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Nanopharmaceuticals (Au-NPs) after use: Experiences with a complex higher tier test design simulating environmental fate and effect

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ABSTRACT

The current environmental hazard assessment is based on the testing of the pristine substance. However, it cannot be excluded that (nano)pharmaceuticals are excreted into sewage during the use phase followed by entry into wastewater treatment plants (WWTPs). Sorption to sewage sludge or release via effluent can result in modified ecotoxicological effects which possibly can only be detected with a modified test approach. The objective of our study was to investigate a realistic exposure scenario for metallic nanoparticles (NPs) in pharmaceutical products, excreted into effluent, and released into the environment after treatment in WWTPs. The test approach was illustrated by using gold (Au) NPs. Effluent from model WWTPs were investigated in aquatic tests (*Daphnia magna*, fish cell lines). Sewage sludge was used as a sole food source (*Eisenia fetida*) or mixed with soil and used as test medium (soil microorganisms, *Folsomia candida*, *Enchytraeus crypticus*). To cover the aspect of regulation, the test systems described in OECD-test guidelines (OECD TG 201, 211, 220, 232, 249, 317) were applied. Modifications and additional test approaches were included to meet the needs arising out of the testing of nanomaterials and of the exposure scenarios. The results were assessed regarding the suitability of the test design and the toxicity of Au-NPs. Except for activated sludge as a sole food source for *E. fetida*, the selected test approach is suitable for the testing of nanomaterials. Additional information can be gained when compared to the common testing of the pristine nanomaterials in the standardized test systems. Effects of Au-NPs were observed in concentrations exceeding the modeled environmental.

1. Introduction

To avoid or minimize environmental impacts, environmental hazard properties of chemicals must be assessed. Depending on the kind of substance and the intended use, various regulations exist such as REACH (Registration, Evaluation and Authorization of Chemicals) for chemicals (ECHA, 2017b, 2017c) or regulations for human medicinal products (EMA, 2018). The assessment regarding environmental effects is usually based on the assessment of the active ingredient as synthesized,

added to water, soil or sediment. These approaches are described in test guidelines published by the Organisation for Economic Co-operation and Development (OECD) or standards developed by the International Organization for Standardization (ISO). The risk assessment for human medicinal products within the European Union is specified by the "Guideline on the Environmental Risk assessment of Medicinal Products for Humans" (EMA, 2018). The focus is only on the active ingredient and transformations or additional compounds are not necessarily considered.

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Nanomedicine is a comparatively new field but of growing interest, as the nanoscale materials exhibit new possibilities due to their structural, mechanical, chemical, physical and biological properties. In human medicinal products, nanomaterials (NMs) can be used among others as active ingredient, carriers for targeted drug delivery or as an excipient (Patra et al., 2018). Excretion of biomaterials used in nano-carrier drug delivery systems is likely and cannot be excluded (Su et al., 2019). In this case, the products are released into wastewater and end up in wastewater treatment plants (WWTPs), where transformations are not excluded and exposure scenarios with increased environmental relevance can be of interest. Berkner et al. (2016) stated that particularly for inorganic NMs, persistence in the environment has to be expected, and thus long-term effects in all relevant environmental compartments should be covered. They request that consideration be given to transformation (e.g., loss of coating) and aged forms of the nanosized compounds. Potential concerns of nanopharmaceuticals have to be addressed to avoid environmental damage and that the future use is constrained.

NMs accumulate in activated sludge (Sun et al., 2016, 2014) which is used in various countries as fertilizer due to its high content of N and P. In 2012, the European Commission published that the EU-27 produced about 10 million tons of activated sludge (dry solids) a year. Nearly 40% of this was used as fertilizer and spread on agricultural land (European Commission, 2012). Model WWTPs are described (OECD, 2001) to determine the elimination and degradability of organic substances. The test approach has also been found to be appropriate for the investigation of NMs in particular for assessing the distribution of the particles in WWTPs (ECHA, 2017a) and they proved to be suitable for the simulation of environmental relevant exposure scenarios regarding the assessment of NMs. Using this approach, the impact of silver nanoparticles (Ag-NPs) on soil microorganisms and fish embryos simulating environmental relevant exposure scenarios was assessed (Muth-Köhne et al., 2013; Schlich et al., 2017, 2013). The effluent had been used for the aquatic studies and the activated sludge had been mixed with soil for the soil studies.

The objective of the present study was to extend the approach for the simulation of the environmental fate and effect of nanopharmaceuticals such as gold nanoparticles (Au-NPs) which are used for drug delivery (Patra et al., 2018). Schlich et al. (2021) spiked synthetic wastewater with Au-NPs and used it as influent for model WWTPs to study the effect on the microbial community of the activated sludge. In the present study the effect of the activated sludge with adsorbed Au-NPs as well as the effluent with the remaining Au-NPs was investigated in various terrestrial and aquatic ecotoxicological test systems. The applied test systems differed in their complexity and addressed various trophic layers and exposure routes. Three kinds of test systems were taken into account. First, selected test systems described in OECD-test guidelines were used to cover the aspect of regulation. Second, modifications of these test systems were studied. The modifications aimed to address specificities of nanomaterials such as transformation during a prolonged exposure in soil. Third, considering fish cells and microbial respiration activities in multiwell plates, miniaturized approaches were included. These approaches are less time consuming than the standardized methods, avoid invertebrate testing in the case of fish and consider an approach addressed by EFSA (EFSA_PPR_Panel et al., 2017) for microorganisms. The assessment of the experiments was performed with two objectives. First, the suitability of the various test organisms and test approaches in the presence of activated sludge and effluent was considered. Second, the hazard and risk of the used Au-NP were evaluated. The material was available as a dispersion, thus the product (active ingredient and dispersant) as well as the dispersant without Au-NP were considered. Additionally, the pristine substance was tested in selected approaches to compare the suitability of both approaches with respect to regulation.

Overall, the study provides information on the suitability and advantages of the test approach with increased environmental relevance and lists issues to be considered.

2. Material and methods

2.1. Nanomaterials

The Au-NPs were available as dispersion. The properties of the Au-NPs and their respective dispersant are briefly described in the Supporting Information Table S1, and both were provided by Colorobbia Consulting S.r.l (Sovigliana, Vinci (Florence), Italy). The dispersant was an aqueous solution of sodium citrate (1 mM) containing polyvinylpyrrolidone (PVP) as stabilizer (0.27 ± 0.5 [wt%]). In the following the term “Au-NPs” refers to the dispersion of Au-NPs. The term “dispersant” is used for the pure dispersing solution.

2.2. Study design

Activated sludge and effluent produced in four model wastewater treatment plants (Schlich et al., 2021) were used for the experiments. The sludge was mixed with soil or food for the terrestrial tests. The effluent was used for the aquatic tests. Additional set-ups were performed for comparative purposes. Following test approaches were considered:

- WWTPs treated with Au-NPs and WWTPs treated with dispersant
 - Sludge
 - Mixed with soil and used as incubation medium for the studies with soil microorganisms, enchytraeids and collembolans
 - Used as food for earthworms
 - Effluent
 - Used as incubation medium for fish cells and daphnids
- Set-ups for comparative purposes
 - Standard approach – soil mixed with pristine Au-NPs or dispersant for studies with soil microorganisms
 - Activated sludge of the local WWTP for studies with enchytraeids and collembolans
 - Pristine Au-NP directly dispersed in cell medium for fish or daphnia medium

Details of the tests are described below.

2.3. Preparation of activated sludge and treated synthetic wastewater

The preparation of activated sludge and treated synthetic wastewater used for the terrestrial and aquatic ecotoxicological tests is described in detail in Schlich et al. (2021). In brief, the WWTP simulation was carried out following OECD guideline 303A (OECD, 2001). Four laboratory WWTPs (behrst® laboratory wastewater treatment plant KLD 4N, Düsseldorf, Germany) equipped with a denitrification tank (anaerobic), a nitrification tank (aerobic), and a settling tank were used. The total volume of the laboratory WWTP was 10 L. Activated sludge was taken from a municipal WWTP of Schmallingenberg, Germany. The sludge fulfilled the criteria as stated in the German Sewage Sludge Ordinance (AbfKlärV §6 Absatz 1, 1992) regarding the permitted heavy metal thresholds. The activated sludge was poured equally into the nitrification and denitrification tanks to achieve a dry matter content of 2.5 g dm sludge/L after addition of tap water to a final volume of 10 L. Synthetic sewage was continuously dosed into the WWTPs denitrification vessel. After an acclimatization phase of 8 days, the main phase of the test started with the addition of Au-NPs and its dispersant for 10 days. Two single replicates per treatment (Au-NPs and dispersant) were tested. At test end, the sludge of the denitrification and nitrification chambers were mixed for every WWTP and dewatered.

Additionally, fresh activated sludge was available as untreated control and used for selected experiments.

The sludge was mixed with soil or used as food for the terrestrial tests. Effluent from the last day was used for the aquatic tests.

2.4. Gold analysis

The Au concentration in activated sludge, soil and soil sludge/samples was measured, carrying out an aqua regia extraction following the guideline DIN EN 16174:2012-11 (DIN EN 16174, 2012) with an open extraction under reflux. Half a gram of the dried and finely ground sample was transferred to a de-composition vessel and moistened with two drops of water; 6 ± 0.1 mL 37% hydrochloric acid, 7.4 ± 0.1 mL 30% hydrochloric acid, and 2 ± 0.1 mL nitric acid (69%) were added in succession and mixed well. The temperature of the extraction mixture was increased to 175 ± 5 °C at a rate of about 10 °C/min to 15 °C/min and held at 175 ± 5 °C for 10 ± 1 min before allowing the vessel to cool to room temperature. After cooling, the extract was transferred to a 25 mL volumetric flask and then filled up to 25 mL with ultrapure water. If necessary, the extract was filtered through a 0.2 µm membrane filter to remove insoluble residues. Furthermore, aqueous samples were measured after acidification with nitric acid (1% HNO₃).

The Au concentrations were measured in the digested samples and in the aqueous samples via Inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700 ICP-MS, Agilent Technologies, Germany GmbH, Waldbronn, Germany) and ICP-MS/MS (Agilent 8900 ICP-MS/MS; Agilent Technologies) using the isotope ¹⁹⁷Au in different gas modes (7700 ICP-MS: no Gas, Helium and HiHelium; 8900 ICP-MS/MS: no-Gas, Oxygen and NH₃). Each measurement series was verified using quality control standards (certified standard not used for calibration) as well as recalibration samples.

