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Bioaccumulation and effects of sediment-associated gold- and graphene oxide nanoparticles on *Tubifex tubifex*

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ABSTRACT

With the development of nanotechnology, gold (Au) and graphene oxide (GO) nanoparticles have been widely used in various fields, resulting in an increased release of these particles into the environment. The released nanoparticles may eventually accumulate in sediment, causing possible ecotoxicological effects to benthic invertebrates. However, the impact of Au-NPs and GO-NPs on the cosmopolitan oligochaete, *Tubifex tubifex*, in sediment exposure is not known. Mortality, behavioral impact (GO-NP and Au-NP) and uptake (only Au-NP) of sediment-associated Au-NPs (4.9 ± 0.14 nm) and GO-NPs (116 ± 0.05 nm) to *T. tubifex* were assessed in a number of 5-day exposure experiments. The results showed that the applied Au-NP concentrations (10 and 60 $\mu\text{g Au/g}$ dry weight sediment) had no adverse effect on *T. tubifex* survival, while Au bioaccumulation increased with exposure concentration. In the case of GO-NPs, no mortality of *T. tubifex* was observed at a concentration range of 20 and 180 $\mu\text{g GO/g}$ dry weight sediment, whereas burrowing activity was significantly reduced at 20 and 180 $\mu\text{g GO/g}$ dry weight sediment. Our results suggest that Au-NPs at 60 $\mu\text{g Au/g}$ or GO-NPs at 20 and 180 $\mu\text{g GO/g}$ were detected by *T. tubifex* as toxicants during short-term exposures.

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Introduction

Engineered nanoparticles (ENPs) are widely applied in diverse fields, such as medicine, cosmetics, renewable energy, food industry, electronic devices and environmental remediation (Dong and Feng, 2007; Fabrega et al., 2011; Kachynski et al., 2008; Lens, 2009; Pavasupree et al., 2006; Tungittiplakorn et al., 2004; Wei et al., 2008). Among various engineered nanomaterials, gold (Au) and graphene oxide (GO) nanoparticles are widely used. Both Au-NPs and GO-NPs are unique materials for nano-medicine applications such as drug delivery (Dykman and Khlebtsov, 2012; Zhang et al., 2010) and thermodynamic therapy (Lyttton-Jean and Mirkin, 2005;

Wang et al., 2011). Au-NPs have been used in materials science, electron microscopes and biological sensors (Dreaden et al., 2012; Lim et al., 2011; Panyala et al., 2009; Zeng et al., 2011), and GO-NPs have been applied in energy storage, electronics and bioenvironmental materials (Park and Ruoff, 2009; Wang et al., 2011; Zhao et al., 2012). The widespread use of Au-NPs and GO-NPs is likely to increase their release into the aquatic environment via wastewater discharges. Once these nanoparticles are released into the aquatic environment, they will likely undergo transformation processes including dissolution, aggregation, agglomeration, and eventually settle into the sediment (Thit et al., 2015; Zhao et al., 2014). Therefore, sediment may become an ultimate

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reservoir for ENPs. As a result, nanoparticles may be ingested by deposit-feeding benthic invertebrates and potentially be bio-magnified within the food chain (Ferry et al., 2009; Judy et al., 2011), which may pose a high risk to invertebrates and higher trophic level organisms.

Toxicity and bioaccumulation of sediment-associated Ag-NPs and CuO-NPs to sediment-dwelling invertebrates have been investigated (Cong et al., 2011, 2014; Dai et al., 2013; Pang et al., 2012, 2013; Ramskov et al., 2014; Thit et al., 2015). CuO-NPs with concentrations ranging from 30 to 240 μg CuO/g dry weight sediment negatively affected the specific growth rate, feeding rate, bioaccumulation and reproduction of the freshwater snail *Potamopyrgus antipodarum*, whereas the survival of *P. antipodarum* was not affected (Pang et al., 2012, 2013; Ramskov et al., 2014). It was reported that Ag-NPs of 1 to 50 μg Ag/g dw sediment caused DNA damage and genotoxicity in the marine polychaete *Nereis diversicolor* (Cong et al., 2014). Furthermore, the burrowing behavior of *N. diversicolor* was impaired by Ag-NPs at a concentration of 150 μg Ag/g dw sediment without affecting survival (Thit et al., 2015). Dai et al. (2013) investigated the toxic effects of sediment-associated Ag-NPs and CuO-NPs on the mussel *Macoma balthica* and found no negative effects on genotoxicity, mortality, condition index, or burrowing behavior at concentrations from 150 to 200 μg Ag or CuO/g dw sediment (Dai et al., 2013).

