Du, B. *et al.* 2014. Bioaccumulation and trophic dilution of human pharmaceuticals across trophic positions of an effluent-dependent wadeable stream. *Phil. Trans. R. Soc. B.* **369** doi: 10.1098/rstb.2014.0058

Supplementary material

Plasma and tissue extractions

Plasma sample extraction followed a slightly modified methodology [1]. An aliquot of fish plasma (up to 1 mL), combined with the same mixture of internal standards that was used for remaining analyses, were diluted to 5 mL using 0.1% (v/v) aqueous formic acid. The mixture was subsequently loaded on pre-conditioned (5 mL of methanol and 5 mL of nano-pure water) HLB SPE cartridges (200mg, Waters Corp, Milford, MA, USA). Each cartridge was air-dried and then eluted with 5 mL of methanol.

Whole issue sample extraction protocol generally followed previously developed methodologies [2, 3]. Whole individual invertebrates were grouped into composites up to 1 g (w/w). Soft tissue was rinsed in DI water and gently dried prior to weighing and composite grouping. In regard to fish and periphyton, samples were treated individually and approximately 1 g of sample was weighed prior to homogenization. All samples were homogenized using a Tissuemiser. A mixture of 23 isotopically-labeled standards (corresponding to deuterated analogs of each target analyte) was added to each sample prior to extraction. Resulting concentrations of labeled standards in each sample were approximately 100 ng/g. Samples were equilibrated by gentle end-over-end inversion for 20 minutes at 25 ± 0.1 ^{o}C.

LC-MS/MS and stable isotope analysis

Analytes were separated on a 15 cm × 2.1 mm (5μ m, 80 Å) Extend-C18 column (Agilent Technologies, Palo Alto, CA) connected with an Extend-C18 guard cartridge 12.5 mm x 2.1 mm (5 μm, 80 Å) (Agilent Technologies, Palo Alto, CA), employing a Varian ProStar Model 212 pump system equipped with a Model 410 autosampler. A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to achieve chromatographic separation. Eluted analytes were monitored by MS/MS using a Varian model 1200L triple-quadrupole mass analyzer equipped with an electrospray interface (ESI). Calibration curves were constructed by plotting the analyte response factor, which is the ratio of the target analyte signal to the signal of the corresponding isotopically-labeled standard, versus analyte concentration and used to determine analytical concentrations of target analytes. Linear regression with $1/x^2$ weighting resulted in $r^2 \geq 0.998$ for all compounds. Each analytical sample batch included one method blank and duplicate matrix spikes. Instrument calibration was monitored by analyzing continuing calibration verification (CCV) samples between every 5 samples with an acceptability criterion of ±20% [2]. Periphyton and whole biological tissue samples were dried to constant weight (for 24 h at 95 °C in a drying oven) and crushed to a fine powder using a mortar and pestle. These samples were weighted approximately at 1 mg and wrapped in Sn capsules following with the instrumental analysis using a dual-inlet gas-source Stable Isotope Mass Spectrometer and an Elemental Analyzer.

Table S1. List of standards of target analytes, corresponding internal standards, and vendors which compounds were purchased from.

Standards		Vendor[a]
Analgesic	Acetaminophen	Cerilliant
	Acetaminophen d_4	Cerilliant
	Codeine	Cerilliant
	Codeine d_3	Cerilliant
Antihypertensive	Atenolol	Cerilliant
	Atenolol d_7	TRC
	Propranolol.HCl	Cerilliant
	Propranolol d_7	TRC
	Diltiazem.HCl	Cerilliant
	Diltiazem d_3.HCl	TRC
Stimulant	Caffeine	Cerilliant
	Caffeine d_9	TRC
	Methylphenidate.HCl	Cerilliant
	Methylphenidate d_9.HCl	Cerilliant
Anti-seizure	Carbamazepine	Cerilliant
	Carbamazepine d_{10}	Cerilliant
Benzodiazepine	Diazepam	Cerilliant
	Diazepam d_5	Cerilliant
Antihistamine	Diphenhydramine.HCl	Cerilliant
	Diphenhydramine d_3	Cerilliant
Antidepressant	Fluoxetine.HCl	Cerilliant
	Fluoxetine d_3.Oxalate	Cerilliant
	Paroxetine.Maleate	Cerilliant
	Paroxetine d_6.Maleate	Cerilliant
	Sertraline	Cerilliant
	Sertraline d_3.HCl	TRC
Antidepressant metabolite	Norfluoxetine.Oxalate	Cerilliant
	Norfluoxetine d_6.Oxalate	Cerilliant
	Desmethylsertraline.HCl	TRC
	Desmethylsertraline d_4.HCl	TRC
Antilipemic	Gemfibrozil	Sigma-Aldrich
	Gemfibrozil d_6	TRC
Antibiotic	Sulfamethoxazole	Cambridge
	Sulfamethoxazole d_4	TRC
	Trimethoprim	Cambridge
	Trimethoprim d_9	TRC
	Erythromycin	Sigma-Aldrich
	Erythromycin $^{13}Cd_3$	TRC
Anti-inflammatory	Diclofenac sodium	Sigma-Aldrich
	Diclofenac d_4	TRC
	Celecoxib	TRC
	Celecoxib d_4	TRC
Anticoagulant	Warfarin	Cerilliant
	Warfarin d_5	TRC
Artificial sweetener	Sucralose	Sigma-Aldrich
	Sucralose d_6	Santa Cruz

