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A prescription for drug-free rivers: uptake of pharmaceuticals by a widespread streamside willow

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Abstract

Following human excretion and limited removal with wastewater treatment, pharmaceuticals are accumulating in rivers worldwide. These chemicals can challenge the health of fish and aquatic organisms and since rivers provide drinking water sources, there is concern for cumulative exposure to humans. In this study, we discovered that sandbar willow (*Salix exigua*), a predominant riparian shrub along streams throughout North America, has the capacity to quickly remove pharmaceuticals from aqueous solutions. Our study tracked [^{3}H]- or [^{14}C]-labeled substances including 17α-ethynylestradiol (EE2), a synthetic estrogen in oral contraceptives; the antihypertensive, diltiazem (DTZ); and the anti-anxiety drug, diazepam (DZP); and for comparison, atrazine (ATZ), a root-absorbed herbicide. In growth chambers, willow saplings removed 40–80% of the substances from solutions in 24 h. Following uptake, the EE2 and DTZ were retained within the roots, while DZP and ATZ were partly passed on to the shoots. The absorbed EE2 was unextractable and apparently bound to the root tissue, while DTZ, DZP, and ATZ remained largely soluble (extractable). The uptake and translocation of the pharmaceuticals, reflected in the transpiration stream and root concentration factors, were reasonably predicted from their physicochemical properties, including octanol-water partitioning coefficients. These findings suggest the removal of pharmaceuticals as an unrecognized ecosystem service provided by riparian vegetation and especially the inundation tolerant sandbar willow. This encourages the conservation of riparian willows that line riverbanks, to remove pharmaceuticals and other contaminants. This phytoremediation also encourages the preservation of complex, braided channels and islands, which increase the extent of stream shorelines and riparian willows.

Keywords Atrazine · Diazepam · Diltiazem · Ethynylestradiol · Phytoremediation · *Salix exigua*

Introduction

With increasing and aging human populations, there have been progressive increases in pharmaceutical drug production and use, worldwide. With expiration or health recovery, some drugs are flushed down drains and much larger quantities are contributed to wastewater systems with human excretions (Kolpin et al. 2002; Petrie et al. 2015; Ebele et al. 2017). These pharmaceuticals are only partially removed with wastewater treatment and consequently the drugs are released with municipal effluent into rivers (Jackson and Sutton 2008; Verlicchi et al. 2012; Grassi et al. 2013). As a result, pharmaceuticals are detectable as low-level contaminants in most surface fresh waters that have been sampled from throughout North America and Europe and in other regions worldwide (Heberer 2002; Ebele et al. 2017). While the trace occurrence diminishes the prospects for acute impact on humans, there is concern about chronic exposure over the human lifetime and for interactions of these water-borne pharmaceuticals with other substances, including prescribed medicines (Carvalho et al. 2014).

There are also growing indications that chronic exposure to some drugs, and particularly the endocrine disrupting compounds, is harming aquatic vertebrates (Adeel et al. 2017). As with the concern for chronic human exposure, there are undoubtedly interactions across the various water-borne pharmaceuticals and other contaminants, as well as with naturally occurring substances and environmental conditions (Ebele et al. 2017).

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Fig. 1 Flooded sandbar willow (sw) in the Kootenai River, Montana

Following from these concerns, research has investigated wastewater treatments that could destroy or remove the pharmaceuticals and other compounds such as personal care products (Dong et al. 2015). There are also applications to utilize wetland systems for biological remediation, as aquatic plants in addition to sand, substrate and microbial filter systems are developed to remove pharmaceuticals and other contaminants (Carvalho et al. 2014; Zhang et al. 2014). These constructed wetlands, vary dramatically in efficacy and there is a continuing search for complementary approaches and particularly inexpensive, broad-scale measures.

There have also been investigations of phytoremediation of pharmaceuticals, with a range of terrestrial crop and native plants (Carvalho et al. 2014). Willows and poplars are among the plants that have been used successfully in phytoremediation of other compounds (Marmiroli et al. 2011), but there has been limited investigation into their use for the phytoremediation of pharmaceuticals (Iori et al. 2013; Bircher et al. 2015). As a novel complement to the current approaches for pharmaceutical removal from wastewater, we hypothesized that there could be a widespread and cost-effective opportunity with native riparian or streamside willows for the phytoremediation of pharmaceuticals from rivers and other freshwater.

Around the Northern Hemisphere, riparian woodlands are frequently dominated by plants of the Salicaceae (willow family), including willow (*Salix* spp.) shrubs, and cottonwood or riparian poplar (*Populus* spp.) trees (Karrenberg et al. 2002; Rood et al. 2003). The willows and cottonwoods are often obligate riparian plants, and thus restricted to the streamside zones, especially in semi-arid ecoregions. There, the plants are phreatophytes, with roots extending down to the shallow alluvial groundwater that is tightly linked to the streamflow (Rood et al. 2011a). The roots of the riparian plants are thus exposed to the shared surface and groundwater systems and are also directly contacted with river water during periods of high river flow, when the riparian willows are often inundated (Fig. 1), and growth and transpiration persist (Amlin and Rood 2001).

Of the willows, the sandbar, coyote, or narrowleaf willow (*Salix exigua;* syn. *S. interior*, *S. melanopsis, S. sessilifolia*) is the species complex that generally occurs at the lowest elevations and closest to the stream and is consequently most commonly inundated (Rood et al. 2011b). It is widespread through North America, being one of the continent's most widely-distributed woody plants (USDA, NRCS 2018), and is commonly the dominant shoreline shrub in riparian environments.

Sandbar willow is fast growing and expands vigorously through clonal suckering (Rood et al. 2011b). It reaches 3–4 m in height with fine branching and an extensive shoot system and leaf area, and is flood-tolerant, providing substantial capacity for water uptake and transpiration. It provides a range of important ecosystem services, providing rich wildlife habitat, contributing leaf litter and supporting invertebrates that contribute to the aquatic food webs, stabilizing stream banks, reducing erosion, and intercepting agricultural chemicals and nutrients. In addition to those valuable environmental services, we hypothesized that it might contribute to the removal of pharmaceuticals from stream systems. To investigate this prospect, we examined the capacity of sandbar willow saplings to remove pharmaceuticals from aqueous solutions in controlled environmental conditions.

