Regulatory testing of ZnO nanoforms – Fate (OECD 29, 318) and aquatic toxicity (OECD 201, 211) testing of representative nanoforms found on the EU market

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Background

In 2019, the ECHA requested an update of the dossier for zinc oxide requiring the addition of fate and effect data for zinc oxide nanoforms. Most of the tested ZnO nanoforms covered by literature targeted primary particles sizes around 30 nm and featured no coating, thus, they only resemble some of the registered ZnO nanoforms. Based on this data it was hypothesized that nanoforms of ZnO are less or equally toxic than the dissolved form inducing Zn^{2+} cations (hypothesis that zinc ions represent the worst-case). However, there were studies concluding that nano-ZnO seemed to be more toxic than the ionic form. Therefore, there was a concern for nano-ZnO which had to be clarified. A test scheme was specified by ECHA to determine the specific effect of the nano-ZnO. In a first step, fate data regarding the particle transformation/dissolution (TD) and dispersion stability (OECD TG 29, OECD TG 318) were requested for 28 zinc oxide nanoforms, which represented the nanoforms available at the European market at this time. The fate of the 28 nanoforms was screened to classify the particles in terms of their solubility and stability. In the second step, based on the generated fate data, representative nanoforms were chosen to investigate their chronic toxicity to aquatic organisms using the freshwater algal growth inhibition test (OECD TG 201) with *Desmodesmus subspicatus* and *Daphnia magna* reproduction test (OECD TG 211) (considering nano-specific test adaptations as available at this time, when the OECD GD 317 was not available yet). The ECHA test scheme included that ecotoxicological studies should be performed for the nanoform with the highest, lowest and a mean dissolved Zn^{2+} concentration based on the results from the TD test (OECD TG 29) and with a low dispersion stability, high dispersion stability and with condition-depending dispersion stabilities based on the results from the dispersion stability testing (OECD TG 318), respectively.

Fate (OECD GD 29, TG 318)

The **dispersion stability** was tested according to the OECD test guideline 318

- **Test item characterization:** Test items were characterized for their hydrodynamic diameter by DLS and for their Zeta potential at relevant pH.
- **Test performance:** Standard and alternative test media as described in the guideline were used for testing depending on Zeta potentials of the test items in the relevant pH range. The test media cover ranges of hydrochemical parameters relevant for nanoparticle agglomeration behavior such as pH, DOC, divalent ions. Test concentrations were set to 10^{12} particles / L. The screening test was performed with all materials at 3 pH and 3 salt concentrations with DOC present in the media. Dispersion stability was assessed after 6h. The results were used for selection of test materials for ecotoxicological testing. The extended test was performed with selected samples (only condition depending stability)
- **Test results:** Most samples (27/28) were found to be of condition depending stability with only one test item being stable at all conditions in screening step. 11 test items showed low miscibility with water due to hydrophobicity. These samples also showed high variability in terms of recovery due to inhomogeneous dispersions. Guidance how to deal with this issue was missing at time of test performance. High fractions of dissolved material were found for nearly all test items. Especially for pH 4 the results have to be considered being false positives as the material was dissolved to nearly 100% leading to high stability. Using this low pH value is questionable with regard to environmental realism.

The **solubility** of all 28 test items was also assessed by performing a modified transformation/dissolution test according to OECD GD 29

- **Test performance:** A loading of 100 mg/L was applied to test medium with pH 7.6. samples were taken after 0h, 6h and 24h. The aliquots were filtered through 0.2 µm and additionally subjected to ultrafiltration (MWCO 3kDa). The ultrafiltrates were considered as being the operationally defined dissolved fraction.
- **Test results:** Only low amounts were found dissolved with <10% dissolution observed for all test items. These results have to be assessed independently of the dissolved fractions observed in the OECD 318 test due to different total test item concentrations and different hydro chemical conditions. In addition test items in OECD 318 tests were dispersed by ultrasonication, while test material is applied to the test media without any further treatment in OECD GD 29 tests.

