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Bioaccumulation of ^{14}C-17α-ethinylestradiol by the aquatic oligochaete *Lumbriculus variegatus* in spiked artificial sediment

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Abstract

A bioaccumulation study was performed with the endobenthic freshwater oligochaete *Lumbriculus variegatus* MÜLLER exposed to the radiolabelled synthetic steroid 17α-ethinylestradiol (^{14}C-EE$_2$) in a spiked artificial sediment. Concentration of total radioactivity increased constantly and almost linearly during 35 days of exposure. The accumulation factor normalised to worm lipid content and sediment TOC ($AF_{lipid/OC}$) was 75 at the end of the uptake period, but a steady state was not reached. Uptake kinetics were calculated fitting the measured AFs to a kinetic rate equation for constant uptake from sediment using iterative non-linear regression analysis. After 10 days of elimination in contaminant-free sediment 50% of the accumulated total radioactivity was excreted by the worms. Extracts from *L. variegatus* sampled at the end of the uptake phase were analysed by thin layer chromatography (TLC). The results showed that 6% of the total radioactivity incorporated by the worms was ^{14}C-EE$_2$. After treatment of extracts with β-glucuronidase the amount of ^{14}C-EE$_2$ increased to 84%. These results suggest that *L. variegatus* has the potency to accumulate high amounts of conjugated EE$_2$. Hence, a transfer of EE$_2$ to benthivores and subsequent secondary poisoning of predators might be possible.

Keywords: Benthivores; Bioaccumulation; ^{14}C-17α-ethinylestradiol; *Lumbriculus variegatus*; Sediment-associated contaminants; Secondary poisoning; Synthetic estrogens

1. Introduction

The synthetic estrogen 17α-ethinylestradiol (EE$_2$) is a widely used contraceptive agent (Schwabe and Paffrath, 2000). After application in humans it is excreted mainly in conjugated forms with the urine and, hence, it reaches the aquatic environment via sewage effluents (Adler et al., 2001). Its high octanol–water partition coefficient ($\log K_{ow}$ 4.2; Schweinfurth et al., 1997) indicates a tendency to adsorb to organic material and to accumulate in biota or sediments. Contaminated sediments may become a secondary source of exposure to benthic organisms (e.g., Suedel et al., 1994). Accumulation in benthic organisms may cause concern, because these organisms constitute an important link in the aquatic food web. Benthic macroinvertebrates are the prey of various secondary consumers, e.g. crabs, bottom-feeding

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fish and sediment-probing birds (Wootton, 1984; Wallace and Webster, 1996). Thus, transfer of accumulated contaminants to predators may occur and result in secondary poisoning. Since EE_2 is a potent synthetic estrogen, endocrine disrupting effects in fish caused by secondary poisoning cannot be ruled out.

The present bioaccumulation study was performed in order to assess the bioaccumulation of EE_2 in endobenthic organisms. The study was conducted with ^{14}C-radiolabelled EE_2 using the benthic freshwater oligochaete *Lumbriculus variegatus* Müller based on a method developed by Egeler et al. (1997, 1999). In order to ensure standardised conditions and reproducibility an artificial sediment and reconstituted water as overlying medium were used.

2. Materials and methods

2.1. Sediment and overlying water

The artificial sediment consisted of 75% fine quartz sand (type F36, average grain size 0.17 mm; Quarzwerke GmbH, Frechen, Germany), 20% kaolinite clay (type TEC1, Ziegler & Co. GmbH, Wunsiedel, Germany) and 4.5% sphagnum peat powder (Floratorf®, Floragard Vertriebs GmbH, Oldenburg, Germany). The sediment was prepared according to a method described by Egeler et al. (1997). 0.5% fine powder of stinging nettle *Urtica* sp. (Caesar & Loretz GmbH, Hilden, Germany) was added as nutrition for the test animals. During the test further food was not provided since the addition of uncontaminated food may change exposure conditions (Riedhammer and Schwarz-Schulz, 2001). The water content of the sediment was approximately 46% (in addition to sediment dry weight) using deionised water and pH was adjusted with $CaCO_3$ to approximately 7.0. Reconstituted water as described by OECD (1992) was used as overlying medium.

2.2. Test substance application, sample treatment and radioactivity measurement

^{14}C-radiolabelled 17α-ethinylestradiol (EE_2, CAS-No. 57-63-6) dissolved in ethanol was provided by Schering AG, Berlin, Germany. The specific radioactivity was 5.54 MBq mg^{-1} with a radiochemical purity of >99%. The molecule was labelled twice at the position of the 17α-ethinyl group (Fig. 1).