2.5. Terrestrial approaches

2.5.1. Description of soil

The experiments were carried out using sandy soil whose physico-chemical properties are listed in the [Supporting Information Table S2](#).

2.5.2. Soil microorganisms

2.5.2.1. Spiking and incubation of the soil

The treatments included controls (non-spiked soil), dispersant spiked soil (two test concentrations), Au spiked soil (soil spiked with pristine Au; two test concentrations), dispersant-sludge (soil spiked with sludge treated with the dispersant vehicle; one test concentration), Au-sludge (soil spiked with Au-treated sludge; one test concentration). In the control treatments (pure soil; soil with sludge including dispersant). Au background concentration was around 10 µg Au per kg soil dry matter (dm).

For the set-ups with pristine Au concentrations at a low and a high concentration differing by a factor 10 were used. For the sludge-amended soil we aimed at a concentration close to the lower concentration. According to the German Sewage Sludge ordinance ([AbfKlärV §6 Absatz 1, 1992](#)) 5 tons per hectare over 3 years can be spread on agricultural areas corresponding to 1.7 tons per year or 0.56 g of dry matter activated sludge per kg soil dm under the assumption of a soil depth of 20 cm and soil density of 1.5 g/m³. It was decided to spike the amount for two years applied in one dose corresponding to 1.10 g sludge dm per kg soil.

For every approach, batches of 3 kg soil dm were prepared. For spiking, soil with a water content of about 20% of the maximum water holding capacity (WHC_{max}) was used. For the application of the pure dispersant or Au-NPs the required amount of substance was mixed with 3 g air dried soil. This mixture was added to 2997 g soil dm and carefully mixed. For the approaches with activated sludge, 9 g dewatered sludge dm was mixed with 3 kg soil dm resulting in a concentration of 3 g activated sludge/kg soil dm. By adding deionized water and careful mixing, the soil was brought to a moisture content of 45% WHC_{max}. The soil was incubated under aerobic conditions at 20 ± 2 °C for 12 weeks. Every 14 days, any evaporated water was replaced, and the soil was

thoroughly mixed. On day 0 the untreated control soil was investigated using all microbial tests. Microbial activity was determined after 7, 28, 56 and 84 days of incubation for the set-ups spiked with pristine material. Previous experiments with soil/sludge mixtures had shown that the activity in the replicates of the same treatment differed significantly on day 7. Even with intensively mixing of the soil/sludge mixture at test start, a sufficient homogenous mixture could not be achieved. Standard deviations comparable to the other set-ups and sampling dates were obtained on day 28 presumably by the adjustment of the water content every two weeks with intensively mixing which increased the homogeneity. Therefore, for the set-ups with activated sludge the measurements started on day 28. Genomic diversity was measured after an incubation period of 28, 56 and 84 days in all approaches.

2.5.2.2. Experimental procedure. Potential ammonium oxidation (PAO) test: The short-term PAO test was performed according to international standard [ISO 15685 \(2012\)](#). The nitrite levels were determined by spectrophotometry at 530 nm.

Microbial respiration: The MicroResp system (<https://www.microresp.com/>) was used for the determination of the microbial respiration activity according to the manufacturer's protocol. In addition to the usual procedure of MicroResp, a plate was filled glass pearls instead of soil. This plate provided information on the CO₂ background. Due to the low microbial activity in the sandy soil the preparation of a specific calibration curve as recommended by the protocol was not considered to be of value. Therefore, the calibration used by the manufacturer was used and the background values of CO₂ were subtracted to compensate for the abiotic color development.

Data analysis for the functional approaches: Statistical analysis (Student *t*-test, Kruskal–Wallis, ANOVA, PERMANOVA) was performed in ToxRat (version 3.3.0, ToxRat Solutions GmbH, Alsdorf, Germany). For multiple testing, *p* < 0.05 was considered to be statistically significant, and for the Students' *t*-test a one-sided analysis was applied.

Microbial genomic diversity: Soil samples were collected at test start (control) and after an incubation period of 28, 56 and 84 days (all approaches). Total genomic DNA (gDNA) was extracted from each sample, in duplicate, using the DNeasy® PowerSoil® Kit (Qiagen, Germany) according to the instructions of the provider. The concentration and purity of the extracts was measured using a Nano Drop™ 2000 (ThermoScientific, USA). The DNA of the replicates was pooled and the concentration adjusted to 20 ng/µL before sequencing.

16S rDNA next generation sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (South Plainfield, NJ, USA). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and DNA quality was checked on a 0.6% agarose gel. A sequencing library was constructed using a MetaVx™ 16S rDNA Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). Briefly, the DNA was used to generate amplicons that cover V3 and V4 hypervariable regions of bacterial and archaeal 16S rDNA. Indexed adapters were added to the ends of the 16S rDNA amplicons by limited cycle PCR. Sequencing libraries were validated using a DNA chip for the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument. Initial taxonomy analysis was carried out on Illumina Basespace cloud computing platform.

Sequence data resulting from the sequencing process were processed using a proprietary analysis pipeline from GENEWIZ. The QIIME data analysis package was used for 16S rRNA data analysis. The forward and reverse reads were joined and assigned to samples based on barcode and truncated by cutting off the barcode and primer sequence. Quality

filtering on joined sequences was performed and sequence which did not fulfill the following criteria were discarded: sequence length < 200 bp, no ambiguous bases, mean quality score ≥ 20 . Then the sequences were compared with the reference database (RDP Gold database) using UCHIME algorithm to detect chimeric sequence, and then the chimeric sequences were removed. The effective sequences were used in the final analysis. Sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) against the Silva 119 database pre-clustered at 97% sequence identity. The Ribosomal Database Program (RDP) classifier was used to assign a taxonomic category to all OTUs at a confidence threshold of 0.8. The RDP classifier uses the Silva 119 database which has taxonomic categories predicted to the species level. Sequences were rarefied prior to calculation of alpha and beta diversity statistics.

β -diversity measures (Bray–Curtis (BC) dissimilarity) were generated using R (R version 4.0.2) and the package Vegan (Oksanen et al., 2019). PCoA (Principal Coordinate Analysis) was done using the function *pcor* of the R package *ape* and PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance Matrices) was performed using the function *adonis* by the community ecology R package *Vegan*.

2.5.3. Soil invertebrates

Three standard test species were used, *Enchytraeus crypticus*, *Folsomia candida* and *Eisenia fetida*.

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) cultures were kept in agar. Cultures were synchronized to obtain 17–18 days old organisms (for details see Bicho et al., 2015).

Folsomia candida (Collembola) were kept in culture on a moist substrate of plaster of Paris and activated charcoal. Cultures were synchronized to obtain 10–12 days old juveniles.

Eisenia fetida (Oligochaeta: Lumbricidae) were originally obtained from ECT Oekotoxikologie GmbH (Germany), kept in culture in soil (Danish agricultural loam soil) and fed on cow dung ad libitum. The selected worms for the test were adult with visible clitellum with a wet weight between 400 and 500 mg. The worms were acclimatized in uncontaminated test soil (LUF 2.2) for 24 h prior to the test.

All animals are kept under controlled conditions of temperature ($20 \pm 2^\circ\text{C}$), photoperiod (16:8 h light:dark) and food.

2.5.3.1. Test media and spiking. Soil: For *E. crypticus* and *F. candida* tests, the treatments included a control (un-spiked soil), control-sludge (soil spiked with control sludge), dispersant-sludge (soil spiked with sludge treated with the dispersant vehicle), Au-sludge (soil spiked with Au-treated sludge). Sludge was added to achieve a final concentration of 2000 mg sludge /kg soil (dm/dm). This amount slightly exceeds the maximum concentration according to the German Sewage Sludge ordinance and the amount used for the microbial tests (see Section 2.5.2) and simulates that larger amounts or activated sludge may be added. The process consisted of directly mixing the activated sludge with the dried soil per individual replicate, to ensure total raw amounts per replicate. Moisture was adjusted to 50% of soils' WHC_{max}, adding deionized water. The soil was left to equilibrate for 24 h prior to the start of the tests.

Food: For *E. fetida*, exposure was via the food, i.e. the sludge treatments were provided as food source. A control with 1 g dried cow dung food source was run in parallel. Two replicates were used and the food source (1 g dry weight, with food having a 50% water content) was added to the soil surface once a week (Simonsen and Scott-Fordsmand, 2004).

2.5.3.2. Experimental procedures. Enchytraeid reproduction test (OECD and OECD extension): The standard guideline for the Enchytraeid Reproduction Test (ERT) (OECD, 2016a) was followed (28 days), plus the OECD extension, as described in Ribeiro et al. (2018), i.e. extending the test 28 more days (56 days in total) and adding extra monitoring

sampling times at days 7, 14, 21, (28) and 56 days. Endpoints included survival for all sampling times, reproduction at days 28 and 56, i.e. number of juveniles and population, respectively, and size at days 28 and 56. Four replicates per treatment were conducted. Due to the limitation in the amount of sludge, samples for days 7, 14 and 21 sampling were conducted in duplicate but with twice the number of organisms (20), to ensure equivalent sampling.

In short, 10 synchronized age animals (17–18 days old) were introduced in each test container with moist soil (\varnothing 4 cm with 20 g of soil for exposure up to day 28, and \varnothing 5.5 cm with 40 g of soil for exposure up to day 56) and food supply (22 ± 2 mg, autoclaved rolled oats). The test ran for up to 56 days at $20 \pm 1^\circ\text{C}$ and 16:8 h photoperiod. Food and water were replenished every week.

At sampling days (7, 14, 21 and 28) adults were carefully removed from the soil and counted (survival). At day 28, reproduction was assessed by counting the juveniles, after being fixed with ethanol and stained with Bengal rose (1% in ethanol). Adult organisms were photographed for posterior determination of size (area) using the software Image J (Schneider et al., 2012). For the replicates that continued until day 56, adults were carefully removed from the soil at day 28, and the rest proceeded accordingly. At day 56, population numbers were counted, following the procedure described. Among the largest organisms (expected to belong to the first laying), 10 were photographed for posterior determination of size (area) using the software Image J (Schneider et al., 2012).

