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Accumulation potential and aquatic toxicity of relevant groups of nanomaterials and product formula

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Table of Content

1 Description of task

Task 4.6 Biokinetics and toxicity in aquatic organisms (TEKNIKER, SINTEF, NMBU, HWU, FURG, UFMG, GNano/IFSC-USP, UOS, BfR, EMBRAPA).

Accumulation potential and toxicity of manufactured nanomaterials (MNMs) at low concentrations in the aquatic environment are largely unknown. Systematic studies with relevant species, such as *Daphnia magna*, should be employed to investigate the effects of relevant selected nanomaterials in comparison to commercially available products.
According to their relevance, selected granular biodurable nanoparticles, carbon nanotubes and soluble nanoparticles have been tested. In parallel to the examination of toxicological effects, particle accumulation has been determined by appropriate analytical techniques. Sample preparation and dispersion protocols as well as toxicity and particle kinetics, should be related to other in vivo studies within WP4 and to in vitro studies within WP5.

Task 4.6.1 Toxicity studies in aquatic systems (TEKNIKER, SINTEF, NMBU, HWU, FURG, UFMG, GNano/IFSC-USP, UOS, EMBRAPA): Aquatic toxicity studies have been carried out for the MNMs selected and systematic in vivo/in vitro testing within NANoREG. Three priority species representing different trophic levels have been selected for testing with NANoREG core MNMs: the unicellular green algae *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the soil nematode *Caenorhabditis elegans*. The fish *Danio rerio*, the aquatic midge *Chironomus riparius* and the bacteria *Vibrio fischeri* have also been tested. For the three priority organisms, existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs).

Task 4.6.2 Biokinetic studies in aquatic systems (TEKNIKER, NMBU, BfR, SINTEF): The aim is to determine the aquatic toxicity and biokinetics of selected MNMs and to confirm grouping approaches for relevant nanomaterials. Particle accumulation in *Daphnia magna* has been analyzed using inductively coupled plasma mass spectrometry (ICP-MS). Furthermore, the uptake, accumulation and hepatic gene expression in Brown trout (*Salmo trutta*) were analyzed through water and diet by means of plasma ion analysis, liver and quantitative polymerase chain reaction (qPCR) analysis.

2 Description of work & main achievements

2.1 Summary

Task 4.6 has focused on the definition of methodologies to overcome the limitations in the standardization of manufactured nanomaterials (MNMs) ecotoxicity testing. Special attention has been devoted to the development of methods to prepare dispersions of MNMs for toxicological studies in aquatic systems, in order to address the reproducibility of such studies, through round robin-style testing. Existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs). Once the dispersion and ecotoxicity SOPs have been defined, the partners have tested the ecotoxicity tests with the NANoREG core MNMs. These tests have included several species representing different trophic levels (*Pseudokirchneriella subcapitata*, *Daphnia magna*, *Caenorhabditis elegans*, *Chironomus riparius*, *Danio rerio* and *Salmo trutta*), which have been exposed to the NANoREG core MNMs, such as multiwalled carbon nanotubes (MWCNTs) and Ag, ZnO, SiO_2, TiO_2 and CeO_2 NPs. Finally, concrete guidance and recommendations to design and conduct the ecotoxicity experiments have been proposed in the form of decision trees and degrees of toxicity based on specific cut off values (Section 2.5.3). According to these diagrams, some of the NANoREG core MNMs have been classified into hazard categories.

Silver nanoparticles have shown a high ecotoxic potency, and lower adverse effects have generally been observed in the test systems for MWCNTs and titanium, cerium, silicon and zinc oxide MNMs. In most cases, the different partners have reported comparable findings for the same MNM types with the same species. Despite a standardized MNM dispersion method and careful attention to the SOPs, some variation in the 50% effective concentration (EC) values has been determined between partners conducting the same tests, indicating the difficulty in achieving fully reproducible ecotoxicity data with MNMs.

Tables 1 to 5 summarise the main ecotoxicity results obtained. The EC50 values are marked with a colour coding in order to enable the interpretation of the toxicity levels:

	EC50 > 100 mg/L

	10 mg/L < EC50 < 100 mg/L
	1 mg/L < EC50 < 10 mg/L
	EC50 < 1 mg/L

Table 1. Test results obtained for the NANoREG core MNMs and the crustacean *Daphnia magna* (neonates)

Type of MNM	Code	Partner	NOM	EC50 (mg/L)	Stock (2.56 mg/mL)		Start-Highest concentration tested		End-Highest concentration tested	
					Z_{ave}	PDI	Z_{ave}	PDI	Z_{ave}	PDI
TiO_2	JRCNM01000a	Gnano/IFSC-UCP	No	**131.14 (89.32 - 281.47)** (72 h-test)	10.97	0.216	1115	0.192	1807	0.502
		SINTEF	No	**>100** (72 h-test)	236.5	0.200	961.4	0.200	15208	0.600
		SINTEF	No	**>100** (48 h-test)	236.5	0.200	961.4	0.200	2946	0.800
	JRCNM01001a	UOS	Yes	**>100** (72 h-test)	457.1	0.221	958.4	0.154	755.7	0.400
		SINTEF	No	**>100** (48 h-test)	489.9	0.300	2026.3	0.500	2099	0.900

	JRCNM01003a	UOS	Yes	**127.69 (89.81-303.19)** (72 h-test)	145.6	0.200	835.8	0.197	401.2	0.224
		SINTEF	No	**1399** (48 h-test)	155.3	0.300	2416	0.400	1167.7	0.600
SiO_2	**JRCNM02000a**	IK4-TEKNIKER	No	**2.05 (1.74-2.42)** (72 h-test)	252.1	0.424	246.0	0.402	301.8	0.563
		UOS	Yes	**28.28 (28.28-47.24)** (72 h-test)	373.3	0.236	448.9	0.292	304.2	0.202
ZnO	**JRCNM01100a**	Gnano/IFSC-UCP	No	**1.44 (0.11-18.28)** (72 h-test)	362.98	0.147	554.4	0.486	631.9	0.584
CeO_2	**JRCNM02102a**	IK4-TEKNIKER	No	**33.84 (32.76-34.95)** (72 h-test)	190.4	0.309	1666	0.267	4175	0.374
Ag	**JRCNM03000a**	Gnano/IFSC-UCP	No	**0.076 (0.071-0.083)** (72 h-test)	70.16	0.536	465.95	0.801	528.8	0.866
		IK4-TEKNIKER	No	**0.028 (0.020-0.039)** (72 h-test)	81.54	0.236	66.64	0.283	216.2	0.426
		UOS	No	**0.021 (0.017-0.026)**	152.9	0.173	873.7	0.538	2621.9	1.000
		UOS	No	**0.032 (0.019-0.066)**	212.7	0.202	2092.4	1.000	2642.5	1.000
MWCNTs	**JRCNM04000a**	SINTEF	No	**152.6** (48 h-test)	36057.8	1.000	24650	0.700	366	1.000
	JRCNM04001a	IK4-TEKNIKER	No	**47.87 (38.09-30.17)** (72 h-test)	1602	0.591	308.1	0.569	3915	0.659
		SINTEF	No	**>100** (48 h-test)	*	-	4925	0.500	32739.3	0.700
	JRCNM04100a	SINTEF	No	**49.7** (72 h-test)	*	-	43180	0.700	1101	0.700
		SINTEF	No	**108.7** (48 h-test)	*	-	43180	0.700	1553.8	0.900

*Stock solution too concentrated for instrument – error value returned.

Table 2. Test results obtained for the NANoREG core MNMs and algae *Pseudokirchneriella subcapitata*

Type of MNM	**Code**	**Partner**	**NOM**	**EC50 (mg/L)**	**Stock (2.56 mg/mL)**		**Start (0h)-Highest concentration tested**		**End (72h)-Highest concentration tested**	
					Z_{ave}	**PDI**	**Z_{ave}**	**PDI**	**Z_{ave}**	**PDI**
TiO_2	**JRCNM01000a**	SINTEF	No	**661.7 (0.839-521991)**	236.5	0.200	317.0	0.200	361.5	0.300
	JRCNM01001a	Gnano/IFSC-UCP	No	**50.57 (19.24-132.93)**	13.0	0.222	421.4	0.239	420.1	0.299
		SINTEF	No	**43.09 (3.7-505)**	489.5	0.300	519.9	0.400	700.8	0.500
	JRCNM01003a	IK4-TEKNIKER	No	**0.27 (0.15-0.48)**	151.6	0.334	2389.3	0.244	2697	0.203
		IK4-TEKNIKER	Yes 8 mg/L	**>100**	151.6	0.334	175.3	0.259	178.7	0.270
		IK4-TEKNIKER	Yes 20 mg/L	**>100**	151.6	0.334	152.6	0.245	173.8	0.316
		SINTEF	No	**11.34 (3.29-39.17)**	162.4	0.300	3592.7	0.400	628.9	0.600

ZnO	**JRCNM01100a**	Gnano/IFSC-UCP	No	**0.07 (0.021-0.23)**	328.62	0.257	279.7	0.600	954.3	0.375
CeO_2	**JRCNM02102a**	IK4-TEKNIKER	No	**1.24 (1.07-1.43)**	196.8	0.345	2558	0.301	1940	0.279
		IK4-TEKNIKER	Yes 8 mg/L	**31.9 (10.2-99.5)**	196.8	0.345	176.4	0.234	235.3	0.219
		IK4-TEKNIKER	Yes 20 mg/L	**74.3 (18.0-305.9)**	196.8	0.345	167.9	0.215	259.4	0.235
Ag	**JRCNM03000a**	Gnano/IFSC-UCP	No	**0.025 (0.001-0.940)**	54.03	0.501	321.2	0.587	411.3	0.801
		IK4-TEKNIKER	No	**0.021 (0.004-0.121)**	91.69	0.212	92.11	0.214	94.27	0.227
		IK4-TEKNIKER	No	**0.021 (0.013-0.035)**	-	-	82.31	0.312	82.30	0.316
		NMBU	No	**0.008 (0.005-0.010)**	73.6	0.284	-	-	250	0.535
		SINTEF	No	**0.010 (0.002-0.062)**	74.49	0.300	67.6	0.300	66.9	0.300

Table 3. Test results obtained for the NANoREG core MNMs and nematode *Caenorhabditis elegans*

Type of MNM	**Code**	**Partner**	**NOM**	**EC50 (mg/L)**	**Stock (2.56 mg/mL)**		**Start (0h) - highest concentration tested**		**End (72h) - Highest concentration tested**	
					Z_{ave}	**PDI**	**Z_{ave}**	**PDI**	**Z_{ave}**	**PDI**
TiO_2	JRCNM01001a	UFMG	No							
		NMBU	No	**16.31 (7.77-21.10)**	499.3±13.25	0.394±0.014	1072±27	0.188±0.049	198±13.2	0.405±0.045
		UOS	Yes	**>50**	379.3±7.67	0.167±0.01	1606.90±48.06	0.352±0.028	4800.67±829.99	1.303±0.072
		EMBRAPA	No	**42.53 (27.24-64.52)**						
	JRCNM01003a	UOS	Yes	**>50**	158.43±4.13	0.189±0.02	807.67±149.09	0.203±0.013	1820.90±28.03	0.470±0.032
CeO_2	JRCNM02102a	NMBU	No	**>50**	184.2±7.17	0.257±0.017	1116±46	0.166±0.081	213±11.7	0.407±0.225
		UOS	Yes	**>50**	170.10±3.37	0.236±0.01	2077.77±122.81	0.558±0.044	1809.20±185.71	0.397±0.054
Ag	JRCNM03000a	UFMG	No	**<18**						
		NMBU	No	**0.96 (0.95-0.97)**	75.8±1.16	0.292±0.035	623±41	0.465±0.083	520±91.8	0.716±0.158
		UOS	Yes	**Growth 3.35<EC20>9.00 Reproduction 0.78<EC20>1.13**	166.63±2.87	0.247±0	193.17±3.79	0.170±0.009	206.67±0.91	0.233±0.009
		EMBRAPA	No							

	JRCNM03002a	NMBU	No	**>100**	902.4± 226.2	0.592± 0.112	1265± 51.6	0.223±0. 139	305.5± 19.2	0.333±0 .267
SiO_2	JRCNM02000a	UFMG	No	*Non- linear resonse						
		UOS	Yes	**>50**	220.77 ±4.69	0.153± 0.01	741.83 3±120. 98	0.452±0. 061	1133.2 3±234. 71	0.523±0 .090
	JRCNM02003a	UFMG	No	*Non-linear response						
ZnO	JRCNM01100a	UFMG	No	<10**						
MWC NTs	JRCNM04000a	UFMG	No	**<100**						

*Non-linear dose response. Enhanced effects at low compared to high concentrations, possibly due to aggregation

**Non-reproducible effects between tests.

Whilst the data for many of the tests did not allow the EC50 to be calculated, in many cases due to a non-linear dose-response, some minor effects in endponts were seen for some of the MNMs. To enable comparison between organisms and particles, these are summarised below.

- **MWCNTs (JRCNM04000a)** - UFMG: Recovery, fertility and growth **not affected by any of the concentrations.** Slight reduction in reproduction at (≥ 10 mg/L)
- **ZnO NPs (JRCNM01100a)** - UFMG: Recovery **slightly affected at 100 mg/L** and **0.001 to 0.1 mg/L**. Fertility and reproduction reduction between **0.032 and 100 mg/L.**
- **SiO_2 NPs** - UFMG: JRCNM0**2000a** MNM caused **reduction in reproduction** over a larger range of concentrations (**0.1 to 50 mg/L**), compared to **JRCNM02003a (0.1 to 0.32 mg/L**).
- **Ag NPs (JRCNM03000a):** The highest concentrations (**2 and 4 mg/L**) tested by NMBU resulted in a **significant reduction in growth (~50 %),** and **fertility and reproduction were completely inhibited**. UFMG and UOS reported considerable reduction in reproduction.
- **Ag nanorods (JRCNM03002a)** - UFMG: Recovery, fertility, reproduction and growth **not affected** by any of the concentrations tested.
- **TiO_2 NPs (JRCNM01001a)** - NMBU: The most sensitive endpoint was **reproduction**, especially at the highest concentrations (**17-50 mg/L).**
- **CeO_2 NPs (JRCNM02102a)** - NMBU: Growth and fertility **slightly affected** at **intermediate concentrations** (5.5-17 mg/L).

Table 4. Test results obtained for the NANoREG core MNMs and fish *Danio rerio*

Type of MNM	Code	Partner	NOM	EC50 (mg/L)	Stock (2.56 mg/mL)		Start (0h)-Highest concentration tested		End (96h)-Highest concentration tested	
					Z_{ave}	PDI	Z_{ave}	PDI	Z_{ave}	PDI
Ag	**JRCNM03000a**	Gnano/IFSC-UCP	No	**10.07 (4.57-22.22)**	60	0.548	150.1	0.342	561.9	0.584

		UOS	No	**0.588 (0.162-2.143)**	151.16	0.240	150.2	0.141	216.8	0.150
SiO_2	**JRCNM02000a**	UOS	Yes	**>100**	283.7	0.185	515.5	0.323	1447.3	0.805
TiO_2	**JRCNM01000a**	EMBRAPA	No	**>100**	-	-	-	-	-	-
		EMBRAPA	Yes	**>100**	-	-	-	-	-	-
	JRCNM01001a	Gnano/IFSC-UCP	No	**>100**	37.52	0.218	493.8	0.289	413.7	0.480
ZnO	**JRCNM01100a**	EMBRAPA	No	**>100**	-	-	-	-	-	-
		EMBRAPA	Yes	**>100**	-	-	-	-	-	-

Table 5. Test results obtained for the NANoREG core MNMs and aquatic midge *Chironomus riparius*

Type of MNM	Code	Partner	NOM	EC50 (mg/L)	Stock (2.56 mg/mL)		Start (0h)-Highest concentration tested		End (48h)-Highest concentration tested	
					Z_{ave}	PDI	Z_{ave}	PDI	Z_{ave}	PDI
TiO_2	**JRCNM01001a**	UOS	Yes	**>100**	390.1	0.238	412.4	0.266	2168.0	0.817
	JRCNM01001a	UOS	No	**>100**	435.7	0.243	1093.5	0.145	2579.9	1.000
	JRCNM01003a	UOS	No	**>100**	137.9	0.198	2256.3	0.230	6288.3	1.000
	JRCNM01003a	UOS	No	**>100**	186.1	0.233	2694.6	0.689	6175.9	1.000
SiO_2	**JRCNM02000a**	UOS	No	**>100**	266.6	0.232	658.9	0.412	406.2	0.264
		UOS	No	**>100**	268.1	0.195	475.0	0.303	318.5	0.211
CeO_2	**JRCNM02102a**	UOS	No	**>100**	169.4	0.321	-	-	-	-
ZnO	**JRCNM01100a**	UOS	Yes	**>100**	211.1	0.112	193.3	0.110	1467.67	0.617
				163.31	248.2	0.166	655.8	0.227	2154.4	1.000
Ag	**JRCNM03000a**	UOS	No	**5.42 (2.169-19.718)**	172.1	0.229	207.47	0.206	204.63	0.201
		UOS	No	**7.44 (2.946-24.7)**	156.1	0.262	194.5	0.210	181.8	0.185

2.2 Background of the task

Among the multiple pathways of MNMs into the ecosystems, aquatic organisms constitute one of the most important for their entrance and transfer throughout the food web. Surface waters receive pollutants from atmospheric deposition, leaching from soil and through direct inputs, such as run-off and wastewater discharges from domestic and industrial sources [1]. Surface water bodies can also import water from groundwater reservoirs, transporting with it MNMs [2]. Furthermore, the aquatic environment has been targeted for some nano-scale environmental remediation techniques [3]. Despite some methods for detecting and characterizing MNMs in natural waters being developed, the information on levels in aquatic

environments is scarce [2]. There are still major knowledge gaps on production, application and release of nanomaterials that affect the modeled values, though an agreement on the order of magnitude of the environmental concentrations can be reached. According to a study by Gottschalk et al. [4], modeled and analytical concentrations of TiO_2, ZnO, CeO_2 and Ag NPs, fullerenes and CNTs in surface waters are in the µg/L range. These MNMs are also included in the priority lists for risk assessment considering production volumes [5], and Garner et al. [6] classified some of these MNMs as the most persistent in freshwater systems, although the type of water was a key factor in their environmental fate. In 2014, the OECD published an expert meeting report [7] discussing the applicability of existing OECD Test Guidelines (TGs) to the fate and ecotoxicity of MNMs, in order to identify whether there was a need to amend current TGs or to develop new ones. It was concluded that a better understanding of the complex interactions between MNMs and environmental compartments should be achieved in order to give specific recommendations for their ecotoxicological assessment. Specifically, the advice regarding aquatic ecotoxicity covered three main issues: the preparation of the stock suspensions, the preparation of the exposure solution, and the test requirements.

Task 4.6 is specifically related to the following questions and needs set within NANoREG: Q1/Q2 (Measurement and characterization), Q3 (Characterization/Transformation), Q4 (Metrology and dose metrics), Q5 (Extrapolation and Grouping), Q9 (Mode of action), Q10 (Hazard) and Q14 (Risk assessment). The close collaboration with partners in WP2 for the definition of methods to prepare dispersions of MNMs for ecotoxicity assessment has helped to obtain the results reported in the present deliverable.

The analysis of the ecotoxicity test results will provide answers towards the regulatory questions specified above, and will also enable to offer regulators modified OECD and ISO SOPs specially tailored for the testing of MNMs. The results of Task 4.6 can be used by regulators for (i) selection of ecotoxicity tests which offer a more accurate assessment of MNM effects, (ii) establishing safe environmental concentrations of MNMs (or at least for specific MNMs), and (iii) for highlighting which MNM types are of more/less concern from an environmental perspective.

2.3 Description of the work carried out

2.3.1 Toxicity studies in aquatic systems

Subtask 4.6.1 Toxicity studies in aquatic systems has developed several Standard Operational Procedures (SOPs) to overcome the limitations in the standardization of manufactured nanomaterials (MNMs) ecotoxicity testing. Given the small number of partners involved in aquatic toxicity studies (9 in total), a fully comprehensive analysis of the NANoREG core MNMs with a broad range of species and endpoints has not been possible. Nevertheless, significant advances in the standardization of nanoecotoxicity testing have been made.

Special attention has been devoted to the standardization of methods to prepare dispersions of MNMs for ecotoxicity assessment. The calibration of the acoustic power and de-agglomeration efficiency delivered by probe sonicators has been completed, following the *'SOP for probe sonicator calibration of delivered acoustic power and de-agglomeration efficiency for ecotoxicological testing'*. This calibration is a key requirement in comparative testing, being necessary for harmonization of MNM dispersion preparation and characterization procedures. Moreover, the *'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media'* has been used to generate benchmark data on size distributions and stabilities of batch dispersions of MNMs. This method has led to improve the dispersion state of MNMs in Milli-Q water, which are subsequently diluted into specific media necessary for the ecotoxicity tests. The MNMs tested were SiO_2 NPs (JRCNM02000a, JRCNM02003a), CNTs (JRCNM04000a, JRCNM04001a, JRCNM04100a), Ag NPs (JRCNM03000a, JRCNM03002a), TiO_2 NPs (JRCNM01000a, JRCNM01001a and JRCNM01003a), CeO_2 NPs (JRCNM02102a), $BaSO_4$ NPs (JRCNM02020a) and ZnO NPs (JRCNM01100a and JRCNM01101a). This work has been conducted by SINTEF, NMBU, TEKNIKER and other partners of WP2, according to the SOPs developed by Andy Booth and Keld Alstrup Jensen (WP2). One of the key issues in ecotoxicity testing of MNMs is their reproducible dispersion in the different culturing media required for each organism. A specific ecotoxicity dispersion SOP has been prepared in collaboration with WP2, which includes the optional addition of environmentally representative concentrations of Suwannee River natural organic matter (SR-NOM) where MNM dispersions are unstable (20 mg/L in the dispersion media or 10 mg/L in the subsequent step in the exposure media). The SOPs mentioned above are available on CIRCABC (Library > C-NANoREG results > NANoREG developed improved SOPs and Methods > WP2 developed improved SOPs and Methods).

The specific SOPs for MNM ecotoxicity assessment generated within Task 4.6.1 are included in the present deliverable in Annexes 1 to 4 and have also been uploaded to CIRCABC (Library > C-NANoREG results >

NANoREG developed improved SOPs and Methods > WP4 developed improved SOPs and Methods). The most relevant modifications made to standardized procedures (OECD, ISO) in these SOPs are detailed below:

- Standard Operating Procedure: Toxicity Test with *Daphnia magna* for NANoREG core nanomaterials, based on the OECD-Guideline 202 "Daphnia sp., Acute Immobilization Test" [8] and the ISO 6341:2012 "Water Quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus. Acute toxicity test" [9]. An alternative test procedure has been established, which includes increasing the age of the test organisms (4 days) and the duration of the exposures (72 h). These modifications allow control of the effect of organism age on the ecotoxicity results and provide a better overview of the MNM behavior over a longer period of time. The optional addition of environmentally representative concentrations of SR-NOM where MNM dispersions are unstable (20 mg/L in the dispersion media or 10 mg/L in the subsequent step in the exposure media) is another major modification introduced in this SOP with respect to ISO and OECD Guidelines.

- Standard Operating Procedure: Toxicity Test with Microalgae *Pseudokirchneriella subcapitata* for NANoREG core nanomaterials, based on the OECD-Guideline 201 "Algal growth inhibition test" [10] and a chlorophyll fluorescence technique referenced in this Guideline [11] to estimate the biomass concentrations. Extracted chlorophyll is used as a means of deriving the biomass of an algal culture in the presence of MNMs, which interfere with measurements of culture density normally made by optical absorbance. The particulates and cell debris settles to the bottom of the extraction tubes while the chlorophyll is in solution and is measured fluorometrically. Apart from the optional addition of SR-NOM, some modifications of the standard chlorophyll extraction procedure have been adopted due to the nature of the tested substances.

- Standard Operating Procedure: Toxicity test with the nematode *Caenorhabditis elegans* for the NANoREG core nanomaterials, based on the International standard ISO 10872:2010 "Water Quality- Determination of the toxic effects of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)" [12]. Apart from the optional addition of SR-NOM, the main change to the ISO protocol has been that all test exposures are performed in low ionic strength aqueous media, rather than in soil or on agar plates. This is because the agar or the standard nematode growth media can cause MNM agglomeration and reduce its toxicity [13].

- Standard Operating Procedure: Fish, Acute Toxicity Test for NANoREG core nanomaterials, based on the OECD-Guideline 203 "Fish, Acute Toxicity Test" [14]. The optional addition of environmentally representative concentrations of SR-NOM where MNM dispersions are unstable is the main modification introduced in this SOP with respect to the OECD-Guideline.

Following the finalization of the dispersion and ecotoxicity SOPs, the partners have tested the ecotoxicity tests with the NANoREG core MNMs, according to the test matrix included in Table . Three priority species representing different trophic levels have been exposed to NANoREG core MNMs: the crustacean *Daphnia magna*, the unicellular green algae *Pseudokirchneriella subcapitata* and the soil nematode *Caenorhabditis elegans*. The fish *Danio rerio* and the aquatic midge *Chironomus riparius* have also been tested. Existing OECD and ISO standard methods for ecotoxicity assessment have been adapted specifically for MNM testing and developed into defined Standard Operational Procedures (SOPs).

Table 6. Aquatic ecotoxicity test matrix of NANoREG core MNMs (version 5, actualized on 17 December 2015)

		PARTNER RESPONSIBLE				
		IK4-Tekniker		NMBU	GNano/IFSC-USP	University of Seoul
Type of MNM	MNM ID code	Algal growth inhibition (*P. subcapitata*) OECD-201	*D. magna* Acute Im. test OECD-202	*C. elegans* (ISO 10872)	Fish acute toxicity test *D. rerio* OECD 203	*Chironomus riparius* Mortality test OECD-235
TiO_2	JRCNM01000a	S	G, S			
	JRCNM01001a	G, S	S, US	N, E, US	G, E	US
	JRCNM01003a	T, S	US, S	US		US
SiO_2	JRCNM02000a		T, US	U, US	US	US
	JRCNM02003a			U		
ZnO	JRCNM01100a	G	G	U		US
	JRCNM01101a				E	
CeO_2	JRCNM02102a	T	T	N, US		US
Ag	JRCNM03000a	G, T, N	T, G, US	U, N, US	G, US	US
	JRCNM03002a	N		U, N		
MWCNTs	JRCNM04000a	S	S	U		
	JRCNM04001a	S	T, S			
SWCNTs	JRCNM04100a	S	S			
		Main priority species			Other species	

T=TEKNIKER; S=SINTEF; N= NMBU; G=GNano/IFSC-USP; U=UFMG; US=University of Seoul; E=EMBRAPA

The WP1 tasks have been supported by providing the templates to collect the ecotoxicity data generated in task 4.6.1. The templates for *D. magna*, *P. subcapitata*, *C. elegans* and *D. rerio* ecotoxicity tests have been created by TEKNIKER, NMBU and IFSC-USP/GNano and have been sent to Task 1.5 leader and uploaded on CIRCABC.

Another significant advance made in this period is the coordination and development by SINTEF and NMBU of a guidance document in collaboration with WP2: *'Technical guidance document on procedures for the quantification of manufactured nanomaterials exposure and fate in dispersions for aquatic ecotoxicological studies'* (CIRCABC location: Library > C-NANoREG results > NANoREG developed_improved SOPs and Methods > WP2 developed_improved SOPs and Methods). The guidance document focuses on improved characterization of the exposure solutions used in toxicity tests. It was submitted to the NANoREG project group and has been distributed and implemented in some of the ecotoxicity tests performed in NANoREG. Importantly, the guidance document has been evaluated by SINTEF as part of the work conducted with WP2 and WP4. A copy of the guidance document is included as an appendix in NANoREG Deliverable Report D2.8 '*Test item preparation, exposure, dose and fate for regulatory purposes and toxicology*', and a detailed overview of the evaluation process and associated data provided in the main text of D2.8.

2.3.2 Biokinetic studies in aquatic systems

Biokinetic studies have been conducted for the quantification of CeO_2 NPs (JRCNM02102a) and Ag NPs (JRCNM03000a) in *Daphnia magna* by BfR, in cooperation with SINTEF, NMBU and TEKNIKER. The technique used for this purpose was inductively coupled plasma mass spectrometry (ICP-MS). Both types of nanoparticles were applicated in five different concentrations and one control was run without nanoparticle treatment.

Furthermore, NMBU has analyzed the uptake, accumulation and hepatic gene expression in Brown trout (*Salmo trutta*) exposed to Ag (ions, and JRCNM03000a MNM) through water and diet. Juvenile trout were obtained from the hatchery Bjørkelange Settefisk (1940 Bjørkelangen) and originated from a local wild Brown trout strain in Aurskog-Høland (Norway). The fish were acclimatized in the water used for the experiment (local drinking water) for two weeks at 10 °C before the 96 h exposure period. The organisms were exposed, through water and diet, to silver as Ag ions (Ag^+) (added as $AgNO_3$) and Ag NPs (JRCNM03000a). In the dietary exposure the fish were exposed to fodder contaminated with silver (ions or NPs) to a nominal Ag concentration of 12.5 µg/g fodder, and the control group was fed uncontaminated fodder. The number of fish in each of the three dietary groups was 20. The dietary exposure was conducted in a flow through system, and fish were fed twice a day. In the waterborne exposure the fish were exposed to three different concentrations, 2, 5 and 10 µg/L of Ag as Ag^+ (added as $AgNO_3$) and Ag NPs. The water exposed fish were fed uncontaminated fodder. The waterborne exposure was conducted in a semistatic setup, where 30 of the 50 L were exchanged once a day and the exposure were renewed. In these groups, there were only 6 fish per group and they were fed only once a day before the renewal of the exposure water. Control groups were included for both the dietary and waterborne exposures, The fish were sacrificed after 96 h. Blood were sampled for plasma ion analysis, liver and gills were sampled for accumulation and qPCR analysis.

2.4 Results

2.4.1 Toxicity studies in aquatic systems

2.4.1.1 Validation of the dispersion protocol for MNMs in environmental exposure media.

Five NANoREG partners participated in a round robin style benchmarking of the NANoREG-ECOTOX Standard Dispersion SOP (WP4 partners SINTEF, NMBU, TEKNIKER and WP2 partners SINTEF, UdL and LEITAT). A suite of 14 MNMs selected from the NANoREG core material list were chosen for use in this process. Detailed data about the results obtained have been provided by NRCWE, SINTEF and CNR in the deliverable D 2.6 '*Validated protocols for test item preparation for key in vitro and ecotoxicity studies*'. As an example of the characterization conducted, Figure 1 provides a summary of the Z_{ave} values obtained in the benchmarking study by each partner for each of the 14 core nanomaterials studied (N.B not all partners were able to provide data for all 14 of the core nanomaterials). Briefly, the data show that reproducible dispersions for most of the 14 core nanomaterials could be achieved at both the intra- and inter-laboratory levels. However, results for certain nanomaterials such as the high aspect ratio nanomaterial (HARN) carbon nanotubes (NM4001, NM401 and NM411) were limited owing to the use of dynamic light scattering (DLS) as the selected method for dispersion characterization. Alternative characterization techniques such as transmission electron microscopy (TEM) are needed for an improved dispersion characterization of HARNs. However, the work shows that reproducible nanomaterial dispersions can be achieved and implemented as part of aquatic ecotoxicity studies such as those reported here.

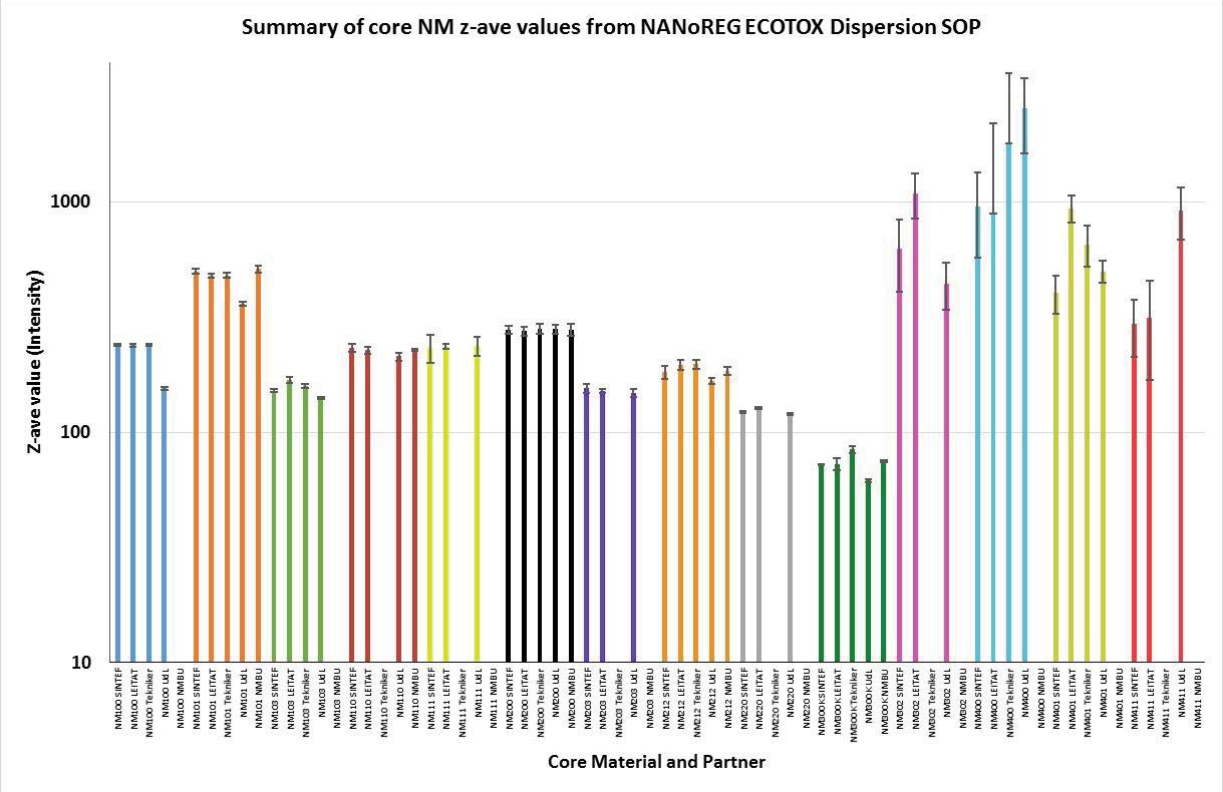

Figure 1. Individual hydrodynamic mean Z_{ave} values in batch dispersions for each of the 14 selected NANoREG core MNMs generated by each laboratory participating in the round robin benchmarking study. The individual data points presented are average values determined by individual partners from triplicate samples (n=10*). *Same sample analyzed x10.

2.4.1.2 *Daphnia magna* Acute Immobilization Tests

<u>72h-pretest studies using neonates (<24h) and 4-day old individuals for specific NANoREG core MNMs</u>

The exposure times were increased to 72 h in order to have a better overview of the NMs behaviour during a longer period of time. Despite this increment, no additional feeding was carried out to avoid introducing test variables that could interact with the NMs. The effect of the age of the organisms in the ecotoxicity results was also analyzed, using both 24h neonates and 4 days organisms.

The available standard ecotoxicity tests for *Daphnia magna* include:

- OECD-Guideline 202 "Daphnia sp., Acute Immobilization Test" [8]
- ISO 6341:2012 "Water Quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus. Acute toxicity test" [9].

Based on previously published data and personal experience, the NANoREG partners in Task 4.6.1 observed that the existing standard tests were not optimal for assessing the potential acute toxicity of nanomaterials. As part of the work conducted in Task 4.6.1, partners Tekniker, SINTEF, GNano/IFSC-USP and UoS conducted a pre-test to evaluate the following parameters within the standard tests:

- Test duration – the viability of extending the test duration from 48 h to 72 h was investigated. This was based on studies showing that the acute effects of nanomaterial exposure may be delayed past the standard 48 h test period.

- Organism age – the viability of using 4-day-old *D. magna* instead of 24 h old *D. magna* was investigated. This was based on studies indicating that 24 h old organisms may become entrapped

in HARN nanomaterials such as CNTs whereas older organisms are larger and stronger which prevents this.

- Use of NOM – the inclusion of NOM into the exposure media was investigated. This was based upon previous studies showing that many nanomaterials exhibit increased dispersion concentration and dispersion stability in the presence of NOM, and that NOM is ubiquitous in natural aquatic environments.

GNano/IFSC-USP

GNano/IFSC-USP reported that both <24h and 4-day old daphnids were adequate to perform assays with JRCNM01000a (TiO_2 NPs), since no mechanical issues were observed. Neonates were more sensitive to JRCNM01000a than 4 day-old organisms, during exposure periods of 48 and 72 h. They observed agglomeration of NPs in all test concentrations and all measured time intervals. However, the DLS analysis results obtained showed Z_{ave} of ~240 nm for stock dispersions, which were outside the determined for JRCNM01000a in the benchmarking study.

A) Assays with 24 h old *Daphnia magna* individuals
There was no immobile animal after the exposure to TiO_2 NPs for 24 h (Table 7). After 48 h of exposure (Table 8), the percentage of mobility of the animals was 95% at 100 mg/L of TiO_2 NPs whereas for 72 h of exposure, percentages of mobility of the animals were 90, 90 and 65% at 1, 10 and 100 mg/L of TiO_2 NPs, respectively (Table 9).

Table 7. Total number of mobile animals (< 24 h old) and its percentage of mobility for each tested concentration after an exposure period of 24 h. R = replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	5	5	5	5	20	100
0.01	5	5	5	5	20	100
0.1	5	5	5	5	20	100
1	5	5	5	5	20	100
10	5	5	5	5	20	100
100	5	5	5	5	20	100

Table 8. Total number of mobile animals (< 24 h old) and its percentage of mobility for each tested concentration after an exposure period of 48 h. R = replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	5	5	5	5	20	100
0.01	5	5	5	5	20	100
0.1	5	5	5	5	20	100
1	5	5	5	5	20	100
10	5	5	5	5	20	100
100	5	4	5	5	19	95

Table 9. Total number of mobile animals (< 24 h old) and its percentage of mobility for each tested concentration after an exposure period of 72 h. R = replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	5	5	5	5	20	100
0.01	5	4	5	5	19	95
0.1	5	5	5	5	20	100
1	5	4	4	5	18	90
10	4	4	5	5	18	90
100	2	3	4	4	13	65

The 72 h EC_{50} was 131.14 ± 32.08 (SE) mg/L of TiO_2 NPs, with confidence interval between 89.32 and 281.47 mg/L of TiO_2 NPs (Figure 2). For 72 h EC_{20} was 61.20 ± 16.10 (SE) mg/L of TiO_2 NPs, with confidence interval between 32.32 and 116.03 mg/L of TiO_2 NPs (Figure 2). The 48 h EC_{50} and 48 h EC_{20} values could not be estimated.

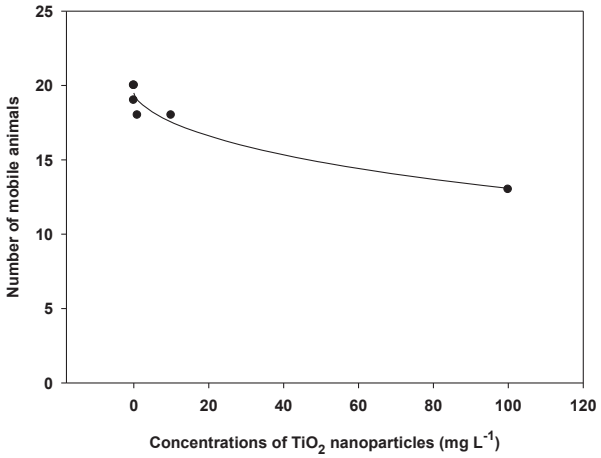

Figure 2. Concentration-response curve of the number of mobile *Daphnia magna* individuals (< 24 h old) obtained after 72 h of exposure to five tested concentrations of TiO_2 NPs (0.01, 0.1, 1, 10 and 100 mg/L).

The chemical and physical parameters measured during the assay are shown in Table 10.

