DELIVERABLE REPORT

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Executive Summary

In this work for the NanoMILE deliverable report entitled "Pre-validated new model system (terrestrial isopods)" we first revised the existing data on validated test system with isopods established in a previous EU funded project (NanoValid project). Second, we implemented this knowledge in the NanoMILE project by testing the SOP: "Feeding and bioaccumulation assay with isopods" with the nanoparticles (NPs) obtained within NanoMILE. The model terrestrial organism *Porcellio scaber* (Isopoda, Crustacea) is described and the strength of the selected model is presented. Third, we developed a novel maternal transfer model system with isopods, conducting four experiments with two different NanoMILE NPs. We observed classical toxicological parameters, survival and feeding activity of females. The silver NPs did not cause any effect upon NP ingestion. The exposure of females on Ag NPs also did not affect the number of produced juveniles. The bioaccumulation studies revealed that silver was accumulating in the females and not in the juveniles after 3 days of feeding experiment with Ag NPs. For the conclusion, the weakness and strength of the used protocols are discussed and ways of improvement are proposed. We found the assay suitable for further intralaboratory testing of NanoMILE NPs.

1. Introduction

Within the Task 6.4 of the NanoMILE project we are investigating maternal transfer and possible transgenerational effect of nanoparticles (NPs) on juvenile isopods. On the basis of validated feeding assay with isopod *Porcellio scaber* and established SOP for bioaccumulation studies in NanoValid project (Annex 1 and 2) we have developed a new protocol for studying bioaccumulation and effect of NPs on juvenile isopods developing within females. The additional experiments for pre-validating this new protocol were done with NanoMILE nanoparticles (Figure 1).

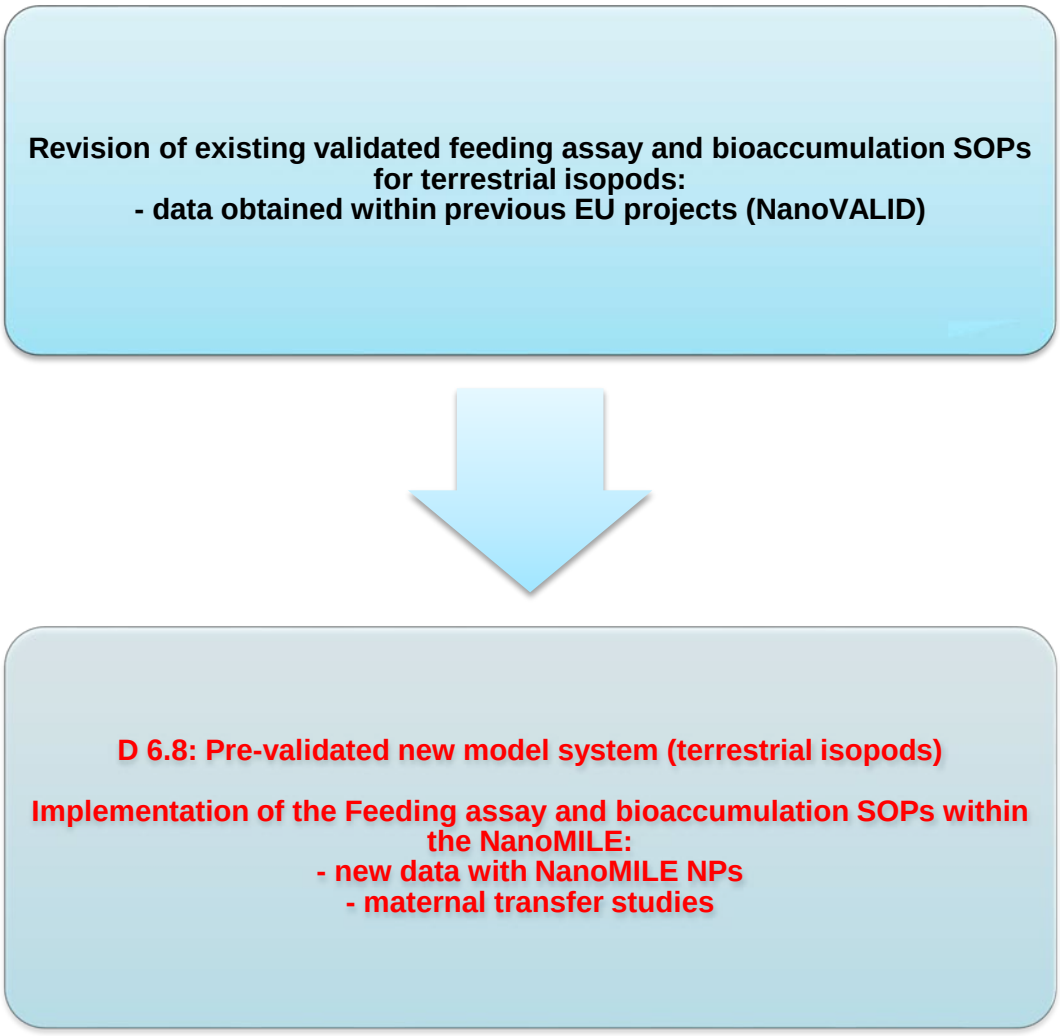

Figure 1: The organization of work represented in Deliverable 6.8. The two SOPs for the new model system were validated within NanoValid project and implementation of these protocols was done in NanoMILE project.

Single-species toxicity testing is highly important for characterization of chemical impact and only a few animal species fulfill the criteria for being used as test organisms for testing the effects of pollutants in the terrestrial environment. The biology of the species must be well known and some of the most important criteria are organism size (possible to maintain laboratory cultures and perform measurements on whole body), abundance, simple identification, sublethal responses to chemicals and information that enables differentiation of measured effects from natural background variability (Drobne, 1997).

Isopods are terrestrial invertebrates which are often a species of choice for toxicity studies. Their digestive system is the main route for contaminant intake and their accumulation in the body is attributed largely to dietary exposure (Drobne, 1997). Exposure of isopods (prominently *P. scaber*) to contaminant-treated food was established as a standard toxicity testing protocol by Drobne and Hopkin (Drobne and Hopkin, 1994, 1995) and was later adapted for nanotoxicity testing (Golobič et al., 2012). The species has been used in a variety of tests on the effects of elevated concentrations of metals (Drobne and Hopkin, 1994, 1995; Jereb et al., 2003; Zidar et al., 2005), biocides (Staak et al., 1998; Stanek et al., 2006), veterinary drugs (Kolar et al., 2010), and also nanoparticles (NPs) (Jemec et al., 2009; Drobne et al., 2008, 2011, 2012). In these tests several sensitive endpoints have been measured, including biochemical biomarkers, histopathological changes, behavioral response and physiological measures as well as organism level responses. The selected biomarkers vary in sensitivity, duration of feeding before the effect is detected and their ecological relevance.

Isopods have been shown to accumulate the highest concentrations of metals such as zinc, cadmium, lead, and copper so far recorded in any soft tissue (Hames and Hopkin, 1989; Vijver et al., 2004). Therefore, data on stored amounts of metals gives insight into bioavailable amounts of metals ingested with food and so the isopods are a useful model in bioaccumulation studies (Hassall et al., 2005). Metal accumulation in digestive glands, which are the major digestive organ with intestinal, hepatic, and pancreatic functions, is explained also as a detoxifying mechanism, which diminishes the potential adverse effect of ingested metal ions (Hames and Hopkin, 1989).

One of the adaptations of these crustaceans on terrestrial environment is the development of the marsupium, the brood pouch where females are carrying eggs. It is provisioned with fluid from the mother and allows early development to take place independently of an external water source (Surbida and Wright, 2001). During embryonic development in the marsupium the eggs are nourished by the female (Hoese and Janssen, 1989; Warburg and Rosenberg, 1996). They provide nutrition to the developing mancae (Hoese and Janssen, 1989) and they can also regulate pH and osmolality of the marsupial fluid (Surbida and Wright, 2001). It has been shown that diet of females can affect

phenotypic variation of juveniles (Kight, 2009) and that was motivation to study effect of female oral exposure to NPs for implementation of established model isopod system for testing of NPs.

2 Basic biology of *Porcellio scaber* (Isopoda, Crustacea)

Isopods inhabit the upper layer of the soil and surface leaf litter in a variety of urban and natural habitats. They belong to both meso- and macrofauna with an average body length between 8 and 12 mm. Some representative of the species can grow up to 20 mm long and usually have a grey colour, paler underneath, although brown, yellow or orange hues may also be observed (Figure 2).

Figure 2: A population of *Porcellio scaber (*Isopoda, Crustacea) in the natural habitat (photo: A. Jemec).

Their body is equipped with a number of types of appendages, like antennae, mouthparts, thoracopods, pleopods, and uropods. These provide a large surface area, and as such they lose water rapidly (Kostanjsek et al., 2006). They feed mainly on dead organic material, are distributed throughout the world, and are abundant in many different terrestrial environments (Van Capelleveen, 1987). They also play an important role in decomposition processes, mainly as fragmentors of dead plant material. Their choice of food depends largely on the quality of the leaves, the stage of decomposition and the moisture level. Isopods also eat their own feces and those from other soil animals so they are often regarded to be coprophagous (Kostanjsek et al., 2006). The body of terrestrial isopods is covered by a cuticle. Frequent moulting is an important characteristic of both juvenile and adult isopods and is related to growth, renewal of the cuticle and reproduction in mature females. Isopods moult in two phases, first shedding the posterior and then the anterior half of the body.

The food consumption of terrestrial isopods varies according to species (Warburg, 1987) and is approximately 0.4 mg dry food per mg dry wt. of animal per day (own observ.). Food assimilation efficiency is between 30 and 50% in *P. scaber* (Nair et al., 1994; Drobne and Hopkin, 1995). Food can remain in the gut for 4−17 h (Hartenstein, 1964) and is digested and absorbed in a 24 h digestive cycle (Hames and Hopkin, 1989). When the feeding rate is lower, food remains in the gut for a longer time. Animals produce 10−35 faecal pellets per day and one pellet is about 1. 5 ± 0.2 mm long. About five to seven pellets fill the entire gut. Daily faecal production is independent of the animal's size (own observation, Kostanjsek et al., 2006).

The digestive system of the terrestrial isopod *P. scaber* is composed of a stomach, four blind-ending digestive gland tubes (hepatopancreas), and a gut (Figure 3). Food enters the digestive glands directly *via* a short stomach or after the reflux from the gut, and ingested material is mixed with digestive fluids. The cuticles of the foregut and hindgut are completely renewed during exuviation (Štrus and Blejec, 2001). The pH in the digestive system varies from acidic in the midgut glands and anterior chamber of the hindgut to slightly acidic in the posterior part (Zimmer, 2002).

