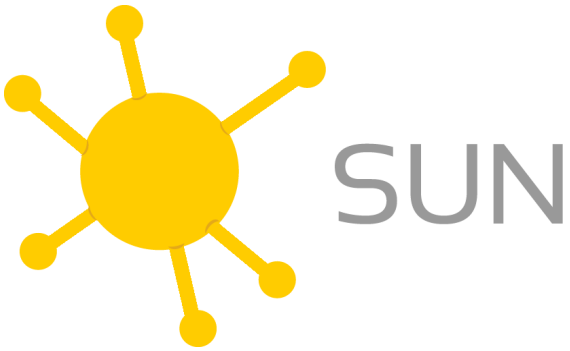

Deliverable report for

SUN

Sustainable Nanotechnologies

Grant Agreement Number 604305

Deliverable D 4.3
Integrated risk evaluation of the consequences of NOAA on ecosystem services

Due date of deliverable: 31/01/2017
Actual submission date: 20/04/2017

Lead beneficiary for this deliverable: ***Aarhus University – AU***

Dissemination Level:		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Table of Contents

Description of Task

DOW text: We will base the tools for risk analysis on the biological function of sewage treatment plants (including studies on the NOAA partitioning within the treatment plant and the effluent and waste) on data from Tasks 4.1, T 4.2 and WP3. This will be linked to the soil, sediment, and water risk analysis.
The models and tools from Tasks 4.1, 4.2 and WP3 on the structure and function (including trophic transfer) of soil, sediment and water will result in the first fully probabilistic ecological Risk Assessment tool, including probabilistic evaluation of environmental distribution, - exposure scenarios, and species sensitivity distributions. The importance of multiple stressors will be included in the risk analysis, comprising the related uncertainty analysis. The probabilistic ecological RA tool will be included in the SUNDS as a specific module, which results will be integrated with both LCA and human health RA to support the selection of most suitable risk reduction measures. As the RA tools (for both exposure and effects) will include probabilistic assessment in most facets, the evaluation of the potential risk can enlighten where the most effective mitigation action can be performed e.g. where emission is best reduced in a cost-benefit analysis (with LCA) compared to risk.

Long-term risks based on epigenetic changes are not included in the European Risk Assessment, hence the task is to develop tools to evaluate whether such change has sufficient grounds for being incorporated and, in case, how this could be achieved. The influence of using rapid tools as predictors of long-term effects in RA will be evaluated, as well as their use as screening tools to group or classify NOAA prior to Risk Assessment. This will be performed together with WP6 on human health to cross-extrapolate relevant information.

Evaluation of tools to estimate NOAA trophic transfer, including risk evaluation for secondary poisoning of fish, birds and small mammals [fish (see Task 4.1), birds (toxicity and assimilation models from literature), and small mammals (toxicity values from WP6]. For both birds and small mammals secondary poisoning NOAA tools will be developed for several tiers, e.g. worm bioaccumulation and small mammal food ingestion. For human exposure the fish and plant bioaccumulation will be combined with relevant food consumption/assimilation (see Task 4.1) done in combination with WP6. Studies on long-term and trophic transfer has only been performed to a very limited extend, however models will be applied on the collected and newly produced data. This latter part will include development of probabilistic models for bioaccumulation-trophic transfer.

Description of work & main achievements

The reporting in this deliverable has integrated information from Del 4.1 and Del 4.2 into a risk assessment perspective. There may be some overlapping to avoid that the reader has to go back Del 4.1 and 4.2.

The eco-toxicological results from the tools developed in Tasks 4.1, 4.2 has been included in the first fully probabilistic ecological Risk Assessment tool in the SUNDS model (see WP8). Hence, in this report, we will not discuss the probabilistic risk assessment in further detail, but we will take a closer look to how the individual tests/tools systems can provide a valuable information for the risk assessor.

In general the main achievement for the WP4 is that tools developed will make the assessor able to understand what are the long-term hazards, and in addition to this where and how will toxicity occur and what is the likely mechanism. The latter is in line with Adverse Outcome Pathway requested by regulators. By having such information the risk assessor will be able to define the appropriate risk mitigate strategy. The producers (companies) will be able to use the tools to design materials safer by design e.g. by understanding the toxicity mechanisms and linking tis to

the material characters.
The tools developed (incl. Del 4.1 and 4.2) cover the function of the Sewage Sludge Treatment Plant, and the Soil, water and Sediment ecosystem. In case there is a requirement for both a generic and local risk assessment, then the tools can be applied to other species.

Within SUN several case studies were performed, the degree of testing mainly depending on amount of material available. The most complete dataset is for CuO nanomaterials; hence, example of CuO NP results will be included below.

[A note to the reader: In the following pages – the text volume per area does not reflect its importance nor the amount of work performed. It is a balance between the reporting of each item among the three deliverables (4.1, 4.2, and 4.3), and it reflects the difference in reporting style/word use for the various partners. The total number of pages has been minimised, more data is available in the publications.]

Develop tools to estimate long-term effects

Sewage treatment plants (STP)

Sewage Treatment Plants and Microbial studies
Fate and effects in a STP and long-term effects of nanomaterials in soil under enhanced environmental conditions.

In this part, nanomaterials were added continuously with the synthetic sewage to a model STP, while deriving the effect on the microbial degradation activity. The simulated STPs were equipped with a denitrification, nitrification and secondary clarifier. The experiments were conducted as described in the OECD guideline 303A with a control treatment and treatments with different influent concentrations of nanomaterials varying from 0.04 to 1.0 mg/L. Here, the concentrations represent environmental relevant concentrations (0.04 mg/L) and a worst-case scenario (1 mg/L). The nanomaterials were dosed into the denitrification section of the STP continuously over 10 days. Beforehand the nanomaterials were mixed within a tube system with synthetic sewage to allow chemical reaction of the nanomaterials with the surrounding media, to simulate most realistic the fate of the nanomaterials in a sewer system before they reach the STP. The amounts of nanomaterials in the effluent and sludge scenarios were determined exemplarily.

After 10 days continuous addition of the nanomaterial, the sludge was dewatered and added to the soil in accordance with the German sewage sludge ordinance, which states 5 tons per hectare over 3 years can be spread on agricultural areas. Respectively 1.67 g of dry matter sludge could be introduced into the soil, under the assumption of a soil depth of 20 cm and soil density of 1.5 g/m^3.

After the application of the sewage sludge into soil, samples were taken, for chemical analysis to verify the nanomaterial concentration in the soil-sludge mixture. In addition, the soil was incubated at 20°C in an incubation chamber in the dark. Frequently the soil was circulated to avoid anaerobic areas and the water loss due to evaporation was compensated. The long-term effect of the nanomaterials added via sewage sludge on the soil microorganisms was investigated after 30, 60, 100 and 140 days of incubation.

Due to the high concentration of nitrate from the sewage sludge used in the long-term effect tests measurement of the nitrate concentration as described in OECD guideline 216 was not performed. Instead, the nitrite concentration was determined with a short-term potential ammonium oxidation test performed in accordance with the ISO guideline 15685.
There was no impact on the biological function/microbial activity of the sewage sludge due to any of the tested nanomaterials (Pristine CuO, Fe_2O_3 P.Red101, WCCo-1NP-SYN and the fragmented

Fe_2O_3_PE_FP 2) neither on the elimination of dissolved organic carbon nor on the denitrification and nitrification processes.

During the 10 days of nanomaterial addition, daily samples were taken from the influent, effluent and of the sewage sludge after test termination to determine the fate of the nanomaterials. By testing WCCo-1NP-SYN, samples were sent to the partner Veneto Nanotech that analysed the samples for their tungsten/cobalt concentration in sludge and afterwards in soil-sludge-mixtures.

In general, for WCCo-1NP-SYN and copper oxide nanomaterials we found a high sorption rate of the nanomaterials to sewage sludge. For the WCCo-1NP-SYN 91% of the nanomaterial at the low concentration of 0.04 mg/L was adsorbed to the sludge, whereas the sorption at the higher concentration of 1 mg/L was slightly lower with 74%. A similar result was found for the CuO nanomaterials. Here, 105% of the CuO was recovered in the sludge at the environmental relevant concentration of 0.04 mg/L, whereas around 71% was found at a high influent concentration of 1 mg/L. No determination of the fate was done in this first test, since another test was planned for the pristine and fragmented Fe_2O_3 nanomaterials.

In the long-term experiments to determine the effect of the pristine and fragmented products over 140 days the pristine Fe_2O_3 P.Red101 showed no effect on the activity of the ammonium oxidizing bacteria over the whole test period. For WCCo-1NP-SYN we found a slight inhibition of 33.5% and 29.9% at measured concentrations of 0.30 and 5.4 mg/kg dm soil, respectively, after 30 days incubation. However, after 60, 100 and 140 days no statistically significant difference to the control treatment including uncontaminated sludge was found. The test was performed repeated afterwards with the same sludge applied into soil and the effect after 15, 30 and 60 days was determined. Here, after 15 days 20.8% and 10.0% inhibition at measured concentrations of 0.3 and 5.4 mg/kg dm soil were found but no statistically significant differences to the control after 30 and 60 days. Therefore, the effects were reproducible but they were not concentration dependent and short-term effects. The pristine WCCo-1NP-SYN applied without sewage sludge induced no effects on the activity of the ammonium oxidizing bacteria after 28 days. The treatment of the WCCo-1NP-SYN in the STP and the application via sewage sludge into soil might have modified or transformed the nanomaterial followed by a slight short-term inhibition. This fact indicates the importance of a testing of nanomaterials under enhanced environmental conditions.

For the pristine CuO NMs long-term tests were performed twice. In the first long-term test an inhibition of the activity of the ammonium oxidizing bacteria was determined after 100 days, ranging from 20.1% to 28.3% at measured concentrations of 0.31 and 5.57 mg CuO/kg dm soil compared to the control. There was no effect determined after 30 and 60 days incubation. After test termination of the STP the sludge was frozen at -20°C for three month, afterwards melted, and again used for a long-term test over 140 days. In this repetition of the first test from day 60 to day 140 an inhibition of 24.2 up to 75.5% was found in the lower test concentration of 0.31 mg/kg dm soil, whereas it was statistically significant different (28.3%) from the control treatment only after 140 days at the highest test concentration of 5.57 mg/kg dm soil. The results in the highest test concentration were comparable, while the effect of nanomaterials in the lower test concentration increased many times. Due to this, we performed a second STP simulation with the influent concentrations of 0.04, 0.4 and 1 mg/L, dewatered the sludge after 10 days and applied it into test soil. Here, again we found different effects with high effects (20.8 and 34.6% inhibition) on the activity of the ammonium oxidizing bacteria at 0.04 and 1 mg/L after 30 days. After 60 days incubation we found no inhibition of the soil microflora at any of the tested concentrations.

Differences between the first test and its repetition can be explained by the storage of the sewage sludge in a freezer. The storage conditions and the process of melting before the application can

influence the nanomaterial and enhance the release of ions from the pristine CuO nanomaterial. Therefore, we found a higher toxicity with increasing incubation duration.

The formation of agglomerates that reduced the toxicity at a higher concentration, whereas smaller agglomerates or single nanoparticles were available at the lower concentration can explain the higher toxicity at the lower concentration.
The lowest inhibition found in the test performed after the second STP simulation can be explained due to a shift in the soil pH. There were around 6 month between the first tests and the last test and the soil used was stored in stainless steel vessels outdoor. Due to this, the start pH in the first tests was ~4.5, whereas it was ~5 in the last test. The pH is an important factor for the toxicity of ion releasing nanomaterials. We found a stronger toxicity in the tests with a lower pH, which supports this suggestion.

To improve the case study for pristine and fragmented CuO nanomaterials a simulated sewage treatment plant with fragmented CuO nanomaterials and subsequent long-term test regarding the effects of these fragmented CuO nanomaterials over 140 days should have been started in September 2016. Unfortunately, the fragmented CuO product was not available in a sufficient amount and time and therefore, a STP simulation with a laboratory scale STP and a subsequent long-term test investigating the effects of the pristine Fe_2O_3 P.Red 101 and the corresponding fragmented product Fe_2O_3_PE_FP_2 were conducted. Over 10 days adequate amounts of the test material corresponding to influent concentrations of 0.4 and 1.0 mg/L were added into the denitrification vessel of the STP. Both, the pristine and the fragmented iron oxide nanomaterial, did not affect the biological function of the sewage treatment plant. Sewage sludge samples were analysed for the iron content to determine whether the nanomaterials were adsorbed to the sewage sludge or passed the STP by the effluent. Unfortunately, due to the high background of iron in the sewage sludge (4.5 g/kg dm sludge) and the soil (5 g/kg dm soil) which was used for the subsequent long-term test, an evaluation of the fate of the two Fe_2O_3 nanomaterials was not possible. There is evidence that suggests that the pristine nanomaterial was adsorbed to the sewage sludge by around 60%. For the fragmented product, an evaluation of the data was not possible. Here, additionally the polypropylene matrix of the fragmented product hampers the chemical analysis of the iron in the environmental sample.

The sewage sludge was dewatered and parted. One-half of the sewage sludge was immediately applied into soil and the subsequent long-term test was performed. The second half was treated with an anaerobic digestion at 35°C for 28 days. The sludge was again dewatered and applied into soil. The second treatment aimed to observe possible transformations of the Fe_2O_3 nanomaterials due to the anaerobic digestion that might alter effects on the soil microorganisms.

For the evaluation of the test, a worst-case scenario was considered in which 100% of the nanomaterials would adsorb to the sewage sludge and will be applied into soil. Therefore, concentrations of 4.85 mg/kg dm soil and 12.1 mg/kg dm soil were used as nominal concentrations for the evaluation the test.

The tested pristine and fragmented Fe_2O_3 nanomaterials Fe_2O_3 P.Red 101 and Fe_2O_3_PE_FP_2 had no effect on the activity of the ammonium oxidizing bacteria during the whole test period of 140 days. No influence due to the additional anaerobic digestion treatment could be detected. Therefore the EC50 values were set to be above the highest test concentration of 12.1 mg/kg dm soil and the NOEC values were set to be at the highest test concentration of 12.1 mg/kg dm soil or above.

It can be concluded that there is no effect on the observed soil microorganisms due to the tested pristine and fragmented Fe_2O_3 nanomaterials independent if applied pure into soil or via sewage sludge. Even, if due to the anaerobic digestion of the sludge the nanomaterials were transformed,

the transformation did not change the toxicity of the nanomaterials from non-toxic to toxic.

The results of all long-term tests are summarized in Table 1.

Table 1. Results obtained for the case study with the pristine CuO product and three other products, using tool I and tool III (potential ammonium oxidation activity).

Test item	Date	EC50 [mg/kg dm soil]	NOEC [mg/kg dm soil]	Comments
CuO (First test)*	D30	> 5.57	≥ 5.57	No effects at both conc.
	D60	> 5.57	-	Inhibition of 45% at 0.31 mg/kg; no effect at 5.57 mg/kg
	D100	> 5.57	-	20% and 28% at 0.31 and 5.57 mg/kg, respectively
	D140	> 5.57	≥ 5.57	No effects at both conc.
CuO (Second test)*	D30	> 5.57	-	21% and 35% at 0.31 and 5.57 mg/kg, respectively
	D60	> 5.57	≥ 5.57	No effects at both conc.
	D100	> 5.57	≥ 5.57	No effects at both conc.
	D140	> 5.57	≥ 5.57	No effects at both conc.
Overall conclusion: Test 1 was performed at a low pH in soil between 4.0 and 4.5, whereas the second test was performed at a higher pH between 4.5 and 5.0. Effect concentrations therefore are related to the respective soil pH.				
WCCo-1NP-SYN**	D30	> 5.40	-	34% and 30% at 0.30 and 5.40 mg/kg, respectively
	D60	> 5.40	≥ 5.40	No effects at both conc.
	D100	> 5.40	≥ 5.40	No effects at both conc.
	D140	> 5.40	≥ 5.40	No effects at both conc.
Fe_2O_3P.Red101***	D30	> 12.1	≥ 12.1	No effects at both conc.
	D60	> 12.1	≥ 12.1	No effects at both conc.
	D100	> 12.1	≥ 12.1	No effects at both conc.
	D140	> 12.1	≥ 12.1	No effects at both conc.
Fe_2O_3_PE_FP_2***	D30	> 12.1	≥ 12.1	No effects at both conc.
	D60	> 12.1	≥ 12.1	No effects at both conc.
	D100	> 12.1	≥ 12.1	No effects at both conc.
	D140	> 12.1	≥ 12.1	No effects at both conc.