The nominal concentration of the test substance was set to 300 ng EE_2 g^{-1} wet weight (ww) or 556 ng EE_2 g^{-1} dry weight (dw), which corresponds to 100 000 disintegrations per minute (dpm) g^{-1} sediment ww. This concentration was selected in order to quantify potential metabolites at a minimal rate of 10% of parent compound at an assumed default accumulation factor of 1

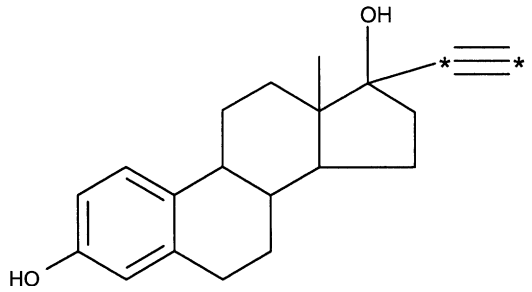

Fig. 1. Chemical structure of ^{14}C-17α-ethinylestradiol; radiolabelled atoms are marked with an asterisk.

in the given sample size of 1 g biota by thin layer chromatography (TLC). In a previously conducted 28-day sediment toxicity study under comparable conditions, the No Observed Effect Concentration (NOEC) referring to the sublethal parameters reproduction and biomass was determined at 31.6 mg EE_2 kg^{-1} dw (own unpublished results). The concentration used in this study was 100 times below the NOEC, therefore, toxic effects were not expected.

A small portion of the quartz sand was spiked with the test substance dissolved in acetone. Prior to mixing the spiked sand with the other sediment constituents, the acetone was allowed to evaporate. After spiking, the concentration was confirmed by measuring the radioactivity of the spiked quartz sand and of the complete sediment. Total radioactivity was measured in the overlying water, sediment and biota samples by Liquid Scintillation Counting (LSC; TriCarb 2500 TR, Canberra-Packard, Dreieich, Germany). Water samples were analysed directly using 1 ml sample mixed with 4 ml scintillation cocktail (Perkin-Elmer Life Sciences GmbH, Rodgau-Jügesheim, Germany). Sediment samples were analysed throughout the experiment by two different methods: 0.2–0.5 g ww sediment samples were thoroughly mixed with 1 ml acetone, mixed with 10 ml LSC cocktail, and subsequently analysed by LSC. Additionally, sediment sub-samples were combusted in a TriCarb 307 Sample Oxidizer (Canberra Packard, Dreieich, Germany) prior to LSC. The latter method resulted in recovery rates of 101 ± 9% ($n = 3$), hence results of combustion were used for further calculations. Biota samples were treated with 1 ml tissue solubiliser (Soluene 350, Packard, Dreieich, Germany) and incubated for 5 h at 50 °C. Subsequently and after neutralisation with 100 µl HCl, the samples were measured in 12.5 ml Hionic Fluor LSC cocktail (Packard, Dreieich, Germany). The recovery rate of this method was 95 ± 2% ($n = 3$).

Dry weight (dw) and lipid content of the animals, and dw and total organic carbon (TOC) of the sediment were determined using sub-samples of controls at the end of the exposure period. Dry weight was determined after heating the samples for 12 h at 105 °C in a drying oven.

After homogenisation by ultrasonication of approximately 0.5 g ww biota samples, lipid content was measured based on a method described by de Boer et al. (1997). TOC of the sediment was determined via LOI (loss on ignition) according to DIN (1985) after drying overnight at 105 °C and subsequent combustion at 600 °C for 2 h. The TOC was calculated based on the LOI using the conversion factor of 1.72 which corrects the LOI for other fugacious organic components lost during combustion of the sample (Atkinson et al., 1958, in McKeague, 1976).

2.3. Test animals

The sediment dwelling oligochaete *L. variegatus* is a suitable test organism for bioaccumulation studies, since the worms consist of sufficient tissue mass for radiochemical analysis and, in principle, uptake of contaminants via body surface and ingestion is possible (Phipps et al., 1993). However, according to Leppänen and Kukkonen (1998) exposure due to sediment ingestion appears to be the predominant route of exposure for *L. variegatus*. The worms are easily cultured and handled under laboratory conditions (Phipps et al., 1993; Drewes, 1996). The animals used in the test were taken from in-house cultures. They are cultured in 3.5 l glass containers, and fed with a suspension of finely ground TetraMin® fish food twice per week. To synchronise the physiological status of the worms their body was intersected with a scalpel (Brust et al., 2001). The posterior part was then transferred to vessels containing water and quartz sand. After 6 days the oligochaetes regenerated a new head and began to burrow into the sand. 14 days after synchronisation the animals were transferred to the test vessels containing spiked sediment.