We tested three widely used pharmaceuticals: 17α-ethynylestradiol (EE2), a synthetic estrogen used in birth control pills and an endocrine disrupting compound; the antihypertensive, diltiazem (DTZ; e.g., Cardizem®); and an anti-anxiety medication, diazepam (DZP; e.g., Valium®). As a positive control or reference compound, we also tested atrazine (ATZ), a C3 selective herbicide that is known to be root-absorbed and transported to shoots (Burken and Schnoor 1997). These compounds have been repeatedly reported from wastewater and surface waters, although at highly variable concentrations (Kolpin et al. 2002; Verlicchi et al. 2012), and represent a range of chemical classes and properties (Trapp 2004), and were consequently suitable for this study with *Salix exigua*.

Our objectives were to determine short-term uptake rates for these four compounds by the abundant and widely-distributed streamside shrub, and the subsequent distributions in the plants, as assessed by following radioisotope-labeled analogs. We were able to assess common measures of distribution, the root concentration factor (RCF) and transpiration stream concentration factor (TSCF, Briggs et al. 1982; Trapp 2000), to compare behavior among compounds in relation to their chemical properties (Shone and Wood 1974; Burken and Schnoor 1998), and to provide a more general framework for the applicability of these analyses (Lamshoeft et al. 2018).

Materials and methods

Plant materials and growth conditions

Sandbar willow (*Salix exigua* Nutt.) stems ('whips') were cut from native plants growing around a floodplain pond adjacent to the Oldman River below the University of Lethbridge in Lethbridge, Alberta (49°41'10.29"N; 112° 51'36.68"W). This location was upstream from the wastewater outflow from Lethbridge, the first city along the Oldman River which drains relatively pristine regions of the Rocky Mountains (Koning et al. 2006). Collection was in mid-March after winter chilling had relieved dormancy and before spring flushing commenced. The stems were stored wrapped in moist paper towels in a dark refrigerator at 4 °C until needed. For each experiment, apparently healthy stems were chosen and cut into 10 cm long segments with relatively uniform diameter of about 0.5 cm and a healthy bud below the apical cut. The cuttings were placed upright in pans of water at room temperature and after rooting commenced, were transferred to a hydroponic system within a growth chamber.

The system was adapted from Gibeaut et al. (1997), with 38 l opaque plastic tubs 60 cm L × 47 cm W × 22 cm H for the reservoirs (Fig. 2). Forty, 1.5 cm diameter holes were drilled in each opaque lid for the plants, and a continuous reservoir aeration system used an aquarium pump (Petcetera Air Pump AP-3800, Richmond, BC, Canada) and 12.7 cm long diffusing stone. Reverse osmosis purified water was used and following the addition of 2.5 ml/l Dutch Nutrient Formula Gro A & B solution (Homegrown Hydroponics, Peterborough, ON, Canada), the pH was 6.4. One rooted cutting was inserted through each hole and a strip of basalt fiber rock wool (FibrGrow Horticultural Products, Sarnia, ON, Canada) was wrapped around the cutting to provide support and block light while allowing reservoir aeration.

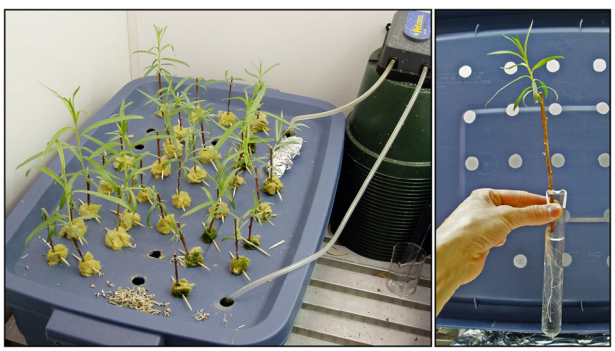

Fig. 2 The study set-up with the hydroponic system for growing the willow saplings (left) and the test tube treatment to assess chemical uptake (right)

The hydroponic apparatus was positioned in a Conviron Model E15 growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada) with temperature of 20 °C, 70% relative humidity, and a 16 h day/8 h night cycle, with 306 µmol s^{-1} m^{-2} photosynthetically active radiation at plant height from 400 W metal halide bulbs (Sylvania Metalarc, Mississauga, ON, Canada). Apparently healthy and fairly uniform 20–25-day-old willow cuttings with shoots 8–12 cm long were used for the uptake studies, and randomly assigned to the treatments.

Chemicals

As indicated, we investigated three pharmaceuticals: 17α-ethynylestradiol (EE2); diltiazem (DTZ); and diazepam (DZP), along with the herbicide, atrazine (ATZ) as a positive reference, a substance already known to be absorbed (Table 1). The non-radiolabeled substances (all ≥98% purity) were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON); DTZ as the hydrochloride salt, the pharmaceutical formulation of that drug, which will dissociate to DTZ in solution. The radiolabeled forms, cis-(+)-[N-methyl-^{3}H]-diltiazem (specific activity: 74.5 Ci $mmol^{-1}$; radiochemical purity > 97% by High Performance Liquid Chromatography), [methyl-^{3}H]-diazepam (86.0 Ci $mmol^{-1}$; radiochemical purity > 97%), and 17α-[6,7-^{3}H (N)]-ethynylestradiol (40.0 Ci $mmol^{-1}$; radiochemical purity > 97%) were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA). [Ring-U-^{14}C]-atrazine (10.35 mCi $mmol^{-1}$; radiochemical purity 96%) was obtained from Sigma-Aldrich Canada Ltd. The [^{3}H]-DTZ apparently degraded fairly quickly and was included only in the main experiment. Chemicals were dissolved in ethanol and then diluted with water for the treatment solutions, as indicated.

The uptake of substances from aqueous solution by plants depends strongly upon their chemical properties (Table 1). These include lipophilicity, or conversely hydrophobicity, as represented by the octanol-water partition coefficient (K_{ow}, expressed as log K_{ow}) and we obtained log K_{ow} values from ECOSAR™ (Ecological Structure Activity Relationship Model Class Program, MS-Windows Version 2, US Environmental Protection Agency, Washington, DC, USA). The coefficients are established with compounds in their neutral (uncharged) state, and given the acidic or basic character of the compounds, and their acid dissociation constants (K_a, expressed as pK_a; Table 1), EE2, DZP and ATZ can be expected to be neutral at the pH 6.4 of the experimental solution. In contrast, DTZ is likely to be ionized and its K_{ow} will not reflect the effects of its positive charge (Boxall et al. 2012; Carvahlo et al. 2014, Miller et al. 2016), which is recognized instead in the pH-adjusted K_{ow} (D_{ow}; Table 1).

