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Bioaccumulation of the synthetic hormone 17 α -ethinylestradiol in the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*

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ABSTRACT

The present study investigated the bioaccumulation of the synthetic hormone 17 α -ethinylestradiol (EE2) in the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*, in water-only and spiked sediment assays. Water and sediment residue analysis was performed by LC/MS–MS, while biota extracts were analyzed using both LC/MS–MS and a recombinant yeast estrogen receptor assay. At the lowest exposure concentration, *C. tentans* accumulated less EE2 than *H. azteca* in the water-only assays ($p = 0.0004$), but due to different slopes, this difference subsided with increasing concentrations; at the exposure concentration of 1 mg/L, *C. tentans* had a greater body burden than *H. azteca* ($p = 0.02$). In spiked sediments, *C. tentans* had the greatest EE2 accumulation (1.2 ± 0.14 vs. 0.5 ± 0.05 $\mu\text{g/g dw}$, $n = 4$). Measurements in *H. azteca* indicated a negligible contribution from the sediments to the uptake of EE2 in this species. These differences were likely due to differences in the behavior and life history of the two species (epibenthic vs. endobenthic). Water-only bioaccumulation factors (BAFs) calculated at the lowest exposure concentration were significantly smaller in *C. tentans* than in *H. azteca* (31 vs. 142, respectively; $p < 0.0001$). In contrast, the sediment bioaccumulation factor (BSAF) of *C. tentans* was larger than that of *H. azteca* (0.8 vs. 0.3; $p < 0.0001$). Extracts of the exposed animals caused a response in a recombinant yeast estrogen receptor assay, thus confirming the estrogenic activity of the samples, presumably from EE2 and its estrogenic metabolites. The results of the present study suggest that consumption of invertebrate food items could provide an additional source of exposure to estrogenic substances in vertebrate predators.

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1. Introduction

Concerns over exposure to chemicals capable of altering endocrine function have stimulated significant research on their effects on aquatic biota. Several studies have reported incidences of intersex, physiological abnormalities, and varying degrees of hormonal imbalance in fish exposed to sewage treatment effluent (Jobling and Sumpter, 1993; Purdom et al., 1994; Jobling et al., 1998), and although a number of classes of contaminants have been shown to affect the endocrine function of vertebrates, natural and synthetic estrogens have generally been implicated (White et al., 1994; Desbrow et al., 1998; Routledge et al., 1998). These hormones have been detected in sewage treatment effluents, surface waters, and sediments, following incomplete removal from sewage treatment facilities (Ternes et al., 1999; Kolpin et al., 2002a; Ternes et al., 2002; López de Alda et al., 2002).

The synthetic hormone 17 α -ethinylestradiol (EE2), used in birth control and hormone replacement therapy, has been

reported to exhibit *in vitro* biological activities similar to that of the natural hormone 17 β -estradiol, due to its strong affinity for the estrogen receptor (Pawlowski et al., 2004; Van den Belt et al., 2004). Several studies have shown that exposure to concentrations of EE2 in the range of those detected in the environment (0.001–0.273 $\mu\text{g/L}$) (Ternes et al., 1999; Kolpin et al., 2002b) elicits the synthesis of the egg-yolk precursor vitellogenin, increases incidences of histological aberrations, and causes the feminization of male fish (Harries et al., 1996; Länge et al., 2001; Parrott and Blunt, 2005; Palace et al., 2006). In contrast to aquatic vertebrates, in which estrogens are involved in sexual function, unambiguous evidence of sex receptors in invertebrates has not been demonstrated (Köhler et al., 2007). For example, the development and differentiation of sexual characteristics of insect is not under hormonal control, while the sexual differentiation in crustaceans is mediated by the androgenic hormone (Defur et al., 1999; Nijhout, 1994). Thus, aquatic invertebrates would not necessarily be expected to be sensitive to EE2. In previous studies, we investigated the effects of a chronic EE2 exposure to the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*, and showed that adverse effects occur at concentrations several orders of magnitude greater than those detected in the environment, and

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that the risks associated with exposure to benthic invertebrates are small (Dussault et al., 2008).

The octanol–water partition coefficient of EE2 ($\log K_{OW} = 4.0$; Kasim et al., 2004) indicates a potential for sorption to organic matter and bioaccumulation in organisms, yet the latter, to date, has been the subject of few studies. Benthic invertebrates play an important role in aquatic food webs, and can be exposed to contaminants present in sediments and overlying water (US EPA, 2000). A previous study in our laboratory revealed that EE2 sorbed to sediments is considerably less bioavailable to benthic invertebrates compared to EE2 dissolved in water (Dussault et al., submitted). However, through trophic transfer, benthic invertebrates could be a source of secondary contamination to vertebrate predators, which are more sensitive to EE2 (Länge et al., 2001; Liebig et al., 2005). In the present study, the bioaccumulation of EE2 was investigated and quantified in the midge *C. tentans* and the freshwater amphipod *H. azteca*, in water-only and spiked sediment assays, to compare and contrast the relative contribution of EE2 from sediments with that of water. In addition, to estimate the bioavailability and the estrogenic potential of these organisms to vertebrate predators, the estrogenic potency of biota extracts was evaluated using a recombinant yeast estrogen receptor assay.

