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Bioconcentration of the antidepressant fluoxetine and its effects on the physiological and biochemical status in *Daphnia magna*

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ABSTRACT

The aim of this study was to evaluate the bioconcentration potential of fluoxetine and its biological effects in *Daphnia magna*. After 48 h of waterborne exposure, the bioconcentration of fluoxetine in *D. magna* was determined to be 460.61 and 174.41 L kg^{-1} for nominal exposure concentrations of 0.5 and 5 μg L^{-1}, respectively. Moreover, various biological endpoints, including physiological responses (filtration and ingestion rates), enzymatic biomarkers related to neurotoxicity [acetylcholinesterase (AChE)] and antioxidant defense [superoxide dismutase (SOD)], and an oxidative stress damage marker [malondialdehyde (MDA)], were assessed. Fluoxetine exposure increased the filtration rate of daphnia, while the ingestion rate was not obviously modified. AChE activity was significantly inhibited, highlighting the neurotoxicity of fluoxetine on *D. magna*. However, with some alterations in the SOD activity and MDA content, no obvious oxidative damage was observed in *D. magna* exposed to fluoxetine at the tested concentrations. These results indicate that fluoxetine can be accumulated and consequently induce physiological and biochemical perturbations in *D. magna*.

1. Introduction

Selective serotonin reuptake inhibitor (SSRI) antidepressants are a class of human pharmaceuticals for treating clinical depression and other mood disorders, such as attention-deficit disorder, obsessive-compulsive disorder, panic disorder, and social phobia (Schultz and Furlong, 2008). These antidepressants function by inhibiting reuptake of the neurotransmitter serotonin, and they ultimately increase the serotonin concentration in the central nervous system in humans (Nutt et al., 1999). Due to the increased prevalence of mental health disorders in modern society, the use of SSRIs, particularly in developed nations, is massive. As a result, SSRIs have been found in sewage and surface water worldwide (Fong and Ford, 2014; Silva et al., 2012, 2015).

Fluoxetine is one of the most commonly detected SSRIs in the aquatic environment, with concentrations ranging from 0.01 to 3.5 μg L^{-1} in raw sewage waters (Lajeunesse et al., 2012; Salgado et al., 2011), 0.007–0.93 μg L^{-1} in wastewater treatment effluents (Kostich et al., 2014; Lajeunesse et al., 2012; Martínez Bueno et al., 2007), and 0.004–0.14 μg L^{-1} in surface water (Alonso et al., 2010; Metcalfe et al., 2010; USEPA, 2007). Like other pharmaceuticals present in the aquatic environment, fluoxetine can have adverse effects on aquatic flora and fauna. In fact, it has been classified as one of the most toxic pharmaceuticals in non-target organisms (Oakes et al., 2010). Previous studies have demonstrated that fluoxetine exposure can lead to neuroendocrine disruptions, reproductive effects, and changes in behavior or life history traits in various fish species (Ansai et al., 2016; Dzieweczynski et al., 2016; Pelli and Connaughton, 2015; Schultz et al., 2011; Weinberger and Klaper, 2014). Regarding invertebrates, some studies have demonstrated that fluoxetine could affect reproduction, development and locomotion in mollusks and crustaceans (Campos et al., 2012, 2016; Fong and Ford, 2014; Silva et al., 2015).

As a keystone planktonic crustacean, *Daphnia magna* is widely distributed in the freshwater environment, and it is often used as a model species in toxicology studies. Brooks et al. (2003) determined that the acute median lethal concentration (LC_{50}) of a 48 h fluoxetine exposure was 820 μg L^{-1} for *D. magna*. An acute toxicity study demonstrated that the half maximal effective concentration (EC_{50}) of fluoxetine in *D. magna* (immobilization test, 48-h exposure) was 5.91 mg L^{-1} (Minguez et al., 2014), while Stanley et al. (2007) reported immobilization lowest observed effect concentrations (LOECs) of 429–444 μg L^{-1} for chronic (21 d) *D. magna* exposure to fluoxetine enantiomers. Péry et al. (2008) found that the newborn length in *D. magna* was significantly impacted by fluoxetine, with a LOEC of 31 μg L^{-1}. Moreover, chronic (30 d) exposure to 36 μg L^{-1} fluoxetine significantly increased the fecundity of *D. magna* (Flaherty and Dodson, 2005). However, the endpoints applied in these studies were limited to

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physiological levels. Information on the biochemical responses of *D. magna* to fluoxetine, such as enzyme activities, is scarce.