Table 1 Chemical properties of the three pharmaceuticals and the herbicide used in the study of uptake by sandbar willow

Chemical	CAS number	Molecular mass (*g/mol*)	Log K_{ow}[a]	pK_a[b]	Acid/base character	Water solubility[a] (*mg/l*)
Ethynylestradiol (EE2)	57-63-6	296.4	3.67	10.4	weak acid	11.3
Diltiazem (DTZ)	42399-41-7	414.5	2.70[c]	8.0	base	465
Diazepam (DZP)	439-14-5	284.7	2.82	3.4	weak base	50
Atrazine (ATZ)	1912-24-9	215.7	2.61	1.7	weak base	34.7

[a]Log K_{ow} and solubility values from published values in ECOSAR™

[b]NCBI. PubChem Compound Database

[c]Log D_{ow} for DTZ at pH 6.4 is 1.1, from the equation $\log D_{ow} = \log K_{ow} + \log [1 + 10^{(pKa - pH)}]^{-1}$

Chemical uptake by whole plants

The main, whole plant experiment investigated the rates of removal from solution of the radiolabeled chemicals and then their subsequent distribution in the willow saplings. For each treatment, 1 µg of unlabeled compound and 1.67 kBq of the radiolabeled form were dissolved in ethanol and added to nutrient solution from the hydroponic system to produce 24 ml of solution with 42 ng/ml of EE2, DTZ, or DZP, or 81 ng/ml of ATZ (because the specific radioactivity of [^{14}C]-ATZ was lower), and 4.2 µl/ml ethanol in a 30 ml glass culture tube. These concentrations are higher than typically found in streams (Kolpin et al. 2002). In a preliminary study, we observed similar rates of [^{3}H]-EE2 uptake with the additions of 0, 1, or 10 µg non-labeled EE2 (Franks, 2006), indicating that results would be applicable over a range of concentrations and that the study dosage would not approach saturation.

Uniform rooted cuttings (saplings) were selected and for each treatment, one sapling was inserted into the test tube so that the roots that grew from the cutting base were largely submerged but the woody stem did not contact the solution (Fig. 2). Each culture tube was wrapped in aluminum foil to exclude light, and at the top the foil was wrapped tightly around the cutting to maintain its position and prevent evaporation. The wrapped tubes with willow saplings were placed in racks and returned to the growth chamber for the 24 h study. We independently developed this study system which is very similar to a system recommended by Lamshoeft et al. (2018) as a standard to determine chemical uptake factors by plants.

There were six replicates for EE2, DZP, and ATZ, and four for DTZ and we sampled the solution of each test tube at 0, 2, 4, 8, and 24 h. Before each sampling, the volume of nutrient solution was adjusted to 24 ml and the added volume was recorded as a measure of transpiration. After replenishment, an aliquot was removed and assessed with liquid scintillation counting (LSC) to determine the amount of remaining radiolabeled chemical in the solution. Aliquots were counted in Ecolite+ (MP Biomedicals, Santa Ana, CA, USA) scintillation cocktail, using a Beckman LS 5000 instrument (Beckman Coulter Inc., Brea, CA, USA).

Additionally, blank controls with 24 ml of each chemical solution chemical were included with foil-wrapped culture tubes. The volume drop in these blank controls was negligible (<1%), indicating minimal evaporation. Aliquots were sampled from the blank controls at each interval to consider aspects such as possible binding to glassware but this was also negligible.

After the 24 h sampling, the cuttings were removed from solution and the roots were rinsed in fresh solution and blotted dry. Each plant was then divided into three components: (1) the roots, (2) the woody stem cutting, and (3) the green shoot consisting of the new green stem and leaves. These were weighed fresh, frozen and stored at −20 °C prior to tissue combustion or solvent extraction.

Chemical uptake without transpiration

To determine chemical uptake without transpirational water flux, a subsequent experiment was undertaken but with (1) decapitated saplings, the roots and woody stem cuttings after the new shoot was cut off; or (2) only the roots, which were excised from the cuttings and then immersed in the solutions (3 replicates each). DTZ was omitted from this experiment because the [^{3}H]-DTZ had degraded. The treatment plants were selected for apparently similar root masses and one was placed in each culture tube containing 1 µg of non-labeled chemical and 0.83 kBq of the radiolabeled form, producing concentrations of 42 ng/ml in the nutrient solution for EE2 and DZP, and 61 ng/ml for ATZ. Each culture tube was foil-wrapped and these were placed in racks in the growth chamber and control blanks again indicated negligible evaporation or binding to glass. Without transpiration, the solution loss from the experimental tubes was minimal.

For this experiment, sampling was undertaken at 0, 1, 2, 4, 8, and 24 h. At 24 h, the willow tissues were harvested and roots were rinsed and blotted, similar to the whole plant study. The roots were excised from the cutting in the roots and cutting treatment, and the organs from each tube were

weighed fresh and frozen prior to combustion or solvent extraction.

Analyses of chemical distributions and extractability

Two procedures, combustion or solvent extraction, were undertaken to determine the distribution of the radiolabeled compounds across the harvested organs. For combustion, subsamples of the plant organ components (0.50–0.75 g fresh weight from roots and shoots, 0.20 g from cuttings) were combusted (oxidized) to determine the radioisotope distributions and this also provided total radioactivity levels to assess the recoveries with the solvent extraction procedure. For combustion, we used a Biological Material Oxidizer OX-500 (R.J. Harvey Instrument Corporation, Tappan, NY, USA). Following combustion of the [^{14}C]-ATZ samples, the [^{14}C]O_2 was trapped in Carbo-Sorb E and added to Permafluor E+ for LSC (PerkinElmer, Boston, MA, USA; 1:2 v/v; 15 ml for each LSC vial). Alternately, for the [^{3}H]-pharmaceuticals, the tritiated water from combustion was trapped in the scintillation cocktail Ecolite+, and assessed with LSC. Radioactivities recovered from combustion and determined by LSC were corrected for losses in the procedure based on parallel analyses of known quantities of the radiolabeled compounds.