2. Materials and methods

2.1. Chemicals

Acetonitrile (MeCN, HPLC grade), methanol (MeOH, HPLC grade), acetone ($\text{CH}_3)_2\text{CO}$, distilled in glass), and ammonium carbonate were acquired from Caledon Laboratories (Georgetown, ON, Canada), while 17α -ethinylestradiol (EE2; purity $\geq 98\%$) and isotopically labelled $^2\text{H}_2$ - 17β -estradiol ($^2\text{H}_2\text{E}_2$; atomic purity $> 98\%$) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Yeast nitrogen base (without amino acids) and bactoagar were obtained from Difco Laboratories (Detroit, MI, USA). Lysine, histidine, monobasic sodium phosphate and dibasic sodium phosphate were acquired from Sigma-Aldrich (Oakville, ON, Canada). Dextrose, copper sulfate and potassium chloride were purchased from VWR (Mississauga, ON, Canada), magnesium sulfate was obtained from EM Science (Cherry Hill, NJ, USA), β -mercaptoethanol was acquired from Fisher Scientific (Whitby, ON, Canada), and sodium dodecyl sulfate was obtained from Mallinckrodt Specialty Chemicals (Mississauga, ON, Canada). Oxaliticase was purchased from EnzoGenetics (Corvallis, OR, USA), and *o*-nitrophenyl- β -galactopyranoside was obtained from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Bioaccumulation

2.2.1. Water-only exposures

Water-only bioaccumulation studies were conducted in reconstituted water (ASTM, 2004; Borgmann, 2002), using nominal concentrations ranging from 0.01 to 5 mg/L in a continually aerated gravity-fed flow-through system, which ensured the maintenance of constant concentrations of EE2, its aqueous half-life being 10 d (Jürgens et al., 2002). The assays were performed in 300 mL tall-form beakers, to which 50 mL of sand (particle size 250–500 μm) was added in the *C. tentans* experiment, and a small piece of cotton gauze was added in the *H. azteca* experiment, to serve as substrate (Borgmann, 2002). The beakers were held within larger aquaria containing 16 L (*C. tentans*) or 8 L (*H. azteca*) of water, as previously described (Dussault et al., 2008). Water circulation within and between the beakers was ensured through two water-exchange ports, previously described by Benoit et al. (1997). Complete water renewals were performed daily, using freshly made solutions. Water characteristics were as follows: pH, 7.9 ± 0.03 ($n = 24$, *H. azteca*) and 8.1 ± 0.04 ($n = 42$, *C. tentans*); water temperature, 19.8 ± 0.08 ($n = 24$, *H. azteca*) and 22.5 ± 0.03 ($n = 42$, *C. tentans*); conductivity, 366 ± 0.9 ($n = 24$, *H. azteca*) and 376 ± 0.3 ($n = 42$, *C. tentans*); dissolved oxygen, 8.5 ± 0.07 ($n = 24$, *H. azteca*) and 7.1 ± 0.17 ($n = 63$, *C. tentans*) mg/L; ammonia levels were maintained below 1 mg/L. Water was sampled weekly for residue analysis.

Upon test initiation, 12 *C. tentans* larvae (<24 h post-hatch) or 15 *H. azteca* (21–28 d) were added to each beaker ($n = 4$ at each concentration). A test duration of 21 days was selected to represent a chronic exposure scenario, due to the short larval life stage of *C. tentans*, which emerges after 21 d (US EPA, 2000). In contrast, due to the smaller size of *H. azteca*, larger animals were selected at test initiation to ensure sufficient samples were available for tissue analysis. Each container was fed either 1 mL of a 6 g/L fish food slurry daily (*C. tentans*) (US EPA, 2000), or 2.5 mg of dried Tetra Min food flakes every other day (*H. azteca*) (Borgmann, 2002). Exposure

to EE2 via ingestion of contaminated food may represent an additional source of exposure, but was not quantified in the present study. At the end of the exposure period, the surviving animals from each beaker were counted, and transferred into 2–4 mL of acetone (pooled by beaker), refrigerated and extracted within 48 h ($n = 4$ at each concentration).