2.4. Experimental procedure

For the bioaccumulation experiment a lid-covered glass aquarium, which contained the test vessels, was installed in a climate chamber and kept at 20 ± 2 °C (Fig. 2). Each test vessel (100 ml glass tubes) for the uptake and elimination phase of the experiment was filled with 26 g ww spiked sediment and 60 ml reconstituted water as overlying medium, resulting in a sediment to water ratio of 1:4 (v/v). Unspiked sediment was used in controls. The spiked and unspiked water-sediment systems were incubated under test conditions for 4 days prior to the addition of the oligochaetes. The exposure was started adding 10 worms into each test vessel. Continuous aeration of the overlying water with moistened air was provided by a pressure pump via pasteur pipettes for each individual test vessel. After passing the overlying water, the air was removed from the system by a vacuum pump and passed through washing bottles filled with triethyleneglycol (TEG) and 10% potassium hydroxide (KOH) solution to retain any evaporating radioactivity. Light intensity was 100–300 lx at a 16:8 h light–dark cycle. Temperature, dissolved oxygen and pH were measured in the overlying water of the controls at regular intervals.

Samples for the uptake phase were taken after 0.3, 1, 3, 7, 14, 21, 28 and 35 days of incubation to determine the total radioactivity in the overlying water, sediment and worms. Control samples were taken after 0.3 and 35 days. At each sampling point four replicates were used. From each sampled test vessel the overlying water was decanted carefully and kept separately. Then, two sub-samples of sediment without worms were removed from the test vessel using a steel spatula. One of these two sediment sub-samples was transferred to combusto-cones® (Canberra-Packard, Dreieich, Germany), weighed and stored at −20 ± 2 °C until further analysis. The second sediment sub-sample taken from each test vessel was analysed directly by LSC for total radioactivity as described above. About 10 ml of reconstituted water were added to the remaining sediment, which was vigorously shaken by using a vibraxer for a few seconds. The slurry was transferred into a petri dish, from where the worms were collected and counted. The animals were then rinsed with reconstituted water to

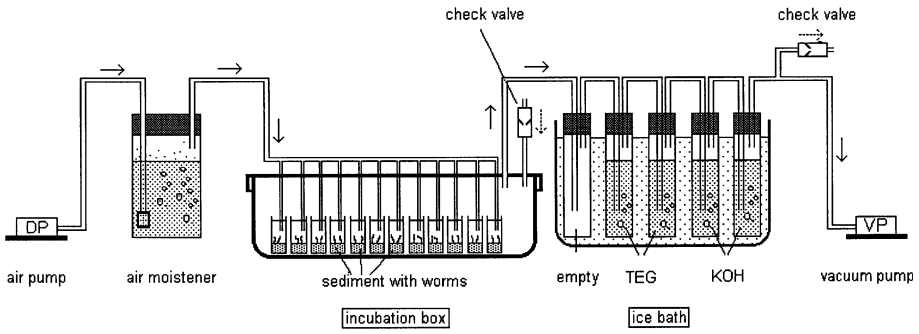

Fig. 2. Schematic test setup (TEG = triethyleneglycol; KOH = potassium hydroxide); modified following Egeler et al. (1997).

remove adherent sediment particles. A brief contact of the worms with the brim of the glass dish removed excess water. Finally, the worms of each test vessel were pooled and transferred to empty LSC vials, weighed and stored at -20 ± 2 °C until further analysis. The worms were analysed including their gut content, since for benthivorous predators, the whole worms are considered relevant as potential contamination source.

After 35 days, the elimination phase of the experiment was started by transferring the worms of the remaining 20 test vessels from spiked into uncontaminated sediment. Thereby, the number of animals from each test vessel and the four controls were counted. Samples of overlying water, sediment and biota were taken 1, 2, 4, 7 and 10 days after start of the elimination phase, and analysed for total radioactivity as described above.

2.5. Evaluation of uptake and elimination kinetics

The ratio of total radioactivity in biota (dpm g^{-1}) to total radioactivity in sediment (dpm g^{-1}) was calculated for each replicate at each sampling point. The mean accumulation factor (AF) was calculated using the measured values of the four replicates for each sampling point. The kinetic rate equation for uptake from sediment (Eq. (1)) using iterative non-linear regression and the computer software SPSS (version 7.5.2G, SPSS Inc., 1997) was fitted to these AFs to predict the time course of uptake until steady state, the uptake rate constant (k_s) and the elimination rate constant (k_e):

$$C_b(t) = \frac{k_s}{k_e} \times C_s\left(1 - e^{-k_e \times t}\right), \quad (1)$$