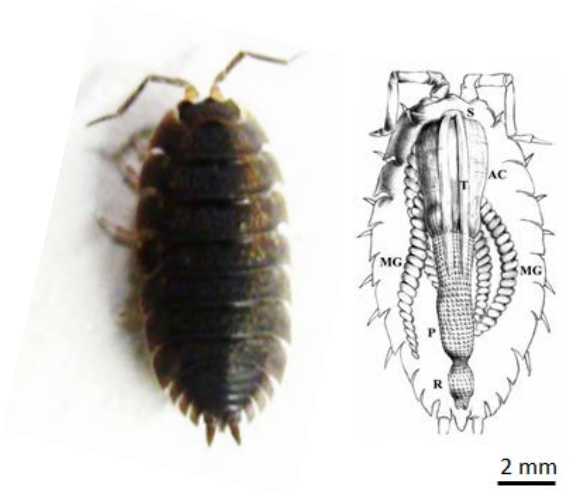

Figure 3: Anatomy of the digestive system of *Porcellio scaber.* a Dorsal view of stomach (S), midgut glands (MG) and hindgut consisting of anterior chamber (AC) with typhlosole (T), papillate region (P) and rectum (R) (photo: Kostanjšek et al., 2006; Jemec A.).

Hepatopancreatic tissue has intestinal, hepatic and pancreatic functions and is directly exposed to substances in the food (Zimmer, 2002).The hepatopancreatic epithelium contains two cell types, the large secretory and absorptive B cells that project into the lumen of the hepatopancreas and the wedge-

shaped S cells that lie between the B cells and accumulate large amounts of ions such as calcium, and salts of uric acid (Wägele, 1992). A link between the physiological condition of isopods and the histological appearance of the hepatopancreas and the biochemical stress markers has been confirmed (Jemec et al., 2012).

After fertilization of female isopods, the egg is moved to the brood pouch (marsupium). The terrestrial marsupium is filled with fluid from the mother and this allows early development to take place independently of an external water source (Surbida and Wright, 2001). In the marsupium embryo is developing and this yellowish developing egg is visible through the brood pouch (Figure 4). During embryonic development in the marsupium the eggs are nourished by the female through cotyledons (Hoese and Janssen, 1989; Warburg and Rosenberg, 1996).

Figure 4: brood pouch (marsupium) on the ventral side of *P. scaber* where juveniles are developing. On right photography the developed juveniles are hatching from the marsupium.
(photo:http://www.botanikbochum.de/pflanzenbilder_tiere/jpg/Porcellio_scaber_Kellerassel_BORobertMueser070609_ja02.jpg)

Intramarsupial development of *P. scaber* lasts about 35 days under laboratory conditions and includes embryonic development, from fertilized egg to the early-stage embryo, the mid-stage embryo and the late-stage embryo, and development of the marsupial larva manca until release to the external environment (Milatovic et al., 2010). Females provide nutrition to the developing mancae (Hoese and Janssen, 1989; Warburg and Rosenberg, 1996) and they can also regulate pH and osmolality of the marsupial fluid (Surbida and Wright, 2001). Diet of females can affect phenotypic variation of juveniles (Kight, 2009). Donker et al. (1993) showed that females collected from a lead contaminated area start to reproduce earlier which resulted in a fewer young per female.

Terrestrial isopods are among the most promising organisms in terrestrial ecotoxicology and *Porcellio scaber* is one of the widely studied terrestrial isopods. Therefore *P.scaber* is already proposed as the organism of choice for testing the toxicity of metals in the terrestrial environment (Drobne and

Hopkin, 1995). The advantage of using terrestrial isopods in toxicity studies compared to other terrestrial organisms is that a battery of parameters (e.g. organism feeding activity, digestive cells histopathology changes, biochemical parameters…) can be tested at different levels of biological complexity. Organisms have acquired different feeding strategies, which allow them to respond to spatial and temporal variations in the quality and quantity of food (Zimmer, 2002).

3 *In vivo* feeding exposure of isopods (*Porcellio scaber*, Isopoda, Crustacea) to NPs

Feeding exposure is the most important means for *in vivo* exposure of *P. scaber* to nanoparticles; moreover, it enables assessment of the exact exposure dose (Drobne, 1997), which is harder to estimate with other terrestrial invertebrates (e.g. earthworms, collembolans). Any changes in feeding rates of terrestrial isopods affect the decomposition process and subsequently matter and energy flux through ecosystems (Drobne, 1997). Feeding exposure represents the normalizing factor for all other measurements on *P. scaber* tissues, as established in standard toxicity testing protocol (Drobne and Hopkin 1994, 1995), and adapted for nanotoxicity testing (Golobič et al., 2012).

The isopod test with *Porcellio scaber* was first developed by (Drobne and Hopkin, 1994) and later adapted for exposure to NPs in our laboratory (Golobič et al., 2012, Pipan-Tkalec et al., 2010). In the test, the animals are fed with NP-contaminated leaves over a period of 14 days (Annex 1: SOP *In vivo* feeding exposure of isopods (*Porcellio scaber*, Isopoda, Crustacea) to nanomaterials, established in the NanoValid project).

A well characterised suspension of nanoparticles (in distilled water) with known concentration is applied on abaxial uncontaminated dry hazelnut (*Corylus avellana*) leaves surface. Leaves are weighting approximately 100 mg. In the control group, the leaves are treated only with distilled water. Each individual animal is then placed in a 9-cm Petri dish. One hazelnut leaf treated with distilled water or NPs suspension placed in each Petri dish is the animal's only food source. Humidity in the Petri dish is maintained by spraying tap water on the internal side of the lid every day. All Petri dishes are kept in a large glass container under controlled conditions in terms of air humidity, temperature and light regimen (16:8h light:dark photoperiod) (Figure 5).

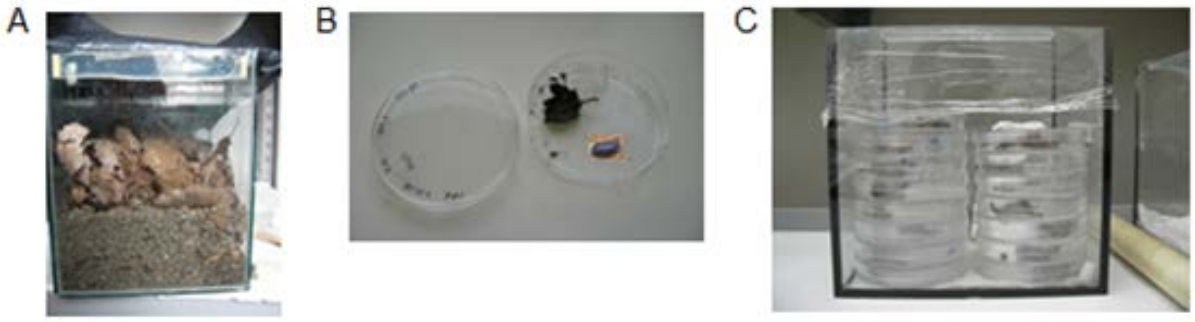

Figure 5: A laboratory culture of *P. scaber* in glass container filled with soil and dry leaves: (A) Petri dish with leave treated with nanoparticles as animal only food source (B) and glass container with Petri dishes with leaves and animals, which is kept under controlled conditions (C).

After the animals are exposed for 14 days to treated leaves, the fecal pellets and leaves are removed from the Petri dishes, dried at room temperature for 24 h, and weighed separately. The feeding rate of isopods is calculated as the mass of consumed leaves per animal wet weight per day. The food assimilation efficiency is calculated as the difference between the mass of consumed leaves and the mass of fecal pellets divided by the mass of consumed leaves. The amount of NPs consumed can be also calculated on the basis of the quantity of leaf consumed and the amount of particles applied on the leaf, with the assumption that the suspension is applied evenly on the leaf surface. The weight change of an animal is calculated as the difference in its mass from the beginning to the end of the experiment.

After the feeding exposure the animals are sacrificed and the tissues are analysed. After 14 days of feeding the total amount of uptake of NPs (metal) in the tissue is measured with atomic absorbtion spectroscopy (AAS) method (Golobič et al, 2012, Annex 2), biodistribution of metals is observed with proton induced x-ray emission (PIXE) (Novak et al, 2012) or Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICPMS). Assays have also been developed for studying cell membrane integrity and biochemical and molecular changes after NPs digestion (Valant et al., 2009; Romih et al., 2015).

3.1 Validation of the feeding assay with isopod *Porcellio scaber*

3.1.1 Presentation of the assay

University of Ljubljana was a project partner in NanoValid, a project where the reference methods for toxicological testing, NPs exposure and effect assessment have been developed. Within the project the protocol for exposure and effect of nanomaterials on terrestrial invertebrate organism *Porcellio scaber* was validated. Data on the intra- and inter- laboratory comparisons have provided evidence, that the assay exhibits good repeatability and has the potential to be further validated to reference method for nanomaterial induced terrestrial invertebrate toxicity and bioaccumulation.

In the laboratory comparison studies, NanoValid CuNPs were used as testing nanoparticles. In addition, also copper salt was tested. All partners tested the following concentrations: 2000 µg Cu (CuO NPs) /g leaf, 5000 µg Cu (CuO NPs) /g leaf, and 5000 µg Cu^{2+}/g leaf. Results showed that the measured concentrations of Cu on leaves that have been sent by the participants were close to nominal values as reported also by the reference laboratory, UNI-LJ. Greater variability of Cu content was observed in case of 5000 µg CuO NPs/g leaf in comparison to lower concentration (Figure 6).