Soil pH (0.01 M CaCl_2) was measured at all the sampling times.

Folsomia candida reproduction test (OECD and OECD extension): Tests followed the standard OECD guideline 232 (OECD, 2016b) (28 days), plus the OECD extension, as described in Guimarães et al. (2019), i.e. extending the test 28 more days (56 days in total) and adding extra monitoring sampling times at days 7, 14, 21, (28) and 56 days.

At all sampling points, endpoints included survival and reproduction, i.e. number of juveniles (or population at day 56). Additionally, organisms' size was assessed at days 28 and 56. Four replicates per treatment were conducted. Due to the limitation in the amount of sludge, samples for day 7, 14 and 21 sampling were conducted in duplicate but with twice the number of organisms (20), to ensure equivalent sampling.

In short, 10 synchronized age animals (10–12 days old) were introduced in each test container with moist soil (\varnothing 5.5 cm with 30 g of soil) and food supply. The test ran for up to 56 days at $20 \pm 1^\circ\text{C}$ and 16:8 h photoperiod. Food and water were replenished every week.

At each sampling day (7, 14, 21, 28 and 56 days), the test vessels were flooded with water and the content was transferred to a crystallizer dish. The surface was photographed for further analyses (count and measure (size, area)) using the software ImageJ (Schneider et al., 2012).

For the replicates that continued until day 56, after similar flooding and photographing for counting and measuring, the sampled juveniles, at day 28, were transferred with a spoon to a box with a layer of plaster of Paris to absorb extra water from the spoon. For exposure of the next generation, ten of the biggest juveniles (ca. 11 days old) were transferred to new test vessels containing soil (spiked at day 0), representing an F1 exposure. The test ran under the same conditions. At day 56, survival (F1) and reproduction (F2) were counted and measured, following the described procedure. Soil pH (0.01 M CaCl_2) was measured at all the sampling times.

Eisenia fetida bioaccumulation test - via food (OECD adapted): The test followed the standard guideline for Bioaccumulation in Terrestrial Oligochaetes OECD 317 (2010) with some adaptations. In short, the worms were randomly assigned to a test vessel, and were in the same weight range (400–500 mg). One worm was introduced per test container (\varnothing 72 mm, 42 cm high, with 100 g of moist soil), the sludge treatment (1 g) was placed on the surface (in a small hole) and provided as food. The procedure was the same for the control where cow dung was provided. Two replicates per treatment were conducted (due to the limited amount of material). The test ran at $20 \pm 1^\circ\text{C}$ and a 16:8 h photoperiod. Food (1 g)

and water were replenished every week. Sampling days included day 0, 14, 21, 28 and 42. At the sampling days the earthworms were hand-sorted from the soil, depurated and weighed, recording survival and weight of each replicate.

Data analysis: One-way analysis of variance (ANOVA) followed by the post-hoc Holm-Sidak method ($p < 0.05$) was used to compare the endpoints between treatments (SigmaPlot 11.0).

2.6. Aquatic approaches

The short and long-term effects of the effluents collected from the WWTPs treated with the dispersant vehicle (Disp-E) or with the Au-NP (Au-E) was studied in fish cell lines and *Daphnia magna*. In addition, the short-term toxicity of the pristine Au-NP was assessed in both organisms.

2.6.1. Fish cell lines

Three cell lines derived from rainbow trout (*Oncorhynchus mykiss*) were used. RTL-W1 (liver) cell line was a generous gift from Drs. Lee and Bols, who obtained it from normal liver tissue (Lee et al., 1993). RTgill-W1 (branchial arc) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RTS-11 (spleen) cells were developed by Ganassin and Bols (1998) and kindly provided by Dr. Bols.

The cell lines were cultured in Leibovitz's L-15 medium (ThermoFisher Scientific Inc., Madrid, Spain). Depending on the cell line, this medium was added with (1%) penicillin and streptomycin (P/S) (10,000 U/mL/10 mg/mL) (Lonza Group, Barcelona, Spain) and with different amounts of fetal bovine serum (FBS) (10% for RTL-W1 and RTgill-W1, and 20% for RTS-11) (Lonza Group).

2.6.1.1. Short-term exposure. The three cell lines were exposed for 24 h directly to the Au-E and Disp-E, and to a range of 7 dilutions of the effluents (dilution factor: $\frac{1}{2}$). Additionally, a positive control treated with a concentration range (66–500 μ M) of sodium dodecyl sulfate (Sigma-Aldrich, Madrid, Spain) was also included in each 96-well plate. Cells were also exposed for 24 h to a range of concentrations (0.78; 1.56; 3.125; 6.25; 12.5; 25; 50; 100 mg/L) of the pristine Au-NP directly dispersed in cell medium, and to the vehicle control (highest concentration of the dispersant present in the NP dispersion). Three experiments were performed each in triplicate.

2.6.1.2. Long-term exposure. For the long-term exposure (28 d), both effluents were completely dried and reconstituted in the respective cell media. The RTL-W1 and RTS-11 cells were selected for this experiment, as a representation of less and more sensitive cell lines, respectively, according to the short-term exposure results.

24-well plates were used to develop the long-term experiments, following the protocol described by Galbis-Martínez et al. (2018), with modifications. Briefly, cells were seeded and maintained at 20 °C for 24 h to obtain a confluent cell monolayer. Then cells were exposed to the respective reconstituted effluent (in triplicate). At days 7, 14 and 21, cells were rinsed with PBS, trypsinized with trypsin-EDTA (Lonza Group) and $\frac{1}{4}$ of cells were sub-cultured in a new 24-well plate. They were maintained in the specific L-15 medium without treatment for 24 h to allow them to attach to the bottom of the wells. Then, the medium was replaced by new reconstituted effluent. Five plates were initially established for each experiment ($n = 3$), and cytotoxicity was evaluated after 1, 7, 14, 21 and 28 days, each time in a different plate in triplicate.

2.6.1.3. Cytotoxicity assays. To monitor different endpoints of viability a fluorometric-based assay system that facilitates the simultaneous use of three assays was employed. The assays were conducted following the protocol described by Dayeh et al. (2005), with the modifications reported by Lammel et al. (2013). This triple assay studied the cell

viability on the basis of mitochondrial activity with the Alamar Blue (AB) test; the damage to the plasmatic membrane with the 5-carboxy-fluorescein diacetate, acetoxymethyl ester (CFDA-AM) assay; and the accumulation of neutral red (NR) dye in the lysosomes of viable uninjured cells by means of the neutral red uptake (NRU) assay. Briefly, cells were first washed with PBS, to remove any remaining exposure medium. Then, they were treated with 100 μ L (short-term) or 400 μ L (long-term) of a solution of AB (1.25% v/v) (Invitrogen; ThermoFisher Scientific, Madrid, Spain) and CFDA-AM (4 μ M) (Sigma-Aldrich, Madrid, Spain) prepared in MEM(-) (ThermoFisher Scientific Inc.), supplemented with non-essential amino acids (Lonza Group), for 30 min under dark conditions. Fluorescence intensity of the AB and CFDA-AM conversion products (resorufin and carboxyfluorescein, respectively) was measured at 532 and 590 nm or 485 and 535 nm excitation (exc.) and emission (em.) wavelengths, respectively, using a microplate reader (Tecan Spark 20M, Tecan Group Ltd., Männedorf, CH). Afterwards, the reagents were removed and the cells were washed once with PBS and treated for 1 h with 100 μ L (short-term) or 400 μ L (long-term) of a NR working solution (0.03 μ g/mL) prepared in the supplemented MEM(-), again under dark conditions. After this incubation period, the NR solution was removed, and cells washed with PBS. To extract the potentially retained NR, 100 μ L (short-term) or 400 μ L (long-term) of a 1% glacial acetic acid (Sigma-Aldrich)/50% ethanol (Panreac AppliChem, Barcelona, Spain)/49% Milli-Q water solution were added, and fluorescence was measured at 532 nm exc. and 680 nm em.

2.6.2. *Daphnia magna*

2.6.2.1. Short-term exposure: acute immobilization assay with *Daphnia magna*. The effect of the aged Au-NP present in the effluent versus the effect of the pristine Au-NP was studied following the OECD guideline 202 (OECD, 2004). To perform the assay, the Daphtoxkit (Micro-BioTests, Gent, Belgium) distributed by Ecotest (Valencia, Spain) was used. Different groups were tested: (1) control group, no treatment; (2) daphnids exposed to a range of 5 concentrations (0.1, 6.25, 12.5, 25, 50 and 100 mg/L) of the pristine Au-NP in the daphnia medium, (3) vehicle group, highest concentration of the dispersant in the Au-NP treated group; (4) daphnids exposed to the dispersant-effluent (Disp-E) and four $\frac{1}{2}$ serial dilutions; (5) daphnids exposed to Au-effluent (Au-E, 0.1 mg Au/L) and four $\frac{1}{2}$ serial dilutions, and (6) daphnids exposed to a range of 5 concentrations (0.1, 6.25, 12.5, 25, 50 and 100 mg/L) of the pristine Au-NP in the Disp-E. Four replicates per concentration of material were conducted.

2.6.2.2. Long-term exposure: *Daphnia magna* reproduction test. The reproduction test was performed according to the protocol of OECD guideline 211 (OECD, 2012). The different treatments included long-term exposure (21 d) to: (1) M4 medium (control), (2) dispersant-effluent (Disp-E) and (3) Au-effluent (Au-E). To obtain a homogeneous suspension, the effluents were sonicated with a Vibra-CellTM (VCX 750, Sonics) sonicator for 5 min at 20% amplitude in an ice bath, delivering approximately 4000J. The exposure suspensions were renewed three times per week. Before each medium renewal, a sonication step was applied during 2 min at 20% amplitude, delivering approximately 1500J. *Daphnia* were fed with *Chlorella vulgaris* (1st week: 0.1 mg/ C/*Daphnia*/day, 2nd and 3rd weeks: 0.15 mg/ C/*Daphnia*/day). For the two effluents, the combined vitamin stock solution of M4 medium was provided to the parent animals once a week. Due to the limited volume of effluent available, the volume of test solution was reduced to 20 mL per parent animal instead of 50 – 100 mL as recommended in the test guideline. The endpoints recorded were: total number of living offspring, total number of living offspring per parent animal alive, number of broods, number of dead offspring, dead parent animals, parent animals alive at the end of test and mean number of offspring. Calculations were oriented to define the inhibition of reproduction (%)

of animals exposed to any of the effluents compared to M4 medium control, and inhibition of reproduction (%) in animals exposed to Au-E compared to Disp-E.