where C_b is the concentration of total radioactivity in biota (dpm g^{-1}), C_s is the concentration of total radioactivity in sediment (dpm g^{-1}), k_s is the sediment uptake rate coefficient (day^{-1}) and k_e is the elimination rate constant (day^{-1}). A constant for loss of bioavailability was not considered in this calculation, because the ratio of acetone-extractable total radioactivity in the sediment (measured immediately after sampling) to total radioactivity analysed after combustion of the samples remained constant throughout the experiment. When a steady state of the uptake is reached, i.e. when uptake and elimination by the animals is balanced, the accumulation factor normalised to biota lipid content and sediment TOC is defined as biota-sediment accumulation factor BSAF (Eq. (2)):

$$\mathrm{BSAF} = \frac{C_{b,ss}}{C_{s,ss}} = \frac{k_s}{k_e}, \quad (2)$$

where $C_{b,ss}$ and $C_{s,ss}$ are the concentration of total radioactivity in biota and concentration of total radioactivity in the sediment at steady state, respectively (ASTM, 2000). The elimination of accumulated radioactivity from the worms in contaminant-free sediment can be described plotting the measured total radioactivity in biota against time with non-linear regression analysis using the one-compartment model (Eq. (3)) as described by Kukkonen and Landrum (1994):

$$C_b(t) = C_{b\,day\,35} \times e^{-k_e \times t}, \quad (3)$$

where $C_{b\,day\,35}$ is the concentration of total radioactivity in worms at the end of the uptake phase at day 35 (dpm g^{-1}). The two-compartment model (Eq. (4)) calculates the elimination of the accumulated substance with different elimination rate constants considering the biota as two compartments:

$$C_b(t) = A \times e^{-k_a \times t} + B \times e^{-k_b \times t}. \quad (4)$$

A and B represent the size of the two different compartments, whereby A is the compartment with faster and B the one with slower elimination of the accumulated compound. k_a and k_b represent the corresponding elimination rate constants. All calculations were performed based on wet weight, dry weight and normalised to worm lipid content and sediment TOC.

2.6. Determination of the amount of conjugated ^{14}C-EE$_2$ in worm tissues by TLC

For determination of the amount of conjugated ^{14}C-EE$_2$ in worm tissues, eight 1-l glass vessels containing 260 g ww sediment, 1 g animals and 600 ml reconstituted water as overlying medium were prepared. Four test vessels were spiked at the same ^{14}C-EE$_2$ concentration as the test vessels for the kinetic experiment, and 4 test vessels were kept as controls. All test vessels were sampled after 35 days of exposure as described above. After the exposure the worms were lyophilised (LDC-1, Christ, Germany), then homogenised in 4 ml methanol with an ultrasonication baton (Labsonic 2000, B. Braun, Melsungen, Germany) at 20 kHz for 10 min. Then the samples were centrifuged for 10 min at 1761g. This extraction was repeated once with 3 ml methanol and twice with 3 ml acetone. All supernatants of the respective sample were pooled and the solvents evaporated in a stream of nitrogen until dryness. The residues were dissolved in 1 ml methanol. The extracts were stirred on a vibraxer and ultrasonicated for 10 min to desorb radioactive material from the surface of the test-tube. Thereafter, the extracts were filtered (0.2 μm PTFE-filter, Roth, Karlsruhe, Germany) into 3-ml glass vessels and stored at 4 °C. The recovery rate of the extraction procedure was $65 \pm 2\%$ ($n = 4$).

These extracts from worms exposed to ^{14}C-EE$_2$ were analysed by thin layer chromatography (TLC) to measure the amount of conjugated and free ^{14}C-EE$_2$ contained in *L. variegatus*. Therefore, aliquots of the extracts were treated with β-glucuronidase prior to TLC analysis. For incubation of the extracts, 300 μl

aliquots corresponding to 600 000–700 000 dpm total radioactivity were evaporated and resuspended in 5 ml 0.2 M sodium-acetate buffer (pH 5). Then, 100 µl of β-D-glucuronide glucuronosohydrolase (β-glucuronidase, type HP-2, product no. G7017, Sigma, Germany) were added and the suspension was incubated overnight at 37 °C. The suspension was extracted three times with ethyl acetate. The organic phases of the respective samples were pooled, concentrated almost to dryness and dissolved in 1 ml methanol. The recovery rate of the incubation procedure was determined as 89 ± 5% (n = 3).