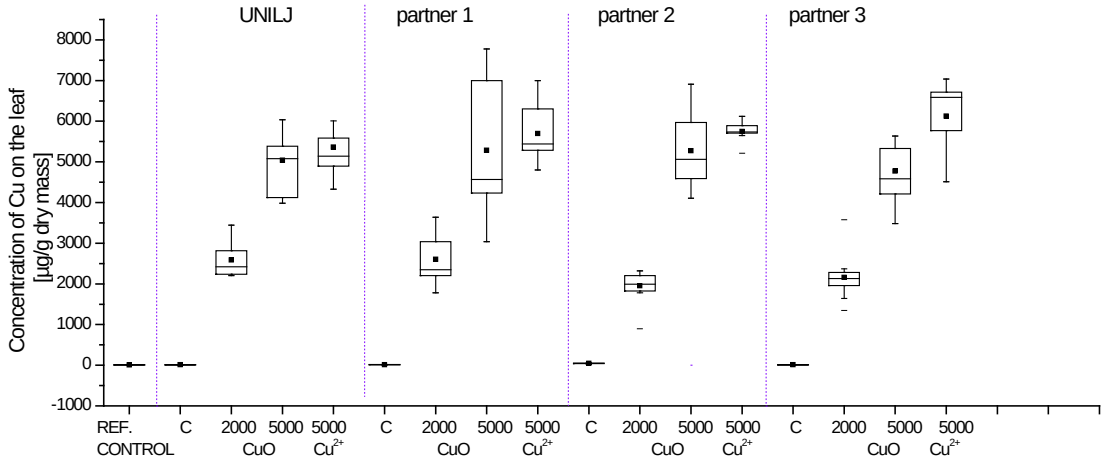

Figure 6: Copper concentration on the leaves (µg Cu/g leaf) in the reference laboratory (UNILJ), and three participants. Symbols on the box plot represent value: maximum and minimum value (whiskers: ⊥), mean value (■), outliers (-).

The median values for feeding rate from all participants were in the interval of reference feeding value for control population [0.032, 0.073] mg/g (Drobne and Drobne, 2014). The participants obtained the same 2 weeks LOEC[1] $_{\text{feeding rate}}$ as in our laboratory (5000 µg Cu/g leaf exposure) (Figure 7).

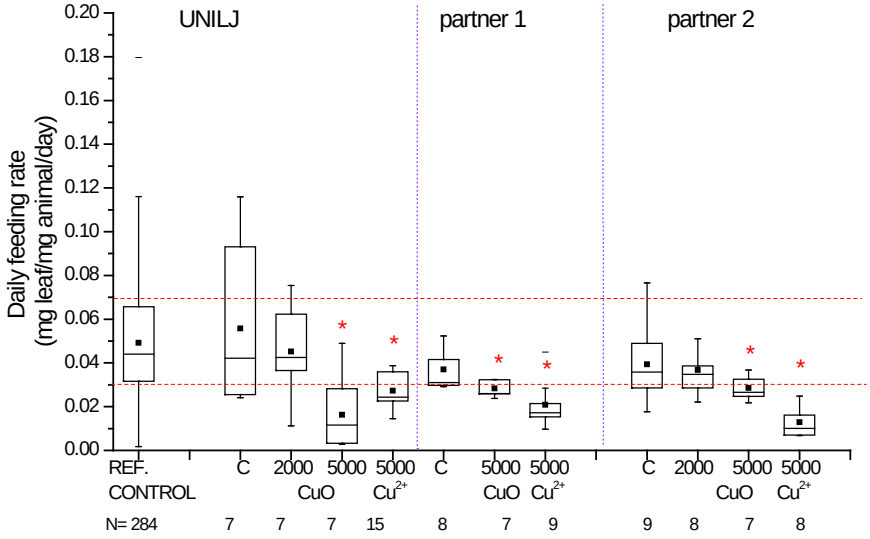

Figure 7: Feeding rate of animals exposed to 2000 and 5000 µg Cu/g leaf. Nominal exposure concentrations of Cu are provided on x-axis. Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (■), outliers (-), and $p<0.05$ (*; Mann-Whitney). Red dotted lines are reference feeding value for control population (Drobne and Drobne, 2014).

In conclusion, the validation study with the isopods within the NanoValid project revealed that the "In vivo feeding exposure of isopods (*Porcellio scaber*, Isopoda, Crustacea) to nanomaterials" is repeatable and reproducible and can thus serve as a basis for the development of other test assays with this test model.

[1] LOEC: Lowest Observed Effect Concentration

3.1.2 Implementation of validated feeding assay with isopod *Porcellio scaber* – exposure to NPs produced in NanoMILE project

In the NanoMILE project, a range of nanoparticles were produced, and a selection of these were used in WP6 in order to establish *in vivo* reactions between NPs and selected organisms. We used the established validated SOP for feeding experiments to expose model organisms to the NPs. We implemented the validated data with the results on how these tested NPs affect the feeding behavior of isopods. One of the objectives of WP6 was to establish if specific features, like ageing of NPs confer significant alteration in biological system. In addition the aged NPs were tested to assess what could possibly result in different feeding activity of animals.

In one experiment, animals were exposed to Ag NPs and to same, but aged NPs, with exposure concentration 1000 µg Ag/g leaf. In a separate experiment, animals were exposed to aged (done by NanoMILE partner, University of Birmingham) and non-aged CeO_2 NPs with exposure concentration 1000 µg Ce/g leaf. The results showed that there was no statistically significant effect of tested materials on feeding behavior of isopods (Figure 8). We compare the obtained values with reference control values for feeding rate and conclude that obtained data were in the interval of reference feeding value for control population (0.032, 0.073) mg/g (Drobne and Drobne, 2014) as in the validation study of project NanoValid.

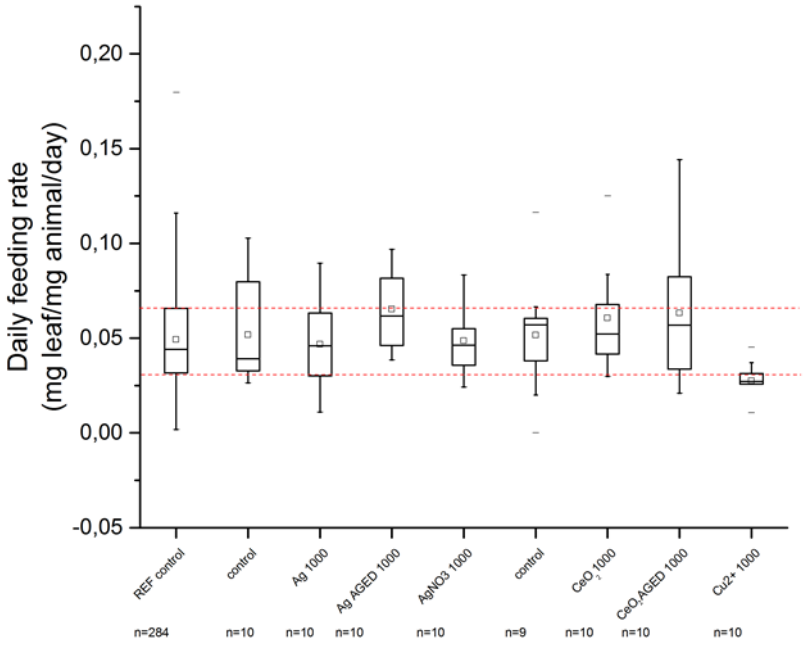

Figure 8: Feeding rate of animals exposed to 1000 µg Ag and CeO_2 (non-aged and aged)/g leaf. The $AgNO_3$ and Cu^{2+} are positive controls. Nominal exposure concentrations (1000 µg NPs/g of food) and number of animals

per group are provided on x-axis. Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (□), outliers (-). Red dotted lines are reference feeding value for control population (Drobne and Drobne, 2014).

3.2 Validation of the NPs (or metals dissolved from them) bioaccumulation assay with isopods

The second commonly applied assay with isopods is the study of bioaccumulation of NPs. The study relies on the 14 days of feeding on NPs, and then the isopods are sectioned, digestive glands are isolated and the concentration of metals is measured with one of the techniques to study the body burden of NPs in organisms, by AAS or ICPMS. The assay on the bioaccumulation of metals dissolved from CuO NPs has also been validated within the NanoValid project. The data of copper analysis of animals were comparable to the reference laboratory and revealed that the Cu-exposed isopods accumulated significantly more Cu than control animals (Figure 9).

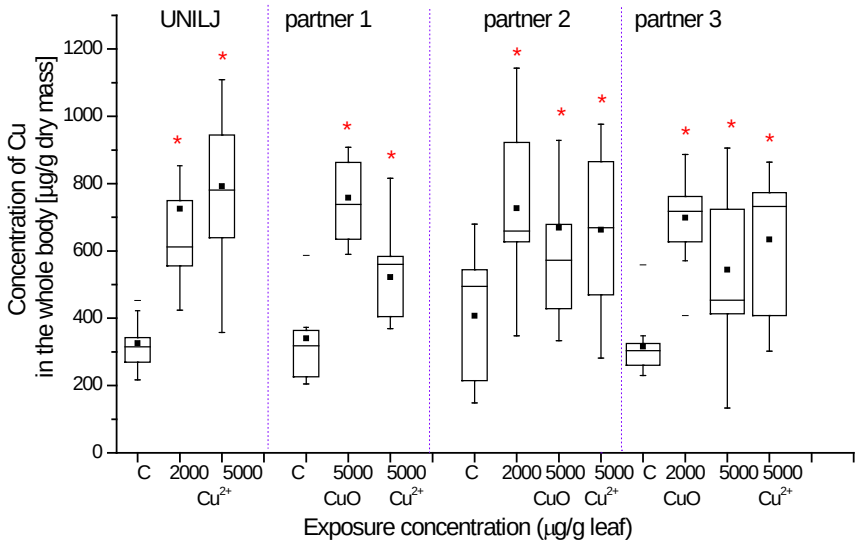

Figure 9: Bioaccumulation of copper in the whole body of isopods. Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (■), outliers (-), and $p<0.05$ (*; Mann-Whitney).

The protocol will be implemented with the results on bioaccumulation of NPs produced in the NanoMILE project and data will be represented in other deliverables (work in the Task 6.1 and 6.3) of WP6.

4 Maternal transfer studies in the NanoMILE project

The knowledge gained within the validation of the "Feeding and bioaccumulation assay with isopods" tested with NanoMILE NPs was used to develop a new model assay for the NPs maternal transfer studies. Here we present the development of this assay.

The females of isopods with marsupium from laboratory cultures were exposed to selected NPs. Their feeding behavior was observed, the number of juveniles hatched after female exposure to food treated with metal NPs was counted and the bioaccumulation of the metals in the juveniles and the females was measured (feeding method based on: Annex 1: SOP *In vivo* feeding exposure of isopods (*Porcellio scaber*, Isopoda, Crustacea) to nanomaterials, established in the NanoValid project). We conducted four experiments:

	Nanoparticles	**Exposure concentration**	**Number of exposed females/group**	**Duration of experiment**
Experiment 1	TiO_2 and TiO_2 with PVP (NNM PROm-TiO_2un-10nm-121113c and NNM PROm-TiO_2PVP-10nm-121113c)	1000 µg of TiO_2 NP/g of food	10	2 – 28 days
Experiment 2	Ag NPs (Silver(JRC)-NM300K)	1000 µg of Ag NP/g of food	10	1-19 days
Experiment 3	Ag NPs (Silver(JRC)-NM300K)	1000 µg of Ag NP/g of food	5	7-14 days
Experiment 4	Ag NPs (Silver(JRC)-NM300K)	1000 µg of Ag NP/g of food	5	3 days

Table 1: Feeding exposure of females with developing marsupium experiments conducted with NanoMILE nanoparticles.

