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Bioaccumulation and Bound-Residue Formation of a Branched 4-Nonylphenol Isomer in the Geophagous Earthworm *Metaphire guillelmi* in a Rice Paddy Soil

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Nonylphenols (NPs) are the breakdown products of the nonionic surfactants nonylphenol ethoxylates and are toxic pollutants. Here we studied the bioaccumulation, elimination, and biotransformation of NP (12.3 mg kg^{-1} soil dry weight) in a typical Chinese geophagous earthworm, *Metaphire guillelmi,* in a rice paddy soil, using 4-[1-ethyl-1,3-dimethylpentyl]phenol (4-NP_{111}), the main constitute of technical NP, radiolabeled with ^{14}C. Earthworms rapidly bioaccumulated ^{14}C-4-NP_{111} following a two-compartment first-order kinetics model. At steady state (after 20 days exposure), the normalized biota-soil accumulation factor amounted to 120, and 77% of the accumulated radioactivity were present as nonextractable bound residues. The total radioactivity was eliminated from the earthworm following an availability-adjusted decay model and controlled by the elimination rate of the bound residues (half-life = 22.6 days). The extractable residues consisted mainly of one less-polar metabolite (37%) and polar compounds (50%), including glucuronide conjugates of 4-NP_{111} and the metabolite; and free 4-NP_{111} accounted for only 9% of the total extractable residues. This study provides the first results of the toxicokinetics and biotransformation of 4-NP in a terrestrial organism, and underlines the significant underestimation of the bioaccumulation and risk assessment based only on free NP in earthworms.

Introduction

Nonylphenol (NP) is a breakdown product of nonionic nonylphenol ethoxylate surfactants used worldwide. NP is found in wastewater treatment plants and in the environment, and is more persistent, lipophilic, and toxic than the parent ethoxylates (*1*). NP is ubiquitous in the environment (*2–4*), tends to adsorb to soil and sediment, and accumulates easily in organisms owing to its high hydrophobicity ($\log K_{ow}$ = 4.48) (*5–7*).

NP occurs in biosolids (i.e., treated sludge of sewage and wastewater treatment plants) in large quantities (up to several thousand mg kg^{-1}) (*8*), and is persistent in these biosolids (*9*). Increasing land application of the biosolids as fertilizer can lead to the release of large amounts of NP into the soil (*8*). Much attention has been paid to the sorption and degradation of NP in soil (*6, 10, 11*), but the bioaccumulation and transformation of NP within soil invertebrates has surprisingly not yet been studied, except in a field survey of micropollutants in earthworms, in which 5.2−7.7 mg NP kg^{-1} soil dry weight were detected (*12*). In contrast, bioaccumulation of NP in various aquatic organisms, such as *Lymnaea stagnalis*, *Lumbriculus variegatus*, and *Tapes philippinarum,* has been widely investigated (*5, 13–17*), and one metabolite in the form of a glucuronide conjugate has been identified (*14*).

Earthworms are the predominant biomass in most temperate terrestrial ecosystems. Earthworms, and particularly geophagous earthworms, process large amounts of soil through their feeding and burrowing activities (*18*). Earthworms can accumulate many organic and inorganic soil pollutants and are ideal testing organisms for the bioaccumulation of pollutants in soil (*12, 19*). The bioaccumulation of organic pollutants in earthworms depends on both the hydrophobicity and the availability of the pollutants in the soil environment (*20–23*). The bioavailability of NP would control the biodegradation and mass transfer of NP in the environment (*24, 25*).

Most studies on bioaccumulation of micropollutants have been based on the measurement of the pollutants in the free form (*12, 23*); very few studies have evaluated the transformation and metabolites of micropollutants within the earthworm body. Organisms might detoxify the pollutant via conjugation, for example, in the form of glucuronides to facilitate excretion, or via formation of bound, that is, nonextractable, residues (*26, 27*). Such residues and conjugates might be accumulated in or eliminated from the organisms and are easily quantified by using radioactive tracer techniques (*14, 28*). This technique, however, has not yet been used for NP in soil organisms.