a. TRC-Toronto Research Chemicals (Toronto, Ontario, Canada); Cambridge-Cambridge Isotope Laboratories (Andover, MA, USA); Santa Cruz-Santa Cruz Biotechnology (Dallas, Texas, USA)

Table S2. Summary of tissue samples from the North Bosque River, Texas, USA.

Organism (N)	Weight (g) (mean±SD)	Length (cm) (mean±SD)	Composite	If composite, no. of organisms used
H. azteca (~4000)	<0.001	NA	Yes	~1000
R. elongate (16)	0.08±0.01	NA	Yes	5
Planorbis sp. (68)	0.11±0.01	NA	Yes	9
Small *C. fluminea* (165)	0.07±0.01	NA	Yes	17
Medium *C. fluminea* (46)	0.21±0.02	NA	Yes	5
Large *C. fluminea* (10)	1.4±0.26	NA	No	NA
U. imbecilis (2)	1.2	NA	No	NA
U. tetralasmus (1)	30	NA	No	NA
G. affinis (200)	0.06±0.01	NA	Yes	20
Small *L. megalotis* (10)	9.6±3.4	7.7±0.73	No	NA
Medium *L. megalotis* (10)	25±7.5	10±1.1	No	NA
Large *L. megalotis* (9)	68±20	15±1.4	No	NA
M. salmoides (2)	42	15	No	NA
A. natalis (1)	110	20	No	NA
I. punctatus (1)	51	16	No	NA
L. yanellus (2)	51	16	No	NA

Table S3. Water chemistry data from a 3-day sampling event (June 17-19, 2013) of the North Bosque River, Texas, USA, downstream from the Stephenville, Texas, wastewater treatment plant discharge.

	Ammonia[a]	Nitrate/Nitrite	Total Phosphorous	Phosphate	pH	DO (mg/L)	Specific Conductance (mS/cm)	T (°C)
Day 1	75.4	6840	1110	843	7.93	8.40	1.09	30.0
Day 2	85.1	6745	1140	745	7.89	8.92	1.02	26.2
Day 3	78.3	6830	950	581	7.87	8.18	1.06	26.5

[a] Nutrient data are expressed in µg/L.

Table S4. Method detection limits (MDLs) and occurrence of analytes in water and *Lepomis megalotis* plasma from the North Bosque River, Texas, USA.

Compounds	Surface Water (ng/L; mean±SD; n=3)				Fish Plasma (µg/L) (mean±SD; n=15)	
	MDL	Day 1	Day 2	Day 3	MDL	Detected
Acetaminophen	2.9	ND	ND	ND	4.7	ND
Codeine	8.3	ND	ND	ND	6.9	ND
Atenolol	4.3	ND	ND	ND	2.9	ND
Propranolol	1.8	ND	ND	ND	0.58	ND
Diltiazem	0.24	33±10	27±4.3	23±11	0.12	0.16±0.14
Caffeine	4.5	15±5.5	15±1.8	11±2.6	4.7	ND
Methylphenidate	0.30	ND	ND	ND	0.27	ND
Carbamazepine	0.53	360±8.3	370±4.4	370±14	0.53	4.1±3.8
Diazepam	4.6	ND	ND	ND	2.8	ND
Diphenhydramine	0.22	19±12	19±3.0	20	0.12	3.0±2.2
Fluoxetine	8.4	ND	ND	ND	5.5	ND
Paroxetine	11	ND	ND	ND	1.8	ND
Sertraline	6.1	ND	ND	ND	3.7	ND
Norfluoxetine	8.5	ND	ND	ND	3.9	ND
Desmethylsertraline	5.4	ND	ND	ND	8.3	ND
Gemfibrozil	2.1	35±11	31±4.5	31±10	6.9	ND
Sulfamethoxazole	1.3	98±19	92±11	87±1.6	1.9	ND
Trimethoprim	1.3	ND	ND	ND	2.8	ND
Erythromycin	8.6	ND	ND	ND	8.6	ND
Diclofenac	2.8	79±43	71±29	86±55	0.84	ND
Celecoxib	11	130±49	81±10	79±27	7.9	ND
Warfarin	0.78	ND	ND	ND	0.93	ND
Sucralose	36	20000±5500	20000±4800	16000±1500	37	ND

Table S5. Predicted therapeutic hazard values and fish plasma levels, and observed surface water concentrations and *Lepomis megalotis* plasma concentrations for carbamazepine, diltiazem and diphenhydramine from the North Bosque River, Texas, USA.