The time of exposure in standard feeding assay is 14 days. In the four maternal feeding experiments conducted, the time of exposure varied because of different stages of intramarsupial development of juveniles. We observed the developing embryos through the brood pouch with stereomicroscope.
In the first experiment we collected females which appeared to have a late stage marsupium, but some of the juveniles underwent hatching from marsupium after only two days of feeding females on NPs. The longest period of exposure to hatching was 28 days. In the second experiment we tried to choose population of females in more uniform stage of intramarsupial development but again the hatching of some juveniles had already started after one day of exposure; some of the animals in the experiment did not hatched before 19 day of exposure. In a third experiment the juveniles hatched between 7 and 14 days.

When the animals are hatched from the marsupium (which can happen at night time) they can immediately consume the leaves treated with NPs (the only food source in the Petri dish), and this in turn could be the source of measured metals in their bodies. We concluded that we could not establish the intramarsupial development states of juveniles in the marsupium without isolating them. With isolation of even one or two juveniles the marsupium would be disrupted and maternal transfer could be interrupted. Based on this observations we decided to adopt the feeding protocol and expose females with marsupium only for 3 days (Experiment 4) and then isolate the juveniles directly from the marsupium and prepare them for bioaccumulation studies. In that experiment animals were exposed to silver NPs and positive control ($CoCl_2$ with concentration 2000 µg of Co/g of food). We were unable to do more experiments at the time because of the lack of fertilized females in the laboratory culture.

In the experiments survival of females and their feeding behavior (feeding rate) was observed. In the experiment 3, when hatching from the marsupium occurred, juveniles were counted.

4.1 Results on feeding behavior, survival of females and number of hatched juveniles

The survival of females in the experiment 1 and 2 was low in controls where 50% of control animals die in the experiment 1 and 30% of animals did not survive in the experiment 2. In the Experiment 3 and 4 survivals of animals in control and in all exposure groups was 100%. In the group exposed to Ag NPs in experiment 4 the survival rate was 80% (Figure 10).

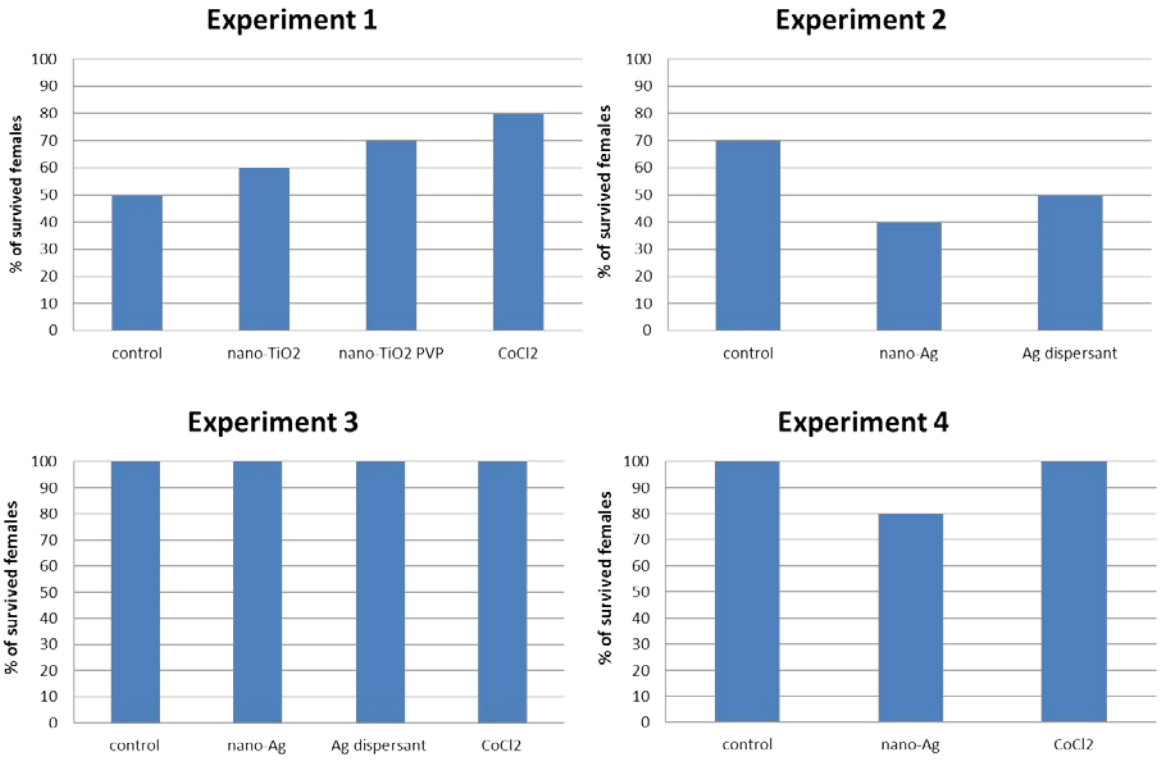

Figure 10: Percentage of females surviving in each experiment. In experiment 1 and 2, 10 animals per group were exposed and in experiment 3 and 4, 5 animals were exposed to food dosed with nanoparticles. The nominal exposure concentrations were 1000 µg of TiO_2 NP or Ag/g of food and for positive control 2000 µg of Co/g of food.

The first two experiments were performed with animals from the same laboratory culture and after the experiments we observed high percentage of animals with digestive glands bacterial infection (Figure 11) in the culture and this invalidated the results from these experiments. Only experiments 3 and 4 are considered as valid.

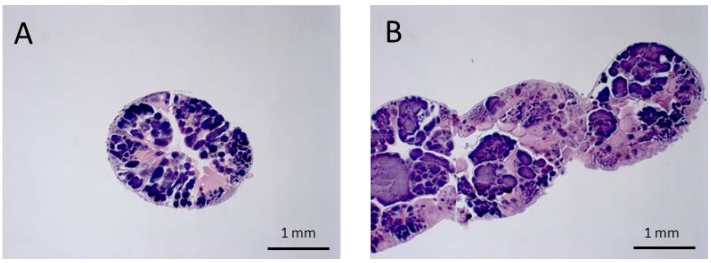

Figure 11: Cross section (A) and longitudinal section (B) of digestive gland of control animal. Hematoxylin and eosin staining. Hypertrophed cells seen as violet dark regions stained by hematoxylin (stains nucleic acids), filled with intracellular bacteria in different developmental stages.

Feeding rate (calculated per day of exposure) in two valid experiments (exp. 3 and 4) was comparable to feeding value for control population median values (0.032mg/g, 0.073mg/g respectively) of animals fed with Cu NPs, established in the work of Drobne and Drobne (2014). There was no statistical differences among control and exposure groups in the case of exposure to NPs (experiment 3 and 4) or only Ag dispersant (Experiment 3). A statistically significant smaller feeding rate was observed with positive control ($CoCl_2$) in both experiments (Figure 12).

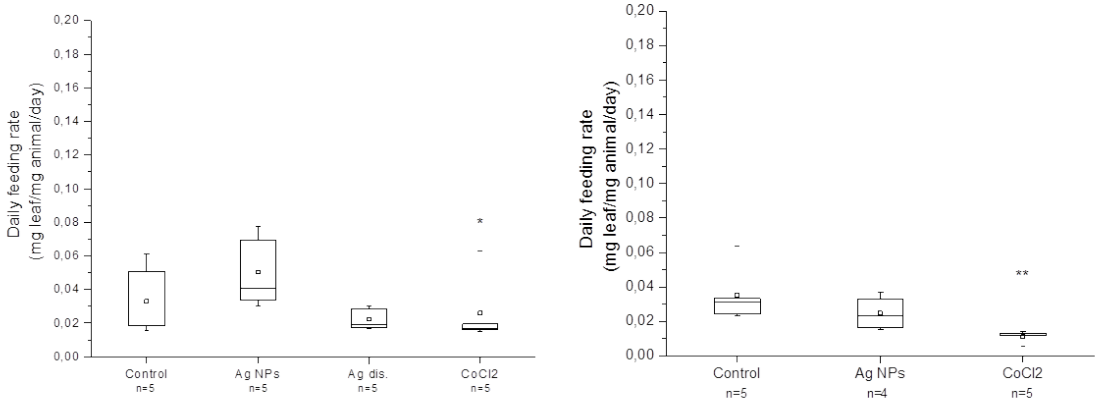

Figure 12: Feeding rate of animals exposed to 1000 Ag/g leaf (for Ag NPs and Ag dispersant) and 2000 and µg Co/g leaf for the positive control. Number of survived animals are represented x-axis (n). Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (□), outliers (-) and $p<0.05$, $p<0.01$ (*;**, Mann-Whitney).

In the literature it has been reported that exposure of females to metal pollutants in the environment can affect the number of young per female (Surbida and Wright, 2001). In experiment 3 we have not observed changes in the number of juveniles after NPs ingestion (Figure 13). In experiment 4 the juveniles were removed directly from marsupium and some of them were in the too early development state to be counted, therefore the data for number of juveniles in this experiment are missing.

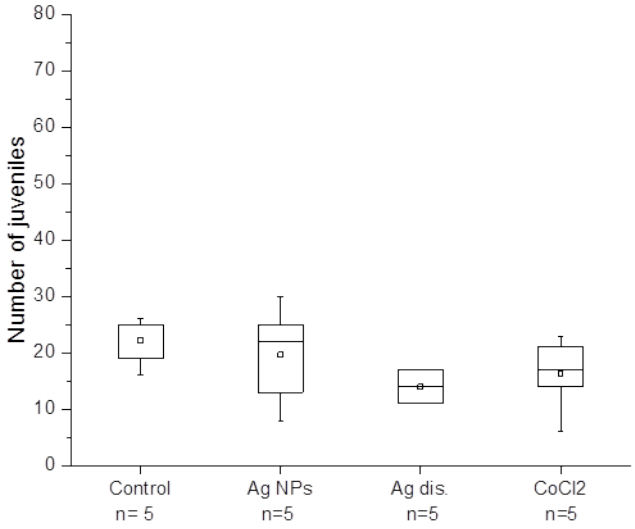

Figure 13: Number of survived juveniles per female exposed to 1000 µg of Ag or Ag dispersant/g leaf and 2000 and µg Co/g leaf for the positive control. Number of survived animals are represented x-axis (n). Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (□), outliers (-).