^{14}C-EE_2-stock solution, incubated and original extracts were plotted on TLC plates pre-coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany). The eluent used was $CHCl_3$:ethyl acetate (8:2) according to Molnár et al. (1982). The eluent was chosen based on prior experiments which demonstrated that separation of the following unlabelled substances similar to EE_2 was satisfactory: EE_2, norethynodrel, norethisterone and mestranol were separated with retention factors (R_f) of 0.52, 0.61, 0.43 and 0.74, respectively. Each TLC plate was run with radiolabelled EE_2 (stock solution) as a reference. Developed TLC plates were scanned with a digital autoradiograph scanner (Berthold, Type 3052, Wildbad, Germany) and analysed quantitatively with a signal analyser (Berthold, Type 3061, Wildbad, Germany).

3. Results

The exposure of *L. variegatus* at 300 ng g^{-1} ww sediment to ^{14}C-EE_2 did not affect the reproduction and growth of the worms compared to controls (Table 1). Dry weight (dw) and lipid content of worms was determined as 19 ± 1.5% of ww and 8 ± 0.4% of dw, respectively. TOC of the sediment was 2.4 ± 0.1% of sediment dry weight (Table 1). Throughout the accumulation test no radioactivity was found in the TEG traps, and less than 0.02% of the initially introduced radioactivity was found in the KOH traps, indicating that ^{14}C-EE_2 did not volatilise, and only negligible amounts were mineralised.

3.1. Uptake kinetics

Fig. 3a shows the measured, almost linear, uptake of total radioactivity in the worms during 35 days of exposure to ^{14}C-EE_2-spiked sediment. After 35 days the measured accumulation factor ($mAF_{worm/sediment}$) was 75 based on total radioactivity and normalised to worms lipid content and sediment TOC (Table 2). The concentration of the radiolabelled compound in the sediment decreased to 68% of the initial concentration at day 35. Until day 21 of exposure the total radioactivity in the overlying water increased up to approximately 1% of the initial radioactivity in the sediment and remained constant thereafter.

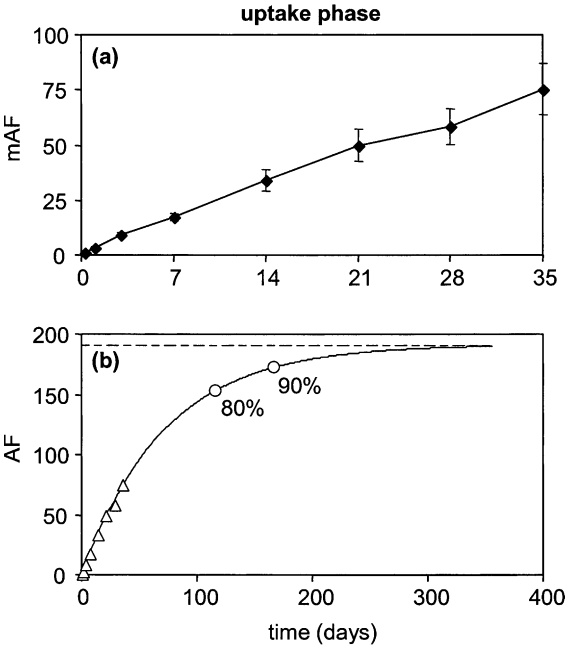

Fig. 3. (a) Measured accumulation factors (mAF) for ^{14}C-EE_2 spiked to sediment (n = 4, standard deviation). (b) Calculated accumulation factors (cAF) of ^{14}C-EE_2 in *L. variegatus* (solid line) by extrapolating the non-linear regression model applied to the measured AF (△).The open circles indicate when 80% and 90% of the calculated steady state (BSAF) is reached (r^2 > 0.99).

Table 1
Reproduction, biomass and lipid content of *L. variegatus* exposed to ^{14}C-EE_2-spiked and unspiked (controls) sediment for 35 days and TOC of the sediment (mean ± standard deviation; n = 4, if not otherwise indicated)

	Number of animals per test vessel[a]	Total biomass per replicate [g ww][b]	Worm dry weight [%][b]	Worm lipid content [% of dw][b]	TOC [% of dw]
Control	23.5 ± 4.2	1.29 ± 0.12	19 ± 1.5	8 ± 0.4	2.4 ± 0.1 (n = 5)
^{14}C-EE_2-spiked sediment	22.4 ± 5.8 (n = 24)	1.33 ± 0.13	–	–	–

[a] Values determined for worms kept in small 100-ml glass vessels during the uptake phase.
[b] Values determined for worms kept in large 1-l glass vessels.

