Title: 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; $\delta^{13}\text{C}$) were measured among treatments ranging from 0–15000 nmol/L-sucralose and 0–323 nmol/L-fluoxetine. Contrary to our predictions, sucralose significantly increased green leaf area, photosynthetic capacity, and $\delta^{13}\text{C}$ of *L. minor* at environmentally relevant concentrations. The increase of $\delta^{13}\text{C}$ 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.

Keywords: artificial sweetener; bioremediation; duckweed; pharmaceuticals and personal care products (PPCP); phytoremediation; selective serotonin reuptake inhibitor; water quality

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 $\mu\text{g/L}$ (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 $\mu\text{g/L}$ (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 $\mu\text{g/L}$ (Kolpin and Meyer, 2002; Silva et al., 2012).

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 >1,000 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-hour 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. EPA 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.

Materials & Methods

1.1 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.

1.2 Plant Collection & Laboratory Acclimation

Lemna minor collection and acclimation methods were identical for the laboratory experiments with sucralose and 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 hours of light ($348 \mu\text{mol m}^{-2} \text{s}^{-1}$) 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 hours 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.

1.3 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 account for any spatial effects from flask placement within the environmental chamber). Each experiment included six treatment levels (i.e., six concentrations of PPCP) and five replicates per treatment level (i.e., flasks containing *L.*

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4 161 *minor*). The treatment levels of sucralose ranged from 0-15089 nmol/L and for fluoxetine ranged
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6 162 from 0-323.28 nmol/L (Table 1). The treatment levels contained an order of magnitude
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9 163 difference of the added compound and are hereafter referred to as “control”, “low”, “medium”,
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12 164 “high”, “very high”, and “extreme” treatments. The treatment concentrations were based on
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16 166 (Kolpin and Meyer, 2002; Oppenheimer et al., 2011; Silva et al., 2012). Pure sucralose powder
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19 167 (Guardian Biosciences, Phoenix, Arizona, USA) and fluoxetine powder (Sigma Aldrich, Inc, St.
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21 168 Louis, Missouri, USA; CAS 56296-78-7) was used to make the test solutions, and both are
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24 169 highly stable in aqueous solutions for >30 days (Knight, 1994; Kwon and Armbrust, 2006). The
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26 170 solution was not fortified with sucrose because we wanted to be able to detect sucralose uptake
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29 171 using isotopes; specifically, sucralose is a derivative of sucrose and may not be discernable via
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31 172 isotopic signature. 125 ml sterilized flasks that held 100 ml of solution were used as the
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34 173 experiment units, for which there were five replicates per treatment. We placed 10 *L. minor*
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36 174 fronds in each experimental unit. Parafilm was used on the neck of the flask in order to reduce
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39 175 environmental contamination and evaporation of the solution. Measurements were taken on the
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41 176 individual fronds and calculated averages per flask (experimental unit) are reported for the
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43 177 graphics and statistics. Toxicity tests were conducted for a total of 21 days in order to: (1) allow
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46 178 enough time for plants to uptake compounds and to react to the different concentrations; (2)
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49 179 conclude the experiment prior to *L. minor*’s life expectancy of 28 days; and (3) differ from other
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51 180 experiments lasting <1 week in duration (Lillicrap et al., 2011; Soh et al., 2011).

52 53 181 **1.4 Plant Growth and Physiological Measurements**

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55 182 We manually counted the number of fronds on days 1 and 21. Absolute daily growth rates
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58 183 were calculated as the average number of new fronds (via asexual budding) per day and
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4 184 compared across treatments. The *per capita population growth rate* (r), or the biotic potential of
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$$r = (\delta N / \delta t) / N_0$$

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12 187 where $(\delta N / \delta t)$ was the change in population size from the start to finish of the experiment (21
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14 188 days), and

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16 189 N_0 was the beginning population size at the start of the experiment (day 1)

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19 190 The “ r ” was the exponential rate of population increase without density-dependence and
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21 191 represents the theoretical maximum rate of change of population size. We also measured the
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24 192 change in “green leaf area” of individual fronds (the dark green photosynthetic area of a leaf) by
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26 193 analyzing photographic images of fronds on days 1, 7, 14, and 21. The frond’s green leaf surface
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29 194 area was analyzed using ImageJ software (U.S. National Institutes of Health, Maryland, USA).

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31 195 We measured the length of the *L. minor* roots in the fluoxetine experiment because the root
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36 197 21. Root number and length were measured for five roots per fluoxetine treatment using
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41 199 We visually observed and documented the relative amount of bacterial colonies on the fronds
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43 200 and roots at the end of the experiment (day 21). First, we independently estimated the relative
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45 201 abundance of bacterial colonies on a scale of “no bacteria”, “some bacteria” and “high bacteria”,
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48 202 that was based on observable bacterial colonies in the form of a biofilm on the water’s surface
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51 203 and root systems. Secondly, we discussed our estimates and agreed on the relative amount of
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53 204 bacteria to report herein. This qualitative measure was chosen because this approach was time
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55 205 and cost effective and was able to highlight trends associated with sucralose.