The solvent extraction procedure sought to redissolve and extract the radiolabeled compounds and this produced two outcomes, the extractable or 'soluble' versus the unextractable or 'bound' fractions. This could indicate the likelihood that the pharmaceutical or its metabolites could be returned to the water system. The extractable fraction would probably be more readily leached from abscised (fallen) leaves than the bound fraction, although natural leaching with water would be lower than extraction with the aqueous solvent that was used. The frozen root or shoot tissue was ground in a mortar to a fine pulp using sand and 80% aqueous methanol (by volume) was added, the suspension was centrifuged, and the supernatant decanted. The residue was then re-extracted twice with 100% methanol. For the shoots, a fourth extraction with methanol was carried out if any green pigmentation remained. The supernatants were pooled, and aliquots were bleached using a 5.25% sodium hypochlorite solution and analyzed by LSC. After solvent treatment, the bound fraction remained and this tissue residue was combusted, followed by [^{14}C]O_2 or [$^{3}H_2$]O trapping and LSC to quantify that component.

Solvent extraction was more difficult with the woody stem cuttings. These were first ground to a fine powder in a mortar with sand and liquid nitrogen. A sub-sample was removed for combustion and another sub-sample underwent further grinding with 80% aqueous methanol and this was then homogenized with sonication using a Polytron (Kinematica CH-6010, Brinkmann Instruments, Westbury, NY, USA). This homogenized sample was then subjected to a similar solvent extraction procedure as that for the shoots, again including bleaching and LSC, and combustion of the final residue.

The two procedures, combustion or solvent extraction, revealed the distributions of the radiolabel [^{3}H] or [^{14}C] from the applied substances but their chemical status was not determined. Following uptake, there could be some metabolism of these substances in willow, as occurs in the closely related poplar (Burken and Schnoor 1997; Bircher et al. 2015) and other species (Carter et al. 2018), and the radiolabel tracking would thus reveal the distribution of the original substance and prospective radiolabeled metabolites.

Root and transpiration stream concentration factors

The RCF and TSCF provide measures of the chemical uptake by roots (RCF) or uptake and translocation with the transpiration stream into the shoot (TSCF; Briggs et al. 1982, Trapp 2000). These are assessed relative to the external concentrations, and standardized for root mass or transpiration volume to allow comparisons across samples and studies. The RCF values were calculated for each substance with the results from the experiment with the excised roots, and these could also be determined from the experiments with the roots attached to the cutting, or from the whole plant uptake study. The RCF equation was from Shone et al.(1974):

$$\mathrm{RCF} = \frac{[\text{concentration in the root } (\mu\text{g compound/g fresh root mass})]}{[\text{external solution concentration } (\mu\text{g/ml})]}$$

We calculated the TSCF for the four compounds from the whole plant uptake studies with the equation from Briggs et al. (1982):

$$\mathrm{TSCF} = \frac{[\text{concentration in transpired water } (\mu\text{g compound/ml volume transpired}]}{[\text{external solution concentration } (\mu\text{g/ml})]}$$

The concentration of the compound in transpired water was assessed indirectly (Briggs et al. 1982). The quantity of compound was assumed to be the amount determined from the radioactivity recovered from the shoot and cutting. We used results from oxidized plant parts, as these yielded the most consistent recoveries across samples. For the external solution concentration, the average of the initial and final values was used (Briggs et al. 1982). Although transpiration slowed through the dark phase of the diurnal cycle, we again used the 24 h results to provide consistency with prior studies. For earlier sampling points, the values were higher, but the relative rankings were similar across the chemicals and the correspondences with the K_{ow} were also similar for the different sampling intervals.

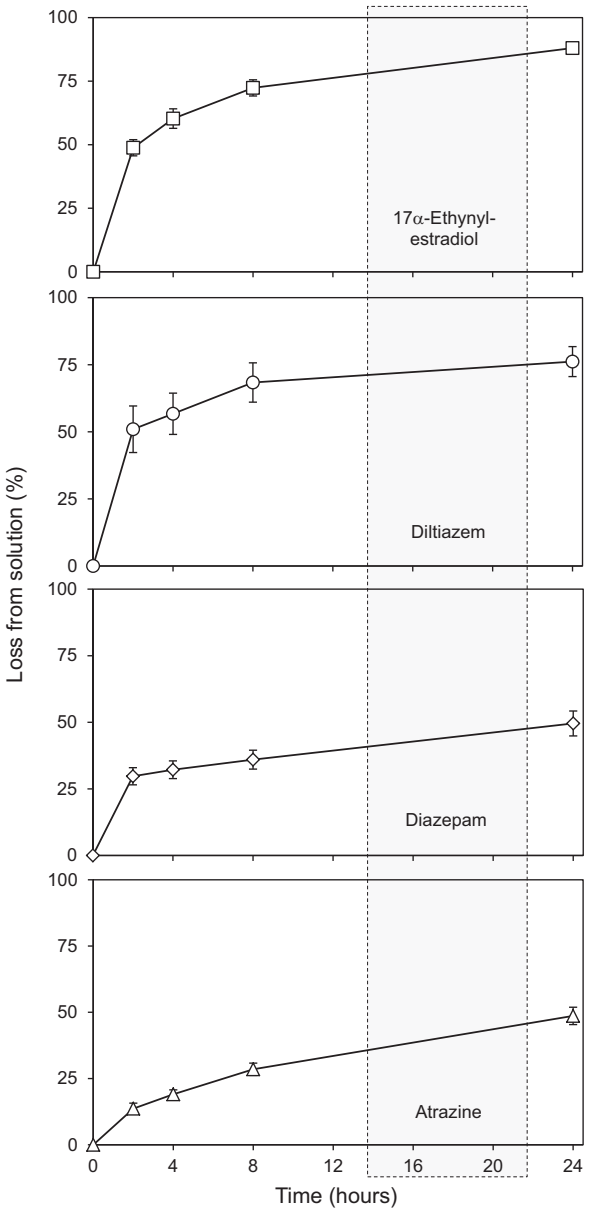

Fig. 3 Loss of radioactivity from solutions of [^{3}H]-17α-ethynylestradiol, [^{3}H]-diazepam, [^{3}H]-diltiazem, or [^{14}C]-atrazine, with plants of *Salix exigua*. Means ± SE, $n = 6$, diltiazem $n = 4$. The 16 h day and 8 h night (shaded) are indicated

Statistics

We used IBM SPSS Version 21 (Armonk, NY, USA) for analyses of variance (ANOVA) or covariance (ANCOVA) and calculation of Pearson correlation coefficients (r).