Measured concentration:
*CuO: 0.31 and 5.57 mg CuO/kg dm soil
**WCCo:0.30 and 5.40 mg WCCo/kg dm soil
Calculated nominal concentrations:
***Fe_2O_3: 4.85 and 12.1 mg Fe_2O_3/kg dm soil

Conclusion second part – fate and long-term effects in STP and soil

The combination of a simulated sewage treatment plant with long-term studies on the effects of

various nanomaterials provides a good tool to determine both fate and effect of the nanomaterials in a STP and the terrestrial environment. This tool can quickly display the whereabouts of various nanomaterials in the STP to predict the corresponding exposure to the environment. In addition, it is possible to simulate the exposure of the aquatic or terrestrial environment, depending on the fate of the nanomaterials, and to determine the effects within the environment using standardized OECD or ISO guideline. Nanomaterials tested within SUN were mainly adsorbed to sewage sludge at environmental relevant concentrations. Therefore, IME simulated within its developed tool a sewage sludge application for fertilization of agricultural land using a variety of ecotoxicological test methods to test the effect of nanomaterials within a realistic exposure scenario.

The tests with the pristine CuO nanomaterial showed, that the test conditions (e.g. soil pH), the storage conditions (4°C or -21°C) and the choice of an adequate test concentration (low to high concentrations; no limit test) are important factors for the performance of ecotoxicological tests with nanomaterials.

Terrestrial media

Tool I (Simulation of a sewage treatment plant)
Adapted test procedure based on the OECD guideline 303A for the simulation of a STP to investigate the fate of nanomaterials in STP and to determine the effects on the degradation capacity in a STP under environmental relevant conditions.

The tool can be combined with the additional performance of long-term tests in soil (Sub-Tool Ia). For this purpose, the nanomaterials are applied via the sewage sludge of the STP into soil to simulate the usage of sludge as agricultural fertilizer. In addition, the tool can be combined with aquatic toxicity tests that are performed with the STP effluent (Sub-Tool Ib) to provide information on an impact on the aquatic environment.

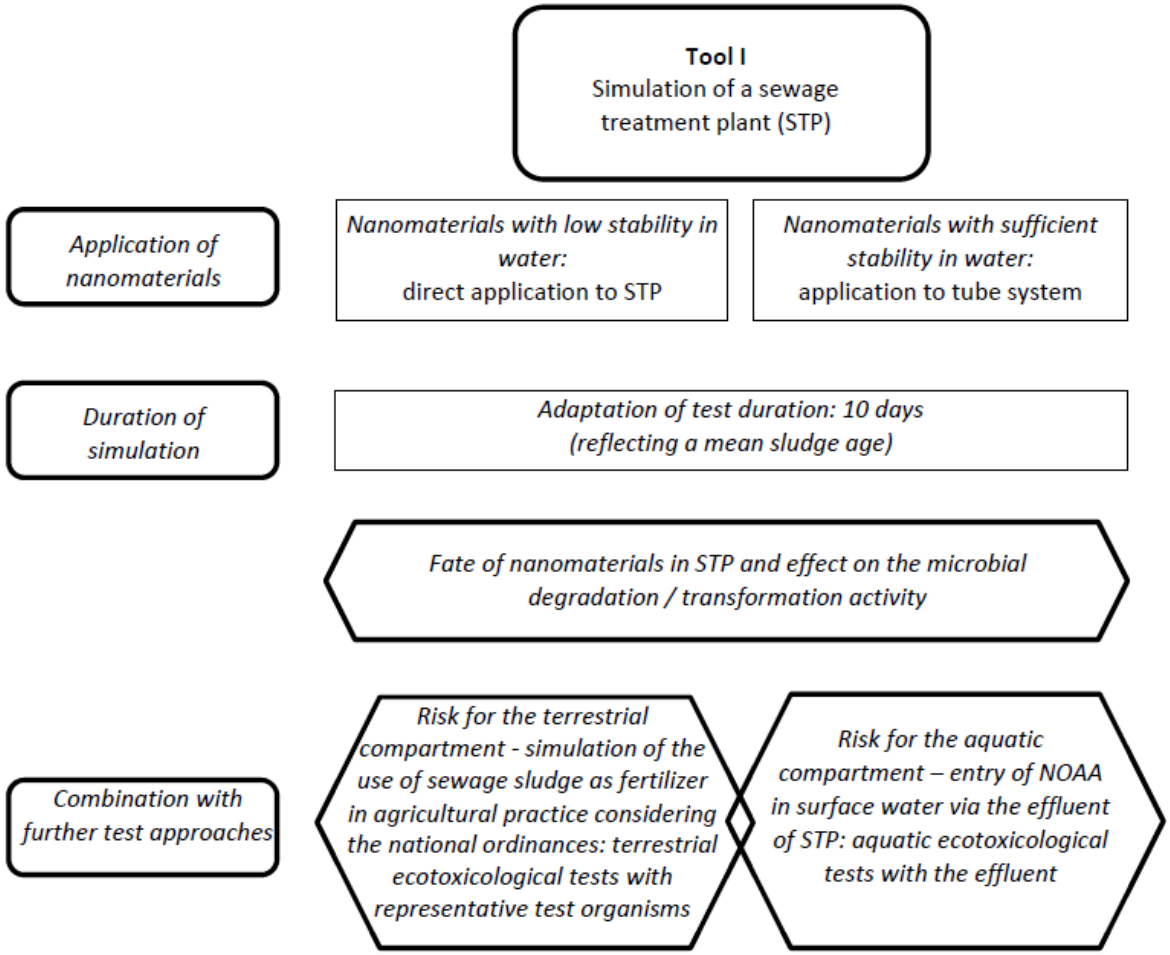

Figure 1: Scheme for the proposed Tool I.

To achieve the different aims and to evaluate the different proposed tools, the work was divided in three parts.

First part: How are effect concentrations of pristine and fragmented nanomaterials in soil at long-term exposure?

Based on the results of standard screening experiments on the effect of the pristine and fragmented nanomaterials in soil and sewage sludge the nanomaterials that were tested in STP simulations and subsequent long-term tests over 140 days in soil were chosen. Therefore, soil was spiked with 10 – 1000 mg/kg dm soil of the nanomaterial (1000 mg/kg dm soil corresponds to the highest test concentration allowed in the OECD guideline 216/ISO 15685). After an incubation period between 24 hours and 84 days, the effect on the microbial activity was measured in accordance to the ISO guideline 15685 (2012).
The effect on the sewage sludge was tested in accordance to the OECD guideline 209 at concentrations between 6 and 100 mg/L (100 mg/L corresponds to the highest test concentration allowed in the OECD guideline 209).

Nanomaterials, which had an effect on the sewage sludge and the soil microorganisms, were treated with priority as the nanomaterials that have been identified as such within the project. Here, these were CuO, WCCo, Fe_2O_3 and CNTs.

In the **first part** of our testing approach, the general toxicity of the nanomaterials on the activity

of sewage sludge and the activity of soil microorganisms was investigated.
In the sludge respiration inhibition test (OCED 209) the pristine CuO and Fe_2O_3 P.Red101 lead to a significant different respiration compared to the control at concentrations between 50 and 100 mg/L. There was no effect determined for the pristine WCCo-1NP-SYN.

Ammonium oxidizing bacteria revealed to represent a very sensitive group of soil microorganisms towards nanomaterials (NM), hence the main focus has been placed on this group, using ISO Guideline 15685 (Potential ammonium oxidation). No effects on the ammonium oxidizing bacteria occurred for a range of different pristine nanomaterials (e.g. orgP.Red254, WCCo-1NP-SYN, MWCNT or Fe_2O_3 P.Red101) independent from the exposure duration (0 - 84 days) at concentrations up to 1000 mg/kg dm soil. However, the pristine copper oxide nanomaterial inhibited the ammonium oxidizing bacteria at a high concentration of 1000 mg/kg dm soil already at test start with an increasing inhibition until test termination and appeared to be the most toxic pristine nanomaterial to the soil microorganisms (tested at IME).

To differentiate between effects caused by nanoparticles of fragmented products the effect of the corresponding matrix was investigated. The effect of the fragmented reference products PP_FP_2, PP_FP_3, PE_FP_2 and Epoxy FP on the activity of the ammonium oxidizing bacteria was observed. For PP_FP_2, PP_FP_3 and Epoxy FP test concentrations of 10 and 1000 mg/kg dm soil were chosen. Unfortunately, the available amount of the fragmented reference matrix PE_FP_2 was limited and test concentrations had to be changed to 10 and 50 mg/kg dm soil. The effect was observed at test start and after 7 and 28 days incubation. None of the chosen reference matrices affected the ammonium oxidizing bacteria at any of the chosen time points or concentrations. Therefore, for PP_FP_2, PP_FP_3, PE_FP_2 and Epoxy FP the EC50 was above the highest test concentrations of 1000 mg/kg dm soil and for PE_FP_2 the EC50 was above 50 mg/kg dm soil.

For the fragmented products OrgPig_PP_FP 2, OrgPig_PP_FP 3, Fe_2O_3_PE_FP 2 and CNT_epoxy_FP no effect on the activity of the ammonium oxidizing bacteria at concentrations up to 1000 mg/kg dm soil were observed after the application into soil and the subsequent incubation period. For the Fe_2O_3_PE_FP 2 and CNT_epoxy_FP an inhibition of 22.9 and 38.3%, respectively, compared to the control was found one day after the application at a concentration of 1000 mg/kg dm soil. However, after 28, 56 and 84 days incubation there were no statistically significant differences to the control even at these replicates.

The long-term effect of Cu-carbonate and Cu-amine and the associated dispersant on the ammonium oxidizing bacteria was observed in two additional tests with an incubation period of 84 days to provide detailed information for the copper case study.
For the first test the dispersant for both materials was not available and therefore the test was conducted without dispersant control. At test concentrations of 100, 250, 500 and 1000 mg test item/kg dm soil the effect on the ammonium oxidizing bacteria at test start and after 28, 56 and 84 days incubation was observed. For the Cu-carbonate a concentration response relationship was found for concentrations from 100 to 500 mg/kg dm soil, whereas the inhibition found at the highest test concentration of 1000 mg/kg dm soil was always less pronounced. The inhibition at 100, 250 and 500 mg/kg increased from test start with 22%, 34% and 52% up to 42%, 76% and 72% at test end, respectively. At the highest test-concentration of 1000 mg/kg dm soil the inhibition remained steady with 63% at test start and 57% at test end.

For the Cu-amine a concentration response relationship was found at test start. The activity of the ammonium oxidizing bacteria was inhibited by 12%, 52%, 83% and 79% at test concentrations of 100, 250, 500 and 1000 mg/kg dm soil. Afterwards a comparable course of the effect due to the Cu nanomaterial as for the Cu-carbonate was found. There was no effect at 100 mg/kg dm soil; however, a strong inhibition was determined at 250 and 500 mg/kg dm soil increasing from test

start until test end. The inhibition found at 1000 mg/kg dm soil remained steady with 79% at test start and 73% at test end.

After the first test, a second test with less test concentrations but with the associated dispersant was performed to evaluate the effect caused by the dispersant in the first test. Based on the results of the first test, test concentrations of 100 and 250 mg/kg dm soil were chosen. The associated dispersant was tested at the same concentrations to differentiate between effects caused by the nanomaterial and the dispersant.

At test start no effect on the ammonium oxidizing bacteria due to the Cu-carbonate material was found at test concentrations of 100 and 250 mg test item/kg dm soil. After 28 days, the activity of the ammonium oxidizing bacteria was inhibited by 49% at the highest test concentration of 250 mg test item/kg dm soil. The associated dispersant did not affect the soil microflora at the same concentration level and therefore the effect can be related to the Cu-carbonate nanomaterial. By an increasing incubation time, the inhibition of the activity of the ammonium oxidizing bacteria increased steadily. At test end (84 days) the activity of the ammonium oxidizing bacteria was inhibited by 30% and 80% at 100 and 250 mg test item/kg dm soil, respectively. An effect due to the associated dispersant was not determined throughout the whole test period.

For the Cu-amine nanomaterial the results were different from the results obtained with Cu-carbonate nanomaterials. At test start, the ammonium oxidizing bacteria were affected by the Cu-amine at a test concentration of 250 mg test item/kg dm soil. However, the inhibition of the activity of the ammonium oxidizing bacteria was comparable between the Cu-amine and it is associated dispersant with 44% and 33% inhibition, respectively. After 28 days incubation the inhibition of the associated dispersant remained stable at 34%, whereas the inhibition of Cu-amine at a concentration of 250 mg/kg dm soil increased to 100%. At test end (84 days) the activity of the ammonium oxidizing bacteria due to the Cu-amine nanomaterial and the associated dispersant at 250 mg/kg dm soil appeared to be comparable at 69% and 78%, respectively. The test concentration of 100 mg/kg dm soil did not affect the ammonium oxidizing bacteria during the whole test. Summarily, a clear effect due to the associate dispersant was found in the second test including the dispersant as test material. However, the inhibition caused by the Cu-amine including the dispersant and the nanomaterials was more pronounced, indicating that there is an effect mainly caused by the Cu nanomaterial.

The EC50 and NOEC values of all performed tests are summarized in Table 1-4.

Conclusion first part - screening tests

Based on the screening test results it was concluded that the chosen test concentrations of 0.04 to 1 mg/L are appropriate for the STP simulation and subsequent long-term tests in soil.

In addition it could be shown, that there can be slight toxic effects of the fragmented nanomaterials at high concentrations, but that these effects are short-term effects that decrease during the subsequent incubation.

The Cu-carbonate and Cu-amine nanomaterials strongly affected the soil microflora, however, at least for the Cu-amine it appeared that the associated dispersant in a low degree affected the ammonium oxidizing bacteria.

The test systems used for the determination of effects of pristine and fragmented nanomaterials on the sludge and soil microorganism community were sufficient.

Table 2: EC50 and NOEC values obtained from the potential ammonium oxidizing activity with different fragmented nanomaterial products.

Test item	Date	EC50 [mg/kg dm soil]	95% confidence interval	NOEC [mg/kg dm soil]	Comments
OrgPig_PP_FP_2	D0	> 1000	n.d.	≥ 1000	-
	D28	> 1000	n.d.	≥ 1000	-
	D56	> 1000	n.d.	≥ 1000	-
	D84	> 1000	n.d.	≥ 1000	-
OrgPig_PP_FP_3	D0	> 1000	n.d.	≥ 1000	-
	D28	> 1000	n.d.	≥ 1000	-
	D56	> 1000	n.d.	≥ 1000	-
	D84	> 1000	n.d.	≥ 1000	-
Fe_2O_3_PE_FP_2	D0	> 1000	n.d.	10.0	23% inhibition at 1000 mg/kg dm soil
	D28	> 1000	n.d.	≥ 1000	-
	D56	> 1000	n.d.	≥ 1000	-
	D84	> 1000	n.d.	≥ 1000	-
CNT_epoxy_FP	D0	> 1000	n.d.	n.d.	15 and 38% inhibition at 10 and 1000 mg/kg dm soil, respectively
	D28	> 1000	n.d.	≥ 1000	-
	D56	> 1000	n.d.	≥ 1000	-
	D84	> 1000	n.d.	≥ 1000	-
Cu-carbonate	D0	566	[453 – 504]	< 100	-
	D28	n.d.	n.d.	< 100	Poor dose response. Calculation of EC50 not possible. Effects at 250 and 500 mg/kg dm soil were above 70%.
	D56	n.d.	n.d.	< 100	
	D84	n.d.	n.d.	< 100	
Cu-amine	D0	251	[235 – 267]	100	Inhibition caused by Cu-amine NM and associate dispersant at test start. After d28 influence of dispersant less pronounced.
	D28	167	[144 – 190]	100	
	D56	316	[189 – 589]	100	
	D84	176	[162 – 190]	100	

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Table 3: EC50 and NOEC values obtained from the potential ammonium oxidizing activity with different fragmented reference matrices.