Table 10. Chemical and physical parameters obtained from acute toxicity test with TiO_2 NPs using as test organism 24 h old *Daphnia magna* individuals. The parameters were collected at 0 and 72 h. T (temperature in °C); DO (dissolved oxygen in mg/L); TH (total hardness in mg $CaCO_3$/L)

Concentrations (mg/L)	pH		T		DO		TH	
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
0	7.90	8.57	19.5	20.1	6.57	5.39	220	220
0.01	7.90	8.06	19.6	20.1	6.81	4.56	220	220
0.1	7.90	7.92	19.8	20.2	6.76	4.29	-	-
1	7.90	7.98	19.7	20.1	6.75	4.56	212	212
10	7.90	8.13	19.9	20.1	6.69	5.11	-	-
100	7.90	7.93	19.8	20.2	6.72	4.32	204	204

The total hardness was only measured at the low, intermediate and high tested concentrations.

The DLS analysis obtained after the sonication of stock solution of TiO_2 prepared in Milli-Q water (2.62 mg/L) showed that mean hydrodynamic diameter (Z_{ave}) was 10.97 ± 0.32 nm (± standard deviation), with mean PDI value of 0,216 ± 0,007 nm (± standard deviation).
For the test concentrations of 0.01 mg/L (low concentration), 1 mg/L (intermediate concentration) and 100 mg L^{-1} (high concentration), the Z_{ave} values at the start of the test (0 h) were 56.93 ± 7.19 nm (mean PDI of 0.468 ± 0.069), 4835 ± 1048 nm (mean PDI of 1.0) and 1115 ± 157.9 nm (mean PDI of 0.192 ± 0.061), respectively. After 48 h, the Z_{ave} values were 3578 ± 2608 nm (mean PDI of 0.584 ± 0.295), 1846 ± 249 nm (mean PDI of 0.806 ± 0.168) and 1448 ± 86.2 nm (mean PDI of 0.547 ± 0.154), respectively. Finally, after 72 h (the end of test), the Z_{ave} values for three test concentrations above cited were 1538 ± 1192 nm (mean PDI of 0.858 ± 0.208), 1647 ± 770.7 nm (mean PDI of 0.941 ± 0.115) and 1807 ± 119.1 nm (mean PDI of 0.502 ± 0.123), respectively. There was, therefore, agglomeration of TiO_2 NPs in all test concentrations, and all measured time intervals.

B) Assays with 4 days old *Daphnia magna* individuals
It was not observed immobile animals after 24, 48 and 72 h of exposure to TiO_2 NPs (Tables 11, 12 and 13).

Table 11. Total number of mobile animals (4 days old) and its percentage of mobility for each tested concentration after an exposure period of 24 h. R means replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	0	0	0	0	20	100
0.01	0	0	0	0	20	100
0.1	0	0	0	0	20	100
1	0	0	0	0	20	100
10	0	0	0	0	20	100
100	0	0	0	0	20	100

Table 12. Total number of mobile animals (4 days old) and its percentage of mobility for each tested concentration after an exposure period of 48 h. R means replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	0	0	0	0	20	100
0.01	0	0	0	0	20	100
0.1	0	0	0	0	20	100
1	0	0	0	0	20	100
10	0	0	0	0	20	100
100	0	0	0	0	20	100

Table 13. Total number of mobile animals (4 days old) and its percentage of mobility for each tested concentration after an exposure period of 72 h. R means replicate

Concentrations (mg/L)	R1	R2	R3	R4	Total mobile animals	% Mobility
control	0	0	0	0	20	100
0.01	0	0	0	0	20	100
0.1	0	0	0	0	20	100
1	0	0	0	0	20	100
10	0	0	0	0	20	100
100	0	0	0	0	20	100

The 48 h EC_{50} and 48 h EC_{20} values could not be estimated as well as the 72 h EC_{50} and 72 h EC_{20} values, since all animals remained mobile during the entire test.
The chemical and physical parameters measured during the assay are shown in Table 14.

Table 14. Chemical and physical parameters obtained from acute toxicity test with titanium dioxide (TiO_2) using as test organism 4 days old *Daphnia magna* individuals. The parameters were collected at 0 and 72 h. T (temperature in °C); DO (dissolved oxygen in mg/L); TH (total hardness in mg $CaCO_3$/L)

Concentrations (mg/L)	pH		T		DO		TH	
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
0	7.41	7.63	21.4	22.3	4.84	5.30	240	240
0.01	8.28	7.89	21.0	22.6	4.68	4.98	240	240
0.1	8.35	7.86	21.0	22.8	5.31	5.10	-	-
1	8.36	7.98	20.9	22.8	5.49	5.91	232	232
10	8.36	7.95	20.9	22.8	5.72	5.89	-	-
100	8.04	7.90	21.0	22.8	5.44	5.59	222	222

The total hardness was only measured at the low, intermediate and high tested concentrations.

The DLS analysis obtained after the sonication of stock solution of TiO_2 prepared in Milli-Q water (2.58 mg/L) showed that mean hydrodynamic diameter (Z_{ave}) was 9,794 ± 0,255 nm (± standard deviation), with mean PDI value of 0,213 ± 0,006 nm (± standard deviation).

For the test concentrations of 0.01 mg/L (low concentration), 1 mg/L (intermediate concentration) and 100 mg/L (high concentration), the Z_{ave} values at the start of the test (0 h) were 1421 ± 1393 nm (mean PDI of 0.799 ± 0.185), 1613.2 ± 249.34 nm (mean PDI of 0.605 ± 0.182) and 1881 ± 279.31 nm (mean PDI of 0.479 ± 0.135), respectively. After 48 h, the Z_{ave} values were 620.22 ± 580.68 nm (mean PDI of 0.581 ± 0.209), 1333.7 ± 686.2 nm (mean PDI of 0.760 ± 0.114) and 1777 ± 115.3 nm (mean PDI of 0.403 ± 0.137), respectively.
After 72 h (the end of test), the Z_{ave} values for three test concentrations above cited were 2214 ± 1214.4 nm (mean PDI of 0.990 ± 0.025), 1654.5 ± 239.7 nm (mean PDI of 0.611 ± 0.239) and 1541 ± 205.4 nm (mean PDI of 0.216 ± 0.08), respectively. There was agglomeration of TiO_2 NPs in all test concentrations and all measured time intervals.

SINTEF

SINTEF observed limited stability for JRCNM01000a over time in the exposure media (MHRW), in the absence of SR-NOM. JRCNM01000a showed a dose dependent effect only for adult daphnia at 48 h. Exposed neonates had an increased mortality in lower exposure concentrations at a 24 and 48 h exposure duration. Whilst neonates in the control group exhibited 72 h mortality of ≤ 10%, 3-day old *D. magna* exceeded the validity threshold at 72 h, indicating a limited applicability of 3-day old organisms in 72 h testing. Furthermore, different results from the data obtained by GNano for JRCNM01000a were observed. GNano could calculate an EC50 for the neonates after 72 h (but not at 48 h), and they found no mortality in the 4-day olds at 48 h and 72 h exposure. In contrast, SINTEF observed that neonate daphnia were impacted more significantly by lower exposure concentrations, while in adult daphnia a dose-response was observed after 48 h exposure.

In stability tests, the JRCNM01000a showed a good dispersibility, although over time limited stability in the exposure media medium hard reconstituted water (MHRW). No natural organic matter (NOM) was used in this test.

- For JRCNM01000a exposures, a dose dependent effect was only observable for adult daphnia at 48 h in the performed tests. Exposed neonates had an increased mortality in lower exposure concentrations at a 24 and 48 h exposure duration.
- Whilst neonates in the control group exhibited 72 h mortality of ≤ 10%, 3-day old *D. magna* exceeded the validity threshold at 72 h, indicating a limited applicability of 3-day old organisms in 72 h testing.
- Reference substance exposures showed that neonate *D. magna* had an EC50 of 1.7 mg/L, which is within the required validation limits (according to protocol 0.6 and 2.1 mg/L). Three-day old individuals were slightly less sensitive with a calculated EC50 of 2.48 mg/L, exceeding the validation limits.
- Dissolved oxygen, temperature and pH values were as required.

A) JRCNM01000a toxicity pre-test with neonate *D. magna*

Results from the JRCNM01000a toxicity pre-test with neonate old *D. magna* are presented in Table 15 (24 h), Table 16 (48 h) and Table 17 (72 h). There was a 15% immobilization of animals in the 0.1 mg/L exposure concentration (Table 15). As this immobilization had occurred already after 24 h, it might be due to mechanical damage. After 72 h of exposure (Table 17), the percentage of immobile animals was 15% at 10 and 100 mg/L exposure concentrations, 20 % at 0.1 mg/L and 10 % in the control groups.

Tables 15-17. Total number and percent of mobile animals (neonates) and percentage of immobilization at 24, 48 and 72 h. *n*=20.

Table 15: 24 h exposure

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control	5	5	5	5	20	100	0
0.01					Not Tested		
0.1	3	4	5	5	17	85	15
1	5	5	5	4	19	95	5
10	5	5	5	5	20	100	0
100	5	5	5	5	20	100	0

Table 16: 48 h exposure

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (total)	Immobilization %
Control	4	5	5	5	19	95	5
0.01					Not Tested		
0.1	3	4	5	5	17	85	15
1	5	5	5	4	19	95	5
10	5	5	5	5	20	100	0
100	5	5	5	5	20	100	0

Table 17: 72 h exposure

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (total)	Immobilization %
Control	4	5	5	4	18	90	10
0.01					Not Tested		
0.1	3	4	5	4	16	80	20
1	5	5	5	4	19	95	5
10	4	5	4	4	17	85	15
100	5	4	4	4	17	85	15

B) JRCNM01000a toxicity pre-test with 3-day old *Daphnia magna*.

Results from the JRCNM01000a toxicity pre-test with 3-day old *D. magna* are presented in Table 18 (24 h), Table 19 (48 h) and Table 20 (72 h). No immobilization was observed at 24 h exposure; whilst the highest concentration exposure (100 mg/L) caused a 30 % immobilization at 48 h. Data from 72 h are not valid due to a high mortality in the control group.

Tables 18-20. Total number and percent of mobile animals (3-day old) and percentage of immobilization at 24, 48 and 72 h. n=20.

Table 18: 24 h exposure

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control	5	5	5	5	20	100	0
0.01					Not Tested		
0.1	5	5	5	5	20	100	0
1	5	5	5	5	20	100	0
10	5	5	5	5	20	100	0
100	5	5	5	5	20	100	0

Table 19: 48 h exposure; control animal in R4 was killed by mechanical interaction

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (total)	Immobilization %
Control	5	5	5	4	19	95	5
0.01					Not Tested		
0.1	5	4	4	5	18	90	10
1	5	4	5	5	19	95	5
10	4	4	5	5	18	90	10
100	3	2	5	4	14	70	30

Table 20: 72 h exposure

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (total)	Immobilization %
Control	4	4	3	3	14	70	30
0.01					Not Tested		
0.1	3	3	2	3	11	55	45
1	4	3	5	5	17	85	15
10	3	4	4	4	14	70	30
100	2	2	4	3	11	55	45

C) $K_2Cr_2O_7$ toxicity validation test with neonate and 3-day old *D. magna*.

Results from the $K_2Cr_2O_7$ toxicity validation test with neonate and 3-day old *D. magna* are presented in Table 21 (neonates) and Table 22 (3-day old). *D. magna* were exposed for 24 h to $K_2Cr_2O_7$ in the concentration range 0.32 to 3.2 mg/L. Tables 21 and 22 show number and percentage of mobile animals and percent immobilization (*n*=20). EC50 values of the neonates are in the required concentration range (0.6 – 2.1 mg/L), being 1.7 mg/L (95% confidence interval 1.54-1.882). Three-day old individuals were less sensitive, and had

a calculated EC50 value of 2.48 mg/L (95% confidence interval 1.985-3.099), which is slightly higher than the required concentrations.

Table 21: 24 h exposure of neonates

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (total)	Immobilization %
Control	5	5	5	4	19	95	5
0.01	5	5	5	5	20	100	0
0.1	5	5	3	5	18	90	10
1	5	5	5	5	20	100	0
10	2	3	2	1	8	40	60
100	1	0	0	0	1	5	95

Table 22: 24 h exposure of 3-day old individuals

Concentration (mg/L)	R1	R2	R3	R4	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control	5	5	5	5	20	100	0
0.32	5	5	5	5	20	100	0
0.56	4	5	5	5	19	95	5
1	3	4	4	5	16	80	20
1.8	5	4	3	4	16	80	20
3.2	1	1	3	1	6	30	70

D) Comparison of immobilization in control individuals kept for 24, 48 and 72 h.

Results from the comparison of immobilization in 3-day old control individuals kept for 24, 48 and 72 h are presented in Table 23 (24 h), Table 24 (48 h) and Table 25 (72 h). No immobilization occurred during 24 h (Table 23). Two and one individuals out of 25 were immobilized in the live and dry feed groups, respectively after 48 h (Table 24). In both the live and dry feed groups, mortality exceeded 10% at 72 h (Table 25).

Table 23: 24 h exposure

Concentration (mg/L)	R1	R2	R3	R4	R5	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control/life algae	5	5	5	5	5	25	100	0
Control/dry algae	5	5	5	5	5	25	100	0

Table 24: 48 h exposure

Concentration (mg/L)	R1	R2	R3	R4	R5	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control/life algae	4	4	5	5	5	23	92	8
Control/dry algae	5	5	5	5	4	24	96	4

Table 25: 72 h exposure

Concentration (mg/L)	R1	R2	R3	R4	R5	Mobile animals (total)	Mobile animals (%)	Immobilization %
Control/life algae	3	4	5	4	4	20	80	20
Control/dry algae	2	1	3	3	2	11	44	56

UOS

UOS performed the acute toxicity tests using Ag NPs (JRCNM03000a) and CeO_2 NPs (JRCNM02102a). They estimated 48h-EC50 of Ag NPs as 0.022 mg/L and 0.03 mg/L using 24h old neonate and 4-day old *D. magna*, respectively. No toxicity was observed for CeO_2 NPs until 100 mg/L concentrations using 24h old neonates (see Tables 26-28).

Table 26. 48 h acute toxicity of Ag NPs (JRCNM03000a) on *Daphnia magna* (Neonates, n=3, Mean ± SE)

Batch dispersion (2.56mg/mL)	Exposure Conc (mg/L)	Start(0h)	End(48h)	Effective concentrations [mg/L]

Z(ave)	P.D.I		Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	(95% CL)
140.8±0.9	0.203±0.006	0.005	2617.1 ±50.0	1.489± 0.018	-11.8 ±0.4	7.95	20	7.64	2812.2 ±308.8	1.543± 0.193	0.8 ±1.2	7.83	20	6.77	EC50 : 0.022
		0.017	2140.8 ±117.4	1.217± 0.064	-6.6 ±0.3	7.94	20	7.56	3009.8 ±445.1	1.539± 0.171	11.1 ±1.0	7.81	20	6.67	0.019< EC50 <0.028
		0.05	1138.5 ±115.3	0.682± 0.059	13.9 ±27.8	8.1	20	8.31	2642.5 ±335.8	1.448± 0.174	-10.5 ±0.5	7.8	20	7.39	

Table 27. 72 h acute toxicity of Ag NPs (JRCNM03000a) on *Daphnia magna* (4 days, n=4, Mean ± SE)

Batch dispersion (2.56mg/mL)		Exposure Conc (mg/L)	Start(0h)							End(48h)							Effective concentrations [mg/L] (95% CL)
Z(ave)	P.D.I		Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	Hardness (mg/L)	Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	Hardness (mg/L)	
247.9±2.2	0.242 ±.0.005	0.005	6222 ±1299.0	3.028 ±0.629	-10.5 ±0.4	7.71	20	7.25	215	1256.3±197.2	0.6 ±0.0	-23.8 ±0.4	7.52	20	6.92	235	EC50 : 0.030 0.024 < EC50 < 0.039
		0.016	3499.1±283.2	1.861 ±0.122	-13.6 ±0.1	7.74	20	7.51	225	2279.2±185.6	1.2 ±0.1	-21.0 ±0.6	7.88	20	7.03	250	
		0.05	2850.6±286.2	1.524 ±0.177	-9.2 ±0.5	7.76	20	6.94	225	2011.1±73.3	0.8 ±0.0	-13.2 ±1.2	7.76	20	6.97	250	

Table 28. 72 h acute toxicity of CeO_2 (JRCNM02102a) on *Daphnia magna* (Neonates, n=4, Mean ± SE)

Batch dispersion (2.56mg/mL)		Exposure Conc (mg/L)	Start(0h)							End(48h)							Effective concentrations [mg/L] (95% CL)
Z(ave)	P.D.I		Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	Hardness (mg/L)	Z(ave)	P.D.I	Zeta Potential [mV]	pH	T [°C]	DO [mg/L]	Hardness (mg/L)	
218.2± 12.9	0.264 ± 0.007	10	958.1±50.0	0.475±0.029	-18.7 ±0.4	7.74	20	7.65	210	346.4± 4.0	0.2±0.0	-20.5 ±1.0	7.97	20	7.66	255	No EC50 was estimated from the range of exposure concentrations (1-100mg/L)
		32	716.4±15.4	0.326±0.009	-19.9 ±0.1	7.76	20	7.13	210	582.4± 5.1	0.3±0.0	-17.1 ±0.5	8.04	20	7.89	250	
		100	1314.7±24.1	0.289±0.024	-18.4 ±0.3	7.9	20	7.21	205	446.7± 3.1	0.2 ±0.0	-17.6 ±1.1	7.93	20	8.24	220	

IK4-Tekniker

IK4-Tekniker performed the pre-testing for MWCNTs (JRCNM04001a) in the presence of 10 mg/L SR-NOM. Similarly to the results reported by SINTEF for the pre-test using JRCNM01000a, the control group of 4-day organisms exhibited 72 h mortality considerably higher than 10%, thus exceeding the validity threshold (Tables 29-31). In the case of the neonates, the 72 h mortality in the control group was lower but still higher than 10% (Tables 32-34). Hence, the applicability of 4-day old organisms and the 72 h testing was limited. However, for 24h-exposures to MWCNTs, a dose dependent effect was observable for both neonate and adult daphnids, and specifically, adults were more sensitive to MWCNTs.

Reference substance $K_2Cr_2O_7$ exposures (Tables 35-36) showed that neonate *D. magna* had a 24h-EC50 of 1.35 mg/L, which is within the required validation limits (between 0.6 and 2.1 mg/L). Adults were slightly less sensitive with a calculated 24h-EC50 of 1.65 mg/L.

Dissolved oxygen, temperature and pH values were as required.

A) JRCNM04001a toxicity pre-test with neonate *D. magna*

Table 29. Total number of immobile neonates and percentage of immobilization at 24 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	1	0	0	0	1	5
2	0	0	0	0	0	0
5	0	0	0	0	0	0
10	0	0	0	0	0	0
20	1	0	0	0	1	5
50	0	0	0	2	2	10

Table 30. Total number of immobile neonates and percentage of immobilization at 48 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	1	1	0	1	3	15
2	0	0	1	1	2	10
5	1	1	0	0	2	10
10	0	0	0	0	0	0
20	1	0	0	0	1	5
50	2	2	1	1	6	30

Table 31. Total number of immobile neonates and percentage of immobilization at 72 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	3	3	3	3	12	60
2	1	1	2	2	6	30
5	3	1	0	1	5	25
10	0	0	1	2	3	15
20	1	1	0	0	2	10
50	3	5	2	1	11	55

B) JRCNM04001a toxicity pre-test with 4-day old *D. magna*

Table 32. Total number of immobile 4d-organisms and percentage of immobilization at 24 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	0	1	0	0	1	5
2	0	2	0	1	3	15
5	2	2	2	2	8	40
10	1	3	1	0	5	25
20	2	2	2	3	9	45
50	1	2	3	3	9	45

Table 33. Total number of immobile 4d-organisms and percentage of immobilization at 48 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	4	3	3	4	14	70
2	2	3	2	2	9	45
5	5	2	5	5	17	85
10	4	5	5	5	19	95
20	5	5	3	5	18	90
50	5	5	5	4	19	95

Table 34. Total number of immobile 4d-organisms and percentage of immobilization at 72 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	4	4	4	4	16	80
2	3	4	4	3	14	70
5	5	3	5	5	18	90
10	5	5	5	5	20	100
20	4	5	4	5	18	90
50	5	5	5	5	20	100

C) $K_2Cr_2O_7$ toxicity validation test with neonate and 4-day old *D. magna*

The control group of neonates and 4-day organisms exhibited 24 h mortality within the required validation limit (<10%).

Table 35. Total number of immobile neonates and percentage of immobilization at 24 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	0	0	0	0	0	0
0.32	0	0	0	0	0	0
0.56	0	0	0	0	0	0
1	0	1	0	0	1	5
1.8	2	4	4	5	15	75
3.2	5	5	5	5	20	100

Table 36. Total number of immobile 4d-organisms and percentage of immobilization at 24 h.

Concentration (mg/L)	R1	R2	R3	R4	Immobile animals (total)	Immobilization %
Control	0	0	1	0	1	5
0.32	0	0	0	1	1	5
0.56	1	0	1	0	2	10
1	0	2	0	1	3	15
1.8	1	2	1	2	6	30
3.2	5	5	5	5	20	100

Definitive studies - *Daphnia magna* Acute Immobilization Tests

GNano/IFSC-USP

The results for acute immobilization tests performed by GNano/IFSC-USP with *Daphnia magna* are summarized in Figures 3 to 6, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	JRCNM01000a		Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	10.97	0.216			0.01
											1
											100
1	JRCNM01100a		Powder	JRC	ZnO	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	362.98	0.147			0.1
											0.9
											8.1
1	JRCNM03000a		Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	70.16	0.536			0.3
											0.9
											1.2

Figure 3. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. magna* by GNano/IFSC-USP, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test											
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01000a	Supernatant	56.93	0.468						7.90	19.6	6.81
	Supernatant	4835	1.0						7.90	19.7	6.75
	Supernatant	1115	0.192						7.90	19.8	6.72
JRCNM01100a	Supernatant	420.9	0.647	-9.43					8.04	23.7	5.10
	Supernatant	777.3	0.874	-10.9					7.92	23.4	5.22
	Supernatant	554.4	0.486	-9.54					8.00	23.4	5.77
JRCNM03000a	Supernatant	<Detection Limit	<Detection Limit	<Detection Limit					7.79	22.0	5.72
	Supernatant	<Detection Limit	<Detection Limit	<Detection Limit					7.75	21.8	5.85
	Supernatant	465.95	0.801	-8.39					7.85	21.8	5.93

Figure 4. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. magna* by GNano/IFSC-USP, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01000a	Supernatant	1538	0.858						8.06	20.1	4.56
	Supernatant	1647	0.941						7.98	20.1	4.56
	Supernatant	1807	0.502						7.93	20.2	4.32
JRCNM01100a	Supernatant	251.5	0.551	-11.6					7.43	23.1	5.49
	Supernatant	532.6	0.611	-7.99					7.43	23.3	5.74
	Supernatant	631.9	0.584	-14.7					7.27	23.6	4.95
JRCNM03000a	Supernatant	<Detection Limit	<Detection Limit	<Detection Limit		415 nm	0.14		7.49	21.7	4.88
	Supernatant	<Detection Limit	<Detection Limit	<Detection Limit			0.32		7.52	21.9	4.52
	Supernatant	528.8	0.866	-10.5			0.51		7.75	21.6	4.68

Figure 5. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. magna* by GNano/IFSC-USP, compiled according to the *D. magna - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
JRCNM01000a	EC50 EC20	D. magna Acute Toxicity Test	252	SOP-Toxicity Test with Daphnia magna	10/01/2016	GNano/IFSC-USP	72	EC50 = 131.14 EC20 = 61.20
JRCNM01100a	EC50 EC20	D. magna Acute Toxicity Test	256	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	19/04/2016	GNano/IFSC-USP	72	EC50 = 1.44 (0.11-18.28) EC20 = 0.60 (0.04-8.72)
JRCNM03000a	EC50 EC20	D. magna Acute Toxicity Test	256	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	12/04/2016	GNano/IFSC-USP	72	EC50 = 0.76 (0.71-0.83) EC20 = 0.57 (0.52-0.63)

Figure 6. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. magna* by GNano/IFSC-USP, compiled according to the *D. magna - Ecotoxicity test data entry template*.

EC50 value for TiO_2 NPs (JRCNM01000a) NPs was higher than 100 mg/L.

ZnO NPs (JRCNM01100a) were difficult to disperse at the stock solution. It was not possible to disperse the recommended concentration for standard dispersion protocol (2.56 g/L). Therefore, an additional dilution step was necessary to achieve the concentration of 0.256 g/L, which was suitable for dispersion. However, stability parameters showed aggregation of ZnO NPs in the stock solution and in exposure media. Water quality parameters remained stable during exposure. Figure 7 shows ZnO NP concentrations as a function of effect percentages, and the linear regression equations used for estimative of effective concentrations of ZnO NPs to *D. magna*. The mean effective concentration of ZnO NPs for *D. magna* was 1.44 mg/L.

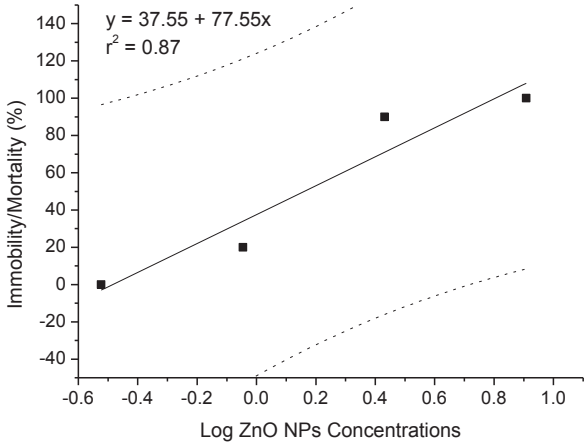

Figure 7. Linear regression equation after logarithmic transformation for estimation of Mean Effective Concentration (EC50) of ZnO NPs for *D. magna*, after 72 h of exposure. Dotted line means upper and lower limits of confidence (95%).

Ag NPs (JRCNM03000a) was the most toxic nanomaterial in *D. magna* assay. Compared to the other MNMs, Ag NPs presented the lowest values of EC50 during 72 h exposure. UV-Vis analyses were performed on Ag NPs exposure media for determination of Ag concentrations after 72 h exposure. A linear regression equation was used for Ag concentration estimative: y = -0.001 + 0.065x (r^2 = 0.99). According to the data, it is possible to infer that Ag NPs determined concentrations were lower than nominal Ag NPs added to the exposure media. These significant Ag losses were probably due to adsorption to the test containers during exposure span. These losses were higher than 30%. Stability values for lower and intermediate concentrations were not accurate due to equipment features. Ag NPs were nicely dispersed in the stock solution; however, there was nanomaterial aggregation in the exposure media during the assay. Figure 8 presents Ag NPs concentrations (logarithmic scale) in function of effect percentages, and the linear regression equation used for effective concentrations assessment during *D. magna* exposure to AgNPs. The mean effective concentration of AgNPs for *D. magna* was 0.76 mg/L. The sensitivity test with potassium dichromate presented an $EC_{50\text{-}24h}$ of 0.86 mg/L with lower limit of 0.74 mg/L and upper limit of 0.98 mg/L. This value was within the recommended range by the *D. magna* test SOP.

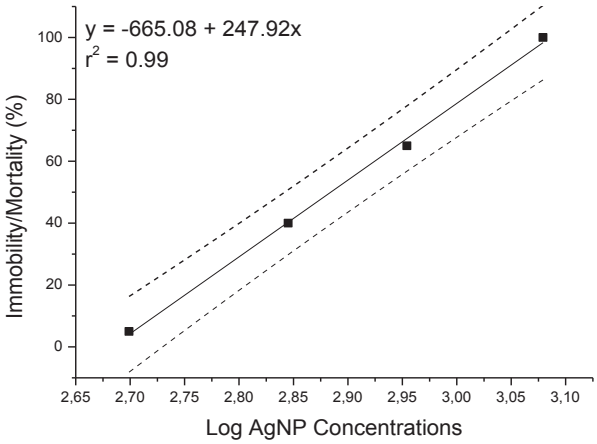

Figure 8. Linear regression equation after logarithmic transformation for estimation of Mean Effective Concentration (EC50) of *D. magna* after 72 h of Ag NPs exposure. Dotted line means upper and lower limits of confidence (95%).

IK4-Tekniker

The results for acute immobilization tests performed by IK4-Tekniker with *Daphnia magna* are summarized in Figures 9 to 12, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion				
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	JRCNM03000a	07206	Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	81.13	0.241	NO		0.003 0.033 0.325
2	JRCNM03000a	07206	Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	81.54	0.236	NO	SEM	0.003 0.033 0.325
1	JRCNM02000a	990488	Powder	JRC	SiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	252.10	0.424	NO	SEM	0.01 1 100
1	JRCNM02102a	990005	Powder	JRC	CeO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	190.40	0.309	NO	SEM	0.01 1 100
2	JRCNM02102a	990005	Powder	JRC	CeO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	180.10	0.262	NO		1 10 100
1	JRCNM04001a	990678	Powder	JRC	MWCNTs	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	1602.00	0.591	NO	SEM	2 10 50

Figure 9. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. magna* by IK4-Tekniker, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM03000a	Homogenized medium								8.00	20.9	6.0
	Homogenized medium								8.00	20.9	6.0
	Homogenized medium								8.00	20.9	6.0
JRCNM03000a	Homogenized medium	182.30	0.377	-8.20	SEM	450	0.15	-	7.97	20.0	6.0
	Homogenized medium	67.25	0.364	-9.91	SEM	450	0.17	-	7.97	20.0	6.0
	Homogenized medium	66.64	0.283	-11.90	SEM	450	0.31	-	7.97	20.0	6.0
JRCNM02000a	Homogenized medium	909.20	0.462	-6.65	SEM	210		-	7.96	20.6	6.0
	Homogenized medium	301.70	0.423	-14.20	SEM	210		-	7.96	20.6	6.0
	Homogenized medium	246.00	0.402	-17.60	SEM	210		-	7.96	20.6	6.0
JRCNM02102a	Homogenized medium	944.00	0.598	-6.22	SEM	400	0.00	-	7.97	20.0	6.0
	Homogenized medium	1649.00	0.775	-16.60	SEM	400	0.00	-	7.97	20.0	6.0
	Homogenized medium	1666.00	0.267	-5.81	SEM	400	158.93	-	7.97	20.0	6.0
JRCNM02102a	Homogenized medium								8.00	20.9	6.0
	Homogenized medium								8.00	20.9	6.0
	Homogenized medium								8.00	20.9	6.0
JRCNM04001a	Homogenized medium	2792.00	0.910	-13.00	SEM	500	0.43	-	7.96	20.6	6.0
	Homogenized medium	2557.00	0.896	-18.00	SEM	500	3.71	-	7.96	20.6	6.0
	Homogenized medium	308.10	0.569	-15.50	SEM	500	7.63	-	7.96	20.6	6.0

Figure 10. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. magna* by IK4-Tekniker, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM03000a	Homogenized medium									20.6	
	Homogenized medium									20.6	
	Homogenized medium									20.6	
JRCNM03000a	Homogenized medium	443.10	0.453	-12.20	SEM	450	0.54	-	7.70	20.4	4.1
	Homogenized medium	384.20	0.518	-9.96	SEM	450	0.27	-	7.83	20.4	4.1
	Homogenized medium	216.20	0.426	-13.20	SEM	450	0.59	-	7.81	20.4	4.2
JRCNM02000a	Homogenized medium	573.70	0.563	-12.80	SEM	210		-	7.73	20.4	4.2
	Homogenized medium	458.10	0.455	-8.60	SEM	210		-	7.93	20.4	4.7
	Homogenized medium	301.80	0.563	-19.40	SEM	210		-	7.89	20.4	4.3
JRCNM02102a	Homogenized medium	549.10	0.473	-13.80	SEM	400	0.00	-	8.01	20.4	4.2
	Homogenized medium	299.00	0.452	-15.10	SEM	400	0.00	-	7.93	20.4	4.5
	Homogenized medium	4175.00	0.374	-8.98	SEM	400	50.84	-	7.90	20.4	4.7
JRCNM02102a	Homogenized medium									20.6	
	Homogenized medium									20.6	
	Homogenized medium									20.6	
JRCNM04001a	Homogenized medium	1956.00	0.952	-20.60	SEM	500	0.00	-	7.72	20.4	4.7
	Homogenized medium	2430.00	0.765	-25.10	SEM	500	2.76	-	7.77	20.4	4.6
	Homogenized medium	3915.00	0.659	-24.70	SEM	500	8.33	-	7.80	20.4	4.5

Figure 11. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. magna* by IK4-Tekniker, compiled according to the *D. magna - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
JRCNM03000a	EC50 EC20		0.325	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	11/05/2016	IK4-TEKNIKER	48	0.028(0.018-0.043) 0.006 (0.004-0.009)
JRCNM03000a	EC50 EC20		0.325	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	14/04/2016	IK4-TEKNIKER	72	0.028 (0.020-0.039) 0.006 (0.005-0.008)
JRCNM02000a	EC50 EC20		100	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	21/04/2016	IK4-TEKNIKER	72	2.05 (1.74-2.42) 0.03 (0.02-0.03)
JRCNM02102a	EC50 EC20		100	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	14/04/2016	IK4-TEKNIKER	72	33.84 (32.76-34.95) 0.01 (0.01-0.01)
JRCNM02102a	EC50 EC20		100	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	11/05/2016	IK4-TEKNIKER	48	N.D. (not determined: effective concentration values could not be calculated because of the low toxicity levels).
JRCNM04001a	EC50 EC20		50	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	21/04/2016	IK4-TEKNIKER	72	47.87 (38.09-60.17) 5.66 (4.38-7.32)

Figure 12. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. magna* by IK4-Tekniker, compiled according to the *D. magna - Ecotoxicity test data entry template*.

SINTEF

The results for acute immobilization tests performed by SINTEF with *Daphnia magna* are summarized in Figures 13 to 16, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

Replicate number	NM ID code	Vial number	NM supplier (from NANoREG)	Sample Name	Batch dispersion				Nominal concentration [mg/L] (low-intermediate-high)
					Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	
1	JRCNM01000a	06956	Fraunhofer	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	236.5	0.2	NO		0.1 10 100
2	JRCNM01000a	06956	Fraunhofer	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	236.5	0.2	NO		0.1 10 100
1	JRCNM01001a	990941	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	489.9	0.3	NO		0.1 10 100
2	JRCNM01001a	990941	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	489.9	0.3	NO		0.1 10 100
1	JRCNM01003a	07001	Fraunhofer	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	155.3	0.3	NO		0.1 10 100
2	JRCNM01003a	07001	Fraunhofer	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	155.3	0.3	NO		0.1 10 100
1	JRCNM04000a	990414	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	36057.8	1	NO		2 20 50
2	JRCNM04000a	990414	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	36057.8	1	NO		2 20 50
1	JRCNM04001a	990735	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure			NO		2 20 50
2	JRCNM04001a	990735	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure			NO		2 20 50
1	JRCNM04100a	06157	Fraunhofer	SWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure			NO		2 20 50
2	JRCNM04100a	06157	Fraunhofer	SWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure			NO		2 20 50

Figure 13. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. magna* by SINTEF, compiled according to the *D. magna - Ecotoxicity test data entry template*. Samples in green are from the 48 exposures and samples in blue are from the 72 h exposures.

NM ID code	Start of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01000a	Homogenized medium	689.3	0.4	-20.4		-	-	0.06	7.9	19.7	7.66
	Homogenized medium	381.8	0.3	-7.2		-	-	5.18	7.93	19.7	7.67
	Homogenized medium	961.4	0.2	0.5		-	-	56.36	7.91	19.7	7.72
JRCNM01000a	Homogenized medium	689.3	0.4	-20.4		-	-	0.06	7.9	19.7	7.66
	Homogenized medium	381.8	0.3	-7.2		-	-	5.18	7.93	19.7	7.67
	Homogenized medium	961.4	0.2	0.5		-	-	56.36	7.91	19.7	7.72
JRCNM01001a	Homogenized medium	1136.7	0.5	-12.1		-	-	0.06	7.7	19.7	7.72
	Homogenized medium	970.7	0.5	-5.4		-	-	4.63	7.91	19.7	7.59
	Homogenized medium	2026.3	0.5	-5.9		-	-	46.65	7.95	19.7	7.58
JRCNM01001a	Homogenized medium	1136.7	0.5	-12.1		-	-	0.06	7.7	19.7	7.72
	Homogenized medium	970.7	0.5	-5.4		-	-	4.63	7.91	19.7	7.59
	Homogenized medium	2026.3	0.5	-5.9		-	-	46.65	7.95	19.7	7.58
JRCNM01003a	Homogenized medium	775.2	0.6	-8.9		-	-	0.05	7.75	19.7	7.75
	Homogenized medium	641.4	0.5	3.5		-	-	5.63	7.9	19.7	7.54
	Homogenized medium	2416	0.4	7.3		-	-	54.04	7.87	19.7	7.5
JRCNM01003a	Homogenized medium	775.2	0.6	-8.9		-	-	0.05	7.75	19.7	7.75
	Homogenized medium	641.4	0.5	3.5		-	-	5.63	7.9	19.7	7.54
	Homogenized medium	2416	0.4	7.3		-	-	54.04	7.87	19.7	7.5
JRCNM04000a	Homogenized medium	35846	0.9	-9.6		800	1	-	7.74	19.7	7.69
	Homogenized medium	62407	0.9	-10.9		800	14	-	7.82	19.7	7.72
	Homogenized medium	24650	0.7	-		800	35	-	7.83	19.7	7.72
JRCNM04000a	Homogenized medium	35846	0.9	-9.6		800	1	-	7.74	19.7	7.69
	Homogenized medium	62407	0.9	-10.9		800	14	-	7.82	19.7	7.72
	Homogenized medium	24650	0.7	-		800	35	-	7.83	19.7	7.72
JRCNM04001a	Homogenized medium	9810.2	0.8	-7.77		800	0	-	7.71	19.7	7.65
	Homogenized medium	6316.7	1	-9.9		800	11	-	7.78	19.7	7.57
	Homogenized medium	4925	0.5	-		800	35	-	7.81	19.7	7.69
JRCNM04001a	Homogenized medium	9810.2	0.8	-7.77		800	0	-	7.71	19.7	7.65
	Homogenized medium	6316.7	1	-9.9		800	11	-	7.78	19.7	7.57
	Homogenized medium	4925	0.5	-		800	35	-	7.81	19.7	7.69
JRCNM04100a	Homogenized medium	25389.7	0.9	-3.35		800	0.4	-	7.78	19.7	7.66
	Homogenized medium	47790	0.8	-6.84		800	9	-	7.8	19.7	7.56
	Homogenized medium	43180	0.7	-		800	22	-	7.82	19.7	7.66
JRCNM04100a	Homogenized medium	25389.7	0.9	-3.35		800	0.4	-	7.78	19.7	7.66
	Homogenized medium	47790	0.8	-6.84		800	9	-	7.8	19.7	7.56
	Homogenized medium	43180	0.7	-		800	22	-	7.82	19.7	7.66

Figure 14. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. magna* by SINTEF, compiled according to the *D. magna - Ecotoxicity test data entry template*. Samples in green are from the 48 exposures and samples in blue are from the 72 h exposures.

