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Ecotoxicogenomic assessment of diclofenac toxicity in soil

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ABSTRACT

Diclofenac is widely used as nonsteroidal anti-inflammatory drug leaving residues in the environment. To investigate effects on terrestrial ecosystems, we measured dissipation rate in soil and investigated ecotoxicological and transcriptome-wide responses in *Folsomia candida*. Exposure for 4 weeks to diclofenac reduced both survival and reproduction of *F. candida* in a dose-dependent manner. At concentrations ≥200 mg/kg soil diclofenac remained stable in the soil during a 21-day incubation period. Microarrays examined transcriptional changes at low and high diclofenac exposure concentrations. The results indicated that development and growth were severely hampered and immunity-related genes, mainly directed against bacteria and fungi, were significantly up-regulated. Furthermore, neural metabolic processes were significantly affected only at the high concentration. We conclude that diclofenac is toxic to non-target soil invertebrates, although its mode of action is different from the mammalian toxicity. The genetic markers proposed in this study may be promising early markers for diclofenac ecotoxicity.

1. Introduction

In recent decades, the global consumption of pharmaceuticals and personal care products (PPCPs) has led to increased levels of these compounds in the environment and possible adverse effects on wildlife. In North America, Europe and Asia, PPCPs have been detected in surface water and in the solid end products of wastewater treatment (biosolids) (Kinney et al., 2006). Non-steroidal anti-inflammatory drugs (NSAIDs) are among the list of the most commonly detected PPCPs in surface water (Buser et al., 1998; Corcoran et al., 2010). The NSAID diclofenac (2-[(2,6-dichlorophenyl)amino] benzene acetic acid) is consumed in hundreds of tons annually (Buser et al., 1998). This pharmaceutical agent is applied to reduce inflammation and pain by blocking the enzyme cyclooxygenase, which catalyzes the synthesis of prostaglandins from arachidonic acid (Vane and Botting, 1998). As a consequence of its widespread use, diclofenac has been detected in many waste treatment plant effluents and biosolids (Rosal et al., 2010; Stulten et al., 2008). Diclofenac will also end up in soils amended with sewage sludge. Moreover, diclofenac may be released into soil from the corpses, feces and urine of livestock containing diclofenac residues (Oaks et al., 2004).

Diclofenac seems to have the highest level of acute toxicity among the NSAIDs tested (Fent et al., 2006). The lowest observed effect concentration (LOEC) in water at which both renal lesions and alterations of rainbow trout gills occurred was 5 μg/L (Schwaiger et al., 2004). Populations of Indian vulture (*Gyps indicus*) have collapsed due to bioaccumulation of diclofenac residues taken up from cattle corpses (Oaks et al., 2004). However, little is known about the potential effects of diclofenac on non-target species in the terrestrial ecosystem, such as soil invertebrates. To address this, we report ecotoxicological effects and gene expression data on diclofenac-exposed springtails in this paper.

Springtails (Collembola) are soil-dwelling arthropods which have a role as detritivores in the soil ecosystem and contribute to decomposition processes and the recycling of nutrients. Since they are abundant in soil and sensitive to soil pollutants, the springtail species *Folsomia candida* is often used as an indicator in soil ecotoxicological tests to assess the effects of soil pollutants (Fountain and Hopkin, 2005). A standardized protocol has been developed

Abbreviations: nAChR, nicotinic acetylcholine receptor; NSAIDs, non-steroidal anti-inflammatory drugs; GO, gene ontology.

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for assessing the toxicity of (single) chemicals as well as for soil-quality assessment using this species (ISO, 1999). Recently, new genomics tools are integrated into existing standardized toxicity tests to enhance the assessment (Snape et al., 2004; Van Straalen and Roelofs, 2008). By measuring gene expression, we can elucidate the mode of action of the pollutant and determine the nature of adverse disturbances of the physiological state of the organism. *F. candida* was employed in several ecotoxicogenomics studies using spiked soil samples as well as natural polluted soils (Nota et al., 2008; Roelofs et al., 2012; Chen et al., 2014; Nota et al., 2009).

In the present study we evaluate the effect of diclofenac on the survival and reproduction of *F. candida* in soil. Moreover, a microarray experiment was applied to measure the transcriptome-wide response, in order to unravel molecular response pathways invoked and to identify the biological processes affected by diclofenac. The concentrations of diclofenac used in our transcriptomic analysis are 50 and 200 mg/kg soil which correspond with low and high exposure levels. Combining measurements on key life-history traits (survival and reproduction) and transcriptional profiles, we aim to elucidate the modes of action of diclofenac in this soil invertebrate.

2. Materials and methods

2.1. Experimental soil, compound and spiking

For all experiments, the natural LUFA 2.2 soil (Speyer, Germany) was used. Diclofenac-sodium (CAS Number 15307-79-6, Sigma–Aldrich) was spiked into the soil as a solution in deionized water, to reach concentrations ranging from 50 to 3200 mg per kg dry weight soil. Moisture content of the soil was adjusted to 22% (w/w), corresponding to 50% of the maximum water hold capacity. The spiked soils were thoroughly mixed and exposures started immediately afterwards. All the exposures were performed in a climate room at 20 °C, 75% relative humidity and a 12/12 h light/dark cycle.