Test item	Date	EC50 [mg/kg dm soil]	NOEC [mg/kg dm soil]	Comments

PP_FP_2	D0	> 1000	≥ 1000	-
	D7	> 1000	≥ 1000	-
	D28	> 1000	≥ 1000	-
PP_FP_3	D0	> 50	≥ 50	-
	D28	> 50	≥ 50	-
	D56	> 50	≥ 50	-
PE_FP_2	D0	> 1000	≥ 1000	-
	D28	> 1000	≥ 1000	-
	D56	> 1000	≥ 1000	-
Epoxy_FP	D0	> 1000	≥ 1000	-
	D28	> 1000	≥ 1000	-
	D56	> 1000	≥ 1000	-

Table 4: EC50 and NOEC values obtained from the potential ammonium oxidizing activity for the case study with the pristine CuO product.

Application via powder				
Date	EC10 [mg/kg]	EC20 [mg/kg]	EC50 [mg/kg]	NOEC
D28	3.27 [0.990 – 7.31]	30.9 [15.9 – 50.9]	2279 [1252 – 5334]	100
D56	396 [n.d.]	538 [n.d.]	965 [n.d.]	100
D84	181 [47.5 – 306]	287 [109 – 426]	697 [500 – 848]	100
Application via dispersion				
	EC10 [mg/kg]	EC20 [mg/kg]	EC50 [mg/kg]	NOEC
D28	n.d. [n.d.]	0.896 [0.496 – 1.46]	91.6 [69.5 – 123]	1.00
D56	93.7 [42.0 – 151]	219 [131 – 306]	1112 [830 – 1661]	100
D84	312 [n.d.]	424 [n.d.]	762 [n.d.]	100

n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000.

Table 5: Results obtained with five different other pristine products (potential ammonium oxidation activity).

Test item	EC50 [mg/kg dm soil]	NOEC [mg/kg dm soil]	Comments
NM-403	> 100	≥ 100	Results after 28 days incubation
orgP.Red254	> 1000	≥ 1000	
Fe2O3P.Red101	> 1000	≥ 1000	
MWCNT	> 1000	≥ 1000	
WCCo-1NP-SYN	> 1000	≥ 1000	

Based on the results of the first and second part of the investigation the following tool was developed:

Third part: Single application versus repeated application

In the third part, the short and long-term effect on soil microorganisms of copper nanomaterials applied at once and in a stepwise approach into soil was investigated. Therefore, pristine nanomaterials were applied to soil in a single application procedure and a repeated application, which was carried out three times (at 4-week intervals) within a period of 84 days (Table 6). The approach is presented in Figure 2 showing exemplarily the test design used for the investigation of the short and long-term effects of copper nanomaterials on soil microorganisms in the scope of SUN.

Table 6: Test design to observe differences in the effect of CuO NMs on the soil microflora using different application methods.

Concentration 1	333 mg/kg	111 mg/kg
Concentration 2	1000 mg/kg	333 mg/kg
Application	At day 0	At day 0, 28, 56
Sampling	Day 28, 56, 84	Day 28, 56, 84 Sampling at day 28 and 56 was performed before the next application
Microbial endpoints	Structural (DNA/RNA by ARISA) and functional endpoints (microresp® and ISO 22939), potential ammonium oxidation acitivity as reference endpoint)	

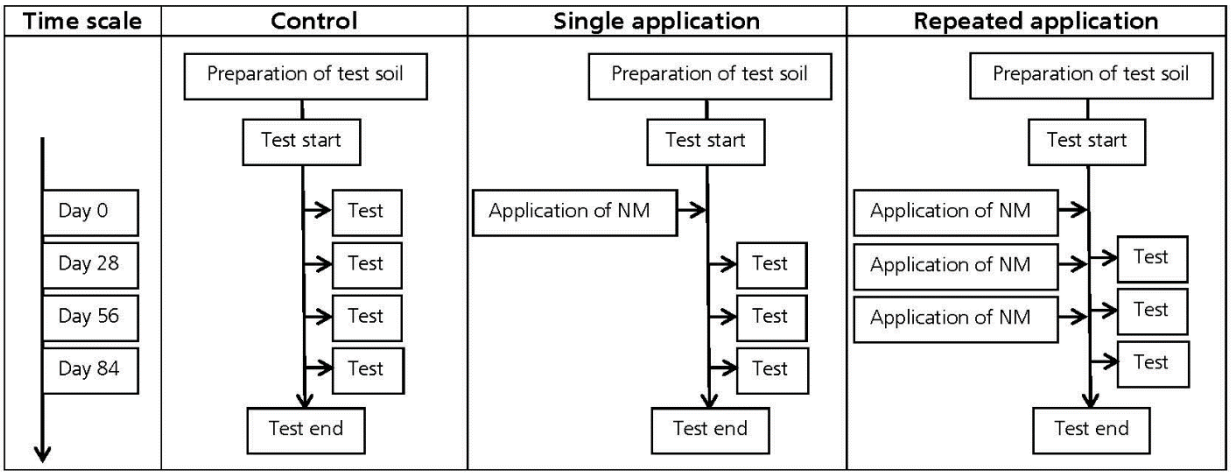

Figure 2: Time schedules of the single and stepwise repeated applications and the subsequent ecotoxicological experiments.

A defined amount of soil was removed from the incubation vessels at each time point (1% of the soil mass for the CuO-NM application). The removed soil was air dried for three days and then used as the carrier soil for the subsequent application. Samples were taken for the ecotoxicological studies immediately before the next application step. In addition to the ISO 15685 the influence on the exo- and endoenzymatic activity (ISO 22939 and MicroResp) was observed in this test set up. The procedures and the obtained results were published in Schlich et al. (2016).

The results of the **third part (III)** of our testing approach regarding the short and long-term effect of copper nanomaterials applied at once and in a stepwise approach into soil on soil microorganisms are already published in the peer review journal Environmental Pollution. Here, it could be shown that the comparability of single and repeated applications of ion-releasing nanomaterials depends on the test endpoint and duration. Repeated applications ultimately

resulting in the same test concentrations as a single application do not provide further information if nitrifying microorganisms and exoenzymes are tested. However, differences between single and repeated applications become apparent when substrate-induced respiration is considered. The toxicity of ion-releasing nanomaterials is specific to the material, and is likely to be based on parameters such as the rate of ion release and agglomeration behaviour.

Regarding the used test systems, the potential ammonium oxidation activity was the test parameter with the highest sensitivity among the three test systems and this provides important information concerning the toxicity and bioavailability of the test material. The MicroRespTM approach indicates the effect of nanomaterials on the microbial community and can report the potential recovery of microbial populations due to the replacement of sensitive, damaged microorganisms with more resistant ones. The enzyme activity patterns reveal the activity of exoenzymes in the bulk soil, and this indicates the rate of ion release by nanomaterials because exoenzyme activity adapts more slowly than the microbes producing the enzymes.

Conclusion third part – repeated exposure
For regulatory purposes, the test duration is an important factor and should be extended to observe the effects of long-term ion release from nanomaterials.

The three test systems (Potential ammonium oxidation, MicroRespTM, enzyme activity patterns) together provide comprehensive information about the impact of different nanomaterials on the soil microflora and its diversity. Information on microbial exoenzymes can be received with fluorescent substrates. Due to the large fluorescent molecule attached to carbon sources, the complete molecule is too large for uptake into the microbial cell. Therefore, the substrate can only be transformed by exoenzymes. These enzymes are stabilized in soil. The stabilization can also be a kind of protection against a toxic impact. The degree of inhibition of these enzymes by nanomaterials indicates the availability of these enzymes for the nanomaterials. A limitation of this test approach is the availability of fluorescent marked carbon sources. Only a limited number of fluorescent-labelled substrates is available. From the seven tested chemicals addressing the C-, N-, S- and P-cycle a minimum set comprising of an amino acid (L-alanine), a carbon source (nonanoate) and a P-containing substance (phenylphosphate disodium salt) is recommended.

Additionally, with this method information on the ion release of the nanomaterials can be received. In the scope of SUN, it became obvious that the exoenzyme activity adapts more slowly than the microbes and the endoenzymes that are determined by the MicroRespTM approach. Therefore, a continuous release of toxic ions results in an increased toxic effect. The speed with which the extent of inhibition increases can be used as indicator for the velocity of the ion release and hence for the stability of the nanomaterials.

Based on the results of the third part of the investigation the following tools were proposed:

Tool II (Simulation of repeated pollutant entry)
Test set up with a repeated exposure approach simulating the periodical entry of lower (environmental more relevant) concentrations into a test system and the subsequent effects on the soil microflora.

A single exposure approach can be performed at the same time to observe relevant differences between both approaches.

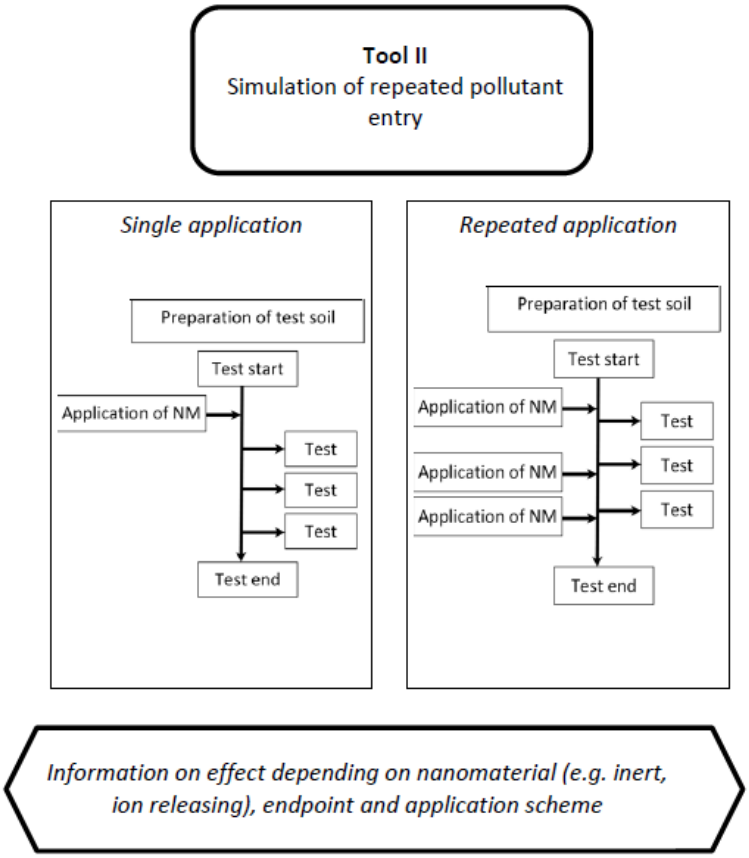

Figure 3: Scheme for the proposed Tool II.

Tool III (Measuring approach: Detection tool regarding microbial activity)
The combination of three test approaches providing together detailed information of the impact of nanomaterials on the soil nutrient cycles, the location of the impact (impact on endo- or exoenzymes) and a potential recovery or replacement of soil microbial groups.

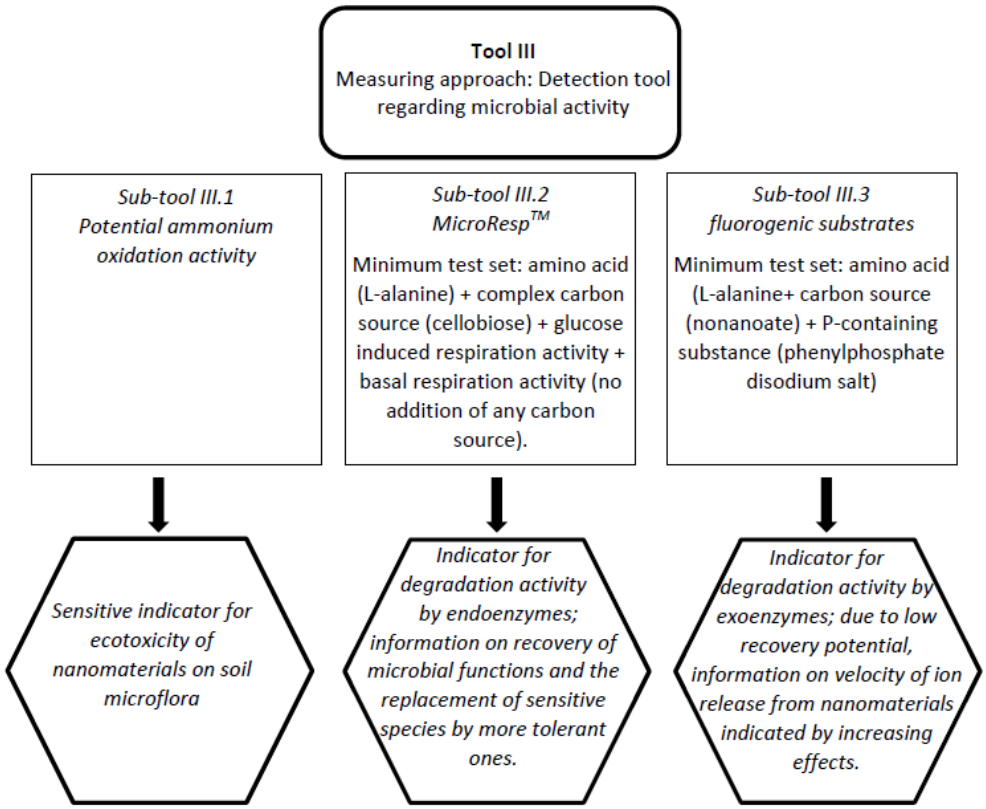

Figure 4: Scheme for the proposed Tool III.

Soft-bodied organisms

UAVR developed test tools/systems for enchytriaeds; the novel tools are fully compliant with the OECD test-guidelines and the environmental risk assessment. They however provide the required advancement in order to get a deeper understanding of possible long-term toxicity. The tools included full life-cycle tests, longevity test, and multiple generation test design (see Figure 5, see also Del 4.2).

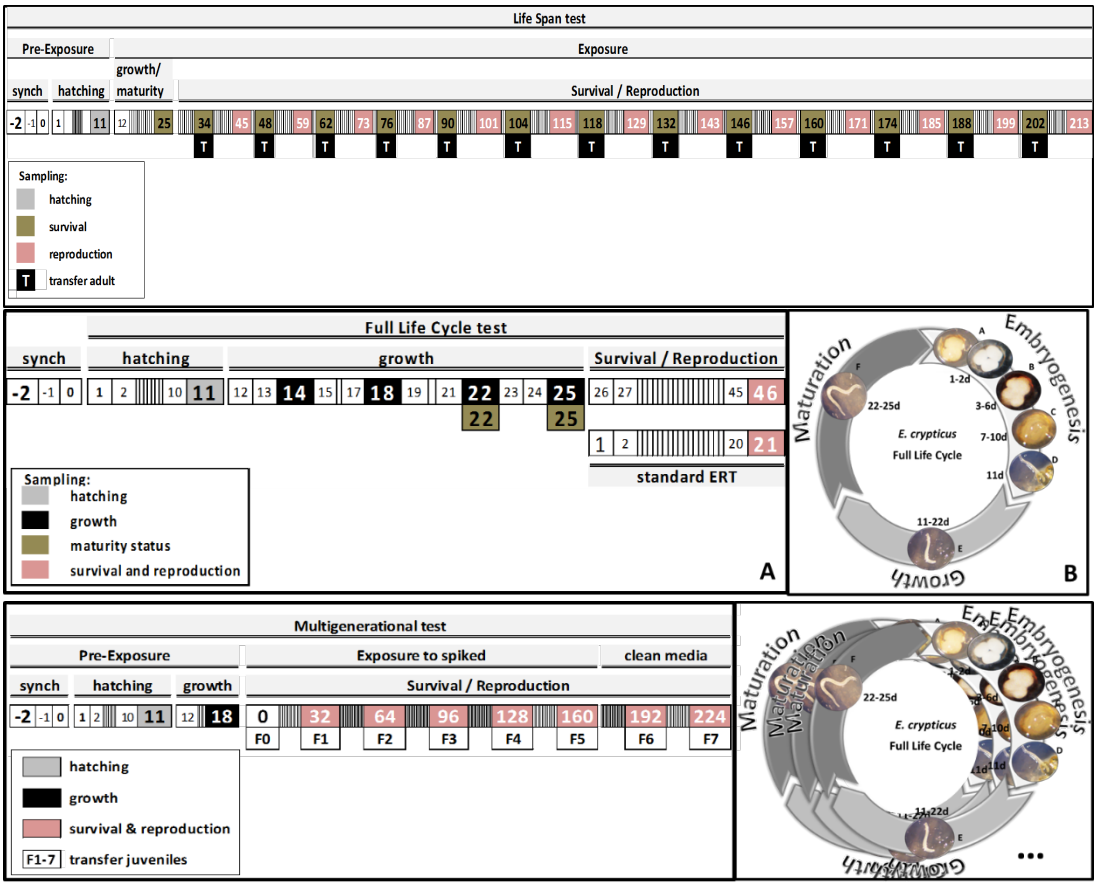

Figure 5: *Test tools developed in Sun for Enchytraeus crypticus, covering longevity, full life cycle, and multi-generation measures.*

A wide range of both pristine and fragmented products was tested. The result from these tools can (as the original OECD 220) be used directly in the probabilistic risk assessment, obviously considering comparability of endpoints and test duration. Hence, the relevant data were provided and included in the probabilistic risk assessment tools developed in WP8.