NM ID code	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01000a	Supernatant	Particles settled out of	Particles settled out of	Particles settled out of		-	-	0.04	7.83	20.1	7.78
	Supernatant	1089.1	0.4	-13.9		-	-	0.43	7.8	20.1	7.87
	Supernatant	2946	0.8	-5.8		-	-	0.24	7.19	20.1	7.87
JRCNM01000a	Supernatant	Particles settled out of	Particles settled out of	Particles settled out of		-	-	0.03	7.83	20.1	7.78
	Supernatant	2074.4	0.6	-1.6		-	-	0.09	7.8	20.1	7.87
	Supernatant	15208	0.6	aborted by instrument		-	-	0.03	7.19	20.1	7.87
JRCNM01001a	Supernatant	1509	0.7	-8.1		-	-	0.04	7.84	20.1	7.85
	Supernatant	1299	0.5	-4.4		-	-	0.43	7.86	20.1	7.88
	Supernatant	2099	0.9	-4.6		-	-	0.24	7.8	20.1	7.75
JRCNM01001a	Supernatant	795.5	0.4	-6.4		-	-	0.02	7.84	20.1	7.85
	Supernatant	1442.1	0.5	-4.6		-	-	0.1	7.86	20.1	7.88
	Supernatant	1101.3	0.7	-3.2		-	-	0.2	7.8	20.1	7.75
JRCNM01003a	Supernatant	545.5	0.4	-2.2		-	-	0.02	7.84	20.1	7.67
	Supernatant	1311.8	0.6	0.6		-	-	0.24	7.86	20.1	7.72
	Supernatant	1167.7	0.6	3.9		-	-	0.33	7.81	20.1	7.77
JRCNM01003a	Supernatant	655.1	0.5	-4.7		-	-	0.01	7.84	20.1	7.67
	Supernatant	1346.7	0.7	-0.4		-	-	0.13	7.86	20.1	7.72
	Supernatant	1665	0.6	0.5		-	-	0.22	7.81	20.1	7.77
JRCNM04000a	Supernatant	1029.5	0.7	-		800	0.25	-	7.77	20.1	7.76
	Supernatant	2546.3	1	-		800	0.28	-	7.84	20.1	7.78
	Supernatant	366	1	-		800	0.26	-	7.82	20.1	7.75
JRCNM04000a	Supernatant	609	0.6	-2.75		800	0.23	-	7.77	20.1	7.76
	Supernatant	933.6	0.7	-5.15		800	0.31	-	7.84	20.1	7.78
	Supernatant	1944.8	0.8	-		800	0.27	-	7.82	20.1	7.75
JRCNM04001a	Supernatant	497.7	0.5	-		800	0	-	7.71	20.1	7.81
	Supernatant	39788	1	-		800	0.4	-	7.74	20.1	7.93
	Supernatant	32739.3	0.7	-		800	0.36	-	7.81	20.1	7.78
JRCNM04001a	Supernatant	480.5	0.6	-1.36		800	0	-	7.71	20.1	7.81
	Supernatant	27101	0.8	-4.75		800	0	-	7.74	20.1	7.93
	Supernatant	25244	0.8	-		800	0	-	7.81	20.1	7.78
JRCNM04100a	Supernatant	1014.5	0.8	-2.41		800	0.16	-	7.75	20.1	7.75
	Supernatant	1933.5	0.9	-4.7		800	0.16	-	7.73	20.1	7.77
	Supernatant	1553.8	0.9	-		800	0.16	-	7.75	20.1	7.56
JRCNM04100a	Supernatant	1077.9	0.7	-4.01		800	0.21	-	7.75	20.1	7.75
	Supernatant	2288.7	1	-7.69		800	0.16	-	7.73	20.1	7.77
	Supernatant	1101	0.7	-		800	0.1	-	7.75	20.1	7.56

Figure 15. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. magna* by SINTEF, compiled according to the *D. magna - Ecotoxicity test data entry template*. Samples in green are from the 48 exposures and samples in blue are from the 72 h exposures.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
JRCNM01000a	EC50			SOP-Toxicity Test with Daphnia magna	08.03.2016	SINTEF	48	>100
	EC20		100					>100
JRCNM01000a	EC50			SOP-Toxicity Test with Daphnia magna	08.03.2016	SINTEF	72	>100
	EC20		100					>100
JRCNM01001a	EC50			SOP-Toxicity Test with Daphnia magna	07.03.2016	SINTEF	48	>100
	EC20		100					>100
JRCNM01001a	EC50			SOP-Toxicity Test with Daphnia magna	07.03.2016	SINTEF	72	3.95 (mortality in controls above accepted limit)
	EC20		100					20.41 (mortality in controls above accepted limit)
JRCNM01003a	EC50			SOP-Toxicity Test with Daphnia magna	08.03.2016	SINTEF	48	103.9
	EC20		100					1399
JRCNM01003a	EC50			SOP-Toxicity Test with Daphnia magna	08.03.2016	SINTEF	72	3.58 (mortality in controls above accepted limit)
	EC20		100					22.26 (mortality in controls above accepted limit)
JRCNM04000a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	48	108.8
	EC20		100					152.6
JRCNM04000a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	72	63.4 (mortality in controls above accepted limit)
	EC20		100					106.6 (mortality in controls above accepted limit)
JRCNM04001a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	48	>100
	EC20		100					>100
JRCNM04001a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	72	>100 (mortality in controls above accepted limit)
	EC20		100					>100 (mortality in controls above accepted limit)
JRCNM04100a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	48	94.7
	EC20		100					108.7
JRCNM04100a	EC50			SOP-Toxicity Test with Daphnia magna	20.04.2016	SINTEF	72	4.40
	EC20		100					49.7

Figure 16. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. magna* by SINTEF, compiled according to the *D. magna - Ecotoxicity test data entry template*. Samples in green are from the 48 exposures and samples in blue are from the 72 h exposures.

UOS

The results for acute immobilization tests performed by UOS with *Daphnia magna* are summarized in Figures 17 to 20, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1.00	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	170.6±0.8	0.225±0.007			0.00
											0.01
											0.05
2.00	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	152.9±2.8	0.173±0.006			0.01
											0.02
											0.05
3.00	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	152.9±2.8	0.173±0.006			0.01
											0.02
											0.05
4.00	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	212.7±1.1	0.202±0.005			0.01
											0.02
											0.05
5.00	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	212.7±1.1	0.202±0.005			0.01
											0.02
											0.05
1.00	JRCNM01001a		Powder	JRC-IHCP	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	457.1±6.5	0.221±0.018			10.00
											32.00
											100.00
1.00	JRCNM01003a		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	2834.6±142.7	1.423±0.124			6.25
											25.00
											100.00
2.00	JRCNM01003a		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	145.6±1.5	0.2±0.0			10.00
											32.00
											100.00
3.00	JRCNM01003a		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	145.6±1.5	0.2±0.0			10.00
											32.00
											100.00
1.00	JRCNM02000a		Powder	JRC-IHCP	SiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	373.3±19.07	0.236±0.011			10.00
											32.00
											100.00

Figure 17. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. magna* by UOS, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K		4028.7±915.3	1.845±0.348						7.94	20.00	7.46
		2831.4±227.4	1.474±0.117						7.93	20.00	7.43
		2136.5±83.5	1.187±0.053						7.95	20.00	7.25
NM300K		2373.5±482.4	1.319±0.236	-117.50±139.00					7.81	20.00	7.34
		1906.1±108.4	1.080±0.049	-1.82±10.50					7.77	20.00	7.39
		873.7±83.4	0.538±0.048	-14.56±0.49					7.92	20.00	7.50
NM300K		2373.5±482.4	1.319±0.236	-117.50±139.00					7.78	20.00	8.65
		1906.1±108.4	1.080±0.049	-1.82±10.50					7.86	20.00	8.21
		873.7±83.4	0.538±0.048	-14.56±0.49					7.92	20.00	8.15
NM300K		3603.3±305.6	1.194±0.16	-23.14±0.62					7.81	20.00	7.34
		3181.0±175	1.7±0.074	-20.96±0.36					7.77	20.00	7.39
		2092.4±134.8	1.167±0.086	-57.84±58.54					7.92	20.00	7.50
NM300K		3603.3±305.6	1.194±0.16	-23.14±0.62					7.81	20.00	7.34
		3181.0±175	1.7±0.074	-20.96±0.36					7.77	20.00	7.39
		2092.4±134.8	1.167±0.086	-57.84±58.54					7.92	20.00	7.50
JRCNM01001a		1020.0±19.1	0.448±0.005	-22.47±1.22					7.98	20.00	7.71
		821.9±7.4	0.275±0.012	-18.18 ±0.26					7.92	20.00	7.60
		958.4±0.154	0.154±0.029	-5.87±12.31					7.80	20.00	7.53
JRCNM01003a		621.1±11.1	0.297±0.004	-5.36±1.12					7.94	20.00	7.31
		724.5±17.4	0.331±0.014	-27.83±0.46					8.02	20.00	7.33
		2278.6±102.8	0.295±0.050	-17.04±0.11					8.00	20.00	7.46
JRCNM01003a		616.1±115	0.294±0.011	-21.21±0.60					7.94	20.00	7.45
		583.6±4.7	0.259±0.005	-19.24±0.06					7.89	20.00	7.12
		835.8±6.2	0.197±0.012	-19.52±0.05					7.95	20.00	7.08
JRCNM01003a		616.1±115	0.294±0.011	-21.21±0.60					7.94	20.00	7.45
		583.6±4.7	0.259±0.005	-19.24±0.06					7.89	20.00	7.12
		835.8±6.2	0.197±0.012	-19.52±0.05					7.95	20.00	7.08
JRCNM02000a		1038.633±120.757	0.6077±0.058	-26.227±0.970					7.41	20.00	7.78
		567.3±72.820	0.356±0.044	-22.61±0.403					7.47	20.00	7.60
		448.9±28.543	0.292±0.017	-21.423±0.944					7.29	20.00	7.60

Figure 18. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. magna* by UOS, compiled according to the *D. magna - Ecotoxicity test data entry template*.

	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K		3297.0±235.1	1.216±0.125						8.10	20.00	6.63
		2347.6±516.1	1.741±0.256						8.03	20.00	6.73
		1849.8±367.9	1.039±0.197						8.12	20.00	6.87
NM300K		3959.8±197.9	2.025±0.083	-154.84±93.87					7.97	20.00	7.60
		3199.5±84.6	1.760±0.058	0.39±19.34					7.91	20.00	7.91
		1872.0±159.8	1.048±0.078	-19.84±41.32					7.87	20.00	7.87
NM300K		2778.3±273.0	1.565±0.14	-22.10±0.52					7.95	20.00	7.65
		1504.2±149.5	0.846±0.093	-23.66±0.85					7.99	20.00	7.75
		2621.9±75.6	1.337±0.035	-57.91±42.17					7.98	20.00	7.89
NM300K		2132.9±180.9	1.128±0.088	-15.49±0.71					7.66	20.00	7.05
		2157.5±91.5	1.136±0.031	-22.91±1.01					7.61	20.00	7.31
		3629.9±89.5	1.853±0.027	-22.37±0.84					7.61	20.00	7.48
NM300K		2812.2±308.8	1.543±0.193	0.8±1.2					7.79	20.00	6.59
		3009.8±445.1	1.539±0.171	11.1±1.0					7.85	20.00	7.40
		2642.5±335.8	1.448±0.174	-10.5±0.5					7.88	20.00	7.01
JRCNM01001a		435.3±3.8	0.240±0.005	29.82±3..50					8.21	20.00	7.43
		266.0±112.5	0.238±0.008	-18.24±0.73					8.13	20.00	7.34
		755.7±42.8	0.400±0.029	-24.10±0.52					8.04	20.00	7.21
JRCNM01003a		7641.7±678.9	2.698±0.218	26.48±2.90					8.10	20.00	7.24
		3479.0±225.6	1.348±0.086	-11.57±2.95					8.05	20.00	7.23
		5144.7±219.3	1.401±0.069	-7.40±1.94					8.06	20.00	7.16
JRCNM01003a		232.2±10.2	0.150±0.009	-26.17±1.00					7.99	20.00	6.94
		246.5±2.5	0.155±0.006	-25.78±0.62					8.09	20.00	7.40
		503.3±13.9	0.254±0.016	-19.68±0.96					8.12	20.00	7.41
JRCNM01003a		210.1±1.1	0.125±0.004	-24.35±0.58					8.08	20.00	6.65
		250.0±1.0	0.138±0.009	-23.06±0.64					8.03	20.00	7.23
		401.2±8.8	0.224±0.008	-18.18±0.69					8.04	20.00	7.15
JRCNM02000a		609.633±77.367	0.385±0.046	-15.88±0.539					7.74	20.00	7.80
		511.033±6.372	0.3193±0.005	11.56±30.127					8.01	20.00	7.95
		304.167±60.090	0.202±0.039	-16.993±0.591					7.81	20.00	7.80

Figure 19. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. magna* by UOS, compiled according to the *D. magna - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
NM300K	immobilization	Daphnia immobilisation test(OECD TG 202)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2015.9.30	UOS	48h	EC50 0.01875 0.01607< EC50 <0.2187 EC20 0.00907 0.00603< EC20 <0.01172
NM300K	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2016.1.21	UOS	48h	EC50 0.02452 0.02206< EC50 <0.03186 EC20 0.01445 0.00982< EC20 <0.01820
NM300K	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2016.1.21	UOS	72h	EC50 0.02085 0.01722< EC50 <0.02581 EC20 0.01067 0.00597< EC20 <0.01427
NM300K	immobilization	nanoreg sop-Daphnia magna V3 (4days)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2016.1.23	UOS	48h	EC50 0.02567 0.02185< EC50 <0.03106 EC20 0.01638 0.01199< EC20 <0.02010
NM300K	immobilization	nanoreg sop-Daphnia magna V3 (4days)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2016.1.23	UOS	72h	EC50 0.03216 0.01905< EC50 <0.06612 EC20 0.02288 -0.01184< EC20 <0.03583
JRCNM01001a	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	i) SOP-Toxicity Test with Daphnia magna (with NOM)	2016.04.28	UOS	72.00	No toxicity (up to 100mg/L)
JRCNM01003a	immobilization	Daphnia immobilisation test(OECD TG 202)	2560.00	ii) SOP-Toxicity Test with Daphnia magna (without NOM)	2016.03.05	UOS	48h	EC50 128.62 94.90< EC50 < 229.45 EC20 58.15 38.07< EC20 < 89.66
JRCNM01003a	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	i) SOP-Toxicity Test with Daphnia magna (with NOM)	2016.03.05	UOS	48h	EC50 190.29 118.14< EC50 <5367.48 EC20 113.09 73.28< EC20 <2383.33
JRCNM01003a	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	i) SOP-Toxicity Test with Daphnia magna (with NOM)	2016.03.05	UOS	72h	EC50 127.69 89.81< EC50 <303.19 EC20 56.34 29.96< EC20 <99.52
JRCNM02000a	immobilization	nanoreg sop-Daphnia magna V3 (neonate)	2560.00	i) SOP-Toxicity Test with Daphnia magna (with NOM)	2016.04.27	UOS	72h	EC20 15.33214 8.58277< EC20< 20.22692 EC50 28.282 28.282 < EC50 < 47.235

Figure 20. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. magna* by UOS, compiled according to the *D. magna - Ecotoxicity test data entry template*.

2.4.1.3 *Pseudokirchneriella subcapitata* Growth Inhibition Tests

GNano/IFSC-USP

The results for algal growth inhibition tests performed by GNano/IFSC-USP are summarized in Figures 21 to 24, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	JRCNM01001a		Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	13.0	0.222			3.5
											128
											820
1	JRCNM01100a		Powder	JRC	ZnO	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	328.62	0.257			0.05
											0.25
											0.40
1	JRCNM03000a		Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	54.03	0.501			0.025
											0.08
											0.8

Figure 21. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *P. subcapitata* by GNano/IFSC-USP, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test											
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01001a	Supernatant	523.17	0.652	-26.0					8.25	23.1	6.03
	Supernatant	470.6	0.282	-23.2					8.33	23.6	5.81
	Supernatant	421.4	0.239	-24.2					8.38	23.5	5.93
JRCNM01100a	Supernatant	269.1	0.649	-24.5					7.52	22.0	6.56
	Supernatant	334.4	0.542	-23.2					7.54	21.9	5.67
	Supernatant	279.7	0.600	-29.5					7.54	21.9	5.70
JRCNM03000a	Supernatant	305.73	0.497	-27.9		415 nm	< Detection Limit		7.31	23.4	4.86
	Supernatant						< Detection Limit		7.40	23.4	4.99
	Supernatant	321.2	0.587	-27.4			0.56		7.48	23.3	4.91

Figure 22. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *P. subcapitata* by GNano/IFSC-USP, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM01001a	Supernatant	584.3	0.604	-27.5					7.42	23.2	4.26
	Supernatant	859.6	0.396	-22.1					7.42	23.5	4.33
	Supernatant	420.1	0.299	-21.9					7.40	23.4	4.92
JRCNM01100a	Supernatant	685.5	0.662	-27.2					7.79	21.7	6.21
	Supernatant	659.4	0.819	-22.1					7.75	21.6	5.84
	Supernatant	954.3	0.375	-16.9					7.81	21.6	5.61
JRCNM03000a	Supernatant	881.50	0.602	-18.4		415 nm	< Detection Limit		7.16	23.9	5.81
	Supernatant						< Detection Limit		7.30	23.9	5.62
	Supernatant	411.3	0.801	-18.4			< Detection Limit		7.39	23.9	5.52

Figure 23. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *P. subcapitata* by GNano/IFSC-USP, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (D)	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Effective concentrations [mg/L] (95% CL)**
JRCNM01001a	EC50 EC10	P. subcapitata Acute Toxicity Test	256 mg/L	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	10/05/2016	GNano/IFSC-USP	72	EC50 = 50.57 (19.24-132.93) EC10 = 4.38 (1.48-12.98)
JRCNM01100a	EC50 EC10	P. subcapitata Acute Toxicity Test	256 mg/L	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	17/05/2016	GNano/IFSC-USP	72	EC50 = 0.07 (0.021-0.23) EC10 = 0.02 (0.004-0.089)
JRCNM03000a	EC50 EC10	P. subcapitata Acute Toxicity Test	256 mg/L	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	29/04/2016	SGNano/IFSC-USP	72	EC50 = 0.25 (0.006-9.40) EC10 = 0.05 (0.001-2.35)

Figure 24. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *P. subcapitata* by GNano/IFSC-USP, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

TiO_2 NPs (JRCNM01001a) stock solution dispersion was good; however there was nanomaterial aggregation in the exposure medium. Water quality parameters at the beginning and end of exposure were suitable for assays execution. Figure 25 presents TiO_2 NPs concentrations (logarithmic scale) as a function of percentages of effect, and the linear regression equations used for the calculation of mean inhibition growth concentrations during *P. subcapitata* exposures to TiO_2 NPs. The value for EC50 of TiO_2 NPs and *P. subcapitata* was 50.57 mg/L.

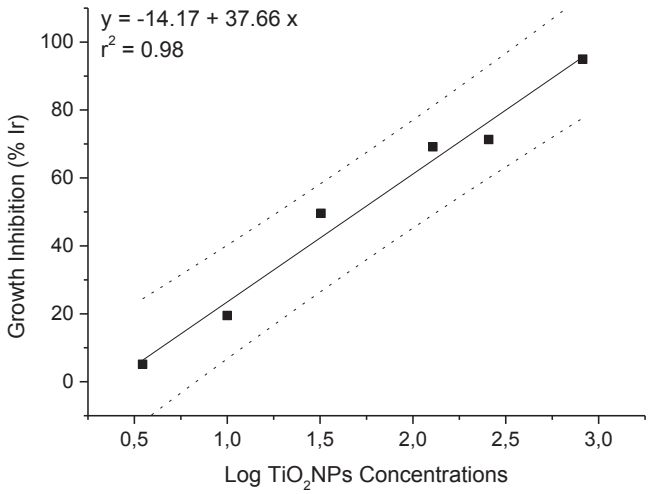

Figure 25. Linear regression equation after logarithmic transformation for estimation of Mean Inhibition Growth Concentration (EC50) of *P. subcapitata* after 72 h of TiO_2 NPs exposure. Dotted line means upper and lower limits of confidence (95%).

ZnO NPs (JRCNM01100a) was the most toxic nanomaterial in *P. subcapitata* assay since it presented the lowest value of EC50 during 72 h of exposure. The same issues in the dispersion of ZnO NP stock solutions observed in the *D. magna* assays occurred for the *P. subcapitata* experiments. Thus, additional dilution step was necessary to achieve the concentration of 0.256 g/L, which was suitable for dispersion. There was nanomaterial aggregation in the exposure media during the exposure. Water conditions were maintained suitable for assays execution. Figure 26 presents ZnO NPs concentrations (logarithmic scale) in function of percentages of effect, and the linear regression equations used for the calculation of inhibition growth concentration during *P. subcapitata* exposures to ZnO NPs. The value obtained for EC50 of ZnO NPs and *P. subcapitata* was 0.07 mg/L.

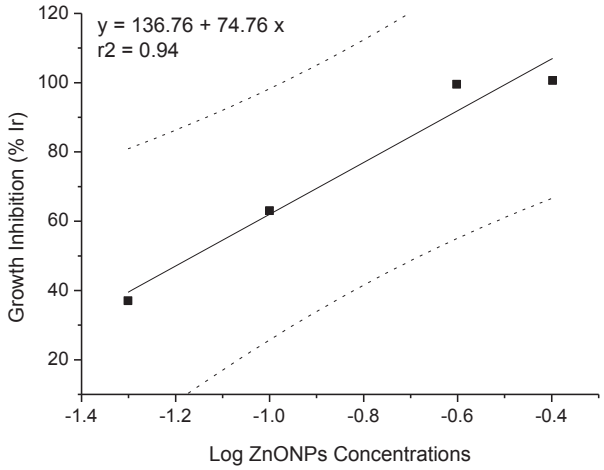

Figure 26. Linear regression equation after logarithmic transformation for estimation of Mean Inhibition Growth Concentration (EC50) of *P. subcapitata* after 72 h of ZnO NPs exposure. Dotted line means upper and lower limits of confidence (95%).

UV-Vis analyses were performed on Ag NPs (JRCNM03000a) exposure media for determining Ag concentration after 72 h of exposure. It was not possible to assess Ag NP concentration in the exposure media; probably because of the Ag NPs adsorption to the test containers and/or microalgae cells during exposure. The Ag NPs and *P. subcapitata* assays were hardly reproducible: four tests were performed and the results of growth inhibition were not comparable. Sorensen and Baun (2015) observed the same issues when testing Ag NP toxicity to algae. According to the latter, aging of AgNP test solution in algal medium for 24h yielded test reproducibility and concentration-response patterns, being possible to infer that NP undergo

time-dependent processes when diluted in test solutions, which should be considered in nanoecotoxicological experiments. We believe that Ag NPs adsorption to the glass flasks or algae cells during the assays, or even the use of freshly prepared Ag NP test solution (without aging), might have influenced our results. Ag NPs presented good dispersion in the stock solution. Nevertheless, there was nanomaterial aggregation in the exposure medium during the assay. Water quality parameters obtained were suitable for the assay development. Figure 27 presents Ag NPs concentrations (logarithmic scale) as a function of inhibition growth percentages, and the linear regression equations used for assessing effective concentrations of Ag NPs to *P. subcapitata*. The mean inhibition growth value was 0.25 mg/L.

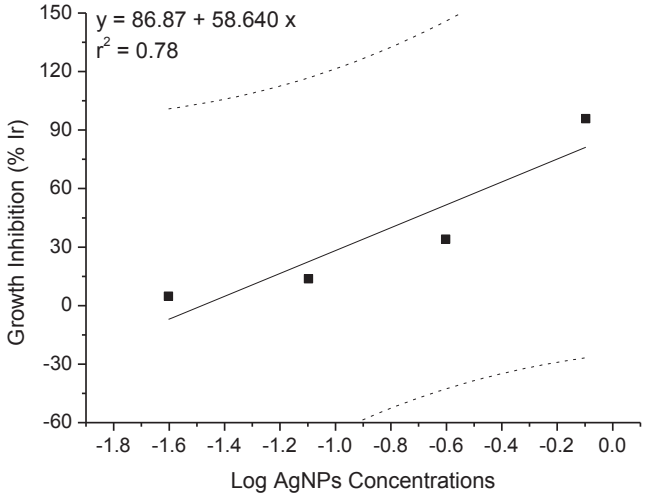

Figure 27. Linear regression equation after logarithmic transformation for estimation of Mean Inhibition Growth Concentration (EC50) of *P. subcapitata* after 72 h of Ag NPs exposure. Dotted line means upper and lower limits of confidence (95%).

IK4-Tekniker

The results for algal growth inhibition tests performed by IK4-Tekniker are summarized in Figures 28 to 31, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion				
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	JRCNM03000a	07206	Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	-	-	NO	SEM	0.01 0.10 1.02
2	JRCNM03000a	07206	Dispersion (Tween 20/PEG)	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	91.69	0.212	NO	SEM	0.01 0.05 0.51
1	JRCNM01003a	001286	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	151.60	0.334	NO	SEM	0.63 10.00 160.00
2	JRCNM01003a	001286	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	151.60	0.334	NO	SEM	0.63 10.00 160.00
3	JRCNM01003a	001286	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	151.60	0.334	NO	SEM	0.63 10.00 160.00
1	JRCNM02102a	001242	Powder	JRC	CeO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	196.80	0.345	NO	SEM	0.63 10.00 160.00
2	JRCNM02102a	001242	Powder	JRC	CeO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	196.80	0.345	NO	SEM	0.63 10.00 160.00
3	JRCNM02102a	001242	Powder	JRC	CeO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	196.80	0.345	NO	SEM	0.63 10.00 160.00

Figure 28. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *P. subcapitata* by IK4-Tekniker, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM03000a	Homogenized medium	-	-	-	SEM	-	-	-	-	21.6	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	21.6	-
	Homogenized medium	82.31	0.312	-	SEM	450	1.31	-	-	21.6	-
JRCNM03000a	Homogenized medium	208.30	0.323	-18.7	SEM	450	0.15	-	-	21.8	-
	Homogenized medium	93.31	0.216	-20.2	SEM	450	0.18	-	-	21.8	-
	Homogenized medium	92.11	0.214	-22.4	SEM	450	0.65	-	-	21.8	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	2389.30	0.244	-3.3	SEM	400	151.92	-	-	24.2	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	22.4	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	22.4	-
	Homogenized medium	175.30	0.259	-25.8	SEM	400	199.06	-	-	22.4	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	152.60	0.245	-24.6	SEM	400	189.50	-	-	22.7	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	21.6	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	21.6	-
	Homogenized medium	2558.00	0.301	-19.9	SEM	400	361.70	-	-	21.6	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	22.4	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	22.4	-
	Homogenized medium	176.40	0.234	-29.7	SEM	400	202.75	-	-	22.4	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	167.90	0.215	-28.5	SEM	400	175.99	-	-	22.7	-

Figure 29. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *P. subcapitata* by IK4-Tekniker, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
JRCNM03000a	Homogenized medium	-	-	-	SEM	-	-	-	-	23.1	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	23.1	-
	Homogenized medium	82.30	0.316	-	SEM	450	0.97	-	-	23.1	-
JRCNM03000a	Homogenized medium	727.50	0.665	-13.6	SEM	450	0.15	-	-	22.9	-
	Homogenized medium	102.10	0.331	-19.0	SEM	450	0.22	-	-	22.9	-
	Homogenized medium	94.27	0.227	-22.2	SEM	450	0.59	-	-	22.9	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	22.7	-
	Homogenized medium	2697.00	0.203	-	SEM	400	175.76	-	-	22.7	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	23.2	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	23.2	-
	Homogenized medium	178.70	0.270	-	SEM	400	201.35	-	-	23.2	-
JRCNM01003a	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	173.80	0.316	-	SEM	400	196.49	-	-	24.2	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	23.1	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	23.1	-
	Homogenized medium	1940.00	0.279	-	SEM	400	305.82	-	-	23.1	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	23.2	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	23.2	-
	Homogenized medium	235.30	0.219	-	SEM	400	217.21	-	-	23.2	-
JRCNM02102a	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	-	-	-	SEM	-	-	-	-	24.2	-
	Homogenized medium	259.40	0.235	-	SEM	400	197.52	-	-	24.2	-

Figure 30. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *P. subcapitata* by IK4-Tekniker, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (D)	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Effective concentrations [mg/L] (95% CL)**
JRCNM03000a	EC50 EC10		1.02	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	10/03/2015	IK4-TEKNIKER	72	0.021 (0.013-0.035) -
JRCNM03000a	EC50 EC10		0.51	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	18/03/2016	IK4-TEKNIKER	72	0.021 (0.004-0.121) 0.011 (0.002-0.052)
JRCNM01003a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	13/04/2015	IK4-TEKNIKER	72	0.27 (0.15-0.48) -
JRCNM01003a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	15/06/2015	IK4-TEKNIKER	72	No inhibition (increase in the growth rate)
JRCNM01003a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	30/04/2015	IK4-TEKNIKER	72	No inhibition (increase in the growth rate)
JRCNM02102a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	10/03/2015	IK4-TEKNIKER	72	1.24 (1.07-1.43) -
JRCNM02102a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	15/06/2015	IK4-TEKNIKER	72	31.9 (10.2–99.5) 16.2 (4.8–54.4)
JRCNM02102a	EC50 EC10		160.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	30/04/2015	IK4-TEKNIKER	72	74.3 (18.0–305.9) 38.0 (8.4–171.6)

Figure 31. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *P. subcapitata* by IK4-Tekniker, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*. The SR-NOM concentrations selected to carry out the tests were 8 mg/L (replicates 'number 2') and 20 mg/L (replicates 'number 3').

NMBU

In addition to the toxicity tests according to the SOPs (Figure 32), NMBU carried out an extensive characterization of the exposure media using size fractionation techniques, in order to improve understanding of some of the factors influencing toxicity.

Regarding the exposure characterization, the results clearly show the change in the size fractions of Ag present in the exposure suspension over time for the JRCNM03000a MNM (Figure 32). At time 0h almost 100 % of the Ag can be found as colloids/NPs (defined as <0.22 µm, > 10 kDa), at time 72h almost all Ag are present as particulate matter >0.22 µm. This change is also the most probable explanation to the reduction in growth inhibition seen in the algae after 72h compared to 24h.

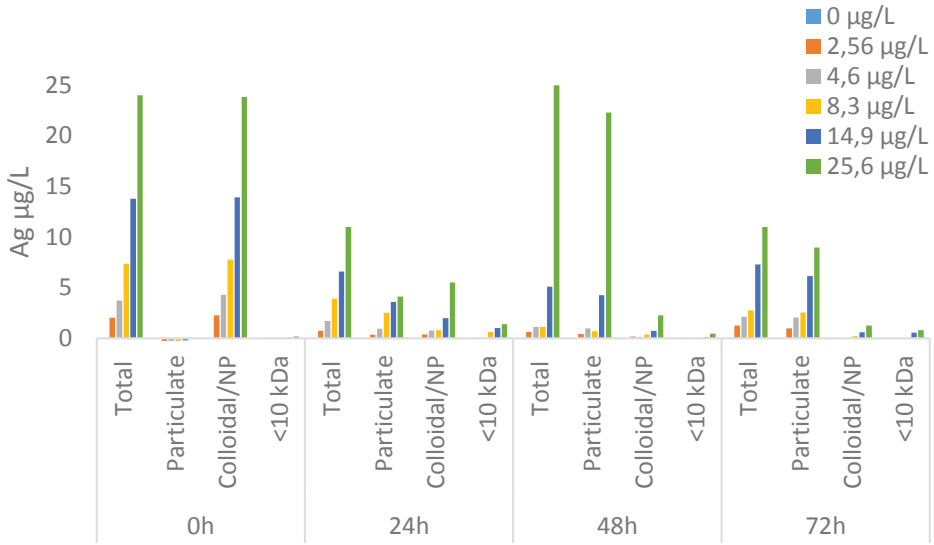

Figure 32. Size fractionation in combination with total analysis on ICP-MS of the exposure suspensions of Ag NPs (JRCNM03000a) over time. The fractions are defined as: particulate >0.22 µm, colloidal/NP <0.22 µm and >10kDa, LMM <10 kDa.

In addition to stock characterization, dynamic light scattering (DLS) was used for characterizing particle size in exposure suspensions. The highest exposure concentration of Ag NPs, 25.6 µg Ag/L, was analyzed with the DLS at time 24 and 72 h (Table 37). The presence of algae in the suspension resulted in a Z_{ave} most likely not representative of the real particle size. Thus, the algae were removed from suspension by pre-treating the sample with low-speed centrifugation. This however, did not seem to reduce the Z_{ave} or the PDI. Taking also the number based size distribution into account, gives a very different picture. One, the mean particle size is much smaller, and two, pre-treating the sample to remove algae from suspension do reduce the mean particles size. It is important to keep in mind that both pre-treating the samples with low-speed centrifugation and not doing the pre-treatment, both potentially give a skewed picture of the actual particle size. Either the algae led to a bigger particle size or the bigger particles were removed together with the algae in the low-speed centrifugation, thus resulting in smaller mean particle size.

Table 37: Size distribution of JRCNM03000a in exposure media for *P. subcapitata* over time, with and without pre-treatment to remove the algae from suspension.

25.6 µg/L of Ag from JRCNM03000a	Z_{ave} (d.nm)	PDI	Intensity mean (d.nm)	Number mean (d.nm)
24h, without algae	118±12	0.185	127.8±34.3	88±9
72h, without algae	347±136	0.766	1012±817	38±8
72h, with algae	250±136	0.535	700±869	55±9

Dynamic light scattering was not a suitable method of size characterization of Ag nanorods (JRCNM03002a), given their shape and large size. Thus, for these particles only TEM was used for size characterization of stock suspensions. The ECs during the experimental period were far from the nominal concentrations. For the three highest concentrations the effective concentrations was < 1 % on nominal, and for the two lowest concentrations it was around 10 %, when algae were included. However, the concentrations seemed relatively stable over time. There were not performed and size fractionation on these exposure suspensions.

Toxicity results obtained for Ag NPs and nanorods is provided in Figures 33 to 37, and Table 38. It was not possible to give clear EC values for nanorods due to large variability in the data over time. After 24 h there was a dose response in the growth inhibition (Figure 34), however this changed drastically over time, and after 72 h only the highest concentration produced growth inhibition (50 % growth inhibition, effective concentration 89 and 72 µg Ag/L when algae were included and excluded, respectively). The speciation of

the Ag present in suspension/solution were not analyzed, thus it was not possible to say if there were any NM left in exposure suspensions, or if it was dissolved Ag^{+}.

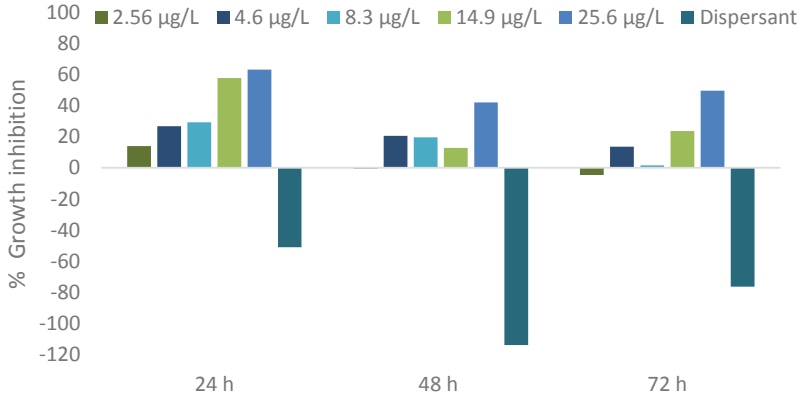

Figure 33. Growth inhibition in *P. subcapitata* after exposure to Ag NPs (JRCNM03000a).

Table 38. Toxicity of AgNPs (JRCNM03000a) dispersions on *P. subcapitata* (µg Ag/L).

EC50 (95%CL)	EC10 (95%CL)
7.7 (4.68-9.82)	4.9 (1.76-8.26)

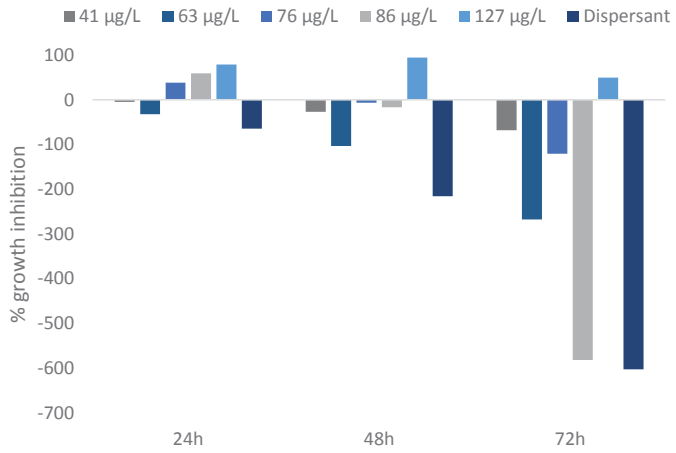

Figure 34. Growth inhibition (%) of *P. subcapitata* exposed to the Ag nanorods (JRCNM03002a). The dispersant equals the amount present in the highest concentration of the NM (nominal concentration 25.6 mg/L).

Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Batch dispersion					
						Dispersion protocol (*)	Hydrodynamic size (nm)	PDI	¿Out of range?	Microscopy verification (**)	Concentration [mg/L] (low-intermediate-high)
1	JRCNM03000a	7455	Dispersion (Tween 20/PEG)	Fraunhofer	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	73,6±0,5	0,284±0,006		TEM	0.0256
											0.0149
											0.0083
											0.0046
											0.00256
1	JRCNM03002a	6408	Dispersion (Tween 20/PEG)	Fraunhofer	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	Method considered not suitable for this NM			TEM	25.6
											8
											2.5
											0.8
											0.26

Figure 35. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *P. subcapitata* by NMBU, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	Initial exposure medium						Final exposure medium					
	Hydrodynamic size [nm]	PDI	ph	T [°C]	O_2 concentration [mg/L]	Microscopy verification (**)	Hydrodynamic size [nm]	PDI	ph	T (°C)	O_2 concentration [mg/L]	Microscopy verification (**)
JRCNM03000a			7.9	20			250±136	0,535±0,157	8.0	20		
			7.9						7.8			
			7.9						7.8			
			7.9						7.8			
			7.9				1083±670	0,792±0,177	7.8			
JRCNM03002a			7.8						7.9			
			7.8						7.8			
			7.8						7.8			
			7.8						7.8			
			7.8						7.8			

Figure 36. Quality experimental parameters of the NANoREG core MNMs at the start and end of the tests conducted with *P. subcapitata* by NMBU, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Effective concentrations [mg/L] (95% CL)**	Parameter value **Effective concentrations as % of nominal concentration 0h;72h**
JRCNM03000a	**EC50** (95 % CL): 7.7 (4.68-9.82) µg Ag/L **EC10** (95 % CL): 4.9 (1.76-8.26) µg Ag/L		0,025±0,0057	SOP-Toxicity Test with Pseudokirchneriella subcapitata	09/03/2015	NMBU	72	0,01775 (0,010101;0,025399)	94;43
			0,014±0,0028					0,00820 (0,004437;0,011953)	92;49
			0,007±0,0015					0,00378 (0,001182; 0,006368)	89;33
			0,004±0,0007					0,00217 (0,001057; 0,003273)	81;46
			0,002±0,0004					0,00115 (0,00052;0,00178)	79;49
JRCNM03002a	**No growth inhibition was observed in any of the exposure groups. EC50 EC20**		0.25	SOP-Toxicity Test with Pseudokirchneriella subcapitata	02/03/2015	NMBU	72	0,12758 (0,04758;0,20758)	1;0
			0.11					0,08583 (0,06991;0,10175)	1;1
			0.086					0,07633 (0,06991;0,08272)	3;3
			0.079					0,06308 (0,05016;0,07600)	10;8
			0.082					0,04050 (0,0126;0,06842)	32;9

Figure 37. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *P. subcapitata* by NMBU, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

SINTEF

The results for algal growth inhibition tests performed by SINTEF are summarized in Figures 38 to 41, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion				
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	NM300K (JRCNM03002a)	07730	Dispersion (Tween 20/PEG)	Fraunhofer	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	74.49	0.300	NO		0.0063 0.03 0.10
1	JRCNM01000a	06956	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	236.50	0.200	NO		0.10 10.00 100.00
1	JRCNM01001a	990941	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	489.50	0.300	NO		0.10 10.00 100.00
1	JRCNM01003a	07003	Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	162.40	0.300	NO		0.32 8.00 200.00
1	JRCNM4000a	990414	Powder	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	36057.80	1.000	NO		2.00 20.00 50.00
1	JRCNM4001a	990649	Powder	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	874.90	0.800	NO		2.00 20.00 50.00
1	JRCNM4100a	06157	Powder	JRC	MWCNTs	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	-	-	NO		2.00 20.00 50.00

Figure 38. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *P. subcapitata* by SINTEF, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	Start of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K (JRCNM03002a)	Homogenized medium	127.30	0.5	-9.1		-	-	0.007	7.84	22	-
	Homogenized medium	72.70	0.4	-9.8		-	-	0.022	7.83	22	-
	Homogenized medium	67.60	0.3	-11.6		-	-	0.08	7.85	22	-
JRCNM01000a	Homogenized medium	279.70	0.4	-23.0		-	-	0.02	8.63	21.7	-
	Homogenized medium	246.60	0.2	-27.3		-	-	1.16	8.09	21.7	-
	Homogenized medium	317.00	0.2	-22.1		-	-	50.91	7.95	21.7	-
JRCNM01001a	Homogenized medium	505.00	0.6	-20.4		-	-	0.02	8.43	21.7	-
	Homogenized medium	402.70	0.4	-21.9		-	-	3.67	7.95	21.7	-
	Homogenized medium	519.90	0.4	-23.4		-	-	45.77	7.91	21.7	-
JRCNM01003a	Homogenized medium	551.6	0.6	-24.30		-	-	0.04	8.27	22.7	-
	Homogenized medium	455.8	0.3	-16.60		-	-	2.7	8.18	22.7	-
	Homogenized medium	3592.7	0.4	-7.50		-	-	89.6	8.15	22.7	-
JRCNM4000a	Homogenized medium	2412.60	0.5	-11.8		800	0.00	-	7.98	21.35	-
	Homogenized medium	9959.30	1.0	-19.2		800	11.90	-	8.02	21.35	-
	Homogenized medium	7215.00	0.9			800	29.70	-	7.52	21.35	-
JRCNM4001a	Homogenized medium	756.20	0.4	-11.9		800	0.00	-	8.06	21.35	-
	Homogenized medium	6138.00	0.9	-23.2		800	6.30	-	8.06	21.35	-
	Homogenized medium	7633.70	1.0			800	9.70	-	7.18	21.35	-
JRCNM4100a	Homogenized medium	15665.30	0.5	-18.9		800	0.30	-	8.58	21.35	-
	Homogenized medium	57943.30	1.0	-11.9		800	8.50	-	8.52	21.35	-
	Homogenized medium	55724.30	1.0			800	20.50	-	7.52	21.35	-

Figure 39. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *P. subcapitata* by SINTEF, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	End of the ecotoxicity test										
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium					
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K (JRCNM03002a)	Supernatant	218.00	0.40	-1.8		-	-	0.005	7.91	22.7	-
	Supernatant	82.40	0.40	-5.3		-	-	0.02	7.92	22.7	-
JRCNM01000a	Supernatant	66.90	0.300	-16.3		-	-	0.075	7.97	22.7	-
	Supernatant	1944.70	1.00	aborted by machine		-	-	0.01	8.07	21.2	-
	Supernatant	232.50	0.20	-25.5		-	-	0.47	8.05	21.2	-
JRCNM01001a	Supernatant	361.50	0.300	-22.0		-	-	0.25	7.86	21.2	-
	Supernatant	846.60	0.600	-16.0		-	-	0.01	8.04	21.2	-
	Supernatant	515.50	0.500	-18.1		-	-	0.23	7.89	21.2	-
	Supernatant	700.80	0.500	-21.1		-	-	0.25	7.7	21.2	-
JRCNM01003a	Supernatant	598.7	0.4	-19.00		-	-	0.03	8.78	22.6	-
	Supernatant	2315.7	0.5	-15.90		-	-	2.3	7.92	22.6	-
	Supernatant	628.9	0.6	-18.10		-	-	87.4	7.85	22.6	-
JRCNM4000a	Supernatant	131.00	0.300	-3.3		800	0.23	-	7.95	20.9	-
	Supernatant	589.20	0.400	-22.6		800	0.58	-	7.9	20.9	-
	Supernatant	1300.00	1.000	-		800	0.30	-	7.93	20.9	-
JRCNM4001a	Supernatant	554.00	0.60	-26.9		800	0.00	-	7.87	20.9	-
	Supernatant	18864.30	0.90	-25.1		800	0.04	-	7.94	20.9	-
	Supernatant	5008.30	1.000	-		800	0.15	-	7.86	20.9	-
JRCNM4100a	Supernatant	1491.70	0.900	-17.4		800	0.00	-	7.92	20.9	-
	Supernatant	1456.00	0.900	-9.7		800	0.00	-	7.91	20.9	-
	Supernatant	2043.00	0.900	-		800	0.00	-	7.85	20.9	-

Figure 40. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *P. subcapitata* by SINTEF, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (D)	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Effective concentrations [mg/L] (95% CL)**
NM300K (JRCNM03002a)	EC50 EC10		0.10	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	13/06/2016	SINTEF	72	0.01 0.0097 (0.0015-0.062)
JRCNM01000a	EC50 EC10		100.00	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	04/04/2016	SINTEF	72	661.7 (0.839-521991) 0.867 (0.0089-841.8)
JRCNM01001a	EC50 EC10		100.00	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	04/04/2016	SINTEF	72	43.09 (3.7-505) 0.0931 (0.0005-15)
JRCNM01003a	EC50 EC10		200.00	SOP-Toxicity Test with Microalgae P.subcapitata (without NOM)	20/04/2016	SINTEF	72	11.34 (3.29-39.17) 0.112 (0.043-2.90)
JRCNM4000a	EC50 EC10		100.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	11/04/2016	SINETF	72	method not appropriate absorbance of Chl to CNT
JRCNM4001a	EC50 EC10		100.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	11/04/2016	SINTEF	72	method not appropriate absorbance of Chl to CNT
JRCNM4100a	EC50 EC10		100.00	SOP-Toxicity Test with Microalgae P.subcapitata (with NOM)	11/04/2016	SINTEF	72	method not appropriate absorbance of Chl to CNT

Figure 41. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *P. subcapitata* by SINTEF, compiled according to the *P. subcapitata - Ecotoxicity test data entry template*. The SR-NOM concentration selected to carry out the tests was 20 mg/L.

2.4.1.4 Toxic effects on growth, fertility and reproduction of *Caenorhabditis elegans*

NMBU, UFMG, EMBRAPA.

This section includes the toxicity results obtained by the different partners for a range of different NANoREG core MNMs.

A) MWCNTs (JRCNM04000a)

Two definitive tests were conducted by UFMG with the nominal exposure concentrations of 10, 18, 32, 56, and 100 mg/L (Test A) and 0.1, 0.32, 1.0, 3.2, and 10 mg/L (Test B) (Table 39).

Table 39. Results from toxicity testing of MWCNTs (JRCNM04000a) to *C. elegans*. (Test conducted by UFMG).

Nominal concentration mg/L	Definitive test DT	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	27	100	100	21 (<1)	100

56	DT (A)	27	100	100	20 (<1)	100
32	DT (A)	26	100	100	48 (1.2)	100
18	DT (A)	--	--	--	--	--
10	DT (A)	--	--	--	--	--
10	DT (B)	27	100	100	86 (3.2)	100
3,2	DT (B)	23	100	100	144 (6.3)	100
1	DT (B)	24	100	100	128 (5.3)	100
0,32	DT (B)	24	100	100	124 (5.2)	100
0,1	DT (B)	30	100	100	192 (6.4)	100
Control		19	100	100	70 (3.7)	100

B) ZnO NPs (JRCNM01100a)

Two definitive tests were conducted by UFMG with the nominal exposure concentrations of 10, 18, 32, 56, and 100 mg/L (Test A) and 0.001, 0.0032, 0.01, 0.032, and 0.1 mg/L (Test B) (Table 40). In Test A (10-100 mg/L), recovery and growth were reduced at the highest concentration. However, in Test B (0.001-0.1 mg/L) recovery was affected in all exposure concentrations and growth was reduced at the two highest concentrations, 0.032 and 0.1 mg/L. A reduction in both fertility and reproduction could be observed at all concentrations tested in Test A, as well as the two highest concentrations, 0.032 and 0.1 mg/L, in Test B. The exposure in Test B clearly led to more toxicity than in test A, despite the lower nominal exposure concentrations used. This could possibly be explained by high concentrations leading to increased aggregation and subsequent sedimentation, thus in reality lower effective exposure concentrations than in Test B.

Table 40. Observed toxicity of the ZnO nanomaterial JRCNM01100a to *C. elegans* (Test conducted by UFMG).

Nominal concentration mg/L	Definitive test (DT)	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	19	80	0	3 (<1)	0
56	DT (A)	19	100	10	4 (<1)	100
32	DT (A)	19	100	10	3 (<1)	100
18	DT (A)	19	100	30	10 (<1)	100
10	DT (A)	19	100	30	15 (<1)	100
0,1	DT (B)	3	0	0	0	0
0,032	DT (B)	14	80	0	7 (<1)	80
0,01	DT (B)	22	80	>90	13 (<1)	100
0,0032	DT (B)	21	90	>90	77 (3.6)	100
0,001	DT (B)	26	90	>90	141 (5.4)	100
Control		19	100	100	70 (3.7)	100

C) SiO_2 NPs (JRCNM02000a)

Two definitive tests were conducted by UFMG with the nominal exposure concentrations of 10, 18, 32, 56, and 100 mg/L (Test A) and 0.1, 0.32, 1.0, 3.2, and 10 mg/L (Test B) (Table 41). Recovery, growth and fertility were not affected at any of the exposure concentrations tested in the two tests (A and B). Reproduction seemed to be significantly reduced in all concentrations, except in the highest concentration in each of the two tests (100 mg/L in Test A, 10 mg/L in Test B). There is a discrepancy in the results in test A and B when it comes to reproduction. The same nominal exposure concentration 10 mg/L, lead to differences in reproduction.

Table 41. Observed toxicity of the SiO_2 nanomaterial JRCNM02000a to *C. elegans* (Test conducted by UFMG).

Nominal concentration mg/L	Definitive test (DT)	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	23	100	100	159 (6.9)	100
56	DT (A)	31	100	100	50 (1.6)	100
32	DT (A)	28	100	100	34 (1.2)	100

18	DT (A)	28	100	100	34 (1.2)	100
10	DT (A)	28	100	100	64 (2.3)	100
10	DT (B)	27	100	100	174 (6.4)	100
3,2	DT (B)	16	100	90	7 (<1)	100
1	DT (B)	18	100	100	21 (1.2)	100
0,32	DT (B)	16	100	100	9 (<1)	100
0,1	DT (B)	26	100	100	25 (<1)	100
Control		19	100	100	70 (3.7)	100

D) SiO_2 NPs (JRCNM02003a)

Two definitive tests were conducted by UFMG with the nominal exposure concentrations of 10, 18, 32, 56, and 100 mg/L (Test A) and 0.1, 0.32, 1.0, 3.2, and 10 mg/L (Test B) (Table 42). Recovery and fertility was not affected at any of the test concentrations in any of the two tests conducted. Growth were however reduced in the two highest concentrations (3.2 and 10 mg/L) in Test B. Reproduction was the most sensitive endpoint and seemed to be significantly reduced in the two highest test concentrations (56 and 100 mg/L) in test A, and the two lowest concentrations (0.1 and 0.32 mg/L) in Test B.

Table 42. Observed toxicity of the SiO_2 nanomaterial JRCNM02003a to *C. elegans* (Tests conducted by UFMG).

Nominal concentration mg/L	Definitive test (DT)	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	28	100	100	82 (2.9)	100
56	DT (A)	32	100	100	62 (1.9)	100
32	DT (A)	30	100	100	200 (6.6)	100
18	DT (A)	26	100	100	120 (4.6)	100
10	DT (A)	28	100	100	220 (7.8)	100
10	DT (B)	19	100	100	130 (6.8)	80
3,2	DT (B)	25	100	100	88 (3.5)	80
1	DT (B)	32	100	100	104 (3.2)	100
0,32	DT (B)	25	100	100	18 (<1)	100
0,1	DT (B)	27	100	100	8 (<1)	100
Control		19	100	100	70 (3.7)	100

E) Ag NPs (JRCNM03000a)

The UFMG reported no effect on recovery or growth in any of the test concentrations (Table 43). The fertility as well as the reproduction were reduced at the three highest test concentrations in Test A (32, 56, and 100 mg/L). In Test A, effects could only be seen for reproduction at the two lowest test concentrations (0.1 and 0.32 mg/L). However, this might not be significant.

For JRCNM03000a, NMBU observed an effect at 2 mg Ag/L for size and growth. Fertility was reduced at 1 mg Ag/L and reproduction at 0.5 mg Ag/L. However, this reduction in reproduction at 0.5 mg Ag/L was not significant compared to control, this first appears at 1 mg Ag/L. At the two highest concentrations, 2 and 4 mg/L, there was no reproduction (Table 44).

Table 43. Observed toxicity of the Ag nanomaterial JRCNM03000a to *C. elegans*. (Test conducted by UFMG).

Nominal concentration mg/L	Definitive test (DT)	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	24	100	80	1 (<1)	100
56	DT (A)	17	100	70	2 (<1)	100
32	DT (A)	22	100	80	1 (<1)	100
18	DT (A)	28	100	100	114 (4.1)	100
10	DT (A)	30	100	100	126 (4.1)	100

10	DT (B)	25	100	100	112 (4.5)	100
3,2	DT (B)	21	100	100	80 (3.8)	100
1	DT (B)	--	--	0	3	0
0,32	DT (B)	24	100	100	55 (2.3)	100
0,1	DT (B)	22	100	100	58 (2.6)	100
Control		19	100	100	70 (3.7)	100

Table 44. Results from toxicity testing of the Ag nanomaterial JRCNM03000a to *C. elegans* (Test conducted by NMBU).

Nominal concentration mg Ag/L*	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
0.1 (1)	12		92	19	99
0.25 (2.5)	15		95	20	95
0.5 (5)	14		100	13	106
1 (10)	17		72	2	104
2 (20)	12		8		49
4 (40)	15				46
Control			100	11,3	100

* The Ag nanoparticles are dispersed in 4 % each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween20), and only 10.16 % (w/w) are the Ag nanoparticles. Thus, the concentrations are given as the nominal concentration of Ag, numbers in brackets are the appx. NM concentration (AgNPs + dispersant).

F) Ag nanorods (JRCNM03002a)

Effects were not observed at any of the test concentrations in any of the tests conducted by UFMG (Table 45) or NMBU (Table 46).

Table 45. Observed toxicity of the Ag nanomaterial JRCNM03002a to *C. elegans* (Tests conducted by UFMG).

Nominal concentration mg/L	Definitive test (DT)	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
100	DT (A)	31	100	100	83 (2.7)	100
56	DT (A)	27	100	100	60 (3.7)	100
32	DT (A)	26	100	100	80 (3.8)	100
18	DT (A)	21	100	100	108 (5.1)	100
10	DT (A)	23	100	100	87 (3.8)	100
10	DT (B)	30	100	100	293 (9.8)	100
3,2	DT (B)	22	100	100	129 (5.9)	100
1	DT (B)	23	100	100	185 (8.0)	100
0,32	DT (B)	25	100	100	184 (7.2)	100
0,1	DT (B)	27	100	100	212 (7.8)	100
Control		19	100	100	70 (3.7)	100

Table 46. Observed toxicity of the Ag nanomaterial JRCNM03002a to *C. elegans* (Tests conducted by NMBU).

Nominal concentration mg Ag/L*	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
0.1 (1)	14		100	21	107
0.25 (2.5)	13		100	28	101
0.5 (5)	15		88	10	103

1 (10)	14	88	17	103
2 (20)	16	100	18	103
4 (40)	16	100	14	104
Control		100	11,3	100

*The silver nanoparticles are dispersed in 4 % each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween20), and only 10.16 % (w/w) are the Ag nanoparticles. Thus, the concentrations are given as the nominal concentration of Ag, numbers in brackets are the appx. NM concentration (AgNPs + dispersant).

G) TiO_2 NPs (JRCNM01001a)

NMBU observed some toxicity at the two highest concentrations 17 and 50 mg/L, giving a slight reduction in growth and fertility (this might not be significant). The most sensitive endpoint was again reproduction, where the two highest concentrations gave reduced reproduction (Table 47). Embrapa Environment did not report any toxicity effects in any of the exposure concentrations, except at the highest nominal concentration of 100 mg/L where a reduction in reproduction was observed (Table 48).

Table 47. Observed toxicity of the TiO_2 nanomaterial JRCNM01001a to *C. elegans* (Tests conducted by NMBU).

Nominal concentration mg/L	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
0.2	15		66	29	92
0.6	12		74	16	94
1.8	12		82	17	96
5.5	14		87	19	99
17	14		77	7	89
50	12		71	2	90
Control			100	11,3	100

Table 48. Observed toxicity of the TiO_2 nanomaterial JRCNM01001a to *C. elegans* (Tests conducted by Embrapa Environment).

Nominal concentration mg/L	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)
0.01	50	96	95	57	101
0.1	44	96	100	59	128
1.0	47	100	100	54	104
10	46	96	96	57	104
100	32	92	93	42	84
Control	46	95	98	54	115

H) CeO_2 NPs (JRCNM02102a)

The tests conducted by NMBU showed that growth and fertility were affected at nominal concentration of 5.5 and 17 mg/L (Table 49). In the highest test concentration, similar effects were not observed. This was probably caused by the increased aggregation and sedimentation of particles at this high concentration. Within the test concentration range, there also seemed to be a reduction in reproduction. However, compared to control this reduction was not significant.

Table 49. Observed toxicity of the CeO_2 nanomaterial JRCNM02102a to *C. elegans* (Tests conducted by NMBU).

Nominal concentration mg/L	No of worms	Recovery (%)	Fertility (%)	Reproduction (no of offspring/worm)	Growth (%)

0.2	14	100	26	100
0.6	19	100	21	92
1.8	13	100	23	91
5.5	15	81	20	85
17	13	66	17	88
50	14	96	13	97
Control		100	11,3	100

UOS

The results for toxicity tests performed by UOS on *C. elegans* are summarized in Figures 42 to 44, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (*)	Hydrodynamic size (nm)	PDI	¿Out of range?	Microscopy verification (**)	Concentration [mg/L] (low-intermediate-high)
1	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	166.63±2.87	0.247±0			0.5
											10
											50
1	JRCNM01001a		Powder	JRC-IHCP	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	379.3±7.67	0.167±0.01			0.5
											10
											50
1	NM103		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	158.43±4.13	0.189±0.02			0.5
											10
											50
1	JRCNM02000a		Powder	JRC-IHCP	SiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	220.77±4.69	0.153±0.01			0.5
											10
											50
1	JRCNM02102a		Powder	JRC-IHCP	CeO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	170.10±3.37	0.236±0.01			0.5
											10
											50

Figure 42. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *C. elegans* by UOS, compiled according to the *C. elegans - Ecotoxicity test data entry template*.

NM ID code	Initial exposure medium					Final exposure medium				
	Hydrodynamic size [nm]	PDI	ph	T [°C]	Microscopy verification (**)	Hydrodynamic size [nm]	PDI	ph	T (°C)	Microscopy verification (**)
NM300K	492.70±130.58	0.288±0.053	6.35	18.0		1791.53±209.76	1.020±0.110	7.74	20.8	
	221.83±2.83	0.219±0.031	6.35	17.8		323.83±25.78	0.210±0.015	7.03	20.0	
	193.17±3.79	0.170±0.009	6.70	18.0		206.67±0.91	0.233±0.009	7.89	20.2	
JRCNM01001a	4444.57±631.12	1.954±0.285	6.57	19.3		12354.97±354.28	4.690±0152	6.72	20.2	
	1437.0±28.92	0.582±0.011	6.64	19.1		1453.27±111.89	0.563±0.008	6.72	20.5	
	1606.90±48.06	0.352±0.028	6.58	19.1		4800.67±829.99	1.303±0.072	6.63	20.5	
NM103	968.20±106.52	0.547±0.061	6.56	18.9		3852.70±311.98	1.797±0164	6.59	21.6	
	620.17±68.23	0.301±0.055	6.61	18.9		5203.87±629.81	1.893±0.248	6.51	20.4	
	807.67±149.09	0.203±0.013	6.91	18.8		1820.90±28.03	0.470±0.032	6.57	20.2	
JRCNM02000a	1540.20±382.36	0.870±0.209	6.56	19.0		2076.17±179.90	1.132±0.115	6.54	20.6	
	904.27±118.69	0.543±0.057	6.86	18.6		467.13±82.65	0.301±0.050	6.85	20.4	
	741.833±120.98	0.452±0.061	6.78	19.1		1133.23±234.71	0.523±0.090	6.74	20.3	
JRCNM02102a	5970.93±258.90	2.506±0.063	7.05	19.0		11495.47±1908.58	4.135±0.618	7.08	20.0	
	2057.40±48.51	0.833±0.031	7.19	18.7		1450.83±83.03	0.608±0.03	7.03	20.1	
	2077.77±122.81	0.558±0.044	7.01	18.9		1809.20±185.71	0.397±0.054	6.95	20.3	

Figure 43. Quality experimental parameters of the NANoREG core MNMs at the start and at the end of the tests conducted with *C. elegans* by UOS, compiled according to the *C. elegans - Ecotoxicity test data entry template*.

NM ID code	**Growth**	**Reproduction**	Initial concentration [mg/L]	Protocol REF	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Effective concentrations [mg/L] (95% CL)**
NM300K	Length of worm at 48hr from L1	Offspring number of worm at 72hr from YA	2560	SOP-Toxicity Test with C. elegans		UOS	Growth (48hr) Reproduction (72hr)	Growth 3.35 <EC20< 9.00 Reproduction 0.78 <EC20< 1.13
JRCNM01001a	Length of worm at 48hr from L1	Offspring number of worm at 72hr from YA	2560	SOP-Toxicity Test with C. elegans		UOS	Growth-48 from L1 larva	
NM103	Length of worm at 48hr from L1	Offspring number of worm at 72hr from YA	2560	SOP-Toxicity Test with C. elegans		UOS	Growth-48 from L1 larva	
JRCNM02000a	Length of worm at 48hr from L1	Offspring number of worm at 72hr from YA	2560	SOP-Toxicity Test with C. elegans		UOS	Growth-48 from L1 larva	
JRCNM02102a	Length of worm at 48hr from L1	Offspring number of worm at 72hr from YA	2560	SOP-Toxicity Test with C. elegans		UOS	Growth-48 from L2 larva	

Figure 44. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *C. elegans* by UOS, compiled according to the *C. elegans - Ecotoxicity test data entry template*.

2.4.1.5 *Danio rerio* Acute Toxicity Tests

GNano/IFSC-USP

The results for acute toxicity tests performed by GNano/IFSC-USP with *D. rerio* are summarized in Figures 45 to 48, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size (nm)	PDI	¿Out of range?	Microscopy verification (B)	Nominal Concentration [mg/L] (low-intermediate-high)
1	JRCNM01001a		Powder	JRC	TiO2	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	37.52	0.218			6.25
											25
											100
1	JRCNM03000a		Powder	JRC	Ag	i) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Standard Procedure	60	0.548			6.0
											12.0
											16.0

Figure 45. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. rerio* by GNano/IFSC-USP, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test												
			Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Test procedure (static, semi-static, flow-through)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	pH	T [°C]	O_2 concentration [mg/L]	Total Hardness (mg $CaCO_3$/L)
JRCNM01001a	Supernatant	static	649.0	0.784						7.01	22.5	5.09	11
	Supernatant	static	525.6	0.513						7.29	22.1	4.62	12
	Supernatant	static	493.8	0.289						7.17	21.9	4.91	11
JRCNM03000a	Supernatant	static	122.61	0.505	-18.6					7.40	24.0	5.32	24.0
	Supernatant	static	196.10	0.473	-19.3					7.45	23.8	4.73	25.0
	Supernatant	static	150.09	0.342	-25.9					7.50	23.4	4.74	24.0

Figure 46. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. rerio* by GNano/IFSC-USP, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

	End of the ecotoxicity test											
	Stability of MNMs in exposure medium					Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	pH	T (°C)	O_2 concentration [mg/L]	Total Hardness (mg $CaCO_3$/L)
JRCNM01001a	Supernatant	780.3	0.849	-21.7					7.06	21.2	5.36	11
	Supernatant	573.4	0.701	< Detection Limit					7.16	21.0	5.30	12
	Supernatant	413.7	0.480	-24					7.11	21.2	5.74	15
JRCNM03000a	Supernatant	358.60	0.799	-34.8		415 nm	5.59		6.76	23.1	4.11	26.0
	Supernatant	255.93	0.526	-26.2			9.58		6.75	22.8	3.73	26.0
	Supernatant	561.90	0.584	-28.8			16.75		6.67	22.7	3.97	26.0

Figure 47. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. rerio* by GNano/IFSC-USP, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	**Number of fish/replicate**	Initial concentration [mg/L]	Protocol REF (D)	Date of acquisition	Operator	Exposure time [hours]	Parameter value **Lethal concentrations [mg/L] (95% CL)**
JRCNM01001a	LC10 LC50	Danio rerio	5	256	SOP-Toxicity Test with fish Danio rerio	20/02/2016	GNano/IFSC-USP	96	LC50 >100
JRCNM03000a	LC10 LC50	Danio rerio	6	256	SOP-Toxicity Test with fish Danio rerio	20/04/2016	GNano/IFSC-USP	96	LC10 = 5.48 (2.68-15.65) LC50 = 10.07 (4.57-22.22)

Figure 48. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. rerio* by GNano/IFSC-USP, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

UV-Vis analyses were performed on Ag NPs (JRCNM03000a) exposure media for estimative of Ag concentration after 96 h of exposure. A linear regression equation was used for estimating Ag concentration: $y = -0.020 + 0.045x$ ($r^2 = 0.99$). It was possible to infer that Ag NPs determined concentrations were not significantly different from AgNPs nominal concentrations added in the exposure media (lower than 20%). The stability parameters obtained showed that Ag NPs dispersion in stock solution was appropriate; nonetheless there was nanomaterial aggregation in the exposure media. The water quality parameters at the beginning and the end of exposure (0 and 96 h) were suitable for the experiments conducted. Figure 49 presents Ag NP concentrations (logarithmic scale) in function of lethality percentages, and the linear regression equation used for lethal concentration assessment during *D. rerio* exposures to Ag NPs. The value of LC50 estimated was 10.07 mg/L.

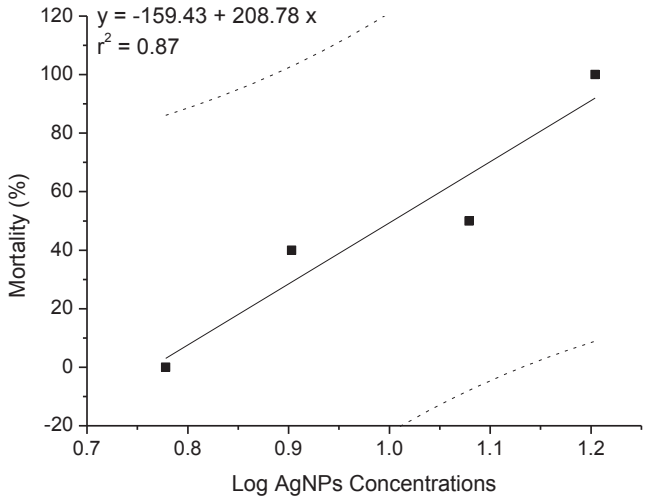

Figure 49. Linear regression equation after logarithmic transformation for estimation of Mean Lethal Concentration (LC50) of *D. rerio* after 96 h of Ag NPs exposure. Dotted line means upper and lower limits of confidence (95%).

D. rerio exposure to TiO_2 NPs (JRCNM01001a) did not result in toxic effect (mortality). And the LC50 value obtained was higher than 100 mg/L.

EMBRAPA

Since limit test could be achieved at 100 mg/L in order to demonstrate that the estimated LC50 was greater than this concentration, the maximum dose tested was this threshold dose with and without SR-NOM in a concentration of 10 mg/L. Animals were evaluated concerning abnormalities (e.g. loss of equilibrium, swimming behavior, respiratory function, pigmentation, etc.) after 24, 48, 72 and 96 hours.

There were no incidents during the test or abnormal behavior of fish. The suspension of TiO_2 NPs (JRCNM01001a) had milky appearance after sonication, while ZnO NPs (JRCNM01101a) remained partly in clusters on the surface (Figure 50). Neither in the groups exposed only to TiO_2 and ZnO NPs nor in the groups exposed to TiO_2 and ZnO NPs plus NOM, there were no deaths at the maximum dose tested and sublethal effects were not observed. Therefore, it was not possible to calculate the LC50 limit at the dose tested of 100 mg/L, and the estimated LC50 was greater than this concentration. Also, it was also not possible to calculate the LC10 due to lack of deaths among fish exposed to MNMs.

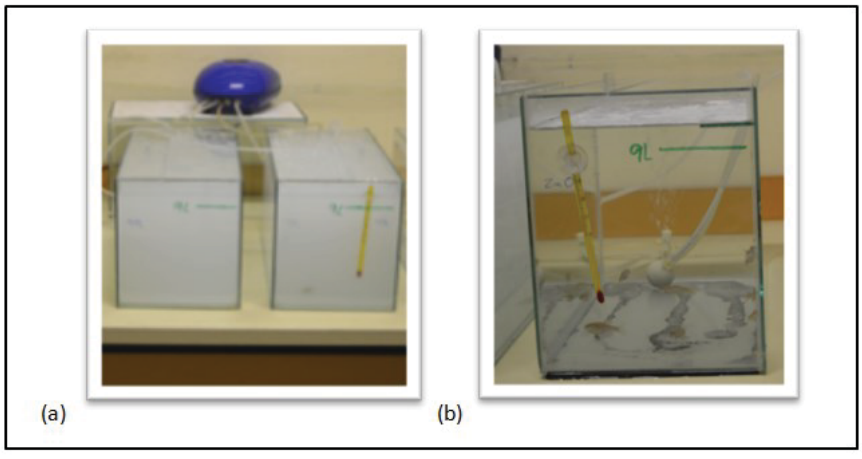

Figure 50. Zebrafish exposed to (a) TiO_2 NPs and (b) ZnO NPs after sonication without NOM.

UOS

The results for acute toxicity tests performed by UOS with *D. rerio* are summarized in Figures 51 to 54, which represent the data obtained using the Ecotoxicity test Data entry templates sent to Joint Research Centre-JRC in order to support Task 1.5.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v10) - Enhanced Procedure	151.16±35.95	0.2402±0.05			0.01
											1
											100
1	JRCNM02000a		Powder	JRC-IHCP	SiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v11) - Enhanced Procedure	283.70±79.125	0.185±0.051			10
											32
											100

Figure 51. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *D. rerio* by UOS, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test											
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K		558.066±166.66	0.355±0.099	7.99±2.22					7.81	25	7.47
		206.166±57.498	0.144±0.0401	10.33±2.88					7.74	25	7.58
		150.166±41.882	0.141±0.039	11.13±3.10					7.81	25	7.44
JRCNM02000a		838.76±238.26	0.505±0.143	11.17±3.11					7.65	25	7.81
		750.80±218.334	0.454±0.131	9.77±3.11					7.88	25	7.84
		515.467±146.37	0.323±0.09	9.77±3.26					7.8	25	7.81

Figure 52. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *D. rerio* by UOS, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

NM ID code	End of the ecotoxicity test											
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]
NM300K		3618.8333±1080.221	1.785±0.4999	12.45±3.71					8.31	25	6.88
		2007.433±560.537	0.867±0.242	7.58±2.26					8.72	25	7.53
		216.766±60.483	0.15±0.041	6.35±1.77					8.65	25	7.04
JRCNM02000a		1310.73±367.134	0.758±0.212	9.69±2.70					7.81	25	7.24
		1116.2±311.79	1447.27±403.94	-20.26±5.82					7.98	25	7.23
		1447.27±403.94	0.805±0.224	9.69±2.70					7.83	25	7.39

Figure 53. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *D. rerio* by UOS, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
NM300K	mortality	nanoreg sop-Fish V1	2560	i) SOP-Toxicity Test with fish (with dispersant)	20160120-24	UOS	96h	LC50 0.588 0.162 < LC50 < 2.143
JRCNM02000a	mortality	nanoreg sop-Fish V2	2560	i) SOP-Toxicity Test with fish (with NOM)		UOS	96h	Low toxicity (up to 100 mg/L)

Figure 54. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *D. rerio* by UOS, compiled according to the *D. rerio - Ecotoxicity test data entry template*.

2.4.1.6 *Chironomus riparius* Acute Toxicity Tests

UOS

Acute toxicity test was conducted on *C. riparius* following a modified OECD TG235 [17] and the *Daphnia magna* SOP.

Ag NPs (JRCNM03000a) were more stable than other materials, i.e. ZnO NPs (JRCNM01101a), TiO_2 NPs (JRCNM01001a and JRCNM01003a) and SiO_2 NPs (JRCNM02000a) in test media. ZnO NPs were exposed to *C. riparius* in the presence of 10 mg/L of SR-NOM. TiO_2 NPs (JRCNM01003a) were dispersed without NOM. TiO_2 NPs (JRCNM01001a) were dispersed in the presence of NOM and were stable at the start of test, though they aggregated at the end. SiO_2 NPs (JRCNM02000a) were dispersed without NOM and were stable at the highest concentration (100 mg/L) until the end of the test. The results for acute immobilization tests performed by UOS with *C. riparius* are summarized in Figures 55 to 58.

						Batch dispersion					
Replicate number	NM ID code	Vial number	Material State	NM supplier (from NANoREG)	Sample Name	Dispersion protocol (A)	Hydrodynamic size [nm]	PDI	¿Out of range?	Microscopy verification (B)	Nominal concentration [mg/L] (low-intermediate-high)
1	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	156.1±1.17	0.262±0.005			1
											10
											20
2	NM300K		Dispersion (Tween 20/PEG)	Fraunhofer	Ag	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	172.1±7.73	0.229±0			0.3
											3
											30
1	JRCNM01001a		Powder	JRC-IHCP	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	390.1±2.88	0.238±0.01			0
											100
2	JRCNM01001a		Powder	JRC-IHCP	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	435.7±9.2	0.243±0.013			1
											10
											100
1	NM103		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	186.1±2.02	0.233±0			0
											100
2	NM103		Powder	Fraunhofer	TiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	137.9±2.78	0.198			1
											10
											100
1	JRCNM02000a		Powder	JRC-IHCP	SiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	266.6±27.55	0.232±0			0
											100
2	JRCNM02000a		Powder	JRC-IHCP	SiO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	268.1±21.63	0.195±0.014			1
											10
											100
1	NM110		Powder	Fraunhofer	ZnO	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	248.2±3.7	0.166±0.01			25
											50
											100
2	NM110		Powder	Fraunhofer	ZnO	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	211.1±2.75	0.112±0.01			1
											10
											100
1	JRCNM02102a		Powder	JRC-IHCP	CeO2	ii) 20150429 NANoREG-Ecotox Dispersion SOP (v6) - Enhanced Procedure	169.4±0.97	0.231±0.003			0
											100

Figure 55. Sample information and batch dispersion characterization of the NANoREG core MNMs used in the tests conducted with *C. riparius* by UOS, compiled according to the *C. riparius - Ecotoxicity test data entry template*.

	Start of the ecotoxicity test												
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium							
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]	Hardness (mg/L)
NM300K	Supernatant	142.27±2.65	0.191±0.025						8.06	20	7.21	
	Supernatant	138.43±2.80	0.154±0.027						8.06	20	7.12	
	Supernatant	194.53±1.74	0.21±0.012						8.1	20	7.21	
NM300K	Supernatant	684.1±8.63	0.417±0.01	-21.02±0.36					7.72	20	7.09	73
	Supernatant	292.2±4.8	0.19433333	-18.97±0.72					7.81	20	7.26	71
	Supernatant	207.47±1.62	0.206±0.01	-15.75±1.21					8.02	20	7.32	64
JRCNM01001a	Supernatant								7.98	20	7.39	68
	Supernatant											
	Supernatant	1093.5±40.34	0.145±0.01						7.87	20	7.25	63
JRCNM01001a	Supernatant	1259.5±40.4	0.697±0.22	-19.4±0.73					7.71	20	8.83	
	Supernatant	490.8±6.45	0.283±0.005	-24.74±0.41					7.85	20	9.25	
	Supernatant	412.37±3.43	0.266±0.007						7.62	20	8.69	
NM103	Supernatant								7.88	20	7.42	
	Supernatant											
	Supernatant	2694.6±243.92	0.689±0.12									
NM103	Supernatant	6929.10±405.83	2.79±0.14	-201.23±205.68					7.88	20	7.67	
	Supernatant	3708.23±173.53	1.21±0.07	1.40±17.75					8.03	20	8.07	
	Supernatant	2256.33±69.44	0.23±0.08	-216.44±254.26					7.81	20	8.25	
JRCNM02000a	Supernatant								7.98	20	7.39	68
	Supernatant											
	Supernatant	475±11.64	0.303±0.01						8	20	7.4	50
JRCNM02000a	Supernatant	2448.8±222.3	1.336±0.110	-224.01±211.83					8.04	20	7.35	64
	Supernatant	1046.3±104.1	0.619±0.06	-21.5±0.30					8.03	20	7.42	55
	Supernatant	658.9±25.0	0.412±0.014	-21.73±0.52					7.84	20	7.52	54
NM110	Supernatant	453.4±9.19	0.206±0.02						8.06	20	7.21	
	Supernatant	492.7±0.88	0.245±0.01						8.06	20	7.12	
	Supernatant	655.8±10.24	0.227±0.02						8.1	20	7.21	
NM110	Supernatant	1011.5±67.43	0.605±0.03	-30.65±4.06					7.63	20	7.42	74
	Supernatant	201.4±1.94	0.133±0	-29.04±0.11					7.87	20	7.46	77
	Supernatant	193.3±0.84	0.110±0.01	-29.47±1.07					7.85	20	7.85	98
JRCNM02102a	Supernatant								7.98	20	7.39	68
	Supernatant											
	Supernatant								7.94	20	7.44	64

Figure 56. Quality experimental parameters of the NANoREG core MNMs at the start of the tests conducted with *C. riparius* by UOS, compiled according to the *C. riparius - Ecotoxicity test data entry template*.

	End of the ecotoxicity test											
		Stability of MNMs in exposure medium				Quantification of MNMs in exposure medium						
NM ID code	Exposure medium analyzed (C)	Hydrodynamic size [nm]	PDI	Zeta Potential [mV]	Microscopy verification (B)	UV/Vis Wavelength [nm]	UV/Vis Total concentration [mg/L]	ICP Total concentration [mg/L]	ph	T [°C]	O_2 concentration [mg/L]	Hardness (mg/L)
NM300K	Supernatant	162.367±24.450	0.154±0.058						8.06	20	7.21	
	Supernatant	137.1±1.552	0.158±0.011						8.06	20	7.12	
	Supernatant	181.833±1.966	0.185±0.010						8.1	20	7.21	
NM300K	Supernatant	444±28.8	0.269±0.02	-21.02±0.36					7.81	20	5.92	84
	Supernatant	271.6±11.9	0.176±0.01	-18.97±0.72					7.79	20	6.09	78
	Supernatant	204.63±2.66	0.201	-15.75±1.21					7.75	20	6.25	86
JRCNM01001a	Supernatant								6.7	20	6.05	75
	Supernatant											
	Supernatant	4673±701.62	2.003±0.27						7.93	20	6.49	70
JRCNM01001a	Supernatant	2579.93±191.84	1.256±0.10						7.47	20	5.86	
	Supernatant	1159.5±95.18	0.643±0.049						7.52	20	5.78	
	Supernatant	2167.97±150.82	0.817±0.06						7.69	20	6.92	
NM103	Supernatant								7.6	20	7.32	
	Supernatant											
	Supernatant	6175.87±483.8	2.1±0.14									
NM103	Supernatant	3823.4±502.81	1.62±0.21	-18.05±0.91					7.78	20	6.64	
	Supernatant	4911.03±424.38	1.88±0.16	-18.98±0.35					7.97	20	6.66	
	Supernatant	6288.33±742.87	2.52±0.26	-20.81±0.16					8.02	20	6.7	
JRCNM02000a	Supernatant								6.7	20	6.05	75
	Supernatant											
	Supernatant	318.5±21.58	0.211±0.01						7.77	20	5.14	73
JRCNM02000a	Supernatant	1448.267±97.54	0.773±0.046	-19.11±1.11					7.61	20	5.75	73
	Supernatant	664.73±42.0	0.401±0.027	-2.44±3.29					7.72	20	5.9	72
	Supernatant	406.2±14.42	0.264±0.007	-17.47±0.87					7.82	20	6.06	72
NM110	Supernatant	1570.2±92.9	0.81±0.03						6.99	20	6.66	
	Supernatant	1882±95.56	0.959±0.04						7.19	20	7.09	
	Supernatant	2154.4±149.47	1.031±0.08						7.2	20	6.64	
NM110	Supernatant	1231.2±42.01	0.692±0.02	-18.27±2.52					7.65	20	6.32	80
	Supernatant	268.3±9.5	0.18	-21.62±1.13					7.72	20	6.3	86
	Supernatant	1467.67±41.37	0.617±0.01	-17.40±0.43					7.71	20	6.35	89
JRCNM02102a	Supernatant											
	Supernatant											
	Supernatant								6.7	20	6.05	75

Figure 57. Quality experimental parameters of the NANoREG core MNMs at the end of the tests conducted with *C. riparius* by UOS, compiled according to the *C. riparius - Ecotoxicity test data entry template*.

