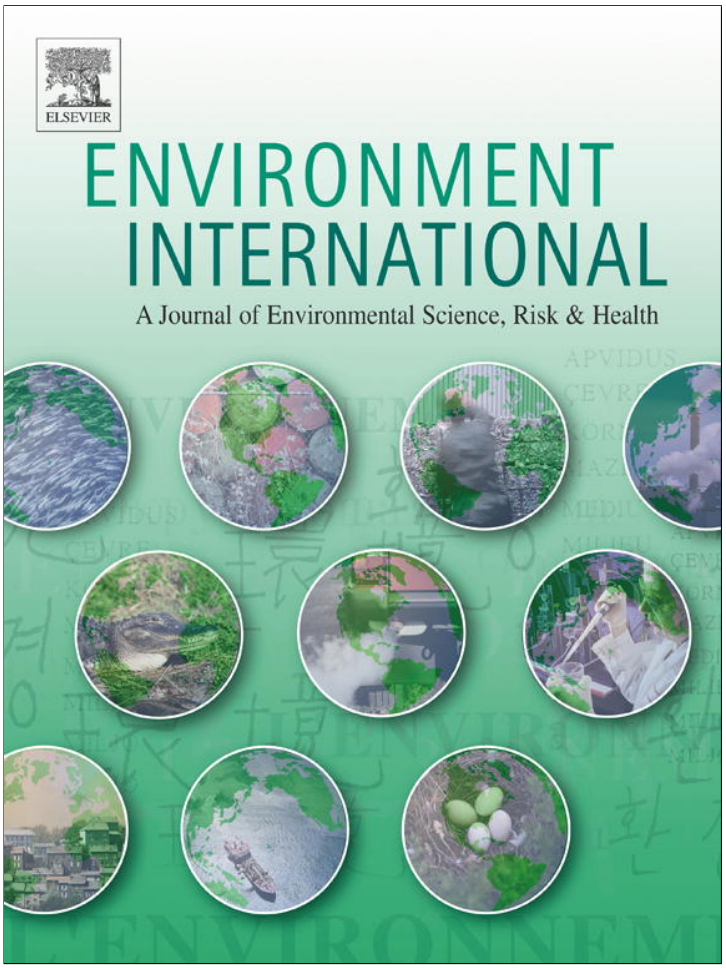

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Environment International 45 (2012) 22–31

Contents lists available at SciVerse ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Changes in concentrations of hydrophilic organic contaminants and of endocrine-disrupting potential downstream of small communities located adjacent to headwaters

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ARTICLE INFO

Article history:
Received 7 January 2012
Accepted 4 April 2012
Available online xxxx

Keywords:
Androgen
Dioxin-like activity
Estrogen
In vitro assay
POCIS
Waste Water Treatment Plant

ABSTRACT

Endocrine-disruptive potential and concentrations of polar organic contaminants were measured in seven headwaters flowing through relatively unpolluted areas of the Czech Republic. Towns with Wastewater Treatment Plant (WWTP) discharges were the first known sources of anthropogenic pollution in the areas. River water was sampled several kilometers upstream (US) and several tens of meters downstream (DS) of the WWTP discharges, by use of Pesticide and Pharmaceutical Polar Organic Integrative Samplers (POCIS-Pest, POCIS-Pharm). Extracts of passive samplers were tested by use of a battery of *in vitro* bioassays to determine overall non-specific cytotoxicity, endocrine-disruptive (ED) potential and dioxin-like toxicity. The extracts were also used for quantification of polar organics. There was little toxicity to cells caused by most extracts of POCIS. Estrogenicity was detected in all types of samples even though US locations are considered to be background. At US locations, concentrations of estrogen equivalents (EEq) ranged from less than the detection limits (LOD) to 0.5 ng EEq/POCIS. Downstream concentrations of EEqs ranged from less than LOD to 4.8 ng EEq/POCIS. Concentrations of EEq in POCIS extracts from all DS locations were 1 to 14 times greater than those at US locations. Concentrations of EEq measured in extracts of POCIS-Pest and POCIS-Pharm were in a good agreement. Neither antiestrogenic nor anti/androgenic activities were detected. Concentrations of 2,3,7,8-TCDD equivalents (TEq_{bio}) were detected in both types of POCIS at concentrations ranging from less than the LOD to 0.39 ng TEq_{bio}/POCIS. Nearly all extracts of POCIS-Pharm contained greater concentrations of TEq_{bio} activity than extracts of POCIS-Pest. Concentrations of pesticides and pharmaceuticals in extracts of POCIS were generally small at all sampling sites, but levels of some pharmaceuticals were significantly greater in both types of POCIS from DS locations. Chemical analyses along with the results of bioassays documented impacts of small towns with WWTPs on headwaters.

1. Introduction

Municipal and industrial waste waters can be sources of compounds that are able to cause acute toxicity as well as sublethal chronic abnormalities including disruption of hormonal balance in aquatic organisms (endocrine disruption, ED). Persistent and bioaccumulative organic chemicals have been conventionally monitored, but less persistent and less hydrophobic organic compounds are currently used as pesticides, prescription and non-prescription drugs and personal care products. Despite their lesser bioconcentration potential, relatively large fluxes of some of these compounds into aquatic systems might be acutely toxic and/or induce sublethal chronic abnormalities (Alvarez

Abbreviations: AEq, androgenic equivalent; AhR, Aryl hydrocarbon receptor; DS, downstream; E1, estrone; E2, 17β-estradiol; E3, Estriol; EC, effective concentration; ED, endocrine disruption; EDCs, endocrine disruptive compounds; EE2, 17α-ethynylestradiol; EEq, estrogenic equivalent; HpOCs, hydrophilic organic compounds; K_{ow}, octanol–water partition coefficient; LOD, limit of detection; LOQ, limit of quantification; NR, Neutral Red; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzodioxins; PCDFs, polychlorinated dibenzofurans; PNEC, Predicted No Effects Concentration; POCIS, Polar Organic Chemical Integrative Sampler; POCIS-Pest, Polar Organic Chemical Integrative Sampler optimized for polar Pesticides; POCIS-Pharm, Polar Organic Chemical Integrative Sampler optimized for most Pharmaceuticals; R_s, sampling rate (L/day); TEq_{bio}, dioxin-like equivalent obtained in bioassay; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; US, upstream; WWTP, Waste Water Treatment Plant.

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doi:10.1016/j.envint.2012.04.001

et al., 2007). Furthermore, some of these chemicals (particularly pharmaceuticals) can be highly potent, such that even concentrations at or near analytical detection limits may have biological activity.

Concentrations and/or ecotoxicological effects of hydrophilic organic compounds (HpOCs, contain one or more polar functional groups or a significant molecular dipole moment) have been reported in discharges of Waste Water Treatment Plants (WWTP) and/or downstream receiving waters (Aguayo et al., 2004; Bolong et al., 2009; Caliman and Gavrilescu, 2009). Downstream reaches of rivers have been shown to be polluted by compounds of both industrial and communal origin (Bolong et al., 2009), and therefore it is difficult to evaluate contributions and effects of pollutants released by individual towns. There are fewer sources of HpOC pollution in the headwaters and their potential impacts are not easy to assess, since there is limited information on concentrations of pollutants in the background areas.

Although different groups of HpOCs can contribute to adverse effects, xenoestrogens and xenoandrogens have emerged as environmental issues due to their ability to mimic or otherwise adversely affect functions of natural reproductive hormones, which could result in impaired reproduction of aquatic organisms (Matthiessen and Johnson, 2007). Even though the efficiencies of conventional WWTPs with activated sludge systems to remove estrogenic and androgenic compounds seem to be relatively high (88–>99% for estrogens and 96–>99% for androgens (Korner et al., 2000; Leusch et al., 2010; Murk et al., 2002; Svenson and Allard, 2004), concentrations of these endocrine disruptive compounds (EDCs) in some effluents are sufficient to cause ED (Kirk et al., 2002). Since some EDCs can cause adverse effects at small concentrations (ng/L), it is difficult and expensive to detect them by instrumental analyses (Korner et al., 2000). Moreover, because they occur in mixtures, even if they can be quantified, it is difficult to predict the potential effects of these compounds (Leusch et al., 2005). Therefore, *in vitro* bioassays can serve as cheaper and more environmentally relevant alternative to screen for the combined effects of mixtures on specific biological endpoints (Kinnberg, 2003).

