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Ecotoxicological assessments show sucralose and fluoxetine affect the aquatic plant, *Lemna minor*

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ABSTRACT

Pharmaceuticals and personal care products (PPCP) are prevalent in aquatic systems, yet the fate and impacts on aquatic plants needs quantification for many compounds. We measured and detected sucralose (an artificial sweetener), fluoxetine (an antidepressant), and other PPCP in the Portneuf River in Idaho, USA, where *Lemna minor* (an aquatic plant in the environment and used in ecotoxicology studies) naturally occurs. Sucralose was hypothesized to negatively affect photosynthesis and growth of *L. minor* because sucralose is a chlorinated molecule that may be toxic or unusable for plant metabolism. *A priori* hypotheses were not created for fluoxetine due to lack of previous studies examining its impacts on plants. We conducted laboratory ecotoxicological assessments for a large range of concentrations of sucralose and fluoxetine on *L. minor* physiology and photosynthetic function. Frond green leaf area, root length, growth rate, photosynthetic capacity, and plant carbon isotopic composition (discrimination relative to a standard; $\delta^{13}C$) were measured among treatments ranging from 0 to 15000 nmol/L-sucralose and 0–323 nmol/L-fluoxetine. Contrary to our predictions, sucralose significantly increased green leaf area, photosynthetic capacity, and $\delta\ ^{13}C$ of *L. minor* at environmentally relevant concentrations. The increase of $\delta\ ^{13}C$ from sucralose amendments and an isotope-mixing model indicated substantial sucralose uptake and assimilation within the plant. Unlike humans who cannot break down and utilize sucralose, we documented that *L. minor*—a mixotrophic plant—can use sucralose as a sugar substitute to increase its green leaf area and photosynthetic capacity. Fluoxetine significantly decreased *L. minor* root growth, daily growth rate, and asexual reproduction at 323 nmol/L-fluoxetine; however, ambiguity remains regarding the mechanisms responsible and the applicability of these extreme concentrations unprecedented in the natural environment. To our knowledge, this was the first study to show aquatic plants can uptake and metabolize sucralose as a carbon source. This study further supports the common notion that *L. minor* can be useful in bioremediation of PPCP from wastewaters.

1. Introduction

Anthropogenic compounds, such as pharmaceuticals and personal care products (PPCP), are commonly detected in aquatic systems worldwide (Kolpin and Meyer, 2002; Pal et al., 2010; Yoon et al., 2010). These compounds are biologically active for human use but also have the potential to effect non-target organisms when released to aquatic systems via wastewater treatment plants (Rosi-Marshall and Royer, 2012). Immediately downstream of outlets of wastewater treatment plants (WWTP), the concentrations of PPCP typically range from ng/L to several μg/L (Pal et al., 2010). Many PPCP are ubiquitous in the environment but we still lack understanding of the fate and ecological impacts to aquatic organisms (Boxall et al., 2012; Brooks et al., 2009; Daughton and Ternes, 1999; Rosi-Marshall and Royer, 2012).

Sucralose and fluoxetine are two prevalent PPCP found downstream of most urban aquatic systems (Kolpin and Meyer, 2002; Silva et al., 2015). Sucralose (an artificial, no-calorie sweetener under the trade name "Splenda") entered the market in 1991 and is currently used in over 4000 products in many countries (U.S. Food and Drug Administration, 1998; European Union, 2004). Greater than 95% of ingested sucralose is excreted in urine, degraded <2% at wastewater treatment plants, and exported unaltered to rivers via effluent (Soh et al., 2011; Torres et al., 2011). Sucralose has been detected in surface waters at concentrations up to 11 μg/L (Oppenheimer et al., 2011; Tollefsen et al., 2012). Fluoxetine (a selective serotonin reuptake inhibitor, commonly prescribed for depression under the trade name "Prozac") has been used since the 1980′s. Fluoxetine is metabolized <10% (Hiemke and Härtter, 2000), excreted, and detected in surface waters at concentrations up to 9 μg/L (Kolpin and Meyer, 2002; Silva et al., 2012).

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The fate and impacts of sucralose to the aquatic environment and biota remain unclear. Because sucralose is a chlorinated molecule, it is persistent in the environment and may be toxic (Naumann, 2000; U.S. Environmental Protection Agency, 1999). Further, sucralose structurally resembles sucrose-sugar (Knight, 1994) and therefore could possibly be used by plants. Several sucralose toxicity studies concluded that sucralose does not bioaccumulate or affect the survival, growth, or reproduction of green algae at concentrations greater than environmental detections (Hu et al., 2016; Lillicrap et al., 2011; Soh et al., 2011). The U.S. Environmental Protection Agency's *Ecological Structural Activity Relationship Model* (ECOSAR) suggests sucralose may be toxic to aquatic plants at >1000 mg/L and the risk quotient for sucralose is low (Tollefsen et al., 2012). However, concerns remain due to a lack of aquatic plant ecotoxicology studies (Walker et al., 2012) and the possibility that sucralose may negatively affect plant carbon relations (Lubick, 2008; Reinders et al., 2006). Sucralose may affect plant carbon relations, such as uptake and photosynthesis. Reinders et al. (2006) showed that 8 mM of sucralose disrupted sugar cane's sucrose uptake gene, ShSUT1, which inhibited sucrose uptake and transport within the plant. Conversely, sucralose did not inhibit sucrose uptake in *Lemna gibba* after 24-h exposure to sucralose at 1000 mg/L, nor did it impact *L. gibba* wet weight or growth rate after 7-day exposure (Soh et al., 2011). The contrasting conclusions of these previous studies may be due to various reasons, including the differences in: plant physiology (e.g., vascular versus non-vascular), the duration of toxicity experiments (most studies were <7 days in duration), and the metrics chosen (e.g., ecological structure, function, behavior, or mortality). It was also speculated that sucralose can inhibit photosynthesis (Kessler, 2009; Lubick, 2008), though data supporting this claim are hard to substantiate in the existing literature. The determination of whether sucralose can affect plant carbon relations is a priority because aquatic plants comprise a large portion of the environment's total biomass and are a primary carbon source for higher trophic levels.