NM ID code	**Endpoint**	**Assay name**	Initial concentration [mg/L]	Protocol REF (E)	Date of acquisition	Operator	Exposure time [hours] (D)	Parameter value **Effective concentrations [mg/L] (95% CL)**
NM300K	mortality	modified OECD TG 235	2560	i) SOP-Toxicity Test with chironomid (with dispersant)	2015.09.11-09.13	UOS	48	7.44 2.946<LC50<24.7
NM300K	mortality	dispersion-nanoreg sop Daphnia magna V3/acute test-modified OECD TG235	2560	i) SOP-Toxicity Test with chironomid (with dispersant)	2016.01.19-01.23	UOS	48	5.42 (2.169<LC50 <19.718)
JRCNM01001a	mortality	modified OECD TG 235	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2015.11.04-11.06, 2016.01.27	UOS	48	No toxicity (up to 100 mg/L)
JRCNM01001a	mortality	dispersion-nanoreg sop Daphnia magna V3/acute test-modified	2560	i) SOP-Toxicity Test with chironomid (with NOM)	2016.04.07-4.9	UOS	48	No toxicity (up to 100 mg/L)
NM103	mortality	modified OECD TG 235	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2015.11.04-11.06	UOS	48	No toxicity (up to 100 mg/L)
NM103	mortality	dispersion-nanoreg sop Daphnia magna V3/acute test-modified	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2016.02.11-02.13	UOS	48	No toxicity (up to 100 mg/L)
JRCNM02000a	mortality	modified OECD TG 235	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2015.10.12-10.14, 2016.01.27	UOS	48	No toxicity (up to 100 mg/L)
JRCNM02000a	mortality	dispersion-nanoreg sop Daphnia magna V3/acute test-modified	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2016.02.29-3.2	UOS	48	No toxicity (up to 100 mg/L)
NM110	mortality	modified OECD TG 235	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2016.01.07, 01.14	UOS	48	LC10=12.436, LC50=163.31 (95% Confidence Limits could not be calculated)
NM110	mortality	dispersion-nanoreg sop Daphnia magna V3/acute test-modified OECD TG235	2560	i) SOP-Toxicity Test with chironomid (with NOM)	2016.01.25-01.27	UOS	48	Low toxicity (100 mg/L exposure - 10% effect)
JRCNM02102a	mortality	modified OECD TG 235	2560	ii) SOP-Toxicity Test with chironomid (without NOM)	2015.11.6-11.08	UOS	48	No toxicity (up to 100 mg/L)

Figure 58. Endpoints and effective concentrations obtained in the tests conducted with the NANoREG core MNMs and *C. riparius* by UOS, compiled according to the *C. riparius - Ecotoxicity test data entry template*.

2.4.2 Biokinetic studies in aquatic systems

NMBU (*Salmo trutta* tests)

In the water exposure the measured concentration of Ag in the different exposures were found to be 1.6±0.18, 4.4±0.30, and 7.6±1.89 µg/L for the three Ag NP exposure groups, which were 80, 88 and 76 % of nominal concentration, respectively. For the Ag^+ exposure groups the measured concentrations were

2.0±0.42, 4.8±0.40, and 9.6±0.37 µg/L, thus >95 % of nominal concentrations for all three exposure groups. The discrepancy between nominal and measured concentrations could be a result of sorption of Ag to the walls of the exposure container. However, the most likely explanation is aggregation and sedimentation out of the water column, at least for the NPs. The silver concentrations of the two contaminated fodders were 17±1.0 and 12±0.9 µg/g for the Ag^+ and Ag NP contaminated fodders, respectively.

Uptake of Ag into liver, gill and kidney of both the dietary and the waterborne exposed fish was analyzed. For all organs and exposure routes the uptake was greater for Ag+ ions than for Ag NPs. For the waterborne exposure to Ag^+ a clear dose-response was observed in all three sampled organs, gill, liver and kidney (Figure 59). In the waterborne exposure, the small amounts of Ag detected in organs were not significantly different from controls, suggesting that the Ag NPs were less bioavailable than Ag+ ions. Dietary exposure to both Ag+ and Ag NPs resulted in accumulation in both liver and kidney, but no significant accumulation of Ag on gills, regardless of source (Ag^+ and NPs). Liver showed slightly higher accumulation of Ag from ions compared to Ag NPs, but the transfer to kidneys was similar. Given the high concentrations of Ag NPs in sediments and organic material as compared to water, the dietary exposure could be an important exposure route in the environment.

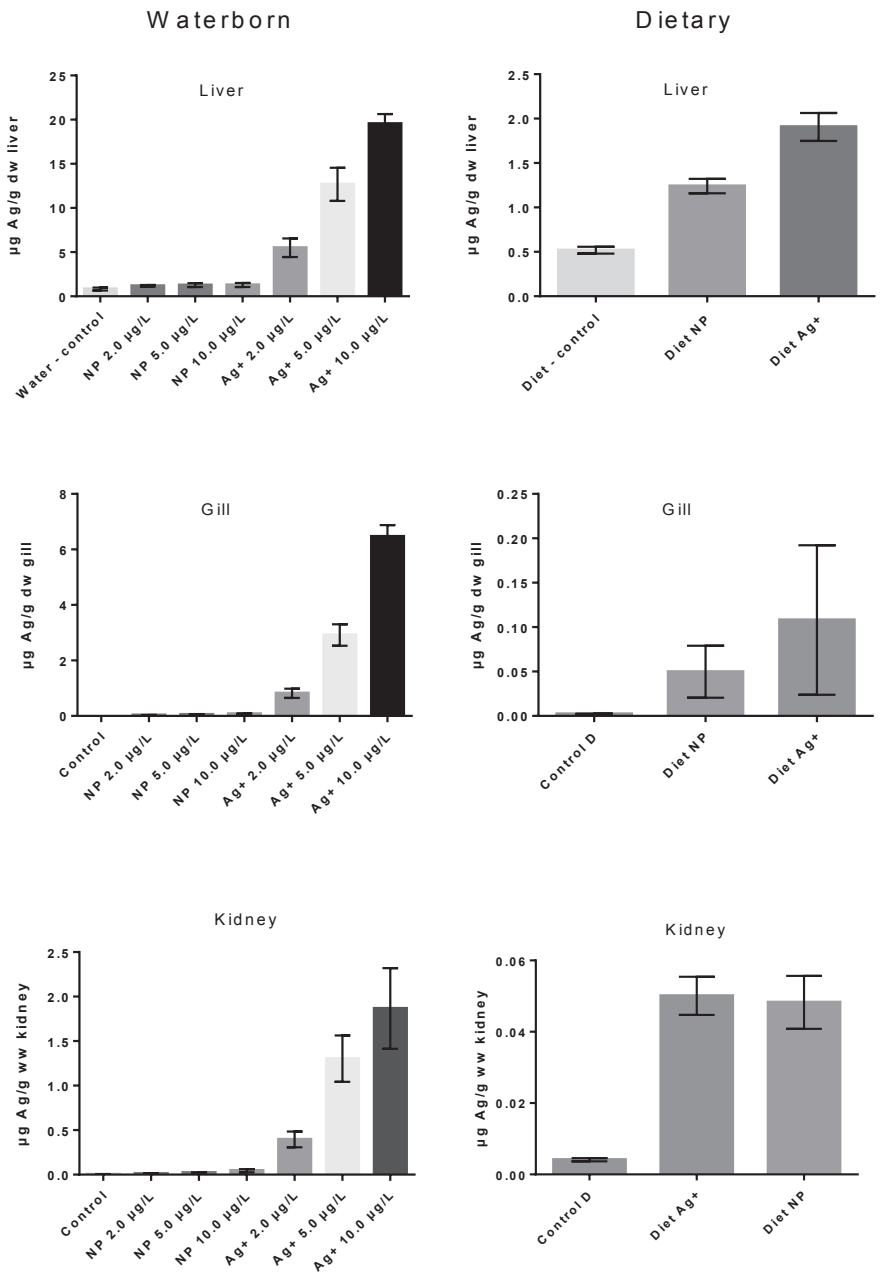

Figure 59. Accumulation in liver, gill and kidney in *Salmo trutta* after waterborne and dietary exposure to silver ions (Ag^+, added as $AgNO_3$) and Ag NPs nanoparticles (JRCNM03000a). Be aware of the different scales on the y axis, and that the accumulation in kidney is given as µg Ag/g wet weight kidney, and not µg Ag/g dry weight organ like for gill and liver.

BfR (nanoparticle uptake by *Daphnia magna*)

Daphnia magna were exposed to 5 different concentrations of cerium dioxide (JRCNM02102) and silver (JRCNM03000a) nanoparticles. The test concentrations where established according to the EC50 values for both materials. While for cerium dioxide concentrations of 100, 32, 10, 3.2 and 1 mg/L were tested, silver was amended to 3.25, 1, 0.32, 0.1 and 0.032 mg/L. The exposure and the subsequent wash of the daphnids was performed at IK4-Tekniker in Eibar/Spain. Samples were shipped to BfR in Berlin/Germany on dry ice. Upon arrival, the samples were digested in separate vessels, each with an mixture of 2.5 mL H_2O, 2 mL HNO_3 (69 %) and 1 mL H_2O_2 (30 %).

The gathered solutions were diluted further with ultrapure water including the addition of two internal standards. For determination of cerium, indium (^{115}In) and lutetium (^{175}Lu) were selected as internal standards, for determination of silver, rhodium (^{103}Rh) was employed alongside with lutetium (^{175}Lu). Cerium

was quantified based in the isotopes ^{140}Ce and ^{142}Ce, silver by analyzing ^{107}Ag and ^{109}Ag. Quantification was carried out with a quadrupole Thermo Fisher X Series II instrument.

While cerium dioxide at concentrations of 100, 32 and 10 mg/L was internalized by the animals at almost the same percentage of 0.92 to 0.95, the uptake was clearly reduced at the lowest concentrations of 3.2 and 1 mg CeO_2 per mL with 0.35 to 0.37 respectively 0.13 to 0.14 % (Figure 60). The observed differences could be due to the occurance of larger agglomerates in the culture medium at higher CeO_2 concentrations. These might be more efficiently retained by the animals in comparison to smaller particles that were expected at low concentrations. In the case of silver nanoparticles, the maximum concentration measured was lower than 0.006 µg/mL, a value close to the detection limit of the applied method. For this reason, no conclusions could be drawn on the uptake of silver nanoparticles by *Daphnia magna.* As a reason for the low recovery of silver, a precipitation of the substance in the form of insoluble $AgCl_2$ was considered.

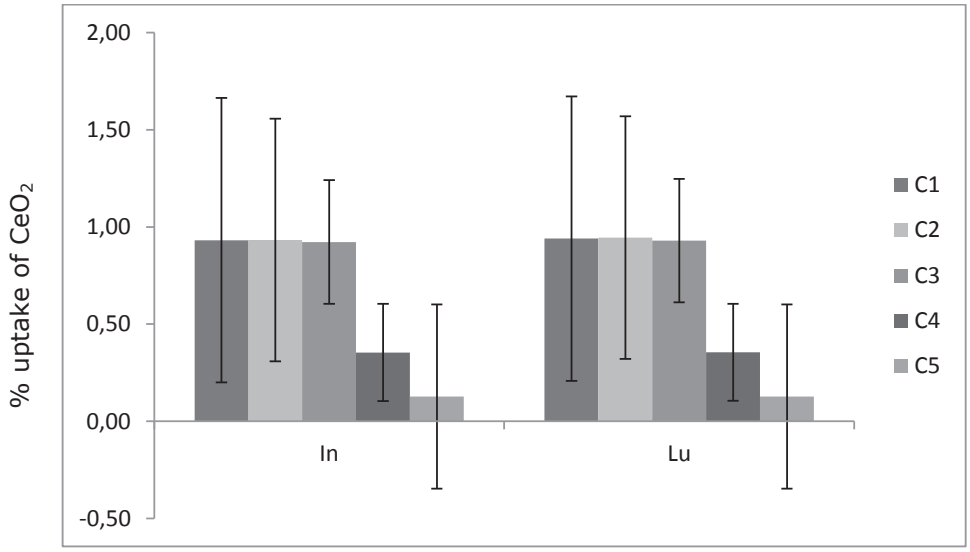

Figure 60. Percentual uptake of cerium dioxide nanoparticles from the culturing medium by *Daphnia magna*: displayed from the highest to the lowest concentration (C1: 100 mg/L; C2: 32 mg/L; C3: 10 mg/L; C4: 3,2 mg/L; C5: 1 mg/L). Uptake rates were calculated with indium (left) and luthetium (right) as internal standards.

2.5 Evaluation and conclusions

2.5.1 Toxicity studies in aquatic systems

2.5.1.1 Validation of the dispersion protocol for MNMs in environmental exposure media.

The study conducted to benchmark the NANoREG-ECOTOX Standard Dispersion SOP showed the feasibility of generating comparable and reproducible MNM dispersions in different laboratories. For those MNMs which exhibit a spherical or near-spherical shape, the NANoREG-ECOTOX Standard Dispersion SOP appears to be both reproducible within the same laboratory and also across different laboratories. In general, standard deviations for individual data sets and for combined data sets were low. In contrast, DLS analysis of high aspect ratio MNMs (HARNs) such as nanosilver rods/fibres (JRCNM03002a) and carbon nanotubes (JRCNM04000a, JRCNM04001a and JRCNM04100a) proved to be more problematic. Such limitations in the applicability of DLS for quantifying HARN dispersions are well-known, and they led to larger standard deviations within the data sets generated by each partner compared to non-HARN MNMs. However, there was a general comparability across all laboratories for most of the HARN materials included in the benchmarking activity. Furthermore, it was inferred from the available data for spherical/near-spherical MNMs that HARN dispersions could be reproducibly prepared using the NANoREG-ECOTOX Standard Dispersion SOP. Alternative quantification techniques, such as optical microscopy, may afford more accurate quantification methods of HARN dispersions.

This dispersion benchmarking study represented an important step towards the standardization of MNM dispersion preparation for environmental and human health hazard and exposure studies. The DLS analysis method for quantification of the MNM dispersions exhibited both suitability and limitations for MNM dispersion characterization and quantification depending on the type of MNM.

2.5.1.2 *Daphnia magna* Acute Immobilization Tests

72h-pretest studies using neonates (<24h) and 4-day old individuals for specific NANoREG core MNMs

Taking into account the results obtained, the final tests were conducted with neonates and extended exposure periods of 72h, reporting both the EC50 at 48h and at 72h. Concerning the use of SR-NOM, each partner decided whether using NOM or not depending on the stability of the MNMs in the exposure media.

GNano/IFSC-USP: Both ages (24 h old and 4 days old *D. magna* individuals) were adequate to perform assays with TiO_2 NPs, since we do not observe mechanical issues when they were exposed to this MNM. As expected, 24 h old *D. magna* individuals were more sensitive to TiO_2 NPs than 4 day old ones, during a time period of exposure of 48 and 72 h.

SINTEF: The percentage survival of neonate and 3-day old *D. magna* following 72 h exposure to concentrations of JRCNM01000a in the concentration range (0.1-100 mg/L) are summarized in Figures 61a and 61b. No valid calculation of EC50 values was possible for either the neonates (Figure 61a) or the 3-day old *D. magna* (Figure 61b). The percentage survival of neonate and 3-day old *D. magna* following 24 h exposure to concentrations of $K_2Cr_2O_7$ in the concentration range (0.32 to 3.2 mg/L) are summarized in Figures 62a and 62b). EC50 values could be determined for both neonates and 3-day old *D. magna* in this validation test. The EC50 value for the neonates was found to be in the range acceptable, whilst that for the 3-day olds was slightly higher. This is to be expected as the target range for the standard test has been established using neonates. The results show that the neonate *D. magna* were adequate when prolonging the standard 48 h assay to 72 h. However, 3-day old *D. magna* cannot be used in 72 h assays. Neonate *D. magna* were impacted more significantly by lower exposure concentrations, while in adult D. magna a dose-response was observed after 48 h exposure. Visual observations showed that D. magna were covered in, and internalized, JRCNM01000a TiO_2 NPs. Furthermore, they appeared "heavier" at high TiO_2 exposure concentrations. No direct mechanical issues, such as trapping, were observed. No significant difference was found between 3-day old *D. magna* fed on dried algae and those fed on live algae. After 72 h, significant immobilization was observed in both study groups indicating that the type of food source used for feeding the culture prior to conducting the assays did not improve the ability of the 3-day old organisms to survive for 72 h.

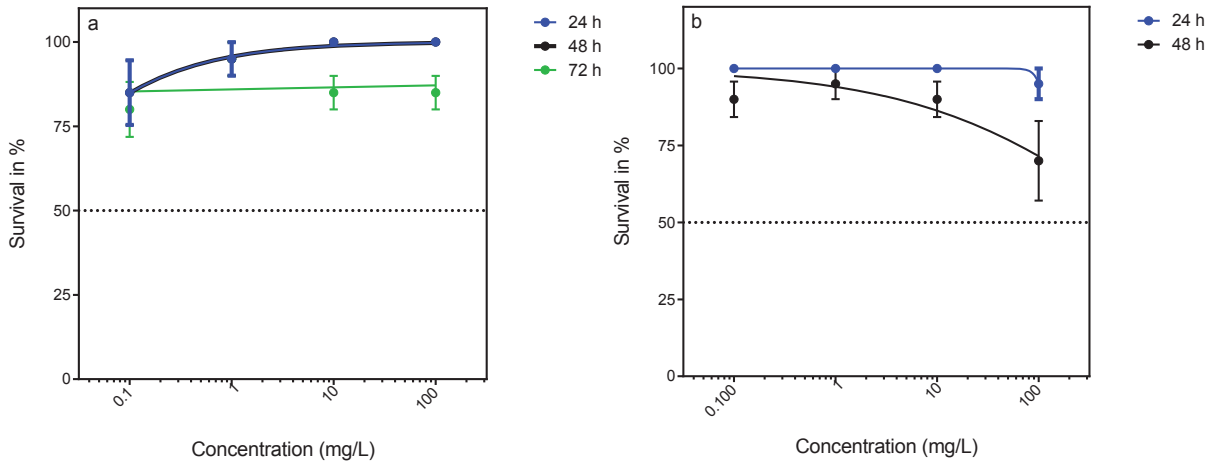

Figure 61. Percent survival of neonate (a) and 3-day old (b) *D. magna* after JRCNM01000a concentration.

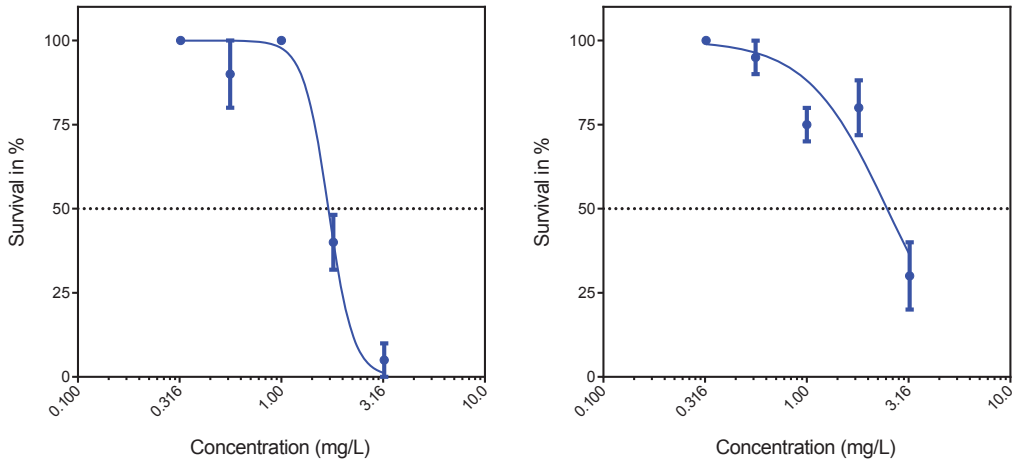

Figure 62. Percent survival of neonate (a) and 3-day old (b) *D. magna* after 24 h exposure to the reference substance $K_2Cr_2O_7$.

Definitive studies - *Daphnia magna* Acute Immobilization Tests

Ag NPs (JRCNM03000a): GNano/IFSC-USP has reported that Ag NPs produced an EC50 of 0.76 mg/L for *D. magna*. IK4-Tekniker has reported lower 72h-EC50 values than GNano/IFSC-USP for daphnids in the case Ag NPs (JRCNM03000a). Nevertheless, it was assumed that GNano/IFSC-USP nominal concentrations were based on the whole NM dispersion 10.16 % Ag nanoparticles + dispersants (~90%). If this was the case, the effective concentration causing 50% immobilization would be in the same order of magnitude (0.028 mg/L for IK4-Tekniker and ~0.076 mg/L for GNano/IFSC-USP). The differences observed between them might be explained considering the variability in the stability of the dispersions prepared. For instance, the hydrodynamic sizes obtained by both partners, specially the high polydispersity indexes reported by GNano/IFSC-USP due to their equipment properties, pose a relevant aspect. The EC50 values reported by UOS were remarkably similar to the obtained by IK4-Tekniker, both for the 48-h and 72-h exposures, ranging from 0.019 to 0.032 mg/L.

ZnO NPs (JRCNM01100a): GNano/IFSC-USP has reported that ZnO NPs were slightly less toxic than Ag NPs for daphnids, although the effective concentrations were approximately in the same range (EC50 = 1.44 mg/L).

SiO_2 NPs (JRCNM02000a): IK4-Tekniker reported an EC50 = 2.05 mg/L, which was consistent with the EC50 value of 28.28 mg/L obtained by UOS in the presence of SR-NOM.

CeO_2 NPs (JRCNM02102a): IK4-Tekniker reported an EC50 = 33.84 mg/L.

MWCNTs: The results reported by SINTEF showed some adverse effect. In the case of 48-h exposure, they can be ordered as follows considering decreasing toxicity: JRCNM04100a (EC50 = 94.7 mg/L), JRCNM04000a (EC50 = 108.8 mg/L) and JRCNM04001a (EC50 > 100 mg/L). The organisms exposed to the MWCNTs during 72 h showed higher effects and JRCNM04100a produced an EC50 of 4.40 mg/L; the rest of tests were not valid since mortality in controls exceeded the accepted limits. Nonetheless, IK4-Tekniker reported an EC50 = 47.87 mg/L for JRCNM04001a for a valid 72-h test.

TiO_2 NPs: GNano/IFSC-USP has reported an EC50 values of 131.14 mg/L for TiO_2 NPs (JRCNM01000a) on *D. magna*. Similarly to GNano/IFSC-USP, SINTEF obtained low toxicity levels for TiO_2 NPs, with JRCNM01000a, JRCNM01001a and JRCNM01003a showing EC50 values higher than 100 mg/L. In the case of 72-h exposure, JRCNM01001a and JRCNM01003a MNMs produced EC50 in the range of 3-4 mg/L, but mortality in controls exceeded the accepted limits in those tests, thus those results were not valid. Similarly, UOS obtained EC50 values higher than 100 mg/L for TiO_2 NPs, also in the case of the tests conducted using SR-NOM during 72 h with the MNMs JRCNM01001a and JRCNM01003a.

Considering all the ecotoxicity data generated for *D. magna*, even taking into account the differences on the stability parameters obtained by the partners during the exposures, we can classify the MNMs analyzed in order of decreasing toxicity as follows: Ag NPs > ZnO NPs > SiO_2 NPs > CeO_2 NPs > MWCNTs >TiO_2 NPs.

These results are relevant to the knowledge of MNMs effects on aquatic organisms, and crucial to evaluate the concentrations that could be considered safe during eventual environmental contaminations.

Despite the implementation of a common, benchmarked dispersion preparation method and a standard method for conducting the *D. magna* acute toxicity studies, the results highlight the difficulty in achieving reproducible ecotoxicity data across different laboratories.

2.5.1.3 *Pseudokirchneriella subcapitata* Growth Inhibition Tests

Ag NPs (JRCNM03000a): The test conducted by GNano/IFSC-USP resulted in EC50 = 0.25 mg/L. In addition to toxicity, Ag NPs exhibited some issues concerning the reproducibility of tests when algae were exposed to them. Ag losses might have occurred due to the nanomaterial adsorption either to the exposure containers or to microalgae cells. In agreement with Sorensen and Baun [15], GNano/IFSC-USP recommended to consider Ag NP transformation processes in test solutions in nanoecotoxicity testing, aiming to overcome issues in adsorption to the test containers, time-dependent interactions and decreased reproducibility of tests. IK4-Tekniker reported lower 72h-EC50 values than GNano/IFSC-USP for algae in the case Ag NPs (JRCNM03000a). Nevertheless, it was assumed that GNano/IFSC-USP nominal concentrations were based on the whole NM dispersion 10.16 % Ag nanoparticles + dispersants (~90%). If this was the case, the effective concentration causing 50% immobilization would be quite similar (0.021 mg/L for IK4-Tekniker and ~0.025 mg/L for GNano/IFSC-USP). Although these values were in the same order of magnitude, the differences observed between them might be explained considering the variability in the stability of the dispersions prepared. For instance, the hydrodynamic sizes obtained by both partners, specially the high polydispersity indexes reported by GNano/IFSC-USP due to their equipment characteristics, pose a relevant aspect. NMBU obtained an EC50 of approximately 0.008 mg/L (concentrations of Ag) for Ag NPs (JRCNM03000a), even lower that the reported by the rest of the partners. For the Ag nanorods (JRCNM03002a), EC values could not be obtained due to the NM instability in the media and drastic changes over time, which also affected the concentrations. There was considerable sorption to algae, aggregation and removal from the media, which underlined the importance of exposure characterization and robust SOPs in toxicological studies. The resuls obtained by SINTEF are in the same line as the rest of partners, since the EC50 values reported were 0.01 mg/L for the two assays performed.

ZnO NPs (JRCNM01100a): GNano/IFSC-USP results showed that ZnO NPs were slightly less toxic than Ag NPs for algae (as occurred in the case of daphnids), since the effective concentration producing 50% inhibition in growth was 0.07 mg/L.

CeO_2 NPs (JRCNM02102a): The tests conducted by IK4-Tekniker resulted in an effective concentration causing 50% inhibition of 1.24 mg/L. Increasing SR-NOM concentrations in the algal medium (8 and 20 mg/L) alleviated partially their adverse effects on *P. subcapitata* growth (EC50 = 31.9 mg/L after adding 8 mg/L SR-NOM; EC50 = 74.3 mg/L after adding 20 mg/L SR-NOM).

TiO_2 NPs: GNano/IFSC-USP obtained an EC50 value of 50.57 mg/L for TiO_2 NPs (JRCNM01001a) on *P. subcapitata*. The tests conducted by IK4-Tekniker produced an EC50 = 0.27 mg/L for MNM JRCNM01003a. Increasing SR-NOM concentrations in the algal medium (8 and 20 mg/L) alleviated completely their adverse effects on *P. subcapitata* growth, with no inhibition observed. More details regarding the 'camouflage' of the adverse effects of TiO_2 (and CeO_2 NPs as well) on algae in the presence of SR-NOM are provided by Cerrillo et al. [16]. The results obtained by SINTEF for JRCNM01001a were quite similar to those reported by GNano/IFSC-USP, with an EC50 = 43.09 mg/L. They reported the highest inhibitions for JRCNM01003a, in the same line as the results reported by IK4-Tekniker, although in this case the adverse effect observed was lower (EC50 = 11.34-18.77 mg/L).

CNTs: Upon close examination of the CNT data determined for NM400, NM401 and NM411 using *P. subcapitata*, SINTEF highlighted a significant limitation with the adopted/proposed ecotoxicity test methodology. The approach used to remove the nanomaterials from dispersion and to release the chlorophyll in the algal cells for measurement and quantification led to the chlorophyll molecules being exposed to the test nanomaterials. In the case of the highly adsorbent CNTs, over 90% of the released chlorophyll was observed to bind directly to the CNTs, efficiently removing the chlorophyll for the dissolved aqueous phase. As a result, all studies with CNTs indicated significant reduction in algal growth and therefore high toxicity, but it was an artefact of the chlorophyll adsorption to the CNTs. It should also be considered that any other nanomaterial which has the capacity to adsorb chlorophyll (to a small or large extent) would also create false positives for algal toxicity. It is therefore recommended that an alternative method is developed for the accurate quantification of chlorophyll, but this was beyond the scope of the current project.

Despite the variability observed in the quality of the dispersions prepared by the partners, the ecotoxicity data generated for *P. subcapitata* showed different adverse effect levels for the MNMs analyzed. They can

be classified in order of decreasing toxicity as follows: Ag NPs > ZnO NPs > CeO_2 NPs ≈ TiO_2 NPs. It is worth mentioning that SR-NOM alleviated the adverse effects of MNMs on *P. subcapitata* growth, completely in the case of TiO_2 NPs and partially in the case of CeO_2 NPs. Previous studies have evidenced this behavior for other algal species and types of NOM. In addition, this type of natural organic matter increased significantly the stability of these NPs in the exposure media and also the reproducibility of the toxicity test results [16]. These results are relevant to the knowledge of MNMs effects on aquatic organisms, and crucial to evaluate the concentrations that could be considered safe during eventual environmental contaminations.

Despite the implementation of a common, benchmarked dispersion preparation method and a standard method for conducting the *P. subcapitata* acute toxicity studies, the results highlight the difficulty in achieving reproducible ecotoxicity data across different laboratories.

2.5.1.4 Toxic effects on growth, fertility and reproduction of *Caenorhabditis elegans*

ZnO NPs (JRCNM01100a): The tests performed at low concentrations led to more adverse effects. This could possibly be explained by high concentrations leading to increased aggregation and subsequent sedimentation, thus in reality lower effective exposure concentrations. Recovery was slightly affected at 100 mg/L and 0.001 to 0.032 mg/L, and completely eliminated at 0.1 mg/L. Considerable reduction in fertility and reproduction was observed between 0.032 and 100 mg/L.

Ag NPs (JRCNM03000a): It was assumed that UFMG's nominal concentrations were based on the whole NM dispersion 10.16 % Ag nanoparticles + dispersants (~90%). If this was the case, two concentrations could be directly compared UFMG's 1 and 10 mg/L with NMBU's 0.1 (1) and 1 (10) mg/L. UFMG test showed an adverse effect mainly on the reproduction endpoint, and NMBU reported a reduction in fertility, reproduction and growth. At the two highest exposure concentrations tested by NMBU, 2 (20) and 4 (40) mg/L, there was a significant reduction in growth (~50 %), and no observed fertility or reproduction. Adverse effects on reproduction and growth were also observed in the tests performed by UOS. Although the effective concentrations were variable in the different tests performed by the partners, it seems clear that Ag NPs affect especially the reproduction and growth of *C. elegans*.

SiO_2 NPs (JRCNM02003a): The silica nanomaterial JRCNM02000a caused a reduction in reproduction over a larger range of concentrations (0.1 to 56 mg/L), compared to JRCNM02003a (0.1 to 0.32 mg/L). The rest of endpoints analyzed (recovery, fertility and growth) were not affected by these MNMs.

TiO_2 NPs (JRCNM01001a): The most sensitive endpoint was reproduction, especially at high concentrations, both in the tests performed by NMBU (17-50 mg/L) and Embrapa Environment (100 mg/L). In addition, NMBU reported a moderate reduction in fertility and slight in growth in the whole range of concentrations analyzed (0.2-50 mg/L), whilst Embrapa observed this behaviour only for the highest concentration (100 mg/L).

CeO_2 NPs (JRCNM02102a): Growth and fertility were affected at intermediate concentrations (5.5-17 mg/L), but the highest concentrations did not produce increased effects. This was probably caused by the higher aggregation and sedimentation of NPs at the highest concentrations.

MWCNTs (JRCNM04000a): *C. elegans* were not affected by any of the concentrations in any of the definitive tests. Although there seemed to be a reduction in reproduction at concentrations ≥ 10 mg/L, it might not be significant compared to the results obtained with the controls.

Ag nanorods (JRCNM03002a): Recovery, fertility, reproduction and growth were not affected by any of the concentrations tested.

Although the data show differences between nanomaterial and partner test, some general conclusions can be drawn. The highest toxicity was seen for Ag NPs, and Ag nanorods showed much lower effect. Media characterization indicated that this was due to the instability of the Ag nanorods, and rapid removal from the test media. Ag NPs were more stable, although they also showed a change in size distribution over time. TiO_2 NPs (JRCNM01001a) gave EC50s in the range of 16-43 mg/L without NOM. In the presence of NOM, both of the tested TiO_2 nanomaterials (JRCNM01001a and JRCNM01003a) gave EC50s of >50 mg/L. Characterization of stock solutions showed reasonable agreement between laboratories, and similar results with and without NOM, but after 72 h the solutions with NOM showed a significantly higher Z_{ave} and PDI. CeO_2, SiO_2 and MWCNTs showed EC50>50 mg/L. In some tests, ZnO NPs gave EC50<10 mg/L, but these were not reproducible. Many of the materials tested showed non-monotonic dose response, with increases in effects seen at low concentrations as compared to high concentrations, which might be explained by aggregation or removal at higher concentrations. Some MNMs (e.g. CeO_2) increased reproduction compared to controls at low concentrations. It is unclear whether this is a result of size distribution, loss of MNMs to

walls at low concentrations, or a defense response of the organism. Further work is necessary on media and NP characterization during exposure to improve understanding of the factors influencing MNM ecotoxicology.

Considering all the ecotoxicity data generated for *C. elegans*, even taking into account the differences on the stability parameters obtained by the partners during the exposures, we can classify the MNMs analyzed in order of decreasing toxicity as follows: Ag NPs > ZnO NPs > SiO_2 NPs > TiO_2 NPs > CeO_2 NPs > MWCNTs > Ag nanorods. As it was observed in the case of daphnids and microalgae, the nematode *C. elegans* showed more adverse effects when was exposed to Ag and ZnO NPs. TiO_2 NPs, which resulted in lower adverse effects than the rest of NPs studied specially in the case of *Daphnia magna*, showed higher toxicity towards *C. elegans*.

2.5.1.5 *Danio rerio* Acute Toxicity Tests

Ag NPs (JRCNM03000a): The tests performed by GNano/IFSC-USP produced EC50 values of 10 mg/L. Nonetheless, it was assumed that GNano/IFSC-USP nominal concentrations were based on the whole NM dispersion 10.16 % Ag nanoparticles + dispersants (~90%). If this was the case, the effective concentration causing 50% immobilization would be 1 mg/L. UOS obtained an EC50 of 0.59 mg/L, which was in the same range as the effective concentration reported by GNano/IFSC-USP.

TiO_2 NPs (JRCNM01001a): GNano/IFSC-USP reported that TiO_2 NPs (JRCNM01001a) were non-toxic for *D. rerio*, since the effective concentrations were above 100 mg/L. Similarly, Embrapa Environment observed neither lethal nor sublethal effects on fish for this MNM. During the exposures, which were conducted in the presence and absence of 10 mg/L SR-NOM, incidents or abnormal behaviour of fish at the maximum doses tested (100 mg/L) did not occur.

ZnO NPs (JRCNM01101a): The tests conducted by Embrapa Environment showed neither lethal nor sublethal effects on fish for these NPs. They did not observe incidents or abnormal behaviour of fish during the exposures at the maximum doses tested (100 mg/L), which were conducted in the presence and absence of 10 mg/L SR-NOM.

SiO NPs (JRCNM02000a): UOS reported low toxicity for these NPs on *D. rerio*, since the effective concentrations were above 100 mg/L. It is worthwhile mentioning here that in this case the exposures were conducted in the presence of SR-NOM.

Therefore, the only MNM that clearly produced an adverse effect was Ag NPs (JRCNM03000a), which on the other hand were those showing the highest toxicity levels for the rest of organisms studied.

2.5.1.6 *Chironomus riparius* Acute Toxicity Tests

Ag NPs (JRCNM03000a): The tests performed by UOS produced EC50 values of 5.42 and 7.44 mg/L.

ZnO NPs (JRCNM01100a): EC50 values could be calculated for the organisms exposed to ZnO NPs. The tests performed in the absence of SR-NOM resulted in an EC50 = 163.31 mg/L. However, the adverse effects were considerably reduced in the presence of SR-NOM, where only EC10 could be calculated (in the range of 100 mg/L).

TiO_2 NPs: MNM JRCNM01001a was non-toxic for *C. riparius*, since the effective concentrations were above 100 mg/L, both in the absence and in the presence of SR-NOM. The NPs corresponding to JRCNM01003a showed neither toxic effects up to 100 mg/L in the absence of SR-NOM.

SiO_2 NPs (JRCNM02000a): Toxicity towards *C. riparius* was not observed for these NPs, since the effective concentrations were above 100 mg/L.

CeO_2 NPs (JRCNM02102a): UOS reported no toxicity for these NPs on *C. riparius* for concentrations below 100 mg/L.

Adverse effects on *C. riparius* were observed only in the case of the organisms exposed to Ag NPs (JRCNM03000a). ZnO NPs produced also an effect to some extent, but it was considerably alleviated in the presence of SR-NOM. Thus, these results are consistent with the obtained in the tests performed with *P. subcapitata*, where SR-NOM diminished the toxicity of TiO_2 and CeO_2 NPs. The rest of NPs (TiO_2, SiO_2 and CeO_2) did not show an adverse effect.

2.5.2 Biokinetic studies in aquatic systems

The tests conducted showed differences in uptake and accumulation in organs of brown trout exposed to silver ions and nanoparticles both within and between exposure routs. Exposure to Ag ions is known to result in accumulation and toxicity to a range of aquatic organisms, which was also observed in this case. The accumulation of silver in the waterborne Ag^+ exposure was much higher in all examined organs compared to

the Ag NPs (JRCNM03000a), as well as all groups in the dietary exposure. There was also a significant accumulation of silver in all three organs examined for the dietary exposure, but no difference in transfer between the Ag^+ and Ag NP exposures.

The uptake of CeO_2 NPs (JRCNM02102a) by *Daphnia magna* was relevant at the highest concentrations tested, whilst it was clearly reduced at the lowest concentrations. This fact could be caused by the occurance of larger agglomerates in the culture medium at higher concentrations, which might be more efficiently retained by the animals in comparison to smaller particles expected at low concentrations. In the case of Ag nanoparticles (JRCNM03000a), the maximum concentration measured was lower than the detection limit of the applied method. Hence, conclusions could not be drawn on their uptake by daphnids. The precipitation of the substance in the form of insoluble $AgCl_2$ was considered as a reason for the low recovery of silver.

2.5.3 Guidance to design and conduct the ecotoxicity experiments

Ecotoxicology of MNMs can be subdivided in different areas, including freshwater toxicity, marine toxicity and terrestrial toxicity, where processes such as bioaccumulation, bioconcentration and trophic transfer are also relevant. In additon, environmental fate processes such as biodegradation, and which relates to the environmental persistence of MNMs, are also closely related to ecotoxicology [18]. Ecotoxicity tests are tools used within environmental hazard assessment frameworks to answer questions about their intrinsic dangers, and their potential risks can be characterized by comparing such hazard assessments with exposure assessments. Hazard assessments usually follow a tiered approach and short-term (acute) tests are generally used first, with organism survival the most common effect measured. Longer-term (chronic) tests to observe commonly sublethal effects on organism growth or reproduction are used when results from short-term tests combined with large safety factors suggest that there may be risks to the environment [19]. The use of acute and chronic tests in an ecotoxicity testing strategy is proposed by the EU REACH regulation for aquatic and terrestrial toxicity. These tiered ecotoxicity testing strategies are developed worldwide and supported by international bodies such as the OECD and ISO. Some important considerations for the use of hazard data in risk assessment emerge from these ecotoxicity testing schemes, such as those indicated by Crane et al. [19]:

- Aquatic toxicity data are likely to be the primary, and often the only information on toxicity for assessing risks to both aquatic and terrestrial environments unless physical and chemical data on a substance suggest that partition onto soils or aquatic sediments might occur.
- Short-term aquatic toxicity tests with algae, daphnids and fish will be performed first, with longer-term tests performed only in case that short-term tests suggest that there may be risks (for example were acute toxicity is observed for exposure concentrations exceeding those expected in the environment).
- There is a widespread desire worldwide to minimize vertebrate testing, with several points in the testing schemes at which it is possible to use alternative test approaches, if these are available, or to provide evidence that fish are likely to be less sensitive. Even if these conditions cannot be fulfilled, a fish limit test (i.e., using only one high concentration and a control) can performed first to establish the relative sensitivity of fish compared with algae and Daphnia, before a full fish test with many more animals is considered.
- The ways in which organisms might be exposed to MNMs, rather than the way in which effects are measured, is the most important uncertainty when assessing their ecotoxicity. Research on establishing appropriate test strategies and methods should focus primarily on defining realistic worst-case exposure scenarios for MNMs in the environment and then testing their toxicity under these scenarios. The fate and behaviour of MNMs should be considered with or without the presence of natural and anthropogenic substances and conditions that may influence aggregation state.
- A set of rapid, cost-effective tests should be agreed between regulators, industry and other stakeholders that are primarily able to demonstrate that a MNM has similar hazard properties to other physical forms of a substance. These should include tests to identify overall toxicity (e.g., cell viability assay or microbial population growth test), and specific modes of toxicity that may not be detected by a general toxicity screen, but are relevant for that type of MNM, such as genotoxicity, immunotoxicity assays, and an oxidative stress assay. These rapid tests might establish whether hazard data on demographic endpoints can be read across to nanoscale from macroscale substances. If these tests are unable to demonstrate that a MNM has similar properties to other physical forms, its specific effects on survival, growth and reproduction should be measured, involving both acute and chronic tests, until sufficient confidence has been built in the use of assessment factors to extrapolate from acute to chronic effects.