The most frequently reported effect connected with EDs in surface waters is feminization of male fish downstream of WWTPs (Jobling and Tyler, 2003). Among estrogenic EDCs, the steroidal estrogens estrone (E1), estradiol (E2), and synthetic estrogen analogue, ethinyl estradiol (EE2), are some of the most potent endocrine disruptors in sewage effluents, all having more than thousand times greater potency to cause ED, at least in fish, than most other xenobiotics (Young et al., 2004). Under environmental conditions, steroidal hormones have been identified to be primarily responsible for observed adverse estrogenic effects on fish downstream of WWTPs although other weakly estrogenic compounds, such as alkylphenols and bisphenol A, can contribute to the effects (Desbrow et al., 1998; Gross-Sorokin et al., 2006). Important is also the fact that effluents from WWTPs can contain antiandrogenic chemicals as well. Their presence has been suggested by previous studies as a potential complication in establishing the chemical causation of fish sexual disruption (Tyler and Jobling, 2008). Efforts to identify the contributing antiandrogens are now underway, using a targeted fractionation process combined with screening by recombinant yeast assay and high-quality analytical chemistry. It should also be mentioned that certain compounds may act as both estrogens and antiandrogens (*e.g.* Suzuki et al., 2005).

There are two different approaches of sampling water, either active or passive. We chose to use passive integrative sampling, rather than traditional grab or composite sampling, for two reasons: i) passive sampling permits determination of time-weighted average concentrations of HpOCs in water, which is especially important when concentrations of HpOCs fluctuate over time because of changes in weather or variable diurnal patterns of consumption of products which are primary sources of HpOCs and, ii) the most potent EDCs usually occur at small concentrations (ng/L) and passive integrative samplers serve as an effective alternative to collecting and handling large volumes of water (Alvarez et al., 2007).

One useful passive sampler for HpOCs is the Polar Organic Chemical Integrative Sampler (POCIS). Relatively good correlations have been observed between concentrations of estrogenic equivalent (EEq) determined in bioassays for POCIS and grab water samples (Arditsoglou and Voutsa, 2008; Vermeirssen et al., 2005). POCIS has been shown to sample a wide variety of polar as well as moderate hydrophobic organic compounds with log K_{ow} of less than 4. Two types of adsorbents are considered standard for deployment of POCIS in the field. One of the two standard configurations, POCIS-Pest, preferentially concentrates waterborne HpOCs such as polar pesticides, natural and synthetic hormones, and other wastewater-related contaminants. The other, POCIS-Pharm, incorporates a sorbent optimal for sequestering polar pharmaceuticals (Alvarez et al., 2007).

Both types of POCIS exhibited linear uptake of phenolic and steroid compounds during 28-day tests conducted in laboratory during which concentrations of analytes in water were held constant. The correlation coefficients of the linear regression with respect to timescale were greater than 0.995 for POCIS-Pest and 0.985 for POCIS-Pharm, which suggests that uptake was time-integrative and the rate of uptake was not time-dependent during the exposure period. Moreover, rates of sampling (R_s) were not affected by changes in concentrations of tested compounds (Arditsoglou and Voutsa, 2008; Matthiessen and Johnson, 2007).

In the present study, water quality in terms of HpOCs and EDCs was studied in several headwaters in the Czech Republic. A combination of instrumental analyses of individual chemicals and *in vitro* assays with extracts from POCIS-Pest and POCIS-Pharm was conducted to: i) determine background levels of anti/estrogenic, anti/androgenic and dioxin-like activities in headwater streams upstream of known sources of anthropogenic pollution, and ii) evaluate the impacts of small towns and their WWTP discharges on concentrations of mixtures of EDCs in rivers.

2. Methods

2.1. Collection of samples

One POCIS-Pest and one POCIS-Pharm (Exposmeter AB, Sweden) sampler were deployed at each location. Study locations were upstream and downstream of seven municipal WWTPs, which were situated on small rivers and streams in relatively unpolluted areas of the Czech Republic (Fig. 1). Upstream (US) POCIS were placed from 2 to 5 km upstream of WWTPs in highland forest areas with minimal anthropogenic impact, while downstream (DS) sites were within 150 to 250 m of WWTP effluents. The towns studied, Králíky, Jilemnice, Cvikov, Tachov, Volary, Vimperk and Prachatice, are the upstream-

Fig. 1. Location of the sampling sites on small rivers in the Czech Republic: 1 — River Tichá Orlice near town Králíky; 2 — Stream Roudnický potok (upstream) and Jizerka river (downstream) near town Jilemnice; 3 — Stream Boberský potok near town Cvikov; 4 — River Mže near town Tachov; 5 — River Volyňka near town Vimperk; 6 — Stream Volarský potok near town Volary; 7 — Stream Živný potok near town Prachatice.

most sources of anthropogenic pollution on the assessed rivers/streams. These rivers/streams have natural or seminatural habitats flowing mostly through woodlands but there are agricultural fields or pastures in close proximity (0.2–3 km) to most of the towns. All WWTPs applied mechanical–biological treatment with activated sludge and Cvikov WWTP had an additional stabilizing pond (1.4 ha). All locations were sampled in June 2008, except for Prachatice, which was sampled in January 2008. Duration of deployment of samplers was 2 to 3 weeks. Duration of deployment should be within the linear uptake period for most HpOCs. Characteristics of WWTPs and river/stream conditions are summarized (Table 1).

2.2. Extraction of POCIS

After collection of POCIS, all samples (entire POCIS) were stored at −18 °C until analysis. The exposed POCIS was disassembled; the sorbent was transferred to the glass gravity flow chromatographic column with glass wool plug and analytes were eluted by the appropriate solvent mixture. Methanol was used as the eluent for POCIS-Pharm and a mixture of dichlormethane: methanol: toluene (8:1:1) was used for POCIS-Pest. The eluate was then evaporated to a small volume, the solvent was changed to methanol and the sample volume was adjusted to 2 mL for chemical analyses. Hexane, dichloromethane, acetone, toluene (all in Suprasolv purity), water and methanol (Hypergrade for LC/MS) were purchased from Merck (Darmstadt, Germany). The aliquots of extracts were further concentrated four-fold under a gentle stream of nitrogen to decrease the LOD for *in vitro* assays. The process blank samples were prepared following sample preparation procedure of both POCIS types and they were analyzed together with the other samples.

2.3. Bioassays

Four individual bioassays were used to determine overall cytotoxicity, anti/estrogenicity, anti/androgenicity and dioxin-like potencies of extracts of POCIS-Pest and POCIS-Pharm samplers. The reporter gene assays employed mammalian cell lines MVLN and H4IIE-*luc* and two types of recombinant *Saccharomyces cerevisiae*. MVLN are human breast carcinoma cells stably transfected with luciferase gene under the control of estrogen receptor, which were used for the assessment of cytotoxicity and anti/estrogenicity. Cytotoxicity of the samples was also investigated by recombinant strain of *S. cerevisiae* which expresses genes for enzyme luciferase under standard conditions (Leskinen et al., 2005). The potency of POCIS extracts to modulate androgen receptor-mediated responses was examined by use of recombinant *S. cerevisiae* that were modified to express human androgen receptor along with firefly luciferase under transcriptional control of androgen-responsive element (Michelini et al., 2005). H4IIE-*luc* are rat hepato-carcinoma cells stably transfected with the luciferase gene under control of Aryl hydrocarbon receptor (AhR) and they were used for the assessment of dioxin-like activity (Sanderson et al., 1996). At least two independent experiments were conducted in each bioassay for each exposure variant. All dilutions of POCIS extracts or controls were tested at least in triplicate.

