 g (wet weight, corresponding to 5 g dry weight) of uncontaminated food was spread on the soil surface in each container. After 28 days, the adult earthworms were removed and weighed, and after 56 days, the number of juveniles in each test container was counted.

Statistical calculations were performed with the ToxRat® Pro 2.10 software for ecotoxicity response analysis (ToxRat® Solutions GmbH, Alsdorf, Germany). Statistical significance was calculated using one-sided Williams' multiple *t* test for the evaluation of dose-response curves and using Student's *t* test (homogenous variances) and Welch's *t* test (nonhomogenous variances) for the comparison of the control samples (Figure 1).

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Authors' contributions
KS performed all experiments and drafted the manuscript. KT was involved in the discussions concerning soil protection and helped draft the manuscript. KH-R participated in the design of the study and in the discussion of the results and was involved in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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