We also concluded that the duration of exposure or the female initial weight is not correlated with the number of juveniles hatched in experiment 3 (Figure 14).

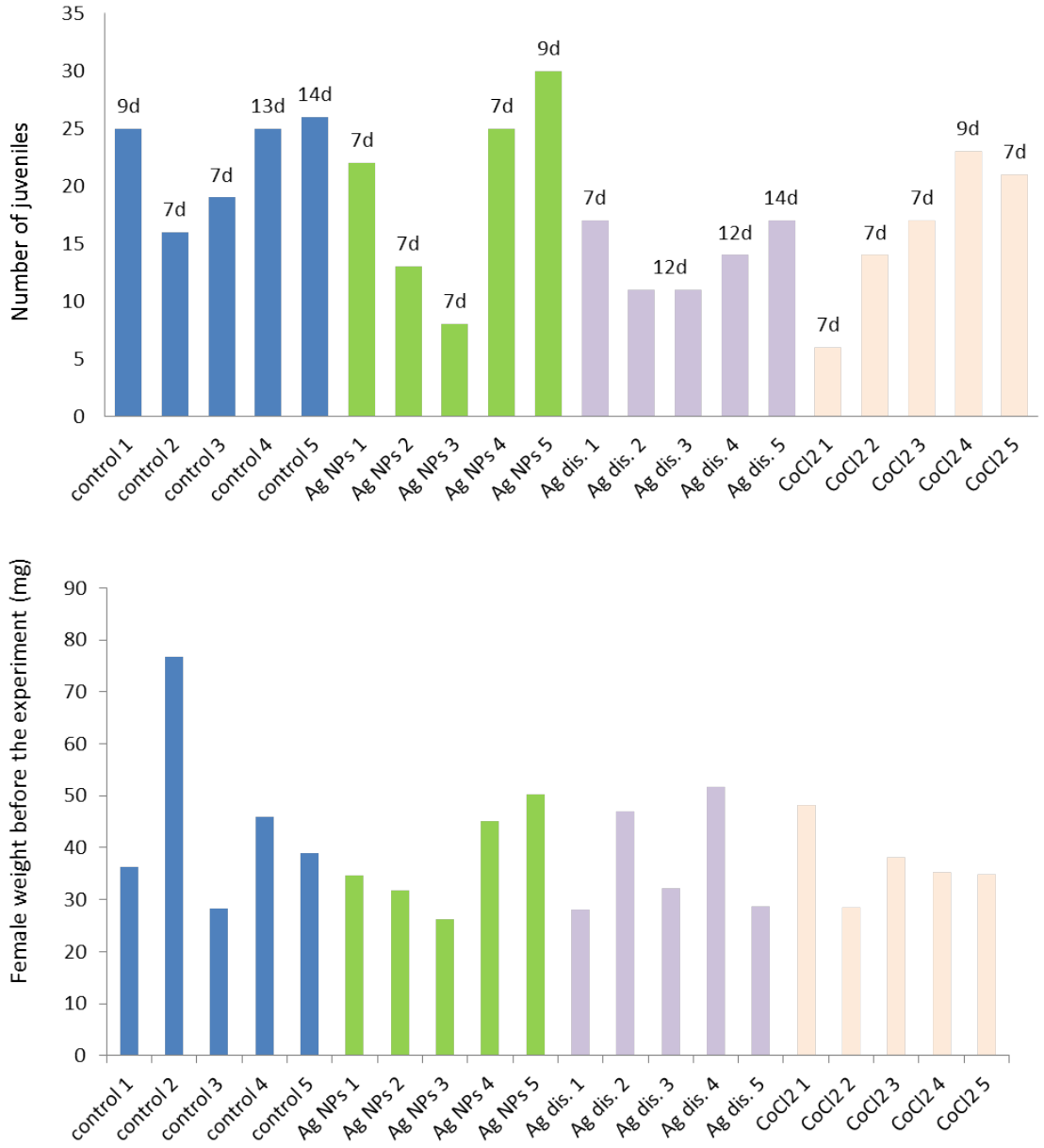

Figure 14: Number of surviving juveniles per female (A) in experiment 3, where each column represent one animal. On each column the number of days (d) of exposure for each female is written. On the lower figure the weight of each female before the experiment is represented (B). Exposure concentrations were 1000 and µg Ag/g leaf and 2000 and µg Co/g leaf for the positive control.

4.2 Bioaccumulation of silver in exposed females and juveniles

We measured the accumulation of metals in animals only after Experiment 4, where females were all exposed for same time period and juveniles were removed directly from the marsupium before they

were hatched. After the experiment females and juveniles were shock-frozen in liquid N_2, using tissue-freezing medium, cryo-sectioned and lyophilised. We combined samples of juveniles from all 5 females from one group in one sample in order to have enough sample volume for the detection of contained silver (level of detection, LOD, is 15 µg/L). Prior to analysis, dry samples were digested in a heating block with a mixture of concentrated nitric (65% HNO_3), and perchloric acid (70% $HClO_4$) (HNO_3:$HClO_4$ = 7:1, v/v). After evaporation of the acid, the residue was dissolved in 0.2% HNO_3. The total Ag concentrations in the samples were analyzed with a flame atomic absorption spectrometer (Perkin Elmer AAnalyst 100). Within each measurement, a certified reference material (TORT-2, National Research Council of Canada) was used to check the accuracy and precision of the analytical procedures. Results showed that silver is accumulating in the females exposed to Ag NPs dosed food, but not in the juveniles (Figure 15).

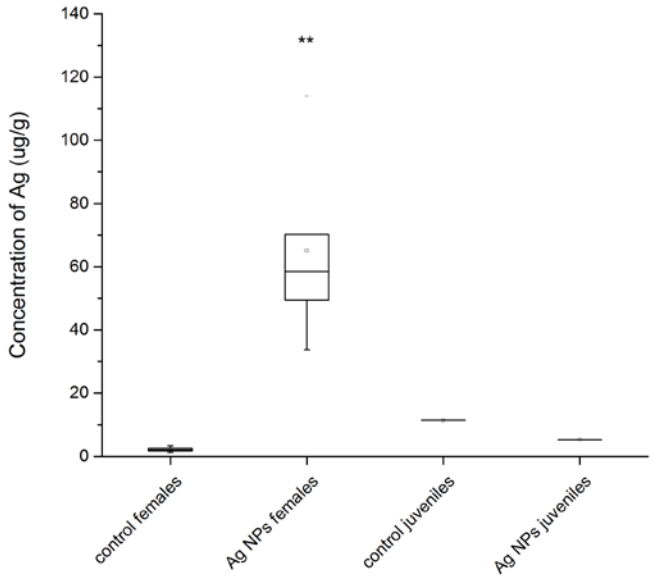

Figure 15: Bioaccumulation of silver in the whole body of females (measurements for 5 females per group) and in juveniles (one sample of combined juveniles from 5 females). Symbols on the box plot represent: maximum and minimum value (whiskers: ⊥), mean value (□), outliers (-), and $p<0.01$ (**; Mann-Whitney). Exposure concentrations were 1000 and µg Ag/g leaf .

5 Discussion and Conclusions

The comparison of results of the feeding assay with isopods obtained within NanoValid project with the new data obtained with NanoMILE NPs, provided evidence, that the assay exhibits good repeatability. The tested NPs (aged and non-aged) did not affect the feeding behavior of model organisms. Control values were in the range of reference feeding values for control population.

Within the NanoMILE project we have tested a new model isopod system for maternal transfer studies. We exposed females with developing marsupium to TiO_2 and Ag NPs produced in the project. We were observing changes in feeding behavior of exposed females, their survival and finally the changes in number of hatched juveniles upon NPs digestion by females. We measured the bioaccumulation of metals originating from NPs in the females and in juveniles in order to establish the maternal transfer of ingested elements into developing eggs in marsupium. We did not observe any effect of NPs on this maternal transfer, however the bioaccumulation of silver in the Ag NPs exposed females was observed.

The problems which can occur observing widely used toxicological endpoints like survival, growth, and reproduction, is in their low sensitivity. Biochemical, histological, and physiological endpoints are often more sensitive and suitable. It is known that isopods can survive large doses of pollutants, in particular metals, so the survival is not necessarily a suitable endpoint for measuring effect of NPs exposure. In our two valid experiments, the feeding rate of isopods exposed to Ag NPs was not affected. Statistically significant decrease of feeding rate was observed only in the case of positive controls. However, the ingestion of NPs is proven to be able to affect the feeding behavior of isopods (Jemec et al., 2008; Novak et al., 2012; Pipan Tkalec et al., 2011) and therefore is considered to be an informative endpoint in the feeding assay.

Another disadvantage of this model is in generally less frequent and less regular breeding in isopods maintained in the laboratory (Steel, 1980) as in their natural environment. Although we are maintaining several laboratory cultures, the reproduction is altered and is not as regular and frequent. For that reason, the experiments for maternal studies are limited with number of females carrying marsupia available for conducting the experiments. The females carrying eggs are also more susceptible to stress and possibly to bacterial infections. We observed high percentage of infection of our laboratory cultures after the period when the first two experiments were conducted; therefore we assumed that infected animals were included in the experiments. We confirmed that by isolating and observing histopathology of digestive glands of survived females in the experiments. Terrestrial

isopods are often invaded by intracellular bacteria with a complex developmental cycle. The outcome of that infection is pathological change in infected hepatopancreatic tissue and in the last stage of the bacterial life cycle, cell death occurs. During the infection, physiological state of animals is changed and because of that we cannot consider the results obtained with infected population as reliable. We propose that laboratory cultures of isopods are regularly checked for intracellular infection and that infected animals are not used in the experiments.

Another endpoint observed after feeding assay with females carrying marsupium was bioaccumulation of metals originating from ingested NPs in the females and possibility for maternal transfer of NPs on juveniles during the intramarsupial development. We did not succeed to visually establish the state of intramarsupial development of juveniles using light microscopy and because of that juveniles were hatched from marsupiums in different time periods, a fact which disabled a 14 day experiment as per the validated feeding protocol. Immediately after hatching juveniles can eat the food dosed with NPs or even maternal feces, so it is important that they are removed from the marsupium after the exposure of females. Therefore we adjusted the time period of exposure for 3 days and after that juveniles were isolated and removed directly from the aqueous brood pouch. The results of the experiment where females were exposed to Ag NPs showed that also in that short period (3 days) silver was accumulating in the females but not in the juveniles. In future experiments, we will optimize the protocol in a way that we will collect the females with early stage of marsupium from laboratory cultures and exposed them to NPs at least for seven days to confirm the result, that maternal transfer of NPs or ions dissolved from NPs is not taking place. As the part of our work in the other tasks in which we are participating (Task 6.4 in the WP6) the maternal transfer protocol will be intra-laboratory tested with NanoMILE NPs and the results will be presented.