Results

Chemical uptake by whole plants

The radiolabeled chemicals were lost quickly from the aqueous solution in the culture tubes containing the willow saplings (Fig. 3). The uptake rate varied across the four substances and was most rapid for the synthetic estrogen, [^{3}H]-EE2, and then [^{3}H]-DTZ, followed by the other pharmaceutical, [^{3}H]-DZP, and finally the herbicide, [^{14}C]-ATZ. About one-half of the EE2 or DTZ was lost within the first 2 h after the saplings were added, while about one-third of the DZP and about one-sixth of the ATZ were lost. The uptake rates subsequently declined and this would have partly reflected the progressive removal of the chemicals, and dilution due to the solution replenishment for the transpiration loss. The uptake rates further declined from 8 to 24 h and this included the dark night phase with reduced transpiration (Fig. 3). After 24 h the uptake did not differ significantly between EE2 and DTZ, but was lower for both DZP and ATZ (Table 2).

No evidence for toxicity of the chemicals was apparent from observation of the plants or suggested by decline of transpiration rates (Clausen and Trapp 2017), which were quite consistent during the light phase and more gradual in the dark. There was no significant difference among the chemical treatments in the total volume transpired after 24 h (Table 2), and the volume transpired per unit shoot weight did not differ among treatments (ANCOVA, treatment × shoot weight, $P = 0.44$)

Although randomly selected, there were differences across the treatment groups for the cutting weights (Table 2). There was no significant difference for either root or shoot fresh weight, however. The total volume transpired

Table 2 Summary of fresh weight, transpiration and chemical uptake in *Salix exigua* plants in the whole plant uptake study

Chemical	Fresh weight (*g*)			Volume transpired (*ml*)	Chemical uptake (%)
	Roots	Cutting	Shoot		
Ethynylestradiol	0.47 ± 0.05a	0.91 ± 0.05ab	0.71 ± 0.05a	10.4 ± 0.6a	88.0 ± 1.0a
Diltiazem	0.41 ± 0.13a	0.61 ± 0.05b	0.64 ± 0.08a	10.0 ± 0.5a	76.2 ± 5.6a
Diazepam	0.53 ± 0.10a	0.76 ± 0.09b	0.93 ± 0.16a	13.7 ± 2.4a	49.6 ± 4.7b
Atrazine	0.48 ± 0.08a	1.23 ± 0.15a	1.00 ± 0.09a	14.3 ± 1.2a	48.6 ± 3.3b

Fresh weights were at the 25 h harvest and total volumes transpired and chemical uptakes were over the 24 h study, as displayed in Fig. 3. Means ± SE; values followed by different letters significantly differ (Tukey's HSD, after ANOVA; $P < 0.05$)

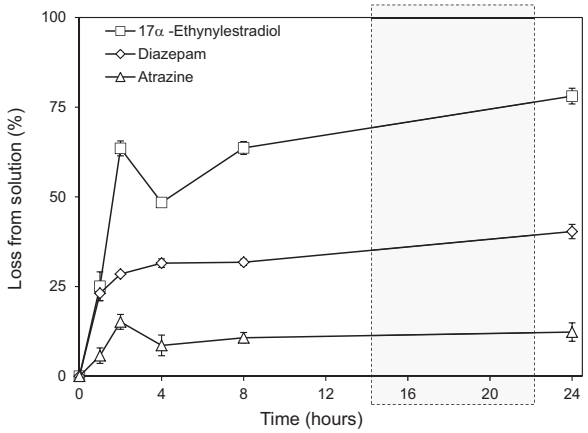

Fig. 4 Loss of radioactivity from solutions of [^{3}H]-17α-ethynylestradiol, [^{3}H]-diazepam, or [^{14}C]-atrazine, with roots excised from plants of *Salix exigua*. Means ± SE, $n = 3$. Loss at 24 h was used to calculate RCF (Fig. 7). The 8 h night period is shaded

after 24 h was correlated best with the shoot weights in each treatment (EE2, DZP, and ATZ, all $n = 6$, $r = 0.94$-0.96, $P < 0.01$; for DTZ, $n = 4$, $r = 0.92$, $P = 0.076$). Uptake was correlated with the volume transpired for ATZ and DZP ($r = 0.94$ and 0.91, $P < 0.05$), but not for DTZ ($r = 0.86$, $P = 0.14$) or EE2 ($r = 0.51$, $P = 0.31$).

Chemical uptake without transpiration

The experiments with excised roots were intended to analyze RCF, and the curves of loss from solution (Fig. 4) also suggest the influence of transpiration on uptake and translocation of the substances. In the absence of transpiration, the uptake of [^{3}H]-EE2 was similar to that observed in whole plants (Fig. 3), while that of [^{3}H]-DZP was reduced and [^{14}C]-ATZ was reduced even further. [^{3}H]-DTZ was not included in this later experiment because it had degraded.

Analyses of chemical distributions and extractability

We next analyzed the distributions of substances in the saplings, with the two procedures of whole tissue combustion, or solvent extraction followed by residue combustion. From the whole tissue combustion, mass balance calculations revealed that 82% of radioactivity originally applied as [^{3}H]-EE2 could be accounted for, with 72% in the plants; for [^{3}H]-DZP, 93%, with 46% in the plants; and for [^{14}C]-ATZ, 87%, with 38% in the plants. For [^{3}H]-DTZ, from solvent extraction, 99% of the applied radioactivity was recovered, with 79% in the plants.

We found that the [^{3}H]-EE2 was primarily in the roots with slight passage into the cutting and shoot (Fig. 5). [^{3}H]-DTZ was also almost only in the roots, and in contrast, [^{3}H]-DZP was slightly more abundant in the shoot than roots and some was in the woody cutting. [^{14}C]-ATZ was