The new test tools enable the risk assessor to get detailed information on hazard, while still keeping the test systems standardized (Figure 2). The novel detailed information deals with which stage of the organism life cycle that is most sensitive and whether effects appear after prolonged exposure.

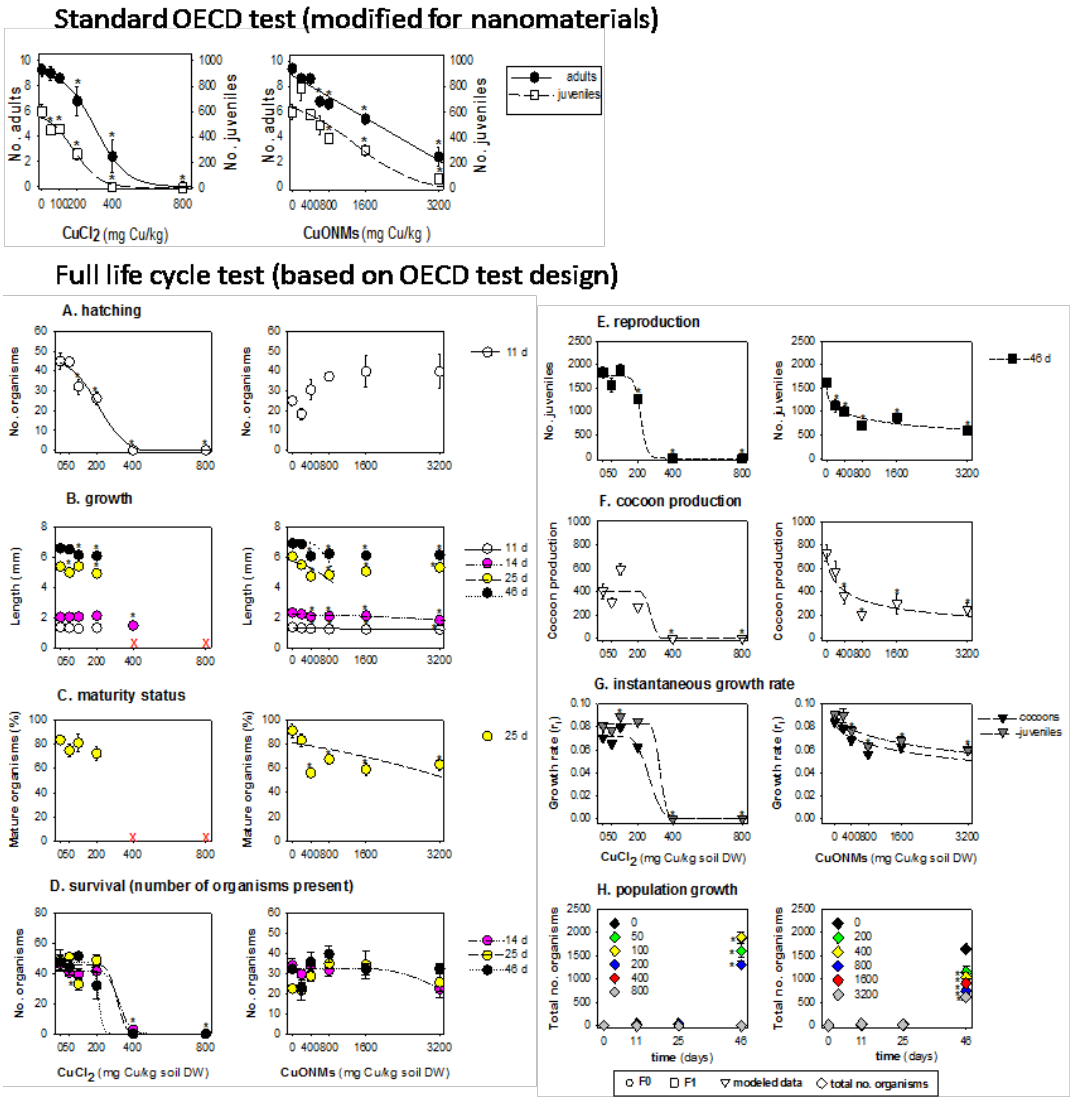

Figure 6. *Example of results from the standard OECD test (top two Figure) compared to the SUN test tools (lower Figures) described in Figure 1. The results are for CuCl2 and CuO nanomaterials, and illustrate the detailed results obtained with the novel technique (Bicho et al. 2017).*

It can be seen from Figure 6, published in Bicho et al 2017), that CuONMs mainly affected growth or juveniles' development, whereas CuCl2 mainly affected embryo development and/or hatching success and adults survival. Compared to the ERT, the FLCt allowed discrimination of effects between life stages and provided indication of the underlying mechanisms; further, the FLCt showed increased sensitivity, e.g. reproductive effects for CuONMs: EC10=8 mg Cu/kg and EC10=421 mg Cu/kg for the FLC and the ERT respectively (Figure 7). Development of the above test tool means that the risk assessment can move from a static short-term "black-box" tool to a tool that provide and include longer-term consequences. "Black-box" referring to current population toxicity information (e.g. OECD 220) where it is not possible to identify the cause of toxicity.

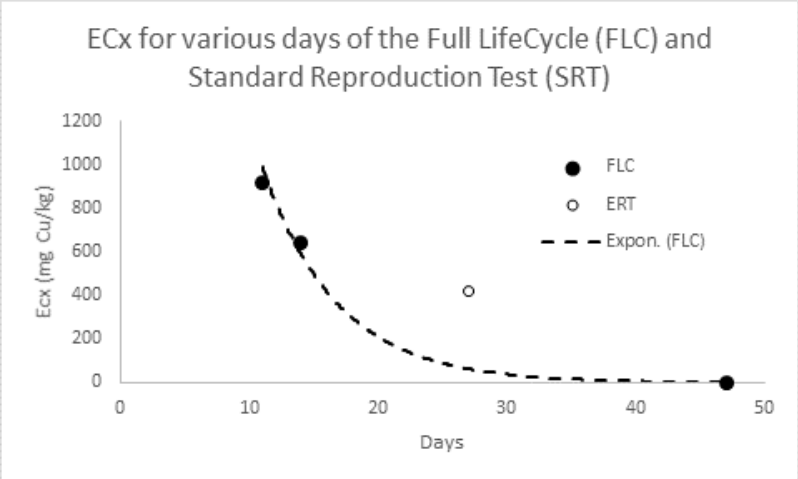

Figure 7: *The figure illustrates how the short-term measures of the full life cycle test may enable a prediction on longer-term toxicity, and how this compares to the traditional reproduction test (used by the OECD).*

The omics studies (i.e. gene-, proteome- and metabolome information) which are anchored in the population effects enables us to develop Adverse Outcome Pathway (AOP) directly linked to population measures (see Figure 8). The AOP approach is requested by regulators as the current risk assessment only provide black-box population toxicity information i.e. where it is not possible to identify the cause of toxicity. However, the AOP provides detailed information and will help the risk assessor to identify why toxicity occurred and how this may be mitigated e.g. by a safer designed material.

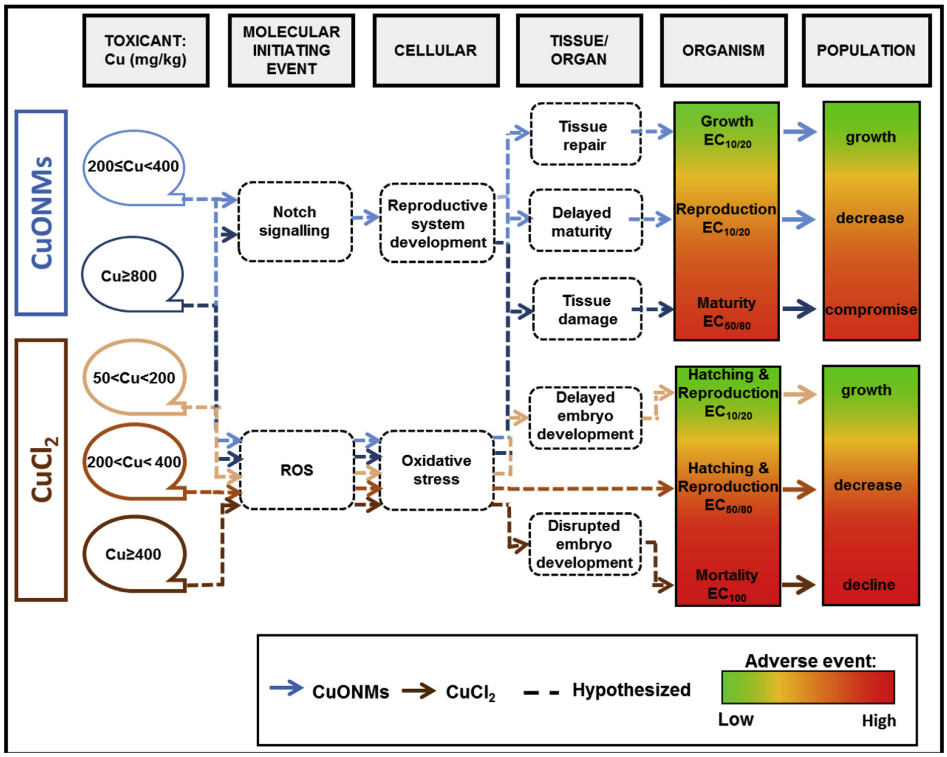

Figure 8: *Example of Adverse outcome pathway (AOP) for CuCl2 and CuONPs, showing the similarities and differences between the two.*

Hence, it is clear that the modified testing tool can be used to evaluate the impact of nanomaterials on soil ecosystem, and that it can be directly accessible to the regulator and allow the producer (companies) to design safer materials.

Similarly, to the microbial studies, there were severe challenges when using the test systems to

study Fragmented Products (FP) rather than pristine materials. The FP was larger than the mouth of the organisms and in some case larger than the organisms (see Figure 9), it changed the soil structure /humidity and weathering was probably in sufficient. Hence, concerning risk assessment care has to be taken when a material show high (compared to expected) ECx/NOEC values, as this may simply be a consequence of reduced/no exposure rather than no toxicity.

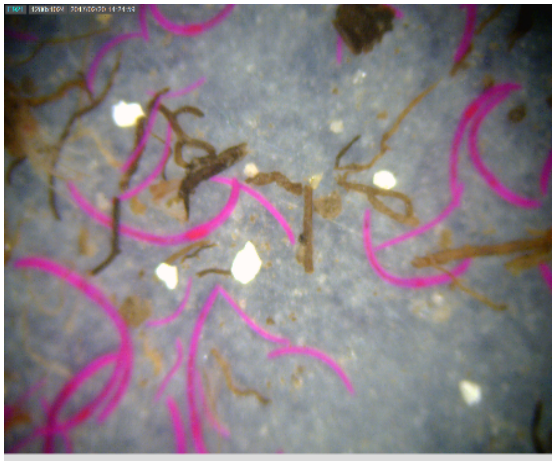

Figure 9: *Enchytraeus crypticus (red lines) with Fragmented Products (white spots).*

This means that it should be reconsidered whether fragmented materials can be tested in standard test systems. One solution seem to evaluate the fragments as micro plastic, since many of the fragments are in micro size range. However, this does not inform on potential toxicity of the nanoform, since the plastics (which contains the nano) are not decomposed in the laboratory test media but likely will be in the environment.

AU developed test systems for earthworm, another soft-bodied key species. The earthworm Eisenia fetida is a larger and longer living worm, which is the key organisms/species in virtually all terrestrial risk assessment globally. In collaboration with UAVR similar longer-term test designs were developed i.e. full life-cycle test and multiple generation tests. The life-cycle test for this species stretched over 84 days. The worms are exposure also in their juvenile growth phase, hence sub-lethal effect arising in this phase will be included, this in not captured in the present OECD design. To compare, in human health it is now known that exposure in prenatal and juvenile phase is critical for adult performance. Hence, the developed tool is a very useful instrument to gain information on long-term consequences, and since life-stage information is available this allow prediction of the impact on the population growth. For example, for the CuONP case the studies showed that the most sensitive endpoint was the production of juveniles, but that cocoon production was less affected (see Figure 10). Hence, when evaluating the longer-term consequences the potential hatchability is the parameter to focus on. The test systems developed for *Eisenia fetida*, also were fully compliant with the corresponding earthworm test (OECD 222), and provide the advantages in longer-term prediction for the hazard and risk assessment.

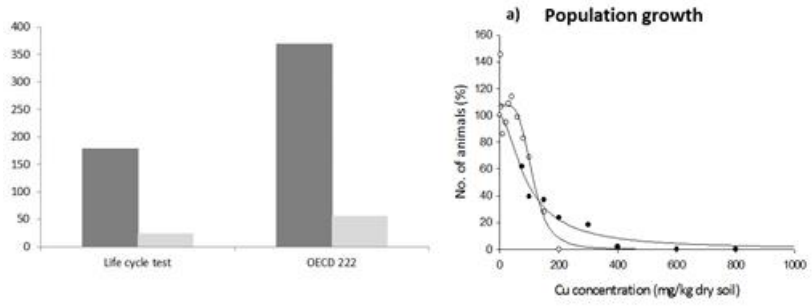

Figure 10: *Comparison between OECD 222 and the new life cycle test: LEFT: dark grey columns are mortality (LC10) and light grey are reproductive (EC10). RIGHT: population growth in the new life cycle test (dark dots CuO-NP and white dots Cu-salt).*

The fragmented material was not tested in the earthworm studies, since insufficient material was available. It is likely that the same general concerns raised above regarding ecotoxicity-testing of fragmented materials counts here. To provide the risk assessor with a rapid tool that informs on sensitivity across species and provide mechanistic insight we developed an array of in vitro test tools; see section later in the document.

Hard-bodied organisms

VUR analysed six nanoparticles for their toxicity by adapting OECD guideline 232, which makes use of the model organism *Folsomia candida* and assesses the effects on survival and reproduction after 4 weeks of exposure. Standard toxicity tests were performed in soil spiked in with a range of concentration of six nanoparticles: WCCo, CuO, Fe2O3, CNT, Organic Pigment Red(OrgPig). As control compounds readily soluble metal salts (CoCl2, CuCl2, and FeCl3) were taken along. All exposures were performed in natural Lufa 2.2 soil.

Time (generations)	WCCo NM	$CoCl_2$ $6H_2O$	CuO NM	$CuCl_2$	Fe_2O_3 NM	$FeCl_3$ $6H_2O$	CNT NM	OrgPig NM
	EC50	EC50	EC50	EC50	EC50	EC50	EC50	EC50
T = 1	6486 (4795-8974)	469 (405-533)	>6400	853 (645-1060)	>6400		>6400	>6400
T = 2	>6400		>6400					
T = 3	2463 (945-3981)		>6400					
T = 4	3835 (2682-4989)		>6400					
T = 5	5668 (5319-6017)		>6400					
T = 6	>6400		>6400					

Table 7: EC50 values for the effect on the reproduction of Folsomia candida after 28-d exposure (T = 1) to Lufa 2.2 soil freshly spiked with nanomaterials (NM) or chlorides and after 2 (T = 2), 3 (T = 3), until 6 (T = 6) generations. EC50 values are presented in mg/kg d.w.). Corresponding confidence intervals are presented in between brackets.