2.6.3. Au-NPs characterization in the effluents and exposure medium

Their presence was studied by TEM using a JEOL JEM 2100 microscope (JEOL Ltd., Japan) with EDX (Oxford Inca, United Kingdom).

2.6.4. Data analysis

The raw data of the cytotoxicity assays were corrected subtracting the background fluorescence (cell-free control) and normalized as a percentage against the control. All results are presented as mean \pm standard deviation of at least three independent experiments performed in triplicate. Raw data from *Daphnia* assays were transformed to percentages of survival, enabling comparisons between each exposed group and the control. Normality and homoscedasticity of all data were checked by the Shapiro–Wilk test and Bartlett's test, respectively. Since the data were normal and homoscedastic, a one-way repeated measures analysis of variance (RM-ANOVA) followed by a Dunnett's post hoc test was performed, allowing the detection of significant difference from control values. A *t*-test unpaired was applied to evaluate significant differences between effluents. All the statistical analyses were performed with the software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The estimation of the $I(E)C_{20/50}$ or $IT_{20/50}$ (concentration or time causing a 20/50% of inhibition/effect with respect to the controls) were done by fitting the results to a regression model equation for a sigmoidal curve using the same statistical software.

3. Results

3.1. Concentration of gold in activated sludge, effluent and soil-gold mixtures

The results on the concentrations in activated sludge and effluent are presented in detail by Schlich et al. (2021). In brief, the mean gold concentrations in the sludge of the two model WWTPs was 4.9 g/kg. The concentrations were additionally determined in the soil-sludge mixtures used for the microbial tests after an incubation period of 56 days. During this incubation period, the soil was turned and mixed several times and thus expecting an improved homogeneity. The concentrations in the soil-sludge mixtures were 4.3 and 5.7 mg/kg. For these tests 1.000 mg of sludge were added per kg of soil dm, resulting in a calculated mean of the gold concentration in the activated sludge of 5.0 g/kg dm which is comparable to the mean concentration measured in the activated sludge samples. The concentrations in the effluents of both replicates were 96 and 104 μ g/L with a mean value of 100 μ g/L. For the invertebrate testing, soil was spiked with 2 g of activated sludge resulting in a calculated concentration of 9.8 mg/kg (Supporting Information Table S3).

Additionally, we determined the concentrations in the soil samples spiked directly with the Au-NPs and used for the microbial tests. Mean concentrations of 2.4 mg/kg for the low concentration and 80.1 mg/kg for the high concentration were measured.

3.2. Terrestrial approaches

3.2.1. Soil microorganisms

3.2.1.1. Functional approaches. On day 7, the pH was 5.4 ± 0.2 for all approaches. However, from day 28, there was a shift between the soil setups with and without activated sludge. While the pH in the control soils and the soils with pure dispersant or Au-NPs, stayed nearly constant, the pH of the soils with activated sludge decreased to a pH of about 4.5 by day 28 (Supporting Information Table S4).

The basal respiration activity slightly decreased during the incubation period from about $1.4\text{--}1.1 \mu\text{g CO}_2 \times \text{g}^{-1} \times \text{h}^{-1}$ (Supporting Information Table S5) for the untreated control. The measured values of the two control replicates showed a good comparability throughout the test. The soil treated with pristine Au-NPs and dispersant showed statistical significant effects on the basal respiration compared to untreated control although the differences were small at about 10% (Supporting Information Fig. S1). Furthermore, there was no conclusive time course and no clear concentration-effect relationships neither for the dispersant nor for the Au-NPs. For Au applied via activated sludge, a 33% inhibition compared to the dispersant was determined on day 28 which decreased slowly by the end of the incubation period. The concentration of Au in the samples with activated sludge were comparable to the low concentration of the pristine materials. However, the inhibitory effects differ between both approaches when the approaches with dispersant are used as reference. Only at the end of the incubation period, in both approaches, with about 6% similar inhibitory effects were determined.

The absolute values of the SIR exceeded those of the basal respiration for all set-ups (Supporting Information Table S6). For the pristine material more pronounced effects were found for the SIR compared to the basal respiration (Supporting Information Fig. S2) but the effects of neither the pristine Au-NPs nor the dispersant showed a time-course. Stimulation and inhibition alternate across the sampling points. As an example, in the vessel with the low gold concentration there is stimulation on day 7 and day 56, but inhibition on day 28 and 84. Furthermore, a similar pattern of stimulation and inhibition over time was observed for both gold concentrations and for both dispersant concentrations. The patterns between gold and dispersant were obviously different. Both gold concentrations resulted in an inhibition on day 28, whereas the set-ups with dispersant showed stimulation. When the dispersant is used as reference for the set-ups with gold (Supporting Information S2; middle section) the missing time course indicates that Au-NPs did not affect the SIR.

The effects in the set-ups with Au-NPs added via activated sludge were comparable for both respiration rates. The SIR in the soil spiked with activated sludge showed on days 28 and 56 statistically significant effects for the Au-NPs compared to the dispersant. The effect decreased with time and at test end (day 84) a value comparable to the dispersant control was determined (Supporting Information Fig. S2, right section).

The potential ammonium oxidation activity showed statistically significant differences at some time points for the approaches with the pristine material and for the material applied via activated sludge. (Supporting Information Table S7, Fig. S3). Significantly lower activities were determined in the approaches with the pristine Au-NPs after an incubation period of 28 days with no activity at all at the higher test concentration. Although no technical defect could be identified, these values should not be overestimated as on day 7 and 56 no effect was obvious. In contrast to the pristine material, a significant stimulation on day 28 was obvious for the Au-NPs added with sludge.

3.2.1.2. Genomic diversity. The mean number of sequences per environmental DNA (eDNA) sample ranged from 44,812 to 75,204 across all samples analyzed. Data are accessible at NCBI (BioProject accession number PRJNA745588).

The mean species diversity, represented by the alpha-diversity indexes Chao1, Shannon and Simpson (Supporting Information Table S8) indicated that there were no significant changes (ANOVA, $p > 0.05$) in the bacterial richness and evenness within the experiment course or by the application of the treatments. To evaluate the differentiation of the community (beta-diversity), principal coordinate analysis (PCoA), displaying dissimilarities among communities (using BC distances), was performed at class level to evaluate changes in the ten different soil setups. The analysis showed that the microbial community from setups using pure soils differ significantly (PERMANOVA, $p < 0.001$) from those which were mixed with activated sludge (Supporting Information

Fig. S4), indicating that the addition of activated sludge changes the bacterial community composition. In this figure, it is also possible to observe changes in the bacterial communities over the experiment course, which are more evident in the samples amended with sludge.

To highlight the effect of application of gold and dispersant, PCoA analysis of the setups using pure soils and soils mixed with activated sludge were performed separately (Fig. 1 and Fig. 2, respectively).

In the case of the setups of pure soils (Fig. 1) it was possible to observe changes in the bacterial community during the experiment course although changes were only significantly different when compared to the control samples from day 0 (PERMANOVA, $p < 0.001$). Regarding the application of dispersant and gold, no significant differences (PERMANOVA, $p = 163$) were observed between controls and dispersant at each respective sampling time. This indicated that the dispersant does not have an effect on the community of the sample. The microbial community of the gold treatments (high and low) were significantly different (PERMANOVA, $p < 0.001$) from controls and dispersant at each respective sampling time. This effect increases significantly with the concentration of gold used with the impact on in the community being much bigger when using higher concentrations of gold. The effect on the community of soils, at the low concentration of gold used is comparable to the effect observed in the soils mixed with sludge in which the concentration is equivalent to the low concentration used in the pure soils.

The results of the setups mixed with activated sludge showed obvious changes in the community over time (PERMANOVA, $p < 0.002$), and also an obvious change in the community after application of gold (PERMANOVA, $p < 0.005$) which becomes more predominant over the experiment course (Fig. 2).

3.2.2. Soil invertebrates

3.2.2.1. Enchytraeid reproduction test (OECD and OECD extension). For the standard 28-day test, no significant differences occurred between the treatments, although there was a tendency to increase the performance, i.e. reproduction numbers when Disp-S and Au-S were added, without major size variation (Fig. 3). Results on day 56 showed a similar tendency of increased performance, this being significant for Au-S (in comparison to 0-control and CT-S) with approximately 6000 organisms, which is approximately twice more than in the control. Additionally, an impact in size was recorded, with animals being smaller in the treatments with higher numbers (Disp-S and Au-S).

The pH did not vary obviously from day 0–28 ($\text{pH } 5.5 \pm 0.2$), but decreased by day 56 (from 4.9 in control to 4.1 in Au-S) (Supporting Information Fig. S5A).

3.2.2.2. Folsomia candida reproduction test (OECD and OECD extension). Comparable to *E. crypticus*, no significant differences occurred

between the treatments for the standard 28 day test. However, there was a tendency for increased size which was more pronounced for the juveniles, in CT-S and Disp-S. In contrast, the test design with sludge (Au-S) resulted in a decrease in (Fig. 4). Results at day 56 showed a tendency for a decrease in performance, i.e. survival and reproduction (population), when sludge was added to the soil. As for the size, the Au-S remains slightly lower than average, but no significant impact.

3.2.2.3. Eisenia fetida bioaccumulation test via food (OECD adapted).

Results from the bioaccumulation test (Fig. 5) show that animals died in all treatments by day 21. Only the organisms in the control which were fed with cow dung, survive. Hence it was not possible to continue up to day 42 as planned. Mass measurements show an overall decrease in sludge treatments (Fig. 5B). The weekly observations showed a clear difference from day 7, where the sludge treatments caused fungal growth (Supporting Information Fig. S6).

3.3. Aquatic approaches

3.3.1. Characterization of Au-NPs in the effluents, daphnia medium and cell medium

The Au-NP concentration in the effluent was 0.1 mg/L. Even when the concentration was low, the images obtained by TEM showed NPs in the matrix that were confirmed as Au by EDX (Supporting Information Fig. S7). The size distribution could not be measured due the low amount of NPs present. The Au-NP were not found in the Disp-E.