2.2.2. Sediment exposures

Spiked sediment studies were performed in 1-L Imhoff sedimentation cones (Borgmann and Norwood, 1999), using a reference sediment (Lake Erie site # 112, pH 8.17, alkalinity 89 mg/L, 36% clay, 55% silt, 9% sand, 1.9% total organic carbon; Lee Grapentine, Environment Canada, Burlington, ON, Canada, personal communication). A spiking concentration of 10 $\mu\text{g/g}$ dw was selected, using a 0.2% v/vw MeOH concentration, while control sediments were spiked with 0.2% v/vw MeOH. Samples were mixed by rotation at a speed of 6 rpm for 72 h, after which 25 mL aliquots of sediment were added to the bottom of each water-filled cone. The water column in each cone was gently aerated throughout the study. The test systems were left to equilibrate with the overlying water for 24 h prior to test initiation. On day 0, 12 *C. tentans* larvae (<24 h post-hatch), or 15 *H. azteca* (21–28 d) were added to each cone and exposed to the experimental conditions for 21 days. Water characteristics were relatively similar to those of the water-only exposure, however water conductivity was greater (397 ± 4.0 ($n = 17$, *H. azteca*) and 622 ± 12.0 mS/cm ($n = 11$, *C. tentans*)), which we attribute to the presence of sediments. Food was added to each cone using the same protocol as the water-only exposures. Given the small size of *H. azteca*, triplicate samples were pooled to obtain sufficient biomass for analysis, hence the number of cones was increased (to 24) to maintain the number of samples analyzed constant. After the exposure period, the animals from half of the cones were counted, removed, rinsed and transferred to 2–4 mL of acetone, while the remaining half of the replicates from the sediment exposure ($n = 4$ (*C. tentans*) or 12 (*H. azteca*)) were submitted to a 24-h gut-clearance period in water free of EE2 for a preliminary estimate of the depuration of EE2. For *C. tentans*, a small amount of clean sand was added to the depuration beakers. Samples were then transferred to 2–4 mL of acetone as detailed above.

In order to investigate the role of sediments in the bioaccumulation of EE2 in *H. azteca*, cages were also installed at the water surface of the cones, thus allowing comparisons between water-only and whole water column (sediment and overlying water) exposures. Because sediment avoidance behavior has been reported in *H. azteca* (Hoke et al., 1995; Call et al., 2001), the number of animals visible from above, and therefore not in direct contact with the sediments, were counted during four feeding events. The experimental design did not permit counting all the animals present in the water column, hence these values represent relative estimates.

2.3. Chemical analysis

2.3.1. Extractions

When necessary, water residue samples were pre-concentrated by solid phase extraction (Supelco ENVI-18, Sigma-Aldrich, Oakville, ON, Canada), and eluted in 3×2 mL MeOH, following the procedures outlined previously (Dussault et al., 2009). If sample pre-concentration was not necessary, samples were filtered through 0.45 μm syringe filters and frozen until sample analysis was completed.

Sediment samples were thawed, and approximately 10–12 g of sediment was analyzed by pressurized liquid extraction in MeOH, explained in more detail in Dussault et al. (2009). An extraction preparation aid (Hydromatrix 15–20% w/vw; Varian, Mississauga, ON) was mixed with the sediments prior to transfer into the extraction cells.

Biota samples were extracted by sonication of whole, pooled organisms in 2–4 mL of acetone (5 min degas, 30 min sonication), followed by centrifugation at 1000 g for 30 min at 4 °C. The supernatant was collected, evaporated to dryness, reconstituted in 250–500 μL of 50:50 MeOH:MQ, filtered through 0.2 mm syringe filters, and stored at -20 °C until chemical analysis was performed (Dussault et al., accepted). The remaining tissues were dried at 60 °C for 48 h and weighed.

2.3.2. LC/MS–MS analysis

Prior to analysis, a 5 mg/L $^2\text{H}_2$ -E2 internal standard was spiked into each sample, by addition of 20% of sample volume (e.g., 50 μL were added to a 250 μL sample), which served to correct for variations in ionization efficiency. Method development and validation are described in detail in Dussault et al. (2009). All analyses were performed by liquid chromatography tandem mass spectrometry, using an Acquity UPLC separation module, and a Quattro Ultima Micromass mass spectrometer (Waters, Milford, MA), in negative electrospray ionization mode. Ammonium carbonate (0.5% w/v in MeOH) was infused post-column at a rate of 10 $\mu\text{L}/\text{min}$, which served to assist the ionization of the target analyte (Dussault et al., 2009).

2.3.3. Recombinant yeast estrogen receptor assay

A recombinant yeast estrogen receptor assay was used to evaluate the binding affinity of the biota extracts for the human estrogen receptor, as an indicator of bioavailability to vertebrate predators. The recombinant yeast estrogen receptor

assay used in the present study employed the recombinant strain BJ3505 hER/2ERE of the yeast *Saccharomyces cerevisiae* as described by Gaido et al. (1997) and Burnison et al. (2003), with minor modifications to allow for use in 96-well microtiter plates.