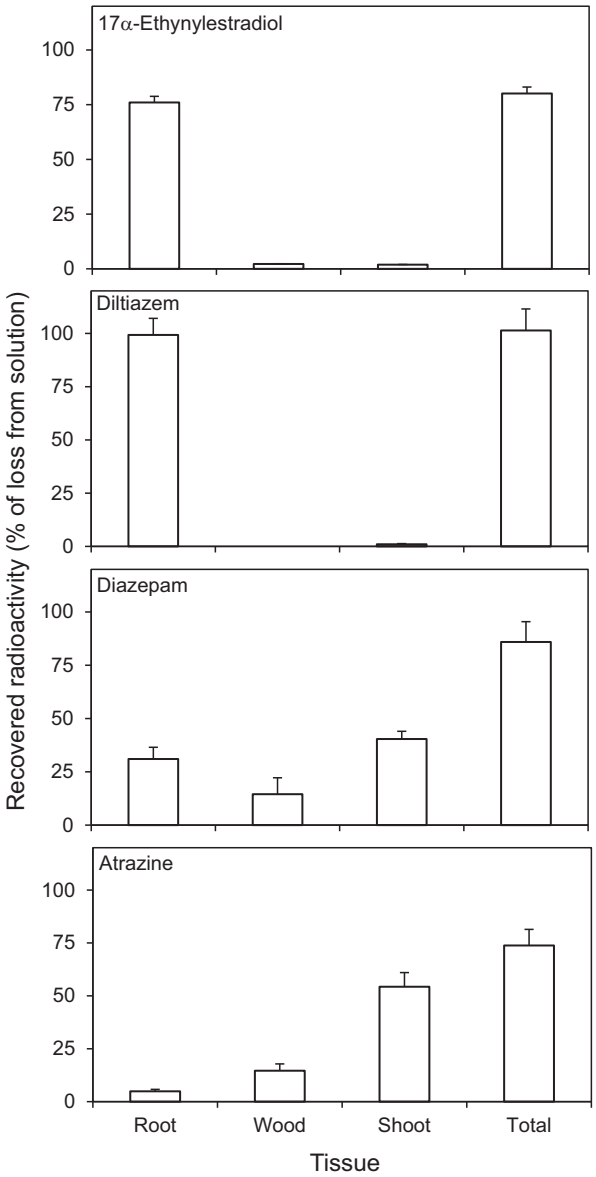

Fig. 5 Distribution of recovered radioactivity from combustion of whole roots, shoots, and cuttings, and total plant recovery (as percent of the loss from solution), for radiolabeled 17α-ethynylestradiol, diazepam, and atrazine added to *Salix exigua*. Means + SE, $n = 3$. For [^{3}H]-diltiazem ($n = 4$), the values are from the extraction

most extensively transported, with increasing proportion in the roots, cutting and shoots (Fig. 5). The distribution patterns contrasted with the uptake rates, as the most limited transport was for [^{3}H]-EE2 and [^{3}H]-DTZ, although they were more quickly removed from the nutrient solution.

The solvent extraction analyses confirmed the differentiation in organ distributions across the chemical treatments and also distinguished extractable (soluble) from unextractable ('bound') radioactivity (Fig. 6). Less than

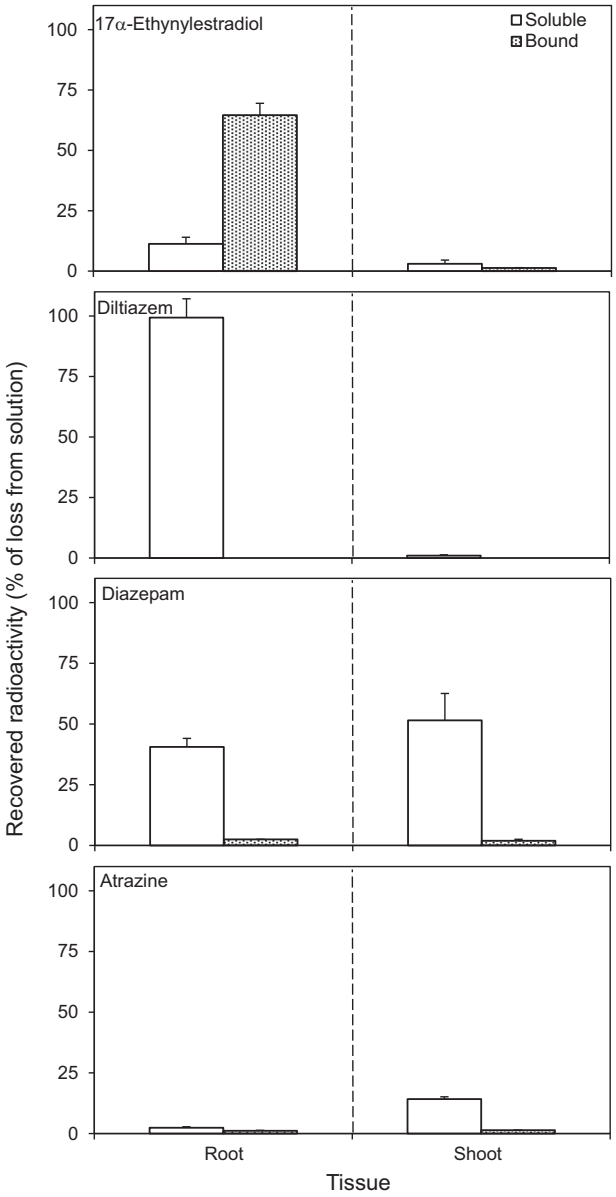

Fig. 6 Distribution of recovered radioactivity, as percent of the loss from solution, among extractable, or soluble, and unextractable, or bound, fractions in roots and shoots for radiolabeled compounds added to *Salix exigua* (means + SE). For 17α-ethynylestradiol, diazepam, and atrazine, $n = 3$. For diltiazem, $n = 4$

one-quarter of the ^{3}H from EE2 was extractable from the roots, while the vast majority remained in the bound fraction within the tissue residue that was combusted. In the shoots, the much lower quantity of ^{3}H from EE2 was largely extractable, and only a small component was in the bound fraction. For the DTZ treatment, the ^{3}H was again almost solely in the root fraction and this was soluble in aqueous MeOH, following the tissue grinding and extraction.

Confirming the patterns from the combustion procedure, the solvent extraction procedure revealed that the radiolabel was more extensively translocated following the [^{3}H]-DZP and [^{14}C]-ATZ treatments (Fig. 6). With [^{3}H]-DZP, there was substantial ^{3}H in the roots and this was mostly extractable. Some radioisotope was in the cutting, and the greatest quantity was in the shoots, and this was mostly extractable (Fig. 6).

The recovery of [^{14}C]-ATZ after solvent extraction was low compared with the combustion analysis (Fig. 5 versus Fig. 6). The majority of the ^{14}C was in shoots, and that was almost entirely extractable (Fig. 6). The minor component in the roots was also mostly extractable.

We similarly analyzed the radiolabel distributions following the experiment with the decapitated saplings and excised roots, and these supported the distributions from the whole plant study. Further results are provided in Franks (2006) and indicate that the ^{3}H from the [^{3}H]-EE2 or [^{3}H]-DZP treatments to the decapitated saplings was primarily retained in the roots, while more of the ^{14}C was passed to the cutting following the [^{14}C]-ATZ treatment. These distributions across the extractable or soluble, versus the unextractable or bound fractions were also supported in the excised roots study. The ^{3}H from [^{3}H]-EE2 was largely unextractable, while the ^{3}H was largely extractable following the [^{3}H]-DZP uptake, and as was the ^{14}C following the [^{14}C]-ATZ treatment.