The hydrodynamic size of Au-NPs in the Au-E indicated a polydispersion index > 0.7 , which led to bad measurements and sizes > 1000 nm. The same was obtained in the Disp-E. Pristine Au-NPs dispersed in Disp-E and in daphnia medium were generally stable with time, with no great differences in size related to the Au-NP concentration and medium (Supporting Information Table S9).

Results from the DLS analyses of both effluents dried and restored in cell medium are shown in the Supplementary Information (Supporting Information Table S10). During the first 24 h, none of the effluents showed differences in size with respect to the control sample (cell medium) as might be expected due to the low amount of Au-NP in the Au-E. However, after 7 days, the polydispersity index (PdI) of the re-suspended effluents was > 0.7 and the Z-Ave increased up to values higher than 500 nm with a main peak of intensity > 900 nm for both effluents. These aggregates maybe due to the presence of other substances in the effluents. When the characteristics of the pristine Au-NP dispersed in cell medium (100 mg/L) were tested by DLS, the NP showed a higher size with respect to the cell medium and effluents at time zero which increased to 510 nm after 7 days. This increase in size could be due to the reaction of the cell medium components with the NP and, therefore, the formation of a biocorona around the Au-NP.

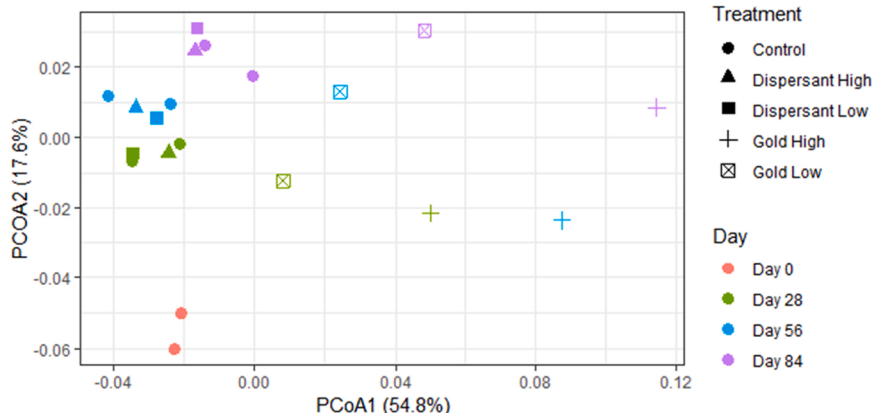


Fig. 1. Principal coordinate analysis (PCoA) of bacterial communities from soil amended with pure substance. Bacterial communities were evaluated to class level, using Bray–Curtis distance. Symbols represent the different setups: control, dispersant low/high and gold low/high, and color codes represent the different sampling times: red (day 28), green (day 56), and blue (day 84). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

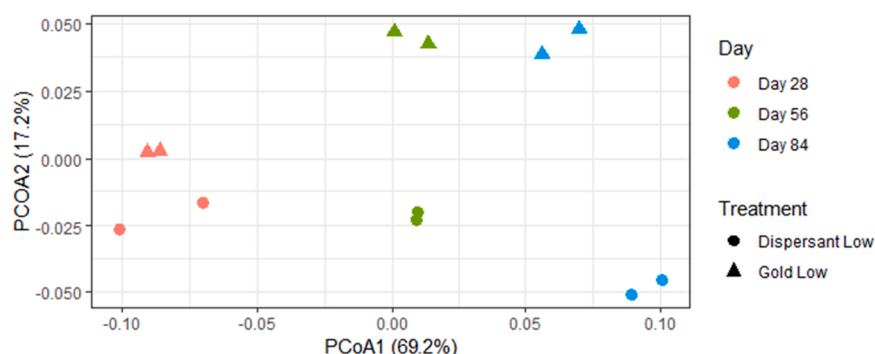


Fig. 2. Principal coordinate analysis (PCoA) of bacterial communities from soil amended with activated sludge (WWTP). Bacterial communities were evaluated to class level, using Bray–Curtis distance. Symbols represent the different setups: dispersant low (●) and gold low (▲), and color codes represent the different sampling times: red (day 28), green (day 56), and blue (day 84). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

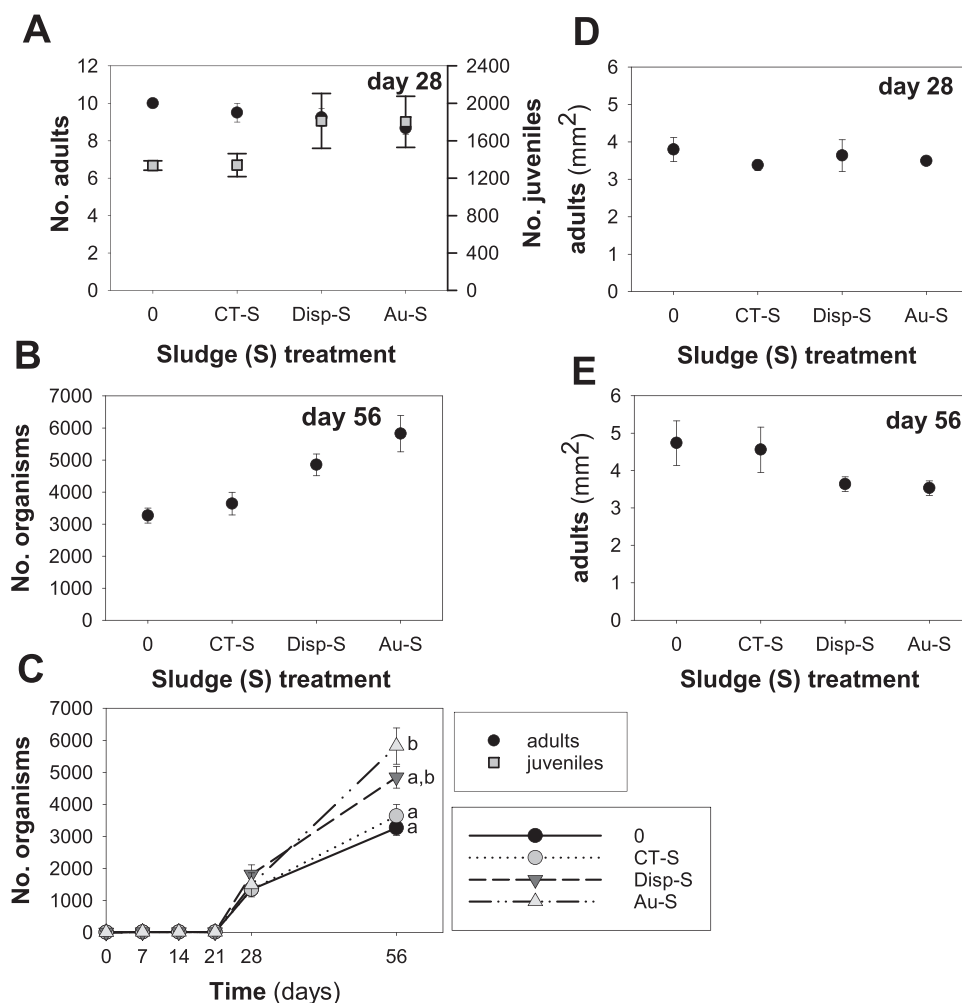


Fig. 3. Results from the standard OECD Enchytraeid Reproduction Test (ERT) and the OECD extension, when *Enchytraeus crypticus* were exposed in LUFA 2.2 soil to the following treatments: 0-control, CT-S: control+sludge, Disp-S: dispersant+sludge and Au-S: Au-NPs+sludge. Sampling included days 7, 14, 21, 28 and 56. Measured endpoints include (A) survival and reproduction (day 28, standard); (B) population numbers (day 56); (C) population numbers (survival + reproduction) in time series; (D) size (day 28); (E) size (day 56) (area, mm²). Values are expressed as average ± standard error. a, b: (p < 0.05, Holm-Sidak method).

3.3.2. Cell lines

A fatal toxic effect was found in the cells after exposure to the pure effluents and their first 1/2 dilution with a total loss of viability (data not shown). This effect was due to the lack of nutrients in the exposure medium. Therefore, it was decided to dry 10 mL of the effluents and resuspend the pellet in the same volume of the cell medium to perform the long-term toxicity assays and verify the toxicity after 1 day of exposure. RTS-11 cells were shown to be valid tools for up to 14 days exposures only whereas RTL-W1 could be used for at least 28 days exposures.

Results from the short-term exposure assays showed that RTS-11 was the most sensitive cell line to the pristine Au-NP, finding an IC₂₀ of 44.6 ± 10.4 mg/L, whereas 100 mg/L did not exert any toxicity in

RTgill-W1 and RTL-W1 cells. Following the same pattern, RTS-11 was the most sensitive cell line to the effluents (Disp-E and Au-E). However, there was not a clear difference between the effects exerted by both effluents.

The results regarding the cytotoxicity of both effluents in RTL-W1 and RTS-11 cells after a long-term exposure are shown in Table 1. The Au-E produced a higher significant toxicity in RTL-W1 after 21 days exposure compared to the toxicity produced by the Disp-E. However, no differences between both effluents were observed in RTS-11 cell lines.