The biota extracts were diluted to concentrations compatible with the recombinant yeast estrogen receptor assay (i.e., concentrations that would cause a quantifiable response), based on the results of the analysis by LC/MS–MS. Duplicate 10 μ L aliquots of extracts were transferred into 96-well microtiter plates (Corning Costar, Corning, NY, USA), and the solvent was evaporated to dryness. A 200 μ L aliquot of cultured yeast cells was added to each well, and incubated at 30 °C for 16 h. A 10 μ L aliquot of the culture solution was then transferred to each well of a new microtiter plate containing 160 μ L of Z buffer+sodium dodecyl sulfate. Cell density (optical density) was measured at 600 nm using a VERSAmax™ kinetic plate reader (Molecular Devices, Sunnyvale, CA, USA) at 30 °C, after which the cells were permeabilized by addition of 40 μ L of Z-buffer containing 500 units/mL of oxalylase, and 4 mg/mL of *o*-nitrophenyl- β -galactopyranoside. Enzymatic cleavage of *o*-nitrophenyl- β -galactopyranoside by β -galactosidase was monitored by measurement of optical density at 420 nm every 15 s for the following 30 min. β -galactosidase activity of each sample was expressed as initial velocity of enzymatic reaction divided by cell density (or V_{max}/OD_{420}). The biota samples were compared to the induction rates of EE2, from a standard curve (0.01–10 nM) added to each plate, and converted to EE2 equivalents using GraphPad Prism (ver. 4.02, GraphPad Software Inc., San Diego, CA, USA).

2.4. Calculations

Uptake from water was modelled using linear and Freundlich isotherms. The Freundlich isotherm yielded the best fit, and was thus selected, while the logarithm form of the isotherm is presented in Fig. 1. These models were then compared with the concentrations in the organisms exposed via sediment, to estimate the fraction of EE2 accumulated from exposure to the overlying water (P_w), and from sediments ($P_s = 1 - P_w$). For water-only exposures, bioaccumulation factors (BAFs) were calculated using the equation $BAF = C_b/C_w$, where C_b and C_w are the concentrations measured in biota and water, respectively. Sediment bioaccumulation factors (BSAFs) were calculated using the equation $BSAF = C_b/C_s$, where C_s is the concentration measured in the bulk sediment.

2.5. Statistical analyses

Differences between the slopes of the regression lines of bioaccumulation with increasing water exposure were analyzed using a two-factor analysis of variance (the variables inspected were species, concentration and concentration*species). Differences between treatments were tested using an analysis of variance. Verification of the assumptions of normality and homoscedasticity was performed using the Shapiro–Wilk statistic and by plotting predicted versus residual values, respectively. The Studentized residuals were compared to the critical values for an approximate test of outliers. Data that did not meet the assumptions of normal distribution of residuals were log-transformed prior to analysis. When the analysis of variance revealed a significant effect, differences were tested using the Tukey HSD test for multiple comparisons. If data transformation did not resolve issues with the assumptions of the analysis of variance, data were analyzed using a Kruskal–Wallis test, followed by pairwise comparisons. The level of significance selected for this series of tests was then adjusted using the Bonferroni method to $\alpha = 0.05/n$, where n is the number of simultaneous comparisons made (Sokal and Rohlf, 1995). All computations were performed using SAS® (ver. 9.1; The SAS Institute, Cary, NC, USA). The selected level of significance was $\alpha = 0.05$.

Statistical analyses for the sediment experiments were complicated by the presence of data below the limit of detection (LOD). During the uptake study, 5/18 samples were below the LOD, all from controls, while 8/18 samples were below the LOD during the depuration study, 5 from controls, and 3 from treatments. Several approaches have been suggested for the use of non-detects, including the substitution of $\frac{1}{2}$ LOD (US EPA, 1989). However, this approach may cause a bias by artificially reducing variance. Since the non-detects were mostly found within the controls, and since comparisons with the controls were not essential to this analysis, the control data were excluded. In order to provide preliminary data on depuration of EE2, the remaining three data points below the LOD were substituted by $\frac{1}{2}$ LOD.

3. Results and discussion

3.1. Survival and growth

Survival and growth in the controls was greater or equal to the minimum test acceptability criteria of 70% and 0.60 mg/organism (*C. tentans*) and 80% (*H. azteca*), in both water-only and sediment assays (US EPA, 2000). With the exception of the greatest exposure