Fluoxetine in aqueous solution can be assimilated into *Lemna* plant tissue (Reinhold et al., 2010), but the effects on plant functions are not established (Silva et al., 2012). A PPCP concoction (ibuprofen, ciprofloxacin, and fluoxetine in high concentrations) caused mortality of *Lemna gibba* (Richards et al., 2004). Standard toxicity tests and hazard quotients suggest little risk of aquatic organisms to fluoxetine exposure, yet more studies from environmentally relevant concentrations of fluoxetine on a variety of aquatic plant metrics are needed (Pal et al., 2010; Rosi-Marshall and Royer, 2012).

Because PPCP have the potential to affect plants, laboratory phytotoxicity tests are commonly conducted on the higher aquatic plant, *Lemna* spp. (U.S. Environmental Protection Agency, 2016). *Lemna minor* and *L. gibba* (common names "duckweed" and "bayroot") are small, freshwater aquatic plants found worldwide. *Lemna* is a buoyant frond with a single root, has rapid asexual reproduction and growth, has a high bio-concentration capacity, and absorbs chemicals from liquid media directly into the leaf (Gorham, 1941). Hence, *Lemna* is a model for ecotoxicology assessment and as a tool for bioremediation (Forni and Tommasi, 2016; Greenberg et al., 1992). The increasing use of PPCP and environmental persistence calls for further ecotoxicological studies, including non-lethal effects such as declines in photosynthetic capacity.

We performed aquatic plant toxicity tests to evaluate how two common PPCP, sucralose and fluoxetine, affected *L. minor* physiology and function. Because sucralose structurally resembles sucrose-sugar and *Lemna* can obtain carbon via uptake of exogenous sugars, we hypothesized that *Lemna* would uptake sucralose from the aquatic medium. Sucralose was predicted to negatively affect plant growth, photosynthetic capacity, and reproduction because sucralose is a chlorinated molecule that would be a toxic and an unusable, exogenous sugar. The influence of fluoxetine on *Lemna* growth and function was also explored without any *a priori* hypotheses due to a lack of information in the literature regarding the impacts of fluoxetine on aquatic plants.

2. Materials & methods

2.1. River water chemistry

We sampled the water column and benthic sediments from the Portneuf River in southeastern Idaho, USA for PPCP on October 23, 2014 to determine the possibility of *L. minor* exposure to PPCP prior to the laboratory experiments. Samples were taken from above the wastewater treatment plant for the city of Pocatello, Idaho, USA (42° 54′ 49.06″N/112° 31′ 16.48″W) and 100 m below the wastewater treatment plant (42° 55′ 10.7″N/112° 31′ 26.33″W). We followed water collection protocols outlined by the Indiana State Department of Health Chemistry Laboratory (Indianapolis, Indiana, USA), who also analyzed the water samples. Specifically, two grab samples were taken from river water and sediments in acid-washed bottles. The water samples were filtered in the field while wearing latex gloves. 60 mL of water was filtered through a syringe fitted with a glass fiber filter (pore size = 0.7 μm) into a 1 L amber glass bottle containing the dechlorinating sodium thiosulfate preservative. All samples were immediately placed on ice and transported to the laboratory. The samples were frozen at −30 °C, shipped overnight on dry ice to the Indiana State Department of Health Chemistry Laboratory, kept frozen at −30 °C, and then thawed for analysis within 2 months. Pharmaceutical concentrations were determined using solid-phase extraction liquid chromatography mass spectrophotometry (SPE/LC/MS/MS) using an Applied Biosystems triple quad API 4000 equipped with an Agilent 1200 high performance liquid chromatograph. Detection limits were 0.5 ng/L and no contamination of analytes were detected within field blank samples used for quality control.

2.2. Plant collection & laboratory acclimation

Lemna minor collection and acclimation methods were identical for the laboratory experiments using sucralose or fluoxetine (described below). Healthy appearing *L. minor* was collected above the city of Pocatello's waste water treatment plant (WWTP) outlet were growing in spring-fed backwaters of the main channel of the Portneuf River. In the laboratory, we separated *L. minor* from other aquatic plants and algae attached to its fronds and rinsed with dechlorinated tap water. Acclimation and toxicity tests were conducted at Idaho State University, Pocatello, Idaho, USA in April 2015. *L. minor* was stored in a single, large container of dechlorinated tap water and placed in *Adaptis* environmental chambers (Conviron, Winnipeg, Manitoba Canada) set at 10 °C and 12 h of light (348 $\mu mol\ m^{-2}\ s^{-1}$) per day, similar to conditions where they were collected. After 3 days of acclimation to these conditions, we increased the temperature to 25 °C and light duration to 14 h per day to enhance growth (Brain and Solomon, 2007). *L. minor* was allowed to acclimate at the new conditions for 3 days prior to toxicity tests.

2.3. Experimental design

We followed OECD guidelines (OECD, 2006) with two exceptions: (1) We conducted a 21 day test (OECD is a 7 day test) and (2) the end points are not reported in ECx. We also followed standard procedures for a 21-day, static-renewal protocol designed for *Lemna* spp. toxicity testing (Brain and Solomon, 2007; Weber et al., 1991), with minor deviations described below. Two separate experiments were conducted using sucralose or fluoxetine, which were randomized, complete-block experimental designs (to