Studies investigating the toxicity of Au-NPs to organisms have mainly focused on water and soil exposure. Soil exposure showed that Au-NP concentrations up to 37.5 μg Au/g dw did not impact the survival and reproduction of the grindal worm *Enchytraeus buchholzi* (Voua Otomo et al., 2014). In contrast, Unrine et al. (2010) showed that Au-NPs were accumulated in earthworms (*Eisenia fetida*) exposed to 5–50 μg Au/g dw soil and caused adverse effects on reproduction (Unrine et al., 2010). In water exposures, 100 μg Au-NP/L induced metallothionein production as a response to metal contamination, and increased activities of catalase, superoxide dismutase and glutathione S-transferase for the bivalve *Scrobicularia plana*. In addition, the burrowing behavior of *S. plana* was impaired when transferred from Au contaminated seawater to clean sediment (Pan et al., 2012). GO-NPs have been found to cause negative effects in aquatic invertebrates during development (Mesarić et al., 2013), and induce significant adverse effects on vertebrates, protozoa and microbial communities (Ahmed and Rodrigues, 2013; Chen et al., 2012; Hu et al., 2015). However, few studies have been conducted with sediment.

The sediment-dwelling Oligochaete, *Tubifex tubifex* was selected as model organism, because they are widely distributed in the freshwater eco-system, and have a feeding behavior that includes ingesting large amounts of fine particles (<60 μm) and extract organic matter associated with ingested sediment. Due to their bioturbation activities (i.e., irrigation and particle mixing), high tolerance for polluted ecosystems, intermediate position in the trophic network and the ease of breeding in the laboratory, they are widely used as a standard model organism in ecotoxicological studies (Bouche et al., 2000; Lagauzere et al., 2009; Mosleh et al., 2007). However, due to difficulties in tracing NPs in the sediment compartment, the ecotoxicity of NPs on benthic invertebrates in sediment exposure media is scarce and studies with Au-NPs and GO-NPs have not been reported

to our knowledge. In the present study, Au-NPs and GO-NPs-induced mortality, avoidance, burrowing behavior and bioaccumulation in *T. tubifex* are investigated via sediment exposure.

1. Materials and methods

1.1. Animal collection and culturing

T. tubifex were reared in aquaria added sediment and freshwater with frequent additions of extra food (mortared Tetramin® Tetra, Germany) in the laboratory at Roskilde University. The body length of *T. tubifex* ranged from 4 to 5 cm. One day before experimental setup, all worms were carefully picked out of the culture and placed in artificial *T. tubifex* media (see below) to empty their guts overnight. During exposure periods, worms were kept in natural sieved sediment without additional food supply.