To develop longer-term tools a multi-generation tool was developed in collaboration with UARV and AU. The multi-generation paradigm for Collembolans followed the design outlined for enchytraeids (above). Two of the six original materials were studied over multiple generations, in which the animals were exposed to NP in the first four generations. After these four generations, two subsequent generations were kept in clean soil under control conditions to assess the potential for recovery. Based on the modified OECD test performed with the first generations, no decrease in survival and reproduction of the springtails was recorded for the CuO, $Fe2O_3$, CNT and OrgPig nanoparticles, not even at the highest concentration of 6400 mg/kg dry soil. Only for WCCo nanoparticles, we were able to calculate an EC50 value of 6486 mg/kg d.w. soil (CI 4795-8974). The control exposures to ionic metals gave results as expected from previous studies reported in the literature. However, in the multi-generation experiment with WCCo nanoparticles we did observe an increase in the sensitivity of *F. candida* upon prolonged exposure. In the third generation, the EC50 dropped from 6486 mg/kg dry soil down to 2463 mg/kg dry soil, meaning an increase in sensitivity by a factor of 2.5. While *F. candida* remained quite sensitive in the 4^{th} generation, we observed a complete recovery in the following two generations under clean/control conditions. The WCCo nanoparticles therefore can have an adverse effect over

subsequent generations, although the concentrations at which we observed adverse effects on reproduction still are very high and far above environmentally relevant concentrations. In a multigenerational study with CuO nanoparticles no changes in the sensitivity of *F. candida* were observed, and since no effects were seen at the highest concentration tested (6400 mg/kg dry soil) no ECx levels could be calculated.

The described studies were also backed up with molecular genomic tools assessing changes in gene expression. Occasionally, we observed slight but significant activation of metallothionein and laminin genes. However, activation levels were low, and could never be correlated to nanoparticle exposure level.

In summary, although little effect were observed for the hard bodied organisms when testing metal nanoparticles then a multi-generation tools was developed, which was combined with mechanistic understanding. As for the soft bodied organisms this tool provide the assessor with information on longer-term consequences e.g. for CuO NPs no change was observed in the following generations whereas this was the case for WCCo NPs.

Multispecies system
– integrating microorganisms, soft- and hard bodied organisms in one test tool
AU developed a multispecies test systems including the soft- and hard-bodied species into an integrated assessment of potential hazard and risk (see Figure 11, also described in Del 4.1). In risk assessment multispecies test (also called mesocosm) is the highest tier testing possible before employing field-testing. For the same reason such test tools are also decisive in European risk assessment, i.e. affecting the final (1-5) assessment factor. The test system developed in SUN integrate the information from the above single species test tools, e.g. in includes the full life cycle of the organisms and consider multiple-generations. It is not a substitute of the individual species tests, since it does not provide the detailed information on each species. However, it does provide an environmental relevant scenario i.e. it sets toxicity estimation in a perspective of species interaction, including mutualism, competition, predation with more.

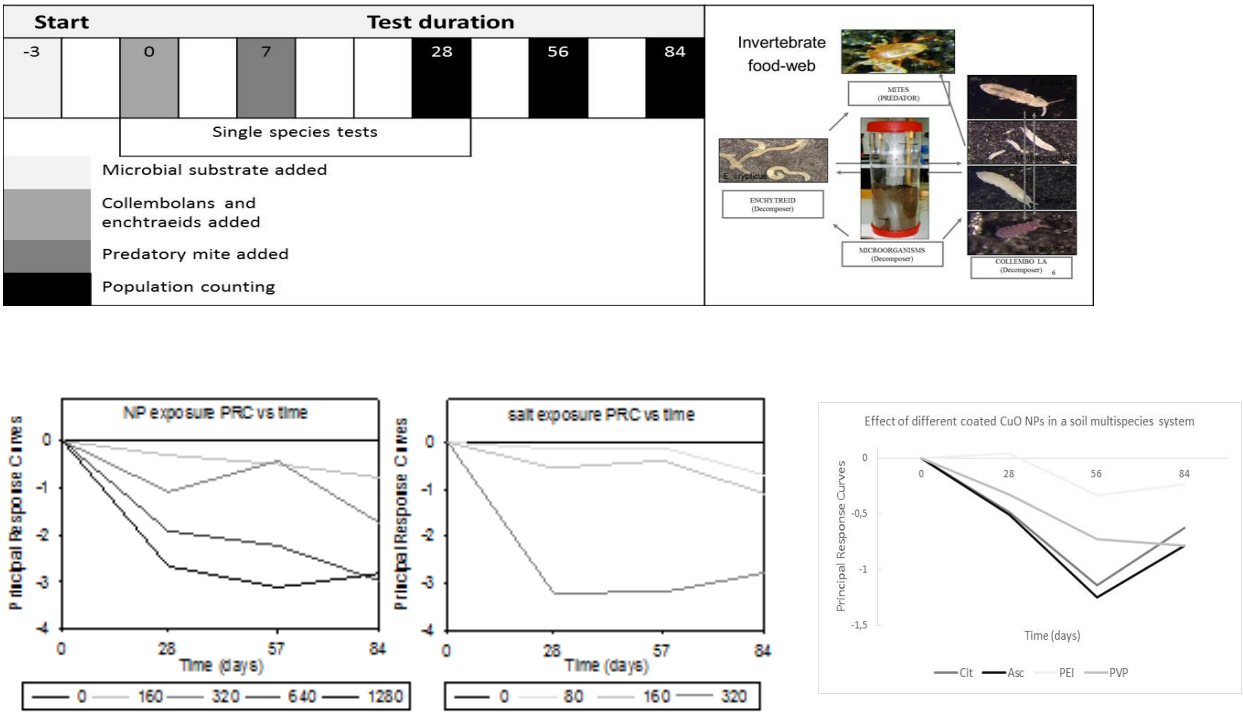

Figure 11: *Top: Multispecies system design. Bottom left: Principle Response Curves (PRC) for the CuONP and the CuNO3 exposure. Bottom right: PRC for the modified CuO NP in the multivariate test system (PRC is multi-variate statistical technique that can determined NOEC/LOEC responses)*

The multispecies system was an effective tool to test nanomaterials in relation to Risk Assessment. It was possible to obtain effect concentration (ECx) for the individual species at each sampling occasion, when the species experienced additional stress, and to obtain a no observed effect concentration (NOEC)/lowest observed effect concentration for the whole system (LOEC), values that can be directly used to predict the Hazard Concentration. This NOEC/LOEC could be compared to the Hazard Concentration (HC5) based on a Species Sensitivity Distribution using the ECx concentration derived for each sampling point. It was observed that although the HC5 remained constant over time, the species sensitivity differed over time i.e. while one species was most sensitive at day 28 another species was most sensitive at day 57 (see Figure 12).

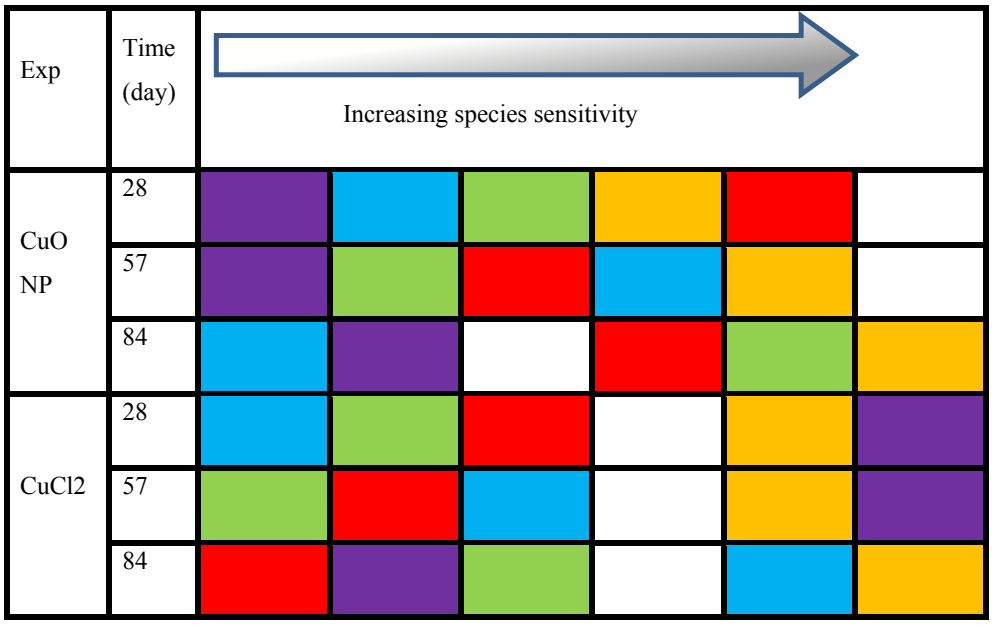

Figure 12: *Shift is species sensitive over time in a multispecies test system. Colours denote the individual species.*

The exact reason for this shift is unknown, but this must be related to the population dynamic of the individual species e.g. the predatory species has a slower population growth, and may hence only be affected at a later time point. Overall, the case study chosen i.e. CuO nanomaterials showed that the soft-bodied organism *Enchytraeus crypticus* was the most sensitive organism. That *E. crypticus* was more sensitive to CuO NP than hard bodied, was also confirmed by the single species studies where the hard bodies was little effected (see e.g. above).

The Fragmented Products (FP) were not tested within the described multispecies test tool, since there was insufficient amount of FP available for testing. However, the modified CuO-NP materials (i.e. coated with citrate, PIE, PVP and Ascorbate) was tested and showed that the coating influence the overall toxicity (PEI coating reducing the toxic effect of the original CuO-NP) and that this remains throughout the 84 days of testing (see example of data in Figure 13).

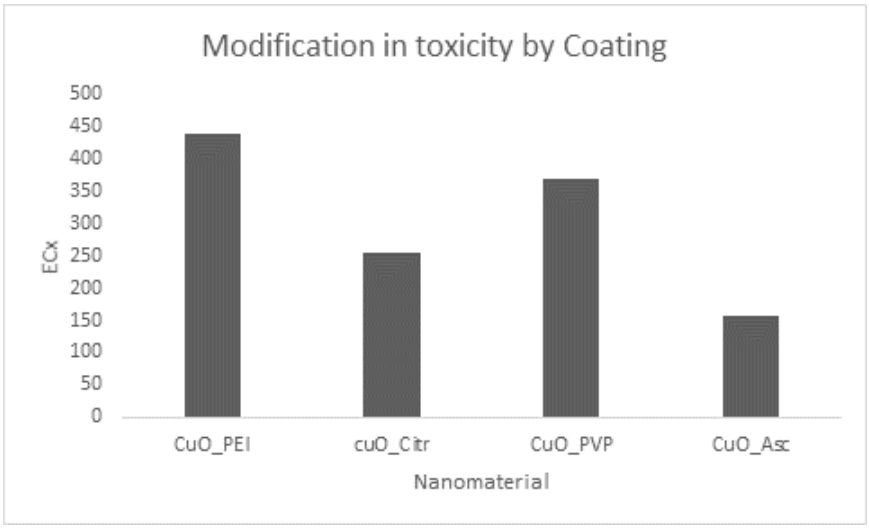

Figure 13: *Invertebrate toxicity for modified CuO nanomaterials, i.e. modified with Ascorbate, PVP, Citrate and PEI.*

Finally, although obvious it is still important to note that the above results e.g. that the soft-bodied was more sensitive than the hard bodied is solely accountable to this particular Cu-based case study, other materials may have the different sensitivity pattern.

Multigenerational studies and epigenetic effects

The multigenerational studies partly inform on the development in toxicity over time (and generations) hence can be used to predict possible longer-term consequences - i.e. recovery, continuous decrease or no changes (Figure 14 , top), For example , For CuO it is observed that for there is an initial increase in toxicity over time followed by a recovery when in clean soil. The same development was not observed for the Cu-salt form. Hence, a multi generation test will provide the risk assessment with ECx (e.g. EC50) values over time; this will allow the assessor to consider such changes for example by increasing the uncertainty factor.

If multi generation population studies are combined with methylation measures (for epigenetic effects) then it enable to explains the observed toxicity changes e.g. if methylation shuts down genes that are affected by Cu exposure (Figure 14, bottom. For example, observing that a pollution causes methylation of specific genes (or the whole genome) indicate the organisms will be affected different in the fooling generation. The importance of this could then be studied in an extended multispecies test system. Similar results for Earthworms are now being finalized and published.

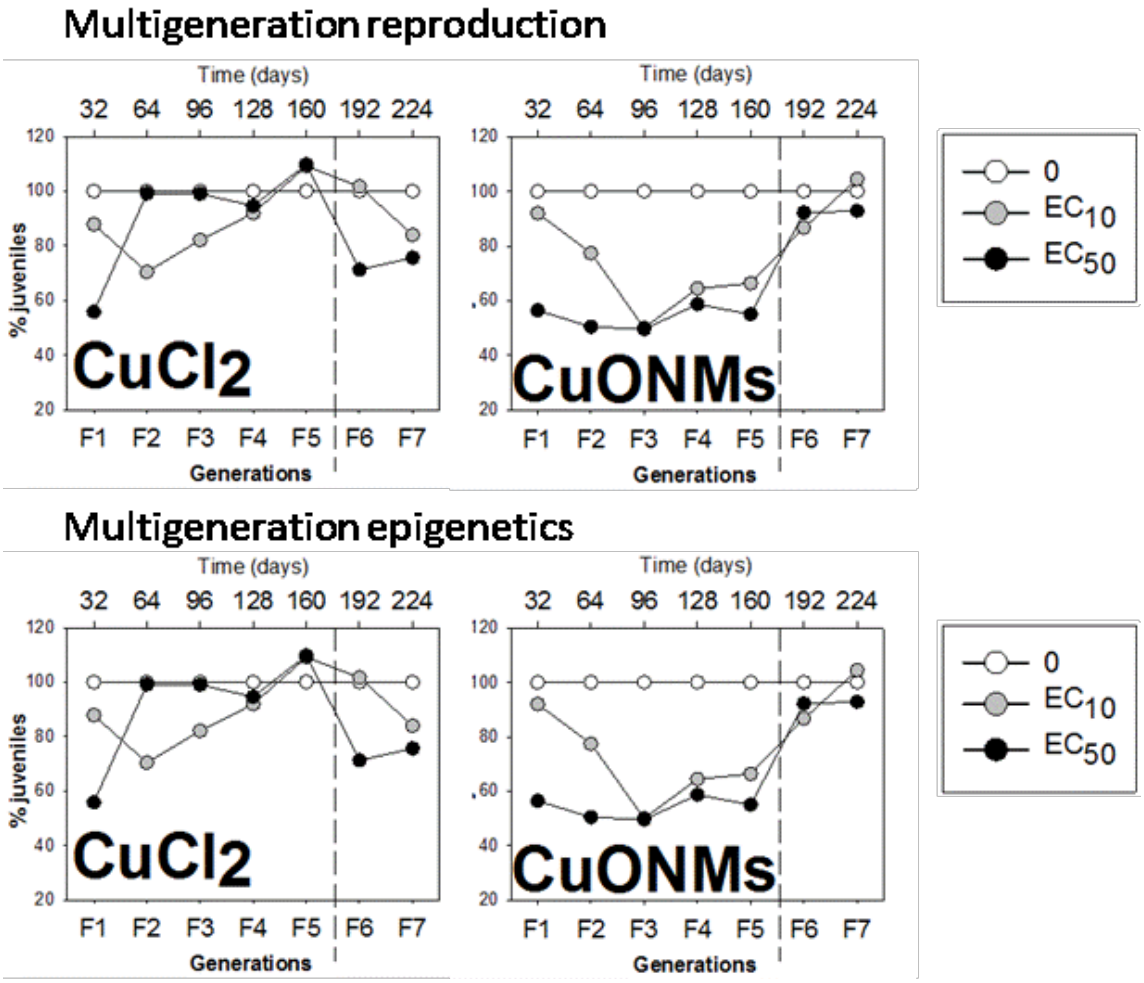

Figure 14: *Top: Reproductive developed over generations, when using the EC10 and the EC50 concentration from the 1st generation. Bottom left: Global methylation development over generations, when using the EC10 and the EC50 concentration from the 1st generation*

Water media

The purpose was to develop tools estimating and predicting the long-term impact in the aquatic ecosystems. In this section, we evaluate the data from the case study to provide a specific example of how data may be used by a risk assessor. Focus was on longer-term testing including repeated exposures. Thus, the repeated exposures tool (Task 4.1) is of direct relevance to this problem. Dissolved metals such as Cu are known to accumulate in sediments, and they may also be transferred into aquatic food chains, so in addition to exposure via the water, dietary uptake is also a concern for CuSO4. The settling behaviours of NPs in water also raise concerns about exposure of sediments and thus the base of the food web in aquatic systems (Klaine et al., 2008). In Task 4.2, fish studies at UoP have tackled the problem of dietary bioavailability of the CuO NPs and the factors altering it, compared to $CuSO_4$. In keeping with the 3Rs, the focus has been on in vitro dietary exposure tools rather than in vivo exposures. Regardless, dietary exposure and the sub-lethal effects of Cu metal salts to fish is well documented (e.g., Clearwater et al., 2002; Handy et al., 2005; Handy et al., 1999; Campbell et al., 2002). The effects of waterborne exposure of trout to dissolved Cu compared to Cu NPs has also been recently reported (Shaw et al., 2012; Al-Bairuty et al., 2013; Al-Bairuty et al., 2016). In these latter studies, the sub-lethal toxicity of the nano form was similar to the metal salt, except the aetiology of the nano exposure took longer to appear, supporting the notion for longer-term test methods. However, the studies were for a Cu metal NP, not a metal oxide as used in the SUN.