The testing schemes below provide a regulatory approach to the results and conclusions obtained in Task 4.6, in order to make them clear and understandable for policymakers. Concrete guidance and

recommendations to design and conduct the ecotoxicity experiments in aquatic organisms are proposed in the form of decision trees and degrees of toxicity based on specific cut off values.

The first step in the ecotoxicological assessment of a MNM in aquatic systems is the preparation of stable dispersions, following the flowchart presented in Figure 63.

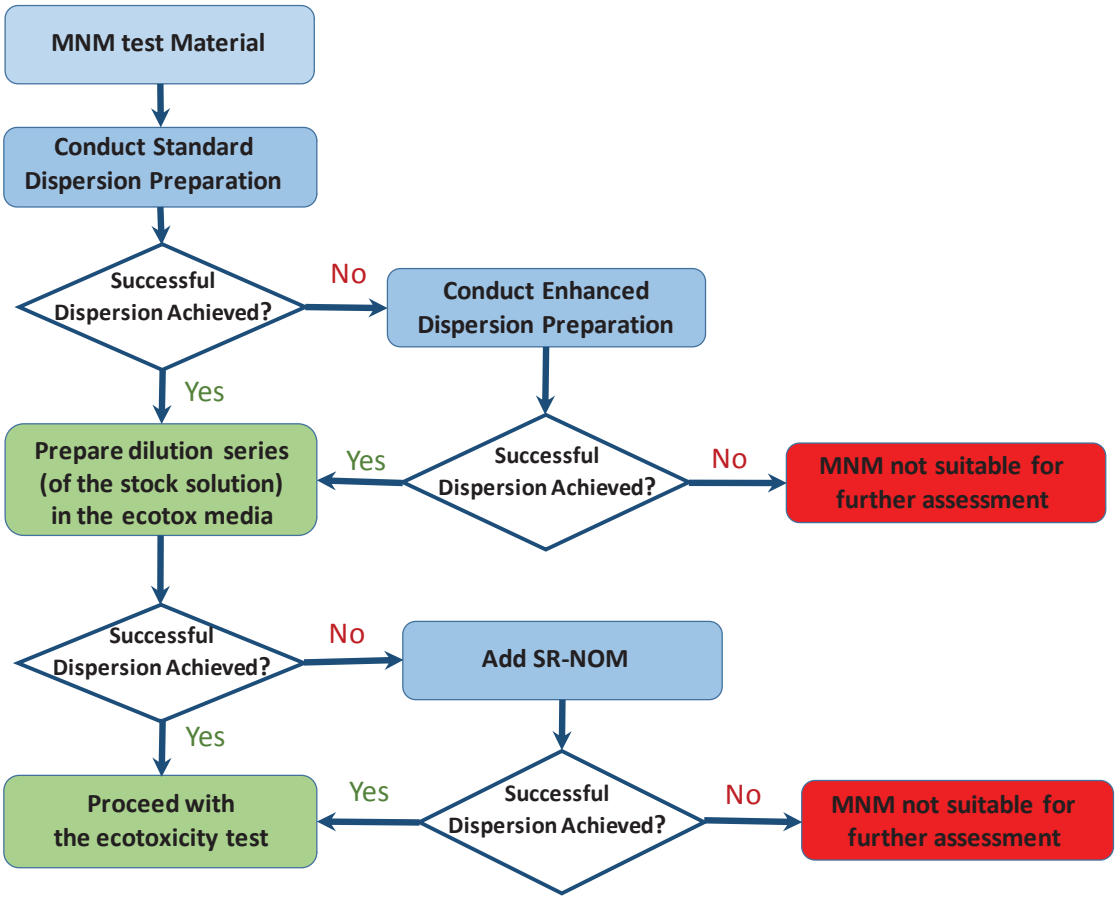

Figure 63. Decision tree for selection of MNM dispersion media and criteria for further environmental assessment. Dispersion preparation is conducted according to the SOP *'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media',* specified in section 2.3.1.

In case that the dispersions prepared are sufficiently stable to obtain reproducible test results, the next step is the selection of the appropriate ecotoxicity tests to perform, according to the decision tree in Figure 64. The main criteria followed in the design of this diagram is to minimize the tests on vertebrates. Hence, a series of tests is proposed starting with the simplest organisms (unicellular green algae). When high effects are observed (EC50<0.1mg/L), it is considered that the MNM has a high hazard for the environment (E category) and it is not necessary to perform further tests. In case of EC50>0.1mg/L, it will be necessary to go to the second trophic level. Applying the precautionary principle, if a particular MNM has several ecotoxicity results on different organisms, the category with the higher hazard will be selected.

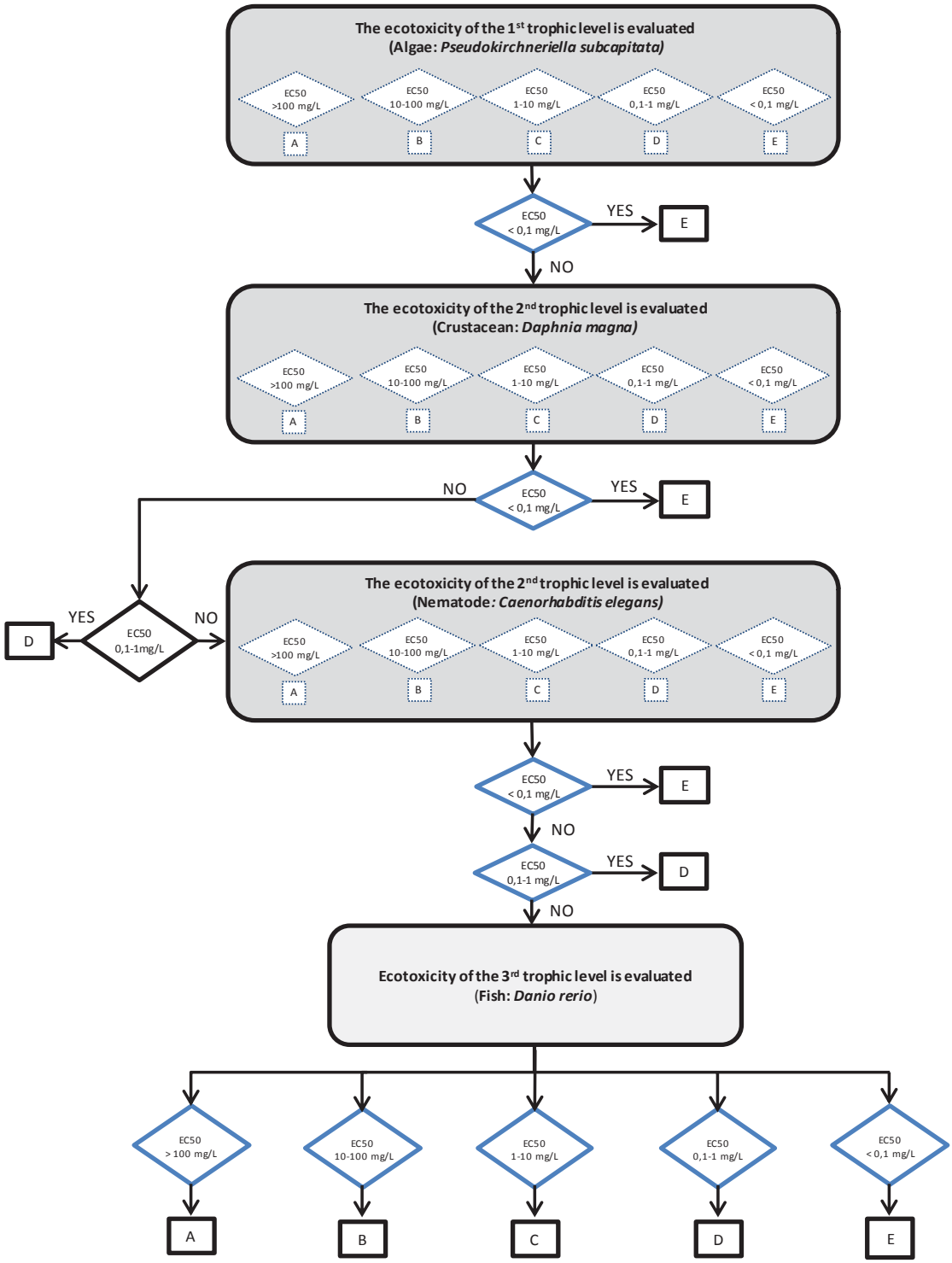

Figure 64. Decision tree for the ecotoxicological assessment of MNMs. Hazard categories established in an arbitrary way, in function of the effective concentrations obtained in the ecotoxicty test. A: not toxic (EC50>100 mg/L); B: harmful (EC50 = 10-100 mg/L); C: toxic (EC50 = 1-10 mg/L); D: very toxic (EC50 = 0.1-1 mg/L); E: extremely toxic (EC50 < 0.1 mg/L).

Considering the toxicity results obtained for aquatic systems in Task 4.6, and following the flowcharts represented in Figures 63 and 64, some of the NANoREG core MNMs have been classified into hazard categories. Further research and experimental toxicity data are necessary to fully determine their effects on aquatic organisms and obtain a complete hazard classification.

Table 50. Classification of NANoREG core MNMs into the hazard categories specified in Figure 64.

NANoREG core MNM	Hazard category	Lowest trophic level required for ecotoxicological assessment	Testing with upper trophic level required?
Ag NPs (JRCNM03000a)	E: extremely toxic (EC50 < 0.1 mg/L)	*P. subcapitata*	No
ZnO NPs (JRCNM01100a)	E: extremely toxic (EC50 < 0.1 mg/L)	*P. subcapitata*	No
CeO_2 NPs (JRCNM02102a)	B: harmful (EC50 = 10-100 mg/L) for *D. magna* and *C. elegans*	Not determined	Yes, fish tests required
TiO_2 NPs (JRCNM01000a)	A: not toxic (EC50>100 mg/L) for *D. magna*	Not determined	Yes, *C. elegans* tests required
TiO_2 NPs (JRCNM01001a)	A: not toxic (EC50>100 mg/L)	*D. rerio*	No
TiO_2 NPs (JRCNM01003a)	A: not toxic (EC50>100 mg/L) for *D. magna* B: harmful (EC50 = 10-100 mg/L) for *C. elegans*	Not determined	Yes, fish tests required

2.6 Data management

The assays and endpoints reported in the present Deliverable have been uploaded to the DET (Data Entry Tool, Diamonds platform) developed by the Netherlands Organization for applied scientific research (TNO). These experimental data were previously reported in the form of Excel templates for the tests performed with *D. magna*, *P. subcapitata*, *C. elegans* and *D.rerio*, which were approved by the Project Office and are available on CIRCABC (Library > C-Workpackages > WP1 > Data logging system > In vivo assays > older version). Additionally, since the templates for reporting the ecotoxicity data on the DET did not finally fit with the pre-defined templates, the complete experimental data has also been uploaded as Excel sheets in CIRCABC (Library > C-Workpackages > WP1 > Data logging system > Logged data > WP 4 - in vivo).

3 Deviations from the work plan

Due to delays on WP2, the final dispersion SOPs for the MNMs in ecotoxicity media were defined late, which created an important accumulative delay on the experimental activities of Task 4.6. A deadline extension of 6th months was requested in order to finalize all the experimental work and include it in the present deliverable D4.12: 'Accumulation potential and aquatic toxicity of relevant groups of nanomaterials and products formula'. The WPL and NANoREG project office were informed about this issue.

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5 List of abbreviations

MNM(s): Manufactured nanomaterial(s)

NP(s): Nanoparticle(s)

CNTs: carbon nanotubes

SWCNTs: Single-walled carbon nanotubes

MWCNTs: Multiwalled carbon nanotubes

SR-NOM: Suwannee River natural organic matter

OECD: Organization for Economic Cooperation and Development

ISO: International Organization for Standardization

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

Annexes

ANNEX 1. Standard Operating Procedure: Toxicity Test with *Daphnia magna* for NANoREG core nanomaterials

1.1. OBJECTIVE AND PRINCIPLE OF THE TEST

This procedure describes how to perform a *Daphnia magna* acute immobilization test to determine the ecotoxicity of specific core nanomaterials in the framework of studies for NANoREG Project.

For this purpose, we base protocol on the OECD-Guideline 202 "Daphnia sp., Acute Immobilization Test" [1] and the ISO 6341:2012 "Water Quality – Determination of the inhibition of the mobility of *Daphnia magna Straus*. Acute toxicity test" [2].

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the NMs dispersions at a range of concentrations over a period of 48 hours in multiwell test plates. Immobilization is recorded at 24 and 48 hours and compared with control values. The test endpoints are the concentrations bringing 50% and 20% immobilization (EC50 and EC20) during the exposure period.

An additional test procedure within the Ecotoxicity Working Group in NANoREG has been established. It consists basically on increasing the age of the test organisms (4 days) and the duration of the exposures (72 h). 4 days daphnids will also be tested in order to check the effect of the organism age on the ecotoxicity results. 72h data will also be collected in order to have a better overview of the NM behavior during a longer period of time. Despite this increment in the test time, no additional feeding will be carried out during the test, in order not to introduce additional test variables that could interact with the NMs.

1.2. MATERIALS AND EQUIPMENT

Beakers and flasks which will come into contact with the test dispersions should be made entirely of glass or other chemically inert material. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may cause adverse effects on daphnids or interfere with the composition of the test dispersions. The following equipment is required:

-HCl (1 M)

-NaOH (1 M)

-Reference substance: $K_2Cr_2O_7$

-Natural organic matter from Suwannee River (SR-NOM) available from the International Humic Substances Society (IHSS).

-Pipettes

-Spatulas

-Beakers

-100 mL, 250 mL and 1000 mL calibrated glass flasks with Teflon-lined caps

-Magnetic stirrer

-Analytical balance (4 digits)

-pH-meter

-Oxygen-meter

-Incubator or temperature controlled room at 20±2 °C, with a constant uniform illumination of minimum 6000 lux at the top.

-Light table or dissection microscope.

-Culture medium: OECD medium with the composition specified in Section 2.1.

-Test organisms: *Daphnia magna* Straus (*Cladocera, Crustacea*) obtained from cultures in parthenogenic reproduction. At the start of the test, the animals should be less than 24 hours/4 days old and, to reduce

variability, it is strongly recommended they are not first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discolored animals, etc.). All organisms used for a particular test should have originated from cultures established from the same stock of daphnids. The stock animals must be maintained in culture conditions (light, temperature and medium) similar to those to be used in the test. If the daphnids culture medium to be used in the test is different from that used for routine culture, daphnids will be maintained in the culture medium and temperature of the test for at least 48 hours prior to the start of the test.

Daphnia magna can also be supplied by specialized companies, in the form of ephippia obtained from laboratory cultures of this crustacean. The Daphtoxkit F^{TM} Magna manufactured by Microbiotests Inc. (Belgium) is an example of a suitable commercially available product, according to ISO Standard 6341:2012. It provides essential material to carry out the toxicity test:

-Vials with ephippia: plastic tubes containing ephippia of *Daphnia magna*, to be stored in a refrigerator at 5±2 °C until use. The number of neonates obtained from each vial suffices for one full toxicity test. Previous experiences have shown that three tests can be performed using two vials.

-Vials with Spirulina microalgae powder: plastic tubes (one for each vial with ephippia) containing a small amount of Spirulina for pre-feeding the test organisms prior to the tests. This feeding provides daphnids with an “energetic reserve” and precludes mortality by starvation (which would bias the test results) during the subsequent 48/72 h test exposure during which the organisms are not fed. Pre-feeding is started 2 h prior to the transfer of the neonates to the test dispersions. For this purpose, fill the Spirulina vial with culture medium and shake the vial thoroughly to homogenize the contents. Pour the algal suspension into the hatching beaker and swirl the contents gently to distribute the food evenly.

-Polycarbonate multiwell test plates composed of 6 rinsing wells and 24 wells for the toxicant dilutions, with Parafilm strips for sealing the plates to minimize evaporation during the incubation period.

-Polyethylene micropipettes for transferring the test organisms, and microsieve for rinsing of the ephippia.

All the non-biological materials provided in the kit are disposable and should only be used once.

NOTE: In the following steps several references to the material provided in Daphtoxkit F^{TM} Magna appear. It can be replaced by other appropriate material which fulfill the requirements specified in the current SOP.

1.2.1. Preparation of the culture medium

The culture medium for *D. magna* will be used as hatching medium for the ephippia and as dilution medium for preparation of the MNM dilution series. The medium selected for the current SOP corresponds to ISO Test water indicated in OECD-202 (ANNEX 3). It is prepared by adding 25 mL of the stock solutions 1-4 (stored in the dark at 4 °C) to 1 L ultrapure water. The stock solutions of nutrients are prepared according to the Table A1.

Table A1. Concentrations of nutrients in solutions for *Daphnia magna* medium.

(Stock solution) Nutrient	Concentration in stock solution	Final concentration in culture medium
(1) $CaCl_2 \cdot 2H_2O$	11.76 g/L	294 mg/L
(2) $MgSO_4 \cdot 7H_2O$	4.93 g/L	123.25 mg/L
(3) $NaHCO_3$	2.59 g/L	64.75 mg/L
(4) KCl	0.23 g/L	5.75 mg/L

Adjust the pH if necessary to 8.1±0.2, with either 1 M HCl or 1 M NaOH. The dissolved oxygen concentration shall be at least 3 mg/L. For this purpose, pre-aeration can be performed by air bubbling for 15 minutes prior to use it.

Two liters of culture medium suffice to conduct three complete *D. magna* bioassays. If the tests are not carried out within a few days after preparation of the medium, it should be stored at 4 °C in darkness. Take care to bring the cooled medium gradually back to room temperature prior to use.

1.3. HATCHING OF THE EPHIPPIA

Hatching of the ephippia must be initiated 3/7 days prior to the start of the toxicity test. Procedure:

1. Pour the contents of one vial with ephippia into the microsieve. Rinse the ephippia thoroughly with tap water to eliminate all traces of the storage medium.

2. Transfer the ephippia into a 100 mL beaker in 50 mL pre-aerated culture medium, and incubate for 72h, at 20±2 °C under continuous illumination of minimum 6000 lux at the top of the beaker.

Important considerations:

The embryonic development of *Daphnia magna* eggs takes about 3 days in optimal conditions. Under the illumination and temperature conditions indicated above the first neonates may even appear before 72 h incubation, but the largest hatching will occur between 72h and 80 h.

Taking into account that a minimum of 120 neonates are needed to perform one complete test and that the neonates should not be older than 24h/4 days at the start of the tests, the organisms must be collected at the latest 90/186 h after the start of the incubation.

1.4. PREPARATION OF TEST DILUTION SERIES

1.4.1. Preparation of aqueous dispersions of NMs

Follow the specific dispersion SOP (Standard or Enhanced dispersion procedure) to prepare the required volume of NM dispersions in ultrapure water. When the "Enhanced dispersion SOP" in not sufficient to achieve the required NM stability, 10 mg/L-Suwannee River natural organic matter (SR-NOM) will be added directly to the test medium before starting the exposures.
When the stability of the nanomaterials is achieved by the addition of NOM, parallel toxicity tests must be performed to determine its effect on daphnids.

1.4.2. Preparation of dilution series

If the approximate toxicity of the NM to *D. magna* is known, a definitive test can be performed immediately. However, if no information is available on its toxicity, two consecutive assays must be performed:
-A range finding test to determine the 0-100% tolerance range of the daphnids to the NM.
-A definitive test to determine with more precision the 50% effect concentration (EC50).

RANGE FINDING TEST

A "tenfold" dilution series must be prepared, starting at the concentration of NMs required as the first dilution level (C1).

Take five 100 mL calibrated flasks and label them as follows: C1 - C2 - C3 - C4 - C5. As an example, Table A2 starts at 100 mg/L as the highest NMs concentration.

Table A2. Dilution series of the NMs

Flask	NMs concentration (mg/L)
C1	100
C2	10
C3	1
C4	0.1
C5	0.01

1. Shake thoroughly the dispersion of NMs prepared in 4.1. and transfer the required volume into the C1 flask to prepare the first dilution. Add culture medium up to the 100 mL mark.
2. Transfer 90 mL culture medium into all the other flasks (C2 to C5).
3. Stopper flask C1 and shake thoroughly to homogenize the dispersion of NMs. Transfer 10 mL of the flask C1 into the flask C2, in order to prepare the second test concentration.
4. Repeat the operation indicated in step 3 for flasks C2 to C5, i.e.:

- 10 mL from C2 to C3
- 10 mL from C3 to C4
- 10 mL from C4 to C5

5. Proceed to section 5: Filling of the test plate.

DEFINITIVE TEST

The dilution series to be prepared spans the range of the lowest concentration producing 100% effect and the highest one producing less than 10% effect in the range finding test. This range can span one order of

magnitude (case A) or two orders of magnitude (case B).The new concentration range to be tested out will again be called C1-C5.

A. C1-C5 spans one order of magnitude

1. Take five 100 mL calibrated flasks and label them as follows: C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 100% effect and C5 the highest that gave less than 10% effect in the range finding test.
2. Take one 250 mL calibrated flask to make up 250 mL of the lowest concentration that produced 100% effect. Transfer the following volumes of this concentration from the 250 mL flask into the other flasks:

- 100 mL to flask C1
- 56 mL to flask C2
- 32 mL to flask C3
- 18 mL to flask C4
- 10 mL to flask C5

Discard the content remaining in the 250 mL flask.

3. Add culture medium up to the 100 mL mark in flasks C2 to C5 (see Table A3).

Table A3. Dilution series C1-C5

Flask	C1 (mL)	Dilution medium (mL)
C1	100	0
C2	56	44
C3	32	68
C4	18	82
C5	10	90

4. Starting from the NMs concentration in flask C1, calculate the actual concentration in each flask (these figures will be needed for the endpoints estimation):

C1 = ...mg/L
C2 = 0.56 x C1 = ...mg/L
C3 = 0.32 x C1 = ...mg/L
C4 = 0.18 x C1 = ...mg/L
C5 = 0.10 x C1 = ...mg/L

5. Proceed to section 5: Filling of the test plate.

B. C1-C5 spans two orders of magnitude

1. Take six 100 mL calibrated flasks and label them as follows: C1 - C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 100% effect and C5 the highest that gave less than 10% effect in the range finding test.
2. Make up the two C1 flasks with 100 mL NMs concentration C1.
3. Transfer the following volumes of NMs dispersion from one of the two C1 flasks to the other flasks :

- 32 mL to flask C2
- 10 mL to flask C3
- 3.2 mL to flask C4
- 1 mL to flask C5

Discard the half empty C1 flask.

4. Add culture medium up to the 100 mL mark in flasks C2 to C5 (see Table A4).

Table A4. Dilution series C1-C5

Flask	C1 (mL)	Dilution medium (mL)
C2	32	68
C3	10	90
C4	3.2	96.8
C5	1	99

5. Starting from the NMs concentration in flask C1, calculate the actual concentration in each flask (these figures will be needed for the endpoints estimation):

C1 = ...mg/L
C2 = 0.32 x C1 = ...mg/L
C3 = 0.10 x C1 = ...mg/L
C4 = 0.03 x C1 = ...mg/L
C5 = 0.01 x C1 = ...mg/L

6. Proceed to section 5: Filling of the Test Plate.

1.5. FILLING OF THE TEST PLATE

1.5.1. Replicates and controls

Each test concentration as well as the control will be assayed in 4 replicates, for a statistically acceptable evaluation of the effects. Each Daphtoxkit test plate includes 4 test wells for the controls and for each NMs concentration.

At least 2 ml of test solution must be provided for each animal. Transfer 10 mL of culture medium into each well of the control row and 10 mL of the respective NMs concentration dilution into each well of the corresponding rows, in the sequence of increasing toxicant concentrations.

The rinsing wells included on the left side of the multiwell plates will be used to prevent dilution of the NMs dispersions during the transfer of the test organisms to the test plate.

1.6. TRANSFER OF THE NEONATES TO THE TEST PLATE AND INCUBATION

Put the beaker with the pre-fed neonates on the stage of the dissection microscope or on the light table. Because of their small size, the transfer will be carried out under a dissection microscope at low magnification (e.g. 10X), or using a light table provided with a dark light strip and a transparent stage. The use of a strip of black paper and a transparent stage enhances the contrast between the test organisms and the white background of the light table, and substantially facilitates the visual observation of the test organisms.

The transfer of the *Daphnia* neonates into the test wells can be performed with a micropipette. Transfer at least 20 (actively swimming) neonates into each rinsing cup in the sequence: control row, row 1, row 2, row 3, row 4 and row 5 (i.e. in order of increasing concentrations of toxicant). Carry over as little as possible culture medium from the hatching beaker to the wells and rinse the micropipette thoroughly with culture medium after each transfer.

Put the multiwell plate on the stage of the dissection microscope or of the light table and transfer 5 neonates from each rinsing well into the 4 wells of each row. Count the neonates as they exit the micropipette to be sure of the transfer of exactly 5 test organisms per well. This transfer will also be performed in the sequence of increasing test concentrations.
Daphnids are quite susceptible to being trapped at the surface of the medium in the wells by the phenomenon of surface tension. They may not be able to free themselves from the surface and may die, thus jeopardizing the outcome of the bioassays. It is of utmost importance to put the tip of the micropipette in the medium during the transfer of the neonates, and not to drop the organisms onto the surface of the medium.

Incubation procedure. The daphnids should not be fed during the test, and the test vessels must not be aerated. Put the Parafilm strip on the multiwell plate and put the cover on tightly. The incubation is conducted in the incubator or the temperature controlled room at 20±2 °C, in darkness. For each single test the temperature should be constant within 19 °C and 21°C.

1.7. MEASUREMENTS AND ANALYTICAL DETERMINATIONS

After 24h, 48h and 72h incubation, put the multiwell plate under the dissection microscope or on the stage of the light table and record the number of dead and immobilized neonates in each well. The neonates which are not able to swim after gentle agitation of the medium for 15 seconds shall be considered to be immobilized, even if they can still move their antennae. In addition to immobility, any abnormal behaviour or appearance should be reported.

Calculate the total number of dead and immobile neonates for each NMs concentration and calculate the % effect. Data will be summarized in tabular form, showing the number of daphnids used and immobilization at each observation for each concentration and control. The percentages immobilized at 24, 48 and 72 hours will be plotted against test concentrations. Data are analyzed by appropriate statistical methods (see Section 8) to calculate the slopes of the curves and the EC50 with 95% confidence limits (p = 0.95). Where the standard methods of calculating the EC50 are not applicable to the data obtained, the highest concentration

causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC50 (this being considered the geometric mean of these two concentrations).

The test dispersions will be analyzed to verify the initial NMs concentrations and maintenance of the exposure concentrations during the test. At the start and end of the test, collect aliquots of the test dispersions and analyze them by the appropriate method (UV/vis absorbance, HR-ICP-MS/ICP-OES, DLS, etc.). These determinations can be performed on several wells at each test concentration for statistical analysis. For unstable NMs, additional sampling for analysis at 24 hour intervals during the exposure period will be prepared in order to better define the loss of the test substance. Analysis of the concentration at the start and end of the test of a low and high test concentration and a concentration around the expected EC50 is sufficient when exposure concentrations vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test will be performed when concentrations do not remain within 80-120 % of nominal. If analysis of the dispersed concentration is required, it may be necessary to separate daphnids from the medium. Separation should preferably be made by filtration. Measure the pH of the test dispersions at the beginning and at the end of the tests.

At the end of the tests, microscopic observations can be performed to verify a normal and healthy appearance of the Daphnia and to observe any abnormal appearance, as may be caused by the exposure to the NMs.

1.8. DATA TREATMENT AND REPORTING

1.8.1. Plotting concentration response curve

Plot the percentage of inhibition against the logarithm of the NM concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier. Fit a line through the data points by computerized statistical method for interpolation.
Depending on the intended usage of data, the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures EC50 and EC20 from the fitted curve. A valid reason for not using a statistical method might be that data are not appropriate for computerized methods to produce any more reliable results than can be obtained by expert judgment. In such situation some computer programs may even fail to produce a reliable solution (iterations may not converge, etc.)

1.8.2. Statistical procedures

The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearizing transformation of the response data (for instance into probit, logit or Weibull units), but non-linear regression procedures allow the handling of unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition, such irregularities may be magnified by the transformation, interfering with the analysis.
Use the concentration-response relationship to calculate point estimates of ECX values. The 95% confidence limits for each estimate must be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically.
Regression analysis should be performed using individual replicate responses, not treatment group means. If, however nonlinear curve fitting is difficult or fails because of too great scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.
Endpoints estimates and confidence limits may also be obtained using linear interpolation with bootstrapping, if available regression models/methods are unsuitable for the data.

1.8.3. Test report

The test report must include the following:

1. Test substance: nanomaterial identification from the suite of NANoREG Project.
2. Test species and supplier or source and the culture conditions used.
3. Test conditions:
    - -Date of start of the test and its duration.
    - -Test concentrations and replicates.
    - -Description of the preparation of test dispersions, including use of dispersants.
    - -Culturing apparatus.

-Temperature. It is measured in control wells or in ambient air and it must be recorded preferably continuously during the test or at least at the beginning and end of the test.

-Concentrations tested: the nominal test concentrations and any results of analysis to determine the concentration of the test substance in the culturing apparatus. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.

4. Results:

-pH values at the beginning and at the end of the test at all test substances.

-Calculated response variables for each test replicate and mean values.

-Graphical representation of the concentration/effect relationship.

-Estimates of toxicity for response variables (EC50 and EC20) and associated confidence intervals.

-Any other observed effects, e.g. any abnormal behaviour or appearance.

-Discussion of the results, including any influence on the outcome of the test resulting from deviations from this procedure.

1.9. VALIDITY OF THE TEST

1. The number of dead + immobile organisms should not exceed 10 % in the controls.
2. 24h-CE50 for the reference substance ($K_2Cr_2O_7$) should be between 0.6 and 2.1 mg/L.
3. The pH in the controls shall not have increased by more than 1.5 units relative to the initial pH in the culture medium.
4. The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L. This value should be measured at least at the controls and the highest test concentration. The contents of each dilution row can be transferred to a beaker to carry out an only measurement, taking care not to modify the dissolved oxygen concentration. If this value is less than 3 mg/L, it shall be measured at the rest of dilution rows. All the test rows with a dissolved oxygen concentration lower than 3 mg/L should not be considered at data treatment.

1.10. REFERENCE TEST

In order to check the correct execution of the test procedure and the sensitivity of the test, it is advised to perform a reference test during the same month in which the test is carried out.
This quality control test may be carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$).

Procedure:

1. A dilution series ranging from 3.2 mg/L to 0.32 mg/L will be prepared following the guidelines given in section 4.2 Definitive test - A. C1-C5 spans one order of magnitude.
2. Take eight 100 mL calibrated flasks, label two of them as 'Stock 1' and 'Stock 2' and the others C0, C1 to C5.
3. Weigh 10 mg potassium dichromate on an analytical balance, transfer it into 'Stock 1' flask and fill to the mark with ultrapure water.
4. Transfer 10 mL "stock 1" solution into "stock 2" flask and fill the latter flask to the mark with culture medium to make up a 10 mg/L toxicant concentration.
5. Transfer the following volumes of toxicant solution from "stock 2" into the other 100 mL flasks:

- 32 mL to flask C1 (3.2 mg/L)
- 18 mL to flask C2 (1.8 mg/L)
- 10 mL to flask C3 (1 mg/L)
- 5.6 mL to flask C4 (0.56 mg/L)
- 3.2 mL to flask C5 (0.32 mg/L)

6. Add culture medium to the mark, stopper the flasks and shake to homogenize the solutions.
7. Fill the multiwell with the toxicant solutions as indicated in Section 1.5: Filling of the test plate.

The 24h EC50 is calculated, and should be within the limits stipulated in Section 9: Validity of the test.

1.11. REFERENCES

[1] OECD 202. GUIDELINES FOR THE TESTING OF CHEMICALS, Daphnia sp., Acute Immobilization Test; Organisation for Economic Cooperation and Development (OECD): Paris, Adopted 13 April 2004.

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ANNEX 2. Standard Operating Procedure: Toxicity Test with Microalgae *Pseudokirchneriella subcapitata*

2.1. OBJECTIVE AND PRINCIPLE OF THE TEST

This procedure describes how to perform an algal growth inhibition test to determine the ecotoxicity of specific core nanomaterials in the framework of studies for NANoREG Project.

For this purpose, we base protocol on the OECD-Guideline 201 "Algal growth inhibition test" [1] and a chlorophyll fluorescence technique referenced in this Guideline to estimate the biomass concentrations [2]. Some modifications of the standard chlorophyll extraction procedure have been adopted due to the nature of the tested substances.
Exponentially growing test organisms, *Pseudokirchneriella subcapitata* (first named as *Selenastrum capricornutum* and *Raphidocelis subcapitata*), are exposed in batch cultures to the NM dispersions over a period of 72 hours. The system response is the reduction of the growth rates in a series of algal cultures exposed to various concentrations of NMs, compared to the average growth of unexposed control cultures. Growth and growth inhibition are quantified as a function of time. The test endpoints are the concentrations bringing 50% and 10% inhibition of growth rate (EC50 and EC10) during the exposure period.

2.2. MATERIALS AND EQUIPMENT

Beakers and flasks which will come into contact with the test dispersions should be made entirely of glass or other chemically inert material. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may interfere with the algal growth or composition of the test dispersion. The following equipment is required:

-HCl (1 M)
-HCl (3 M)
-NaOH (1 M)
-Acetone: HPLC grade, with magnesium carbonate (light powder) added.
-Locust Bean Gum (Galactomannan polysaccharide – Sigma).
-Pipettes
-Spatulas
-100 mL glass flasks with Teflon-lined caps
-250 mL Erlenmeyer glass flasks with air-permeable stoppers, sterilized for the incubation of the test cultures. They allow a sufficient volume of culture for analytical determinations and a sufficient mass transfer of CO_2 from the atmosphere.
-Analytical balance (4 digits)
-pH-meter
-Cool white fluorescent lamps. The light intensity range at the level of the test dispersions must be between 60 and 120 $\mu E \cdot m^{-2} \cdot s^{-1}$ when measured in the wavelength range of 400-700 nm. For light-measuring instruments calibrated in lux, the range of 4440-8880 lux is approximately equivalent.
-Shaking table kept in a temperature controlled room at 23±2 °C with dedicated lighting system. A shaker incubator where shaking, temperature and light system are kept according to specifications is also appropriate.
-Foil-wrapped screw-capped tubes of typical capacity 10 ml to 20 ml.
-Fluorescence spectrophotometer with an excitation wavelength of 430 nm and a measured emission wavelength of 671±10 nm. In case of unavailability of a fluorescence spectrophotometer, a microplate reader may be used. Black microplates are necessary for fluorescence measurements and they must be made from an acetone resistant material. Polypropylene microplates from Greiner Bio One have demonstrated to be suitable for this purpose.
-Test organisms: the strain of *Pseudokirchneriella subcapitata* from the collections listed in Annex I.
-Growth medium: OECD medium with the composition specified in Section 2.2.1.
-Reference substance: $K_2Cr_2O_7$.
-Natural organic matter from Suwannee River (SR-NOM) available from the International Humic Substances Society (IHSS).

2.2.1. Preparation of the growth medium

The algal growth medium is prepared by adding an appropriate volume of the stock solutions 1-4 to sterile ultrapure water. The stock solutions of nutrients are prepared according to the Table A5.

Table A5. Concentrations of nutrients in solutions for *Pseudokirchneriella subcapitata* medium.

Stock solution	Nutrient	Concentration in stock solution	Final concentration in test solution
1: macro nutrients	NH_4Cl	1.5 g/L	15 mg/L
	$MgCl_2 \cdot 6H_2O$	1.2 g/L	12 mg/L
	$CaCl_2 \cdot 2H_2O$	1.8 g/L	18 mg/L
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L
	KH_2PO_4	0.16 g/L	1.6 mg/L
2: Fe-EDTA[a]	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 µg/L
	$Na_2EDTA \cdot 2H_2O$	100 mg/L	100 µg/L
3: trace elements	H_3BO_3	185 mg/L	185 µg/L
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 µg/L
	$ZnCl_2$	3 mg/L	3 µg/L
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 µg/L
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 µg/L
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 µg/L
4: bicarbonate	$NaHCO_3$	50 g/L	50 mg/L

[a] EDTA should be removed when testing metals, to avoid binding on metal ions.

Sterilize the stock solutions by membrane filtration (mean pore diameter 0.2 µm) or by autoclaving (120 °C, 15 min). Store the solutions in the dark at 4 °C.
Do not autoclave stock solutions 2 and 4, but sterilize them by membrane filtration.

Procedure to make 1 L of algal growth medium

1. Transfer 500 ml sterilized ultrapure water into a sterilized 1 L volumetric flask and add an appropriate volume of the stock solutions:
- 10 mL of stock solution 1
- 1 mL of stock solution 2
- 1 mL of stock solution 3
- 1 mL of stock solution 4
2. Make up to 1000 mL with sterilized ultrapure water, stopper the flask and shake thoroughly to homogenize the algal growth medium.
3. Before use, equilibrate the solution by leaving it overnight in contact with air, or by bubbling with sterile, filtered air for 30 min. After equilibration, adjust the pH if necessary to 8.1±0.2, with either 1 M HCl or 1 M NaOH.

2.3. CULTURING OF THE ALGAE

All operations must be carried out under sterile conditions in order to avoid contamination with bacteria and other algae. *P. subcapitata* is generally easy to maintain in various culture media. Information on suitable media is available from the culture collections. The cells are normally solitary, and cell density measurements can easily be performed using an electronic particle counter or microscope.

Stock culture:
The stock cultures are small algal cultures that are regularly transferred to fresh medium to act as initial test material. If the cultures are not used regularly they are streaked out on sloped agar tubes and transferred to fresh medium at least once every two months.
The stock cultures are grown in conical flasks containing the appropriate medium (volume about 100 mL). When the algae are incubated at 20°C with continuous illumination, a weekly transfer is required.
The growth rate of a species can be determined from the growth curve. If this is known, it is possible to estimate the density at which the culture should be transferred to new medium. This must be done before the culture reaches the death phase.

Pre-culture:
The pre-culture is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test (in growth medium) and used when still exponentially growing, normally after an incubation period of 2 to 4 days. When the algal cultures contain deformed or abnormal cells, they must be discarded.

When the tests are conducted without EDTA in the growth medium (see Table A5), the pre-culture must also be EDTA-free.

2.4. PREPARATION OF CULTURE INOCULUM

The initial biomass must be the same in all test cultures and sufficiently low to allow exponential growth throughout the incubation period without risk of nutrient depletion. It should not exceed 0.5 mg/L as dry weight.
In order to adapt the algae to the test conditions and ensure that they are in the exponential growth phase when used to inoculate the test dispersions, a pre-culture is prepared 2-4 days before the start of the test. The algal biomass should be adjusted in order to allow exponential growth to prevail in the inoculum culture until the test starts.
The initial cell concentration recommended for *P. subcapitata* is $5x10^3$ cells/mL. Taking into account the dilution of algal inoculum for the preparation of toxicant dilution series, a concentrated inoculum of $5x10^5$cells/mL is required.
Measure the increase in biomass in the inoculum culture to ensure that growth is within the normal range for the test strain under the culturing conditions. To avoid synchronous cell divisions during the test a second propagation step of the inoculum culture may be required.

