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Effects of short-term exposure to fluoxetine and carbamazepine to the collembolan *Folsomia candida*

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HIGHLIGHTS

- Collembolans showed an avoidance behavior when exposed to carbamazepine.
- Carbamazepine induced oxidative stress, expressed as an increase on LPO levels.
- A pattern of no avoidance characterized fluoxetine exposure, although AChE was inhibited at 4 mg kg^{-1}.

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ABSTRACT

Pharmaceuticals, emerging environmental contaminants, have their ecotoxicological effects to non-target organisms in soil largely unknown. This study assessed short-term effects of two human pharmaceuticals, carbamazepine and fluoxetine, to *Folsomia candida*. Avoidance to spiked soils was assessed after 48 and 96 h exposure and biochemical changes (acetylcholinesterase and glutathione S-transferase activities, and lipid peroxidation levels) after 96 h. *F. candida* avoided soils spiked with 0.04, 0.4 and 4 mg carbamazepine kg^{-1} after 48 h. However, higher number of organisms were found in soils with 40 mg carbamazepine kg^{-1}, a behavior also displayed for 40 mg fluoxetine kg^{-1} spiked soils. After 96 h, *F. candida* showed avoidance behavior to soils with 4 and 40 mg carbamazepine kg^{-1}. Acetylcholinesterase activity decreased in 0.4 mg fluoxetine kg^{-1} exposed organisms. Peroxidative damages were detected in organisms exposed to 4 and 40 mg kg^{-1} carbamazepine and glutathione S-transferase inhibition was observed at 40 mg kg^{-1}. Data suggests that carbamazepine and fluoxetine may pose risk to soil collembolan.

1. Introduction

The presence and possible harmful effects of active pharmaceuticals in the environment is currently an emerging topic of concern in environmental sciences since these compounds, designed to interact with specific pathways and processes in target organisms, are continuously being released into the environment through wastewater treatment plants products like sewage sludge and wastewaters. Non-target organisms may thus be exposed throughout their lives (Okay et al., 2000; Bendz et al., 2005) if their habitats become contaminated with these substances.

Pharmaceutical compounds may partly be excreted as parent compound (not metabolized form) or as metabolites (active or inactive), enter the sewage systems and survive the wastewater treatment process (Buchberger, 2007). After their passage through wastewater treatment plants, the fractions in solution may be disposed as effluents into water bodies (Jones et al., 2005), reused for irrigation in areas with high pressure on water resources (Calisto and Esteves, 2012; Dalkmann et al., 2012) whereas sludge associated pharmaceuticals may be applied for agricultural purposes, to improve soil properties due to its organic matter rich matrix (Kinney et al., 2006a; Wu et al., 2010a; Calisto and Esteves, 2012), or disposed to landfills. Although the use of sewage treatment plant effluents and sludge significantly reduces the demand for water resources and fertilizers, it constitutes the major pathway for the introduction of pharmaceuticals in soils (Calisto and Esteves, 2012), with unforeseen risks in the long-term (Dalkmann et al., 2012). Different classes of pharmaceutically active substances (e.g. antibiotics, non-steroidal anti-inflammatory agents, anticonvulsants, anticoagulants and sex hormones, have been reported in soils in levels up to low mg kg^{-1} levels (Carter et al., 2014). Nonetheless, the knowledge on the ecotoxicological effects of pharmaceuticals to soil organisms in general and to

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soil-dwelling invertebrates (Jensen et al., 2003) in particular, can be considered extremely scarce, despite their crucial role in soil ecosystems (Rusek, 1998) and the recognized recalcitrant nature of some pharmaceuticals in soils (Kormos et al., 2010; Golan-Rozen et al., 2011). The available studies with soil invertebrates suggest a low direct toxicity (effect on reproduction) of antibacterial agents such as tiamulin, olanquindox and metronidazole (Jensen et al., 2003) and antibiotics such as oxytetracycline and tylosin (Baguer et al., 2000). A much higher toxicity was found for antiparasitics such as abamectin and ivermectin (Gunn and Sadd, 1994; Jensen et al., 2003; Kolar et al., 2008) and the antibiotic triclosan (Amorim et al., 2010). This antibiotic has also demonstrated the ability to induce oxidative stress and genetic damage (Lin et al., 2012).

Carbamazepine (antiepileptic) and fluoxetine (antidepressant) are two human pharmaceuticals already found in wastewater treatment effluents, biosolids, surface waters and soils. However, despite the studies on their persistence and low biodegradation in soils, biosolids, and soil–biosolid mixtures (Kinney et al., 2006a; Redshaw et al., 2008; Monteiro and Boxall, 2009), and bioavailability to plants (Wu et al., 2010b; Shenker et al., 2011; Holling et al., 2012), to the authors' knowledge, no studies have focused on the toxicological effects to soil invertebrates.

