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The chronic effects of fullereneC_{60}-associated sediments in the midge *Chironomus riparius* – Responses in the first and the second generation☆

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ABSTRACT

The life cycle parameters of the benthic invertebrate *Chironomus riparius* make it a relevant organism for use in multi-generation chronic ecotoxicology tests. Since studies on chronic exposures with fullerene carbon nanoparticles have revealed adverse effects at lower concentration ranges, it is crucial to gain understanding of the consequences in following generations. The aims of this study were to investigate whether sediment-associated fullereneC_{60} impacts on *C. riparius* emergence and breeding, thus affecting the growth of the second generation. Larvae were exposed to fullerene-spiked sediment at concentrations of 0.5, 10 and 40 mg/kg sediment dw. Total emergence and breeding success were monitored after the first generation and the newly hatched larvae from the first generation exposure were transferred either to continuous exposure or to pristine sediment without fullerene. Findings indicate that the presence of fullerenes has major impacts on the first generation, mainly shown as delayed emergence time of females. Increased larval growth was observed in the second generation, and we conclude that the *C. riparius* response to fullerene exposure indicated significant signs of recovery in second-generation larval growth. The result shows the effects to be important for population dynamics, revealing delayed female emergence time, which leads to situation where adults' breeding is impaired.

1. Introduction

The possible effects of nanoparticles (NPs) on aquatic organisms are not fully understood, partly due to the lack of investigations about chronic effects. A non-biting midge, *Chironomus riparius,* is a potential organism for studying long-term effects through multi-generation studies, due to its metamorphosis after the larval phase leading to the emergence of the adult midges (Armittage et al., 1994). *C. riparius* is an environmentally tolerant species that can resist various changes in environmental conditions, such as pH and oxygen concentration. This tolerance has been studied, and it has been revealed that it stems either from genetic adaptation or from phenotypic plasticity (Havas and Hutchinson, 1982; Redecker and Zebe, 1988). Because of the unusual and unexpected properties of NPs, finding the most suitable methods and organisms for toxicity testing are currently a subject of debate (Kuehnel and Nickel, 2014; Petersen et al., 2015).

C. riparius larval development occurs, under controlled room temperature, within approximately 15 days following the pupal stage. It undergoes complete metamorphosis and the emergence times of the adult midges are strongly dependent on environmental conditions, such as temperature, food availability and stressors or pollution (Armittage et al., 1994). This ability to tolerate long-term environmental stress has been investigated in many studies, under pollution from heavy metals (Winner et al., 1980; Postma et al., 1995a, 1995b; Groenendijk et al., 1999) or from organic chemicals (Gower and Buckland, 1978; Friberg et al., 2011). Multi-generation studies with *C. riparius* or closely related species exposed to

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different toxicants have suggested that this tolerance is due to decreased sensitivity to a certain toxicant in later generations, which is explained as a genetic adaptation (Postma and Davids, 1995; Miller and Hendricks, 1996; Vogt et al., 2007). However, some studies have documented that this originates rather from phenotypic plasticity (Vedamanikam and Shazilli, 2008; Marinkovic et al., 2012), as observations of loss of decreased sensitivity after few generations have been reported.

The toxicity of carbon nanomaterials (CNMs) has been studied with *Daphnia magna* under chronic exposures (Petersen et al., 2009; Tervonen et al., 2010; Pakarinen et al., 2013), but research along multi-generation studies where effects to breeding are investigated, are limited (Arndt et al., 2013, 2014). In one multi-generation study by Arndt et al. (2014), signs of adverse effects were observed using *D. magna* as a test organism. When parental generation was exposed to carbon-based nanomaterials, such as fullerenes and carbon nanotubes, in certain cases a significant decrease in survival or reproduction was observed in the following generations (F_1 and F_2). These findings were strongly related to NPs' surface chemistry. Notably, survival was not affected after exposures to C_{60}, but reproduction decreased in generation F_2, after exposure (50 ppm). In addition, adult size was affected, and a significant decrease after C_{60} exposure was observed at both studied concentrations (10 and 50 ppm). In another study by Arndt et al. (2013), the importance of testing chronic toxicity is highlighted. After an acute experiment, the actual effects may not have been effectively discovered or demonstrated. Thus, it was concluded, when using *D. magna* as a test organism, chronic experiments are vital for identifying potential modes of action for CNMs, since acute experiments do not predict the chronic effects (Arndt et al., 2013).

