Toxicity screening of AgNPs and integrative assessment of soil health through biomarker responses in *Eisenia fetida* earthworm at different levels of biological organization

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In recent years the number of applications and products containing silver nanoparticles (AgNP) has widely increased, mainly due to the antimicrobial properties of silver, thus their release into different environmental compartments such as soil is already occurring. The major source of AgNP deposition onto soil is currently though the disposal of wastewater treatments plant sludge (after land application of the sludge or incineration and posterior deposition) which could modify the terrestrial community. However, the hazards of nanosized silver in soils are poorly investigated despite the great complexity of soil matrix and the potential interactions of its components with pollutants. Besides, little is known about the effects of AgNPs on organisms inhabiting soils. Earthworms have been broadly used for soil health assessment due to their pivotal role in the soil and their quick and measurable responses after exposures to pollutants. Soil health can be assessed measuring these responses in model organisms at different levels of biological organization. Recently, *in vitro* assays with primary cultures of earthworm immune cells, coelomocytes, have been set up as rapid tools for toxicity assessment of chemicals. At organism-level, as earthworms are able to take up chemicals from soil ingestion as well as from soil pore water, through the outer skin, the Paper Contact Toxicity Test (OECD-207) is an initial screening method to identify toxic substances and to obtain relevant toxicity data (LC50 and EC50). Such screening reflects dermal contact exposure while the Artificial Soil Toxicity Test (OECD-207) gives a more representative toxicity data after earthworm exposure though soils. Regarding population-level, the Earthworm Reproduction Test (OECD-222) is designed to be used for assessing the effects of chemicals in soil on the reproductive output. The aims of this work were (a) to determine the toxicity profile of AgNPs with responses achieved at different levels of biological organization, cell-level biomarkers and viability test (Neutral Red Uptake -NRU- and Calcein-AM Viability assays) combined with organism and population level bioassays performed with the aid of Standard Toxicity Tests (OECD-207 and 222) and (b) to establish toxicity threshold of AgNPs which will be helpful to obtain an integrative view of the biological responses in *Eisenia fetida* earthworms. For that purpose, at cell-level, coelomocytes extruded from *E. fetida* were maintained in primary cultures and exposed to PVP-PEI coated Ag-NP (5.5±2 nm, water dispersed) in concentrations ranging 0-100 mg/l, and to PVP-PEI coating agent separately (0.0001-10,4 mg/l) for 24 h. After exposure NRU and Calcein-AM cytotoxicity and viability assays, and flow cytometric analyses were applied in order to decipher coelomocyte subpopulation dynamics and their sensitivity against AgNPs. For the Paper Contact toxicity test *E. fetida* earthworms were exposed to PVP-PEI coated Ag-NPs and to PVP-PEI agent as well, in a range of concentrations (0-200 µg/cm^2). After 48 h mortality and weight loss were assessed, morphological alterations in the digestive tract and in the epidermis were addressed after Alcian Blue staining, and Ag concentrations were quantified by ICP-MS in earthworm tissues. In the Artificial soil test, earthworms were maintained in OECD standard soils spiked with 0-500 mg AgNP/kg for 3 and 14 days. Complementarily NRU and Calcein-AM Viability tests were performed in coelomocytes extruded from exposed earthworms, autometallography was applied on fixed tissue sections (5 µm) to address the distribution of Ag in tissues, and Ag concentrations in soils and tissues were quantified by ICP-MS. Effects on reproduction were assessed after 8 weeks by counting the number of cocoons (hatched/no-hatched) and juveniles present in the soils. PVP-PEI appeared not to be cytotoxic while coated AgNPs exerted an initial stress at low doses and severe toxicity at highest concentrations as revealed NRU and Calcein AM assays. In addition, a clear difference in the sensitivity of the cell-types was detected. Paper Contact test revealed a LC50 of 346.5 ppm Ag-NP and Artificial Soil test of 144.2 mg Ag-NP/kg. Histological and histochemical analyses proved that the primary uptake of AgNPs was via soil ingestion. A decrease in the number of viable cells occurred after 3 d of exposure to 50 mg Ag-NP/kg and after 14 d to 5 mg Ag-NP/kg. Reproduction was severely impaired at high Ag-NP doses. All measurements were integrated in the Integrated Biomarker Response (IBR) index. In conclusion, the combination of *in vitro* test with the Standard Toxicity Tests was useful to establish AgNPs toxicity thresholds and thus this approach can be used for assessing the potential risks of AgNPs in soils. The IBR provided complementary information concerning the mechanisms of biological response to AgNP exposure.

References

[1] Earthworm, Acute Toxicity Tests-207. OECD guideline for testing of chemicals. 1984.
[2] Earthworm Reproduction Test (Eisenia fetida / Eisenia andrei)-222. OECD guideline for testing of chemicals. 2004.

Acknowledgements: Basque Government (Cons. Res. Groups; IT810-13), Univ. Basque Country (UFI 11/37) and Spanish MINECO (Nanosilveromics Project, MAT2012-39372).