Table 2
Comparison of measured accumulation factors (mAF_{35days}) after 35 days of exposure and calculated bioaccumulation factors at steady state (cAF_{SS}) for ^{14}C-EE_2

	ww/ww	dw/dw	lipid/OC
mAF_{35days}	90 ± 14	254 ± 39	75 ± 12
cAF_{SS}	230 ± 60	646 ± 168	191 ± 50

Values are given as the ratios of the wet weights (ww/ww), dry weights (dw/dw) and as the ratio between lipid content of worms to TOC content of sediment (lipid/OC). cAFs are calculated by non-linear regression analysis (±SD); coefficient of determination: $r^2 > 0.99$ ($n = 4$).

The measured accumulation factor after 35 days of exposure corresponds to approximately 40% of the calculated accumulation factor extrapolated to steady state (cAF_{SS}) when applying a non-linear regression model to the measured data (Table 2). According to the regression model, 80% and 90% of the cAF_{SS} would be reached after 116 and 166 days of exposure, respectively. According to the extrapolated values of the non-linear regression model the BSAF is approximately 190 and would be reached after one year (Fig. 3b).

3.2. Elimination kinetics

The elimination of radioactivity from the worms was observed over a period of 10 days (Fig. 4a). Between the observation points at day 7 and day 10 the accumulated radioactivity remained at about 50% of the total radioactivity accumulated by the worms after 35 days of uptake. At the end of the elimination phase (day 10) the total radioactivity in the sediment was low, but detectable (1600 dpm g^{-1} sediment ww). However, the total radioactivity in biota was about 2000 times higher than in the sediment, hence, a significant influence of the radioactivity in sediment on the elimination kinetics can be excluded. The modelled elimination shown in Fig. 4b results from extrapolating the measured data by non-linear regression using the one-compartment model (Eq. (3)). Fitting the two-compartment model to the measured data resulted in one major compartment and another with a negligible small fraction of the major one. According to the extrapolation with the one-compartment model, the total radioactivity accumulated at the end of the uptake phase would decline to 10% and 1% after 30 and 60 days of elimination, respectively.

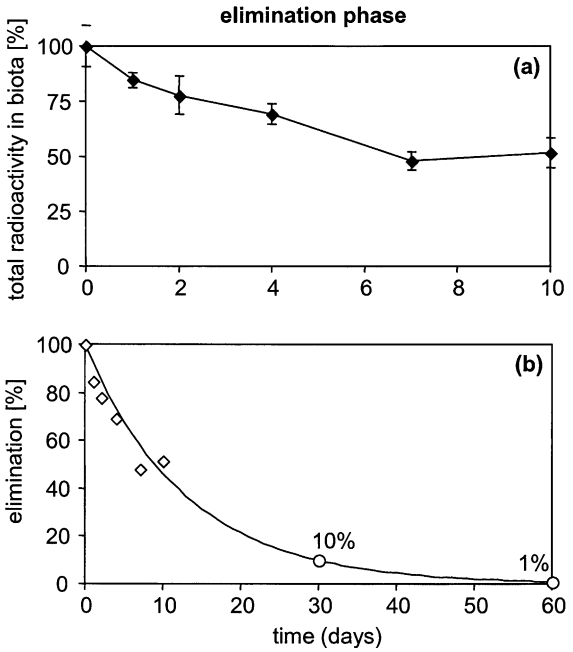

Fig. 4. (a) Measured elimination of ^{14}C-activity from *L. variegatus* over a period of 10 days ($n = 4$, standard deviation). (b) Calculated elimination of ^{14}C-activity over a period of 60 days. Elimination from *L. variegatus* was calculated (solid line) by extrapolating the non-linear regression model applied to the measured elimination values (◇). The open circles indicate when the accumulated total radioactivity has decreased to 10% and 1%, respectively ($r^2 > 0.91$).

3.3. Results from TLC

After TLC the β-glucuronidase-treated and the untreated worm extracts produced two bands containing radioactivity on thin layer plates: the spotting line and the band that corresponded to the ^{14}C-EE_2-standard. Prior to the enzymatic treatment 6% of the extracted total radioactivity in the oligochaetes corresponded to the parent compound ^{14}C-EE_2, whereas 92% remained at the spotting line (Table 3). Two percent of the radioactivity was spread between the spotting line and the

Table 3
Distribution of total radioactivity in % between spotting line and ^{14}C-EE_2-band after development of the TLC plates

	Samples applied on TLC plates		
	^{14}C-EE_2-standard	Extract prior to enzymatic treatment	Extract after enzymatic treatment
^{14}C-EE_2-band	77 ± 8	6 ± 0.5	84 ± 5
Spotting line	13 ± 2	92 ± 1	10 ± 5

Compared is ^{14}C-EE_2-standard with extracts from ^{14}C-EE_2-exposed *L. variegatus* prior to and after β-glucuronidase treatment.