Compound	Predicted THV (ng/L)			Observed Surface Water (ng/L; N =3; ±SE)	Predicted Fish Plasma (µg/L)			Observed Fish Plasma (µg/L; N=15; ±SD)
	K_{OW}	D_{OW}	D_{lipw}		K_{OW}	D_{OW}	D_{lipw}	
Carbamazepine[1]	421897	421897	148249	370±8.6	1.7	1.7	4.9	4.1±3.8
Diltiazem[1]	69	436	265	28±5.4	12	1.9	3.1	0.16±0.14
Diphenhydramine[1]	1952	10547	3706	19±0.76	0.5	0.09	0.26	3.0±2.2

[1]Human C_{max} values for carbamazepine, diltiazem and diphenhydramine are 2000, 30 and 50 µg/L, respectively (Schulz, M., Iwersen-Bergmann, S., Andresen, H., Schmoldt, A., 2012. Therapeutic and toxic blood concentrations of nearly 1000 drugs and other xenobiotics. Critical Care 16, R136). pKa of diltiazem and diphenhydramine are 8.94 and 8.98, respectively. THV – therapeutic hazard value.

Table S6. Stable isotopes and trophic positions of study organisms and periphyton from the North Bosque River, Texas, USA.

common Name	species name	n	$\delta^{13}C$	$\delta^{15}N$	trophic position
Primary Producer					
Periphyton		10	-26.30 ± 2.12	12.34 ± 0.82	1.55 ± 0.24
Invertebrates					
Asian clam	*Corbicula fluminea*	29	-24.79 ± 0.16	14.46 ± 0.50	
small		10	-24.91 ± 0.11	13.88 ± 0.21	2.00
medium		9	-24.74 ± 0.15	14.54 ± 0.14	2.19 ± 0.04
large		10	-24.72 ± 0.17	14.98 ± 0.17	2.32 ± 0.05
Water scorpion	*Ranatra elongata*	3	-24.63 ± 0.80	14.47 ± 1.01	2.17 ± 0.30
Snail	*Planorbis* sp.	8	-28.12 ± 1.13	14.24 ± 0.89	2.11 ± 0.26
Amphipods	*Hyalella azteca*	4	-28.01 ± 0.01	14.31 ± 0.08	2.13 ± 0.02
Paper pondshell	*Utterbackia imbecilis*	2	-24.59	15.76	2.55
Pondhorn mussel	*Uniomerus tetralasmus*	1	-26.20	14.58	2.21
Vertebrates					
Largemouth bass	*Micropterus salmoides*	2	-23.69	19.20	3.56
Mosquito fish	*Gambusia affinis*	10	-25.07 ± 0.18	16.70 ± 0.22	2.83 ± 0.06
Bullhead catfish	*Ameiurus natalis*	1	-24.88	19.49	3.65
Channel catfish	*Ictalurus punctatus*	1	-25.86	15.52	2.48
Green sunfish	*Lepomis cyanellus*	2	-24.27	20.75	4.02
Longear sunfish	*Lepomis megalotis*	29	-24.50 ± 0.50	20.67 ± 0.69	4.00 ± 0.20
small		10	-24.65 ± 0.49	20.79 ± 0.46	4.03 ± 0.14
medium		10	-24.52 ± 0.54	20.96 ± 0.77	4.08 ± 0.23
large		9	-24.30 ± 0.46	20.21 ± 0.65	3.86 ± 0.19

[1] Fick, J., Lindberg, R.H., Parkkonen, J., Arvidsson, B., Tysklind, M. & Larsson, D.G.J. 2010 Therapeutic Levels of Levonorgestrel Detected in Blood Plasma of Fish: Results from Screening Rainbow Trout Exposed to Treated Sewage Effluents. *Environmental Science & Technology* **44**, 2661-2666.
[2] Du, B., Perez-Hurtado, P., Brooks, B.W. & Chambliss, C.K. 2012 Evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for pharmaceuticals in fish. *Journal of Chromatography A* **1253**, 177-183.
[3] Ramirez, A.J., Mottaleb, M.A., Brooks, B.W. & Chambliss, C.K. 2007 Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **79**, 3155-3163.