1.2. Synthesis and characterization of nanoparticles

1.2.1. Synthesis of graphene oxide nanoparticles

Graphene oxide nanoparticles (GO-NPs) were synthesized according to the modified Hummers method (Hummers and Offeman, 1958). Graphite flakes (1 g, 99.8%, Alfa Aesar, China) and NaCl (35 g, Sinopharm Chemical Reagent Ltd., China) were ground into powder with a mortar and pestle. The powder was dissolved in deionized water (18 MPa, Milli-Q water), the solution filtered with filter paper (50 μm) and dried in an oven at 60°C for 24 hr. Subsequently, the dry graphite was dissolved in H_2SO_4 (23 mL, Sinopharm Chemical Reagent Ltd., China), and KMnO_4 (3 g, Sinopharm Chemical Reagent Ltd., China) was slowly added. The mixture was stirred for 30 min at 37°C, followed by 45 min stirring at 70°C. Afterwards, 5 mL deionized water was added, and the solution was heated and stirred for 10 min at 70°C followed by an addition of 40 mL water and heating for 15 min at 100°C. Finally, deionized water (140 mL) was added, followed by H_2O_2 (10 mL, 30%, Sinopharm Chemical Reagent Ltd., China) in order to obtain the brownish graphite oxide. The graphite oxide was purified by centrifugation at 8000 r/min for 5 min, followed by washing with 5% HCl and deionized water 6 times, successively. In order to enhance electrostatic repulsion, NaOH (1.8 g) and deionized water (10 mL) were added to the above solution, then left in an oil bath and stirred for 4 hr. Afterwards, pH was adjusted to <1 by addition of HCl (5 mL, 36%, Sinopharm Chemical Reagent Ltd., China). The solution was centrifuged at 8000 r/min for 5 min five times with DI water. Afterwards, the solution was sonicated for 45 min on ice to obtain GO. Finally, the GO solution was concentrated by centrifugation at 13,000 r/min for 5 min to give a final concentration of 2.09 mg/mL.

1.2.2. Synthesis of gold nanoparticles

Gold nanoparticles (Au-NPs) were synthesized using citrate reduction of HAuCl_4 as described by (Brust et al., 1994, 1995). Briefly, 3 mL of 10 mmol/L HAuCl_4 (AR, Sinopharm Chemical Reagent Ltd., China), 2 mL of 38.8 mmol/L citrate (AR, Sinopharm Chemical Reagent Ltd., China) and 1 mL of

0.075 wt.% NaBH₄ (AR, Sinopharm Chemical Reagent Ltd., China) were successively added to 80 mL deionized water at 1 min intervals with constant stirring. The mixture was stirred for 15 min at room temperature to obtain 5 nm Au-NPs. The theoretical Au-NP concentration was 55 µg/mL.

1.2.3. Characterization of Au-NPs and GO-NPs

The primary particle size of Au-NPs in MilliQ water was assessed by Transmission Electron Microscopy (TEM) (JEM-1011, Japan) operating at 80 kV. The particle size of GO-NPs was examined using Atomic Force Microscopy (AFM) (NanoScope IIIA Veeco, USA). Hydrodynamic diameters (in suspension) and Zeta potential of Au-NP and GO-NP suspensions prepared in deionized water were measured by Zetasizer Nano (ZS90Malven, UK).

1.3. Sediment and *T. tubifex* media preparation

Sediment was collected at Munkholmbroen in Holbæk, Denmark, sieved to <125 µm using deionized water and left to settle for two days. The overlying water was carefully removed through a plastic tube and the sediment was frozen at -20°C in order to kill micro- and macro-organisms. Afterwards, the sediment was thawed, washed with *T. tubifex* media once, left to settle and then overlaying water was removed. The ratio of dry weight to wet weight and organic carbon content in the sediment were measured by first placing the wet sediment in the oven for 24 hr at 105°C, and then heating the dry sediment for 4 hr at 550°C. *T. tubifex* media was prepared with 80 mmol/L CaCl₂·2H₂O, 20 mmol/L MgSO₄·7H₂O, 31 mmol/L NaHCO₃ and 3 mmol/L KCl according to OECD 203, ISO 6341-1982, and then aerated for 48 hr prior to use.