^{14}C-EE_2-band. After treatment with β-glucuronidase, 84% of the extracted total radioactivity showed the same R_f as the ^{14}C-EE_2-standard.

Although the TLC-method does not identify unambiguously chemical substances, the clear separation of the steroid-standards EE_2, norethynodrel, norethisterone and mestranol by TLC and the use of the radiotracer technique provide strong evidence that the radioactive band of the worm extracts which shows the same R_f as the ^{14}C-EE_2-standard contains ^{14}C-EE_2.

4. Discussion

The accumulation of total radioactivity (mAF_{35days} = 75) measured in this study was higher than expected from other bioaccumulation studies with oligochaetes exposed to lipophilic compounds with comparable K_{ow}'s. EE_2 has a $\log K_{ow}$ of 4.2 (Schweinfurth et al., 1997). Pickard et al. (2001) determined mean BSAFs between 0.27 and 1.69 for *L. variegatus* exposed to polychlorinated biphenyls (PCBs) in natural sediments. Brunson et al. (1998) investigated the bioaccumulation of 16 polycyclic aromatic hydrocarbons (PAHs; $\log K_{ow}$: 3.4–6.4) in *L. variegatus* and found BSAFs between 0.97 and 5.3 and between 1.0 and 8.8 for laboratory-exposed and field-collected animals, respectively. Further, Fisk et al. (1998) studied the bioaccumulation of four polychlorinated alkanes (PCAs; $\log K_{ow}$: 6.2–7.4) in *L. variegatus* during 14-day experiments and determined BSAFs between 0.6 and 10. In these experiments the worm lipid and sediment TOC content were similar to the values found in our study. Finally, Landrum et al. (2002) calculated BAFs (based on biota ww and sediment dw) in the range of 1.0–3.3 for *L. variegatus* in fluoranthene-spiked natural sediments. The sediment organic carbon content of 2% was similar to that measured in our study, whereas the lipid content was up to a factor of 2.5 higher in the worms used by Landrum et al. (2002).

According to US EPA (2000) bioaccumulation of lipophilic organic compounds with a $\log K_{ow}$ between 3.5 and 5.0 in organisms exposed to contaminated sediment is expected to reach steady state after 28 days of exposure. On the other hand, Ingersoll et al. (1995) stated that in *L. variegatus* bioaccumulation of lipophilic PAHs with molecular weights (MW: 252–276) similar to that of EE_2 (MW = 296) typically reaches steady state between 28 and 56 days of exposure, whereas low-molecular weight PAHs (MW: 152–178) generally peaked by day 3. In our experiment, 35 days of exposure to ^{14}C-EE_2 was not sufficient to obtain a steady state; the lipid and TOC normalised AF at the end of the uptake phase was measured to be 75. A steady state as calculated by non-linear regression is reached approximately after 360 days of exposure with a calculated BSAF of 190 (Fig. 3b).

In our study, worms were not allowed to purge their gut before measurements of accumulated radioactivity in order to obtain a conservative AF which includes contaminated gut content. In general, after the initial 6 h of elimination, most of the contaminated gut content is assumed to be replaced by clean sediment (Mount et al., 1999). In our experiment, only 15% of the accumulated radioactivity was eliminated by the worms after 24 h in contaminant-free sediment indicating that only a small fraction of accumulated radioactivity was contained in the gut content.

For the elimination phase, it was calculated that 10% of the accumulated ^{14}C-EE_2 still remained in the worms 30 days after transfer into uncontaminated sediment (Fig. 4b). These data suggest that other (active) mechanisms than equilibrium partitioning may be involved in the observed accumulation kinetics. However, the extrapolated parts of the graphs in Fig. 3b and Fig. 4b contain a high degree of uncertainty, since only the initial part of the regression is based on measured data.