The time needed to inhibit 20% and 50% of the viability of the RTL-W1 cells was significantly lower in the cells exposed to the Au-E (Table 1). No differences in these endpoints were noted for the RTS-11

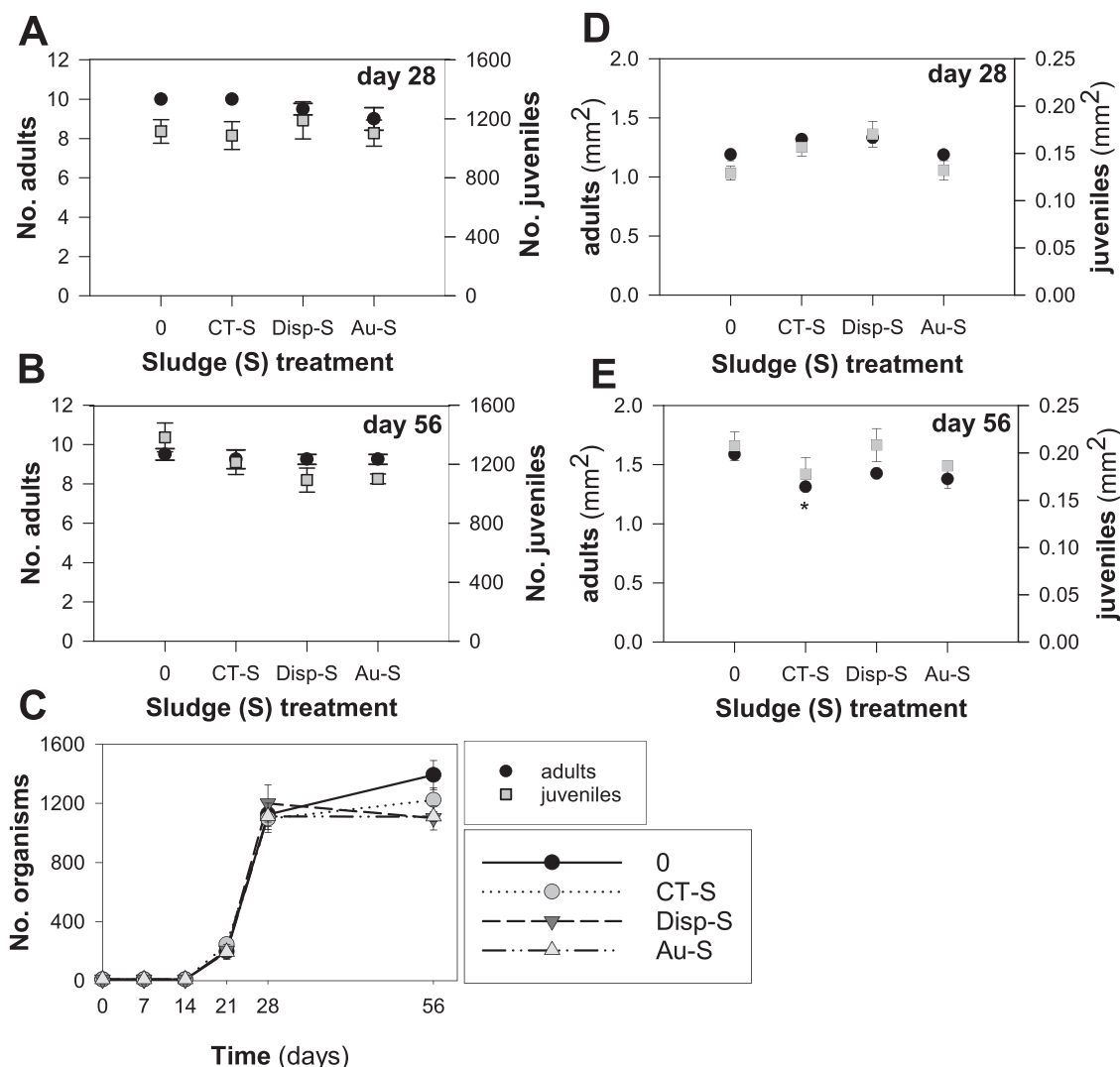


Fig. 4. Results from the standard OECD Collembolan Reproduction Test and the OECD extension, when *Folsomia candida* were exposed in LUFA 2.2 soil to the following treatments: 0-control, CT-S: control+sludge, Disp-S: dispersant+sludge and Au-S: Au-NPs+sludge. Sampling included days 7, 14, 21, 28 and 56. Measured endpoints include (A) survival and reproduction (day 28, standard); (B) survival and reproduction (F2, day 56); (C) population (survival + reproduction) in time series; (D) size (day 28); (E) size (day 56) (area, mm²). Values are expressed as average \pm standard error (AV \pm SE).

cells but a highest sensitivity to both effluents was observed in this fish cell line with an inhibition of 50% of the population reached within the first day of exposure (lysosomal level). This inhibition was attained in the RTL-W1 cells after 14 days exposure (mitochondrial level) (Table 1).

3.3.3. Invertebrates

After 48 h of exposure to 100 mg/L of Au-NPs dispersed in daphnia medium, 50% of the animals were immobilized (Fig. 6). However, no effect was observed at any of the other concentrations. On the other hand, when *D. magna* was exposed to the pristine Au-NP dispersed in the Disp-E, only 25% of immobilization was observed at the highest concentration. There was no toxic effect after exposure of *D. magna* to any of the effluents (data not shown).

After long-term exposure to both effluents, no death was recorded at the end of the experiment for the parent animals (Supporting Information Table S11). No significant differences in terms of the number of living offspring produced by the parent animals and in the number of broods were observed. There was no significant inhibition of reproduction in animals exposed to any of the effluents.

4. Discussion

4.1. Suitability of the test approach

4.1.1. Test design

To investigate the fate and effect of nanomedical products after their use phase, a realistic scenario can be simulated by a combination of model WWTP and aquatic tests as well as terrestrial tests with test systems used in the scope of registration or notification of chemicals. In designing the test, the limited amount of activated sludge has to be taken into account. According to the OECD guideline 303 a sludge concentration at a maximum of 3 g/L in the WWTPs is recommended. This means that with a model WWTP of 10 L, 30 g dm sludge can be obtained. A maximum amount of activated sludge of 40 t per ha and year was identified for an improvement of the soil quality (Roig et al., 2012), but this may not apply to other countries and regions (European Commission, 2012). According to the German Sewage Sludge Ordinance 5 t/ha every three years is allowed (AbfKlärV §6 Absatz 1, 1992) and this can be allocated to the three years. Assuming a soil depth of 20 cm (in accordance to the agricultural practice) and a soil bulk density of 1.5 g/cm³ (OECD, 2000b, 2000c) results, for the 5 and 40 t/ha, in an amount of 1.67 and 13.3 g activated sludge dm per kg soil dm. Based on

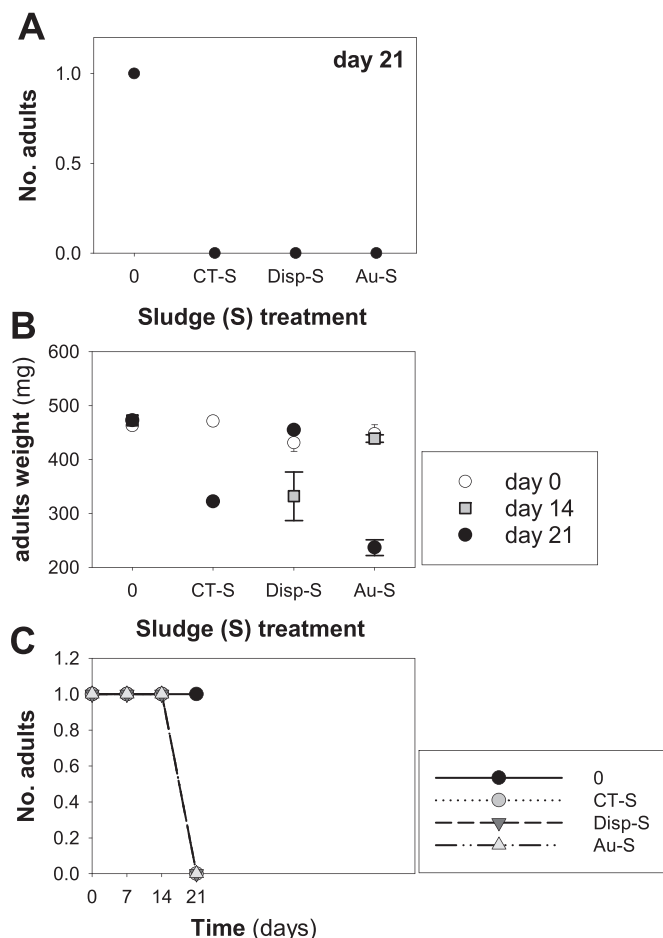


Fig. 5. Results from the adapted standard OECD *Eisenia fetida* bioaccumulation test via food, when exposed in LUFA 2.2 soil to the following treatments: 0-control, CT-S: control+sludge, Disp-S: dispersant+sludge and Au-S: Au-NPs+sludge. Sampling included days 7, 14 and 21. Measured endpoints include (A) survival (B) size (weight, mg); (C) survival in time series; Values are expressed as average \pm standard error (AV \pm SE).

Table 1

Hazard data (inhibition time (IT)) for RTL-W1 and RTS-11 cells after long-term exposure to effluents (Disp-E or Au-E). Measured endpoints include mitochondrial activity (alar blue AB), cell membrane impairment (CFDA-AM) and lysosomal activity (NRU). Mean \pm SD (standard deviation) (n = 3). *, **, *** denote a statistical difference between the exposure to Disp-E and Au-E (t-test, $p < 0.05$, < 0.01 and < 0.001 , respectively).

	IT ₂₀ (days)	IT ₅₀ (days)
RTL-W1 (28 d exposure)		
Disp-E	AB: 14.5 \pm 4.06 CFDA-AM: 23.8 \pm 1.49 NRU: 20.2 \pm 2.48	AB: 21.6 \pm 1.56 CFDA-AM: 27.3 \pm 0.25 NRU: 23.3 \pm 0.79
Au-E	AB: 8.13 \pm 2.04 CFDA-AM: 10.8 \pm 0.29*** NRU: 18.1 \pm 2.28	AB: 15.5 \pm 1.72* CFDA-AM: 20.8 \pm 1.97** NRU: 21.4 \pm 0.60*
RTS-11 (14 d exposure)		
Disp-E	AB: 6.12 \pm 0.03 CFDA-AM: 3.7 \pm 3.35 NRU: 0.14 \pm 0.25	AB: 6.69 \pm 0.04 CFDA-AM: 6.82 \pm 5.42 NRU: 0.51 \pm 0.46
Au-E	AB: 6.14 \pm 0.10 CFDA-AM: 1.39 \pm 2.21 NRU: 0.34 \pm 0.07	AB: 6.72 \pm 0.08 CFDA-AM: 2.65 \pm 3.86 NRU: 0.54 \pm 0.07

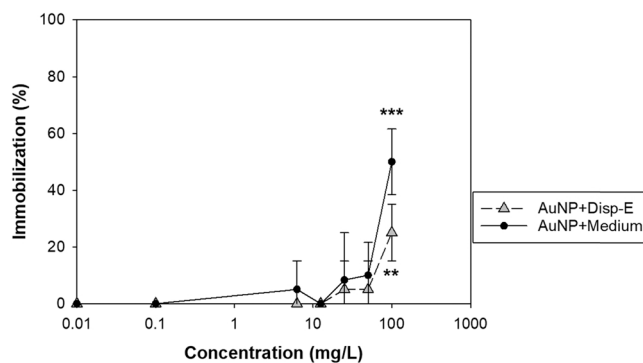


Fig. 6. Immobilization assay for *D. magna* after exposure to 6.25, 12.5, 25, 50 and 100 mg Au-NP/L of daphnia medium or Disp-E for 48 h. Data are presented as mean \pm standard deviation, (n = 4). * represents significant differences related to control (RM-ANOVA followed by a Dunnett's post hoc test; **, $p < 0.01$; ***, $p < 0.0001$).

a total amount of 30 g sludge dm, 2–18 kg soil can be spiked. For the present study, 1–2 g sludge dm/kg soil dm were used. By this, long-term tests with soil microorganisms, enchytraeids and collembolans can be performed. For the earthworm reproduction test according to OECD guideline 222, a minimum of 50–60 g soil per worm and two to four replicates each with 10 worms per test concentration are required. This results in a minimum amount of 1.0–2.4 kg soil dm per test concentration. Hence, earthworm reproduction tests with spiked soil require much activated sludge. An alternative is the bioaccumulation tests using activated sludge as a food source (Simonsen and Scott-Fordsmand, 2004).