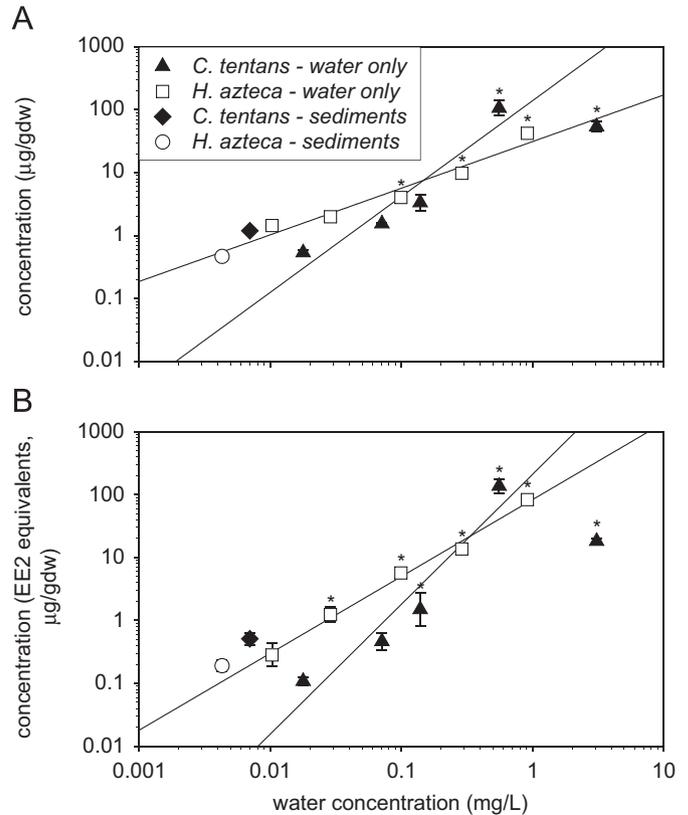


Fig. 1. Bioaccumulation (mean \pm standard error of the mean) of 17 α -ethinylestradiol in *Chironomus tentans* ($y = 1.52x + 2.14$, $r^2 = 0.87$) and *Hyalella azteca* ($y = 0.74x + 1.49$, $r^2 = 0.93$), during a 21-d flow-through water-only exposure, and after a 21-d exposure to spiked sediments: (A) tissue residue analysis by LC/MS–MS; (B) analysis using a recombinant yeast estrogen receptor assay. *Denotes significant bioaccumulation compared to the lowest detectable concentration ($p < 0.05$). Due to poor survival at the highest concentration during the *C. tentans* water-only exposure (35% at 3.1 mg/L), these data were excluded from further analysis.

concentration during the *C. tentans* water-only exposure (3.1 mg/L), where survival was significantly decreased (35% survival) relative to the controls, EE2 exposure did not affect animal survival or growth (Table 1). The 20-d LC50 of EE2 previously estimated in our laboratory was 2.2 mg/L (Dussault et al., 2008), hence we attribute the low survival to the toxicity of EE2. When this treatment was excluded from the statistical analysis, survival was slightly lower during the sediment exposure ($p = 0.04$). Animals from sediment tests were also significantly larger ($p = 0.02$; Table 1), an observation consistent with the literature (Borgmann, 2002). During the *H. azteca* assay, the growth of animals exposed to the sediments was significantly greater than other treatments (water-only and cage exposures; $p \leq 0.02$; Table 1), while the animals kept in the cages had lower survival than other treatments ($p < 0.02$). Animals exposed to the sediments were occasionally observed in amplexus, and subsequently reproduced (data not shown). In contrast, the animals held in the cages had not reached reproductive maturity, as indicated by the absence of amplexus. These disparities between treatments were presumably due to greater availability of food resources for animals exposed to the sediment, compared with those held in the cages, and during water-only exposures.

3.2. Bioaccumulation

3.2.1. Water-only assays

During the 21-d water-only test, *C. tentans* and *H. azteca* accumulated EE2 in a concentration-dependent manner, with

Table 1Survival and growth (mean \pm standard error of the mean) of *Chironomus tentans* and *Hyalella azteca* during a 21-d exposure to EE2 in water and spiked sediment.

	<i>Chironomus tentans</i>		<i>Hyalella azteca</i>		
	Water-only	Sediment	Water-only	Sediment	Cages
Survival (%)	86 \pm 2.2 ^a	74 \pm 5.0	97 \pm 0.9	94 \pm 0.7	79 \pm 3.7 ^b
Dry weight (mg/animal)	0.8 \pm 0.08	1.1 \pm 0.14 ^c	0.3 \pm 0.01	0.6 \pm 0.02 ^c	0.4 \pm 0.01

^a Survival of animals excluding the exposure to the highest treatment (3.1 mg/L, where only 35% survival was observed) was significantly higher than during the sediment-only exposure ($p < 0.05$, Kruskal–Wallis).

^b Value significantly different from other *H. azteca* treatments ($p < 0.017$, Bonferroni-adjusted Kruskal–Wallis).

^c Value significantly larger than other treatments for the same organism ($p < 0.05$).

body burdens varying between 0.5 and 119 $\mu\text{g/g}$ (Fig. 1A). During the *C. tentans* exposure, only 35% of the animals survived in the greatest EE2 treatment (3.1 mg/L). Because exposure to contaminants can result in changes in animal behavior in *C. tentans* (e.g., reduced activity, thus decreasing contaminant exposure), these data were excluded from the analyses. The two-factor analysis of variance revealed significant differences between the slopes of body burden with increasing water concentration ($p < 0.0001$; Fig. 1). At the lowest exposure concentration, the body burden for *C. tentans* was significantly lower than that of *H. azteca* ($p = 0.0004$), and due to a greater slope, higher at a nominal exposure concentration of 1 mg/L ($p = 0.02$; Fig. 1A). If any, differences in uptake between the two species could be due to variations in uptake, depuration and/or conjugation of EE2, however, we are not aware of studies investigating the toxicokinetics of EE2 in aquatic invertebrates. Further investigations would be required to characterize the uptake and metabolism of this chemical in greater detail. Although the number of animals required to characterize the level of bioaccumulation of EE2 at environmental concentrations (several hundreds per sample) did not permit inclusion of these concentrations in the present study, further investigations will be needed to provide a more detailed evaluation of the bioaccumulation of EE2 in these organisms at environmental concentrations.