In humans, EE_2 is excreted mainly after hydroxylation and oxidation as sulfate and glucuronide conjugates (Guengerich, 1990). As shown by TLC analysis in this study, the extracts of *L. variegatus* contained the accumulated ^{14}C-EE_2 almost completely in enzymatically cleavable form(s). *L. variegatus* is apparently not able to excrete these assumed conjugates. One possible explanation is that the worms may "detoxify" EE_2 or its conjugates by sequestration into the chloragogen cells (chloragocytes). Chloragocytes are specialised peritoneal cells of the intestine and dorsal blood vessels in oligochaetes, which, like the liver of vertebrates, are important for metabolic activities, excretion, and the storage of glycogen and lipids (Dhainhaut and Scaps, 2001). Especially in the Lumbricidae these cells can form masses on the mesenteries of the nephridia when nutrition is good (Stephenson, 1972). In terrestrial oligochaetes it has been shown that the chloragocytes are important for the removal of cadmium (Morgan and Morgan, 1998) and organic xenobiotics (Prento, 1994) from other body compartments, and thereby inactivate possible toxicants. Prento (1994) demonstrated, that even highly water soluble natural and synthetic dyes can be accumulated in chloragocytes of terrestrial earthworms due to their hydrophobic parts adsorbed to cellulose granulate when ingested. The author hypothesised that one possible way of disposing of the dye-saturated chloragocytes could be the discharge through the dorsal pores. Such dorsal pores are found only in terrestrial, but not in freshwater oligochaetes (Peters, 1986). This mechanism could explain the high accumulation and low elimination of ^{14}C-EE_2 in *L. variegatus* observed in our experiment. However, the fact that the treatment with β-glucuronidase resulted in an almost complete cleavage of the parent compound ^{14}C-EE_2 from conjugates (and/or proteins) shows, that ^{14}C-EE_2

is accumulated in *L. variegatus* in a form that might be bioavailable for potential predators.

Ternes et al. (2002) measured EE_2 concentrations up to 0.9 ng g^{-1} dw in German river sediments. Assuming 2.4% sediment organic carbon content, 8% lipid fraction in worms, 19% worm dry weight and the lipid/OC normalised measured AF of 75, the exposure of *L. variegatus* for 35 days to the environmentally realistic sediment concentration of 0.9 ng g^{-1} would result in a body burden of 2.8 μg EE_2 g^{-1} lipid corresponding to 43 ng EE_2 g^{-1} worm (ww).

Länge et al. (2001) determined a LOEC (lowest observed effect concentration) of 4 ng l^{-1} in a fish full life-cycle study with fathead minnow (*Pimephales promelas*) related to effects on reproduction and growth. In the same study the authors measured bioconcentration factors (BCFs) of 610 and 660 at different EE_2 concentrations and during different life stages of the fish. Considering the LOEC of 4 ng l^{-1} and the mean BCF of 635, the 'critical body burden' (CBB) in fathead minnow is determined as 2540 ng EE_2 kg^{-1} fish (ww). The CBB is the level in fish at which toxic effects occur. Consequently, a normalised fish of 1 kg would have to consume 59.1 g of worms with the above calculated amount of EE_2 to reach the CBB. Since the mean individual body weight of adult fathead minnow is approximately 3.5 g (Ankley et al., 2001) and the mean individual body weight of *L. variegatus* was approximately 7.4 mg (as measured in this study), a fish would have to consume approximately 28 worms or 5.9% of its own body weight to reach the CBB.

The BCF data for *P. promelas* provided by Länge et al. (2001) were derived from a test where the fish were exposed to concentrations showing toxic effects. This means that the uptake process may have been influenced by effects caused by the test compound. Due to the limitation of the analytical method, lower exposure concentrations could not be applied in their bioconcentration study. Therefore, it cannot be excluded that a BCF for EE_2 in unaffected fish may be different from those reported by Länge et al. (2001).

Up to know, the data on bioconcentration of EE_2 in fish provided by Länge et al. (2001) are the only ones available in literature. The high estrogenic potency of EE_2 towards fish is confirmed when considering the low LOEC values for vitellogenin induction, e.g. 1.67 and 0.3 ng l^{-1} determined for the zebrafish *Danio rerio* (Fenske et al., 2001), and the rainbow trout *Oncorhynchus mykiss* (Sheahan et al., 1994), respectively. Certainly, not all fish species are benthivores and therefore consuming benthic organisms. However, the example above using the fathead minnow illustrates, that transfer of EE_2 via oligochaetes to benthivorous fish should be considered as an additional relevant uptake pathway, which may contribute to the exposure of benthivorous fish.

If *L. variegatus*—like some of its terrestrial relatives—is able to reduce internal physiological bioavailability by sequestration of contaminants into chloragogen tissue as discussed above (e.g. Prento, 1994), acute or chronic sediment toxicity tests might lead to results indicating low sensitivity even at high contaminant concentrations. Consequently, the risk posed by a sediment-associated chemical may be underestimated if looking only at results from toxicity tests with *L. variegatus*. Hence, *L. variegatus* should not be used as sole species in the assessment of sediment toxicity, but rather in a test battery including other test organisms, e.g. *Chironomus* sp. (Riedhammer and Schwarz-Schulz, 2001; EC, 2003). On the other hand, the assumed sequestration mechanism into chloragogen tissue renders *L. variegatus* as a particularly suitable organism for bioaccumulation studies, which could be used to support the assessment of secondary poisoning in food webs.

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