Springtails (Collembola) are common and widespread arthropods in soils throughout the world (Kool et al., 2011), playing an important ecological role as decomposers and detritivores of plant residues (Bai et al., 2010). These organisms have been widely used for ecological impact assessments (Nakamori and Kaneko, 2013), demonstrating sensitivity to the effects of soil contamination. *Folsomia candida*, a widely used ecotoxicological model, has already shown sensitivity to avermectins (Diao et al., 2007; Kolar et al., 2008; Römbke et al., 2010), in terms of survival and reproduction. No studies are currently available on the effects of carbamazepine and fluoxetine to *F. candida*. In this perspective, this study aimed at assessing the short-term effects on *F. candida* exposure to fluoxetine and carbamazepine, using as endpoints the avoidance behavior, the peroxidative damage (lipid peroxidation), the cholinesterases (cholinergic nerve system) and glutathione S-transferase (biotransformation and antioxidant defense) activities.

2. Material and methods

2.1. Chemicals

Fluoxetine HCl was purchased from TCI (Japan) and carbamazepine acquired from Sigma–Aldrich (Germany). All other chemicals were of analytical grade obtained from Sigma–Aldrich (Germany), Bio-Rad (Germany) and Merck (Germany).

2.2. Test organisms

F. candida (Collembola) were maintained in laboratory on a moist substrate of plaster of Paris and activated charcoal (9:1 ratio) at 20 ± 2 °C, under a photoperiod of 16:8 (light:dark). Animals were fed with dried baker's yeast (*Saccharomyces cerevisae*) twice a week, in small amounts to avoid spoilage by fungi. In order to obtain synchronized cultures, adults were transferred into new breeding substrate, stimulating egg release, and after 48 h adults were removed. The eggs hatched after approximately 10–12 d and 22 d old adults were used in this study.

2.3. Test soil and soil spiking

The natural standard soil LUFA 2.2 was used. The properties of this soil can be summarized as follows: pH = 5.5, organic matter = 3.9%, and texture = 6% clay; 17% silt; 77% sand.

A stock solution of fluoxetine was prepared in ultra-pure water and soil spiking, for both avoidance behavior tests and biochemical analysis, was performed just prior to the test start in pre-moistened soil batches (20–40% of the water holding capacity – WHC). Carbamazepine, due to its low water solubility, was thoroughly mixed (as powder) in pre-moistened soil batches (20–40% WHC), preventing the use of a solvent that would be required to prepare a stock solution. Final moisture content for both compound treatments was adjusted to approximately 60% WHC. For both chemicals, nominal concentrations of 0.04, 0.40, 4 and 40 mg kg^{-1} were used.

2.4. Avoidance assay

Avoidance behavior was assessed following the standard avoidance behavior guideline for collembolans (ISO, 2011) using a two-section system with 5 replicates per treatment after 48 h exposure and after a 96 h exposure period. Briefly, plastic circular boxes were physically divided by a removable split and the control soil (30 g wet weight) was placed in one of these sections. The same amount of spiked test soil was placed on the opposite side. A dual control (control soil in both sides) test treatment was also performed. The split was then carefully removed and both soil batches were put into straight contact. Then, 20 organisms were placed on the contact line of the soils. Test chambers were covered with lids containing small holes and kept at 20 ± 2 °C with a photoperiod of 16:8 h (light–dark). At the end of the test periods the plastic splits were re-inserted in the separation line between the two soils and water added to both sides simultaneously. Flooded soils were gently stirred with a spatula, allowing the animals to float on the water surface for counting. Missing organisms were considered dead. The avoidance results were expressed as the percentage of avoidance by the equation (ISO, 2011):

$$A = \frac{C - T}{N} \times 100 \quad (1)$$

A – avoidance (%); C – organisms in the control soil; T – organisms in the treatment soil; N – total number of organisms.

The habitat function of soils is considered to be limited and indicative of an impact on behavior, if the average number of organisms found in the test soil is less than 30% (ISO, 2011).

2.5. Biochemical Responses Assay

Thirty organisms were exposed to carbamazepine and fluoxetine in test containers with 30 g of soil (5 replicates per concentration and control), for 96 h at a room temperature of 20 ± 2 °C and 16:8 light/dark photoperiod, without feeding. After 96 h, test vessels were filled with water and gently stirred with a spatula, allowing the animals to float on the water surface. Animals were immediately and carefully collected, transferred to a container with a substrate of plaster of Paris and activated charcoal, counted, placed in microtubes and immediately frozen in liquid nitrogen. Samples were stored at −80 °C until further biomarker analysis.

2.6. Preparation of biological material for biochemical analyses

Samples were homogenized using a sonicator (Branson S-250A) in potassium phosphate buffer (0.1 M, pH 7.2). An aliquot of the homogenate was collected for the lipid peroxidation assessment and the remaining sample centrifuged (Eppendorf 5810R) for 20 min, at 10000 g, at 4 °C for post-mitochondrial fraction (PMS) isolation.

Protein content of the homogenates and PMS fractions were determined by the Bradford method (Bradford, 1976) adapted to microplate reader (Thermo Scientific Multiskan Spectrum).