We measured the quantum yield of photosystem II (Φ PSII) on days 1, 7, 14, and 21 using a mini-PAM photosynthesis yield analyzer (Heinz Walz GmbH, Effeltrich, Germany) on four fronds per flask. The Φ PSII was a direct measure of the capacity of light capture and conversion to photochemistry of photosystem II and was an indirect metric of overall photosynthetic capacity (Genty et al., 1989). For healthy plants, the maximum values of Φ PSII are ~0.8. Measurements were conducted under ambient (laboratory) light conditions. The mean value per flask was used for treatment comparisons.

1.5 Stable isotopes

On day 21, we destructively sampled *L. minor* to quantify the concentrations of carbon stable isotopes (^{13}C) for the sucralose experiment only. A hypothesis was formed that *L. minor* could uptake sucralose and this would be reflected in the plant's carbon isotopic signature. Each treatment provided two replicates that yielded 3 mg/sample. The samples were dried at 105°C for 24 hours, ground to a fine powder with a clean mortar and pestle, and then packed into tin capsules. The prepared samples were sent to the Isotope Analysis Laboratory at Idaho State University, Pocatello, Idaho USA and analyzed the same day. Samples were analyzed for carbon elemental concentrations using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies, Inc, Valencia, CA, USA) interfaced to a Thermo Delta V Advantage continuous flow isotope ratio mass spectrometer. The elemental analysis uses an evolutionary "flash combustion/chromatographic separation technique." The furnace temperature was kept at 1000°C, while the reduction oven was 650°C. The gases generated from the combustion of the samples are carried in a helium stream into a GC column held at 60°C, separated, diluted in the ConFlo IV, and finally passed to the mass spectrometer for analysis. Isotope ratios of $\delta^{13}\text{C}$ are reported as ‰ values relative to the PeeDee Belemnite (VPDB) scale; whereas $\delta^{15}\text{N}$ values are

reported as ‰ values relative to air-N₂. Three in-house standards (ISU Peptone, Costech Acetanilide and DORM-3), which are directly calibrated against international standards (IAEA-N-1, IAEA-N-2, USGS-25, USGS-40, USGS-41, USGS-24, IAEA-600), were used to create a two-point calibration curve to correct the raw data. A third standard (DORM-3) is used to monitor the accuracy of the data. Data are reported in standard delta notation ($\delta^{13}\text{C}$) relative to the reference standard, VPDB. Analytical precision from standards distributed throughout each run was calculated with precision $\leq \pm 0.2\text{‰}$. Isotopic values are reported in the conventional δ -notation ($\delta = ([R_{\text{sample}}/R_{\text{standard}}] - 1) * 1000$ and were expressed as per mil (‰).

The proportion of frond C derived from sucralose (x) or photosynthesis (1-x) was determined using a mixing model, in which $\delta^{13}\text{C}_{\text{frond}} = x(\delta^{13}\text{C}_{\text{sucralose}}) + (1-x)\delta^{13}\text{C}_{\text{photosynthesis}}$. This model provides a minimum estimate of the proportion of C from uptake because plants grown in liquid media can also potentially reabsorb a fraction of their own respired CO₂. To estimate the upper limit of the proportion of carbon from uptake, the value of $\delta^{13}\text{C}_{\text{photosynthesis}}$ in the mixing model was adjusted by assuming plants relied completely on re-fixation of their own respired CO₂. In this case, the end member for frond photosynthesis was $(\delta^{13}\text{C}_{\text{photosynthesis}}) + (\delta^{13}\text{C}_{\text{sucralose}} - \delta^{13}\text{C}_{\text{air}})$.

1.6 Statistical Analyses

We followed data analysis recommendations by Brain & Solomon (2007) and used SigmaPlot 13.0 (Systat Software, San Jose, CA, USA) for all statistical tests and graphics. Prior to analyses, all data were examined to meet statistical assumptions of normality and homoscedasticity using Shapiro-Wilk's Normality Test and the Brown-Forsythe Equal Variance Test. All data met the assumptions ($p > 0.05$), so no data were transformed. For all statistical analyses, "significance" was determined at a level of $p = 0.05$.

We assessed differences in response variables due to treatment level using one-way analysis of variance (ANOVA). The Dunnett's test was used for post hoc multiple comparisons between means of groups. For the sucralose experiment, the response variables included green leaf area, Φ PSII, count of new fronds (from asexual budding), growth rate, and intrinsic rate of population increase. In the fluoxetine experiment, the response variables were green leaf area, root length, count of new fronds (from asexual budding), growth rate, and intrinsic rate of population increase. We examined the relationships between sucralose concentration (the independent, explanatory variable) with Φ PSII and $\delta^{13}\text{C}$ (the dependent, response variables) using linear regression. One data point in the "Extreme" treatment category contained a statistical outlier, so we removed it from the analyses.