Cytotoxicity of the samples can bias the results of the bioassays, therefore viability of cells was assessed several ways: Viability of MVLN cells was determined by use of the Neutral Red (NR) test where the NR dye is incorporated in the lysosomes of living cells and the uptake of NR is proportional to the number of viable cells. For cytotoxicity testing by NR-test, MVLN cells were seeded at a density of 25 000 cells/well in 96-well microplate ViewPlates™ (Packard, Meriden, CT, USA) and incubated for 24 h at 37 °C under atmosphere enriched with 5% CO_2. During this period cells were grown in DMEM-F12 without phenol red (Sigma Aldrich, USA) containing 10% foetal calf serum previously treated with dextran-coated charcoal to reduce concentrations of natural steroids in the serum. After 24 h, cells were exposed to dilutions of extracts from POCIS and solvent control (methanol, 0.5% v/v). Cytotoxicity was determined after 24 h of exposure, when NR (Sigma-Aldrich, Czech Republic) was added to the exposure medium in microplates to make a final concentration of 0.5 mg/mL. Cells were then incubated for 1 h at 37 °C. Afterwards, the cells were washed twice with phosphate buffered saline and lysed in the presence of acetic acid–ethanol solution (25:25:0.5; ethanol:water:acetic acid) for 15 min on a shaker. Finally, NR uptake was determined spectrophotometrically (Power Wave, BioTek, USA) at 570 nm. Absorbance was related to the response of the solvent control and the percentage of cytotoxicity of each sample dilution (viability of the cells exposed to the sample dilution relative to viability of cells exposed to solvent control (considered as 100%)) was determined. For the other way of assessing the viability, the recombinant strain of *S. cerevisiae* which expresses genes for enzyme luciferase under standard conditions (Leskinen et al., 2005) was used. In the presence of cytotoxic substances in the medium, luminescent light, produced normally by interaction between luciferase and added substrate luciferin, is less. When reaching a linear phase of growth, yeast were seeded into 96-well culture ViewPlates™ (Packard, Meriden, CT, USA) and exposed to vehicle, dilutions of POCIS extracts or to medium alone. Yeast cells were incubated for 2.5 h at 30 °C and then the signal was detected after addition of D-luciferin substrate. Detected luminescence was used to express the percentage of cytotoxicity caused by each sample dilution, as determined by the viability of the cells exposed to sample dilution relative to viability of cells exposed to solvent control, which was assigned a value of 100%.

Exposure for the determination of the anti/estrogenic potency of extracts in MVLN cells was conducted the same way as for the NR cytotoxicity evaluation described above with the following difference: cells were exposed to dilutions of POCIS extracts, calibration of the reference estrogen E2 (dilution series 10^{-12}–0.5×10^{-9} M E2, Sigma-Aldrich, Czech Republic) and solvent control (methanol, 0.5% v/v). After 24 h of exposure, the intensity of luminescence was measured

Table 1
Description of sampling sites, river parameters and sampling dates and duration.

Site no.	Name of town	Inhabitants no.	Name of recipient river(stream)	Effluent %[a]	River Q355 [m^3/s]	River flow velocity [m/s]	Sampling duration [day]	Date of sampling[b]
1	Králíky	4800	Tichá Orlice	20%	0.07	0.23	16	26 May–11 June
2	Jilemnice	6000	Roudnický potok (US)/Jizerka (DS)[c]	5%	0.02	0.08 (US) 0.02 (DS)	16	26 May–11 June
3	Cvikov	1900	Boberský potok	10%	0.08	0.13	21	21 May–11 June
4	Tachov	13000	Mže	15%	0.40	0.17	22	21 May–12 June
5	Vimperk	7650	Volyňka	4%	0.11	0.06	21	22 May–12 June
6	Volary	4000	Volarský potok	5%	0.07	0.12	21	22 May–12 June
7	Prachatice	13000	Živný potok	30%	0.15	0.17	23/16[d]	7/14[d]–30 January

[a] Average contribution of WWTP effluent to the recipient.
[b] All samples were taken in 2008.
[c] US = upstream site, DS = downstream site.
[d] US POCIS-Pest and both DS POCISes have been exposed for 23 days while US POCIS-Pharm for 16 days.

using Promega Steady Glo Kit (Promega, Mannheim, Germany). After subtraction of the response of the solvent control, luminescence in the estrogenicity assay was related to the maximal response of standard ligand (E2max for estrogenicity) and converted to percentages of E2max. Maximal induction as well as the shape of the curve differed among samples, thus equal efficacy or parallelism of the dose–response curves could not be assumed (Villeneuve et al., 2000). To avoid any predictions beyond the measured responses with all samples and to estimate the estrogenic equivalents (EEq) in the samples (expressed in ng E2/POCIS) the EEq_{20} estimate based on the 20% E2max response was reported, since most of the active samples did not reach the 50% E2max. EEq_{20} values were based on relating the amount of E2 causing 20% of the E2max response (EC_{20}) to the amount of sample causing the same response determined from regression analysis (equivalent of amount of E2 per amount of sample). The EC values were calculated by nonlinear logarithmic regression of dose–response curve of calibration standard and samples in Graph Pad Prism (GraphPad Software, San Diego, USA). The anti/estrogenicity was assessed by simultaneous exposure of the sample extract and 17β-estradiol (33 pM E2).

Duration of sampling varied from 16 to 23 days at different locations. Based on the evidence from previous research that uptake of phenolic as well as steroidal estrogens is linear in terms of time and concentration up to at least 28 days (Alvarez et al., 2007; Arditsoglou and Voutsa, 2008), we present our results normalized to 20 days of deployment along with the primary data in Table 3. The normalization was performed to simplify the comparability of our results among different locations and also with other studies in discussion. The data are presented both these ways to demonstrate the possible influence of the somewhat different deployment periods of the samplers on the results and their interpretation.

Concentrations of EEq in water were estimated by use of the sampling rate of E2 (0.09 L/day) previously determined by Matthiessen and Johnson (2007). It is important to stress, that these recalculated values represent approximate estimates of EEq concentrations in water and the values should not be considered as definite concentrations. This estimation will be further discussed in detail.

Concentrations of EEq in water were calculated (Eq. (1)).

$$C_w = C_{POCIS}/R_s t \quad (1)$$

where: C_w is the estimated concentration of EEq in water (ng/L), C_{POCIS} are concentrations of EEq in extracts from POCIS (ng/POCIS; primary not normalized values), R_s is sampling rate (L/day) of E2 previously determined by Matthiessen and Johnson (2007) and t is the sampling period (days).

As it was mentioned, anti/androgenity of POCIS extracts was determined by use of recombinant strain of *S. cerevisiae*. Plating and dosing were the same as for determination cytotoxicity of sample extracts in another strain of *S. cerevisiae* described above, but in this case, yeast cells were exposed not only to POCIS extracts and controls of pure medium and vehicle but also to dilutions of standard (testosterone in a range from 10^{-11} to 10^{-6} M, Sigma-Aldrich, Czech Republic).

The H4IIE-*luc* model was used for analysis of dioxin-like activity of the samples (Sanderson et al., 1996). Cells were seeded at a density of 15000 per well in 96-well microplate ViewPlates™ (Packard, Meriden, CT, USA) and incubated for 24 h under 5% CO_2 at 37 °C, in DMEM-F12 medium with phenol red (Sigma Aldrich, USA) containing 10% foetal calf serum. After 24 h, cells were exposed to the reference compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, with a dilution series of 10^{-12}–0.5×10^{-9} M, Ultra Scientific, USA), or dilutions of POCIS extracts and solvent control (methanol, 0.5% v/v). After 24 h of exposure, the intensity of luminescence was measured using Promega Steady Glo Kit (Promega, Mannheim, Germany). Results from the H4IIE-*luc in vitro* assay were analyzed by the same approach as described for the determination of the EEq above. Presented TEq_{bio} are expressed in ng of TCDD per POCIS. TEq_{bio} values were based on EC_{20} values because most samples did not reach greater EC responses.