2.5. PREPARATION OF TEST DILUTION SERIES

2.5.1. Preparation of aqueous dispersions of NMs

Follow the specific dispersion SOP (Standard or Enhanced dispersion procedure) to prepare the required volume of NM dispersions in ultrapure water. When the "Enhanced dispersion SOP" in not sufficient to achieve the required NM stability, 10 mg/L-Suwannee River natural organic matter (SR-NOM) will be added directly to the test medium before starting the exposures.
When the stability of the nanomaterials is achieved by the addition of NOM, parallel toxicity tests must be performed to determine its effect on algae.

2.5.2. Replicates and controls

The test design includes three replicates at each test concentration (three different dilution series from three different stock dispersions) for a statistically acceptable evaluation of algal growth inhibition. If determination of the NOEC (No Observed Effect Concentration) is not required, the test design may be altered to increase the number of concentrations and reduce the number of replicates per concentration.
The number of control replicates must be twice the number of replicates used for each test concentration, and at least three.
A separate set of test solutions may be prepared for analytical determinations of test substance concentrations (see paragraph 3 in Section 2.7).

2.5.3. Preparation of dilution series

The concentration range in which effects are likely to occur may be determined on the basis of results from range-finding tests. For the final definitive test at least five concentrations, arranged in a geometric series with a factor not exceeding 3.2, should be selected. For test substances showing a flat concentration response curve a higher factor may be justified. The concentration series should preferably cover the range causing 5-75 % inhibition of algal growth rate.
If the toxicity of the NMs to microalgae is approximately known, a definitive test can be performed immediately. If no information is available on its toxicity, two consecutive assays must be performed:
-A range finding test to determine the 0-100% tolerance range of the algae to the NM.
-A definitive test to determine with more precision the 50% inhibition threshold.

RANGE FINDING TEST

A "tenfold" dilution series must be prepared, starting at the concentration of NMs required as the first dilution level (C1).

6. Take five 250 mL Erlenmeyer flasks and label them as follows: C1 - C2 - C3 - C4 - C5. As an example, Table A6 starts at 25 mg/L as the highest NPs concentration.

Table A6. Dilution series of the NMs

Flask	NM concentration (mg/L)
C1	25
C2	2.5
C3	0.25
C4	0.025
C5	0.0025

7. Shake thoroughly the dispersion of NMs prepared in 5.1 and transfer the required volume into the C1 flask to prepare the first dilution. Add growth medium up to the 100 mL mark.
8. Transfer 90 mL growth medium into all the other flasks (C2 to C5).
9. Stopper flask C1 and shake thoroughly to homogenize the dispersion of NMs. Transfer 10 mL of the flask C1 into the flask C2, in order to prepare the second test concentration.
10. Repeat the operation indicated in step 4 for flasks C2 to C5, i.e.:

- 10 mL from C2 to C3
- 10 mL from C3 to C4
- 10 mL from C4 to C5

11. Remove (and discard) 10 mL dispersion from flask C5. Then, remove and discard 1 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
12. Add 0.9 mL of the $5x10^5$cells/mL algal stock suspension to each flask, in order to obtain an initial concentration of $5x10^3$cells/mL. Stopper the flasks and shake them thoroughly to distribute the algal suspension evenly.
13. Proceed to Section 6: Incubation.

DEFINITIVE TEST

The dilution series to be prepared spans the range of the lowest concentration producing 90-100% growth inhibition and the highest one producing 0-10% growth inhibition relative to the control in the range finding test. The new concentration range to be tested out will again be called C1-C5.

A. C1-C5 spans one order of magnitude

6. Take five 250 mL Erlenmeyer flasks and label them as follows: C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 90-100% growth inhibition and C5 the highest that gave 0-10% growth inhibition in the range finding test.
7. Take one 250 mL Erlenmeyer flask to make up 250 mL of the lowest concentration that produced 90-100% growth inhibition. Transfer the following volumes of this concentration from the 250 ml flask into the other flasks :

- 100 mL to flask C1
- 56 mL to flask C2
- 32 mL to flask C3
- 18 mL to flask C4
- 10 mL to flask C5

8. Add growth medium up to the 100 mL mark in the C2, C3, C4 and C5 flasks (see Table A7).

Table A7. Dilution series of the NMs

Flask	C1 (mL)	Growth medium (mL)
C1	100	0
C2	56	44
C3	32	68
C4	18	82
C5	10	90

9. Remove and discard 1 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
10. Add 1 mL of the $5x10^5$cells/mL algal suspension to each flask to obtain an algal density of $5x10^3$cells/mL. Stopper and shake the flasks thoroughly to distribute the algal suspensions evenly.

11. Starting from the concentration in flask C1, calculate the actual concentration of NPs in each flask (these figures will be needed for the endpoints estimation):

C1 = ...mg/L
C2 = 0.56 x C1 = ...mg/L
C3 = 0.32 x C1 = ...mg/L
C4 = 0.18 x C1 = ...mg/L
C5 = 0.10 x C1 = ...mg/L

12. Proceed to Section 2.6: Incubation.

B. C1-C5 spans two orders of magnitude

1. Take six 250 mL Erlenmeyer flasks and label them as follows: C1 - C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 90-100% growth inhibition and C5 the highest that gave 0-10% growth inhibition in the range finding test.
2. Make up two C1 flasks with 100 mL NPs concentration C1.
3. Transfer the following volumes of NPs dispersion from one of the two C1 flasks to the other flasks:

- 32 mL to flask C2
- 10 mL to flask C3
- 3.2 mL to flask C4
- 1 mL to flask C5

Discard the half empty C1 flask.

4. Add growth medium up to the 100 mL mark in the C2, C3, C4 and C5 flasks (see Table A8).

Table A8. Dilution series of the NMs

Flask	C1 (mL)	Growth medium (mL)
C2	32	68
C3	10	90
C4	3.2	96.8
C5	1	99

5. Remove and discard 1 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
6. Add 1 mL of the $5x10^5$cells/mL algal stock to all flasks to obtain an algal density of $5x10^3$cells/mL in each flask. Stopper and shake the flasks thoroughly to distribute the algal suspensions evenly.
7. Starting from the toxicant concentration in flask C1, calculate the actual concentration of toxicant in each flask (these figures will be needed for the endpoints estimation).

C1 = ...mg/L
C2 = 0.32 x C1 = ...mg/L
C3 = 0.10 x C1 = ...mg/L
C4 = 0.03 x C1 = ...mg/L
C5 = 0.01 x C1 = ...mg/L

8. Proceed to Section 6: Incubation.

2.6. INCUBATION

Cap the test flasks with air-permeable stoppers. Place them randomly on a shaker table/incubator, since it is necessary to keep the algae in suspension and facilitate transfer of CO_2 and reduce pH variation.
The surface where the cultures are incubated must receive continuous, uniform fluorescent illumination e.g. of «cool-white» or «daylight» type. Select the light intensity at the level of the test dispersions from the range of 60-120 $\mu E \cdot m^{-2} \cdot s^{-1}$ when measured in the photosynthetically effective wavelength range of 400-700 nm using an appropriate receptor. For light-measuring instruments calibrated in lux, an equivalent range of 4440-8880 lux for cool white light corresponds approximately to the recommended light intensity 60-120 μE $\mu E \cdot m^{-2} \cdot s^{-1}$. Maintain the light intensity within ±15% from the average light intensity over the incubation area. The position of each flask in the incubator may be changed every 24 h in order to compensate a lack of uniformity in the illumination system.
The flasks are incubated for 72±2h and a temperature of 23±2 °C.

2.7. MEASUREMENTS AND ANALYTICAL DETERMINATIONS

The algal biomass in each flask is determined every 24 hours during the test period. The small volumes removed from the test dispersion by pipette to make measurements should not be replaced. Measurement of algal growth is conducted by a chlorophyll extraction procedure to determine the biomass concentrations

(alternative fluorescence method in OECD-201)**Error! Bookmark not defined.**. A detailed procedure to carry out these determinations is specified in Section 2.7.1.
The test dispersions will be analyzed to verify the initial concentrations and maintenance of the exposure concentrations during the test. At the start and end of the test, collect aliquots of the exposure dispersion and analyze them by the appropriate method (UV/vis absorbance, HR-ICP-MS/ICP-OES, DLS, etc.) to quantify the NM concentrations. Measure the pH of the dispersions at the beginning and at the end of the tests. The determination of test substance concentrations can be performed on the three replicate flasks at each test concentration for statistical analysis. For unstable test substances, additional sampling for analysis at 24 hour intervals during the exposure period will be prepared in order to better define loss of the test substance. Analysis of the concentration at the start and end of the test of a low and high test concentration and a concentration around the expected EC50 is sufficient when exposure concentrations vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test will be performed when concentrations do not remain within 80-120 % of nominal. If analysis of the dispersed test substance concentration is required, it may be necessary to separate algae from the medium. Separation should preferably be made by filtration.
The algal growth inhibition test is a more dynamic test system than most other short-term aquatic toxicity tests. As a consequence, the actual exposure concentrations may be difficult to define, especially for adsorbing substances tested at low concentrations. In such cases, disappearance of the test substance from dispersion by adsorption to the increasing algal biomass does not mean that it is lost from the test system. When the result of the test is analyzed, it should be checked whether a decrease in concentration of the test substance in the course of the test is accompanied by a decrease in growth inhibition. To overcome this issue, aliquots of the exposure dispersions are collected and subjected to analysis before and after the algal filtration step to quantify the NM concentration.
At the end of the test, microscopic observation will be performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae, as may be caused by the exposure to the test substance.

2.7.1. Measurement of algal growth

The algal biomass in each flask is determined at 24, 48 and 72 hours by fluorometric determinations of the chlorophyll-a concentrations. Extracted chlorophyll is used as a means of deriving the biomass of an algal culture in the presence of NMs, which interfere with measurements of culture density normally made by optical absorbance. The particulates and cell debris settles to the bottom of the extraction tubes while the chlorophyll is in solution and is measured fluorometrically.

Sampling and storage. Chlorophyll is sensitive to light and oxygen, especially when it is extracted. To avoid oxidative and photochemical destruction, the samples shall not be exposed to bright light or air. Homogenization of the sample may in some cases increase the extraction efficiency.
Procedure. Remove 1 mL of each culture into a foil-wrapped screw-capped tube, add 0.1 mL of Locust Bean Gum suspension (30 mg in 20 mL H_2O), and add 4.4 ml acetone (100%, with magnesium carbonate added). Cap the tubes and invert several times to mix. Place in a dark cupboard at room temperature for 1-7 days before determining the chlorophyll concentration in a fluorescence spectrophotometer. Fluorescence is measured after 24 hours extraction at room temperature and again 7 days later (measurements should be remain stable for that period). *NOTE: In case of unavailability of a fluorescence spectrophotometer, a microplate reader could be used (see specifications in Section 2.2).*
Reagents and comments:
Acetone: HPLC grade, with magnesium carbonate (light powder) added. The $MgCO_3$ acts as a buffer to prevent low pH degrading the chlorophyll, the presence of magnesium ions further protects against degradation by ensuring the magnesium which forms the central ligand of the chlorophyll tetramer is not stripped out. $MgCO_3$ is almost insoluble in acetone. Thus, it is only necessary to add enough to leave a visible deposit on the bottom of the bottle after settling. Resuspension of the acetone before using is not necessary but it does no harm to do so.
Locust Bean Gum (Galactomannan polysaccharide – Sigma) is added to aid in the precipitation of particulate materials. Other polysaccharides may work equally well, although carrageenan has shown to be not suitable. The Locust Gum does not dissolve completely and needs to be stirred and heated on a hotplate at ~100 °C before being dispensed each time (it is made up in a glass Universal tube). If this or another coprecipitant is not available, it may be dispensed with, in which case 4 mL of acetone is added to each tube.
In tests with titanium dioxide nanoparticles, the higher concentrations tested (100 and 1000 mg/L) sometimes produce floating aggregates even in the presence of the locust gum. It helps to agitate the rack of tubes gently (horizontally, by 2 cm approximately, 2-3 times), a short time before determining the chlorophyll concentration.

The acetone is recovered as part of the waste disposal procedure: it is distilled to remove the acetone, and then the residue containing water, salts and nanomaterials is added to a waste disposal stream. The acetone can be further redistilled to a higher purity and reused (usually blended with fresh acetone).
Spectrophotometry. Fluorescence is measured at room temperature in arbitrary units on a fluorescence spectrophotometer/microplate reader with an excitation wavelength of 430 nm and a measured emission wavelength of 671±10 nm. The signal should change less than 0.5% per °C. Fluorescence figures are corrected for background fluorescence measured on solvents mixed with algal medium. The needed sub-sample volume is 0.2 mL with 10-12-mm cuvettes.
In order to obtain the algal biomass values from fluorescence measurements, standard calibration curves can be performed. Mayer et al.**Error! Bookmark not defined.** propose the following procedure: Obtain a single algal culture of $5 \cdot 10^5$ cells/mL and prepare a tenfold dilution series (range $5 \cdot 10^2$-$5 \cdot 10^5$ cells/mL) of each 10 mL. Extract three replicates from each cell density to carry out the fluorescence measurements, and represent the corresponding standard curves (log cells/mL vs. log fluorescence). Averages of three replicate extracts must be corrected with background fluorescence of solvents mixed with growth medium, and standard deviation should not exceed 5%.
To calculate the specific growth rate, prepare twelve control cultures of each 10 mL at an initial nominal density of 10^3 cells/mL. Incubate them for 3 days with daily sampling for fluorescence measurements. A straight line in the time-log (fluorescence) plot will indicate exponential growth with constant chlorophyll content; and low deviations among replicate cultures will indicate that both growth rate and algal chlorophyll content are reproducible among replicates. To obtain this curve it is necessary to maintain constant and homogenous test conditions with regard to temperature and light, as both growth rate and chlorophyll content vary considerably with light and temperature.

2.8. DATA TREATMENT AND REPORTING

2.8.1. Plotting growth curves

The chlorophyll-a concentrations are converted in biomass density in terms of cells/mL, using the calibration curves obtained in Section 7.1.
Tabulate the algae concentration of the test cultures and controls together with the concentrations of test material and the times of measurement, recorded with a resolution of at least whole hours, to produce plots of growth curves. Logarithmic scales are mandatory and generally give a better presentation of variations in growth pattern during the test period. Exponential growth produces a straight line when plotted on a logarithmic scale, and inclination of the line (slope) indicates the specific growth rate.
Using the plots, examine whether control cultures grow exponentially at the expected rate throughout the test. Examine all data points and the appearance of the graphs critically and check raw data and procedures for possible errors, checking any data point that seems to deviate by a systematic error. If procedural mistakes can be identified and/or considered highly likely, the specific data point is marked as an outlier and not included in subsequent statistical analysis. I.e., zero algal concentration in one out of two or three replicate vessels may indicate the vessel was not inoculated correctly, or was improperly cleaned. State reasons for rejection of a data point as an outlier clearly in the test report. Accepted reasons are only (rare) procedural mistakes and not just bad precision. Statistical procedures for outlier identification are of limited use for this type of problem and cannot replace expert judgment. Outliers (marked as such) should preferably be retained among the data points shown in any subsequent graphical or tabular data presentation.

2.8.2. Average growth rate

The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single flask of controls and NMs (1):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (\text{day}^{-1}) \quad (1)$$

where:
μ_{i-j} is the average specific growth rate from time i to j;
X_i is the biomass at time i;
X_j is the biomass at time j
For each test material group and control group, calculate a mean value for growth rate along with variance estimates.
Calculate the average specific growth rate over the entire test duration (days 0-3), using the nominally inoculated biomass as the starting value rather than a measured starting value, because in this way greater

precision is normally obtained. If the equipment used for biomass measurement allows sufficiently precise determination of the low inoculum biomass then the measured initial biomass concentration can be used.
Assess also the section-by-section growth rate, calculated as the specific growth rates for each day during the course of the test (days 0-1, 1-2 and 2-3) and examine whether the control growth rate remains constant (see validity criteria, Section 9). A significantly lower specific growth rate on day one than the total average specific growth rate may indicate a lag phase. While a lag phase can be minimized and practically eliminated in control cultures by proper propagation of the pre-culture, a lag phase in exposed cultures may indicate recovery after initial toxic stress or reduced exposure due to loss of test substance (including sorption onto the algal biomass) after initial exposure. Hence the section-by-section growth rate may be assessed in order to evaluate effects of the test substance occurring during the exposure period. Substantial differences between the section-by-section growth rate and the average growth rate indicate deviation from constant exponential growth and that close examination of the growth curves is warranted.
Calculate the percent inhibition of growth rate for each treatment replicate from equation (2):

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \qquad (2)$$

where:
$\%I_r$: percent inhibition in average specific growth rate;
μ_C mean value for average specific growth rate (μ) in the control group;
μ_T average specific growth rate for the test material.
When the stability of test dispersions is achieved by the addition of dispersants, the dispersant controls rather than the controls without dispersants should be used in calculation of percent inhibition.

2.8.3. Plotting concentration response curve

Plot the percentage of inhibition against the logarithm of the test substance concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier in the first phase. Fit a line through the data points by computerized statistical method for interpolation.
Depending on the intended usage of data, the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures EC_{50} and EC_{10} from the fitted curve. Valid reasons for not using a statistical method may include:
-Data are not appropriate for computerized methods to produce any more reliable results than can be obtained by expert judgment - in such situations some computer programs may even fail to produce a reliable solution (iterations may not converge, etc.)
-Stimulatory growth responses cannot be handled adequately using available computer programs (see Section 8.5 below).

2.8.4. Statistical procedures

The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearizing transformation of the response data (for instance into probit, logit or Weibull units), but non-linear regression procedures are preferred techniques by OECD-201, in order to handle unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition, such irregularities may be magnified by the transformation, interfering with the analysis. Standard methods of analysis using probit, logit, or Weibull transforms are intended for use on quantal (e.g. mortality or survival) data, and must be modified to accommodate growth or biomass data. Specific procedures for determination of EC_X values from continuous data and the use of non-linear regression analysis are detailed and referred by OECD-201.
For each response variable to be analyzed, use the concentration-response relationship to calculate point estimates of EC_X values. When possible, the 95% confidence limits for each estimate should be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically. Regression analysis should be performed using individual replicate responses, not treatment group means. If, however nonlinear curve fitting is difficult or fails because of too great scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.
Endpoints estimates and confidence limits may also be obtained using linear interpolation with bootstrapping, if available regression models/methods are unsuitable for the data.

2.8.5. Growth stimulation

Growth stimulation (negative inhibition) at low concentrations is sometimes observed. This can result from either hormesis ("toxic stimulation") or from addition of stimulating growth factors with the test material to the minimal medium used. The addition of inorganic nutrients should not have any direct effect because the test medium should maintain a surplus of nutrients throughout the test. Low dose stimulation can usually be ignored in EC_{50} calculations unless it is extreme. However, if it is extreme, or an EC_X value for low x is to be calculated, special procedures may be needed. Deletion of stimulatory responses from the data analysis should be avoided if possible, and if available curve fitting software cannot accept minor stimulation, linear interpolation with bootstrapping can be used. If stimulation is extreme, use of a hormesis model may be considered, as specified in OECD-201.

2.8.6. Test report

The test report must include the following:

1. Test substance: nanomaterial identification from the suite of NANoREG Project.
2. Test species: the strain, supplier or source and the culture conditions used.
3. Test conditions:
   - -Date of start of the test and its duration.
   - -Description of test design: test flasks, culture volumes, biomass density at the beginning of the test.
   - -Test concentrations and replicates.
   - -Description of the preparation of test dispersions, including use of dispersants.
   - -Culturing apparatus.
   - -Light intensity and quality (source, homogeneity).
   - -Temperature.
   - -Concentrations tested: the nominal test concentrations and any results of analysis to determine the concentration of the test substance in the test flasks. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.
4. Results:
   - -pH values at the beginning and at the end of the test at all test substances.
   - -Biomass for each flask at each measuring point and method for measuring biomass.
   - -Growth curves (plot of biomass versus time).
   - -Calculated response variables for each test replicate, with mean values and coefficient of variation for replicates.
   - -Graphical presentation of the concentration/effect relationship.
   - -Estimates of toxicity for response variables (EC_{50} and EC_{10}) and associated confidence intervals.
   - -Any stimulation of growth found in any test substance.
   - -Any other observed effects, e.g. morphological changes of the algae.
   - -Discussion of the results, including any influence on the outcome of the test resulting from deviations from this procedure.

2.9. VALIDITY CRITERIA

1. The biomass in the control cultures should have increased exponentially by a factor corresponding to a specific growth rate between 1.5 and 1.7 day^{-1}. In tests performed with EDTA free growth medium this criterion is not applicable.
2. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2 and 2-3) in the control cultures must not exceed 35%. This criterion applies to the mean value of coefficients of variation calculated for replicate control cultures.
3. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%.
4. The pH in the controls shall not have increased by more than 1.5 units relative to the initial pH in the growth medium. For metals and compounds that partly ionize at a pH around the test pH, it may be necessary to limit the pH drift to obtain reproducible and well defined results. A drift of < 0.5 pH units is technically feasible and can be achieved by ensuring an adequate CO2 mass transfer rate from the surrounding air to the test dispersion, e.g. by increasing the shaking rate. Another possibility is to reduce the demand for CO2 by reducing the initial biomass or the test duration.

2.10. ACCURACY OF THE TEST

To prove the validity of the test system, it is recommended to test at least one reference substance (for instance when using a new strain, after changing test conditions, or every 5 to 10 assays). Results should be

compared to those given in Table A9. This table shows the results obtained with the reference substance $K_2Cr_2O_7$ for interlaboratory tests based on the tests specified in the ISO 8692:2012. At the time of publication, review of the reference tests indicated that the sensitivity of the strains had not changed significantly.

Table A9. Interlaboratory test results for EC_{50} in *Pseudokirchneriella subcapitata*

Test substance	Number of laboratories	Outliers	Mean value	Standard deviation	Coefficient of variation
Potassium dichromate	9[a]	4	1.19	0.27	23

[a] The high number of outliers is due to the use of different growth media (with different pH values). Results from the tests using media whose pH deviates from the growth medium specified in ISO 8692 were excluded.

2.11. STRAINS SHOWN TO BE SUITABLE FOR THE TEST

Pseudokirchneriella subcapitata from ATCC 22662 or CCAP 278/4, 61.81 SAG.
This strain is available in unialgal cultures from the following collections:

ATCC: American Type Culture Collection
10801 University Boulevard
Manassas, Virginia 20110-2209
USA

CCAP, Culture Collection of Algae and Protozoa
SAMS Research Services Ltd
Scottish Marine institute
OBAN
Argyll PA37 1QA
Scotland
UK

SAG: Collection of Algal Cultures
Inst. Plant Physiology
University of Göttingen
Nicholausberger Weg 18
D-3400 Göttingen
GERMANY

Appearance and characteristics of *Pseudokirchneriella subcapitata* species:

Appearance	Curved, twisted single cells
Size (L x W) µm	8-14 x 2-3
Cell volume (μm^3/cell)	40-60[1]
Cell dry weight (mg/cell)	2-3 x 10^{-8}
Growth rate[2] (day^{-1})	1.5 -1.7

[1] Measured with electronic particle counter
[2] Most frequently observed growth rate in OECD medium at light intensity approx. 70 µE m^{-2} s^{-1} and 21 °C.

2.12. REFERENCE TEST

In order to check the correct execution and the sensitivity of the test procedure, a reference test must be performed considering the specifications given in Section 2.10. This quality control test will be carried out with the reference chemical potassium dichromate ($K_2Cr_2O_7$).

A dilution series ranging from 2 mg/L to 0.2 mg/L has to be prepared following the guidelines given in section 5.3 Definitive test - A. C1-C5 spans one order of magnitude.

Procedure:

1. Take five 250 mL Erlenmeyer flasks and label them as C1 to C5.
2. Weigh 1 mg potassium dichromate on an analytical balance and transfer it into a 100 mL calibrated flask, adding algal growth medium up to the 100 mL mark and shake to dissolve the chemical and to obtain a 10 mg/L concentration.
3. Transfer the following volumes of toxicant dispersion from the 100 mL flask into the following flasks:

- 20 mL to C1
- 10 mL to C2
- 6 mL to C3
- 4 mL to C4
- 2 mL to C5

4. Add algal growth medium up to the 100 ml mark in the C1, C2, C3, C4 and C5 flasks.
5. Remove and discard 1 mL dispersion from each flask, in order to adjust the volume to prepare the appropriate algal concentrations.
6. Add 1 mL of the $5x10^5$cells/mL algal suspension to each flask to obtain an algal density of $5x10^3$cells/mL. Stopper and shake the flasks thoroughly to distribute the algal suspensions evenly.
7. Proceed to Section 2.6: Incubation.

2.13. REFERENCES

[1] OECD 201. GUIDELINES FOR THE TESTING OF CHEMICALS, Freshwater Alga and Cyanobacteria, Growth Inhibition Test; Organization for Economic Cooperation and Development (OECD): Paris, Adopted 23 March 2006 - corrected 28 July 2011.

[2] Philipp Mayer, Russell Cuhel and Niels Nyholm. 1997. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. *Wat Res* 31:2525-2531.

ANNEX 3. Standard Operating Procedure: Toxicity test with the nematode *Caenorhabditis elegans*

3.1. OBJECTIVE AND PRINCIPLE OF THE TEST

This protocol describes how to perform a chronic toxicity test on growth, fertility and reproduction of Caenorhabditis elegans to determine the ecotoxicity of specific core nanomaterials in the framework of the studies for NANoREG project.

The protocol is based on the International standard ISO 10872:2010 “Water Quality- Determination of the toxic effects of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)” [1].

Juvenile *C. elegans* are exposed to the NM dispersions at a range of concentrations over a period of 96 hours at 20 °C. In controls, the exposed organisms are able to complete a whole life cycle within this period. The endpoints are inhibition of growth, fertility and reproduction of exposed organisms compared to a control. The main change to the ISO protocol is that all test exposures are performed in low ionic strength aqueous media, rather than is soil or on agar plates. This is because the agar, or the standard nematode growth media can cause NM agglomeration and reduce NM toxicity [2].

3.2. MATERIALS AND EQUIPMENT

3.2.1. Reagents and apparatus

Reagents: All reagents used must be of analytical grade.
Water, MQ water (18.2 MΩ·cm at 25 °C).
LB-medium: 10 g Bacto Trypton, 5 g Yeast extract, 10 g NaCl, 1 L MQ water (autoclaved at 121 °C for 20 min).
Cholesterol stock solution: 500 mg of powder cholesterol dissolved in 100 mL absolute ethanol (>99 % purity) by stirring and gentle heating (keep < 50 °C).
S-BASE (with and without cholesterol): 3 g NaCl, 972 mL MQ water, 1 mL 1M $CaCl_2$, 1 mL 1 M $MgSO_4$, 25 mL 1M KPO_4 buffer, and 1 mL Cholesterol stock (excluded in the S-BASE without cholesterol).
Growth medium: There will be a possibility to choose one of two growth mediums.
EPA moderately hard reconstituted water (Table 1).
(http://water.epa.gov/scitech/methods/cwa/wet/upload/2007_07_10_methods_wet_disk2_atx7-10.pdf)
Simulated soil pore water (Table A11) [2].
There will also be possible to add NOM (Suwannee River NOM) if the scientist find it necessary.
Bleaching solution (2 mL Bleach (not germicidal), 7.5 mL 1M NaOH, 5.5 mL MQ water.

M9: 3 g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1 mL 1M $MgSO_4$, adjust total volume to 1 L with MQ water.
Bengal Rose stock solution: Add approximately 300 mg of Bengal Rose to 1000 mL of MQ water and stir thoroughly.
HCl (1 M)
NaOH (1 M)
Reference substance: $K_2Cr_2O_7$
Nanomaterials: The SOP "Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media" will be used in the preparations of the exposure suspensions.
Apparatus:
Autoclave
Incubator or temperature controlled room at 20±2 °C, dark
Spatulas (e.g. Drigalski spatula, glass spatula for distributing bacteria on an agar plate)
Plastic vials (autoclavable and sealed, volume 1.5 mL)
Pipettes
Beakers
Sterile 24 well plates
Analytical balance (4 digits)
Microscope
Shaker
Flame for sterilizing (e.g. Bunsen burner)
Waste bottle
Clean bench
Sterile petri dishes (3 cm, 6 cm or 10 cm in diameter)
Erlenmeyer flasks
Magnetic stirrer
pH meter
50 mL falcon tubes.
Drying oven, approximately 80 °C.
Centrifuge
Inoculating loop.
Waste bottles

3.3. TEST ORGANISMS: CAENORHABDITIS ELEGANS

C. elegans is usually referred to as a widespread, free-living soil nematode extensively used as a test organism in environmental and regulatory testing [2]. It is a bacterial feeder, a hermaphrodite that usually reproduce through self-fertilization, it has four life-stages from hatching to adult stage where the juvenile stages are separated by molts. Under harsh conditions (i.e. starvation), they form a developmentally arrested stage, the dauer larvae, as an alternative third larvae stage. The total life cycle for worms fed on *E. coli* is around 3 days at 20 °C (ISO 10872:2010).

It is of utmost importance to start the test with an age-synchronized population of *C.elegans*. This is achieved by bleaching, leaving only the eggs from the gravid worms as sole survivors.

3.3.1. Preparation of the culture medium

As stated by Tyne et al. [2] and others, the most commonly used growth mediums in *C. elegans* toxicity testing for traditional chemicals are the M9-buffer and K+ media. However, these mediums are not optimal for testing of NMs, due to their high chloride content and high ion strength (234 mM and 254 mM for the M9 and K+, respectively) which increase particle agglomeration. Nor are they ecologically relevant for the test organism [2]. Thus, in this SOP we suggest the use of one of two alternative exposure growth mediums: EPA moderately hard reconstituted water (EPA) or Simulated soil pore water (SSPW). The ion strength (approximately 4 mM and 10 mM for EPA and SSPW, respectively) and the chloride content is much lower for these two mediums, which will reduce particle agglomeration, and especially the SSPW is considered to be ecologically relevant.

Table A10. EPA moderately hard reconstituted water

(Stock solution) Nutrient	Concentration in stock solution g/L	Volume (mL) added of the stock per liter of EPA	Final concentration in culture medium
(1) $CaSO_4 \cdot 2H_2O$	0,5	120	60.0 mg/L / 0.348 mM

(2) $MgSO_4$	10	6	60.0 mg/L /0.498 mM
(3) $NaHCO_3$	10	9.6	96.0 mg/L / 1.143mM
(4) KCl	1	4	4.0 mg/L / 0,054 mM

Table A11. Simulated soil pore water

(Stock solution) Nutrient	Concentration in stock solution (M)	Volume (mL) added of the stock per liter of SSPW	Final concentration in culture medium (mM)
$NaHCO_3$	0.1	40	4
KNO_3	0.1	10	1
$Ca(NO_3)_2$	0.1	12.5	1.25
$MgSO_4$	0.1	5	0.5
Na_2HPO_4	0.1	0.01	0.001
HNO_3	0.1	10	1
Iron standard	0.0179 (1 g/L)	0.559	0.01
Aluminum standard	0.037 (1 g/L)	0.27	0.01
PPFA*	-	50 mg dry weight	

*If dissolved organic carbon component is required Pahokee peat fulvic acid (PPFA) can be used.

3.3.2. Preparation of organisms

Remember always to use sterilized equipment (laminar flow cabinet, bottles, tubes, hands etc.), sterilized using 70 % ethanol or heat (bunsenburner, autoclave).

3.3.3. *E. coli*: OP50

Incubation: Measure 100 mL of LB medium (see attachment for preparation procedure) into a 250 mL Erlenmeyer flask. Using an inoculating loop (sterilized over flame) gently pick one *E.coli* colony off the stock culture plate and transfer it into the LB media. Incubate over night at 37 °C with continuous shaking (200 rpm).

Washing of *E.coli* culture: To prepare the incubated *E.coli* cultures for use as food during pre-culturing and/or in the ecotoxicity test with *C. elegans*, they need to be washed to remove the culture medium (LB medium). When preparing for pre-culturing, these washing steps are performed in S-BASE. However, when preparing the final culture for the toxicity test, the washing steps are performed with the appropriate nematode culture media (in this case EPA or SSPW).

- Transfer the 100 mL incubated *E. coli* culture from the Erlenmeyer flask into two 50 mL Falcon tubes.
- Centrifuge for 10 min at 5000 rpm
- (A) Carefully remove the supernatants, leaving only bacteria pellet in the tube.
- (B) Fill the tubes with 50 mL autoclaved S-BASE (without cholesterol) solution or EPA/SSPW.

- (C) Centrifuge for 5 min at 5000 rpm.
- (D) Remove supernatants, leaving only the bacteria pellet in the tube.
- Repeat A-D once more.
- Place 100 mL S-BASE (with cholesterol) solution or EPA/SSPW into a sterilized Erlenmeyer flask.
- Add ~25 mL of the S-BASE or EPA/SSPW in the Erlenmeyer flask to the centrifugation tube using a pipette. Re-suspend the bacteria by gentle pipetting. Once re-suspended, transfer the suspension back to the Erlenmeyer flask.

3.3.4. *C. elegans*

The strain of *C. elegans* used in these toxicity tests is the wild-type N2. The pre-culturing can be performed either on agar plates according to the ISO 10872:2010, or in an aqueous media (modified S-BASE + cholesterol).

Pre-culturing on agar plate: Inoculate under sterile conditions on agar plate with approximately 200 µL of an overnight culture of E. coli and incubate it for at least 8 h at 37±2 °C. Then cut 2-3 small pieces of out of the starved plate with *C. elegans*, and transfer them under sterile conditions on to an agar plate with a fresh lawn of E. coli. After approximately 3-5 days gravid adults as well as juveniles in stage 1 and 2 will be found on the plate. To achieve an age synchronized population for the start of the test, the pre-culture needs to be bleached, leaving the eggs from the gravid adults as sole survivors.

Pre-culturing in aqueous media: S-BASE, *E.coli*, Nematodes from the main stock.

Transfer aseptically an E. coli colony from LB-agar plate and inoculate 100 mL LB. Incubate at 37 °C with shaking 2000 rpm overnight.

Harvest and wash the E. coli cells from the overnight culture by centrifugation as described (section 3.1).

Re-suspend the E. coli cells in 100 mL S-BASE cholesterol (5mg/L) in a 250 mL Erlenmeyer flask.

Take a NGM plate with a 3-5 days old *C. elegans* culture, add 5 mL of M9-buffer, swirl the liquid gently and transfer the nematodes by pipette to the S-base with E. coli.

Incubate at 20 C, in darkness with shaking (150 rpm) for 3-5 days.

Monitor daily the density and development of the nematodes to obtain high numbers of gravid adults and avoid dauer formation.

On the day before NM exposure, harvest nematodes and bleach as described below.

Bleaching: The bleaching is an effective method to achieve an age-synchronized population of nematodes.

Prepare the bleaching solution and M9 media (autoclaved without $MgSO_4$, add before use when cooled below 50 °C).

When pre-cultured on agar plate rinse the nematodes of the plate with M9 medium into 250 mL glass tubes.

When pre-cultured in aqueous media, split the content in each of the Erlenmeyer flasks (C.elegans+ S-Base) in 25 mL aliquots into 35 mL Corex glass tubes.

Centrifuge for 2 min, 400xg, at 20 °C.

Remove supernatant carefully using a pipette.

- A) Add 25 mL M9 (or MQ water), shake and centrifuge again for 2 min, 400xg, and carefully remove the supernatant.
- Repeat step A).
- Add 15 mL bleaching solution in each tube and leave it for 6 min. IMPORTANT: Shake continuously and vortex 10 sec/min. Check the bleaching efficiency with a stereomicroscope.
- Centrifuge 1 min at 1000xg.
- Carefully remove the supernatant.
- Add 25 mL M9 (gently trickle down the side of the tube, not disturbing the pellet, shake and centrifuge for 2 min at 3000xg. Place the tube so that the pellet is facing outward.
- Carefully remove supernatant.
- B) Add 15 mL of M9 and centrifuge for 2 min at 800xg, again place the tube so that the pellet is facing outward. Carefully remove the supernatant.
- Repeat step B). Increase speed on second centrifugation to 3000xg if the majority of the eggs are still not centrifuged down after the first centrifugation step at 800xg.

- Add 2 mL of M9, using a pasture pipette, carefully mixing the eggs with M9.
- Transfer the eggs and M9 to NMG plates (~1mL/plate).
- Incubate the plates in 20 °C, with gentle shaking (approx. 50rpm).
- These eggs will hatch into L1, which will be the age synchronized population of *C. elegans* used in the test. Needs to be used within 24 h?

3.4. PREPARATION OF TEST DILUTION SERIES

3.4.1. Preparation of aqueous dispersions of NMs

Follow the specific ecotoxicity SOP to prepare the required volume of NMs dispersions in ultrapure water.

When the stability of NMs dispersions is achieved by the addition of natural organic matter (NOM) or other substances, parallel toxicity tests must be performed to determine their effects on the nematode.

3.4.2. Preparation of dilution series

If the approximate toxicity of the NM to *C. elegans* is known, a definitive test can be performed immediately. However, if no information is available on its toxicity, two consecutive assays must be performed:
-A range finding test to determine the 0-100 % tolerance range of the nematode to the NM.
-A definitive test to determine with more precision the 50 % effect concentration (EC50).

The test is conducted in 24 well plates, with a total test volume of 1 mL per well. Each well contains the exposure media of choice (EPA or SSPW), E. coli OP50, NM and 10-20 juvenile L1 *C. elegans*.

Make up suspensions with a NM concentration twice the nominal concentration intended in the test, this will be diluted when mixing it with the *E. coli* suspension (OP50 in EPA/SSPW) and the *C. elegans* juvenile L1 (adding a given volume of a suspension of nematodes with a known density (e.g. 8-15 L1/5 µL, minimize the volume). The total volume of the *E. coli* suspension and the nematode suspension should be 500 µL, the volume of the NM suspension should be 500 µL, giving a total well volume of 1 mL.

RANGE FINDING TEST

A "tenfold" dilution series must be prepared, starting at the concentration of NMs required as the first dilution level (C1).

Take five 10 mL calibrated flasks and label them as follows: C1 - C2 - C3 - C4 - C5. As an example, Table A12 starts at 100 mg/L as the highest NMs concentration.

Table A12. Dilution series of the NMs

Flask	NMs concentration (mg/L)
C1	100
C2	10
C3	1
C4	0.1
C5	0.01

Shake thoroughly the dispersion of NMs prepared in 3.4.1. and transfer the required volume into the C1 flask to prepare the first dilution. Add culture medium up to the 10 mL mark.

Transfer 9 mL culture medium into all the other flasks (C2 to C5).

Stopper flask C1 and shake thoroughly to homogenize the dispersion of NMs. Transfer 1 mL of the flask C1 into the flask C2, in order to prepare the second test concentration.

Repeat the operation indicated in step 3 for flasks C2 to C5, i.e.:
- 1 mL from C2 to C3
- 1 mL from C3 to C4
- 1 mL from C4 to C5
Proceed to section 5: Filling of the test plate.

DEFINITIVE TEST

The dilution series to be prepared spans the range of the lowest concentration producing 100 % effect and the highest one producing less than 10 % effect in the range finding test. This range can span one order of

magnitude (case A) or two orders of magnitude (case B).The new concentration range to be tested out will again be called C1-C5.

A. C1-C5 spans one order of magnitude

Take five 10 mL calibrated flasks and label them as follows: C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 100% effect and C5 the highest that gave less than 10 % effect in the range finding test.

Take one 100 mL calibrated flask to make up 100 mL of the lowest concentration that produced 100 % effect. Transfer the following volumes of this concentration from the 100 mL flask into the other flasks:
- 10 mL to flask C1
- 5.5 mL to flask C2
- 3.2 mL to flask C3
- 1.8 mL to flask C4
- 1.0 mL to flask C5

Discard the content remaining in the 100 mL flask.

Add culture medium up to the 10 mL mark in flasks C2 to C5 (see Table A13).

Table A13. Dilution series C1-C5

Flask	C1 (mL)	Dilution medium (mL)
C1	10	0
C2	5.6	4.4
C3	3.2	6.8
C4	1.8	8.2
C5	1.0	9.0

Starting from the NMs concentration in flask C1, calculate the actual concentration in each flask (these figures will be needed for the endpoints estimation):
C1 = ...mg/L
C2 = 0.56 x C1 = ...mg/L
C3 = 0.32 x C1 = ...mg/L
C4 = 0.18 x C1 = ...mg/L
C5 = 0.10 x C1 = ...mg/L
Proceed to section 5: Filling of the test plate.