The number of model WWTPs determine the number of variants to be tested. Besides variations in the number of concentrations, also variations in the set-ups considered to be controls have to be taken into account. In the case of materials available as a formulation two different kinds of control can be used. Controls with activated sludge or effluent from WWTPs just fed with synthetic wastewater provide information on effects caused by the complete product. Controls with activated sludge or effluent from WWTPs fed with synthetic wastewater spiked with solvent or dispersant, but no test substance, provide information on effects caused by the active ingredient.

The composition of the effluent differs from the composition of the mineral media used for the culture of the aquatic organisms and cell lines, hence effects observed may be due to a relative change in the basic media composition rather than a direct toxic effect and a suitable control is required.

4.1.2. Soil microbial community

Due to the adaptability of the soil microbial community, tests addressing these organisms have no general limitations. The higher activity in the soils with added activated sludge can be explained by the added nutrients, that is why activated sludge is used as fertilizer in many countries (European Commission, 2012). The changes in the microbial community structure (Fig. 2) reflects the adaptation of the microbial community. An example is the composition of the ammonium-oxidizing microbial community which is influenced by the soil pH. With decreasing soil pH, the ratio of ammonium-oxidizing archaeobacteria to ammonium-oxidizing bacteria increases (Hu et al., 2013; Li et al., 2019b).

4.1.3. Soil invertebrates

For *E. crypticus* and *F. candida* the extension of the test up to 56 days proved to be relevant since the impact was more pronounced and

significant with time. The various endpoints (reproduction, size) were differently affected in *E. crypticus* and *F. candida* indicating that the organisms don't provide redundant but additional information regarding hazard in the presence of activated sludge. *E. crypticus* showed an increase in performance (reproduction) and a decrease in size when exposed to the various sludge treatments, especially the Au spiked, as opposed to *F. candida*, where a minor decrease was observed for performance and size. The inverse pattern between sludge exposure and size is a commonly observed strategy where the resources are allocated to reproduction in detriment to other like growth. Organisms' fitness may be assessed by their growth (Pomeroy, 1997) and a minimum size is known to be required for *F. candida* to reproduce (Crommentuijn et al., 1993). Therefore the observed tendency of reduced reproduction, at the same time that adults were smaller, may indicate further risks for the future generations (e.g. as also reported in Amorim et al., 2017).

Between day 28 and 56 the pH decreased during the OECD extension test, i.e. from day 28 (standard) till day 56 (extension) and could be close to the survival limit of the organisms (Jänsch et al., 2005). It is probably more critical for *E. crypticus* than for *F. candida* (Amorim et al., 2005). It remains unknown whether the decrease up to the survival limit of *E. crypticus* is related to the more pronounced effects. However, such a decrease after the addition of sewage is frequently observed (Hooda and Alloway, 1994; Schlich et al., 2013) and reflects an environmental process.

For *E. fetida*, the adapted bioaccumulation design (sampling dates and replicates) would be a good compromise, considering the amount of material available (limited for a reproduction test) and the possibility to test a differentiated source of toxicity, via food as an exposure route (Simonsen and Scott-Fordsmand, 2004). However, the mortality observed in *E. fetida* is a limiting factor. It is assumed that mortality is due to starvation rather than toxicity, i.e. worms may have avoided the food source (sludge treatments). Avoidance of the sludge could be due to several sludge related aspects, general deterrents, nutrients aspects (e.g. C:N, see below), pH etc. This illustrates the difficulties of testing complex media, where many factors may be involved and limits the suitability of feeding experiments with activated sludge. Earthworms, have like other organisms, nutritional requirement of the food. For example, important factors are the C:N ratio and the salt content which should be below 0.5% (Yadav and Garg, 2011). Food with a low C to N ratio (11) was preferred by adult worms whereas a higher C:N ratio (16) was preferred by juvenile and hatchling earthworms (Aira et al., 2006). The C:N content, as well as the electric conductivity as an indicator of the salt content, were determined for activated sludge from the model WWTP and for horse dung which can be used for feeding the stock culture of the earthworms (Hund-Rinke, data not published). With a C:N content of 4.7 and a conductivity of 99.4 $\mu\text{S}/\text{cm}$, the activated sludge considerably differed from the horse dung (C:N 17.0; conductivity 63.6 $\mu\text{S}/\text{cm}$).

4.1.4. Aquatic organisms

4.1.4.1. Fish cell lines. Different fish cell lines were used to assess the toxicity of the pristine Au-NP and the effluents after short- and long-term exposure. Currently there is not an alternative method validated to replace the in vivo assays used to assess the short- and long-term effect of substances in fish. However, very recently a new OECD test guideline has been published, the TG No. 249 "Fish Cell Line Acute Toxicity: The RTgill-W1 cell line assay" (OECD, 2021). This test guideline is designed to predict fish acute toxicity in product testing. The use of different cell lines, different organs and different endpoints of cytotoxicity will provide more information on the toxicity of the tested substances. Here we used three fish cell lines and three endpoints of toxicity to assess the short-term toxicity of the pristine Au-NP and of the effluents. The need to dry a volume of effluent and to re-suspend it in the cell culture medium was identified to maintain the required nutrients and homeostatic conditions for the survival of cells. Survival was also achieved by a 1/4

dilution of the effluents.

To study the effects of a long-term exposure in fish to any substance, OECD guideline 215 on juvenile growth test (OECD, 2000a) should be followed. Here we used two fish cell lines to assess the long-term toxicity of the effluents. We exposed our cells to the dried effluents reconstituted in cell media for 28 days as it is indicated in OECD guideline 215. Different effects were observed depending on the cell line exposed. RTL-W1 showed an IT_{50} of 22 d with the Dis-E and 15 d with the Au-E, whereas RTS-11 was very sensitive ($\text{IT}_{50} < 1\text{d}$) to both effluents. No significant differences in toxicity were observed in RTS-11 cells exposed to both effluents but the Au-E induced a higher toxicity in the RTL-W1 cells. This higher toxic effect after a long-term exposure is difficult to explain taking into account the non-toxic concentration of gold in the Au-E (0.1 mg/L). In fact, as mentioned the acute exposure of these cell lines to this concentration did not induce any toxicity. In addition, in a previous experiment performed in our laboratory (data not published) where the fish cell lines RTgill-W1 were exposed to higher concentrations (50 and 100 mg/L) of the same Au-NP for 28 days, effects were only observed at the end of the exposure period (IT_{50} close to 28 days). The higher toxicity of the Au-E could be the result of the interaction of the Au-NP with the substances of the effluent or of the aging of the NP. This observation supports the use of ecotoxicological tests with increased environmental relevance.

Results from this study indicated that the in vitro assays could be a useful tool in the evaluation of the hazard of complex wastewaters before their release into the environment, at least as a first screening before developing in vivo assays with fish. However, the selection of the cell line and time of exposure is a key point, as shown by the reduced time of viability of RTS-11 compared to RTL-W1.

4.1.4.2. *Daphnia magna*. Our results indicated that OECD guidelines 202 and 211 could be applied to the study of the effects of effluents. We showed that daphnids could be exposed directly to the effluent instead of to the *Daphnia magna* medium for short- and long-term exposures and that the reduction of volume of the test solution didn't impair the performance of the reproduction test.

4.2. Effect of Au-NPs

For the UK and US the mean annual PEC of medical Au-NPs in activated sludge was estimated at 124 and 145 $\mu\text{g}/\text{kg}$, respectively. For the effluents of WWTP the probabilistic modeling resulted in mean concentrations of 440 and 140 pg/L (Mahapatra et al., 2015). Although the medical sector is an important application of Au nanoparticles, due to its unique properties the material is used for further applications such as in electronics, materials, catalysis or decorative fields and the release into the environment can exceed the modeled concentrations. Such PEC values are general values and local site-specific values may be higher. A recent study about the recovery of gold from industrial wastewater with nanoscale zero-valent iron reported that 5000 g of gold was recovered from $\sim 120,000\text{ m}^3$ wastewater in a 12-month period ($\sim 42\text{ }\mu\text{g}/\text{L}$) (Li et al., 2019a).

The mean concentration in the sludge used for the experiments was 4.9 g/kg and exceeded the PEC value for medical applications by a factor of about 10,000. Adding the sludge to soil using an environmental relevant amount such as set out in the German Sludge Ordinance (AbfKlärV §6 Absatz 1, 1992), resulted in concentrations exceeding relevant environmental concentrations. In addition, the concentration of 100 $\mu\text{g Au}/\text{L}$ in the effluent of the WWTPs in the present approach, exceeds the concentration modeled for the medical applications in the UK and US by a factor of 1000. It even exceeds the concentration determined in the industrial wastewater by a factor of 2. For risk assessment, environmental concentrations are compared with hazard data. Based on the testing of a limited number of test organisms or soil functions, also more sensitive, non-tested species in the ecosystem have to be protected.