For each bioassay the limit of detection was determined as the lowest observable effect concentration of standard chemical divided by the greatest non-cytotoxic extract concentration expressed as POCIS equivalent.

Table 2
List of pesticides and pharmaceuticals analyzed in extracts from both POCIS-Pest and POCIS-Pharm and list of perfluorinated organic compounds analyzed in extracts from POCIS-Pest.

Pharmaceuticals	Pesticides		Perfluorinated organics
Carbamazepine	2,4,5-T	MCPA	Perfluoro-1-hexanesulfonate
Cephalexin	2,4-D	MCPP_MECOPROP	2H-perfluoro-2-octenoic acid
Ciprofloxacin	Acetochlor	Metalaxyl	Perfluoro-1-octanesulfonamide
Diaveridine	Alachlor	Metamitron	N-methylperfluoro-1-octanesulfonamide
Diclofenac	Atrazine	Methabenzthiazuron	Perfluorooctanoic acid
Enrofloxacin	Atrazine desethyl	Methamidophos	Perfluorooctane sulfonic acid
Erythromycin	Azoxystrobin	Methidathion	Perfluorononanoic acid
Metronidazole	Bentazone	Metobromuron	
Norfloxacin	Bromacil	Metolachlor	
Ofloxacin	Carbofuran	Metoxuron	
Sulfachloropyridazine	Cyanazine	Metribuzin	
Sulfamethazine	Desmetryn	Monolinuron	
Sulfamethoxazole	Diazinon	Nicosulfuron	
Sulfamethoxypyridazine	Dichlobenil	Phorate	
Sulfapyridine	Dichlorprop	Phosalone	
Trimethoprim	Dimethoate	Phosphamidon	
	Diuron	Prometryn	
	Fenarimol	Propiconazole	
	Fenhexamid	Propyzamide	
	Fipronil	Pyridate	
	Fluazifop-p-butyl	Rimsulfuron	
	Hexazinone	Simazine	
	Chlorbromuron	Tebuconazole	
	Chlorotoluron	Terbuthylazine	
	Imazethapyr	Terbutryn	
	Isoproturon	Thifensulfuron-methyl	
	Kresoxim-methyl	Thiophanate-methyl	
	Linuron	Tri-allate	

2.4. LC/MS/MS analyses

Chemicals such as natrium sulfate, silicagel, methanol *etc.* were purchased from Merck (Darmstadt, Germany). ^{13}C labeled and native perfluorinated compounds were purchased from Wellington Laboratories. ^{13}C labeled Simazine, Sulfamethoxazol, 2.4D and Ciprofloxacin were purchased from Cambridge Isotope Laboratories. Native compounds were purchased from Dr. Ehrenstorfer, AccuStandards and Absolute Standards. All of the standards were purchased from Labicom ltd. (Olomouc, Czech Republic). A list of analyzed compounds is given in Table 2.

A cocktail of internal standards was spiked into each POCIS extract (100 μL of the standard mixture in water was added to 100 μL of POCIS extract). Chemicals were identified and quantified by use of LC/MS/MS. Analyses were performed using three different LC/MS/MS methods.

Chemicals in POCIS extracts were quantified by use of internal standards. A subsample (20 μL for pesticide and 10 μL for pharmaceuticals) was injected onto an analytical column (Phenomenex C18 Aqua, 2 mm × 50 mm, 5 μm particles). The HTS PAL (CTC) autosampler, Rheos2000 (Flux) quaternary pump and TSQ Quantum AccessTM (ThermoScientific, USA) triple quadrupole tandem mass spectrometer were used for analyses of polar pesticides, pharmaceuticals and perfluorinated compounds. Two MS/MS transitions were monitored (where possible) for native analytes to confirm identity. An agreement of results obtained from both transitions better than 30% was accepted as a confirmed result. Isotope dilution and internal standard methods were used for the quantification of target compounds. Quantification limits (LOQs) of analytes were calculated the same way as concentration but peak area corresponding to instrument LOQ was used instead of peak area found in sample. Thus, LOQs are adjusted to internal standards.

Most detected compounds have been shown to be in the linear uptake phase for at least 23 days (the maximal deployment period in our study) (Alvarez et al., 2007). Thus, we present concentrations of those compounds normalized to 20 days of deployment to enable more precise interpretation of our results across different locations and also better comparability with other studies in discussion.

2.5. Statistical analysis

Due to violations of the assumptions of parametric statistical testing, differences between results of the two applied cytotoxicity detection systems as well as between potencies of POCIS-Pest and POCIS-Pharm extracts to induce nonspecific cytotoxicity and act through specific modes of action were evaluated by nonparametric Wilcoxon Matched Pairs test. The same test was applied to assess differences between concentrations of pollutants detected in POCIS-Pest and Pharm extracts. The nonparametric Spearman rank correlation was used to assess the similarity of the potential of POCIS-Pest and Pharm extracts to act through specific modes of action. All statistical analyses were performed with Statistica for Windows® 9.0 (StatSoft, Tulsa, OK, USA), the tests were considered significant at $p<0.05$.

3. Results

There was no response above detection limits observed for blanks in any of the bioassays. The limits of detection in blanks were 0.06 ng EEq/POCIS for estrogenity, 1.29 ng AEq/POCIS for androgenity and 0.03 ng TEqbio/POCIS for dioxin-like activity.

3.1. Cytotoxicity

Most tested concentrations of POCIS extract equivalents (0.00125%–0.25% POCIS/mL) were not cytotoxic to yeast or to MVLN cells. At the greatest tested POCIS extract equivalent concentration 0.5% POCIS extract/mL samples from some locations caused cytotoxicity of as much as 50% (Fig. 2). For both types of POCIS the cytotoxic effects were comparable or greater at DS locations than at US locations with a single exception where the POCIS-Pharm extract at location 5 exhibited greater cytotoxicity at the US location (Fig. 2B). However, the greater cytotoxicity observed DS of WWTPs compared to US was statistically significant only for extracts of POCIS-Pest measured by yeast test. In all other cases, including all extracts of POCIS-Pharm in both bioassays and POCIS-Pest in MVLN cells, the magnitude of differences in cytotoxicity was not statistically significant between US and DS.

Although the yeast test was significantly more sensitive to cytotoxicity of POCIS-Pharm extracts ($p=0.009$) than the MVLN test, the results of the two tests were comparable among POCIS extracts, with no significant difference between the results of the two tests with extracts of POCIS-Pest ($p=0.79$). The yeast test was also significantly more sensitive to POCIS-Pharm extracts than POCIS-Pest extracts ($p=0.01$), whereas there was no statistically significant difference between cytotoxicity of extracts of the two types of samplers in the MVLN test.

3.2. Anti/estrogenicity

Estrogenicity was detected in extracts of both types of POCIS and differences were observed between US and DS locations. No extract showed significant antiestrogenic activity (data not shown). Although samples from DS locations were more estrogenic than those from US locations at all sites, some EEq was detected also in most US samples (Table 3).

Because uptake of the more potent and also some less potent estrogens has previously been demonstrated to be time integrative for more than 25 days (*e.g.* Arditsoglou and Voutsa, 2008), here estrogenic potentials detected in extracts of POCIS are reported also as normalized to 20 days of POCIS deployment. However, differences between data obtained before and after the normalization to 20 days of POCIS deployment were negligible (Table 3).

Concentrations of EEq greater than the LOD (0.1 to 0.6 ng/POCIS) were observed in four out of seven US locations in both types of POCIS. The variation among LOD is caused by slightly different cytotoxicity of extracts. Detected concentrations of EEq in US samples ranged from 0.3 to 0.5 ng/POCIS$_{20\ days}$ in POCIS-Pest as well as in POCIS-Pharm extracts. Since there were no known anthropogenic impacts near US sites, the detected EEq concentrations can be considered as background.