1.4. Sediment spiking and experimental setup

Sediment was spiked by adding a known amount of Au-NP stock suspension, GO-NP stock suspension or MilliQ water (controls) to 6 separate glass beakers containing wet sediment, to final nominal concentrations of 10 or 60 µg Au/g dw for Au-NPs, and 20 or 180 µg GO/g dw for GO-NPs, respectively. There is to the best of our knowledge no published information on environmental sediment concentrations of either Au-NP or GO-NP. The concentrations selected is based on published studies on Au-NP (soil: (Unrine et al., 2010; Voua otomo et al., 2014)) and C₆₀-NP (sediment: (Van der ploeg et al., 2011)) exposures. Sediments were mixed and covered with Parafilm, then left on a shaking table for 24 hr in order to obtain a homogeneous Au-NP and GO-NP distribution, respectively. Hereafter, spiked sediment was transferred to experimental glass beakers (3 replicates for each treatment) and *T. tubifex* media were gently added. The system was left to settle overnight. Hereafter, *T. tubifex* media were removed and 40 mL of fresh *T. tubifex* media were added before introducing *T. tubifex* to the beakers. Worms (20 or 5) were carefully transferred to each beaker to study the toxicities of sediment-associated Au-NPs and GO-NPs on *T. tubifex*, respectively. After 5 days sediment exposure, the worms were transferred to clean *T. tubifex* media and left for 6 hr to empty their gut. The experiment was carried out at (17 ± 2)°C in a controlled climate

room for 5 days. Air was supplied to overlying water from pumps through plastic tubes and pipette tips.

1.5. Sample analysis

Au-NP concentrations in the start-sediment were measured by flame atomic absorption spectrometry (FAAS, SpectrAA-220 VARIAN Mulgrave, Australia). Au-NP concentrations in worm tissue were determined by graphite AAS (GTA 120 VARIAN Mulgrave, Australia). Samples were lyophilized at -50°C overnight, weighted and digested in a microwave oven. Samples were heated in a mixture of HNO₃ (2.25 mL, 35%) and HCl (0.75 mL, 35%) in the microwave oven at 250, 400, 650 and 250 W for 6 min at each step. Afterwards, samples were transferred into a water bath at room temperature and cooled for 30 min. Finally, samples were passed through pre-washed filters (Volume: 35%, HNO₃:MilliQ water = 1:1 (V:V)) into 25 mL volumetric flasks. A series of standard Au solution (0, 5, 10, 20, 40, 60, 80 and 100 µg/L) were used for calibration of Au concentrations.

1.6. Mortality and avoidance response

The ability of *T. tubifex* to avoid sediment spiked with either Au-NPs or GO-NPs was tested by recording the number of worms on the surface of the sediment at different time slots (1 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr and 120 hr) during 5-day exposure. At the end of exposure (day 5), the dead worms were counted. Mortality was calculated using the ratio as the number of dead worms on day 5 divided by the number of worms initially added.

1.7. Burrowing behavior

After 5 days of exposure to GO-NP spiked sediment, worms were transferred to beakers containing 2 cm uncontaminated natural sediment and 40 mL *T. tubifex* media. Burrowing behavior was recorded at 30 min, 1 hr, 2 hr, 12 hr and 24 hr.

1.8. Statistical analysis

Data is presented as mean ± standard deviation (SD) of three replicates except for the data of Au bioaccumulation due to the insufficient biomass of worms. One- and two-way analysis of variance (ANOVA) was employed to detect significant differences among samples. Prior to ANOVA, Levene's Test was used to check homogeneity of variances, and normality of distributions was tested with Kolmogorov-Smirnov. Data were analyzed using SPSS version 19. Significant difference was accepted at a *p* value <0.05. Mortality data were arcsin transformed prior to statistical analyses.

2. Results

2.1. Characterization of Au-NPs and GO-NPs

TEM images of Au-NPs showed an average primary particle size of (4.9 ± 0.14) nm (86% were between 4 and 6 nm) using Nano measure 1.2 software. The particles were spherical and

relatively monodisperse and particle size was normally distributed (Fig. 1a, b). AFM images revealed that the particle size of GO-NPs fragments ranged from 1 to 350 nm with the majority being around 150 nm (Fig. 1c, d). Thus, the thickness of GO-NPs were more than the 0.8 nm which is the typical thickness of single-layer GO sheets (Schniepp et al., 2006), indicating that the synthesized GO-NPs were multi-layered. The hydrodynamic diameter of 50 $\mu\text{g/mL}$ Au-NPs and 200 $\mu\text{g/mL}$ GO-NPs suspensions was (63 ± 0.34) and (121 ± 3) nm, respectively. The zeta potentials for Au-NPs and GO-NPs in MilliQ water were (-34.4 ± 1.2) and (-60.2 ± 1.2) mV, respectively, indicating that both suspensions were stable.

