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Assessing the effects of fluoxetine on *Physa acuta* (Gastropoda, Pulmonata) and *Chironomus riparius* (Insecta, Diptera) using a two-species water–sediment test

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ABSTRACT

Fluoxetine has been tested in a two-species water–sediment system, which allowed a two-generation study with *Chironomus riparius* and a partial life-cycle with the freshwater snail *Physa acuta* to be performed at the same time. The design considered the continuous application of fluoxetine to overlaying water for nominal concentrations of 31.25, 62.5, 125 and 250 μg/L. A fifth treatment (87.5 μg/L) level consisted of pulse applications once a week. Measures of water and sediment concentrations were determined once a week and at the end of experiment (day 44), respectively. The fate study demonstrated that water dissipation can be explained by partitioning of fluoxetine to sediment. At the end of experiment, the percentage of detected fluoxetine was up to 10-fold higher in sediment than in overlaying water. The employed two-species test allowed distinguishing, in the same exposure conditions, effects due to waterborne exposure together ingestion at the sediment surface (freshwater grazing snail *P. acuta*) and exposure by burrowing activities (sediment-dwelling insect larvae *C. riparius*). The effect assessment showed a stimulation of *P. acuta* reproduction at lower concentrations (31.25 and 62.5 μg/L), while the opposite effect was observed at the highest treatment (250 μg/L). Additional studies should be conducted to establish if the statistically significant differences observed in F0 sex ratio at the 62.5 μg/L and F1 adult emergence at 31.25 μg/L of *C. riparius* have a toxicological significance. This study showed that fluoxetine can affect reproduction of freshwater molluscs. The results of the present study may contribute to knowledge on ecotoxicology of pharmaceuticals, about which little data is available. The possible consequences and implications for targeting the environmental risk assessment of fluoxetine are discussed.

1. Introduction

Concern about occurrence of pharmaceuticals in the environment arose two decades ago and initially focused on human health risk. The possibility to long-term exposure of the public through potable water contaminated with pharmaceuticals led to monitoring studies in surface water (e.g.. Richardson and Bowron, 1985; Heberer, 2002; Khetan and Collins, 2007). Although pharmaceuticals have been detected in the ng–μg/L range they are bioactive molecules with specific mechanisms of action which are continuously introduced to surface water. The effects of pharmaceuticals on non-target aquatic organisms are poorly understood, but these emerging pollutants could exert their effects at sublethal concentrations producing imperceptible damage on aquatic life (Daughton and Ternes, 1999). In this context the European Medicines Agency (EMEA) has developed

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the guideline for environmental risk assessment (ERA) of human pharmaceuticals (EMEA, 2006). This guideline considers the same step-wise procedure for all classes of pharmaceuticals in which standardized ecotoxicological tests form the basis for effect assessment.

The EMEA guideline covers the effects of pharmaceuticals on aquatic invertebrates by the standardized *Daphnia* sp. reproduction test and it considers extended effect analysis on sediment dwelling organism only when the pharmaceutical adsorbs to sediment. Pharmaceuticals targeted at specific mammalian receptor systems may also affect aquatic invertebrates. It is possible that other receptor systems occurring in invertebrates may also be sensitive to the actions of pharmaceuticals as pointed out by Pascoe et al. (2003). Considering that invertebrates have unique life strategies that differ from those in vertebrates (i.e. hermaphroditism, parthenogenesis), diversity of receptor and endocrine systems is expected. Mechanistic endpoints to diagnose specific modes of action are still limited in the case of invertebrates. Instead, trends are focussed on developing robust invertebrate chronic test methods (e.g. full life cycle or multigeneration tests) to investigate adverse effects of chemical substances including emerging pollutants of concern (e.g. potential endocrine disrupters) (Gourmelon and Ahtiainen, 2007). Uncertainties in the assessment of invertebrate responses to endocrine-active and other pharmaceuticals can be reduced by combining mechanism-specific biomarkers, multigenerational designs and population growth models (Clubbs and Brooks, 2007). Thus, life-cycle type tests should be more appropriate to assess effects of pharmaceuticals in invertebrates.