Estrogenic equivalents in extracts from DS samples were greater than the LOD at all sites with the single exception of the POCIS-Pest extract at site 2. Concentrations ranged from 0.7 to 4.0 ng/POCIS$_{20\ days}$ for POCIS-Pest and from 0.5 to 4.2 ng/POCIS$_{20\ days}$ for POCIS-Pharm extracts. The greatest concentrations of EEq were observed at DS locations at sites 3 and 7 (Table 3). At site 3 DS samples contained more than 10-fold greater concentration of EEq than the US sample in the case of POCIS-Pest and more than 14-fold greater concentration of EEq than the US POCIS-Pharm. At site 7 DS samples contained more than 7-fold greater concentrations of EEQ than the US sample from POCIS-Pest and more than 5-fold greater concentration than the US sample from POCIS-Pharm, respectively.

Estrogenic potential of water was estimated (Eq. (1)). For US localities sampled by both types of POCIS the calculated water EEq concentrations detected above LOD varied from 0.1 to 0.3 ng/L. Estimated estrogenic potential in water in DS locations sampled by POCIS-Pest ranged from less than 0.4 to 2.2 ng EEq/L and for those sampled by POCIS-Pharm from 0.3 to 2.3 ng EEq/L (Table 3).

There were statistically significant correlations between estrogenic potentials of the pesticide and pharmaceutical POCIS extracts (Spearman rank 0.79, $N=7$, LOD values were replaced by value of 1/2 LOD), despite the discrepancy at the DS location at site 6. At DS location at site 6, repeated evaluation of estrogenic potential confirmed the difference of estrogenicity in extract of POCIS-Pharm compared to POCIS-Pest. The likeness of estrogenicity in extracts of POCIS-Pest and Pharm was also confirmed by nonparametric Wilcoxon Matched Pairs test, which indicated no significant difference between POCIS-Pest and Pharm ($p=0.81$).

3.3. Anti/androgenicity

There was no significant androgenic activity in any extract in the test with recombinant yeast assay (data not shown). Detection limit was 1.29 ng AEq/POCIS. None of the extracts has shown antiandrogenic activity (data not shown).

3.4. Dioxin-like activity

Dioxin-like activity was detected in most extracts. At US locations sampled by POCIS-Pest, concentrations exceeded the detection limit of 0.03 ng TEq$_{bio}$/POCIS in only two cases whereas extracts from the POCIS-Pharm sampler deployed at the same locations had detectable concentrations at six out of seven sites (Fig. 3). Concentrations of TEq$_{bio}$ at US locations ranged from less than the LOD to 0.08 and to 0.22 ng TEq$_{bio}$/POCIS for extracts of POCIS-Pest and POCIS-Pharm, respectively. DS sites mostly showed greater concentrations of TEq$_{bio}$ in extracts from POCIS-Pharm than from POCIS-Pest. Extracts from DS POCIS-Pest contained concentrations of TEq$_{bio}$ that ranged from less than LOD of 0.08 to 0.26 ng TEq$_{bio}$/POCIS and from 0.08 to 0.39 ng TEq$_{bio}$/POCIS in extracts of POCIS-Pharm.

When considering all samples together, significantly greater concentrations of TEq$_{bio}$ were observed in extracts of POCIS-Pharm than extracts of POCIS-Pest (Wilcoxon Matched Pairs test; $P=0.0029$). Nevertheless, similar patterns of greater concentrations of TEq$_{bio}$ at DS locations with similar orders of magnitudes were observed in extracts of both types of POCIS. At most sites, concentrations of TEq$_{bio}$ were greater DS of WWTPs (Fig. 3). Concentrations TEq$_{bio}$ in extracts of DS POCIS-Pest at sites 4 and 7 were greater than those in extracts of POCIS-Pest from US, by 1.4- and 4.9-fold, respectively. Concentrations of TEq$_{bio}$ in extracts of POCIS-Pharm at sites 1, 2 and 5 were approximately equivalent

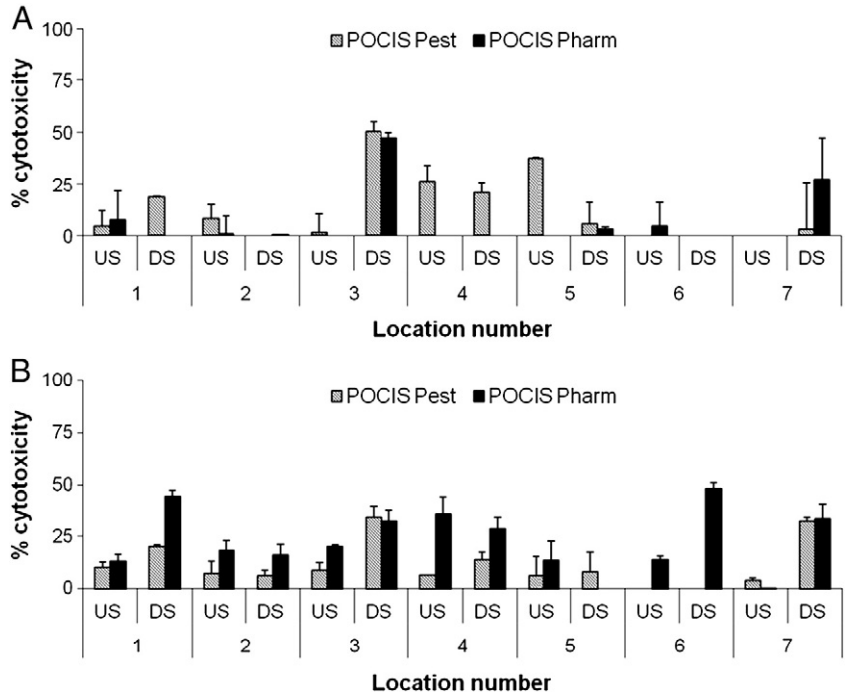

Fig. 2. Cytotoxicity of extracts (concentration of 0.5% POCIS/mL) from upstream (US) and downstream (DS) measured by the yeast screen (A) and by Neutral Red test with MVLN cells (B). Error bars show standard deviations. For samples without any cytotoxic effect, no values are presented.

for US and DS locations, whereas they were about 3-fold greater at the DS location of sites 3 and 4 and at least about 5-fold greater at the DS location at sites 6 and 7.

3.5. Chemical analyses

Although most of the selected chemicals that were monitored were not detected in extracts at concentrations greater than the LOQ (0.1 to 14 ng/POCIS), concentrations of several pharmaceuticals were greater at DS relative to US locations (Table 4). The greatest concentrations of pharmaceuticals were observed at the DS location of site 7. Pharmaceuticals found most frequently and also at the greatest concentrations were carbamazepine and diclofenac. Concentrations of carbamazepine ranged from less than the detection limit (2–8 ng/POCIS) to 9 ng/$POCIS_{20\ days}$ in extracts from US locations and from 13 to 339 ng/$POCIS_{20\ days}$ in extracts from DS locations. The concentrations of diclofenac ranged from less than the LOQ (2–8 ng/POCIS) to 31 ng/$POCIS_{20\ days}$ in extracts from US locations and from 18 to 409 ng/$POCIS_{20\ days}$ in extracts from DS locations. Concentrations in extracts of POCIS-Pest and POCIS-Pharm were comparable with a few exceptions, such as sulfapyridine at sites 3 and 4. Except pharmaceuticals presented in Table 4, a few other compounds — ofloxacin, norfloxacin, ciprofloxacin and erythromycin were detected above the detection limits (LOQ 0.6–14 ng/POCIS), all detected concentrations were lower than 100 ng/$POCIS_{20\ days}$.