3.2.2. Sediment assays

When animals were exposed to spiked sediments, *C. tentans* accumulated significantly more EE2 than *H. azteca* (1.2 ± 0.14 vs. 0.5 ± 0.05 $\mu\text{g/g}$, $p < 0.0001$; Fig. 2), which could be explained by differences in the behavior and life history of the two species. *H. azteca* is a facultative benthic invertebrate, which may or may not be in direct contact with the sediments (Hoke et al., 1995; Call et al., 2001). In contrast, *C. tentans* is an obligate benthic invertebrate inhabiting surficial sediments (\approx top 5 cm), which are used as a substrate to construct a protective case (Reible et al., 1996; Gunnarsson et al., 1999).

There were no significant differences in EE2 bioaccumulation between *H. azteca* exposed to the sediments and those exposed to the overlying water only in cages ($p = 0.08$; Fig. 2), indicating that sediments likely played a negligible role in the bioaccumulation of EE2 by *H. azteca*, which may be due to the behavior and low level of interaction of this organism with sediments. During each observation of the behavior of *H. azteca*, 12–15% of animals were observed in the top 5–10 cm of the water column, and thus not in direct contact with the sediments. The presence of animals in the water column was independent of the presence of EE2 in the sediments ($p = 0.90$), and, hence, probably did not result from EE2-induced avoidance of the sediment. Because only the top 5–10 cm of the sedimentation cone could be inspected, these numbers do not provide an exact estimate of the contact of *H. azteca* with the sediments, but enabled us to test whether the presence of EE2 caused sediment avoidance.

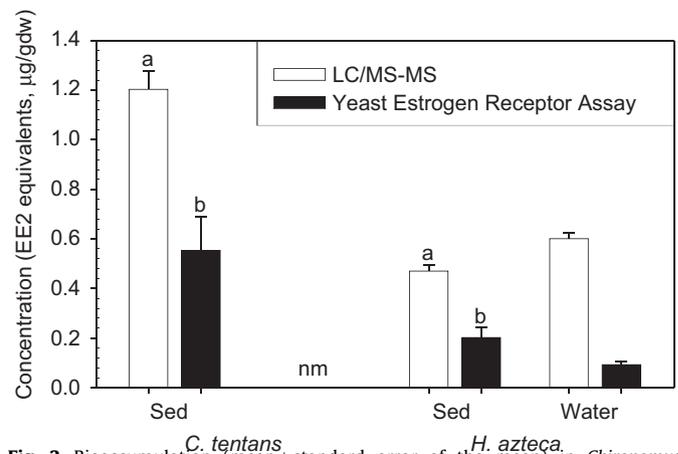


Fig. 2. Bioaccumulation (mean \pm standard error of the mean) in *Chironomus tentans* and *Hyalella azteca*, after a 21-d sediment exposure to the synthetic hormone 17 α -ethinylestradiol, measured by LC/MS-MS analysis and using a recombinant yeast estrogen receptor assay. Exposure was from either sediments (Sed), or the overlying water only (Water). Bars with corresponding letters are significantly different. nm = not measured.

The body burden of EE2 in *C. tentans* exposed via sediment was significantly greater than would be expected from exposure to the EE2 in the overlying water only, in which the EE2 concentration was 0.007 mg/L, as seen by the position of the data point above the water-only regression line for *C. tentans* (Fig. 1A). In contrast, the contribution of the sediment to uptake of EE2 by *H. azteca* was negligible (Fig. 1A). The relative contribution of each matrix to the EE2 accumulated in *C. tentans* during the spiked sediment assay was estimated using the Freundlich model from the water-only assay. During the spiked sediment assay, *C. tentans* was exposed to 0.007 mg/L EE2 in the overlying water, which would result in a tissue concentration of 0.07 $\mu\text{g/g}$, or 6% of the body burden detected in *C. tentans* during the sediment exposure, indicating that a significant proportion (e.g., \sim 94%) of EE2 bioaccumulated came from the sediment (which contained 1.5 $\mu\text{g/g dw}$ EE2). Despite the lower bioavailability of EE2 in sediments compared to that in overlying water, this source of contamination nevertheless appears to be important for deposit-feeders, such as *C. tentans*, which can ingest several times their body weight of sediments daily (Forbes et al., 1998). While specific information on feeding rates for *C. tentans* have not been estimated, the feeding activity of the closely related midge, *C. riparius*, estimated as egestion rates, was reported to be 0.05–0.15 mg dw faeces/g dw larva/h, equivalent to 1.2–3.6 times its body weight daily (Leppänen et al., 1998).