Results

1.7 River Water Chemistry

We detected sucralose in the Portneuf River water above and below the wastewater treatment plant, as well as in the riverine sediments below the plant (Table 2). Sucralose concentrations were two orders of magnitude greater below the wastewater effluent discharge than above the plant (0.60 $\mu\text{g/L}$ and 0.01 $\mu\text{g/L}$, respectively). Fluoxetine was detected only in the river's sediment at 0.03 $\mu\text{g/g}$. Several other PPCP were detected in the Portneuf River at very low concentrations (Appendix 1).

1.8 Sucralose Experiment

The green leaf area of fronds was significantly smaller for the control and low treatments compared to the medium to extreme sucralose treatments (ANOVA; $F(5,29)=4.72$, $p=0.004$). The control and low sucralose treatments experienced chlorosis in up to 50% of its leaf area, which is a reduction of the green photosynthetic pigments of the fronds (Fig. 1). In opposition,

the medium to extreme sucralose treatments (15-15000 nmol/L-sucralose) increased the green leaf area by an average of 30%. Bacterial colonies on *L. minor*'s roots were observed more frequently and were relatively more abundant in the high to extreme sucralose treatments (Appendix 2). All treatments experienced asexual reproduction via budding throughout the 21-day experiment and had a similar individual growth rate (about 2.5 fronds/day) and intrinsic rate of population growth (about $r=0.06$). Sexual reproduction by flowering did not occur during the study.

While Φ PSII initially declined in all treatments (between days 1-14), Φ PSII increased above starting values by day 21 in the high, very high, and extreme treatments (Figure 2). On day 21, Φ PSII of all sucralose treatments were significantly greater compared to the control (ANOVA; $F(5,28)=6.97$, $p < 0.001$; Dunnett's $p < 0.05$; Figure 3). The Φ PSII was positively related to sucralose concentration, and each order of magnitude of sucralose concentration increase resulted in ~1-percentage point increase of photosynthetic capacity.

The $\delta^{13}\text{C}$ was positively related to sucralose concentration (regression; $r^2=0.54$, $p = 0.015$; Fig. 4). Discrimination against the heavier C isotope decreased with increasing sucralose concentration and so the $\delta^{13}\text{C}$ became less negative. Based on a simple mixing model, C that originated from sucralose comprised 27-56% (minimum-maximum values) of frond C by the end of the experiment, indicating substantial uptake and assimilation of exogenous sucralose by *L. minor*.

1.9 Fluoxetine Experiment

Fluoxetine did not influence green leaf area at the conclusion of the experiment on day 21 (ANOVA; $F(5,29)=1.10$, $p=0.384$). The extreme fluoxetine treatment (323 nmol/L-fluoxetine) decreased root lengths by nearly 100% compared to the control (ANOVA; $F(5,29)=3.38$,

$p=0.024$; Fig. 5; Appendix 2). Some roots were completely detached and missing, but many of the roots remained attached to the frond but had shrunk. The extreme treatment also experienced a 50% reduction in asexual reproduction by budding. Thus, the daily growth rate ($F(5)=13.8, p=0.017$) and the intrinsic rate of population growth of *L. minor* in the extreme treatment were about half of *L. minor* in the control treatment. Sexual reproduction by flowering did not occur in any treatment group.

Discussion

Sucralose and fluoxetine are commonly present in aquatic environments and there is need for research studies that assess the fate, uptake and bioavailability, and biological impacts of such compounds (Boxall et al., 2012; Brooks et al., 2009). Our study showed sucralose and fluoxetine both influenced aquatic plant structure (i.e., green leaf area and roots) and function (i.e., photosynthetic capacity, isotopic discrimination).

1.10 Sucralose Experiment

Lemna spp. are mixotrophic plants that obtain C through photosynthesis and/or the uptake of exogenous sugars directly into the leaf (Gorham, 1941). However, it was unknown if *L. minor* would be able to uptake and assimilate C derived from sucralose because humans are unable to metabolize sucralose. In our experiment, two lines of evidence suggested uptake of sucralose from aqueous solution into the plant's cells. First, we observed chlorosis in *L. minor* in the control and low treatment groups and robust health and performance of *L. minor* in the higher sucralose concentrations. Ecotoxicology experiments often fortify the test solutions with sucrose to maintain optimal growth conditions (Brain and Solomon, 2007), but we did not use sucrose solution in order to test uptake ability of sucralose using isotopes. Thus, the observed chlorosis in

the low-sucralose-concentration groups may indicate negative C balance (i.e., carbon starvation). Our results suggest that *L. minor* may be an obligate mixotroph/photoheterotroph that requires uptaking exogenous sugars (sucrose or sucralose) or high gas exchange for optimal growing conditions. Second, although sucralose can disrupt sucrose cellular uptake in sugar cane plants (Reinders et al., 2006), our mixing model data suggested substantial uptake of sucralose (up to 56% C assimilation and a large change in isotopic composition) by *L. minor* from the aqueous solutions. The results from the mixing model are consistent with the increasing (less negative) $\delta^{13}\text{C}$ values with increasing sucralose concentration. This pattern suggests increasing mixotrophy with available sucralose compared to the control treatment, which had no exogenous sugar available, completely autotrophic fronds, and the most negative $\delta^{13}\text{C}$.