Concentrations of most pesticides that were monitored were less than the LOQ (0.1–6.5 ng/POCIS). Most pesticides, which were quantifiable, were triazines, and their concentrations were generally small (<100 ng/$POCIS_{20\ days}$). Concentrations of all detected triazines, including atrazine, atrazine desethyl, hexazinone, simazine and terbuthylazine are summarized in Table 5. Besides triazines, acetochlor at a concentration of 1375 ng/$POCIS_{20\ days}$ was detected in one isolated POCIS-Pest sample from US location of site 2.

Beside the pharmaceuticals and pesticides, perfluorinated organic compounds (listed in Table 2) were also monitored in extracts of POCIS-Pest. However, concentrations greater than the LOQ of 0.21–1.15 ng/POCIS were observed only in a few cases

Table 3
Estrogenic activities in POCIS-Pest and POCIS-Pharm extracts measured by MVLN *in vitro* assay expressed as ng EEq/POCIS, normalized to sampling period of 20 days and recalculated (according to Eq. (1)) to approximate EEq water concentrations.

Site no.	US/DS[a]	POCIS depl.[b] (day)	EEq in POCIS extracts (ng/POCIS)		EEq in POCIS extracts normalized to 20 days of POCIS deployment (ng/$POCIS_{20\ days}$)		Estimated EEq in water derived from E2 R_s[c] and EEq of POCIS extract (ng/L)	
			POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	16	0.2 ± 0.01	<0.2	0.3	<0.3	0.1	<0.1
	DS		1.0 ± 0.1	0.7 ± 0.2	1.3	0.9	0.7	0.5
2	US	16	<0.3	<0.3	<0.4	<0.4	<0.2	<0.2
	DS		<0.3	0.7 ± 0.6	<0.4	0.8	<0.2	0.5
3	US	21	0.4 ± 0.3	0.3 ± 0.1	0.4	0.3	0.2	0.2
	DS		4.2 ± 1.5	4.3 ± 0.4	4.0	4.1	2.2	2.3
4	US	22	0.5 ± 0.2	0.3 ± 0.1	0.5	0.3	0.3	0.1
	DS		0.9 ± 0.2	0.5 ± 0.02	0.8	0.5	0.5	0.3
5	US	21	0.4 ± 0.1	0.5 ± 0.1	0.4	0.5	0.2	0.3
	DS		0.9 ± 0.6	1.0 ± 0.04	0.9	1.0	0.5	0.5
6	US	21	<0.3	<0.3	<0.3	<0.3	<0.2	<0.2
	DS		0.7 ± 0.7	2.3 ± 0.3	0.7	2.2	0.4	1.2
7	US	23/16[d]	<0.6	<0.6	<0.5	<0.8	<0.3	<0.4
	DS		4.5 ± 1.3	4.8 ± 1.0	3.9	4.2	2.2	2.3

[a] US = upstream site, DS = downstream site.
[b] Duration of POCIS deployment.
[c] R_s = sampling rate.
[d] US POCIS-Pest and both DS POCISes have been exposed for 23 days while US POCIS-Pharm for 16 days.

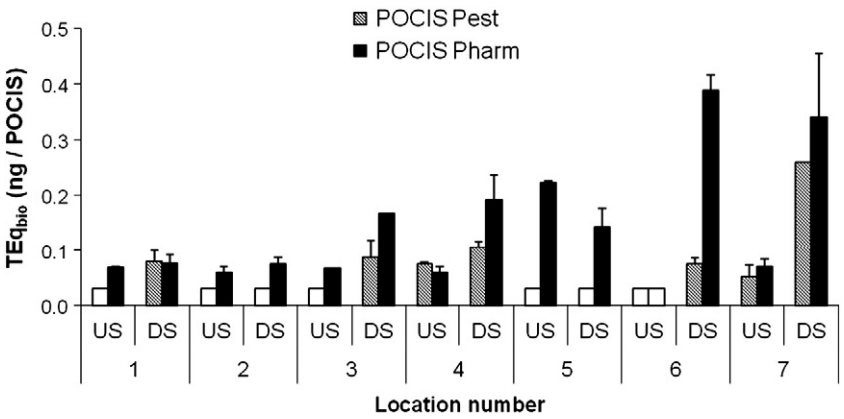

Fig. 3. Dioxin-like activity of upstream (US) and downstream (DS) POCIS-Pest and POCIS-Pharm extracts determined by H4IIE-*luc in vitro* assay. White columns indicate TEq_{bio} concentrations less than our detection limit (0.03 ng/POCIS); error bars show standard deviations.

and were less than 5 ng/POCIS with single exception of perfluorooctane sulfonic acid, which was detected at DS location 2 at concentration 36 ng/POCIS.

4. Discussion

Most previous studies assessing ED contamination of rivers focused on the influence of urbanized areas and larger WWTPs (Kinnberg, 2003), but there is less information on the impact of smaller sources on headwaters where better quality of water would be expected. Our study brings important information on the background levels of ED and HpOCs compounds and the influence of smaller towns without major industrial activities on headwaters pollution. Seven small rivers or streams were sampled by use of POCIS-Pest and POCIS-Pharm passive samplers US and DS of the most upstream sources of anthropogenic pollution, which were small towns with WWTP discharges.

Sampling rates for most compounds, which were investigated by use of POCIS in turbulent conditions, have been reported to range from 0.12 to 0.26 L/day (95%centile of published R_s; Alvarez et al., 2007; Arditsoglou and Voutsa, 2008; Harman et al., 2008; Macleod et al., 2007; Mazzella et al., 2007). This means that in 16 days, which is the minimal time of deployment of POCIS in the study, the results of which are reported here, the amount of the chemicals present in POCIS would be equivalent to 1.92–4.16 L of river water (0.12–0.26 L/day × 16 days). Thus, the least concentration causing cytotoxic effect — 0.5% POCIS/mL, would represent 9.6- to 20.8-fold concentrated river water. Therefore our results suggest little overall cytotoxicity of river water and weak impact of WWTPs onto this unspecific toxicity.

The results of the two systems used to detect cytotoxicity, yeast and mammalian cells, were similar with the exception of greater cytotoxicity of extracts of POCIS-Pharm in the yeast cells. This observation indicates greater sensitivity of the yeast model toward some chemicals that are more concentrated by POCIS-Pharm. Chemical analyses of POCIS-Pest and Pharm extracts did not reveal any significant differences in concentrations of monitored pollutants. However, it has been suggested that some pharmaceuticals have multiple functional groups, which have a tendency to strongly bind to the carbonaceous component of the triphasic adsorbent mixture contained in POCIS-Pest, which results in poor solvent extraction recoveries of some members of this class of compounds during sample processing (Alvarez et al., 2007). Our results demonstrating weak cytotoxicity correspond to another study of Alvarez et al. (2008), who used Microtox® assay to evaluate toxicity of POCIS from surface waters burdened by extensive agriculture. In that study, no extract from passive samplers (POCIS, SPMD) exposed for 29 to 65 days displayed acute toxicity.

Although the study, the results of which are reported here, was conducted in relatively unpolluted areas, some estrogenic activity was detected even at US locations (Table 3). Authors of some other studies had referred to detect concentrations of EEq in reference rivers. Nadzialek et al. (2010), who used active sampling and MCF-7 assay, found EEq concentrations at both tested reference sites in Belgium to be 0.01 and 0.03 ng/L. These concentrations are comparable with those estimated in our study (<0.1–0.3 ng EEq/L) especially if we consider our recalculated results as the worst case scenario. In contrast, Sellin et al. (2009), who used POCIS-Pharm and chemical analyses of their

Table 4
Results of the LC/MS/MS analyses — pharmaceuticals with greatest detected concentrations in extracts from POCIS-Pest and POCIS-Pharm (ng/$POCIS_{20\ days}$). Results are normalized to sampling period of 20 days.