Root and transpiration stream concentration factors

The relative uptake rates across the chemicals were similar for the excised roots, decapitated saplings, and whole plant experiments and from each of these, RCF were calculated. The calculations from the excised roots study avoided the complexity of redistribution and those RCFs are presented in Fig. 7 for the three neutral substances, and these were strongly positively correlated with log K_{ow}. For comparison, the RCF for the ionized compound DTZ, calculated from the whole plant study is also included. It clearly differed from the pattern with the neutral compounds, whether considered in terms of K_{ow} or D_{ow} (pH-adjusted K_{ow}).

From the whole sapling study, the TSCF were calculated, and the neutral compounds displayed strong negative correlation with log K_{ow} (Fig. 7). The TSCF for DTZ also differed from the pattern of the other chemicals. While the linear regressions for RCF and TSCF are plotted, with only three values these relationships are uncertain.

Discussion

Our study priority was to investigate the possible removal of pharmaceuticals from aqueous solutions by sandbar willow. This native riparian shrub is often abundant and generally the lowest-elevation woody plant in riparian zones

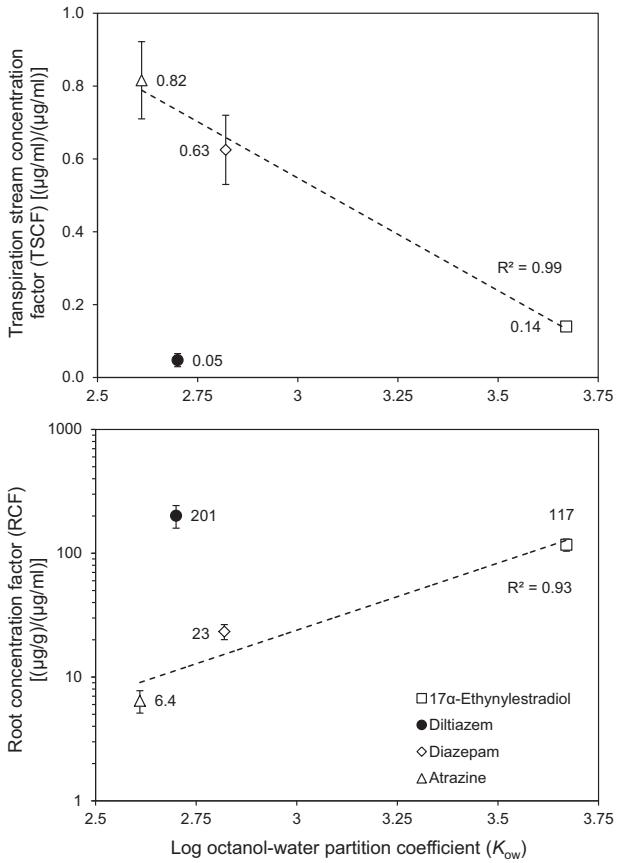

Fig. 7 Transpiration stream concentration factors (TSCF; top) and root concentration factors (RCF, log values; bottom) versus log octanol-water partition coefficients (log K_{ow}) for chemicals provided to *Salix exigua*. For ATZ, DZP and EE2 (neutral, open symbols), TSCF was calculated from translocation of radioactivity to the cutting and shoot in whole plants (as determined by combustion of plant parts), and the volume of water transpired after 24 h of treatments (see Methods). For DTZ (ionized, closed symbol), TSCF was calculated from extraction, where all radioactivity was recovered. RCF was calculated from uptake by excised roots, for ATZ, DZP, and EE2; for DTZ, the value is from the experiment with whole plants, with 99% of the uptake confined to the roots. Means ± SE, $n = 3$ for ATZ, DZP, EE2; $n = 4$ for DTZ. For comparison with log K_{ow} for DTZ, log D_{ow} (pH 6.4) = 1.1

in many regions of North America. At the study onset, we expected modest uptake, and so we included a positive reference with the addition of atrazine (ATZ), a widely used herbicide that kills willows and other C3 plants. This herbicide can be absorbed by roots, and ATZ uptake and metabolism have been studied in poplar trees (*Populus* sp.; Burken and Schnoor 1997), which are in the Salicaceae along with willows. As we thus expected, the [^{14}C]-ATZ was readily absorbed and translocated to shoots by the willow saplings, demonstrating the effectiveness of our study system.

Most importantly, we then observed rapid uptake of each of the three [^{3}H]-labeled pharmaceuticals. The uptake rate was similar to that of ATZ for [^{3}H]-diazepam (DZP), which represents a widely prescribed class of anti-anxiety benzodiazepines. Uptake was even faster for [^{3}H]-diltiazem (DTZ), representing another common class of drugs, antihypertensive calcium channel blockers. The [^{3}H]-17α-ethynylestradiol (EE2) is a synthetic estrogen in oral contraceptives and represents some endocrine disrupting compounds, and its uptake rate was also high. In assessing the rates of uptake and distributions, most of the radiolabel was accounted for during the 24 h studies. There were slight losses, as indicated, especially for [^{3}H]-EE2. It is possible that there were some losses through microbial metabolism in the non-sterile root zone or solution, or as volatile products of radiochemical decomposition or photodegradation, processes that could be explored in future studies (Zhang et al. 2014).

There have been few previous hydroponic studies with these pharmaceuticals, and those typically included longer exposure intervals. Examples include the studies of Wu et al. (2013) with DZP and vegetables, and Maharjan (2014) with DTZ and aquatic plants, and in both cases the chemicals were readily removed from solution, consistent with our findings. Also consistent with our results, Bircher et al. (2015) showed rapid uptake of EE2 and related compounds, in a hydroponic system with hybrid *Populus*. This rapid removal of EE2 provides a promising finding due to serious concern for this class of contaminants that are widespread in rivers and other freshwater resources (Heberer 2002, Kolpin et al. 2002).

Relative to the environmental benefits, removal from the water is critical but there must also be consideration for the fate of the chemicals after uptake. If a substance was readily absorbed but passed with the transpiration stream to the leaves and was then re-released, the contaminant could return to the aquatic system, thus reducing the beneficial phytoremediation. To consider the fates we investigated the distributions across the different plant organs and this revealed that [^{3}H]-EE2 and [^{3}H]-DTZ, or their radiolabeled metabolites, remained in the roots and thus translocation after uptake was limited, similar to the observations of Bircher et al. (2015) with EE2. Since these substances were not substantially translocated to the shoots and especially the leaves, they would not be available for subsequent re-release with the transpiration stream or with leaching from rain, or into the stream following leaf abscission.