The limitation of the small short-term aquatic test battery, which can underestimate the toxicity of pharmaceuticals, has been highlighted by some authors (e.g. Crane et al., 2006), suggesting the need for a test strategy adapted to specific modes of toxic action (Henschel et al., 1997; Länge and Dietrich, 2002; Escher et al., 2005). Although Escher et al. (2005) state the impossibility to include all modes of toxic action in a test battery they defend the use of mechanism-based tests as a complementary tool to prioritizing test procedures. Pharmacodynamic knowledge about pharmaceuticals might confer an advantage to human pharmaceuticals over general chemicals with respect to the environmental risk assessment. Thus, some authors have proposed a case-by-case or "intelligent" testing strategy by obtaining clues about potential targets for environmental toxicity based on database from the mammalian dossier (i.e. pharmaco- and toxicological activity) (Länge and Dietrich, 2002; Seiler, 2002; Huggett et al., 2003).

Recently Fent et al. (2006) have grouped pharmaceuticals in six main classes according to their therapeutic mode of action. Neuroactive compounds, such as antiepileptics and antidepressants are one group of pharmaceuticals commonly used. Fluoxetine is a Selective Serotonin Reuptake Inhibitors (SSRI) prescribed as antidepressant. SSRIs increase the 5-HT (5-hydroxytryptamine or serotonin) neurotransmission by blocking the serotonin reuptake transport proteins. Although serotonin is conserved in all animal phyla, the physiological processes regulated by this neurotransmitter can differ across species. This can be especially relevant for invertebrates, which are genetically very distant from humans. Serotonergic mechanisms regulate somatic process as well as reproductive functions which differ between invertebrate taxa: In bivalves, serotonergic mechanisms regulate reproductive processes (e.g. spawning, oocyte maturation, germinal vesicle breakdown, sperm reactivation and parturition); in freshwater gastropods, they regulate egg laying and induction of penile erection, and, in crustaceans, they regulate ovarian growth (Fong et al., 1998; Fong, 2001; Muschamp and Fong, 2001). Recently, Liang et al. (2006) have elucidated the neuroendocrine pathway through which serotonin modulates the response to environmental and physiological stresses of *Caenorhabditis elegans*. Although 5-HT has been detected in many invertebrates the actions of SSRIs are poorly understood.

We evaluate possible effects of fluoxetine that could not be covered by standard assays using a two-species water–sediment. Long-term reproductive effects on two species of freshwater invertebrates (*Physa acuta* and *Chironomus riparius*) after exposure either through sediment or water column pathways were assessed. The protocol was based on previous experimental designs (Sánchez and Tarazona, 2002; Sánchez et al., 2005). *P. acuta* is a hermaphroditic species and it is expected to respond to fluoxetine as serotonergic mechanisms have been described in freshwater snails. *C. riparius* is included in the EMEA guideline, but we evaluate the effects in a two-generation exposure time. The exposure regimen was designed to maintain relatively constant concentrations over whole test period and simulate realistic exposure conditions of pharmaceuticals to water bodies (i.e. emission via sewage treatment plants). Fate and effects were characterized simultaneously.

2. Materials and methods

2.1. *Culture of test organisms*

C. riparius have been maintained in our laboratory during 25 consecutive generations and are cultured following recommendations described by OECD guidelines 218 and 219 (OECD, 2004) and similar to that described in Sánchez et al. (2005) for *C. prasinus*.

The freshwater snail *P. acuta* (Pulmonata) is a hermaphroditic species that practices both outcrossing and selfing (Bousset et al., 2004), though outcrossing was found predominant by Jarne et al. (2000). *P. acuta* was maintained at our laboratory in a controlled climatic room (20 °C) for several generations prior to the experiment and outcrossing was dominant.

Populations of *P. acuta* are reared in glass beakers (3 L) with spring water, under static flow and aeration, and a photoperiod of 16 h:8 h light/darkness. The spring water was collected from a semi-rural non-polluted site which have been used to culture *C. riparius* in our laboratory for several generations. About 50 adults were placed for breeding and they were fed with 200 mg of fish food flakes, TetraDiscus®, twice a week. Every week adults were moved to clean beakers and deposited egg masses were recovered to initiate new generations. In order to lessen contamination by microorganisms, egg masses were immersed in methylene blue (0.6 μg/L) during 3 h. To allow hatching 20 egg masses were maintained in spring water (100 ml) and gentle aeration. After hatching, (10–15 days) the newly-hatched snails were fed with fine particulate fish food (Sera Micron®) ad