Site no.	US/DS [a]	Sulfapyridine		Sulfamethoxazole		Trimethoprim		Carbamazepine		Diclofenac	
		POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	–	–	–	–	–	–	–	–	–	–
	DS	–	–	74	16	13	9	44	28	60	49
2	US	–	–	–	–	–	–	–	–	–	–
	DS	–	14	9	–	–	–	15	15	18	30
3	US	–	–	11	–	–	–	6	–	31	24
	DS	90	25	27	–	10	8	95	36	133	57
4	US	9	3	–	–	–	–	9	3	–	–
	DS	100	13	59	8	28	10	61	13	100	23
5	US	–	–	–	–	–	–	–	–	–	–
	DS	12	16	–	–	8	14	24	40	31	70
6	US	–	–	–	–	–	–	–	–	–	–
	DS	42	26	30	15	35	32	190	238	181	190
7	US	–	–	–	–	–	–	–	–	–	–
	DS	50	36	200	122	209	209	339	304	391	409

"–" less than LOQ (0.6–14 ng/POCIS).
[a] US = upstream site, DS = downstream site.

Table 5
Results of the LC/MS/MS analyses - concentrations of triazines (ng/POCIS$_{20\ days}$), which were the most frequently detected pesticides at tested sites. Results are normalized to sampling period of 20 days.

Site no.	US/DS[a]	Atrazine		Atrazine desethyl		Hexazinone		Simazine		Terbuthylazine	
		POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm	POCIS Pest	POCIS Pharm
1	US	–	–	–	–	5	7	–	–	15	21
	DS	14	14	8	6	–	–	–	–	2	3
2	US	8	12	18	19	1	–	4	5	1375	1875
	DS	4	7	5	5	4	3	1	1	475	713
3	US	7	7	8	3	32	19	5	4	2	1
	DS	24	11	17	5	49	20	8	4	3	1
4	US	2	3	8	5	6	5	–	–	2	2
	DS	5	2	11	3	8	3	1	–	4	3
5	US	8	7	13	7	18	12	–	–	2	2
	DS	5	11	7	9	12	16	–	1	2	3
6	US	–	–	–	–	1	–	–	–	1	1
	DS	21	31	25	22	20	18	–	1	6	6
7	US	2	2	16	13	9	9	1	2	–	–
	DS	14	11	25	18	10	9	2	1	2	1

"–" less than LOQ (0.1–6.5 ng/POCIS).
[a] US = upstream site, DS = downstream site.

extracts to monitor estrogens in rivers of Nebraska, reported calculated EEq concentrations above detection limit (1 ng/POCIS$_{7\ days}$) in 2 out of 3 reference sites and the concentrations (1.9 and 1.5 ng/POCIS$_{7\ days}$) were at least one order of magnitude greater than those found in our study. Matthiessen and Johnson (2007) evaluated, among others, estrogenic potential of 6 British headwaters with only few sources of estrogenic contamination (isolated houses with septic tanks). They used POCIS, which was previously calibrated in a laboratory study and yeast estrogen screen assay to evaluate estrogenic potential of the POCIS extracts. Their EEq concentrations ranged from less than the LOD (0.08 ng/L) to 1.4 ng/L with a median of 0.3 ng/L (except of 1 site with extremely great EEq value), which are slightly greater but comparable results to ours.

Greater estrogenic potential DS of WWTPs compared to US was detected at all sampled sites (Table 3). Comparable results were obtained by Vermeirssen et al. (2005), who monitored estrogens in POCIS Pest and Pharm extracts deployed US and DS of 5 municipal WWTPs in Switzerland. Four out of the five rivers were, according to earlier DS samples analyses, chosen as moderate to greatly estrogenic whereas one river as less estrogenic. The concentrations of EEq at the least burdened site were very similar to those obtained in our study (0.4 ng EEq/POCIS$_{22\ days}$ in extracts of both types of POCIS placed US and 1.9–2.0 ng EEq/POCIS$_{22\ days}$ in extract of POCIS-Pest and 1.7–1.9 ng EEq/POCIS$_{22\ days}$ of POCIS-Pharm situated DS of the WWTP). In contrast, the river with the greatest estrogenic pollution contained more than 20 ng EEq/POCIS$_{22\ days}$ in both POCIS extracts of US samples and comparable EEq concentrations in DS ones. Similar to our results most DS samples displayed increase of estrogenic activity compared to US ones. Greater concentrations of estrogens in all POCIS samplers deployed DS of municipal WWTPs of smaller towns compared to US sites were also found in Nebraska (Sellin et al., 2009). Those authors determined estrogenic equivalents analytically (based on known potential of steroidal estrogens to cause the effect) and the recalculated EEq concentrations were greater (up to 22.7 ng/POCIS$_{7\ days}$) than those detected by bioassays in our study. However, the greatest EEq concentrations were detected DS of WWTP with trickling filters technology which had been previously proved to be less effective in estrogens removal than activated sludge systems (Svenson et al., 2003) such as those in all WWTPs in our study.

Concentrations of EEq in POCIS extracts were converted to approximate concentrations of EEq in water by use of sampling rate of E2 because: i) in numerous studies steroidal estrogens have been identified to be responsible for most (often more than 90%) of estrogenic activity detected by *in vitro* assays in municipal waste waters effluents (*e.g.* Korner et al., 2001; Routledge et al., 1998) ii) compared to E1, Estriol (E3) and EE2, E2 has the least R_s (Arditsoglou and Voutsa, 2008), which enabled to estimate the worst case scenario (the greatest concentration) and iii) E2 is the standard reference compound used for EEq calculations. For estimating concentrations of EEq in water, R_s for E2 previously established for the same standardized POCIS configuration as used in our study was applied in calculation (0.09 L/day; Matthiessen and Johnson, 2007). From the rates of sampling for E2 given in the literature (Arditsoglou and Voutsa, 2008; Matthiessen and Johnson, 2007), the R_s calibrated at 10 °C was used because the temperature was similar to the conditions in the studied streams and rivers and the application of the lowest R_s value resulted in the worst case scenario estimate. Furthermore, application of the E2 sampling rate calibrated at 23.5 ± 0.5 °C by Arditsoglou and Voutsa (2008) would result in a range <0.1 to 1.8 ng/L EEq, which is similar to the currently presented results (Table 3). Rate of sampling can vary under different environmental conditions (*e.g.* diverse water flow rates, pH or temperature) but all the stations (with exception of location 7) were sampled at the same time eliminating thus at least partially variability. Moreover, the flow rates were always greater than 0.02 m/s and it has been demonstrated that under turbulent conditions sampling rates do not dramatically change as a function of flow velocity (Li et al., 2010). Another line of evidence, which supports the approach of EEq calculation applied in the study, is direct comparison of POCIS with grab samples as reported by Vermeirssen et al. (2005). Those authors measured estrogenic activity in both extracts of POCIS and grab samples and concentrations of EEq in extracts of POCIS were approximately 3-fold greater than the average concentrations of EEq in grab samples. These findings indicated the rate of sampling for estrogenic compounds is approximately 0.14 L/day. This experimentally established R_s is consistent with the results observed in this study where it was assumed that use of R_s for E2 could serve as an approximation to estimate concentrations of EEq in water and that these recalculated results represent a realistic estimate of the worst case scenario.

Even though the most estrogenic extracts came from POCIS exposed DS of Prachatice town (site 7), which has the most inhabitants and the largest proportion of WWTP effluent in relation to the recipient river (Table 1), these two parameters did not correlate with the estrogenic potentials in POCIS extracts from other sites. Other forces, for example different primary sources of estrogens or different WWTP capacity or technology, probably influenced the EEq concentrations in DS samples. Estrogenic activity detected in extracts of POCIS-Pest or POCIS-Pharm was similar, this observation is consistent with previous field as well as calibration studies (Arditsoglou and Voutsa, 2008; Vermeirssen et al., 2005).