Test item	Total recoveries at sampling point 6h (% total concentration of 0h)								
	pH 4			pH 7			pH 9		
	with NOM			with NOM			with NOM		
	0mM	1mM	10mM	0mM	1mM	10mM	0mM	1mM	10mM
T0368-01	103	103	103	95.4	92.1	25.8	93.9	90.7	23.8
T0369-01	104	110	99	105	99.8	90.1	90.7	64.4	65.1
T0371-01	105	85.7	74.1	120	96.1	89.4	78.8	250	59.4
T0373-01	98.8	100	100	89.1	89.1	43.6	85.8	87.3	21.1
T0374-01	103	106	61.1	99.9	82.5	101	101	99.9	96.4
T0375-01	98.6	98.8	99.3	95.7	95.7	51.2	92.9	93.6	38.2
T0376-01	90.8	57.2	43.4	56.2	189	260	125	470	382
T0377-01	101	101	100	96.2	97.8	66.8	94.3	96.2	39.3
T0378-01	106	95	95.5	109	72.5	102	95.8	91.1	83.6
T0379-01	102	101	101	96.8	98.8	60.6	96.5	95.2	20.8
T0385-01	101	101	101	93.3	94.9	52.9	90.4	91.3	24.0
T0387-01	72.9	77.1	64.2	77.5	68.2	50.6	42.7	123	76.9
T0388-01	102	102	103	98.5	99.1	101	93.6	78.3	38.3
T0389-01	100	102	101	72.6	69.3	14.3	68.6	70.4	9.38
T0390-01	208	57.0	62.0	273	69.1	84.1	314	211	136
T0391-01	98.1	96.3	96.5	86.7	43.5	23.9	82.8	38.5	5.26
T0401-01	101	103	99.6	81.3	78.5	86.7	71.5	68.8	69.4
T0409-01	100	102	101	75.6	78.4	72.1	52.2	62.6	33.0
T0410-01	98.9	98	118	99.2	103	102	95.1	94.3	95.9
T0418-01	99.5	99	99.9	95.4	75.4	45.8	92.3	62.6	14.8
T0419-01	101	99.7	99.8	93.7	93.8	31.1	70.0	92.9	44.4
T0420-01	101	101	102	90.2	91.3	38.8	89.5	90.4	20.3
T0421-01	103	99.5	102	95.2	99.3	96.4	97.6	88.9	85.8
T0422-01	96.3	154	74.1	107	146	145	91.6	57.0	81.7
T0423-01	103	105	102	100	98.5	64.8	97.4	96.7	23.4
T0424-01	103	105	104	101	99.5	45.9	98.4	98.6	25.6
T0428-01	106	103	105	82.3	81.4	16.8	79.9	79.1	13.6
T0441-01	45.8	457	269	85.6	106	131	84.8	94.9	90.1

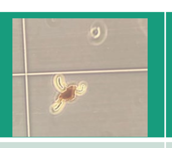
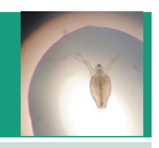
Table 1: Results of the dispersion stability screening test for all 28 test items. Materials selected for ecotox testing are marked in red

Aquatic toxicity testing (OECD 201, 211) – General outcome and lessons learned

A comprehensive set of preliminary tests was performed to adequately address the needs for testing nanoparticles, since the OECD GD 317 was not available at the time.

