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Multigenerational exposure of *Folsomia candida* to silver: Effect of different contamination scenarios (continuous versus pulsed and recovery)

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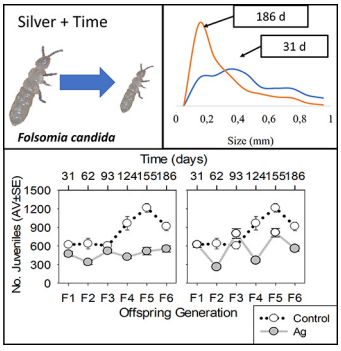
HIGHLIGHTS

- Multigenerational (MG) effects of silver in *Folsomia candida* were assessed.
- Nine different MG regimes were tested including pulses (sludge application mimic).
- Reproduction and size were sensitive endpoints over MG.
- MG exposure caused delay in time for egg laying.
- Effects could be transgenerational due to transference of Ag by maternal generation.

GRAPHICAL ABSTRACT



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ABSTRACT

Effects of pollutants are mostly assessed using standard testing procedures, which cover a fraction of the animals' life cycle. Although, in nature species are exposed during multiple generations of sub-lethal doses of persistent chemicals. In the present study, we focused on the multigenerational (MG) effects of silver in *Folsomia candida* during 6 generations using the EC50 for reproduction as exposure concentration. We tested 9 different exposure scenarios, going from continuous 6 generations Ag exposure over pulse exposure (i.e. one generation clean, next contaminated, next clean etc.) to gradually increasing the number of exposure generations, with a final transfer to clean media. The biological endpoints assessed included survival, reproduction and size, with reproduction being the most sensitive. The biological response depended on the specific MG scenario, e.g. the 6 Ag MG caused a decreased number of juveniles from F4, whereas the pulse exposure experienced an increase in reproductive output when in clean soil. It is uncertain whether Ag causes transgenerational effects, but the reproduction levels in both pulse exposures are lower than in continuous control over the 6 generations which could be due to transference of Ag by the maternal generation. Overall, population size distribution seemed to indicate a delay in time for egg laying, with close relationship between adult survival, organisms size and reproduction output. Size monitoring allowed significant added interpretation possibilities and we strongly recommend the addition of this endpoint to the standard guideline. The smaller observed size range can have implications in terms of adaptation potential, carrying associated increased risk.

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1. Introduction

Hazard of pollutants in the environment is assessed via standard toxicity guidelines (e.g. from OECD (Organization for Economic Co-operation and Development) or ISO (International Standard Organization), using a battery of key organisms. The common standard procedures target only a fraction of the biota life cycle due to a general choice in restraining the testing time, e.g. using a fraction of reproductive period and assessing survival and reproduction (e.g. 28 days for *Folsomia candida* (OECD, 2009). It is known that in reality species can be exposed not only over the full life (which current tests do not cover), but also over many generations, both may cause increased impact on population. This is particularly relevant for the terrestrial environment where soil functions as a sink for many pollutants. Persistent toxics may remain attached to soil particles for years, even when some transformations occur (Petruzzelli et al., 2010). Adaptation may occur when organisms are exposed for long to sub-lethal concentrations of contaminants, facilitated by the organisms' phenotypic to genetic plasticity. Such adaptive changes can involve epigenetic mechanisms, i.e., changes in the gene function but not in the DNA sequence (Schlichting and Wund, 2014). These changes can be transferred from one to the next generations (transgenerational), even when the stressor disappears (Klosin and Lehner, 2016). This means that multigenerational exposure can result in increased resistance or sensitivity to that particular stressor. Obviously, this is not covered by the limited duration of the current standard guidelines, and should receive increased focus.

Few studies focus on the effects of multigenerational (MG) exposure of key organisms, especially when it comes to soil organisms and soil organism exposed to nanomaterials. One example for soil species is the study by Amorim et al. (2017) who studied the MG effect of Cadmium (Cd) exposure over 3-years using the collembolan *Folsomia candida*. Amorim et al. (2017) showed that for the long-term effects the exposure to lower concentration could cause a higher biological impact than exposure to higher concentrations. The authors suggest this could be due to lower activation of defense mechanisms and a variation in the reproduction size strategy. Another study with the same species (Paumen et al., 2008) showed that effects of MG exposure to phenantrene EC50 (10 generations) caused population extinction at F4. Campiche et al. (2007) showed that F0 exposure to insect growth regulators (methoprene, fenoxycarb, teflubenzuron) caused effects in the 2 subsequent generations after the organisms were transferred to clean media. A study with imidacloprid and thiacloprid (van Gestel et al., 2017) showed differences in terms of MG effects with the former causing continuous MG effect and the latter causing effects only in F1. Other soil invertebrate MG studies covered oligochaetes, e.g. *Enchytraeus crypticus and E. albidus.* Studies with *Enchytraeus albidus* (Lock and Janssen, 2002) showed that exposure for two generations to Zn, Cd, Cu and Pb did not increase the sensitivity. For *E. crypticus* exposure to Cu caused an increase in sensitivity in the second generation (Menezes-Oliveira et al., 2013). A recent study (Bicho et al., 2017) followed *E. crypticus* exposed to $CuCl_2$ and CuONM during 4 generations (plus 2 in clean media). Bicho et al. (2017) observed two different MG responses with one Cu form causing an increased sensitivity the other decreased, this depending also on the test concentration. For this species, epigenetic mechanisms may be involved as methylation analysis showed that 1.4% of the 5-methyl cytosine was methylated (Noordhoek et al., 2017).