Bacteria may have either been involved with the breakdown of sucralose to sucrose or assisted *Lemna* with sucralose uptake because we noted greater bacteria presence in high sucralose treatments (Appendix 2). We chose not to sterilize *L. minor* prior to the experiments because it's not common protocol (Brain and Solomon, 2007), we wanted to minimize plant stress, and tested whether the plant was able to uptake sucralose regardless of the mechanism (e.g., frond diffusion or bacterial symbiosis or both). Although we observed more bacterial colonies with sucralose amendments, we did not identify the bacterial type and its functions (e.g., root symbiont, mineralizer, etc). Bacteria are not known to metabolize sucralose (Labare and Alexander, 1993; Omran et al., 2013), but bacteria are often root symbionts that facilitate plants with uptake of various compounds. In addition, some bacteria are able to mineralize sucralose in wet soils and lake water (Labare and Alexander 1993, 1994). The mineralized product of sucralose is similar to its derivative, sucrose (Knight, 1994), which is a plant- and bacteria-accessible sugar. In this experiment, the naturally colonized bacteria may have facilitated

sucralose uptake in *Lemna* by either of these mechanisms, but laboratory studies that isolate *Lemna* from bacteria through plant sterilization would be required to partition the contributions of bacteria in uptake.

Sucralose was likely a usable, exogenous sugar source that increased plant physiology and growth. While our dataset does not reveal the proximal mechanisms, we documented that increased sucralose concentrations led to greater photosynthetic capacity (Φ PSII; Figs. 3 & 4). With more carbon available from uptake, the plants can allocate more carbohydrate resources to their photosynthetic machinery, thus boosting their photosynthetic capacity. Other studies have documented that increased photosynthetic capacity and growth in fern gametophytes was due to the uptake of exogenous sugar (Hill et al., 2011). It is also possible that sucralose is a sugar useful for direct carbon biomass investment, in addition to increasing photosynthetic machinery—two alternative, but not necessarily mutually exclusive mechanisms for the observed increases in green leaf area.

Our statistically and biologically significant *Lemna* responses to sucralose differed from other laboratory studies, which showed no effects. Soh et al. (2011) reported no effects of sucralose on *L. gibba* (weight, frond number, and growth metrics) at concentrations up to 1000 mg/L after 7 days; similarly, a lowest observed effect concentration (LOEC) at 1000 mg/L-sucralose for *L. minor* was found after 7 days (Stolte et al., 2013). Lastly, Hu et al. (2016) did not detect impacts to green algae growth or photosynthetic capacity at 1 mg/L-sucralose and presence of heavy metals. The three studies differ from ours in the concentrations used (mg/L versus μ g/L) and the duration of study (4-7 days versus 21 days). For the first 14 days of this experiment, sucralose appeared to have no impact; however, by day 21, the higher sucralose treatments increased photosynthetic capacity (Fig. 2). We speculate that more time was required for *Lemna* to uptake,

assimilate, and metabolize the exogenous carbon source. Chronic (> 7 day) ecotoxicology studies such as ours are sparse (Stolte et al., 2013), but can reveal longer-term responses that would be experienced by organisms in natural aquatic systems.

1.11 Fluoxetine Experiment

Fluoxetine greatly affected *L. minor* roots and asexual reproduction at 323 nmol/L, but only a maximum concentration of 32 nmol/L has been reported in surface waters to date (Silva et al., 2012). At high concentrations (~65 nmol/L), fluoxetine can also suppress gross primary production rates from riverine biofilms under laboratory conditions (Richmond et al., 2016). Research has not identified threats to aquatic plants from environmentally relevant concentrations (<32 nmol/L-fluoxetine), but a study on green algae found cell deformations at concentrations similar to those detected in the environment (~50 nmol/L-fluoxetine; Brooks et al. 2003). It is possible that fluoxetine caused oxidative stress in *L. minor*, but our data did not evaluate this. Laboratory ecotoxicology studies such as these are informative for exploring non-traditional end points; however, it is unclear the transferability of our results to real ecosystems. For example, what are the consequences of root shrinkage from fluoxetine exposure (Fig. 5 and 6) on a mixotrophic plant like *L. minor*? Do the impacts of fluoxetine on *L. minor* cascade to affect the ecological community? In addition, we continue to lack mechanistic understanding of how fluoxetine affected plant cells, primary production, and asexual reproduction.