Although dioxin-like compounds are usually investigated in less polar matrices such as SPMD or sediments, some recent studies (Dagnino et al., 2010; Reungoat et al., 2010) affirmed this activity also in water phase. In this study, dioxin-like activity was detected in both types of POCIS (0.05–0.39 ng TEq_{bio}/POCIS), even at several US locations. Sampling rates for known AhR active compounds and kinetic of their sampling has not been reported for POCIS yet. Therefore our results cannot be recalculated to water concentrations nor to unified number of days of their deployment. Dioxin-like activity has been traditionally connected with hydrophobic compounds such as polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) or polychlorinated biphenyls (PCBs). Since experimentally-determined values for log K_{ow} range from 6.1 to 8.2 for PCDD and PCDF congeners (Chrostowski and Foster, 1996) and from 4.66 up to 7.44 for PCB congeners, respectively (Zhou et al., 2005), these compounds are not expected to be sampled by POCIS. Our results suggest that less hydrophobic compounds like PAHs, which are also known to bind to AhR, or some unknown compounds might represent non-negligible part of dioxin-like activities in aquatic environment and this issue desires further research.

In this study concentrations of TEq_{bio} in extracts of POCIS-Pharm were approximately 2-fold greater than those in extracts of POCIS-Pest. Up to authors' knowledge, no other comparisons of concentrations of TEq_{bio} in extracts of POCIS-Pest and POCIS-Pharm have been published. However, since the same sorbent mass and membrane were used for both types of POCIS, it seems that different affinity of dioxin-like compounds to the POCIS-Pest *vs.* POCIS-Pharm sorbent might be responsible for the observed difference. Another reason could be the efficiency of extraction methods. However, the most potent and traditionally studied dioxin-like pollutants are hydrophobic substances and POCIS-Pest was extracted by less polar solvent than POCIS-Pharm.

Even though *in vitro* assays revealed some specific potencies of mixtures that might cause effects to the aquatic biota, chemical analyses of a wide range of compounds (Table 2) did not show significant contamination. The greatest effects were observed in estrogenic activity screening assay. However, steroidal estrogens, which have been shown to be responsible for most of the estrogen equivalents in waste waters (Desbrow et al., 1998), were not monitored in this study. Among detected chemicals, some triazines are known to be able to disturb endocrine system of organisms (Danzo, 1997; Vonier et al., 1996). In this study, triazines were detected at concentrations from less than 0.1 to 1875 ng/$POCIS_{20\ days}$ (Table 5) and their previously published sampling rates varied from 0.12 to 0.26 L/day (Alvarez et al., 2007; Mazzella et al., 2007). Estimated concentrations of triazines in water ranged from less than 0.02 ng/L to 781 ng/L, but these compounds are known to be effective at concentrations greater than mg/L (Danzo, 1997; Vonier et al., 1996) and thus their contribution to the responses detected by the *in vitro* systems can be considered negligible.

Concentrations of all monitored chemicals were small compared to the results of other studies (Arditsoglou and Voutsa, 2008; Soderstrom et al., 2009), which was in good agreement with our intention to sample relatively unpolluted areas. Despite the small concentrations of studied contaminants there were obviously increased concentrations of pharmaceuticals in DS samples. This was not so remarkable in case of pesticides. The reason of greater differences of pharmaceuticals concentrations in US and DS extract than pesticides might be the fact that pharmaceuticals are used only in human quarters or farms whereas pesticides are used also in areas distant from towns.

When considering the environmental significance of our results, some of the detected estrogenic equivalents concentrations had been reported to cause adverse effects. Authors of most studies, who observed estrogenic adverse effects on aquatic biota, reported EEq concentrations or corresponding concentrations of estrogens higher than those detected in our study (*e.g.* Sellin et al., 2009; Vermeirssen et al., 2005; Young et al., 2004). However, for example, Vethaak et al. (2005) found elevated levels of yolk protein vitellogenin in male bream (*Abramis brama*) in river with EEq levels determined by *in vitro* ER-CALUX assay as low as 0.17 ng/L. In that study, steroidal hormones were identified as the main contributors to the EEq (Vethaak et al., 2005). To authors' knowledge, the only estrogen, for which LOEC concentrations lower than 0.5 ng/L *in vivo* has been reported, was EE2 (Young et al., 2004). For example, Zha et al. (2008) demonstrated that the reproduction of the F-1 minnows was completely inhibited at EE2 concentration as low as 0.2 ng/L in a multigeneration study with Chinese rare minnows (*Gobiocypris rarus*). In our study, the upstream locations (with estimated EEqs <0.1–0.3 ng/L) were chosen as background sites without any grasslands or human settlements near the catchments and therefore we do not expect steroidal estrogens, particularly the synthetic EE2, to be responsible for the detected EEq. Contrariwise, at downstream locations with estimated EEq <0.2–2.3 ng/L, where municipal waste water effluents were considered as the main sources of estrogens, the presence of highly potent steroidal estrogens would be expected. The relative potency of any estrogens to E2 can differ for *in vitro* and *in vivo* studies (*e.g.* Johnson and Sumpter, 2001). The greatest difference has been reported for EE2. In the *in vitro* assay that we used (MVLN) the estrogenic potency of EE2 relative to E2 is 1.25 whereas in *in vivo* studies concerning production of yolk protein vitellogenin or alteration of ovarian somatic index in fish it has been reported to be approximately 25–30 (Gutendorf and Westendorf, 2001; Young et al., 2004). This indicates that the overall estrogenic equivalents for *in vivo* situation might be even greater that those derived from *in vitro* tests. As far as the authors know, there are no studies available on potential *in vivo* adverse effects in similar locations as examined in our study. Therefore it is not possible to reliably estimate the environmental significance of detected EEq yet.

The levels of vitellogenin in brown trout (*Salmo trutta fario* L.) from US and DS Prachatice (corresponding to our location 7) were investigated in September 2007 by researchers from Faculty of Fisheries and Protection of Waters, University of South Bohemia. There were significantly increased levels of vitellogenin in male brown trout captured downstream compared to the upstream site. The number of examined fish males was 6 at each US and DS location. The median plasma concentration were bellow detection limit of 10 ng/mL in male fish from upstream site and 3035 μg/mL in those from downstream site (Zlabek, personal communication). This corresponds with the results of our study, where the estrogenic activity was bellow detection limit in POCIS exposed upstream of Prachatice, while there were the greatest EEq among all sites in our study detected in POCIS from the Prachatice downstream site (2.3 ng/L). Thus, the increased EEq values from *in vitro* studies might indicate potential *in vivo* effects. Generally, the relevance of *in vitro* determined estrogenic equivalents for *in vivo* situation is a very important issue, which requires further research and which is also in focus of our further studies.

5. Conclusion

The study brought new information about concentrations of polar organic contaminants and endocrine-disruptive potential in relatively unpolluted rivers and about the influence of smaller towns on this type of contamination in affected headwaters. There was an obvious impact on all sites despite the fact that the towns are equipped with municipal WWTPs with advanced activated sludge systems of treatment. Increased exposure potential of estrogenic and dioxin-like compounds (determined by *in vitro* assays) downstream of the towns were demonstrated. Some of the detected estrogenic equivalents concentrations had been reported to cause adverse effects. The study also demonstrated the suitability of passive sampling combined with chemical analyses and *in vitro* bioassays to reveal these impacts.

Acknowledgments

This study has been supported by the projects of Ministry of Education C.R. (ENVISCREEN no. 2B08036 and INCHEMBIOL MSM0021622412), by the project CETOCOEN (CZ.1.05/2.1.00/01.0001) from the European Regional Development Fund, CENAKVA (CZ.1.05/2.1.00/01.0024) and the project SP/2e7/229/07 (Ministry of Environment C.R.). The research was also supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (project # 326415-07) and a grant from the Western Economic Diversification Canada (project # 6578 and 6807). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Prof. Giesy was supported by the Canada Research Chair program, an at large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, The Einstein Professor Program of the Chinese Academy of Sciences and the Visiting Professor Program of King Saud University.

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