These previous studies mostly assumed continuous exposure to the toxic agent, but pollution can occur in pulses or discharges of contaminants, requiring a different design depending on the question and for hazard assessment. For instance Ag occurs naturally in the environment in low concentrations, mainly in the ionic form of the salt such as silver nitrate ($AgNO_3$) (Purcell and Peters, 1998; Ratte, 1999; Wijnhoven et al., 2009).

Silver is known for its high toxicity in soils (Mendes et al., 2015; Ribeiro et al., 2015) with this being relatively lower after an aging period (Diez-Ortiz et al., 2015). Because the release of Ag containing products in the environment can be delivered to the soil via e.g. sewage sludge treatment (Schlich et al., 2013), a realistic scenario will reflect the input to the system in the form of pulses. In terms of sludge amendment application limits are regulated, e.g. once a year in certain countries or dependent on nitrogen/phosphorous content, but there are other sources like the ones resulting from mining (Candeias et al., 2015) or industrial and urban wastewater discharges (Ho et al., 2012; Johnson et al., 2014). Hence, in the present we studied the MG exposure of Ag to the soil organism *Folsomia candida*, when delivered in a range of 9 different scenarios over 6 exposure generations. The endpoints included survival, reproduction and size (extra to the standard) for all generations.

2. Material and methods

2.1. Test organism

Folsomia candida (Collembola) was used. Cultures were maintained in laboratory on a moist substrate of Paris plaster and activated charcoal (8:1 ratio) at 19 ± 1 °C, under a photoperiod regime of 16:8 (light:dark). The organisms were fed once a week with dried baker's yeast (*Saccharomyces cerevisae*). Test organisms were of synchronized age (10–12 days).

2.2. Test chemical, test soil and spiking

Silver nitrate ($AgNO_3$) (99.8% purity, Merck KGaA) was used.

LUFA soil 2.2 natural soil (Speyer) was used. The soil properties can be summarised as follows: pH = 5.5; organic matter = 1.77%; nitrogen content = 0.17%; cation exchange capacity = 10.1 meg/100 g; and texture as 7.2% clay; 8% silt and 84.8% sand.

Test concentrations were 0 and 145 mg Ag/kg soil dry weight (DW), selected as ca. reproduction EC50 based on a previous study (Mendes et al., 2015), with this being a compromise between a sub-lethal concentration and ensuring a viable number of juveniles to continue the test in the next generation. An aqueous solution of $AgNO_3$ was prepared for spiking. Soil equilibrated for 3 days after spiking, this being repeated at each generation transfer (3 days in advance the soil was spiked). Moisture was adjusted to 50% of the maximum water holding capacity (maxWHC). Hence, Ag exposure and speciation within each generation was similar.

2.3. Test procedures

The standard guideline for reproduction test with *Folsomia candida* (ISO, 2014) was used with adaptations. In short, 10 juveniles (10–12 days old) were introduced in the test vessels (Ø 5.5 cm, 250 mL volume) with 30 g wet weight (WW) of soil. Replication consisted of 10 replicates for control and 15 for the Ag spiked soil. Test ran at 20 ± 1 °C, in a 16:8 photoperiod. Food (8 mg) and water were replenished weekly. Test duration was extended to 31 days (instead of 28) to allow juveniles to be 10–12 days, matching the start of the first generation exposure. At test end, the test vessels were flooded with water and gently stirred with a spatula, for organisms to float. The usual procedure for counting was used with a digital picture for analysis using ImageJ Viewer v1.43 image software (Rasband, 1997). To note that besides counting, the size was recorded including body area, length, width and slimness. The juveniles were collected with a spoon, transferred to plaster culture boxes and then selected of similar and larger size (first laid egg clutch). This pool of animals was kept for ca. 2 h in plaster from which they were transferred to new test vessels for the next generation (10 organisms per replicate again). Exposure was done for 5 generations covering different scenarios as shown in Table 1, with all treatments starting at the same time. Although, pulse and continuous Ag exposure may in nature cause different Ag-