Perhaps more concerning, in 2005, Brooks et al. (2005) first reported residues of fluoxetine in fish tissues (muscle, liver and brain) from an effluent-dominated stream. Soon thereafter, a growing number of field and laboratory works confirmed the bioconcentration or bioaccumulation of fluoxetine in various fish species (Ding et al., 2016b; Nakamura et al., 2008; Paterson and Metcalfe, 2008; Schultz et al., 2010; etc.) together with studies addressing fluoxetine accumulation in aquatic invertebrates, such as shrimp (*Gammarus pulex*), water boatman (*Notonecta glauca*) (Meredith-Williams et al., 2012), worm (*Lumbriculus variegatus*) (Karlsson et al., 2016), snail (*Planorbid* sp.) (Du et al., 2015), and mussels (*Elliptio complanata* and *Mytilus galloprovincialis*) (Bringolf et al., 2010; Franzellitti et al., 2014; Silva et al., 2016). However, in *D. magna*, the bioaccumulation or bioconcentration profiles of fluoxetine have yet to be recorded (Puckowski et al., 2016). The accumulation of pharmaceuticals in *D. magna* may form the basis for bioaccumulation through the aquatic food chain (Ding et al., 2015a, b), consequently leading to biological perturbations of aquatic ecosystems. Hence, the importance of investigating the accumulation of pharmaceuticals in *D. magna* should be emphasized (Ding et al., 2016a; Jeong et al., 2016; Kim et al., 2014).

In this study, the bioconcentration profiles of fluoxetine in *D. magna* over 48 h of aqueous exposure were assessed. Moreover, the filtration and ingestion rates of *D. magna* were investigated to evaluate feeding behavior alterations caused by fluoxetine exposure. In addition, to clarify the biochemical effects of fluoxetine on *D. magna*, a battery of molecular biomarkers was detected, including a nervous system enzyme [acetylcholinesterase (AChE)], an antioxidant enzyme [superoxide dismutase (SOD)], and a byproduct of lipid peroxidation (LPO) [malondialdehyde (MDA)]. We expect that this study will expand our knowledge of the risks that fluoxetine poses to aquatic invertebrates.

2. Materials and methods

2.1. Chemicals and reagents

Fluoxetine (fluoxetine hydrochloride, CAS 56296-78-7, purity > 98%, to be referred to as fluoxetine) was purchased from J & K Scientific (Shanghai, China). Hydroxymethyl aminomethane (Tris) was purchased from Nanjing Sunshine Biotechnology Co., LTD. (Nanjing, China), and its purity was > 99%. Bovine serum albumin (BSA) was purchased from Shanghai Huixing Biotechnology Reagent Co., LTD. (Shanghai, China), and the purity was > 98%. Kits for analyzing the AChE and SOD activities and MDA content were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Water was purified using a Milli-Q integral water purification system (Millipore, Milford, MA, USA). Oasis MCX (6 cc, 150 mg) solid-phase extraction (SPE) cartridges were purchased from Waters (Milford, MA, USA).

2.2. Experimental animal

D. magna was obtained from the Wuhan Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and cultured continuously in artificial freshwater (AFW). Briefly, a total of 58.5 mg of $CaCl_2{\cdot}2H_2O$, 24.7 mg of $MgSO_4{\cdot}2H_2O$, 13.0 mg of $NaHCO_3$ and 1.2 mg of KCl were added to 1 L of deionized water (OECD, 2008). The culture was maintained at a constant temperature (22 ± 1 °C) with a 16:8 h (light: dark) photoperiod (15 μE m^{-2} s^{-1}) and renewed three times per week. The daphnia were fed daily with green algae (*Chlorella vulgaris*). Before feeding, the culture media of *C. vulgaris* was collected and centrifuged (1000 × *g*, 10 min), then the supernatants were decanted. The algal pellets were resuspended in the culture media of *D. magna*, and were added to each culture media as food source to give a final cell density of 1×10^6 cells mL^{-1}.