1.12 Field Applications & Conclusions

The fate of PPCP in natural aquatic environments is not well understood (Daughton and Ternes, 1999). Fluoxetine readily leaves the water column and absorbs to sediments, and therefore is predicted to minimally affect floating aquatic plants (Kwon and Armbrust, 2006). *Lemna* can uptake and sorb fluoxetine (Reinhold et al., 2010) but only extreme concentrations

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4 389 affected the plant's physiology (this study; Richards et al. 2004). Conversely, sucralose does not
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6 390 readily sorb to sediments (Table 2; Tollefsen et al. 2012), and this is the first study that showed
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9 391 uptake of sucralose by plants. Field studies, such as reciprocal transplants of *L. minor* above and
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11 392 below wastewater treatment plants, can further assess sucralose uptake and contribute to
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14 393 understanding the fate of sucralose in natural environments. Aquatic plants contribute to the
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16 394 depletion of PPCP in water and are likely important ecosystem compartments for sucralose and
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21 396 *Lemna's* ability to reduce PPCP from the water column can make the plant useful for
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24 397 bioremediation of wastewater (Forni and Tommasi, 2016; Greenberg et al., 1992). From our
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26 398 laboratory experiments, it is not certain how our results will translate to the population-scale;
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29 399 however, an increased photosynthetic capacity can increase *Lemna* population density through
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31 400 time. If true, *Lemna* "blooms" can deplete water column oxygen used by other aquatic biota and
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33 401 shade out submersed algae and plants in wetlands and retention ponds, and thus impact the
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36 402 ecosystem-scale (El-Kheir et al., 2007; Parr et al., 2002). In contrast, retention ponds with highly
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38 403 concentrated fluoxetine can reduce *Lemna* populations and its bioremediation potential. Future
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41 404 studies can examine concoctions of PPCP at the population and ecosystem scales in settings for
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43 405 bioremediation.

46 406 **Declarations**

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References

- Boxall, A., Rudd, M., Brooks, B., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? *Environ. Health Perspect.* 120, 1221–1229.
- Brain, R. a, Solomon, K.R., 2007. A protocol for conducting 7-day daily renewal tests with *Lemna gibba*. *Nat. Protoc.* 2, 979–987. doi:10.1038/nprot.2007.146
- Brooks, B., Huggett, D., Boxall, A., 2009. Pharmaceuticals and personal care products: research needs for the next decade. *Toxicol. Chem.* 28, 2469–2472.
- Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., 2003. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* 142, 169–183. doi:10.1016/S0378-4274(03)00066-3
- Daughton, C., Ternes, T., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ. Health Perspect.* 107, 907.
- El-Kheir, W., Ismail, G., El-Nour, F., Tawfik, T., 2007. Assessment of the efficiency of duckweed (*Lemna gibba*) in wastewater treatment. *Int. J. Agric. Biol.*
- European Union, 2004. No TitlDirective 2003/115/EC of the European Parliament and of the Council of 22 December 2003 amending Directive 94/35/EC on sweeteners for use in foodstuffs. *e. Off. J. Eur. Union* 47, 65–71.
- Forni, C., Tommasi, F., 2016. Duckweed: A Tool for Ecotoxicology and a Candidate for Phytoremediation. *Curr. Biotechnol.* 5, 2–10. doi:10.2174/2211550104666150819190629
- Genty, B., Briantais, J., Baker, N., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta (BBA)-* 990, 87–92.
- Gorham, P., 1941. Measurement of the response of *Lemna* to growth promoting substances. *Am. J. Bot.* 28, 98–101.
- Greenberg, B., Huang, X., Dixon, D., 1992. Applications of the aquatic higher plant *Lemna gibba* for ecotoxicological assessment. *J. Aquat. Ecosyst. Heal.* 1, 147–155.