- **Adjustment of test media:** Get your test medium ready for testing. The EDTA concentration of the OECD growth medium was adjusted to be equimolar with the $FeCl_3$ concentration to avoid reduction of Zn ion concentration.
- **Preparation of test media:** Preparation followed the procedures described in the OECD TG 318 using probe sonication. Test concentrations were achieved using aliquots of the stock dispersion mixed with growth medium. Recoveries of 80 – 120% of the nominal total Zn concentration indicate that the method is suitable.
- **Characterisation of nanoparticles:** ZP and size measurements with a Malvern Zetasizer Nano provide results, however, the ecotoxicological test concentrations and the method for determining the parameters do not really match due to e.g. sedimentation of particles. Interpret the results with the necessary caution.
  It is possible to distinguish between effects caused by nanoparticles, ions and bulk material → Monitoring of the concentrations of the test substance in the aquatic phase (total Zn) and the ratio between particulate (<0.2 µm) and ionic zinc (<0.2 µm + 3kDa centrifugal filtration) should be monitored in samples from the test vessels.
- **Filtration:** Make sure that even simple steps such as filtration are carried out with care. Use small volumes and several filters instead of large volumes with one filter.
- **Determination of cell density:** Direct fluorescence measurements were performed. In addition, a method described by Hund-Rinke et al. (2016) was used. However, not all algae are the same. The method was established for the green algae *R. subcapitata* and results indicate that a simple transfer of the method for the green alga *D. subspicatus* was not possible.
- **Shading:** Despite floating of the particles at the beginning of the test, no influence on algae growth due to shading was observed.
- **Attachment:** During the test attachment of the particles to the algae was observed, which might be a reason for a decrease of the algae growth by shading due to the attachment.
- **Odd observations**: Expect the unexpected - Partial dissolution of the coating of a hydrophobic material lead to an unexpected high release of Zn (nano and dissolved) and Si into the water phase and adhesion to the glass wall (mysterious layer, see Figure 1). Indications for the presence of algae in the layer were found. This was considered as an artefact of the testing set up, which is not environmentally relevant.
- **Toxicity on algae and daphnia:** There is no clear evidence to suggest that nano-ZnO is more toxic than ionic Zn (Table 2).
  While EC10/EC50 values for the nanoforms is sometimes lower than for $ZnCl_2$ it has to be considered that here ion-, nano- and bulk-fraction were tested, which may all contribute to effects including e.g. physical effects by the bulk material.
- **Future Studies:** Next to the ion source ($ZnCl_2$) and complete test item, material that does not contain a bulk fraction (<0.2 µm filtrate) should also be analysed as outlined in the OECD Guidance Document 317, which was published after the testing was completed.

Table 2: Effect concentrations and confidence intervals of $ZnCl_2$ and different ZnO nanoforms on the growth rate of *D. subspicatus* on the reproduction of *D. magna*– EC_{10} and EC_{50} values expressed as total measured Zn.

Test substance	OECD 201 		OECD 211 	
	EC_{10} (mg/L)	EC_{50} (mg/L)	EC_{10} (mg/L)	EC_{50} (mg/L)
$ZnCl_2$	0.68 (0.65 – 0.71)	1.10 (1.06 – 1.16)	0.12 (0.09 – 0.14)	0.20 (0.20 – 0.22)
368	1.08 (0.91 – 1.29)	7.25 (5.93 – 8.86)	0.09 (0.06 – 0.11)	0.15 (0.13 – 0.17)
410	0.45 (0.36 – 0.55)	3.35 (3.04 – 3.70)	0.13 (0.07 – 0.14)	0.16 (0.13 – 0.16)
369	0.24 (0.14 – 0.35)	4.67 (3.68 – 6.37)	No effect at 100 mg/L	

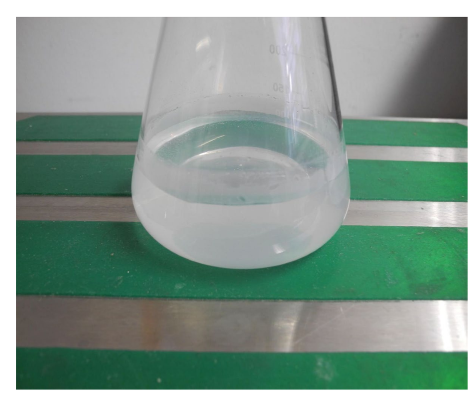


Figure 1: Picture of the layer observed in each Erlenmeyer flask at the highest test concentration.

Hund-Rinke, Kerstin; Baun, Anders; Cupi, Denisa; Fernandes, Teresa F.; Handy, Richard; Kinross, John H.; Navas, José M.; Peijnenburg, Willie; Schlich, Karsten; Shaw, Benjamin J (2016). Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles. Nanotoxicology. DOI: 10.1080/17435390.2016.1229517