2.2. Sediment properties

The ratio of dry weight to wet weight of sediment was 0.38, the organic matter content was 0.9% and the background Au concentration was lower than the detection limit (<5 $\mu\text{g Au/g dw}$ sediment). The concentration of Au in Au-NPs spiked sediment ($n = 4$) was (8.58 ± 1.55) $\mu\text{g Au/g}$ and (70.27 ± 6.70) $\mu\text{g Au/g dw}$, respectively, which was close to the

nominal concentrations (i.e., 10 and 60 $\mu\text{g Au/g dw}$). For GO-NPs, nominal concentrations of 20 $\mu\text{g/g dw}$ or 180 $\mu\text{g/g dw}$ were chosen, and 0.47 or 4.26 mL of the concentration GO-solution was added to the sediment, respectively. The final concentration of GO-NPs in sediment was not determined due to insufficient analysis methods.

2.3. Effects of Au-NPs to *T. tubifex*

2.3.1. Mortality and avoidance behavior

The mortality of *T. tubifex* was 5% in the control, 3.33% in 10 $\mu\text{g/g}$ and 11.67% in 60 $\mu\text{g/g dw}$. No significant difference in mortality between treatments was observed ($p = 0.124$). During 5 day exposure, *T. tubifex* exhibited avoidance behavior to sediment treated with the three concentrations of Au-NPs (i.e., 0, 10 or 60 $\mu\text{g/g dw}$) (Fig. 2). Au concentration and exposure duration did not interact to affect the avoiding behavior ($p > 0.05$). However, there was a tendency that high exposure concentration resulted in a stronger avoiding behavior, especially in the beginning of the exposure period (Fig. 2).

Fig. 1 – Transmission electron microscopy (TEM), atomic force microscopy (AFM) and size distribution of Au-NPs and GO-NPs in MilliQ water: (a) TEM images of 5 nm Au-NPs, (b) size distribution of Au-NPs, (c) AFM images of GO-NPs, (d) size distribution of GO-NPs. Au: gold; NPs: nanoparticles; GO: graphene oxide.

Fig. 2 – Avoidance response of *T. tubifex* during 5-day sediment exposure with Au-NPs ($n = 3$). The bars represent standard deviation (SD).

2.3.2. Bioaccumulation of Au-NPs in *T. tubifex*

Au accumulated in *T. tubifex* tissue during the 5-day sediment exposure. In the control group, the Au body burden was lower than the detection limit. The body burdens of Au in *T. tubifex* after 5 days was 12.49 and 65.84 µg/g dw when exposed to 10 and 60 µg/g dw sediment, respectively. However, to reach above the detection limit in worm tissue, worm biomass was pooled among three replicates leaving one data point per exposure concentration (0, 10, 60 µg AuNP/g dw sediment, $n = 1$ containing up to 20 samples). Thus, no statistical test could be performed, however, the data does show a clear tendency of a concentration-dependent accumulation of Au in *T. tubifex* worms.

2.4. Effect of GO-NPs on *T. tubifex*

2.4.1. Mortality

No worm mortality was observed after 5-day sediment exposure to 20 and 180 µg GO/g dw sediment.

2.4.2. Avoidance and burrowing behavior

Avoidance. There was no interaction between Au concentration and exposure duration on avoidance behavior of *T. tubifex* during 5 days of exposure ($p > 0.05$). During the first hour of exposure to GO-NP spiked sediment, only a few *T. tubifex* were visible at the sediment surface in the control treatment, while 20% and 24% of *T. tubifex* were observed on the surface of the 20 µg GO/g dw sediment and 180 µg GO/g dw sediment, respectively (Fig. 3). Yet, no significant avoidance was detected among treatments ($p > 0.05$).