The ecotoxicity experiments conducted in SUN have also generated data that informs on exposure, especially dissolved and/or apparently bioavailable fractions of metal. The latter is especially important as environmental standards for metals are based on the bioavailable fraction, not the total metal in the aquatic system (e.g., Cu ERA by Brix et al., 2001). In Plymouth

water (a soft freshwater), the CuO NP at neutral pH showed negligible dissolution, but at pH 5 the material can release ~ 5% of the total Cu present in the particle (Figure 14). This may seem inconsequential, but the lethal toxicity of dissolved Cu is around 200-300 µg l^{-1} in Plymouth water depending on the water pH value (Table 8). Thus, a 10 mg l^{-1} dispersion of CuO NP might release 500 µg l^{-1} of metal in a few hours at pH 5, exceeding the lethal concentration to freshwater fishes.

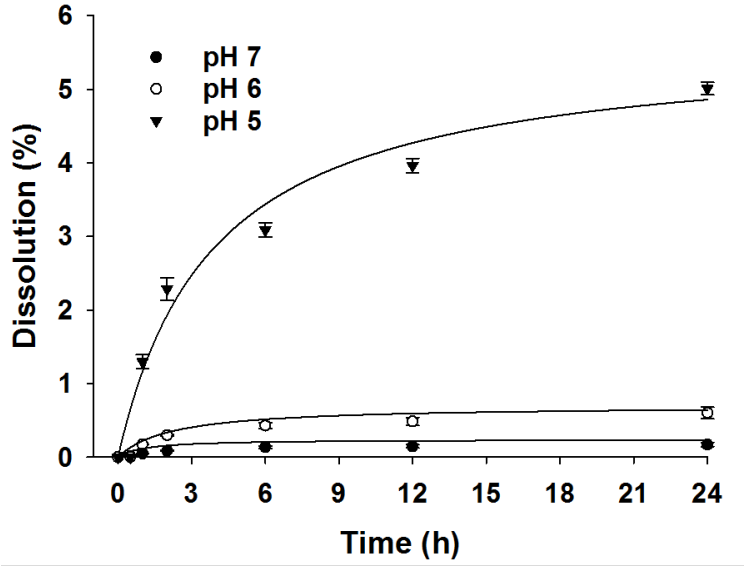

Figure 15: *Dissolution of CuO NPs at different water pH values in Plymouth water.*

Table 8: *96 h LC_{10} and LC_{50} values (mg L^{-1}) for SUN CuO NPs and metal salts in zebrafish embryos exposed from 2-96 hours post fertilisation.*

Material	pH 7		pH 6		pH 5	
	LC_{10}	LC_{50}	LC_{10}	LC_{50}	LC_{10}	LC_{50}
CuO NPs	28.8	142.3	12.2	25.6	2.8	6.6
Cu (as $CuSO_4$)	0.19	0.27	0.15	0.22	0.18	0.36

From the viewpoint of exposure for an ERA, acidic freshwaters (i.e., Scandinavian lakes, salmon rivers in Scotland) would be at risk of metal ion toxicity with low mg l^{-1} exposures to the NP. In Figure 15, the lethal concentrations of $CuSO_4$ and the CuO NP are shown for zebrafish, along with the estimated dissolved metal release. It is clear, that the NP over a range of pH values can present a dissolved metal hazard to fishes. The effect of pH on dissolved Cu toxicity is well known, with lower pH values being protective (Cusimano et al., 1985), due to competition between Cu and H^+ for binding at the gills (Paquin et al., 2002). Thus for $CuSO_4$, the lethal concentration is a higher dose when low pH is protective (Table 8). However, the trend is the exact opposite for the CuO NP (Table 8), presumably because the metal ion dissolution effect in acid conditions greatly out-weighs the ion competition restricting Cu binding at the gill. Exposure modelling for dissolved Cu usually involves the free ion activity model (FIAM) and consideration of the biotic ligand model (BLM) for the fish gill (Paquin et al., 2002). The data here from SUN argues that the BLM in its current form should not be used for a CuO NP, as it may incorrectly under-estimate the bioavailable fraction (i.e., a false negative on the exposure risk).

The lethal toxicity of $CuSO_4$ is expected to be of the order of a few hundred micrograms per litre in soft freshwaters, and this is also confirmed in the metal salt controls here (Table 8), along with expected pH effect of H^+ ions being protective. However, a central question for ERA is whether the hazard from the NP is less than that of the existing metal salts. In such a case, the existing ERA for dissolved metals will also be protective of the metal in nano forms. This notion is generally supported by the lethal toxicity data of the CuO NP to zebrafish, with the nano form being much less toxic than the metal salt (Figure 16A). However, the calculated dissolved metal releases from

the CuO NP exposure at the lethal concentration is shown (Figure 16B). The dissolved metal concentrations from the nano exposure are very similar to the $CuSO_4$ treatment, suggesting that regardless of form, it is the bioavailable dissolved metal fraction that should be used to estimate the hazard for risk assessment purposes.

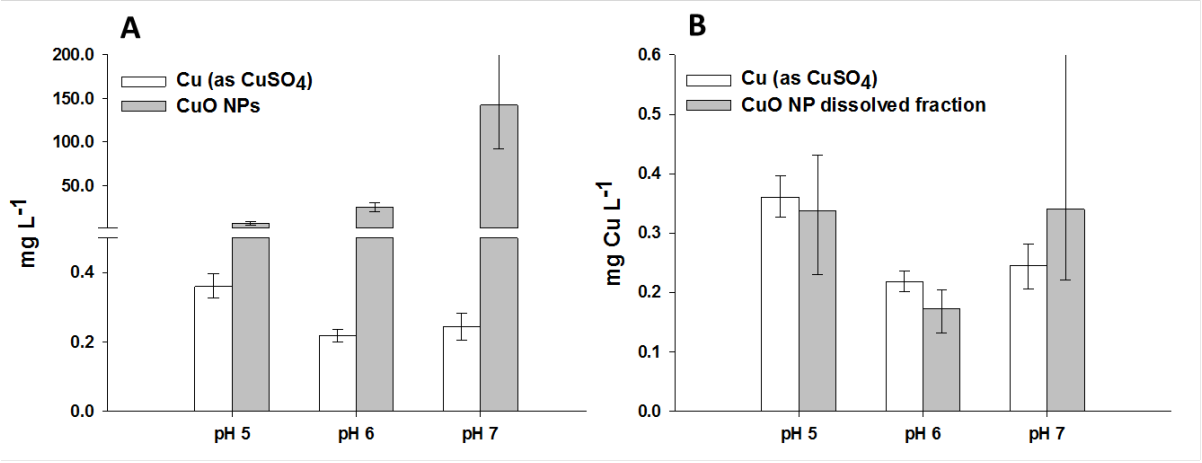

Figure 16: *A) 96 h LC_{50} values for Cu (as $CuSO_4$) and CuO NPs in zebrafish embryos exposed from 2 hours post fertilisation (bars are LC_{50} ± 95% CI; upper CI for CuO NPs at pH 7 is 1894.62 mg L^{-1}). B) 96 h LC_{50} values for Cu (as $CuSO_4$) presented alongside the estimated concentration of dissolved Cu (from data of NP dissolution) at the LC_{50} value for CuO NPs at each pH.*

As for terrestrial environment the aquatic ERA has been criticized for relying too much on lethal endpoints (black-box approach mentioned earlier), when it is the sub-lethal effects that inform on organism health in the long-term and may inform on the mode of action. Furthermore, use of data from continuous exposure studies with single species (i.e., standardised OECD regulatory testing), necessarily lacks the dynamics/complexity of real pollution events. The SUN repeated exposures tool tackles both these problems in ERA. The tool involves exposing early life stage zebrafish from 2-96 hours post fertilisation to calculated LC_{10} concentrations of the NP or metal salt, and comparing continuous with pulse exposure – but using sub-lethal end-points to measure the hazard (Figure 17).

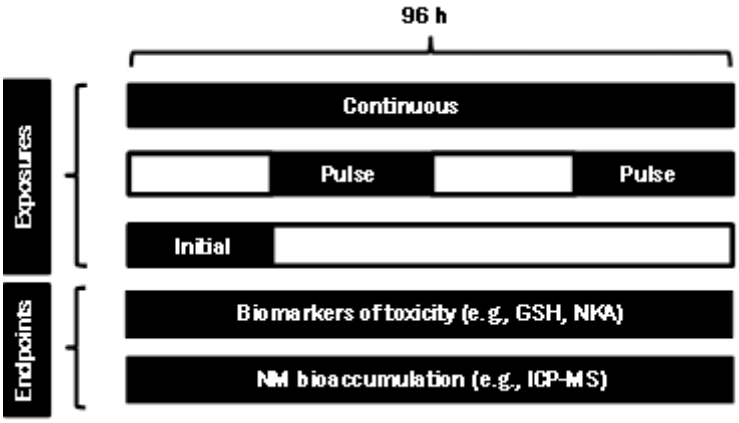

Figure 17*: Pulse exposure tool developed by UoP for use with NPs and including endpoints of relevance to Cu exposures (GSH = glutathione; NKA = Na^+/K^+-ATPase activity).*

Data from the pulse exposure experiments showed that there were treatment related mortalities in early life-stage embryos exposed at LC_{10} concentrations of $CuSO_4$ and CuO NPs; these were higher in fish exposed to the NPs. These mortalities were predominantly observed as occurring in the first 24 h of the experiment. There was close agreement in the effects of CuO NPs and $CuSO_4$ on parameters relating to oxidative stress (total glutathione, GSH) and ionic regulation (Na^+/K^+-

ATPase activity, Figure 18). There is an exception when zebrafish were exposed from 2-24 hours post fertilisation (early exposure). These embryos had significantly decreased Na^+/K^+-ATPase activity and total GSH concentrations at 96 hpf (hours post fertilisation) compared to $CuSO_4$. This was likely due to the strong association of the CuO NPs with the chorion (i.e., surface binding, not true uptake). Regardless, CuO NPs were much less toxic compared to $CuSO_4$ on a total mass basis. There was also no significant differences in effects between continuous or pulse exposure to either materials. This was despite fish in the pulse exposure receiving half the total dose over the course of the 96 h exposure.

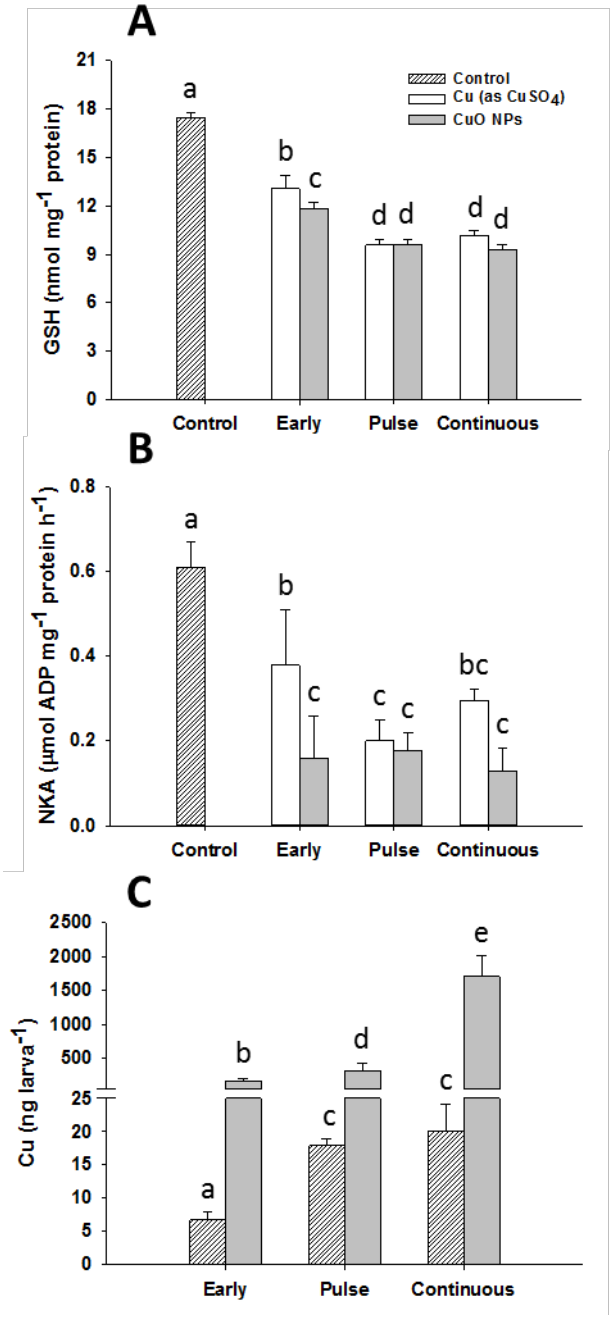

Figure 18: *Effects of CuO NPs and $CuSO_4$ on A) total GSH concentration, B) Na^+/K^+-ATPase activity (NKA) and C) Cu concentrations in chorionated zebrafish embryos at 96 h. Data are means ± SE (n=3/4). Different lower case letters indicate significant differences between treatment groups (One-way ANOVA with post-hoc Holm-Sidak test, p < 0.05). N.B., all control embryos had hatched by 96 h, so no values are presented for Cu concentrations.*

In summary, a number of tools have been developed for fish testing, tools that can be employed for many different nanomaterials. In the specific case of the CuO NP used in SUN showed that waterborne NP likely has a low toxicity to zebrafish. The calculated acutely lethal concentrations of CuO NPs in fish embryos are of the order of 2-30 mg l^{-1} for lethal toxicity (Table 8). The pulse exposure study at the LC_{10} also found sub-lethal effects, and so the probable no effect concentrations are likely to be an order of magnitude below that for sublethal toxicity (circa 100

µg l^{-1}). The environmental concentrations of CuO NPs are unknown, but in general, the predicted environmental concentration of NPs is in the ng l^{-1} – low µg l^{-1} range (e.g., Johnson et al. 2011). The PEC/PNEC for fishes would certainly be < 1, and the difference would be around two orders of magnitude. Thus, the CuO NP would be classified as a low environmental risk for fishes. However, there is uncertainty – the exact proportion of metal release by dissolution is water chemistry dependent, only one species of fish is reported here, and chronic exposure studies have not been conducted with fish. This level of uncertainty would add at least a factor of x10 to any risk assessment, and the margin of safety, after uncertainty may be only one order of magnitude for the CuO NP. Nonetheless, this is an encouraging interpretation of risk for the SUN materials overall, as the other EMNs in the project had negligible toxicity to early life-stage zebrafish (see 36 month report).

Develop long-term predictive tools based on short-term experiments

The purpose was to develop tools estimating and predicting the impact using short-term tests.

So far, current approaches for the environmental risk assessment (ERA) of chemicals in Europe do not take into account data generated using *in vitro, ex vivo* or short-term omics-based approaches or give to them limited value. If we consider as a paradigmatic case the REACH regulation (EC, 2006), for instance, ecotoxicity data necessary in the registration process are generated in different phyla using *in vivo* approaches. However, the same regulation from the very beginning (see for instance the paragraph 40 in the initial considerations) established that the European Commission, the industry and the Member States must contribute to the promotion of alternative methods at the national and international level.