- Hiemke, C., Härtter, S., 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmacol. Ther.* 85, 11–28. doi:10.1016/S0163-7258(99)00048-0
- Hill, J.P., Germino, M.J., Alongi, D.A., 2011. Carbon-use efficiency in green sinks is increased when a blend of apoplastic fructose and glucose is available for uptake. *J. Exp. Bot.* 62, 2013–2022. doi:10.1093/jxb/erq407
- Hu, H., Deng, Y., Fan, Y., Zhang, P., Sun, H., Gan, Z., Zhu, H., 2016. Effects of artificial sweeteners on metal bioconcentration and toxicity on a green algae *Scenedesmus obliquus*. *Chemosphere* 150, 285–293.
- Kessler, R., 2009. Water Treatment: Sweeteners Persist in Waterways. *Environ. Health Perspect.* 117, A438.
- Knight, I., 1994. The development and applications of sucralose, a new high-intensity sweetener. *Can. J. Physiol. Pharmacol.* 72, 435–439. doi:10.1139/y94-063
- Kolpin, D.W., Meyer, M.T., 2002. Pharmaceuticals , Hormones , and Other Organic Wastewater Contaminants in U . S . Streams , 1999 - 2000 : A National Reconnaissance 36, 1202–1211.
- Kwon, J., Armbrust, K., 2006. Laboratory persistence and fate of fluoxetine in aquatic environments. *Environ. Toxicol. Chem.* 25, 2561–2568.
- Labare, M., Alexander, M., 1994. Microbial cometabolism of sucralose, a chlorinated disaccharide, in environmental samples. *Appl. Microbiol. Biotechnol.* 42, 173–178.
- Labare, M., Alexander, M., 1993. Biodegradation of sucralose, a chlorinated carbohydrate, in samples of natural environments. *Environ. Toxicol. Chem* 12, 797–804.
- Lillicrap, A., Langford, K., Tollefsen, K., 2011. Bioconcentration of the intense sweetener sucralose in a multitrophic battery of aquatic organisms. *Environ. Toxicol. Chem.* 30, 673–681.
- Lubick, N., 2008. Artificial sweetener persists in the environment. *Environ. Sci. Technol.* 42, 3125.
- Naumann, K., 2000. Influence of chlorine substituents on biological activity of chemicals: a review. *Pest Manag. Sci.* 56, 3–21.

- 1
- 2
- 3
- 4 482 OECD, 2006. Test No. 221: Lemna sp. Growth Inhibition Test, OECD guidelines
- 5 483 for the testing of chemicals: Lemna sp. Growth Inhibition Test.
- 6 484 doi:10.1787/9789264016194-en
- 7
- 8
- 9
- 10 485 Omran, A., Baker, R., Coughlin, C., 2013. Differential bacteriostatic effects of
- 11 486 sucralose on various species of environmental bacteria. ISRN Toxicol. 2013,
- 12 487 415070. doi:10.1155/2013/415070
- 13
- 14
- 15 488 Oppenheimer, J., Eaton, A., Badruzzaman, M., Haghani, A., Jacangelo, J., 2011.
- 16 489 Occurrence and suitability of sucralose as an indicator compound of
- 17 490 wastewater loading to surface waters in urbanized regions. Water Res. 45,
- 18 491 4019–4027.
- 19
- 20
- 21
- 22 492 Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging
- 23 493 organic contaminants on freshwater resources: Review of recent occurrences,
- 24 494 sources, fate and effects. Sci. Total Environ. 408, 6062–6069.
- 25 495 doi:10.1016/j.scitotenv.2010.09.026
- 26
- 27
- 28 496 Parr, L., Perkins, R., Mason, C., 2002. Reduction in photosynthetic efficiency of
- 29 497 Cladophora glomerata, induced by overlying canopies of Lemna spp. Water
- 30 498 Res. 36, 1735–1742.
- 31
- 32
- 33
- 34 499 Reinders, A., Sivitz, A., Hsi, A., Grof, C., Perroux, J., Ward, J., 2006. Sugarcane
- 35 500 ShSUT1: analysis of sucrose transport activity and inhibition by sucralose.
- 36 501 Plant. Cell Environ. 29, 1871–1880.
- 37
- 38
- 39 502 Reinhold, D., Vishwanathan, S., Park, J., Oh, D., Saunders, F., 2010. Assessment
- 40 503 of plant-driven removal of emerging organic pollutants by duckweed.
- 41 504 Chemosphere 80, 687–692.
- 42
- 43
- 44 505 Richards, S., Wilson, C., Johnson, D., Castle, D., Lam, M., Mabury, S., Sibley, P.,
- 45 506 Solomon, K., 2004. Effects of pharmaceutical mixtures in aquatic microcosms.
- 46 507 Environ. Toxicol. Chem. 23, 1035–1042.
- 47
- 48
- 49
- 50 508 Richmond, E., Rosi-Marshall, E., Lee, S., Thompson, R., Grace, M., 2016.
- 51 509 Antidepressants in stream ecosystems: influence of selective serotonin
- 52 510 reuptake inhibitors (SSRIs) on algal production and insect emergence. Freshw.
- 53 511 Sci. 35, 000–000.
- 54
- 55
- 56 512 Rosi-Marshall, E.J., Royer, T. V., 2012. Pharmaceutical Compounds and
- 57 513 Ecosystem Function: An Emerging Research Challenge for Aquatic
- 58 514 Ecologists. Ecosystems 15, 867–880. doi:10.1007/s10021-012-9553-z
- 59
- 60
- 61
- 62
- 63
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- Silva, L.J.G., Lino, C.M., Meisel, L.M., Pena, A., 2012. Selective serotonin re-uptake inhibitors (SSRIs) in the aquatic environment: An ecopharmacovigilance approach. *Sci. Total Environ.* 437, 185–195. doi:10.1016/j.scitotenv.2012.08.021
- Silva, L.J.G., Pereira, A.M.P.T., Meisel, L.M., Lino, C.M., Pena, A., 2015. Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: Uptake, bioaccumulation and ecotoxicology. *Environ. Pollut.* 197, 127–143. doi:10.1016/j.envpol.2014.12.002
- Soh, L., Connors, K. a, Brooks, B.W., Zimmerman, J., 2011. Fate of sucralose through environmental and water treatment processes and impact on plant indicator species. *Environ. Sci. Technol.* 45, 1363–9. doi:10.1021/es102719d
- Stolte, S., Steudte, S., Schebb, N.H., Willenberg, I., Stepnowski, P., 2013. Ecotoxicity of artificial sweeteners and stevioside. *Environ. Int.* 60, 123–127. doi:10.1016/j.envint.2013.08.010
- Tollefsen, K.E., Nizzetto, L., Huggett, D.B., 2012. Presence, fate and effects of the intense sweetener sucralose in the aquatic environment. *Sci. Total Environ.* 438, 510–516. doi:10.1016/j.scitotenv.2012.08.060
- Torres, C.I., Ramakrishna, S., Chiu, C.-A., Nelson, K.G., Westerhoff, P., Krajmalnik-Brown, R., 2011. Fate of Sucralose During Wastewater Treatment. *Environ. Eng. Sci.* 28, 325–331. doi:10.1089/ees.2010.0227
- U.S. Environmental Protection Agency, 2016. Technical overview of ecological risk assessment analysis phase: Ecological effects characterization. U.S. Environ. Prot. Agency.
- U.S. Environmental Protection Agency, 1999. In Category for Persistent, Bioaccumulative, and Toxic New Chemical Substances. U.S. Environ. Prot. Agency 64, 60194–60204.
- U.S. Food and Drug Administration, 1998. Food additives permitted for direct addition to food for human consumption; sucralose. 21CFR Part 172 [Docket No. 87F-0086]. 63, 16417–16433.
- Walker, C., Sibly, R., Hopkin, S., Peakall, D., 2012. Principles of ecotoxicology, 4th ed. CRC Press.
- Weber, C.I., Environmental Monitoring Systems Laboratory (Cincinnati, O.,