B. C1-C5 spans two orders of magnitude

Take five 10 mL calibrated flasks and label them as follows: C1 - C1 - C2 - C3 - C4 - C5. C1 is the lowest concentration that produced 100 % effect and C5 the highest that gave less than 10 % effect in the range finding test.

Make up the C1 flasks with 100 mL NMs concentration C1.

Transfer the following volumes of NMs dispersion from one of the two C1 flasks to the other flasks:
- 10 mL to flask C1
- 3.2 mL to flask C2
- 1.0 mL to flask C3
- 0.32 mL to flask C4
- 0.1 mL to flask C5

Discard the content remaining in the 10 mL flask.

Add culture medium up to the 10 mL mark in flasks C2 to C5 (see Table A14).

Table A14. Dilution series C1-C5

Flask	C1 (mL)	Dilution medium (mL)
C1	10	0
C2	3.2	6.8
C3	1.0	9.0
C4	0.32	9.68

C5	0.1	9.9

Starting from the NMs concentration in flask C1, calculate the actual concentration in each flask (these figures will be needed for the endpoints estimation):

Proceed to section 3.5: Filling of the Test Plate.

3.5. FILLING OF THE TEST PLATE

Each test concentration will be assayed in 4 replicates, the control in 8 replicates, for a statistically acceptable evaluation of the effects. In the range finder, replicates are not necessary.

The test is conducted in 24 well plates, with a total test volume of 1 mL per well. Each well contains the exposure media of choice (EPA or SSPW), *E. coli* OP50, NM and 10-20 juvenile L1 *C. elegans*.

Start by adding the *E. coli* OP50 suspension (X µL) and the *C. elegans* suspension (Y µL) into all wells (X depends on Y: X+Y = 500 µL, and Y depends on the density of the nematode suspension. Then add 500 µL of the different NM exposure suspension (with a NM concentration 2x the intended nominal concentration) into the four given wells per concentration, and only the growth media into the control wells.

To be able to calculate recovery, a quick screening of all/a representative sub-sample/ under a microscope should be conducted, counting the number of individuals added to each well.

When the plates are filled, seal them with adhesive tape or lids, incubate in the dark at 20±0.5 °C, shaking the plates gently. After 96 h, add 0.5 mL of Rose Bengal stock solution to each test well to stain the nematode cuticle for better recovery. Heat the multidishes with lid in a drying oven for 10 min at 80 °C to terminate the test. The Rose Bengal and heating results in straightened, easily measurable worms. Store the samples at 8±2 degrees until further processing. The samples should be processed within an eight week period.

To be able to estimate the growth in % of control, the size at time 0 is needed. Prepare a plate with *C. elegans* (at least N= 30, 10-20 individuals per well) at day 0, add Rose Bengal, and terminate the test by heating the plate for 10 min at 80 °C as described above.

3.6. MEASUREMENTS AND ANALYTICAL DETERMINATIONS

3.6.1. Recovery

After termination of the test after 96 h, count the number of adult individuals in each well, and calculate recovery by dividing the number of adults by the number of introduced organisms at the beginning of the test and multiply with 100.

3.6.2. Growth and fertility

ISO: Rinse the content of each well into separate petri dishes. Transfer the individuals to a microscopic slide using a stereo microscope (4-20 fold magnification). Measure body length of all adult exposed individuals under microscope using a measurement scale, and determine if eggs are present inside the body of each exposed individual. A worm is considered gravid if the number of eggs inside the body ≥1.

Alternative: Each well is inspected under a microscope and pictures are taken. The body length is then measured using the software ImageJ. The number of eggs in each adult exposed individual in each well is determined under the microscope.

Calculations: Mean body length are calculated for each replicate for further calculations. The replicate growth is the difference of the mean measured body length and mean body length of 30 J1 at the beginning of the test.

Calculate per replicate the % of gravid exposed test organisms in relation to the total number of recovered hermaphroditic exposed test organisms (fertility) by dividing the number of gravid exposed test organisms by the total number of recovered test organisms *100.

3.6.3. Reproduction

Count the number of offspring (second generation juveniles) by using a stereo microscope (4-20 fold magnification). Divide the number of offspring by the number of adults in the well. Express result as offspring per exposed test organism.

3.6.4. Quantitative and qualitative characterization of the NMs

The test dispersions will be analyzed to verify the initial NMs concentrations and maintenance of the exposure concentrations during the test. Aliquots of the test dispersions will be collected at the start and end of the test, and analyzed by the appropriate method (UV/vis absorbance, HR-ICP-MS/ICP-OES, DLS, etc.). Analysis of the concentration at the start and end of the test of a low and high test concentration and a concentration around the expected EC50 is sufficient when exposure concentrations vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test will be performed when concentrations do not remain within 80-120 % of nominal. Measurements of pH need to be conducted of test dispersions at the beginning and end of the test.

3.7. DATA TREATMENT AND REPORTING

3.7.1. Expression of results

Calculate mean and standard deviations for each treatment. Express the results as an inhibition of a test parameter, χt, expressed as % relative to the corresponding value in the control.

Plot the percentage of inhibition against the logarithm of the NM concentration and examine the plot closely, disregarding any such data point that was singled out as an outlier. Fit a line through the data points by computerized statistical method for interpolation. Depending on the intended usage of data, the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures EC50 and EC20 from the fitted curve. A valid reason for not using a statistical method might be that data are not appropriate for computerized methods to produce any more reliable results than can be obtained by expert judgment. In such situation some computer programs may even fail to produce a reliable solution (iterations may not converge, etc.)

3.7.2. 7.2 Statistical procedures

The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearizing transformation of the response data (for instance into probit, logit or Weibull units), but non-linear regression procedures allow the handling of unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total inhibition, such irregularities may be magnified by the transformation, interfering with the analysis.

Use the concentration-response relationship to calculate point estimates of ECX values. The 95% confidence limits for each estimate must be determined. Goodness of fit of the response data to the regression model should be assessed either graphically or statistically.

Regression analysis should be performed using individual replicate responses, not treatment group means. If, however nonlinear curve fitting is difficult or fails because of too great scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.

Endpoints estimates and confidence limits may also be obtained using linear interpolation with bootstrapping, if available regression models/methods are unsuitable for the data.

3.7.3. Test report

The test report must include the following:

Test substance: nanomaterial identification from the suite of NANoREG Project.

Test species and supplier or source and the culture conditions used.

Test conditions:

- Date of start of the test and its duration.
- Test concentrations and replicates.
- Description of the preparation of test dispersions, including use of dispersants.
- Culturing apparatus.
- Temperature. It is measured in control wells or in ambient air and it must be recorded preferably continuously during the test or at least at the beginning and end of the test.

- Concentrations tested: the nominal test concentrations and any results of analysis to determine the concentration of the test substance in the culturing apparatus. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.

Results:
- pH values at the beginning and at the end of the test at all test substances.
- Calculated response variables for each test replicate and mean values.
- Graphical representation of the concentration/effect relationship.
- Estimates of toxicity for response variables (EC50 and EC20) and associated confidence intervals.
- Any other observed effects, e.g. any abnormal behavior or appearance.
- Discussion of the results, including any influence on the outcome of the test resulting from deviations from this procedure.

3.8. VALIDITY OF THE TEST

The criteria for a valid test given in the ISO 10872 are:
- The mean recovery of exposed test organisms from the control is ≥80 % and ≤120 %
- The mean % of males in the control is ≤10 %, the % of males in single control replicates are ≤20 %
- The mean fertility in the control is ≥80 %
- The mean reproduction in the controls is ≥ 30 offspring per exposed test organism

However, due to the changes in growth media, these criteria might not be applicable in all cases.

3.9. REFERENCE TEST

In order to ensure the correct execution of the test procedure and the sensitivity of the test, it is advised to perform a reference test during the same month in which the test is carried out.

This quality control test may be carried out with the reference chemical benzylcetyldimethylammonium chloride monohydrate (BAC-C16), which has been shown to affect the growth of *C. elegans*. Growth inhibition is the only end point tested in the reference test. Perform the test according to ISO 10872, section 7 Reference substance.

3.10. REFERENCES

[1] International Organization for Standardization. 2010. Water Quality - Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda). ISO 10872:2010. Geneva, Switzerland.

[2] Tyne W, Lofts S, Spurgeon DJ, Jurkschat K, Svendsen C. 2013. A new medium for Caenorhabditis elegans toxicology and nanotoxicology studies designed to better reflect natural soil solution condition. *Environmental Toxicology and Chemistry* 32:1711-1717.

ANNEX 4. Standard Operating Procedure: Fish, Acute Toxicity Test

4.1. OBJECTIVE AND PRINCIPLE OF THE TEST

This procedure describes how to perform fish acute exposure to specific core nanomaterials in the framework of studies for NANoREG Project. The fish exposure to the nanomaterials should occur preferably for a period of 96 hours, and the mortalities are recorded at 24, 48, 72 and 96 hours and compared with control values. Concentrations which cause mortality of 50 percent of the fish (LC50) are estimated where possible. Adult fish are exposed to the NMs dispersions at a range of concentrations over a period of 96 h in test containers. A reliable analytical method for the quantification of the nanomaterial in the test solutions must also be available. For this purpose, we base protocol on the OECD-Guideline 203 "Fish, Acute Toxicity Test" [1].

4.2. MATERIALS AND EQUIPMENT

Beakers and flasks which will come into contact with the test dispersions should be made entirely of glass or other chemically inert material. The items should be thoroughly washed to ensure that no organic or inorganic contaminants may cause adverse effects on fish or interfere with the composition of the test dispersions. The following pieces of equipment are necessary:
- Oxygen meter;

- Apparatus for determination of water hardness;
- Adequate apparatus for temperature control;
- Tanks made of chemically inert material and appropriate capacity in relation to the recommended loading.
- Test organisms: species of fish from Table A15 may be used; the testing laboratory is free for choice of the species. It is recommended that the species are chosen taking into account their ready availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological aspects. It is necessary that fish to be healthy and without malformations. The fish species shown in the Table 1 could be cultivated in fish farms or in laboratory conditions, and must be free of disease and parasites, so that the test fish will be healthy and of known parentage. The fish must be maintained in culture conditions (light, temperature and medium) similar to those to be used in the test.

Fish must be maintained in the laboratory conditions and in good quality water for at least 12 days before their use in the tests. It is recommended that the following conditions to be followed at least seven days before testing
-Light: 12 to 16 hours photoperiod daily;
-Temperature: appropriate to the species (see Table A15);
-Oxygen concentration: at least 80 per cent of air saturation value;
-Feeding: three times per week or daily until 24 hours before the test is started.

After 48 hours of acclimatization, mortalities are recorded and the following criteria applied:
- mortalities greater than 10 per cent of population in seven days: rejection of entire batch;
- mortalities between 5 and 10 percent of population: acclimatization continued for seven additional days;
- mortalities less than 5 percent of population: acceptance of batch.

Table A15. Fish species indicated for testing according to OECD 1992.

Recommended species	Recommended test temperature range (°C)	Recommended total length of test fish (cm^{-1})
Brachydanio rerio (Teleostei, Cyprinidae) (Hamilton-Buchanan) Zebra-fish	21 - 25	2.0 ± 1.0
Pimephales promelas (Teleostei, Cyprinidae) (Rafinesque) Fathead Minnow	21 - 25	2.0 ± 1.0
Cyprinus carpio (Teleostei, Cyprinidae) (Linnaeus) Common carp	20 - 24	3.0 ± 1.0
Oryzias latipes (Teleostei, Cyprinodontidae) (Temminck and Schlegel) Ricefish	21 - 25	2.0 ± 1.0
Poecilia reticulata (Teleostei, Poecilidae) (Peters) Guppy	21 - 25	2.0 ± 1.0
Lepomis macrochirus (Teleostei, Centrarchidae) (Rafinesque) Bluegill	21 - 25	2.0 ± 1.0
Oncorhynchus mykiss (Teleostei, Salmonidae) (Walbaum) Rainbow trout	13 - 17	5.0 ± 1.0

Good quality of natural water, reconstituted water (see Table A16) or drinking water (dechlorinated, ifnecessary) may also be used. Values of pH between 6.0 to 8.5, and a total hardness of water between 10 and 250 mg $CaCO_3$ per liter are recommended.
Reagents for reconstituted water preparation should have analytical grade and the deionized or distilled water should have conductivity equal to or less than 10 µScm-1.

Table A16. Example of reconstituted water (ISO 6341-1982).

(Stock solution) Nutrient	Concentration in stock solution
(1) CaCl2·2H2O	11.76 g/L
(2) MgSO4·7H2O	4.93 g/L
(3) NaHCO3	2.59 g/L
(4) KCl	0.23 g/L

-The reconstituted water should be prepared using 25 ml of each solution (1) to (4) as listed in Table A16. They must be mixed with 900 mL of deionized water to reach 1 liter of medium. The sum of the calcium and magnesium ions in this medium is 2.5 mmol/L. The proportion Ca:Mg ions is 4:1 and Na:K ions 10:1. The acid capacity $K_{S4.3}$ of this solution is 0.8 mmol/L. Dilution water must be aerated until oxygen saturation is achieved, and stored for about two days without further aeration before using.

4.3. TEST SOLUTIONS

It is recommended to follow the specific dispersion SOP to prepare the required volume of NM dispersions in ultrapure water. If the NMs dispersion does not exhibit good stability, it is possible the addition of natural organic matter (NOM) according to the "enhanced dispersion SOP" that uses Suwannee River NOM. However, if even using this "enhanced dispersion SOP", the NMs stability is not achieved, it is recommended the use of NOM directly in the test medium. The test laboratory should evaluate if the latter is necessary for a specific NM. The concentration of 10 mg/L of NOM is suggested for the test medium. It worth to mention that if NOM is used, parallel toxicity tests are recommended to determine the effects of the addition of NOM on fish.

It is not necessary to perform adjustment of pH during the test. If any evidence of changes in the pH of the container water becomes visible after the addition of the test nanomaterial, it is recommended to repeat the test, adjusting the pH of the stock solution to that of the tank water before the addition of the test nanomaterial. The pH adjustment should be made in such way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test nanomaterial is caused. Solutions of HCl and NaOH are preferred for adjusting of pH.

If toxicity of the NM to fish is known, a definitive test can be performed immediately. However, if no information is available on its toxicity, two consecutive assays must be performed:
-A range finding test to determine the 0-100% tolerance range of the fish to the NM.
-A definitive test to determine with more precision the 50% lethal concentration (LC50).

4.4. TEST PROCEDURE

- Test should be performed preferably during a period of 96 hours.
- A maximum loading of 1.0 g fish/liter for static and semi-static tests is recommended; for flow-through systems higher loading can be accepted (see Annex 1 for definitions).
- It is recommended a daily photoperiod of 12 to 16 hours of light.
- The appropriate temperature to the species should be maintained (see Table A15) within a range of ±2°C.
- At least 60 percent of air saturation is recommended for the oxygen concentration in test medium. Aeration can be provided since it does not lead to a significant loss of test nanomaterials.
- The animals should not be fed during the test; feeding should be stopped 24 hours before the test.
- Any disturb that may change the behavior of the fish should be avoided.
- It is recommended to use at least seven fish in each test concentration and in the controls.
- Geometric series of at least five concentrations with a factor preferably not exceeding 2.2 should be used.
-Dilutions series should be prepared before the definitive test for the choice of the concentration range that result in the lowest concentration producing 100% of mortality and in the highest one producing less than 10% of mortality.
-One control group (blank) without the addition of nanomaterial and one control containing the dispersing agent (if necessary) should be run in addition to the test series.
-Test should be evaluated at least after 24, 48, 72 and 96 hours. The evaluation consists in recording and removing the dead fish. Dead fish are considered those that do not produce visible gills movement, or that do not produce reaction when touching of the caudal peduncle. It is recommended observations at three and six hours after the start of the test. Observation concerning abnormalities (e.g. loss of equilibrium, swimming

behavior, respiratory function, pigmentation, etc.) should be recorded. Daily measurement of pH, dissolved oxygen and temperature should be carried out.
- Using the proceeding described in this SOP, a limit test may be achieved at 100 mg/L in order to demonstrate that the estimated LC50 is greater than this concentration. A minimum of 7 fish should be performed for the limit test, with the same number in the control(s). If any mortality is observed, a complete study should be conducted. If sublethal effects are observed, these effects should be recorded.

4.5. MEASUREMENTS AND ANALYTICAL DETERMINATIONS

After 96 h of exposure, the total number of dead fish for each NM concentration must be estimated, together with its percentage of lethality. Data may be summarized in tabular form, showing the number of fish used and mortality at each observation for each concentration and control. The percentages of mortalities at 24; 48; 72 e 96 hours should be plotted against test concentrations. Data are analyzed by appropriate statistical methods (see Section 4.6) to calculate the slopes of the curves and the LC50 with 95% confidence limits (p = 0.95). In cases where the standard methods of calculating the LC50 are not applicable to the data obtained, the highest concentration causing no mortality and the lowest concentration producing 100 % mortality should be used as an approximation for the LC50 (considering the geometric mean of these two concentrations).

The test dispersions can be analyzed to verify the initial NMs concentrations and maintenance of the exposure concentrations during the test. At the start and end of the test, collect aliquots of the test dispersions to be analyzed by an appropriate method (UV/vis absorbance, HR-ICP-MS/ICP-OES, DLS, etc.).

These determinations can be performed in several containers at each test concentration for statistical analysis. For unstable NMs, additional sampling for analysis at 24 hour intervals during the exposure period will be prepared in order to better define the loss of the test substance. Analysis of the concentration at the start and end of the test of a low and high test concentration, and a concentration around the expected LC50 is sufficient when exposure concentrations vary less than 20% from nominal values during the test. Analysis of all test concentrations at the beginning and at the end of the test should be performed when concentrations do not remain within 80-120% of the nominal concentration.

4.6. DATA TREATMENT AND REPORTING

4.6.1. Plotting concentration response curve

Plot the percentage of mortality against the logarithm of the NM concentration and examine the plot closely, disregarding any data point that was singled out as an outlier. Fit a line through the data points by computerized statistical method for interpolation.

Depending on the intended usage of data, the quality (precision) and amount of data as well as the availability of data analysis tools, it may be decided (and sometimes well justified) to stop the data analysis at this stage and simply read the key figures LC50 and LC10 from the fitted curve. When the data obtained are not appropriated for LC50 calculation, the highest concentration producing no mortality and the lowest concentration causing 100 percent mortality should be used as an approximation for the LC50 (the geometric mean of these two concentrations could be considered).

4.6.2. Statistical procedures

The aim is to obtain a quantitative concentration-response relationship by regression analysis. It is possible to use a weighted linear regression after having performed a linearizing transformation of the response data (for instance, into probit, logit or Weibull units), but non-linear regression procedures allow the handling of unavoidable data irregularities and deviations from smooth distributions. Approaching either zero or total mortality, such irregularities may be magnified by the transformation, interfering with the analysis.

Use the concentration-response relationship to calculate point estimates of LCX values. The 95% confidence limits for each estimate must be determined. Quality of fitting of the response data to the regression model should be assessed either graphically or statistically.

Regression analysis should be performed using individual replicate responses, instead of treatment group means. However, if nonlinear curve fitting is difficult or fails because of scatter in the data, the problem may be circumvented by performing the regression on group means as a practical way of reducing the influence of suspected outliers. Use of this option should be identified as a deviation from normal procedure because curve fits with individual replicates did not produce a good result.

Endpoints estimates and confidence limits may also be obtained using linear interpolation with bootstrapping, if available regression models/methods are unsuitable for the data.

4.6.3. Test report

The test report must include the following:

1. Test substance: nanomaterial identification from the suite of NANoREG Project.

2. Test fish: scientific name, strain, size, supplier, any pretreatment, supplier or and the culture conditions used.

3. Test conditions:
-Test procedure used (e.g. static, semi-static, flow-through, aeration, fish loading; etc.);
-Water quality characteristics (pH, hardness, temperature);
-Dissolved oxygen concentration, pH values and temperature of the test solutions at 24 hours intervals (in semi-static systems the pH should be measured prior and after water renewal).
-Methods of preparation of stock and test solutions;
-Description of the preparation of test dispersions, including use of NOM in Milli-Q water or in the test medium;
-Test concentrations and replicates;
-Concentrations tested: the nominal test concentrations and any results of analysis to determine the concentration of the test substance in the culturing apparatus. The recovery efficiency of the method and the limit of quantification in the test matrix should be reported.

4. Results:
- maximum concentration causing no mortality within the period of the test;
- minimum concentration causing 100 percent mortality within the period of the test;
- cumulative mortality at each concentration at the recommended observation times;
- LC50 and LC10 values, with 95 percent confidence limits, at each one of the recommended observation times;
- graph of the concentration-mortality curve at the end of the test;
- statistical procedures used for determining the LC50 values;
- mortality in the controls;
- incidents in the course of the test which might have influenced the results;
- abnormal responses of the fish.
-Discussion of the results, including any influence on the outcome of the test resulting from deviations from this procedure.

4.7. VALIDITY OF THE TEST

For a test to be valid the following conditions should be fulfilled:
-the mortality in the control(s) should not exceed 10 percent (or one fish if less than ten are used) at the end of the test;
-constant conditions should be maintained as far as possible throughout the test and, if necessary, semistatic or flow-through procedures should be used (see Section 4.8 for definitions);
-the dissolved oxygen concentration must have been at least 60% of the air saturation value throughout the test;
-there must be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 percent of the nominal concentration throughout the test. If the deviation from the nominal concentration is greater than 20 percent, results should be based on the measured concentration.

4.8. DEFINITIONS (OECD, 1992)

Static test is a test with aquatic organisms in which no flow of test solution occurs. Solutions remain unchanged throughout the duration of the test.

Semi-static test is a test without flow of solution, but with occasional batch wise renewal of the test solution after prolonged periods (e.g. 24 hours).

Flow-through test is a test in which solutions are automatically and continually renewed in the test chambers, the displaced solutions running to waste.

LC50 in this Test Guideline is the median lethal concentration, i.e. that concentration of the test substance in water which kills 50 percent of a test batch of fish within a particular period of exposure (which must be stated).

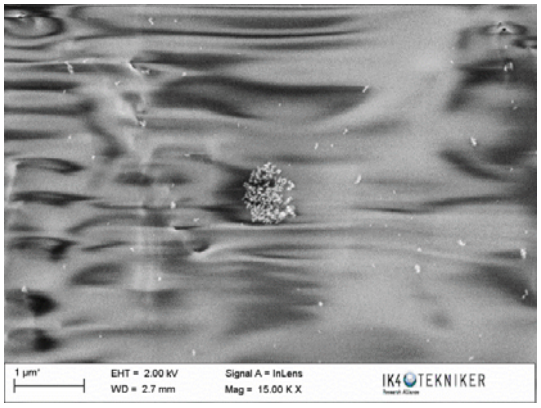
4.9. REFERENCES

[1] OECD 203. GUIDELINE FOR TESTING OF CHEMICALS, Fish, Acute Toxicity Test; Organization for Economic Cooperation and Development (OECD): Paris, 17 July 1992.

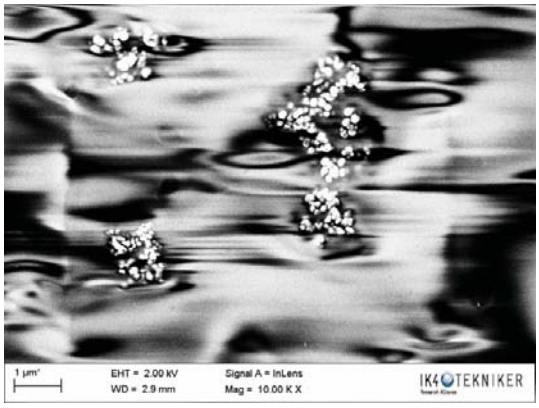
ANNEX 5. Microscopy verification (SEM) of the NANoREG core MNMs during the ecotoxicity tests

Scanning electronic microscopy images corresponding to the tests performed by IK4-Tekniker are provided below:

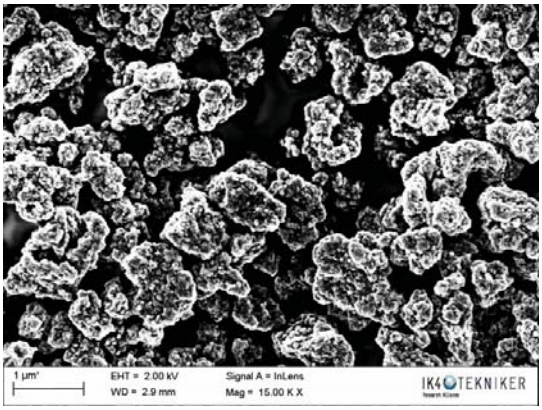
A



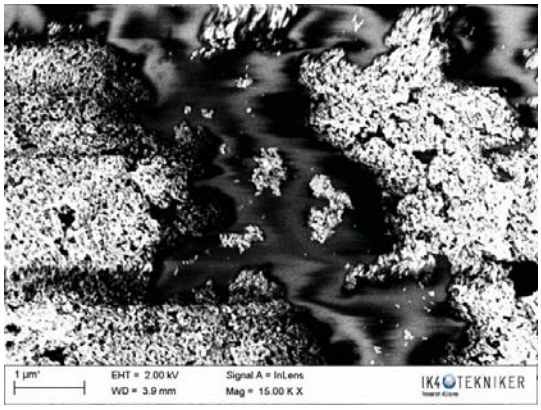
B



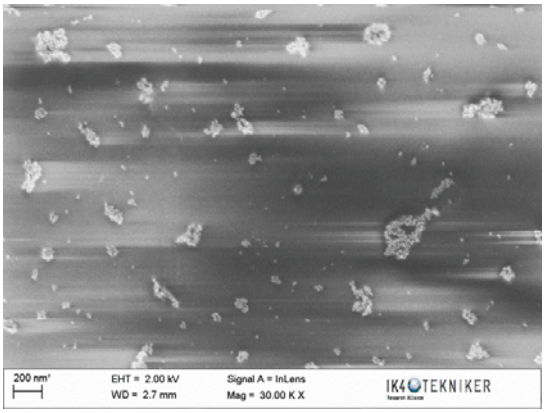
C



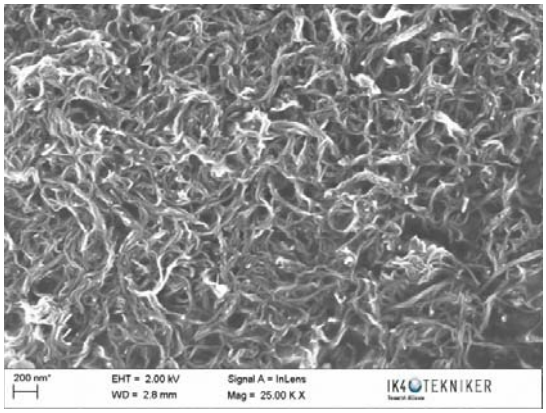
D



E



F



G

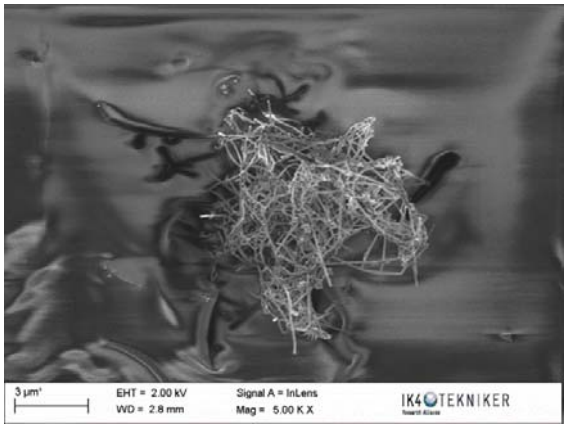
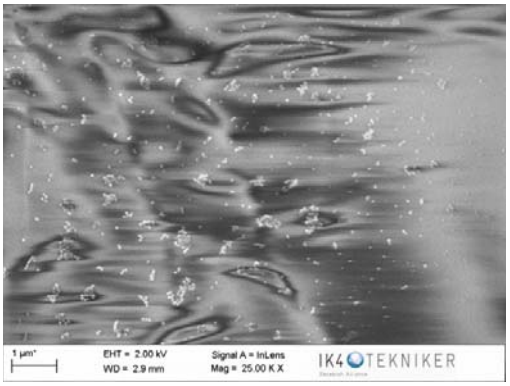


Figure A1. SEM images of NANoREG MNMs dispersions prepared in Milli-Q water for the '*Validation of the dispersion protocol for MNMs in environmental exposure media*': (A) CeO_2 NPs (JRCNM02102a); (B) TiO_2 NPs (JRCNM01000a); (C) TiO_2 NPs (JRCNM01001a); (D) TiO_2 NPs (JRCNM01003a); (E) Ag NPs (JRCNM03000a); (F) MWCNTs (JRCNM04000a); (G) MWCNTs (JRCNM04001a).

A



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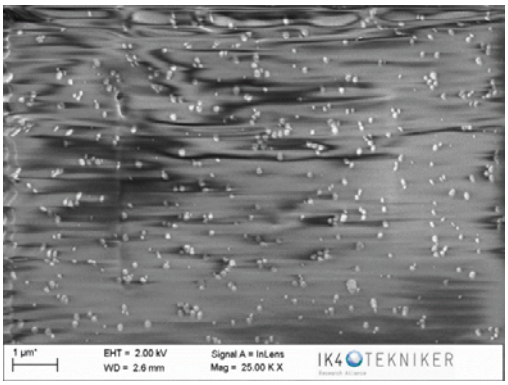
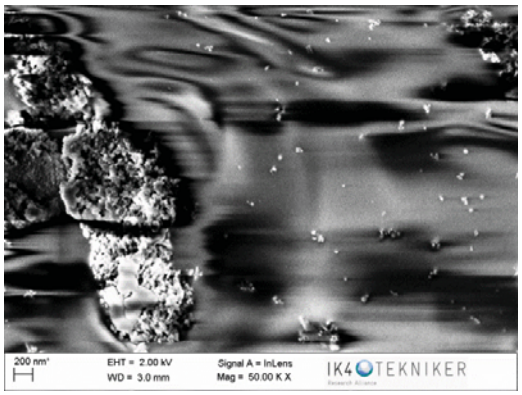


C



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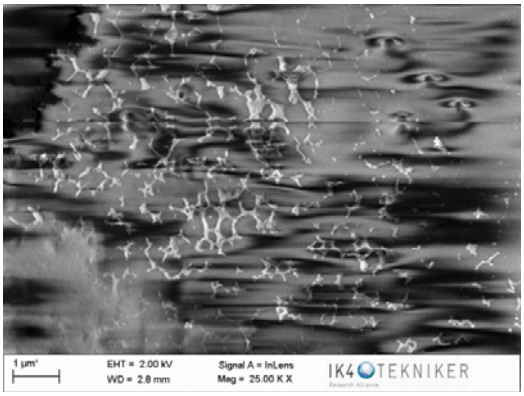

Figure A2. SEM images of NANoREG MNMs dispersions prepared in *D. magna* exposure medium for the tests performed by IK4-Tekniker. Tests corresponding to JRCNM03000a (replicate 2) and JRCNM02000a (replicate 1): (A) 0.003 mg/L Ag NPs-0h, (B) 0.325 mg/L Ag NPs-72h, (C) 100 mg/L SiO_2 NPs-0h, (D) 1 mg/L SiO_2 NPs-72h.

A

B

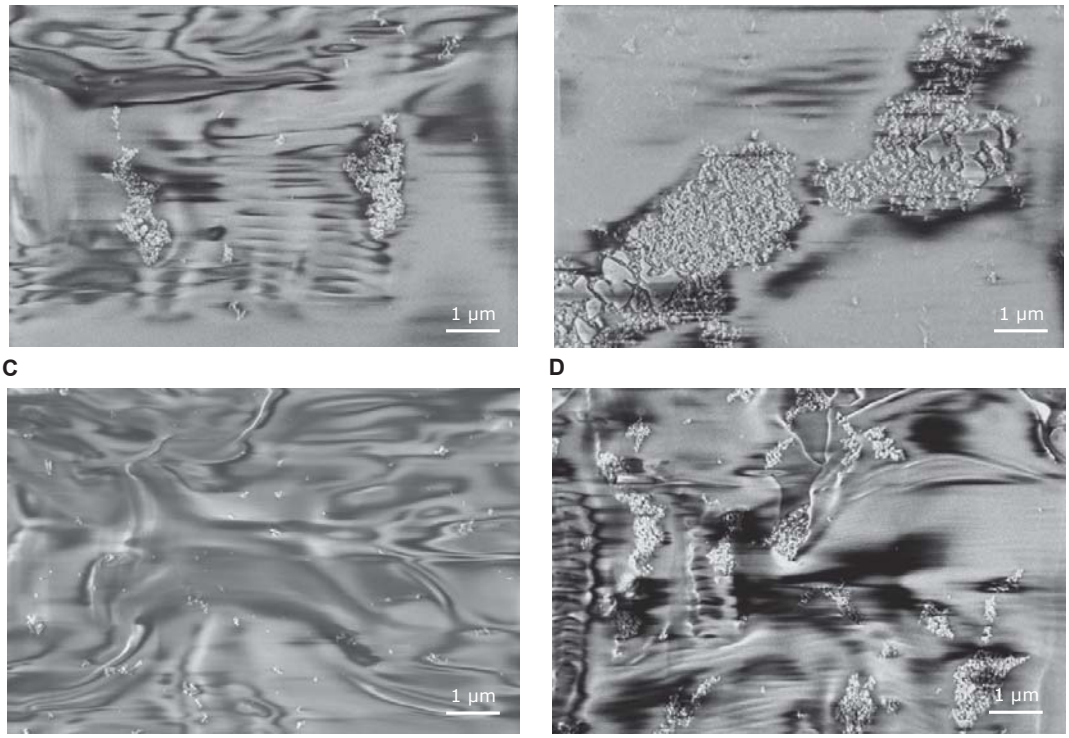

Figure A3. SEM images of NANoREG MNMs dispersions prepared in *P. subcapitata* exposure medium for the tests performed by IK4-Tekniker. Tests corresponding to JRCNM02102a (replicates 1 and 3): (A) 160 mg/L mg/L CeO_2 NPs-0h, (B) 160 mg/L CeO_2 NPs-72h, (C) 160 mg/L CeO_2 NPs + 20 mg/L SR-NOM - 0h, (D) 160 mg/L CeO_2 NPs+ 20 mg/L SR-NOM - 72h.

A B

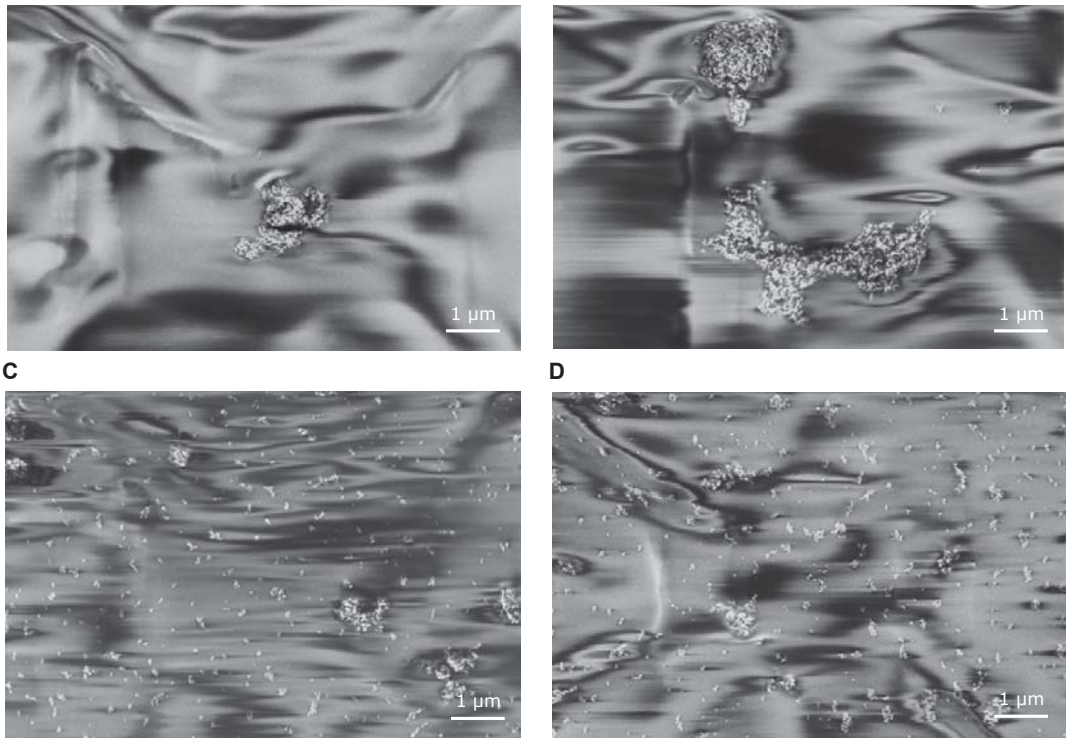

Figure A4. SEM images of NANoREG MNMs dispersions prepared in *P. subcapitata* exposure medium for the tests performed by IK4-Tekniker. Tests corresponding to JRCNM01003a (replicates 1 and 3): (A) 160 mg/L mg/L TiO_2 NPs-0h, (B) 160 mg/L TiO_2 NPs-72h, (C) 160 mg/L TiO_2 NPs + 20 mg/L SR-NOM - 0h, (D) 160 mg/L TiO_2 NPs+ 20 mg/L SR-NOM - 72h.

A **B**

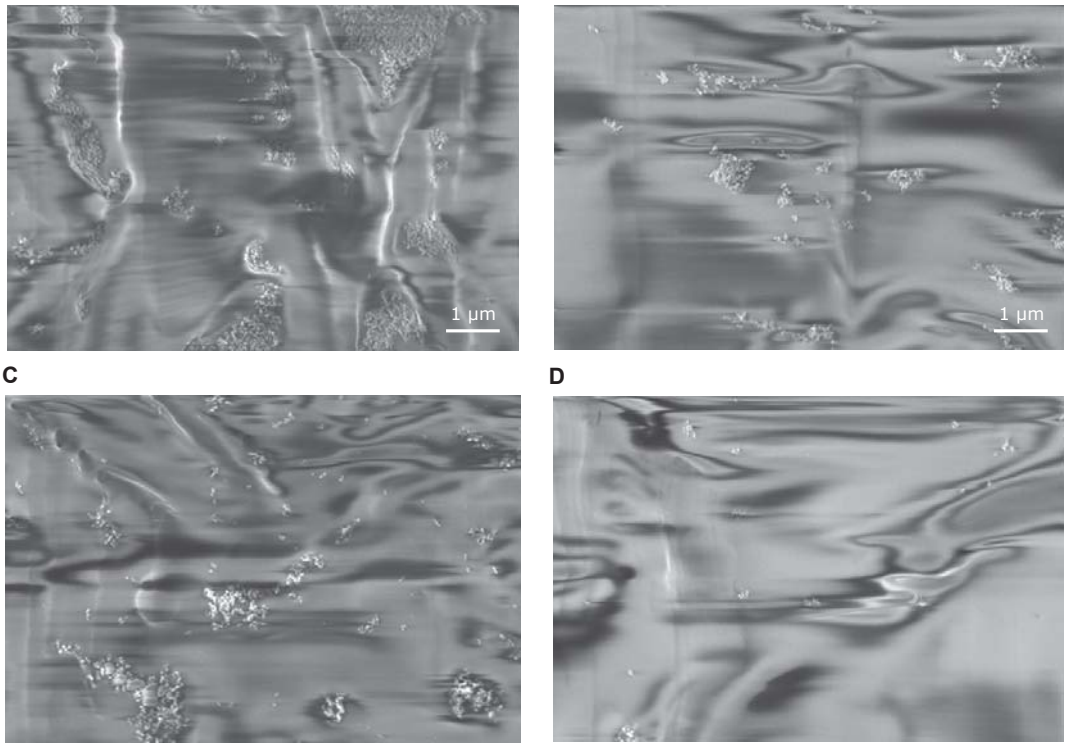

Figure A5. SEM images of NANoREG MNMs dispersions prepared in *P. subcapitata* exposure medium for the tests performed by IK4-Tekniker. Tests corresponding to JRCNM02102a and JRCNM01003a (replicates 2): (A) 160 mg/L mg/L CeO_2 NPs + 8 mg/L SR-NOM - 0h, (B) 160 mg/L CeO_2 NPs + 8 mg/L SR-NOM - 72h, (C) 160 mg/L TiO_2 NPs + 8 mg/L SR-NOM - 0h, (D) 160 mg/L TiO_2 NPs + 8 mg/L SR-NOM - 72h.