In contrast to the root-localization of EE2 and DTZ, DZP was found in the roots, cutting and shoot, indicating some progressive translocation through the sapling, consistent with other studies (Wu et al. 2013; Carter et al. 2018). Diazepam (DZP) is chlorinated and apparently resistant to biodegradation, and is considered a contaminant of high environmental risk (Verlicchi et al. 2012). While it was effectively removed from solution by willows, there is the prospect that some of the DZP or its metabolites, which

could include oxazepam, itself a pharmaceutical (Carter et al. 2018), could be returned to the stream system with leaf loss. Metabolism by the plant or its microbial symbionts should reduce the biological activity and consequently re-release might have reduced environmental impact (Carvalho et al. 2014, Zhang et al. 2014, Bircher et al. 2015).

The herbicide ATZ or its metabolites were more mobile in the saplings, and after uptake was primarily found in the shoots. This is consistent with its mode of action, as it disrupts electron transport in photosystem II of photosynthesis, which must occur in the green shoot tissues. With its transport to the shoots and particularly leaves, there is the prospect that some of the ATZ or its metabolites would be returned to the stream system. While we included ATZ partly as a positive reference, this substance is also of considerable concern relative to environmental and human health. It has been banned in Europe due to groundwater contamination and has been among the most widely detected pesticide contaminants in American drinking water (Gilliom et al. 2006). While less potent than EE2, ATZ can also act as an endocrine disrupting compound in fish and amphibians (Hayes et al. 2011).

In addition to the distribution of the chemicals and their prospective metabolites, we also considered their extractability. We applied procedures that are highly effective for extracting the trace-occurring and diverse phytohormones, which are similar in sizes, solubilities, polarity, and octanol-water partitioning, as some of the pharmaceutical classes (Elias et al. 2012). We thus determined that DTZ, DZP, and ATZ were mostly extractable following tissue grinding and solvent extraction with aqueous methanol or pure methanol. In contrast, most of the EE2 could not be extracted with these or other solvents, even following homogenization with sonication, which is effective for removing a vast range of phytochemicals (Busov et al. 2006), although other extraction procedures could be applied in future studies (Petrie et al. 2017). The EE2 was apparently tightly bound to the root tissue and it would be unlikely that this substance would be re-released through natural leaching.

The distribution, and the related RCFs and TSCFs, and solubility of the compounds on extraction, reflects the effects of their physiochemical properties on uptake and translocation. The most lipophilic, EE2, is the most likely to be taken up or strongly absorbed to root lipids, and being unextractable may be covalently bound in root cells (Miller et al. 2016) and less available for further translocation within the plant. This would result in its high RCF, and low TSCF. The moderately lipophilic, neutral and soluble DZP and ATZ can apparently be readily taken into root cells and then translocated in the transpiration stream to the shoot, and hence their intermediate RCF values and comparatively high TSCF values. Linear correspondences of TSCF and RCF with log K_{ow}, as found in our study, are reasonable over limited ranges of log K_{ow} but the relationships are more complex for a wider range of pharmaceuticals (Miller et al. 2016; Doucette et al. 2018). Over a wide range of log K_{ow}, TSCF may vary in a bell-shaped (Briggs et al. 1982) or sigmoidal manner (Dettenmaier et al. 2009). Our outcomes provide evidence that the uptake of neutral, moderately lipophilic compounds by *Salix exigua* in a hydroponic system is predictable, as has been observed in similar studies with other compounds (Miller et al. 2016).

For some ionized compounds, uptake can be better-predicted on the basis of pH-adjusted K_{ow} (D_{ow}) (Miller et al. 2016; Doucette et al. 2018). For DTZ and RCF this was not the case. The low TSCF for DTZ was, however, consistent with a bell-shaped distribution of TSCF values (Briggs et al. 1982) based on log D_{ow} rather than log K_{ow}. Diltiazem was likely cationic in the conditions of our investigation, and may have been adsorbed to the anionic cell walls of the root, affecting its uptake (Miller et al. 2016), and also apparently leaving it as comparatively extractable.

We thus expect that these and some other pharmaceuticals would be readily taken up and effectively sequestered by riparian willows. We further anticipate that as the older, woody roots and stems die, they would be decomposed by fungi and other saprophytic organisms, which should readily metabolize the various substances. Our study provided an initial, short-term investigation and the future investigation could investigate longer-term distribution, binding, metabolism and decomposition, and particularly in a more natural streamside context.

With the strong likelihood that sandbar willows in natural streamside zones could remove pharmaceuticals from stream systems, we can also provide some guidance on promoting this phytoremediation. Along streams, the willows occur in low-lying positions with shallow alluvial groundwater that is exchanged with the stream water (Rood et al. 2003). The water infiltrates into and out from the alluvial zones and the extent of this exchange should influence the capacity for chemical uptake. Complex, shallow channels with braiding and islands would increase the extent of riverbank shorelines and correspondingly increase the prospect for phytoremediation by riparian willows. Conversely, alterations such as the common bank armoring and channelization would reduce the water exchange and the prospects for contact with willow roots and subsequent contaminant removal.

The patterns of river regulation will also influence the distribution of sandbar willows and other riparian plants as well as the depth to alluvial groundwater and periodic inundation that may increase the contact between riparian willow root systems and the stream water (Fig. 1). For the phytoremediation of aquatic contaminants, willows and especially the sandbar willow are particularly promising.

This shrub occurs in the lowest-elevation zones that are most often inundated and provide the shallowest depths to the alluvial groundwater and the most extensive exchange between that groundwater and the river water. These willows are highly flood- or inundation tolerant and unlike many riparian plants that will reduce or cease root uptake and transpiration when flooded due to anoxia, the willows have aerenchyma that enables oxygenation of root systems (Jackson and Attwood, 1996).

Finally, this finding that the abundant native streamside plant, sandbar willow, can rapidly remove pharmaceuticals from water indicates an unappreciated ecological service. It is known that riparian willows and cottonwoods can intercept and assimilate nutrients and agricultural chemicals from surface and groundwater systems (Burken and Schnoor 1997; 1998) and our new finding would add additional pharmaceuticals to the range of deleterious contaminants that could be removed. This provides yet one more reason to conserve and restore riparian shrubs and trees along rivers throughout North American and around the world.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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