SUPPLEMENTARY INFORMATION for

Multilaboratory evaluation of 15 bioassays for (eco)toxicity screening and hazard ranking of engineered nanomaterials: FP7 project NANOVALID

Nanotoxicology

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The same batches of NMs were used by all partners: SiO₂, TiO₂, Au, MWCNTs, Ag, CuO and ZnO (≥ 99% pure)

Shape, specific surface area and primary size data were centrally provided by NM suppliers.
ζ-potential and dissolution of NMs were measured by NICPB.
The partners prepared the stock suspensions, measured hydrodynamic size in respective test medium and
performed the following (eco)toxicity tests:

- Bacterial growth inhibition assay (Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas putida, P. aeruginosa)
- Vibrio fisheri bioluminescence inhibition assay
- Algal growth inhibition assay (Raphidocelis subcapitata)
- Yeast viability assay (Saccharomyces cerevisiae)
- Protozoan viability assay (Tetrahymena thermophila)
- Crustacean acute immobilization assay (Daphnia magna)

- Isopod (Porcellio scaber) membrane integrity assay

-Zebrafish (Danio rerio) embryo toxicity assay
- Human mesenchymal stem cell (hMSC) membrane integrity assay in vitro
- hMSC mitochondrial activity assay in vitro

-Murine fibroblast BALB/c 3T3 mitochondrial activity assay in vitro
-Murine fibroblast BALB/c 3T3 membrane integrity assay in vitro
-Rat macrophage NR8383 mitochondrial activity assay in vitro

Fig. S1 Experimental set-up of this study. Yellow boxes represent partner institutions involved: National Institute of Chemical Physics and Biophysics (NICPB, Estonia), University of Ljubljana (UL, Slovenia); Centre for Cellular & Molecular Biology (CCMB, India) and University of Tampere (UTA, Finland). Green boxes present bioassays performed by partner institutions.
Fig. S2 Dose-response curves of test organisms to CuO NMs (average from different independent experiments ± standard deviations): 30-min *Vibrio fischeri* luminescence inhibition assay, 72-h algal *Raphidocelis subcapitata* growth inhibition assay, 24-h human mesenchymal stem cell membrane integrity assay *in vitro* (propidium iodide staining), 48-h murine fibroblast BALB/c 3T3 mitochondrial activity assay *in vitro* (WST-1), 48-h crustacean *Daphnia magna* acute immobilization assay and 96-h zebrafish *Danio rerio* embryo toxicity assay.
Fig. S3 Toxicity (EC₅₀) of NMs to different organisms versus hydrodynamic size in the respective test medium (a) and ζ-potential in DI water (b). EC₅₀ >100 mg metal/l were not included. Data are plotted from Table 2, Table 4 and Table S2.
### Table S1 95% confidence limits of EC$_{50}$ values of dissolution-prone NMs (Table 4) and the number of independent experiments performed to obtain these EC$_{50}$ values.

| Tests model | 95% confidence limit | Number of independent experiments |
|-------------|----------------------|----------------------------------|
| **BACTERIA:** | | |
| *Escherichia coli* | 1.36-4.76 not applicable* | 4 2 2 |
| *Staphylococcus aureus* | 1.83-8.55 not applicable* | 12.2-20.2 |
| *Bacillus subtilis* | 2.71-6.33 not applicable* | 8.8-18.8 |
| *Pseudomonas putida* | 2.31-5.33 not applicable* | 26.9-81.6 |
| *Pseudomonas aeruginosa* | 2.0-4.34 not applicable* | not applicable* |
| *Vibrio Fischeri* | 2.48-3.36 3.29-3.82 | 7.88-12.55 |
| **YEAST:** *Saccharomyces cerevisiae* | not applicable** | not applicable** |
| **ALGAE:** *Raphidocelis subcapitata* | 0.00597 - 0.01003 | 0.41-1.07 |
| **PROTOZOA:** *Tetrahymena thermophila* | 3.11-5.11 not applicable* | 3.53-8.12 |
| **Mammalian cell cultures in vitro** | | |
| *human mesenchymal stem cells (PI)* | 1.94-10.74 0.72-6.78 | 0.91-4.29 |
| *human mesenchymal stem cells (MTT)* | 2.80-8.56 not applicable* | 11.4-21.22 |
| *murine fibroblasts BALB/c 3T3 (WST-1)* | 2.7-3.3*** 0.55-0.87 | 6.55-11.6 |
| *murine fibroblasts BALB/c 3T3 (NRU)* | 2.7-2.9*** 0.93-1.11 | 5.95-7.73 |
| *rat macrophages NR8383 (WST-1)* | 9.4-10.2 not determined | not determined |
| **CRUSTACEAN:** *Daphnia magna* | 0.00206 - 0.00266 | 0.82-1.22 |
| **CRUSTACEAN:** *Porcellio scaber* (isolated digestive gland) (AO/EB) | not applicable** | not applicable** |
| **FISH:** *zebrafish (Danio rerio)* embryo | 2.19-4.02 0.84-2.24 | 1.56-1.91 |

*EC$_{50}$ >100 mg/l; **Toxicity endpoint is minimal bactericidal concentration (yeast S. cerevisiae) or LOEC (crustacean P. scaber); ***Published in Zou et al. 2014. AO/EB, acridine orange/ethidium bromide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRU, neutral red uptake; PI, propidium iodide; WST-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.
| Test medium          | Respective assay                        | Hydrodynamic size, nm |
|----------------------|-----------------------------------------|-----------------------|
|                      |                                         | SiO₂      | TiO₂      | Au        | Ag        | CuO       | ZnO       |
| LB medium¹           | Bacterial growth inhibition assay       | 921±138   | 759±48    | 24±2      | 107±1.3   | 1039±141  | 125±2     |
| 2% NaCl              | *Vibrio fischeri* bioluminescence inhibition assay | 1101±76   | 865±354   | 552±34    | 137±0.9   | 1221±250  | 1561±123  |
| Deionised (DI) water | Yeast spot assay; Protozoan viability assay | 854±38    | 367±60    | 23±4      | 132±0.5   | 152±2     | 102±1     |
| Algal growth medium  | Algae growth inhibition test            | 575±65    | 478±44    | not determined          | 83±2.9    | 970±253   | 1476±576  |
| Artificial freshwater² | *Daphnia magna* immobilization assay and zebrfish embryo toxicity assay | not determined | 767±13    | 528±41    | 111±0.6   | 1497±77   | 1733±185  |
| DMEM containing 10% foetal bovine serum (FBS) | Assays with human mesenchymal stem cells | 6858±1120 | 6513±2783 | 489±120   | 87±1      | 171±4     | 277±44    |
| DMEM containing 5.0% newborn calf serum | Assays with murine fibroblasts BALB/c 3T3 | 493±266   | 365±169   | 48±31     | 121±53    | 227±89    | 187±100   |
| Kaighn’s Modification of Ham’s F-12 with 7.5% FBS | Assays with rat alveolar macrophages NR8383 | 620±285   | not determined | 52±43     | 1929±372  | not determined | not determined |

¹ Composition: 10 g tryptone and 5 g yeast extract per liter, pH=7 (NaCl was not added to the medium to reduce the agglomeration of NMs).
² 294 mg/l CaCl²·2H₂O, 123.3 mg/l MgSO₄·7H₂O, 65 mg/l NaHCO₃, 5.75 mg/L KCl dissolved DI water.