Therefore, the measured effect concentrations (e.g. ECx: concentration causing x% effect; NOEC: no observed effect concentration) are reduced by assessment factors resulting in PNECs (predicted no effect concentrations). Depending on the test system and the amount of data, assessment factors of up to 1000 have to be used (ECHA, 2008, 2012). This procedure justifies test concentrations above the predicted environmental concentrations. However, it has to be considered that the concentration can affect the agglomeration behavior of particles (Saengpayb, 2017).

While neither the Au dispersion nor the dispersant added as pristine material or via activated sludge affected the activity over time, an effect on the composition of the **microbial community** even at the lower test concentration (2.4 and 5.0 mg/kg, respectively) was observed. The published effects of nano-gold on the soil microbial community are controversial which can be based on differences in the nanomaterials, soil composition, test conditions such as water content of the soil, incubation duration and methods applied for the characterization of the soil community. Our results are in-line with the detection of toxic effects on the microbial community structure based on denaturing gradient gel electrophoreses at a concentration comparable to our study (3.34 mg/kg) (Nogueira et al., 2012). However, no effect on the function of the microbial soil community are reported at a concentration of 3.3 mg/kg (Maliszewska, 2016) and on structure as well as function at 130 mg/kg (Shah and Belozero, 2009). The results were obtained in laboratory experiments, but it is assumed that similar effects will be observed in the field. It was demonstrated for silver nanoparticles added via activated sludge in a study lasting several years, that laboratory experiments are a time reduced way to indicate what will happen in the field (Schlich et al., 2017). Silver nanoparticles sorbed to activated sludge became bioavailable resulting in toxic effects. Dry and wet periods, freezing and thawing did not result in significant leaching. The increasing effects over time of gold applied via sewage sludge are in-line with the results obtained for silver. For Ag-NPs the release of ions followed by their interaction with microbial structures is considered as an important molecular mechanism. In contrast, Au-NPs can result in a collapse of the membrane potential, a decrease of the ATP level and in an inhibition of the tRNA function (Cui et al., 2012). The increase over time can be explained by an increased bioavailability of gold due to the degradation of the sewage sludge. The expected degradation of the sewage sludge is supported by the decreased microbial respiration activity.

The distribution of microbial abundance in a local microbial community is normally composed by relatively few dominant species and a large number of rare species. The evaluation of taxon-specific changes of abundance and occurrence frequency can provide deeper understanding in the observed changes among conditions. With metabarcoding data, OTU-based approaches are commonly used to identify this changes (Schlich et al., 2021), and several analysis (e.g. TITAN 2 from the R package) are available to identify indicator-taxon responding to the studied conditions. In many cases it is possible to observe positive correlations between microbial diversity and ecosystem function, but the link between ecosystem function and microbial diversity remain still unresolved (Morris et al., 2020).

No approach exists to assess changes in microbial community structure in the scope of regulation. For a first screening we compared our result with the PEC value for Au in a soil / sludge mixture. Using the higher PEC value for Au in sludge of 145 µg/kg and an applied sludge amount of 40 t per year (Roig et al., 2012) results in an Au concentration in a soil/sludge mixture of 1.9 µg/kg. If the highest assessment factor of 1000 is used for our effect data, the resulting predicted no effect value (PNEC) of 5 µg/kg is above, but nevertheless in the range of the PEC value. The PEC value covers only medical applications. If the release from further applications is considered, the PEC-value can increase. Nevertheless, for a robust conclusion, additional investigations with more test concentrations have to be performed to calculate a reliable PNEC-value. Additionally, it has to be discussed which assessment factor is justified for microbial community structures. In doing so, it has to be

taken into account that the adaptation of microbial community structures is a natural process. This implies that an assessment factor of 1000 is not justified, and a lower value seems to be more appropriate. No effect on activity but on the community structure can be an indicator for the adaptation and tolerance of a microbial community, but it can also indicate a reduced resilience. The latter means that the capacity of a community is reduced to persist and maintain or recover their original or stable state in the presence of a second stressor. A differentiation between both conditions is possible by exposing the soil experimentally to a secondary stressor in order to see if there is a reduction in functional capabilities (Azarbad et al., 2016). Microbial community evenness supports functionality under selective stress (Wittebolle et al., 2009). The similar diversity indices in our study support the assumption that the microbial community is still sufficiently stable to provide the soil functions such as the nutrient cycling. But the experimental approach with an additional stressor can provide additional evidence on stability. However, such approaches still have to be developed. This applies, for example, to the decision on the kind of the stressor, which can be a chemical substance, but also a natural impact such as flooding or temperature change. If an additional, environmental relevant stressor results in no functional effect, the modification of the microbial community is considered to be tolerable.

Comparable to the microbial activity, the effect of Au on the tested **invertebrates** was low although the test concentrations exceeded expected environmental concentrations by several orders of magnitude. This is in-line with the results of Voua Otomo et al. (2014) who investigated the effect of Au-NPs on survival and reproduction of *E. buchholzi*. No effect was determined at a concentration of 37.5 mg/kg exceeding the concentration tested in the present study.

Although in our study the feeding of the earthworms with activated sludge failed, more work should be done on this test design. Information on the uptake of NPs which can result in trophic transfer is important. The uptake of Au-NPs by frogs was shown to differ depending on whether the materials were administered as pure substance or via earthworms exposed to the Au-NPs over a period of 60 days (Unrine et al., 2012). Based on the observations for frogs it cannot be excluded that also the uptake by earthworms differs whether the organisms are exposed to the pure particles or adsorbed to activated sludge.

Daphnia magna immobilization and reproduction were not affected after exposure to either the effluent with dispersant or the effluent with Au-NP. This fact was expected, at least regarding the concentration of Au-NP (0.1 mg/L) in the effluent, since the study with the pristine material indicated a non-observed effect concentration (NOEC) of 50 mg/L. The pristine Au-NP was toxic at the highest concentration (100 mg/L) when it was dispersed in standard medium with a 50% of immobilization after a 48 h exposure. This toxic effect was also observed after exposure to the pristine Au-NPs dispersed in Disp-E but with a reduced effect, reaching only 25% of immobilization. The size of the NPs in the exposure medium cannot explain this difference as it has been shown in Table S9. Indeed, the size of Au-NPs dispersed in Disp-E and in daphnia medium was similar during the exposure period. The higher toxicity of Au-NPs dispersed in medium compared to the effluent corresponds to the results with Ag-NPs. Ag-NPs produced a significant negative effect in the number of offspring whereas no effect on reproductive success was observed when animals were exposed to wastewater-borne Ag-NPs (Hartmann et al., 2019). The potential transformation processes during the WWTP procedure and the presence of organic compounds such as humic acids in the effluent have been mentioned as the possible explanation for the observed differences (Galhano et al., 2020; Hartmann et al., 2019; Kühn et al., 2018). The same pattern was also observed during an experiment developed with zebrafish embryos, where control WWTP effluent spiked with Ag-NPs was less toxic than the exposure to Ag-NPs in ISO water (Muth-Köhne et al., 2013). The amount of Au present in the effluent used in our study was very low (0.1 mg/L) compared with the toxic concentration of the pristine Au-NP (100 mg/L) and no toxicity was observed due to possible aging processes. The test

concentration of 0.1 mg/L in the effluent did not induce an effect on the reproduction of daphnids and exceeds the concentration modeled for medical Au-NPs which is in the pg-range (Mahapatra et al., 2015) by several orders of magnitude. Therefore, the risk for daphnids based on Au-NPs from medical applications and released into the environment via effluents of WWTPs is considered to be low. Our results obtained in vitro in *fish cell lines* after a long-term exposure indicated that the aging of Au-NPs during the process in the WWTP induce toxicity in the exposed cells. The results should be checked in fish.

5. Conclusion

Nanopharmaceuticals such as Au-NPs can be released via sewage and enter the environment via activated sludge or effluent of WWTPs. The hazard for organisms of this kind of exposure was successfully investigated in various terrestrial and aquatic test systems. Based on the results following conclusions regarding the suitability of the test design and the effect of Au-NPs were drawn:

5.1. Test design

The effluent is a suitable test medium for *D. magna*. For the tests with fish cells (RTS-11; RTL-W1) a previous drying step of the sample and resuspension in the exposure medium is required to provide sufficient nutrients.

The limited amount of activated sludge has to be considered in the test design. If various terrestrial trophic layers and test organisms are to be addressed, test systems requiring small amounts of soils have to be prioritized. Activated sludge mixed with soil can be used as test medium to investigate soil microorganisms, *E. crypticus*, *F. candida*. However, the nutrient requirements of *E. fetida* are not met with activated sludge as a sole food source and additional studies are required.

The combination of structural and functional approaches for the soil microbial community provide additional information. However, approaches to assess changes in the microbial community structure as far as regulation is concerned still have to be developed. The contradictory behavior of *E. crypticus* and *F. candida* in the various sludge treatments, which becomes more evident with longer exposure periods, justifies the extension of the test performance and the necessity of both organisms for testing.

The addition of activated sludge to soil results in a decrease of the pH during incubation periods exceeding 28 days. While the microbial community can easily adapt, for other soil organisms liming is required if the pH falls below the survival limit of the test organisms.

5.2. Effect of Au-NPs

The effect of the investigated Au-NPs was small. Effects were detected in concentrations exceeding the modeled environmental concentrations. However, the results with the RTL-W1 fish cell line indicate that the aging of Au-NPs during the process in the WWTP induces toxicity in the exposed cells and justifies the use of ecotoxicological tests with increased environmental relevance.

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Kerstin Hund-Rinke: Conceptualization, Methodology, Writing – original draft, Funding acquisition. **Cecilia Diaz:** Methodology, Writing – original draft. **Anne Jurack:** Investigation. **Judith Klein:** Investigation. **Burkhard Knopf:** Writing – original draft. **Karsten Schlich:** Writing – original draft. **María Luisa Fernández-Cruz:** Conceptualization, Methodology, Writing – original draft, Funding acquisition. **David Hernández-Moreno:** Methodology, Writing – original draft, Investigation. **Nicolas Manier:** Investigation. **Pascal Pandard:** Investigation, Writing – original draft. **Bruno Guimarães:** Investigation. **Susana I.L. Gomes:** Investigation. **Janeck Scott-Fordsmand:** Conceptualization, Methodology, Writing – original draft, Funding acquisition. **Mónica J.B. Amorim:** Conceptualization, Methodology, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

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