Actually, cytotoxicity data obtained from *in vitro and ex vivo* approaches could be very useful at different levels, particularly in the framework of Integrated Approaches to Testing and Assessment (IATA). Probably, the two most frequent applications of *in vitro* tests are related with the selection of appropriate concentrations to be applied in further higher tier *in vivo* tests and with the characterization of the cellular and molecular mechanisms underlying the toxic action of chemicals or nanomaterials (NMs). Evidently, cell cultures have intrinsic limitations related with the lack of the metabolic systems present in a whole organism and, in the particular case of cell lines. There can be a modification or even a loss in the transcription of some genes and in the subsequent modifications that render non-functional proteins or enzymes. However, cells in culture can be maintained in a very limited space, with a minimal use of resources and they can be exposed to a number of concentrations of chemicals or NMs in a single culture plate. This makes *in vitro* cell cultures an ideal tool for determining the mechanisms of toxicity that probably are the same at higher organization levels and in the whole organism (Castaño et al., 2003; Fent, 2001).

Although traditionally used for toxicological studies related with chemicals, toxicity tests based on cell lines constitute also an ideal tool for generating basic information about possible deleterious effects of NMs, for the environment also information comparing sensitivity of species. In this case, particular attention must be payed to the generation of stable suspensions of NMs that would need a detailed physico-chemical characterization.
Cell lines are in general less sensitive than the whole organism. However, the selection of appropriate endpoints can lead to an increase in the sensitivity of an assay when generating information about a particular process or mechanism of toxicity. We have applied in the framework of SUN three different cytotoxicity assays on the same set of cells (Lammel and Navas, 2014). These assays inform about general alterations in the cellular metabolism, about malfunctioning of lysosomes and about disruption of plasma membrane integrity. This approach allows us to obtain initial data about the mechanism of toxic action and about toxic concentrations that can be very useful in the hazard assessment of a NM.

An important limitation of *in vitro, ex vivo and short-term* omics toxicity experiments is that they

are conducted as short-term assays, in which data about acute toxicity are generated. However, in a realistic scenario organisms are exposed to xenobiotics (very frequently a very low concentration) for long periods. Considering this, in the SUN project we have tried to develop tools that allow long-term exposure of fish cell lines in an attempt to generate data reflecting better real-life situations.

In vitro

High throughput (HTP) fish cell tool

Fish are the most widely used vertebrates in aquatic risk assessment and regulation (Fent, 2001). However, *in vivo* testing using fish requires an important use of resources and a high number of animals, what is ethically controversial. Therefore, as indicated previously, the European Commission encourages the application of alternative tests (EC, 2006). In this context, the use of fish cell lines appears as suitable for producing the initial data necessary for an appropriate hazard assessment of nanoparticles (NPs).

Rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) are probably two of the most studied species from cold waters. We decided therefore to use cell lines coming from these species. Since liver plays a key-role in detoxification processes and it has been observed that NMs tend to bioaccumulate in this organ (Connolly et al., 2016), we decided to use a cell line of hepatic origin. RTL-W1 cells are probably biliary epithelial cells in origin (Malhão et al., 2013) derived from the normal liver of an adult male rainbow trout (Lee et al., 1993) and they were chosen for our experiments. In addition, since after internalization in the organism NPs could reach the blood and be distributed to the whole organism and here they would interact with blood cells with immunity functions, we selected also a carp macrophage-like cell line (CLC). (Faisal and Ahne, 1990). In our approach, we exposed cells to NPs for a period of 21 days substituting cell culture medium with new medium containing the appropriate concentrations of NM every seven days (see Figure 19). In this way, we were able to observe the effect of a continuous exposure of cells to low concentrations of NM. For the selection of appropriate exposure concentrations, we performed in the first place short-term (24 h) cytotoxicity experiments and selected the used concentrations most close to the calculated EC_{50} and EC_{10} for the long-term exposures. We used CuO NPs as a representative material in our experiments. As a reference chemical, used as a control source of ions, we applied in parallel $CuSO_4$ at concentrations leading to similar Cu concentrations as in the CuO NPs exposures. What we observed was that low concentrations ($\approx EC_{10}$) did not cause an increase of toxicity in RTL-W1 cells after long-term exposures, although an evident increase in toxicity was observed in CLC cells. These results are indicating that the used concentrations of NPs yielded Cu concentrations that RTL-W1 cells can probably detoxify by mechanisms in place. However, concentrations similar to the EC_{50} provoked in both cell lines an important increase of toxicity with time, already observable after 7 days, that at 14 days led already to an important loss of cells that was complete (no viable cells were observable) after 21 days.

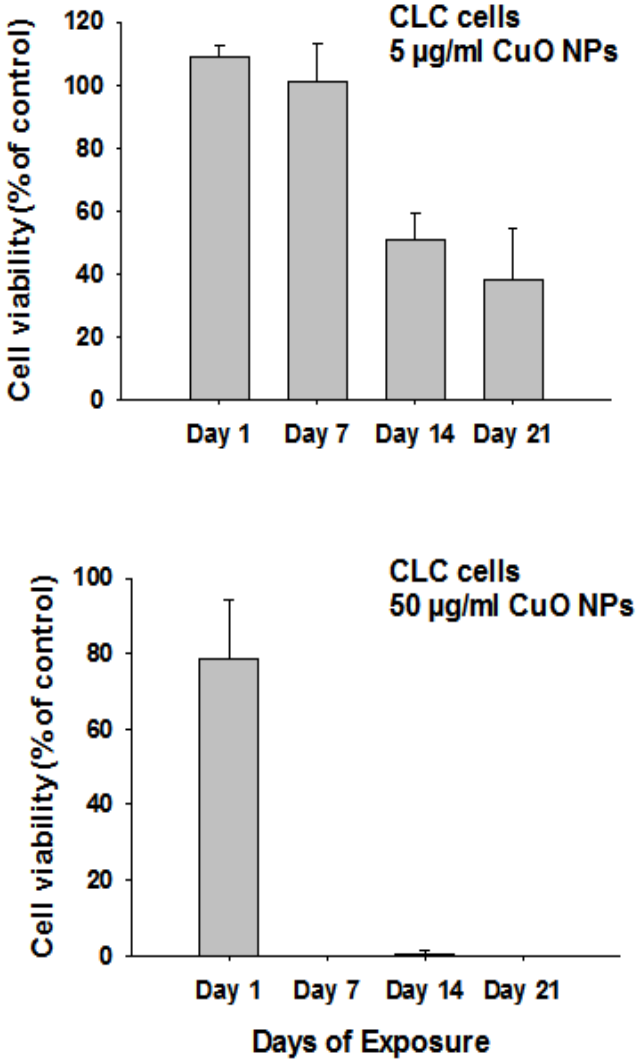

Figure 19: *CLC (Carp Leucocyte like cells) exposed to concentrations similar to the 24 hEC10 and EC50 for 21 days. A decrease in viability with time is evident that could not be detected after 24 h exposure. This can have important implications when using in vitro methods for assessing hazard. (The viability after the first 24 h of exposure, i.e. at day 1, was higher than expected probably due to the differences in the culture conditions between the 24h acute cytotoxicity assays, using 96 well plates, and the 21 days long-term exposure, using 24 well plates).*

The obtained data allows an increase in the sensitivity (since concentrations similar to 24h EC_{50} cause a higher toxicity in the long-term experiments. Even concentrations similar to 24h EC_{10} led to an increase in toxicity in one cell line) and in the specificity (since concentrations similar to EC_{10} did not cause this increase in toxicity with time, at least in one cell line) of the assay. What can be very valuable in the selection of appropriate concentrations to be applied in further more complicate *in vivo* assays.

In addition to this, the increase in sensitivity of the assay, with the corresponding reduction of false negatives, could allow the use of the generated information directly in the hazard assessment of the NMs, permitting in some cases to avoid some *in vivo* assays. The general increase in the accuracy of the assay could also allow a refinement of the assessment with the resulting reduction of the assessment factors.

High throughput (HTP) earthworm cell tool

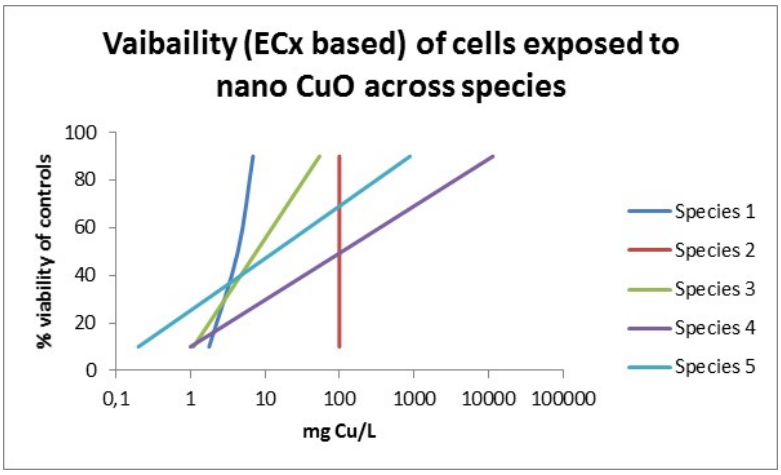

Figure 20: *Outline of the "viability" measures across 5 earthworm species exposed to pristine CuO nanomaterials. The lines represent the linear part of the concentration-response curve.*

As mentioned before, earthworms are the most widely distributed organism in terrestrial risk assessment and regulation of chemicals. In SUN in vitro test systems was developed for 5 earthworm species (Figure 20). The purpose was to include species that are present in European soils, which the standard species *Eisenia fetida* is not. Hence, it is with these in vitro tests possible to obtain a rapid cross- species information i.e. are there indications that one species is more sensitive than another to given nanomaterials. The current results indeed shows that for CuO, there was a large difference in toxicity between the species. In fact, Species 2 seem to be one of the least sensitive species, whereas the grassland species (Species 5) was 10 times more sensitive. This is very important information for a risk assessor, as it suggest performing studies at higher tiers (e.g. in vivo test) to confirm/disconfirm this. It should be remembered that the in vitro test systems, are less likely to suffer differences in "true-exposure" than is in vivo studies as performed in soil, hence they probably more likely express the biological difference between species.

Ex vivo

Fish gut for dietary exposure

Dietary exposure to metal salts are not usually lethal to fishes (Handy et al., 2005), but such experiments do inform on bioaccumulation potential of a substance. For a regulator, the main triggers for concern are persistence in the environment, bioaccumulation potential and toxicity (PBT). Of these, dietary studies are especially relevant to understanding the bioaccumulation potential, as well as the fate of NPs through aquatic food webs. In Task 4.2, UoP developed an in vitro tool to screen intestinal bioavailability of NPs in rainbow trout. Extended details of the methodologies used can be found in the 36 month report. Compartments of the gastrointestinal tract (stomach, anterior, mid- and hind intestine) were filled with 3.177 mg l^{-1} of Cu (as $CuSO_4$) or CuO NPs prepared in physiological saline at pH 7.8. These concentrations of $CuSO_4$ are relevant to Cu concentrations in invertebrate prey items in Cu-impacted rivers (e.g., Woodward et al. 1994; Boyle et al. 2008). Additional gut sacs were filled with physiological saline to account for background concentrations of Cu in the trout tissues. After 4 h incubation, tissue total Cu concentrations were measured in the gut mucosa and the underlying muscle layer of each gastrointestinal tract section. These data are shown in Figure 21.

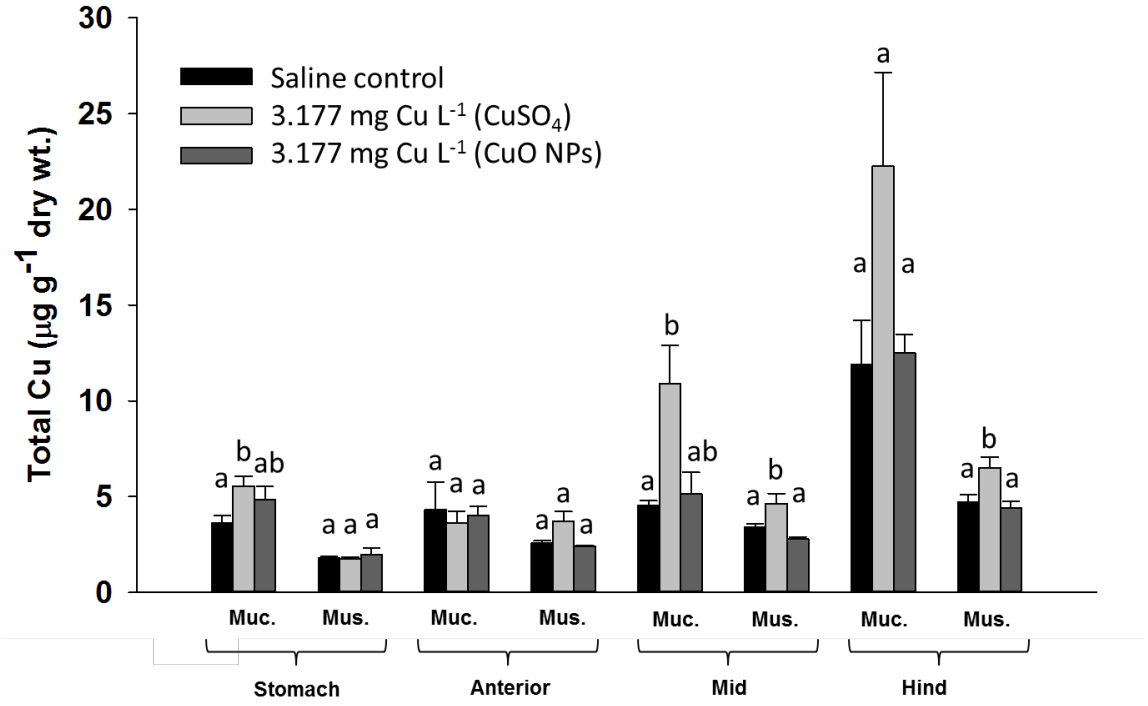

Figure 21: *Cu concentrations in mucosa (Muc.) and muscularis (Mus.) layers of rainbow trout intestine. Data are means ± SE (n = 6). Different lower case letters indicate significant differences between treatment groups and within tissue type (One-way ANOVA with post-hoc Holm-Sidak test, p < 0.05).*

Elevated total Cu concentrations in the mid- and hind-gut of rainbow trout confirmed these regions as the site of accumulation for Cu presented as $CuSO_4$, in keeping with our previous studies on gut sacs (Handy et al., 2000). For the metal salt exposure, some Cu was also evident in the underlying muscularis (Figure 20), indicating progress towards transepithelial uptake of Cu. In contrast, there were no statistically significant increases (One-way ANOVAs $p > 0.05$) in either the gut mucosa or muscularis for exposures to the CuO NP. Experiments are yet to be completed on surface binding to the gut, but data so far suggests low/negligible bioavailability of the CuO NP to trout gut in normal physiological saline.

The gut sac experiments above used of defined physiological salines with no added organic matter or food. The NP was simply dispersed in the saline. However, it is expected that more realistic exposures will give different results. The molecular biology of Cu uptake by cells is relatively well understood, and is Cu accumulation is known to be enhanced by the presence of amino acids in the media, such as histidine (van den Berg and McArdle, 1994). The presence of realistic gut conditions and/or nutrients known to influence bioaccumulation potential are not considered in the ERA for metals or NPs. Consequently, UoP has performed several *in chemico* studies to determine how the gut sacs behave when the CuO NP is presented at low pH (relevant to the stomach) and in the presence of histidine and cysteine, amino acids which will be present in the chyme (Figure 22A). These data indicate that dissolution of the NPs will occur as the CuO NPs pass along the gastrointestinal tract. For the pH effect in gut saline, pH 2 caused ~80 % dissolution, indicating that the metallic NP would be simply dissolved in the stomach, leaving the now dissolved Cu to be absorbed in the intestine. Perhaps more worrying, is that the presence of amino acids such as histidine (Figure 22B) also promote dissolution. These observations point towards a scenario of maximum bioavailability (low pH, followed by the presence of amino acids in food) that would lead to accumulation of dissolved Cu from the NP exposure. This needs to be confirmed in vivo, but it is clear that CuO NPs are not inert in the gut, and will contribute to the total body burden and therefore toxicity. However, there remain other data gaps, including how NPs behave in the trout intestine when presented as a live food, such as *Daphnia magna*. The

invertebrates do show gut impaction with NPs, and even if the invertebrate has not absorbed the ingested particles into its tissues. The fish will receive an NP dose simply be eating the whole animal.