Burrowing behavior. After *T. tubifex* were transferred into clean sediment, time for all organisms to fully burry into the clean sediment was significantly dependent on the pre-exposure concentration, such that time increased with increasing sediment concentration of GO-NPs ($p = 0.005$).

T. tubifex took 1 hr to completely burrow into the sediment in the control treatment, while 8 and 24 hr was needed to completely burrow into the sediment for worms pre-exposed to 20 and 180 µg GO/g dw, respectively (Fig. 4).

3. Discussion

3.1. Au-NP effects and Au bioaccumulation in *T. tubifex*

Generally, sediment-associated Au-NP showed low mortality to *T. tubifex*. Au-NPs with a concentration of 10 and 60 µg Au/g dw had no significant effects on the avoidance behavior of *T. tubifex* during the short-term exposure. However, there was a tendency for higher avoidance for worms exposed to higher concentration of Au-NP and that the lack of significance may be related to a high variation in data. Avoidance responses induced by metal nanoparticles have not been examined greatly in the aquatic environment. Ramskov et al. (2014) reported an avoidance response of the freshwater snail, *P. antipodarum*, exposed to 100 µg Ag-NPs/g dw sediment for 14 days. An avoidance behavior of the polychaete *N. diversicolor* was also observed during exposure to 100 µg Ag-NP/g dw and 150 µg CuO-NP/g dw for 10 days, respectively (Cong et al., 2014; Thit et al., 2015), indicating that benthic invertebrates are able to detect and avoid nanoparticles in sediment exposure, this is in accordance with the present studies on the avoidance behavior of *T. tubifex* to nanoparticles. Furthermore, studies exist examining soil exposures of Oligochaetes, such as the earthworms *Eisenia fetida* and *Enchytraeus albidus*. *E. fetida* consistently avoided soil spiked with Ag-NPs in concentrations of 6.97–54 µg Ag/g dw, Al_2O_3 -NPs with concentrations of 5000–10,000 µg Al_2O_3 /g dw and TiO_2 -NPs with concentrations of 1000–5000 µg TiO_2 /g dw over 48 hr, respectively (Coleman et al., 2010; McShane et al., 2012; Shoults-Wilson et al., 2011). Likewise, *E. albidus* significantly avoided Cu-NPs with a concentration of 43–241 µg Cu/g dw during 48 hr of soil exposure, and the EC_{50} -avoidance was 241 µg Cu/g dw (Amorim and Scott-Fordsmand, 2012).

Metal nanoparticle bioaccumulation in benthic invertebrates in sediment exposure has been studied in a number of benthic invertebrates including the clam *M. balthica* (Dai et al., 2013), the freshwater snail *P. antipodarum* (Pang et al., 2012, 2013; Ramskov et al., 2014), the estuarine worm *H. diversicolor*/*N. diversicolor* (Buffet et al., 2011, 2014; Cong et al., 2014). In sediment exposure, *P. antipodarum* accumulated 40–155 µg Cu/g dw after a long term exposure (8 weeks) to 30–240 µg CuO-NPs/g dry weight sediment, and the Cu body burden increased with increasing exposure concentration (Pang et al., 2012). For Ag-NPs, the clam *M. balthica* were able to accumulate 200–250 µg Ag/g dw after 35 days of exposure to 200 µg/g dw sediment spiked with Ag-NPs (20–80 nm) (Dai et al., 2013). Likewise, in a short-term sediment exposure, *N. diversicolor* accumulated approximately 2–9 µg Ag/g dw tissue after exposure to Ag-NPs (5–100 µg Ag-NPs/g dw sediment), and accumulated Ag increased with exposure concentrations (Cong et al., 2014). These results suggested that benthic invertebrates could accumulate nanoparticles like CuO-NP and Ag-NP. There exist to our knowledge no reported Au bioaccumulation data following sediment-exposure to Au

Fig. 3 – Avoidance response of *T. tubifex* during 5-day sediment exposure with GO ($n = 3$). The bars represent standard deviation (SD).