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ANNEX 1

NANOVALID–TEST METHOD DESCRIPTION FORM

INFORMATION ON TEST METHOD AND PROPOSER / PARTNER				
Name of test method	*In vivo* feeding exposure of isopods (*Porcellio scaber*, Isopoda, Crustacea) to nanomaterials			
Acronym of test method	**IsopodFeeding**			
Status of the SOP	☐ Internal	Draft	☐ Prevalidated	☒ Validated
Proposer - Organisation	University of Ljubljana	Biotechnical faculty	Department of Biology	
Postal address	Večna pot 111, 1000 Ljubljana, Slovenia			
Name of contact person	Prof. Dr. Damjana Drobne, Doc. dr. Anita Jemec			
Tel. no. of contact person	+ 386(1) 320 33 75			
Fax no. of contact person	+ 386(1) 257 33 90			
e-mail of contact person	Damjana.drobne@bf.uni-lj.si; anita.jemec@bf.uni-lj.si			

1.Describe the scientific and technical basis of the test method

Terrestrial isopods are a good choice for toxicity studies since their digestive system is the main route for contaminant intake and their accumulation in the body is attributed solely to dietary exposure (Drobne 1997). Isopods have been shown to accumulate the highest concentrations of metals such as zinc, cadmium, lead, and copper so far recorded in any soft tissue (Hames & Hopkin 1989). Metals are accumulated in digestive gland (hepatopancreas). This phenomenon has been used in variety of ecophysiological, ecotoxicological (reviewed in Drobne 1997) and recently nanotoxicological studies (Pipan-Tkalec et al. 2010, 2011; Golobič et al. 2012; Novak et al. 2013a, 2013b) where bioavailability of metals in the digestive system was of interest.

Exposure of terrestrial isopods (prominently *P. scaber*) to contaminant-treated food was established as a standard toxicity testing protocol by Drobne and Hopkin (1994, 1995) and was later on adapted for nanotoxicity testing (Golobič et al. (2012). Feeding exposure is the most important means for *in vivo* exposure of *P. scaber* to nanoparticles; moreover, it enables assessment of the exact exposure dose (Drobne 1997), which is harder to estimate with other terrestrial invertebrates (e.g. earthworms, collembolans). This protocol serves as a basis for other analyses (e.g. AAS measurements of tissue metal content, AO-EB test for cell membrane integrity, lipid peroxidation assay of digestive gland cells etc.).

In the scope of this feeding assay, feeding rate, fecal production rate and food assimilation are evaluated. This enables assessment of organism-level physiological response to nanoparticles and their potential toxicity (in case of elevated mortality of test animals).

2. Describe the role of the method in context of …

This protocol describes how to set up a feeding experiment (*in vivo* exposure) with terrestrial isopod *Porcelio scaber* (Isopoda, Crustacea) as a pre-requisite for other analyses based on *in vivo* exposure. This protocol needs to accompany all the other SOPs for the analyses based on *P. scaber* tissues.

3. Describe the Standard Operating Procedure (SOP)

A. Reagents and Materials

Materials/Reagents/Equipment	Vendor	Stock Number (content, if self-prepared)
Reagents		
deionized water	own production (by a water-purification system, e.g. Millipore)	as much as necessary
nanoparticles	NanoValid consortium	depends on the aim of the experiment; usually in a mg/mL concentration
P. scaber saline solution	Sigma-Aldrich, Merck or similar; all the chemical must be of a reagent grade	per 0.5 L: 7.265 g NaCl, 0.2984 g KCl, 0.476 g $MgCl_2$, 0.45 g glucose, 0.6 g TRIS Add dH_2O, adjust pH to 7.4
Consumables		
paintbrushes	local vendor	separate brush for each chemical
plastic Petri dishes with vents	e.g. Greiner	separate dish for each test animal
laboratory gloves	e.g. Shield	
pipette tips	e.g. Brand	
Equipment		

vortex shaker/mixer	e.g. Scientific Industries, Inc.
laboratory scale/balance	e.g. Sartorius
pipettes	e.g. Brand, 20-200 µL
stereomicroscope	e.g. Leica, Zeiss
stainless steel tweezers	SPI Supplies, West Chester, PA, USA local vendor
scissors	

B. Safety procedures and precautions

Bioassays are performed taking into consideration the following safety precautions: inhalation of ENM powder and exposure of the skin to ENMs are hazardous. Nanomaterials are weighed in a ventilation hood and liquid phase (dH_2O, buffers) is added right after. Sonication of nanoparticles is performed in a closed containment. Handling of all nanomaterials (and their suspensions) is always performed with laboratory gloves and a lab coat. Applying suspensions of nanomaterials on the leaves is performed in a laboratory.

C. Procedure

C.1. Reagent/Stock preparation

C.1.1 Preparation of nanoparticles stock suspensions

Suspensions of nanoparticles for feeding experiments are always prepared in deionized water (dH_2O). Stirring of aqueous dispersion and sonication is applied when required. Depending on the amount of nanoparticles that needs to be applied on the leaf surface, stock suspension is prepared (e.g. if the desired dosage of nanoparticles was 1000 µg/gram dry weight of leaf, a suspension of 1000 µg/mL should be prepared, 1 mL of which is then applied on the surface of 1 g of leaves).

C.1.2 *P. scaber* saline solution

Prepare the *P. scaber* saline solution by weighing 7.265 g NaCl, 0.2984 g KCl, 0.476 g $MgCl_2$, 0.45 g glucose and 0.6 g TRIS to 500 mL of dH_2O. Adjust pH to 7.4

C.3. Procedure

C.3.1 Feeding experiment: experimental set-up

C.3.1.1. Petri dish labeling

Write the following on the dish cover:

1. experiment Nr.,
2. experiment holder name,
3. particles used and their concentration,
4. Petri dish / test animal Nr.,
5. mass of the leaf,
6. mass of the animal.

Write the following on the bottom of the dish:

1. experiment Nr.,
2. Petri dish Nr.

C.3.1.2 Applying chemicals/suspensions of nanomaterials onto leaves

1. The nanoparticles suspensions should be vortexed before application.
2. Pipettes should be calibrated before use.
3. Cut pieces of dried leaves to 100 ±10 mg.
4. Weigh the pieces of leaves and write down the mass on the Petri dish.
5. For spreading the chemicals/nanomaterials on the leaves, a brush is used.
6. Before the application starts, the brush must be dipped in deionized water.
7. Chemicals/ suspensions of nanomaterials are applied to the abaxial side of the leaves in the volume corresponding to the leaf weight, with a reference volume of 100 µL of the liquid phase per 100 mg of dried leaves.
8. Chemical/suspension of nanomaterials should be spread as evenly as possible, covering the whole surface of the leaf.
9. Avoid prominent leaf veins.
10. After the chemicals/suspensions of nanomaterials are applied, the leaves should be left in Petri dishes for 24 hours at room temperature to dry.
11. After 24 hour period the leaves are weighed. The value obtained is used for further calculations.

C.3.1. 3 Starting the feeding experiments

1. Weigh each animal.

2. Only adult animals exceeding 30 mg are used in the experiments. If moulting or the presence of marsupia is noted, animals are not used in feeding experiments.
3. A single animal along with a treated leaf is placed in a Petri dish.
4. At least 10 animals per concentration should be used.
5. Cover of a Petri dish is sprayed with deionized water from the inside. The sprayed water should be applied in tiny droplets only.
6. Petri dishes are transferred into a glass or plastic made terrarium. The bottom of the terrarium must be covered with a few layers of moist paper towels. The terrarium is then covered with a plastic foil which prevents the water evaporation. A few holes should be punctured in the foil which will allow the air flow.
7. The terrarium is placed in a cultivation chamber which ensures controlled and stable condition with the temperature of 20 +/- 2 ºC and natural light cycles of 16 hours of light, followed by 8 hours of darkness.
8. Duration of exposure depends on further analysis and is usually 14 days.

C.3.1.4 Maintenance of animals during the feeding experiment

Petri dish should be kept moist for the whole period of the experiment. Care should be taken to sustain enough but not too much moist inside the terrarium. If the leaves appear to be wet and there are water drops forming on the bottom of the Petri dish, there is too much moist inside the terrarium.

Animals should be checked every day or at least every second day. Dead animals or females with marsupia should be removed from the experiment.

Once a week, fecal pellets should be picked up from the Petri dish of every animal in a following way:

1. Take new Petri dishes and mark them accordingly. Moist the covers with distilled water and transfer the animals from the original Petri dishes of the experimental group. Add nothing else. Keep them away from heat and direct sun.
2. Leave the original Petri dishes open so that they dry out before removing the fecal pellets.
3. Take Eppendorf tubes, mark and weigh them.
4. Pick up all the fecal pellets from each Petri dish using a brush. Transfer them to Eppendorf tubes in the way that the numbers match.
5. When done, put the animals back to the original Petri dishes and moist them.

C.3.2. Transferring the animals to untreated food (for measurements where NP content in the gut may be an interference, e.g., AAS measurements, see "Microwave acid digestion of organic material")

1. Take new Petri dishes and mark them
2. Put a piece (20–100 mg) of untreated leaf in each dish and moist the covers.
3. Transfer the animals from the original Petri dishes to the new ones.
4. Keep them under controlled conditions (20 +/- 2 ºC and natural light cycles of 16 hours of light, followed by 8 hours of darkness) in a cultivation chamber for 24 hours.

C.3.3. Finishing the feeding experiment

1. Weigh each animal.
2. Leave Petri dishes with fecal pellets and remnants of the leaves for at least 24 hours to dry at room temperature.
3. After at least 24 hours, collect the fecal pellets, put them in Eppendorf tubes and weigh them. Weigh the dry leaves.
4. Mark everything and store away so that it can be found for possible future analysis.
5. If AAS measurements of separate animal parts will be performed or membrane integrity of digestive glands will be assessed (using AO/EB differential staining), the animals need to be dissected (see point C.3.4).

C.3.4. Dissection

1. Before the dissection, prepare a stereomicroscope, scissors, tweezers, some distilled water, some alcohol, and a small Petri dish with *P. scaber* saline solution.
2. Examine the animal under the stereomicroscope and write down any findings.
3. Cut off the head and isolate digestive glands. Describe the glands thoroughly and write down all the findings. Cut off the rear of the animal and remove the gut. Describe the digestive organs thoroughly and write down all the findings (please see *Fig.1.* in annex for structure of isopod digestive system).
4. Depending on desired analyses, store the tissue accordingly.