Research on long-term CNPs exposures to *C. riparius* have only been documented in few studies (Waissi-Leinonen et al., 2012, 2015). The risks of fullereneC_{60} to benthic invertebrates as regards their tolerance, growth and development have already been identified (Waissi-Leinonen et al., 2012, 2015; Pakarinen et al., 2011). In a study with *Lumbriculus variegatus,* fullerene-spiked sediments (10 mg/kg and 50 mg/kg dw) caused loss of epidermal cuticle fibers, but did not affect survival or reproduction (Pakarinen et al., 2011). However, damage to cuticle fibers may affect *L. variegatus*' ability to resist environmental stressors. Interestingly, we found in our previous study (Waissi-Leinonen et al., 2015) that fullerene-associated sediment had the most apparent effect on larval growth in the lower range of the tested concentrations (0.0025–20 mg/kg dw). In that study, *C. riparius* were exposed to fullerene concentrations up to 80 mg/kg dw, but the adverse effects vanished at concentrations of 40 and 60 mg/kg dw, until a dose of 80 mg/kg dw was reached, at which point reduced growth was again observed. In addition, delayed emergence was observed at concentration of 0.5 mg/kg, while in higher treatments (10, 40 and 80 mg/kg), the opposite response was found. *C. riparius* is a tolerant species for environmental stressors and generally, low mortality has been reported even with high C_{60} exposure concentrations (Waissi-Leinonen et al., 2012, 2015). Taking into consideration the observed effects on the larval growth and emergence of the midges with fullerene-treated sediments, even at the low concentrations, questions of the breeding success and viability of the second generation larvae remain open.

This study investigated the chronic effects of carbon NPs, fullereneC_{60}, on *C. riparius* midges at different life stages. In the first generation, survival, reproduction and emergence time were studied. Larvae recovery and potential adaptation were studied in the second generation. Studies on sediment-associated fullereneC_{60} were performed by a 34-day emergence experiment (the first generation), and the growth of the second generation larvae were tested in a 10-day growth test performed in (a) fullerene-associated sediment and (b) pristine conditions. In addition, transmission electron microscopy (TEM) was used to identify histological changes in the second-generation larvae tissues and in microvilli length. The experiments were carried out using artificial sediment under controlled laboratory conditions with nominal concentrations of 0.5, 10 and 40 mg C_{60}/kg dry weight.

2. Materials and methods

2.1. Aqueous nC_{60} suspension characterization and sediment preparation

Crystalline fullerene (C_{60}) powder (99.5%) (Sigma-Aldrich, USA) was added to artificial freshwater (0.5 mM), and the suspension was made as in our previous study (200 mg/L) (Waissi-Leinonen et al., 2015). To measure possible impurities in the fullerene powder, trace metal analyses were performed with inductively coupled plasma optical emission spectrometry (ICP-OES, IRIS Intrepid ll XSP), and only a couple of metal elements were observed (Supplemental Material Table S1). The suspension was stirred for two weeks with a magnetic stir plate at 1000 rpm. Based on UV-VIS absorption at 335 nm, the C_{60} concentration was measured using the standard curve (Cary 50BIO Mulgrave, Australia). Sample preparation was carried out according to our previous study (Waissi-Leinonen et al., 2015), where the standard curve is documented as being linear at concentrations of 0.8–52 mg/L (seven points, $r^2 > 0.999$).

Fullerene agglomerates were characterized using transmission electron microscopy (TEM, Zeiss 900, West Germany, 50 kV incident beam energy), in which the largest dimension of the agglomerates was measured. The imaging software MegaVision by AnalySIS was used for particle size distribution analyses (n = 700) (Supplementary Material Fig. S1). The results were confirmed by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments), and the surface charge of the C_{60} suspension (Zeta potential) was analyzed (Waissi-Leinonen et al., 2015). Before DLS analysis, sediment samples were diluted 1:500 with artificial freshwater and centrifuged for 15 min at 1700 rpm.

In this study, 1000 g of artificial sediment was prepared according to OECD guideline 218 (OECD, 2004), by mixing 20% of kaolinite clay, 75% of quartz sand (50% of the particles in the range of 50–200 μm) and 5% of peat powder (*Sphagnum* sp.) on a dry mass basis. The pH of the sediment was adjusted to 7.0 with $CaCO_3$. The selected food source for the larvae was *Urtica* sp. (0.5% dw, particle size <0.5 mm), which was mixed to the sediments prior the experiment start-up. The amount of *Urtica* was determined according to our previous findings (Waissi-Leinonen et al., 2012, 2015). The sediment's dry mass to wet mass ratio was 0.68, and the total organic carbon content (TOC) was 9.30 ± 1.28 g/kg (Multi N/C 2100 Analytic Jena AG Germany). The test sediments were spiked for 2 h with the fullerene suspension (0.5, 10 and 40 mg fullerene/kg sediment dw) by stirring vigorously with a rotating blade. The fullerene concentrations in the test sediments were quantified for the treatments of 10 and 40 mg/kg using the toluene extraction method for bulk sediment phase, as described in our previous studies (Waissi-Leinonen et al., 2015; Pakarinen et al., 2011). Detailed sample preparation steps are described in the Supplementary Material. Fullerene concentration of 0.5 mg/kg was below the detection limit for the method (<10 mg/kg C_{60}). Recovery, when compared to nominal concentrations, was 100% ± 14.

2.2. Test organisms and exposure conditions

The laboratory culture of *C. riparius* was obtained from the University of Eastern Finland. The experiments were conducted