Agency, U.S.E.P., 1991. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Epa-600/4-90-027.

Yoon, Y., Ryu, J., Oh, J., Choi, B.G., Snyder, S.A., 2010. Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). Sci. Total Environ. 408, 636–643.
doi:10.1016/j.scitotenv.2009.10.049

Tables

Table 1. The gradient of sucralose and fluoxetine concentrations chosen to observe the effects of sucralose and fluoxetine on local duckweed. The Low treatment of sucralose is comparable to recently sampled water below the wastewater treatment plant (Table 1). The highest reported concentrations found in surface water was 11 µg/L sucralose (Oppenheimer et al., 2011; Tollefsen et al., 2012) and 9 µg/L fluoxetine (Silva et al. 2012).

Treatment	Sucralose Concentration (µg/L)	Sucralose Molar Concentration (nmol/L; nM)	Fluoxetine Concentration (µg/L)	Fluoxetine Molar Concentration (nmol/L; nM)
Control	0.00	0.00	0.00	0.00
Low	0.60	1.51	0.01	0.03
Medium	6.00	15.09	0.10	0.32
High	60.00	150.89	1.00	3.23
Very High	600.00	1508.90	10.00	32.33
Extreme	6000.00	15089	100.00	323.28

Table 2. Concentrations of sucralose and fluoxetine above and below the waste water treatment plant (WWTP) on the Portneuf River in Idaho on October 23, 2014. We collected *Lemna minor* from a location above the WWTP to reduce environmental exposure to sucralose and fluoxetine prior to our laboratory experiments.

	Portneuf River Above WWTP	Portneuf River Below WWTP	
	Water Sample (µg/L)	Water Sample (µg/L)	Sediments (µg/g)
Sucralose	0.01	0.60	0.01
Fluoxetine	Below detection	Below detection	0.03

Figure Captions

Figure 1. Comparison of green leaf area (mean and 1 standard deviation) of *Lemna minor* green leaf area after exposure to different concentrations of sucralose for 21 days using a one-way analysis of variance and Dunnett's post-hoc test. The control and low treatments experienced chlorosis and therefore decreased in green leaf area (letter a), while the high to extreme treatments increased significantly in green leaf area (letter b).

Figure 2. Linear regression yields differences in the quantum yield of photosystem II (Φ PSII), organized by treatment and the days Φ PSII was measured during the 21-day sucralose experiment. Each data point represents the mean Φ PSII of an experimental unit (i.e., flask). The six treatments range in concentration from 0-15000 nmol/L-sucralose and are reported in parentheses.