C.4. Data analysis

Write down the following data:

1. mass of leaves before and after feeding experiment,
2. mass of test animals before and after feeding experiment,
3. mass of fecal pellets after the experiment,

4. gender and anomalies (death, moulting of animals) – data from dead and moulting animals are not included in the calculation of feeding rate, fecal production rate and food assimilation efficiency.

Calculate*:

1. feeding rate,
2. fecal production rate,
3. food assimilation efficiency.

*equations are provided in Annex

4. Performance assessment of the method

- Describe the scope of the performance assessment study and how it was undertaken.

Validation of the SOP was done in the scope of D 5.54 deliverable: Reports on development of standard protocols for reference methods: Reports on development of standard protocols for reference methods for (1) toxicological testing, (2) ENP exposure assessment, (3) assessment of the ENP impact on human and environmental health.

- What test substances were used for assessment purposes and what was the basis for their selection?

The reference chemical (positive control) was Cu(NO3)2·3H_2O The chemical was selected on the basis of isopod physiology.

- What were the specific criteria used to judge/describe test method performance?

We consider the test valid when the following criteria have been met:

1. The mortality of control isopods within the exposure period is ≤ 20 %,
2. Therefore, we consider the reference values for our laboratory control *P. scaber* between 0.03 and 0.07 mg/g of feeding rate per day ([0.03<FR<0.07] mg/g) when their weight at the beginning of the test was in the range between 34 and 54 mg ([34<IW<54] mg) (Drobne and Drobne, 2014)

The test animals shall be adults, exceeding 30 mg. If moulting or the presence of marsupia is noted, animals are not used in further feeding experiments and data analysis.

- Was the study conducted under GLP, and/or did it adhere to a recognised quality system?

The study was not conducted according to a recognised quality system.

- What was the reliability of the assay in terms of intra-lab and inter-lab reproducibility?

ADDITIONAL INFORMATION – Expand on to further pages if required

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8. Add here any further information or particular attributes of this Assay/Test and associated datasets that will assist project partners, or a potential third party making use of NANOVALID data, to correctly interpret and analyse the data
(Continue on additional pages if required, or add as addition attachments).

Description and calculation of feeding parameters of *P. scaber*

In isopods, high levels of metals in food are known to depress food consumption; moreover, metal poisoning may be evaded by an avoidance response, by regulation of the consumption rate, by storing metals in hepatopancreas in insoluble form, and/or by fecal and possibly also urinary excretion (reviewed in Drobne and Hopkin 1995).

Generally, decreased feeding rate, decreased fecal production rate and increased food assimilation efficiency denote an adverse effect of a test chemical to the isopods.

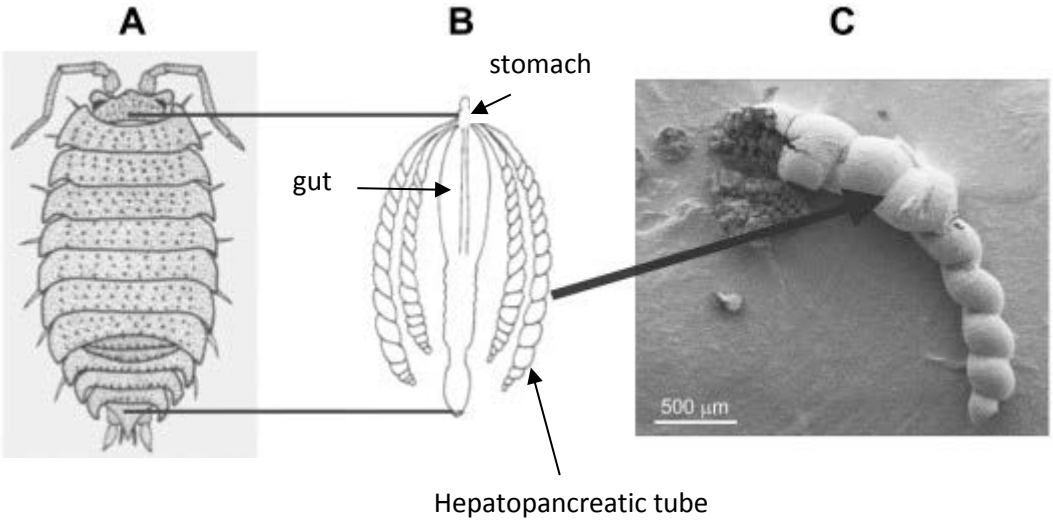
Calculation of feeding parameters (for an individual animal) is as follows:

$$\text{Feeding rate} = \frac{\text{mass of consumed leaves during the experiment (mg)}}{\text{average mass of the animal during the experiment (mg)}}$$

$$\text{Fecal production rate} = \frac{\text{mass of fecal pellets during the experiment (mg)}}{\text{average mass of the animal during the experiment (mg)}}$$

$$\text{Food assimilation efficiency} = \frac{\text{mass of cosumed leaves (mg)-mass of fecal pellets (mg) during the experiment}}{\text{mass of consumed leaves during the experiment (mg)}}$$

Fig.1. Scheme of a terrestrial isopod *Porcellio scaber*: (A and B) digestive system anatomy, (C) scanning electron micrograph of isolated digestive gland tube (Valant et al., 2012).*



*Valant J, Drobne D and Novak S. 2012. Effect of ingested titanium dioxide nanoparticles on the digestive gland cell membrane of terrestrial isopods. Chemosphere Vol 87, Issue 1,Pages 19–25

ANNEX 2

NANOVALID –TEST METHOD DESCRIPTION FORM

INFORMATION ON TEST METHOD AND PROPOSER / PARTNER				
Name of test method	Measurements with flame AAS			
Acronym of test method	**FAAS-measure**			
Status of the SOP	☐Internal	☒Draft	☐Prevalidated	☐Validated
Proposer - Organisation	University of Ljubljana	Biotechnical faculty	Department of Biology	
Postal address	Večna pot 111, 1000 Ljubljana, Slovenia			
Name of contact person	Prof. Dr. Damjana Drobne, Doc. dr. Anita Jemec			
Tel. no. of contact person	+ 386(1) 320 33 75			
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e-mail of contact person	Damjana.drobne@bf.uni-lj.si; anita.jemec@bf.uni-lj.si			

1. Describe the scientific and technical basis of the test method

Atomic absorption spectrometry (AAS) is an analytical technique for qualitative and quantitative determination of chemical elements. AAS enables determination of more than 70 elements (mostly metals and metalloids) in very low concentrations (in the ppm range, i.e. mg/kg or mg/L). The accuracy is very good; the extent of measurement error is about 1-2%. Flame AAS (FAAS) is one of four commonly used AAS techniques, others being graphite furnace AAS (GF-AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS).

FAAS is based on the fact that metal atoms in the ground state absorb light of specific wavelengths. Analyte of interest needs first to be atomized in a flame. A beam of light of a suitable wavelength (in the ultraviolet or visible region of the spectrum) passes through the nebulized sample and converts atoms of the analyte to the excited state. Characteristic excitation wavelengths are specific to each element and precise to 0.01-0.1 nm. Concentration of the element in the sample is determined from the absorption rate, which is compared to the working curve obtained after calibrating the instrument with standards of a known concentration. FAAS is a monoelemental technique.

This protocol describes calibration and measurements with flame atomic absorption spectroscopy (flame AAS) and it is written on the basis of Perkin Elmer AAnalyst 100 spectrometer.

2. Describe the role of the method in context of …

In nanotoxicity studies, this method is applied to determine the amount of metals in aqueous dispersions of nanomaterials and in organic material, for instance *Porcellio scaber* tissues and dry tree leaves (food for feeding experiments with isopods).

This SOP needs to be accompanied by SOP for feeding experiments with isopods and SOP for microwave acid digestion of organic material (*Porcellio scaber* tissues and dry tree leaves).

3. Describe the Standard Operating Procedure (SOP)

A. Reagents and Materials

Materials/Reagents/Equipment	**Vendor**	**Stock Number** (content, if self-prepared)
Reagents		
deionized water	own production (by a water-purification system, e.g. Millipore)	as much as necessary
standards for AAS (containing 1 mg/ml of analyte of interest)	e.g. Perkin Elmer, Merck, Sigma Aldrich	calibration standards prepared by dilution according to the theoretical recommendations for standard concentration
65% HNO_3, reagent grade	e.g. Merck, Sigma Aldrich	prepare stock of 0.2% HNO_3 in deionized water
Consumables		
glassware (beakers, Erlenmeyer flasks, volumetric flasks)	e.g. Brand	
glass test tubes	e.g. Brand	
plastic stoppers for test tubes	e.g. Brand	
plastic Pasteur pipettes	e.g. Brand	

micropipette tips	e.g. Brand	
laboratory gloves, latex and nitrile	e.g. Shield	
Parafilm M	Pechiney Plastic Packaging	
Equipment		
flame atomic absorption spectrophotometer	Perkin Elmer AAnalyst 100	
acetylene supply	Messer	acetylene pressure in the gas cylinder must not fall under 8 bar (800 kPa)!
oxidant (air) supply	own production by an air compressor	
adjustable micropipettes	e.g. Brand	
vortex shaker/mixer	e.g. Scientific Industries, Inc.	

B. Safety procedures and precautions

All handling of concentrated HNO_3 must be performed in a ventilation hood, with a lab-coat, nitrile gloves and safety glasses. Always wear a lab-coat and latex gloves during AAS measurements; when directly measuring samples dissolved in concentrated acid, wear nitrile gloves.

Before each measurement, perform a safety check of the AAS apparatus according to the manufacturer's instructions. Always turn on the ventilation during the measurements. When acetylene supply starts getting low, make sure that acetylene pressure in the gas cylinder does

not fall under 8 bar (800 kPa), otherwise acetone or dimethylformamide solvent could start leaking out the cylinder. Perform regular maintenance check of air and acetylene supply connections to the AAS apparatus (pressure valves, gas hoses) once a year. Provide regular preventive maintenance by a plenipotentiary service every five years.

Collect drainage liquids from the apparatus, acid and sample leftovers in an inert, appropriately labeled container. Take all safety precautions for handling toxic waste. Provide safe storage and deliverance to the specialized institution for destruction.