3.3. Depuration

The presence of non-detects complicated the estimation of 24-h depuration. Preliminary estimates were calculated for the sediment experiment, by substituting non-detects by a value of

$\frac{1}{2}$ LOD, hence these values should be regarded accordingly. Depuration rates of 0.6 and 0.2 $\mu\text{g/g/d}$ were calculated for *C. tentans* and *H. azteca* exposed to the sediments, while a depuration rate of 0.3 $\mu\text{g/g/d}$ was calculated for *H. azteca* exposed to the overlying water. These results represent elimination and/or conjugation rates of 51% and 37% for the sediment exposures, respectively, and 57% for *H. azteca* exposed to the overlying water. It should be noted that only the depuration rate of 0.2 $\mu\text{g/g/d}$ (*H. azteca* exposed to sediments) was calculated without the substitution for non-detects, that these preliminary results are presented for information purposes only, and that further investigations are needed. Liebig et al. (2005) reported a considerably slower depuration rate for EE2 in *Lumbriculus variegatus* with 50% remaining after 10 days, but their calculations also included all conjugation products.

3.4. Bioaccumulation factors

3.4.1. Water-only assays

The water-only bioaccumulation factors calculated for each exposure are presented in Table 2. Direct comparisons between experiments are complicated by the presence of differences in measured water concentrations (and thus, exposure). However, at a water exposure of 0.02 mg/L, common to both species, the BAF was significantly lower in *C. tentans* than in *H. azteca* (31 vs. 142; $p < 0.0001$). The highest BAF obtained for *H. azteca* was at 0.02 mg/L, but a higher BAF of 215 ± 61.1 was obtained at a concentration of 0.56 mg/L for *C. tentans* and was greater than that of *H. azteca* ($p = 0.02$; Table 2). These data suggest that maximum *H. azteca* uptake had been reached at a concentration < 0.03 mg/L (where bioaccumulation was not proportional to concentration, and the BAF decreased with increasing exposure), whereas *C. tentans* uptake a concentration of 0.56 mg/L. Since the bioaccumulation in *C. tentans* was lower than that of *H. azteca* at concentrations below 0.15 mg/L (Fig. 1), these differences may be due to greater conjugation and/or elimination rates, which is in agreement with the preliminary results presented here. In the absence of additional data on the uptake, metabolism and depuration of EE2 in benthic invertebrates, further toxicokinetic studies with these organisms will be required to test this hypothesis.

3.4.2. Sediment assays

Assuming that the source of EE2 was from exposure to sediments only, the BSAFs calculated following exposure to EE2

in spiked sediments were greater in *C. tentans* than in *H. azteca* (0.8 vs. 0.3, respectively; $p < 0.0001$, Table 2). In contrast, the BAF calculated for *H. azteca* held within the cages, and exposed to water concentrations of 0.004 mg/L EE2, was 110 ± 5.6 (Table 2), a value only marginally lower than that determined in the water-only bioaccumulation study (BAF = 142 ± 11.6 at a concentration of 0.02 mg/L). These results indicate that the bioavailability of EE2 sorbed to sediments is substantially reduced, however as noted above, the relative importance of the contribution of sediment to the bioaccumulation of EE2 in *C. tentans* can be significant, due to the life history and behavior of this animal.

Few studies have investigated the bioaccumulation of EE2 in aquatic biota. In a study evaluating the biotransformation and bioconcentration of estrogens in the algae *Chlorella vulgaris*, a bioconcentration factor (BCF) of 27 for estrone was estimated (Lai et al., 2002), but no bioaccumulation of EE2 was detected over a 48-h period. Länge et al. (2001) calculated BAFs of 660 (158 d posthatch, 64 ng/L) and 610 (245 d posthatch, 16 ng/L) for EE2 in the fathead minnow *Pimephales promelas*, however these values were measured at concentrations that caused significant adverse effects, which may have affected uptake and/or depuration kinetics. The BAFs calculated in the present study, 18–215, therefore, appear to be in general agreement with the few values reported in these studies.

A recent study reported a BSAF of 254 in the oligochaete *L. variegatus* after a 35-d exposure to a nominal concentration of 0.5 $\mu\text{g/g}$ ^{14}C -EE2 in spiked sediments (Liebig et al., 2005). The life-history of *L. variegatus*, an endobenthic invertebrate which, in contrast to *C. tentans*, predominantly feeds on sediments, would suggest a greater bioaccumulation from sediments than *C. tentans* (0.8) and *H. azteca* (0.3) investigated in the present study. However, the BSAF determined in the study by Liebig et al. (2005) were calculated using a total sample radioactivity approach, which did not differentiate between EE2 and other transformation products. In fact, further analysis by thin layer chromatography revealed that EE2 corresponded to only 6% of the radioactivity in the biota samples, which may influence the value of their BSAF, unless identical biotransformation was to occur in both invertebrates and sediments. Given the paucity of data and the use of different protocols, more detailed comparisons are not possible.