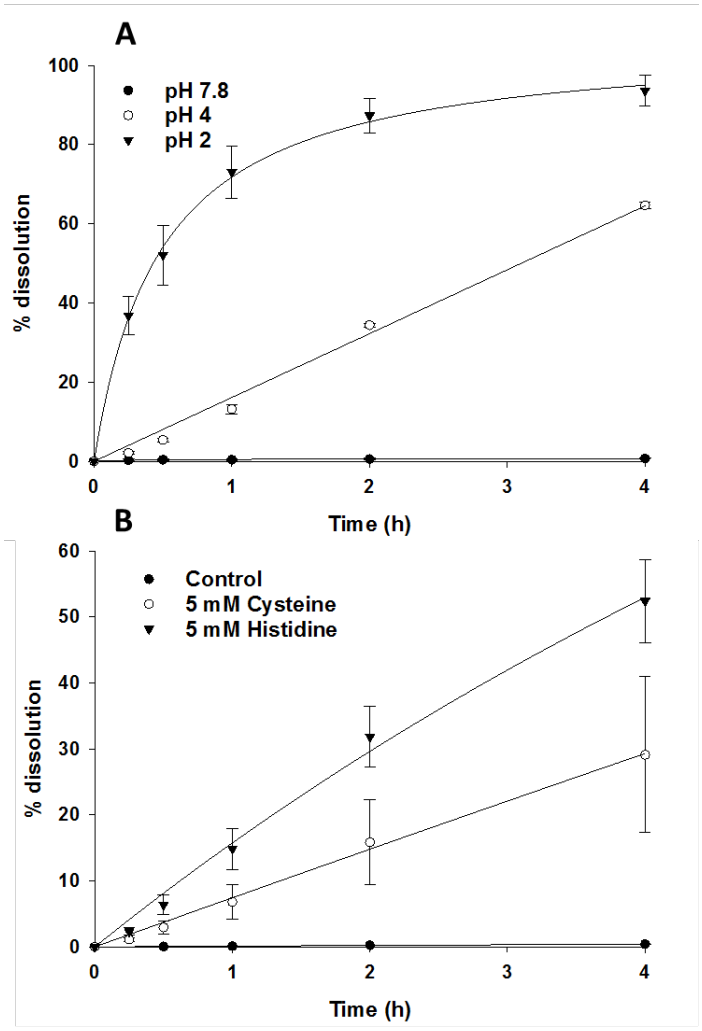

Figure 22: *A) Dissolution of CuO NPs in gut physiological saline at 15 ± 1℃ and at pH 2, pH 4 and pH 7.8 and B) in the presence of amino acids, at pH 7.8 only . Data are means ± SE (n = 3).*

In vivo

Short-term omics-expression testing

Several short-term tools were developed; these consisted of 1-14 day in vivo exposure studies where gene, protein and metabolite expression was measured (see Figure 23). To enhance the predictive capacity these were anchored in population effect concentrations (i.e. concentration for which effect are seen at the population level (reproduction/survival). These short-term studies can when they link the omics to (anchored) population level results be used to make the first AOPs (Figure 24).

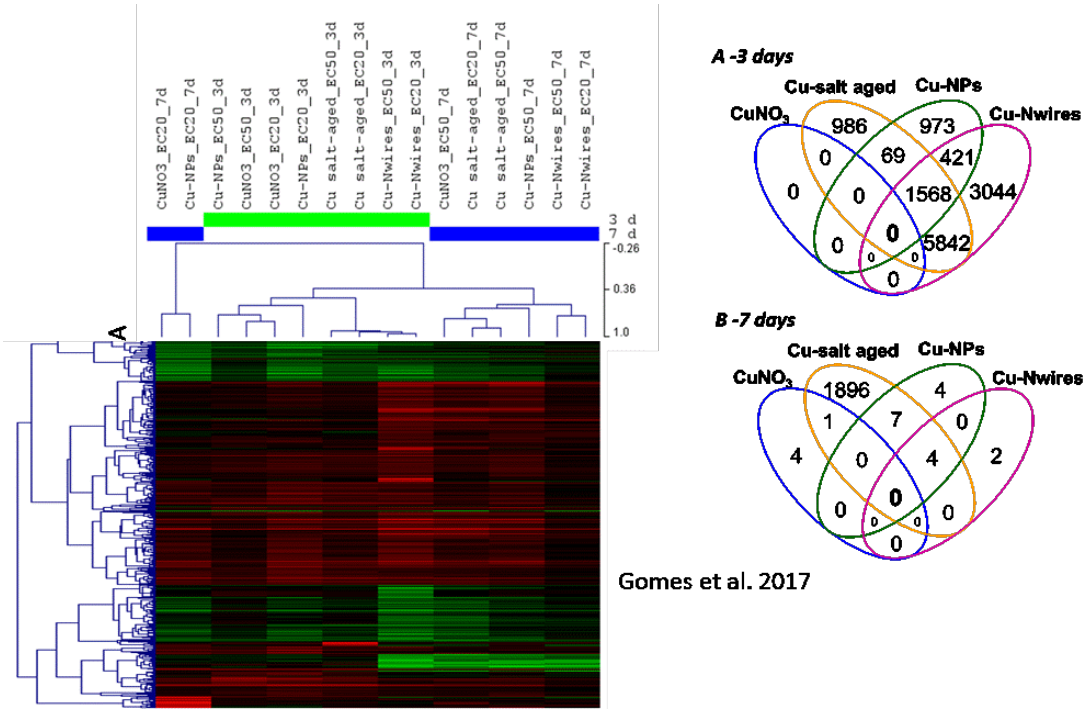

Figure 23: *Gene expression following exposure to Cu-salt or CuONPs for the Enchytraeid E. crypticus.*

These AOPs can then be used as a hypothesis to be further tested and extended. The AOPs can also be used to identify differences in AOPS between different materials (or forms of materials) as seen below, and in this way highlight for the risk assessor whether e.g. materials can be grouped.

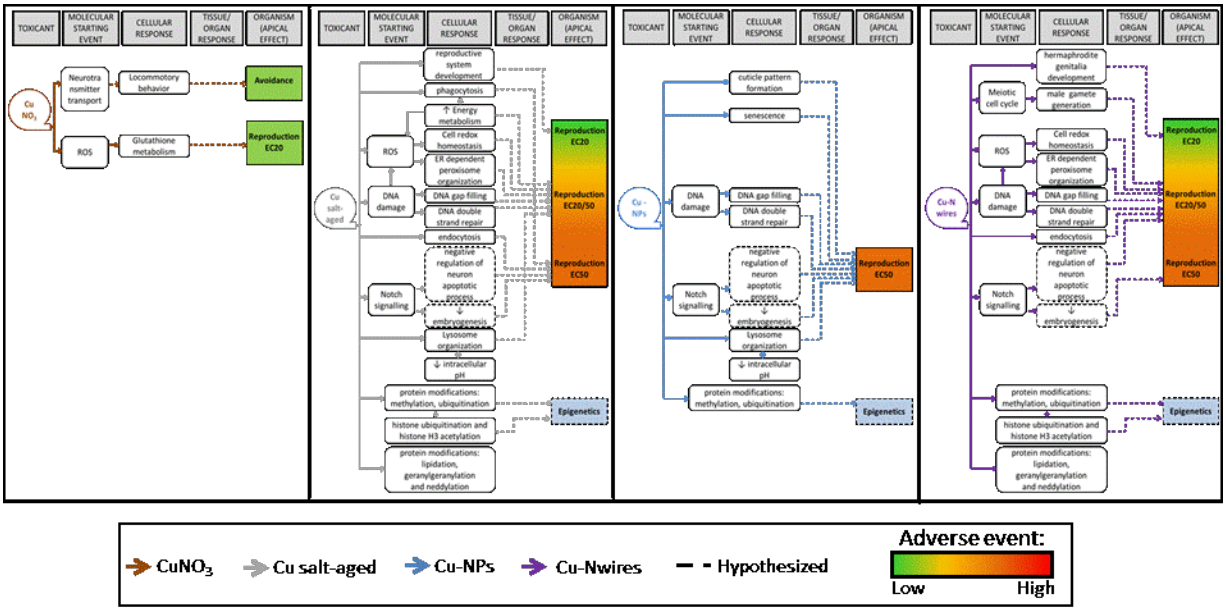

Figure 24: *.Example of Adverse Outcome Pathways (AOPs) developed for different forms of Cu exposure (Gomes et al in press).*

References

Al-Bairuty, G. A., Boyle, D., Henry, T. B. and Handy, R. D. (2016) Sublethal effects of copper sulphate compared to copper nanoparticles in rainbow trout (*Oncorhynchus mykiss*) at low pH: physiology and metal accumulation. Aquatic Toxicology, 174, 188–198.

Al-Bairuty, G.A., Shaw, B.J., Handy., R. D., and Henry, T.B. (2013) Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology, 126, 104–115.

Bicho, R.C., Santos, F.C.S., Scott-Fordsmand, J.J., Amorim, M.J.B. (2017) Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in Enchytraeus crypticus. Environmental Pollution, 224, 117–124.

Boyle, D., Brix, K.V., Amlund, H., Lundebye, A.K., Hogstrand, C. and Bury, N.R., (2008) Natural arsenic contaminated diets perturb reproduction in fish. Environmental Science and Technology, 42, 5354-5360.

Brix, K.V., DeForest, D.K. and Adams, W.J. (2001) Assessing acute and chronic copper risks to freshwater aquatic life using species sensitivity distributions for different taxonomic groups. Environmental Toxicology and Chemistry, 20, 1846-1856.

Campbell, H. A., Handy, R. D. and Sims, D. W. (2002) Increased metabolic cost of swimming and consequent alterations to circadian activity in rainbow trout (*Oncorhynchus mykiss*) exposed to dietary copper. Canadian Journal of Fisheries and Aquatic Sciences, 59, 768-777.

Castaño A, Bols N, Braunbeck T, Dierickx P, Halder M, Kawahara K, Lee LEJ, Mothersill C, Pärt Wood P, Repetto G, Sintes JR, Rufli H, Smith RW, Segner H. (2003) The Use of Fish Cells in Ecotoxicology. Toxicology in Vitro, 31, 317–351.

Clearwater, S.J., Farag, A.M. and Meyer, J.S. (2002) Bioavailability and toxicity of dietborne copper and zinc to fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 132, 269-313.

Connolly M, Fernández M, Conde E, Torrent F, Navas JM, Fernández-Cruz ML. (2016) Tissue distribution of zinc and subtle oxidative stress effects after dietary administration of ZnO nanoparticles to rainbow trout. Science of the Total Environment, 551-552, 334-343.

Cusimano, R.F., Brakke, D.F. and Chapman, G.A. (1986). Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout (Salmo gairdneri). Canadian Journal of Fisheries and Aquatic Sciences, 43, 1497-1503.

EC, European Commission, 2006. Regulation No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/105/EC and 2000/21/EC (2006).

Faisal M, Ahne W. (1990) A cell line (CLC) of adherent peripheral blood mononuclear leucocytes of normal common carp Cyprinus carpio. Developmental and Comparative Immunology, 14, 266-290.

Fent K. (2001) Fish cell lines as versatile tools in ecotoxicology: Assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicology in Vitro, 15, 477–488.

Handy, R. D. (1992) The Assessment of Episodic Pollution I. Uses and limitations of tissue contaminant analysis in rainbow trout (*Oncoryhnchus mykiss*) after short waterborne exposure to Cd or Cu. Archives of Environmental Contamination and Toxicology, 22, 74-81.

Handy, R. D. (1994) Intermittent exposure to aquatic pollutants: Assessment, toxicity and sublethal responses in fish and invertebrates. Mini Review in Comparative Biochemistry and Physiology. C., 107C, 171-184.

Handy, R. D. (Chair), McGeer, J. C., Allen, H. E. Drevnick, P.E., Gorsuch, J. W., Green, A. S. Lundebye-Haldorsen, A-K, Hook, S. E., Mount, D. R. and Stubblefield, W. A. (2005) Toxic effects of dietborne metals: Laboratory studies. In: Toxicity of Dietborne Metals to Aquatic Organisms (editors, J. S. Meyer, W. J. Adams, K. V. Brix, S. N. Luoma, D. R. Mount, W. A. Stubblefield and C. M. Wood). pp 59-112, SETAC Press, Pensacola, USA.

Handy, R. D., Musonda, M. M., Phillips, C. and Falla, S. J. (2000) Mechanisms of gastro-intestinal

copper absorption in the African Walking Catfish: Copper dose-effects and a novel anion-dependent pathway in the intestine. Journal of Experimental Biology, 203, 2365-2377.
ISO 15685 (2012) Soil Quality – Determination of potential nitrification and inhibition of nitrification – rapid test by ammonium oxidation.
ISO 22939 (2010) Soil Quality – Measurement of enzyme activity patterns in soil samples using fluorogenic substances in micro-well plates.
Handy, R. D., Sims, D. W., Giles, A., Campbell, H. A. and Musonda, M. M. (1999) Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. Aquatic Toxicology, 47, 23-41.
Johnson, A.C., Bowes, M.J., Crossley, A., Jarvie, H.P., Jurkschat, K., Jürgens, M.D., Lawlor, A.J., Park, B., Rowland, P., Spurgeon, D. and Svendsen, C. (2011) An assessment of the fate, behaviour and environmental risk associated with sunscreen TiO 2 nanoparticles in UK field scenarios. Science of the Total Environment, 409, 2503-2510.
Klaine, S. J., Alvarez, P. J. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J. and Lead, J. R. (2008) Nanomaterials in the environment, behaviour, fate, bioavailability and effects. Environmental Toxicology and Chemistry, 27, 1825-1851.
Lammel T, Navas JM. (2014) Graphene nanoplatelets spontaneously translocate into the cytosol and physically interact with cellular organelles in the fish cell line PLHC-1. Aquatic Toxicology, 150, 55-65
Lee LEJ, Clemons JH, Bechtel DG, Caldwell SJ, Han K-B, Pasitschniak-Arts IM, Mosser DD, Bols NC. 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome p450-dependent monooxygenase activity. Cell Biology and Toxicology, 9, 279-294.
Malhão F, Urbatzka R, Navas JM, Cruzeiro C, Monteiro RAF, Rocha E. (2013) Cytological, immunocytochemical, ultrastructural and growth characterization of the rainbow trout liver cell line RTL-W1. Tissue and Cell. 45: 159-174.
OECD 220 (2004) Enchytraeid Reproduction test.
OECD 222 (2004) Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei).
OECD 303 (2001) Simulation Test – Aerobic Sewage Treatment – A: Active Sludge Units, B: Biofilms.
Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F. and Gensemer, R.W. (2002) The biotic ligand model: a historical overview. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 133, 3-35.
Shaw, B. J., Al-Bairuty, G. and Handy, R. D. (2012) Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation. Aquatic Toxicology, 116-117, 90-101.
van den Berg, G.J. and McArdle, H.J. (1994) A plasma membrane NADH oxidase is involved in copper uptake by plasma membrane vesicles isolated from rat liver. Biochimica et Biophysica Acta -Biomembranes, 1195, 276-280.
Woodward, D.F., Brumbaugh, W.G., Delonay, A.J., Little, E.E. and Smith, C.E. (1994). Effects on rainbow trout fry of a metals-contaminated diet of benthic invertebrates from the Clark Fork River, Montana. Transactions of the American Fisheries Society, 123, 51-62.

Deviations from the Workplan

In the work plan there was also mentioned bioaccumulation and trophic transfer, although this has been pursued (and measured e.g. long-term earthworm test, Multispecies test, in vivo fish test. The bioaccumulation potential cannot be fully evaluated at present, since the analytical methods of detecting the NP within the organisms are not available (i.e. not developed yet). As mentioned, measurement of total concentrations (e.g. Cu) within the organisms (e.g. earthworm) has been done, these can be used directly in the secondary poisoning scenarios.

Performance of the partners

The partners have performed according to agreement, with a few exceptions of late delierance.

Conclusions

The Sun project was successful in developing a large number of tools (test systems) that enabled the estimation of long-term effects of nanomaterials in the environment. These tools cover the media normally covered in the Environmental Risk Assessment (ERA). The key data have been included in the probabilistic RA and the usefulness of the various methods in comparision to the RA has been discussed in this report.