Figure 3. A linear regression relating sucralose concentrations and photosynthetic capacity quantum yield of photosystem II (Φ PSII) of *Lemna minor*. Data presented are after 21 days of *L. minor* in solutions of sucralose water. An order of magnitude of sucralose concentration resulted in ~1-percentage point increase of photosynthetic capacity.

Figure 4. A linear regression relating $\delta^{13}\text{C}$ to the sucralose solutions. $\delta^{13}\text{C}$ increased with increasing sucralose concentration (i.e., became less negative), indicating less discrimination of the heavier ^{13}C isotope. The statistics reported are with the Extreme data excluded due to outliers. Data presented are after 21 days of *L. minor* in solutions of sucralose water. Note the 2 per mil change in $\delta^{13}\text{C}$, which is statistically and biologically significant.

Figure 5. Comparison of root lengths (mean and 1 standard deviation) of *Lemna minor* after exposure to different solutions of fluoxetine for 21 days. The "Extreme" fluoxetine treatment (323 nmol/L) had significantly shorter root lengths that averaged <1 cm (indicated by letter b).

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Figure 6. Example pictures of control (label: F-C3) and extreme (label: F-E5) root lengths due to fluoxetine treatment. Note the control root lengths are nearly 3x as long as the extreme treatment roots.

Figures

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Appendix

Appendix 1. Results of pharmaceuticals and personal care products detected above and below the wastewater treatment plant (WWTP) on the Portneuf River, Pocatello, Idaho, USA. Data are from a sampling conducted on 24 October, 2014. An * indicates a value below detection.

Compound	Portneuf River Above WWTP			Portneuf River Below WWTP		
	Water Sample (ng/L)	Field Blank (ng/L)	Sediments (ng/g)	Water Sample (ng/L)	Field Blank (ng/L)	Sediments (ng/g)
Acetaminophen	*	*	*	*	*	*
Albuterol	*	*	*	5.8	*	*
Azithromycin	*	*	*	*	*	5.5
Caffeine	*	*	*	40	*	*
Carbadox	*	*	*	*	*	*
Carbamazepine	*	*	*	22	*	*
Chloramphenicol	*	*	*	*	*	*
Cimetidine	*	*	*	*	*	*
Codeine	*	*	*	*	*	*
Cotinine	*	*	*	*	*	*
DEET	5.9	*	*	32	*	*
Diphenhydramine	*	*	*	*	*	12
Erythromycin	*	*	*	*	*	*
Fluoxetine	*	*	*	*	*	29
Gemfibrozil	*	*	*	*	*	*
Ibuprofen	*	*	*	*	*	*
Lincomycin	*	*	*	*	*	*
Naproxen	*	*	*	*	*	6.3
Norcodeine	*	*	*	*	*	*
O-Desmethyl Venlafaxine	*	*	*	*	*	*

Paraxanthine (1,7-Dimethylxanthine)	*	*	*	*	*	*
Rantidine	*	*	*	*	*	*
Sucralose	6.7	*	*	580	*	5.7
Sulfadimethoxine	*	*	*	*	*	*
Sulfamerazine	*	*	*	*	*	*
Sulfamethazine	*	*	*	*	*	*
Sulfamethoxazole	*	*	*	6.4	*	*
Sulfathiazole	*	*	*	*	*	*
Triclocarban	*	*	*	*	*	*
Triclosan	*	*	*	*	*	*
Trimethoprim	*	*	*	*	*	*
Tylosin	*	*	*	*	*	*
Venlafaxine	*	*	*	9.2	*	4.1

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Appendix 2. Visual observation of bacterial colonies growing on *Lemna minor* on Day 21 of the sucralose experiment. The blank columns indicates no bacterial colonies visually observed, the “X” indicates a relatively small abundance of bacteria colonies, and an “XX” indicates a relatively large bacterial colony abundance.

(Concentration of sucralose)		Bacteria on Frond	Bacteria on Root
Treatment: Replicate #			
(0.00 nmol/L)	Control : 1 Control : 2 Control : 3 Control : 4 Control : 5	X X X	X
(1.5 nmol/L)	Low : 1 Low : 2 Low : 3 Low : 4 Low : 5	X X X X X	X X
(15 nmol/L)	Medium : 1 Medium : 2 Medium : 3 Medium : 4 Medium : 5	XX X XX	X X
(150 nmol/L)	High : 1 High : 2 High : 3 High : 4 High : 5	X X	X X X X
(1500 nmol/L)	Very High : 1 Very High : 2 Very High : 3 Very High : 4 Very High : 5	XX X X	X X X X X
(15000 nmol/L)			

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Extreme : 1		X
Extreme : 2	X	X
Extreme : 3	X	X
Extreme : 4	X	X
Extreme : 5	X	X