3.5. Recombinant yeast estrogen receptor assay

Several studies have demonstrated the effectiveness of recombinant yeast estrogen receptor assays for the detection of estrogenic activity of various types of samples extracted from surface water, sediments, fish bile, sewage treatment effluents, biosolids and manure (Desbrow et al., 1998; Burnison et al., 2003; Pawlowski et al., 2004; Peck et al., 2004; Lorenzen et al., 2004; Gibson et al., 2005). In the present study, this approach was used to estimate the relative binding affinity of tissue extracts of *C. tentans* and *H. azteca* for the estrogen receptor, using a recombinant yeast estrogen receptor assay. Because EE2 can be metabolized to other estrogenic compounds (e.g., 17 β -estradiol, estrone, and estriol) (Metcalf et al., 2001; Van den Belt et al., 2004), this assay characterizes the binding affinity of all estrogenic molecules present within each sample. Exposure of the recombinant yeast *S. cerevisiae* to the tissue extracts caused the induction of β -galactosidase activity in samples from biota exposed to EE2, thus confirming the presence of agonists to the estrogen receptor (Fig. 1B, Fig. 2). In contrast, biota samples from the control and methanol control did not induce any response in the recombinant yeast receptor assay.

Because the recombinant yeast estrogen receptor assay is only reactive to molecules that interact with the estrogen receptor, this

Table 2

Bioaccumulation factors (water-BAF; sediment-BSAF; mean \pm standard error of the mean, $n = 4$) in the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*, after a 21-d exposure to the synthetic hormone 17 α -ethinylestradiol in water or spiked sediments.

<i>Chironomus tentans</i>		<i>Hyalella azteca</i>	
Concentration (mg/L)	BAF	Concentration (mg/L)	BAF
<i>Water exposures</i>			
0.02	31 \pm 3.0	0.02	142 \pm 11.6
0.07	22 \pm 0.6	0.03	71 \pm 7.1
0.14	28 \pm 9.1	0.10	41 \pm 2.4
0.56	215 \pm 61.1	0.29	34 \pm 0.7
3.1	18 \pm 3.1	0.91	47 \pm 4.6
<i>Sediment exposures</i>			
–	–	0.004 (cage)	110 \pm 5.6
Concentration ($\mu\text{g/g}$)	BSAF	Concentration ($\mu\text{g/g}$)	BSAF
1.5	0.8 \pm 0.05	1.5	0.3 \pm 0.02

Values in bold are the highest BAFs calculated.

approach may not account for some of the EE2 metabolites, which may be biologically inactive (i.e., which do not bind to the estrogen receptor). For example, EE2 glucuronides are produced by phase II conjugation, which, if located on C3, renders the molecule biologically inactive (Ingerslev and Halling-Sørensen, 2003). In the study by Liebig et al. (2005), approximately 78% of the $^{14}\text{C}_2$ extracted from *L. variegatus* was recovered as EE2 after sample treatment with β -glucuronidase. While values for *C. tentans* and *H. azteca* have not been reported to date, it is likely that some EE2 is present as glucuronides, or as other conjugation products (e.g., sulfate conjugates). Since deconjugation of EE2 glucuronides has been observed in several aquatic vertebrates and invertebrates (Sanborn and Malins, 1980; James, 1986; Tilton et al., 2001), these metabolites could revert to biologically active forms in sensitive vertebrate predators, and could be an additional source of contamination (Liebig et al., 2005). Hence, although the recombinant yeast estrogen receptor assay provided a useful estimate of the estrogenic activity of our test organisms, the potential additional contribution of EE2 conjugates may not have been entirely estimated using this approach. With the exception of the data from the study by Liebig et al. (2005), the degree of conjugation of EE2 in benthic invertebrates has not been fully established, and needs to be investigated further.

In vitro assays provide simple and cost-effective means for elucidating specific mechanisms of action (e.g., estrogen receptor binding), but are not designed to mimic effects likely to occur at higher levels of organization. Due to the limited metabolic capacity of yeast cells, the approach used in the present study does not take into account uptake, bioaccumulation, biotransformation or degradation, nor the complex interactions involved with the regulation of estrogens occurring within an organism (Zacharewski, 1997; Petit et al., 1997; Segner et al., 2003). Hence, whole-organism studies will be required to further establish and estimate the physiological consequences of EE2 exposure through contaminated food.

4. Summary and conclusions

The present study revealed that *C. tentans* and *H. azteca* accumulated EE2 in a concentration-dependent manner in water-only assays. At smaller exposure concentrations, *C. tentans* appeared to bioaccumulate less EE2 than *H. azteca*. In contrast, the uptake of EE2 by *C. tentans* was significantly greater than *H. azteca* when exposure was via spiked sediments. These results indicate that these two organisms were exposed to EE2 through different sources, which may be explained by differences in their life history and behavior. Analysis of biota extracts using the recombinant yeast estrogen receptor assay indicated the presence of estrogenic material, confirming the potential for exposure to EE2 through trophic transfer. Conjugates of EE2 which are biologically inactive were not measured, but could be an additional source of exposure in vertebrate predators through deconjugation processes. Since environmental exposure to EE2 is already a source of concern, exposure through contaminated prey could pose a significant risk to sensitive aquatic vertebrates. Further trophic transfer studies at environmental concentrations will be needed to provide precise estimates of the relative importance of food to the exposure of aquatic vertebrates to EE2.

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