2.2. Measuring diclofenac dissipation in soil

Actual diclofenac concentrations were determined in soils spiked with 200, 500, 1600 and 3200 mg/kg dry soil at 1, 2, 4, 7, 14, and 21 days after spiking. Extraction of diclofenac was performed according to the method of Al-Rajab et al. (2010). In short, at each time point 5 g of soil was vortexed for 10 min with 15 ml ethyl acetate containing 4 mg of mefenamic acid (CAS Number 61-68-7, Sigma–Aldrich) as internal analytical standard. Subsequently, 7 ml of the ethyl acetate extract was centrifuged for 10 min at 3000 rpm (Hettich Rotanta 460R centrifuge) to remove remaining soil particles. Five ml supernatant was evaporated to dryness under a stream of nitrogen and the dried components were reconstituted in 500 μl dimethyl sulfoxide (DMSO). The DMSO solution was diluted 100 times in 50% methanol prior to analysis. Diclofenac and the internal analytical standard mefenamic acid were separated on a Shimadzu high performance liquid chromatography (HPLC) system equipped with a SIL20AC autosampler, two LC-20AD pumps, a SPD20A-UV detector and a Phenomenex Luna C18 column (5 μm, 4.6 × 150 mm) as the stationary phase. For the separation of diclofenac and mefenamic acid, a binary gradient composed of solvent A (acetonitrile 1%, formic acid 0.2%, water 98.8%) and solvent B (acetonitrile 98.8%, formic acid 0.2%, water 1%) was used. The first minute was run with isocratic at 1% of solvent A. From 2 to 20 min, the concentration B increased linearly to 99%, followed by 10 min re-equilibration of the column at the initial conditions. The flow rate was 0.5 ml/min and detection was performed at 280 nm. Seven initial concentrations of diclofenac-spiked soil samples were measured and used for the preparation of a calibration curve to quantify this compound in the dissipation samples (Fig. S1). The sample preparation of the calibration curve and analytical samples was identical. All samples were prepared and measured in triplicate, and the ratio between the peak area of diclofenac and the peak area of the internal standard in the in the UV chromatogram (AUC) was used for quantification.

2.3. Ecotoxicity experiment

The parthenogenetic collembolan *F. candida* (Berlin strain, VU University Amsterdam) was used for all experiments. In mass cultures, maintained in our laboratory for many years, the animal was fed dry baker's yeast (Dr Oetker, The Netherlands) ad libitum. Age-synchronized animals were obtained by transferring adults to new containers where they could lay eggs for 2 days. Two days later, the adult animals were removed. The eggs laid in this period hatched after around 10 days. The hatchlings were used for experiments when they were 10–12 days old.

Toxicity tests were performed following the ISO standard (ISO, 1999). Concentrations of diclofenac of 0-50-100-200-400-800-1600-3200 mg/kg dry soil were spiked to soils and portions of 30 g moist soil were transferred to 100 ml glass jars. In total, five replicates for each treatment were evaluated and each replicate contained ten age-synchronized collembolans. Jars were incubated in a climate room at 20 °C, and opened twice per week for aeration. The moisture levels were adjusted once a week using demineralized water. At the beginning and halfway the experiment, a small amount of food (a few grains of baker's yeast) was added. After 28 d the glass jars were filled with 100 ml water to extract the animals from the soil by means of flotation. The water was decanted in a glass beaker and digital photographs were taken of the water surface. After that, the number of juveniles was counted with Cell^{d} software (Olympus, Germany). Dose-response curves relating juvenile counts to the concentrations of diclofenac were determined by nonlinear regression to estimate effect concentrations The logistic dose–response model described by Haanstra et al. (1985) was applied to fit the data.

2.4. Microarray experiments

For gene expression assessment, LUFA 2.2 soils were spiked with two concentrations of diclofenac, 50 and 200 mg/kg soil, representing low and high exposure levels. Four independent replicates were used for each treatment. Thirty adult (23 d old) collembolans were exposed for 2 d in a jar with 30 g moist soil. After 2 days, the animals were extracted from the soil by means of flotation using 100 ml water, collected in an Eppendorf tube, and snap-frozen in liquid nitrogen. Total RNA was extracted from the pool of 30 animals for each replicate using the SV Total RNA Isolation System (Promega). Then, a Nanodrop ND-100 spectrophotometer (Fisher Scientific) and a Bioanalyzer (Agilent Technologies) were employed to determine the quality and quantity of RNA samples. Five hundred ng total RNA per sample was used as input for amplification and labelling with the Low-Input Fluorescent Liner Amplification Kit, two colors (Agilent Technologies), according to the manufacturer's guidelines. Labeled and amplified complementary RNA was purified using RNeasy (Qiagen) and hybridized by using the Gene Expression Hybridization Kit (Agilent Technologies). Hybridization was performed at 65 °C for 17 h rotation at 10 circulations per minute in an incubator. The RNA samples from different pools of animals were kept separately and considered as biological replicates. For hybridization, a replicated reference design was employed. The four low diclofenac samples and the four high diclofenac samples were mixed with eight unique non-spiked reference samples in the design. For each diclofenac