C. Procedure

C.1. Reagent/Stock preparation

C.1.1 Preparation of calibration standards

C.1.2.1. General information (standards are appropriate for most samples)

In general, calibration standards for measurement by AAS are prepared from certified solutions (e.g. PerkinElmer, Sigma Aldrich, Merck etc.) containing the analyte in concentration 1 mg/mL (1000 mg/L). Certified solution is diluted with deionized water in a volumetric flask to the desired concentration. Prepare at least 4 concentrations of the calibration standards: the highest one is at the limit of linearity for the analyte of interest (data on this can be found in literature) and the other three are relative proportions of the highest concentration.

1. Use calibrated volumetric flasks. Wash them with diluted HNO_3, rinse with deionized water and leave to air dry.
2. Decant a small quantity of the certified solution in an acid-washed beaker, never pipette it directly from the original bottle!
3. Filled the volumetric flask with deionized water up to a few inches below the etched volume label, enough for the tips to reach into the liquid.
4. Add an appropriate volume of certified solution into the water in the flask.
5. Carefully add deionized water up to the engraved tag on the volumetric flask by a Pasteur pipette.
6. Close the volumetric flask with the corresponding stopper and shake well. Store the standards in a refrigerator until use.

C.1.2.2. Special cases

When samples contain high concentrations of acid/base (mineral or organic), are dissolved directly in the concentrated acid/base, or contain a high concentration of organic solvents, it is necessary to prepare specific standards for this purpose. It is known that a high acid content in the sample may decrease the absorbance of the analyte and a high base content may increase the absorbance of the analyte if the instrument calibration standards are prepared in deionized water. The difference in viscosity between the sample and the standard also affects the absorbance of the analyte. These are collectively known as “matrix effects” and are a cluster of

basic interferences in atomic spectroscopy.
In order to avoid the matrix effects, the compositions of the sample and the calibration standard (with the exception of the analyte) need to be as similar as possible. Another option is to use the method of standard additions. More information on this can be found in textbooks of atomic spectroscopy, or the user's manual provided by the manufacturer.

C.2. Preparation of samples

Digest your samples according to SOP for microwave acid digestion of organic material (*Porcellio scaber* tissues and dry tree leaves). Dilute acid digested samples with deionized water until the anticipated analyte concentration falls into the calibration range. Take notes of dilution ratio for further calculations.

C.3. Procedure

C.3.1 Turning on, heating and basic settings of AAS apparatus (PerkinElmer AAnalyst 100)

C.3.1.1. Switching on the apparatus

1. The order of turning on the appliances: AAS apparatus (switch on the bottom right side) → computer, monitor, printer.
2. Carefully and slowly open the valve on the acetylene cylinder and the valve on the air pipe.
3. Run the program *AA WinLab Analyst* and wait for a connection between the computer and AAS apparatus to become established (*Checking Connections* → check mark turns green).
4. Select: *Select Workspace → Use a custom- designed workspace*.
   Open → Manual.flm → OK
5. In the main window, first check whether the green check mark under the label *Safety Interlocks* is on.

   If yes, you can begin to work.

   If not, there are three options:

- An error has occurred in the gas inlet (you forgot to open the acetylene valve, or the gas pressure in the hose is too low for any other reason). Check the proper functioning of the inlet. If everything is in order, check the drainage; otherwise call the maintenance service.
- The drainage tank is full. Decant some of the liquid into the waste container.
- There is a problem with the connection between the drain sensor and the apparatus. Check the contact. If this does not resolve the problem, call the maintenance service.

C.3.1.1. Warming up lamps

Turn on the appropriate hollow cathode lamp for the analyte of interest and the deuterium lamp for background correction. The AAS apparatus automatically centers the lamp and sets the wavelength. The lamp must be left to warm up for at least 15 minutes before the beginning of measurements.

C.3.1.2. Warming up standard solutions

Take the flasks with standard solutions from the refrigerator for at least 30 minutes before the beginning of measurements (even earlier in cold weather or in winter).

C.3.1.3. Preparation of solutions for nebulization between measurements

The apparatus should never be allowed to draw in the air (with the exception of moving the tube from one fluid to another, where this cannot be avoided)! Before lighting the flame, prepare a large beaker or Erlenmeyer flask with deionized water and another one with 0.2 % HNO_3. Dip the nebulizer tube in the liquid to draw in between the measurements or for the purpose of rinsing the nebulizer. 0.2 % HNO_3 is used for rinsing the nebulizer between measurements of samples with high concentrations of metals.

C.3.1.4 . Basic machine settings before measurement

1. Measurement method: *File* → *Open* → *Method* → choose an appropriate method for measuring the analyte of interest.
2. The results file: button *Results set name* in the working window → enter the name of the file, which should contain the essential information, and a longer description.
3. A file with information about the samples: *File* → *Open* → *Sample info file* → select your user directory from the list and find the stored sample data file.

C.3.1.5 . Establishing sample data files

1. *File* → *New* → *Method* → displays an empty data window.
2. Enter the following information :
    - name of the file,
    - volume units: mL,
    - mass units : mg
    - sample volume: 2 mL or more are recommended
    - names and masses of samples
    - the unit of sample concentration (typically mg/g or mg/L).
3. *File* → *Save* → *Method* → choose your user directory and store the method under the appropriate name. Note: The program allows only very short names.

C.3.2 Calibrating the AAS apparatus (PerkinElmer AAnalyst 100)

1. Light the flame (*Flame control* → *ON*).
2. Click the *Blank* button to start the procedure. The apparatus automatically sets the lamp according to the selected method and stores this information.
3. Measure the blank (click the *Blank* button again) to set the "baseline ".
4. Select: *Tools* → *Continuous Graphics*. Draw in the lowest standard and check whether the measured absorbance is of the optimal value. If it is not, adjust the nebulizer flow until

appropriate absorbance is achieved.

5. Measure the standards in the ranking order (click the *Standard* button). The apparatus automatically calculates the linearity of the curve; if the curve is not linear (the warning appears), repeat the procedure. Strive to achieve the correlation coefficient (R^2) as close to 1 as possible.
6. Perform a quality check using the following procedure:
    - Select : *Analyses* → *Characteristic Conc*.
    - Enter the concentration of one of the standards in the top box, and the measured absorbance in the lower box.
    - Click in the upper box. At the bottom of the window the measured concentration and the corresponding characteristic concentration values appear.
    - If the values differ by ≤ 10%, continue with measurements. If the values differ by > 10%, repeat the calibration procedure. Check the accuracy of the standards and prepare new ones if necessary.
7. After completing calibration, measure a blank again.

C.3.3. Performing measurements with the AAS apparatus (PerkinElmer AAnalyst 100)

1. If the samples were prepared by microwave digestion, they can be measured either directly or after dilution with deionized water until the expected concentration of the analyte reaches the calibration range. After each sample dilution, homogenize the sample by vortexing in glass tubes, closed with plastic stoppers.
2. The samples are measured by clicking on the *Sample* button. The measurement lasts as long as the green circle on the button is illuminated. On average, each measurement consumes approximately 1.5 mL sample, which must be considered in their preparation.
3. Measure a blank after every 10 samples. If the content of the analyte in the samples is very low, the blank needs to be measured more frequently. Between measurements, rinse the nebulizer by drawing in deionized water and 0.2 % HNO_3.
4. If the absorbance of the analyte exceeds the absorbance of the highest standard, dilute the sample and repeat the measurement.
5. Finish measurements of samples by measuring a blank.

C.3.4. Shutting down the AAS apparatus (PerkinElmer AAnalyst 100)

1. Turn off the flame (*Flame control* → *OFF*).
2. Close the valve on the acetylene cylinder and the valve on air pipe.
3. Release the remaining gasses from the system by clicking the Bleed button (always twice!).
4. Close the *AA WinLab Analyst* program. The printer will automatically print the remaining data.
5. Turn off the AAS apparatus (switch on the bottom right side) and cover it with plastic foil.

6. Shut down Windows and turn off the computer. Turn off the monitor and printer.

4. Performance assessment of the method

- **Describe the scope of the performance assessment study and how it was undertaken.**

Special performance checks are done at every calibration to ensure the proper functioning of the apparatus. Perkin Elmer AAnalyst 100 has built-in list of characteristic concentration parameters for each element. There is a rule that absorbance value at characteristic concentration and measured absorbance value must not differ for more than 10%. Therefore, before each calibration, the standard solutions are injected in the "Continuous Graphics" mode to check whether the measured absorbance is of the optimal value. If it is not, the nebulizer flow is adjusted until appropriate absorbance is achieved. If this operation still does not yield the optimum conditions, troubleshooting is proceeded.

As a parallel quality check, a multi-element standard (not used for calibration of the machine) is measured right after the establishment of the calibration curve, in the middle of measurements, and at the end of the measurements.

- **What test substances were used for assessment purposes and what was the basis for their selection?**

Substances used are genuine Perkin Elmer single-element instrument calibration standards (see http://www.perkinelmer.com/Catalog/Gallery.aspx?ID=Standards&PID=Atomic%20Absorption%20Consumables&refineCat=Consumables%20Category&N=185%20139%2078925%204293911471&PID=Atomic%20Absorption%20Consumables&TechNVal=4293911471). The basis for their selection is the highest possible compatibility with the manufacturer's apparatus.

- **What were the specific criteria used to judge/describe test method performance?**

As stated above, Perkin Elmer AAnalyst 100 has built-in list of characteristic concentration parameters for each element. The rule – as established by the manufacturer – is that absorbance value at characteristic concentration and measured absorbance value must not differ for more than 10%.

For measured multi-element standard value of the element of interest, relative standard deviation of 10 replicates must not exceed 3% and the certified value must lie in the 95% CI for mean of means of measured values.

- **Was the study conducted under GLP, and/or did it adhere to a recognised quality system?**

No, except for the apparatus manufacturer's (Perkin Elmer) criteria.

- **What was the reliability of the assay in terms of intra-lab and inter-lab reproducibility?**

Intra-lab reproducibility is very good. Based on detailed measurement logs we keep, our lab has always been able to meet abovementioned criteria so far. Moreover, aging of the hollow cathode lamp (decaying energy) has been observed and the lamp was replaced right due to expected absorbances not meeting the described criteria.

Inter-lab reproducibility has not been attempted yet.

ADDITIONAL INFORMATION – Expand on to further pages if required

5. References	
1	Analytical Methods for Atomic Absorption Spectroscopy. Manual Part No. 0303-0152, Release D, September 1996. PerkinElmer, Inc., Waltham, Massachussets, USA http://www.lasalle.edu/~prushan/Intrumental%20Analysis_files/AA-Perkin%20Elmer%20guide%20to%20all!.pdf
2	Atomic Spectroscopy. A Guide to Selecting the Appropriate Technique and System (2008–2010). PerkinElmer, Inc., Waltham, Massachussets, USA

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