2.3. Bioconcentration experiments

Prior to the experiments, daphnids (8 d old) were placed in AFW without algae for at least 1 h to purge the gut contents. The bioconcentration experiments, which consisted of one control group and two exposure concentrations, were conducted in 500 mL glass beakers. According to the concentrations of fluoxetine detected in the aquatic environment (Alonso et al., 2010; Kostich et al., 2014; Lajeunesse et al., 2012; Martínez Bueno et al., 2007; Metcalfe et al., 2010; Salgado et al., 2011; USEPA, 2007) and the previous acute toxicity studies for *D. magna* (Brooks et al., 2003; Minguez et al., 2014), the nominal exposure concentrations were set as 0.5 and 5 μg L^{-1}. Each exposure group was conducted in three replications. At the beginning of the experiments, *D. magna* were dispatched into nine glass beakers. Each beaker contained 500 mL of corresponding exposure media and 100 daphnia individuals. When adding the fluoxetine solutions, methanol was used as a carrier at < 0.1 mL L^{-1} in the final test media. At 0, 3, 6, 12, 24, 36 and 48 h following the beginning of exposure, approximately 10 individuals were sampled by a pipette, rinsed with a fresh culture solution in a culture dish. Then the daphnids were gently dried between two sheets of filter paper, and transferred into a 2-mL centrifuge tube. After freeze at –80 °C for 24 h, the samples were weighed using digital analytical balance to obtain the pooled wet weight of 10 daphnids. The samples were then stored at –80 °C for subsequent processing. At the given time points, approximately 50 mL of exposure media was collected to determine the concentration of fluoxetine and to maintain the density of the daphnids.

2.4. Feeding behavior experiments

Based on a method described by Villarroel et al. (2003), feeding experiments were conducted in darkness. The filtration and ingestion rates were used as measures of the feeding experiments. Ten neonates (< 24 h) were placed in 100-mL glass beakers filled with 50 mL of test solutions. The treatments included a control group and fluoxetine exposure groups at nominal concentrations of 0.5 and 5 μg L^{-1}. During the exposure period, the organisms were fed with 1×10^6 cells mL^{-1} of *C. vulgaris*. After 5 h of exposure, the final food concentrations were measured using a hemocytometer under an optical light microscope (400 × magnification). The filtration rate (*F*) is defined as the volume of medium swept clear by an animal in a unit of time, and the ingestion rate (*I*) is the number of cells consumed by an animal during a specific time interval. The average *F* (μL ind^{-1} h^{-1}) and *I* (cells ind^{-1} h^{-1}) were calculated using the following equations (Gauld, 1951):

$$F = (V/n) \times [(\ln C_0 - \ln C_t)/t] - A, \quad (1)$$

$$A = (\ln C_0 - \ln C_t')/t, \quad (2)$$

$$I = F \times \sqrt{C_0 \times C_t}, \quad (3)$$

where C_0 and C_t are the initial and final food concentrations (cell μL^{-1}), respectively; *t* is the time (h); *n* is the number of animals in volume *V* (μL); and *A* is a correction factor for changes in the control flask without daphnia with a final concentration C_t' after time *t*. The expression $\sqrt{C_0 \cdot C_t}$ represents the geometric mean of the food concentration during time *t*.

2.5. Biochemical tests

Biochemical tests were performed in semi-static conditions with an interval of 1 day in renewed test solutions. The applied exposure concentrations were the same as those in the feeding tests (i.e., 0, 0.5 and 5 μg L^{-1}). A total of 100 neonates were exposed in 500 mL of test solution in each treatment. After 7 d of exposure, all individuals were sampled, rinsed, weighed, and stored at –80 °C, as described above.

The daphnia samples were homogenized in four volumes of cold