NPs, but results are available for Tellinid clams and earthworms following water and soil exposures, respectively. The clam *S. plana* accumulated Au in their soft tissues and the mean concentrations reaching 10.5, 12.0 and 17.7 µg Au/g, respectively for *S. plana* exposed to 100 µg/L of Au-NPs with the size of 5, 15 and 40 nm for 16 days (Pan et al., 2012). The earthworm *E. fetida*, accumulated 0.3–1.5 µg Au/g fresh tissue after 28 days of exposure to 10 µg Au/g dw of Au-NPs, and Au bioaccumulation followed in a dose-dependent manner (Unrine et al., 2010). In the present study, there was a tendency that bioaccumulation of Au-NPs in *T. tubifex* increased with increasing exposure concentration, and bioaccumulated Au in *T. tubifex* was up to 65.84 µg Au/g following exposure to 60 µg Au/g dw sediment of Au-NPs in

Fig. 4 – Time for all *T. tubifex* in each beaker to completely burrow in clean natural sediment after 5-day exposure to GO-NPs. The bars represent standard deviation (SD) ($n = 3$). Different letters indicate statistical differences at $p < 0.05$ level.

a short term period (5 days). Thus, there exist data indicating that benthic invertebrates are able to accumulate CuO-NP, Ag-NP as well as Au-NP on sediment exposure.

3.2. Impaired burrowing behavior of *T. tubifex* following exposure to sediment-associated GO-NPs

The GO-NPs with a concentration of 20 and 180 µg GO/g dw in the sediment did not affect the survival of *T. tubifex* following 5-day exposure in this study. This is in accordance with earlier published results showing low or no mortality in organisms exposed to other carbon nanomaterials (Liu et al., 2014; Pakarinen et al., 2011; Petersen et al., 2008). For example, the mortality of the Oligochaete *Lumbriculus variegatus* was not affected by exposure (28 days) to 50 µg fullerenes/g dw sediment, 0.03 µg SWCNT (single-wall carbon nanotubes)/g dw sediment (SWCNT) or 0.03 µg MWCNT (multi-wall carbon nanotubes)/g dw sediment (Pakarinen et al., 2011; Petersen et al., 2008). GO-NPs has also showed low mortality in other test systems like water exposure, where Liu et al. (2014) reported that 1–100 mg GO/L had no adverse effects on the survival of zebrafish embryos after 96 hr exposure (Liu et al., 2014).

The burrowing behavior of *T. tubifex* in clean sediment was significantly affected following exposure to 20 and 180 µg GO/g dw in the present study. Similar observations were reported for *N. diversicolor*, and *S. plana* exposed to different nanoparticles such as CuO-NP, Ag-NP, CdS-NP and ZnO-NP (Cong et al., 2014; Boldina-Cosqueric et al., 2010; Buffet et al., 2012, 2013a, 2013b, 2014; Thit et al., 2015). Boldina-Cosqueric et al. (2010) discussed the origins of impairments of burrowing behavior in *S. plana*. They found that the reduced burrowing speed of clams from a clean site exposed to contaminated sediment may be interpreted as an avoidance response (Boldina-Cosqueric et al., 2010). In addition, the burrowing behavior was significantly decreased for worms (*H. diversicolor*) exposed to 10 µg Cu/L of CuO NP for 14 days (Buffet et al., 2013a), suggesting that burrowing behavior of benthic invertebrates may be a more sensitive endpoint in behavior tests, and likely of particularly importance when considering NP effects. Since *T. tubifex* plays an important role in biogeochemical processes through its burrowing and irrigation activity, the impairment of burrowing behavior may lead to ecologically detrimental effects, such as an increase in the susceptibility of sediment-dwelling species to predation. This could lead to an increased predation of contaminated worms by fish, possibly biomagnifying NPs up the food chain, thereby affecting the entire ecosystem. However, the concentration range from 20 to 180 µg GO-NPs/g dw used in this study is not expected in the environment, making considerations like this predominantly theoretical.

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