Among the isomers of technical NP, the isomer 4-[1-ethyl-1,3-dimethylpentyl]phenol (4-NP_{111}, according to the IUPAC systematic nomenclature of nonylphenol isomers) is the predominant component, accounting for up to 20% (*29, 30*), and shows the highest estrogenic activity (*31*). We radiolabeled this branched isomer uniformly on the ring of the molecule, and investigated its bioaccumulation, elimination, and transformation in a typical Chinese geophagous earthworm, *Metaphire guillelmi,* in a rice paddy soil. We provide the first information on the transformation and bound-residue formation of NP in terrestrial organisms.

Materials and Methods

Earthworms and Soil. The anecic earthworms *Metaphire guillelmi* were collected from a rice paddy field at the Changshu Experimental Station of the Chinese Academy of Sciences, Jiangsu Province, China. The earthworms, together with soil, were brought to the laboratory in a nylon bag. The soil contained 2.5% total organic carbon (TOC), 46.7% clay, 37.9% silt, and 15.4% sand, and had a pH (0.01 M $CaCl_2$) of 6.31. Before the experiment, the soil was sieved to a particle size of <1 mm and then stored at 4 °C. Active earthworms with a fresh body weight of 2.0−2.5 g were used for the feeding trials.

Chemicals. The uniformly ring-labeled nonylphenol isomer ^{14}C-4-NP_{111} was synthesized via Friedel-Craft alkylation using [U-ring-^{14}C]-labeled phenol and 3,5-dimethyl-3-heptanol as described by Vinken et al. (*32*). ^{14}C-4-NP_{111}

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had a specific radioactivity of 306 MBq mmol^{-1}, a chemical purity of >97% (as analyzed by gas chromatography−mass spectrometry) and a radiochemical purity of 98% (as analyzed by autoradiography, see below). A mixture of β-glucuronidases (type HP-2, from *Helix pomatia*, 116 300 U mL^{-1}) was purchased from Sigma (Shanghai, China).

Bioaccumulation and Elimination Experiments. *Uptake Experiment.* A ^{14}C-4-NP$_{111}$ methanol solution (0.54 mL, 3.42 mg mL^{-1} and 1.0 MBq mL^{-1}) was homogenously distributed in 5.0 g air-dried soil. This soil was then thoroughly mixed with an additional 145 g air-dried soil (diameter <1 mm) in a 3 L flask. The methanol solvent was evaporated by storing the flask at room temperature overnight. The homogeneity of the ^{14}C-4-NP$_{111}$ distribution in the soil was proven by determining the radioactivity of soil subsamples (0.5−1.0 g) from the flask (recovery = 95.6 ± 3.5%, n = 3). After the methanol was evaporated, the soil−water content was adjusted to 60% of the maximal water-holding capacity by adding 36.0 mL distilled water. The ^{14}C-4-NP$_{111}$ in the soil had a chemical concentration and radioactivity of 12.3 mg kg^{-1} and 3.67 MBq kg^{-1} soil dry weight, respectively.

Bioaccumulation and elimination experiments were performed according to Jager et al. (*33*) with slight modifications. Immediately after the soil−water content was adjusted, the uptake experiment was started by adding six adult earthworms into the flask, which was then incubated in a climate chamber at 20 ± 1 °C. $^{14}CO_2$ released from the soil during the exposure was trapped by 1 M NaOH (1 mL) in a vial suspended from the bottom of the flask stopper, and determined by liquid scintillation counter (LSC, see below). The flasks were opened once each day to refresh the headspace. At exposure times of 1.5, 4, 8, 12, 16, and 20 days, one earthworm individual was removed from the flask, washed with deionized water, weighed, and transferred to another 1-L flask containing 100 g soil in the dark to replace the gut contents with soil lacking radioactive ^{14}C-4-NP$_{111}$. Preliminary experiments showed that 24 h was sufficient for this process. The earthworms were then washed again with deionized water, freeze-dried, and weighed. The extractable and bound radioactivity in the earthworms was analyzed (see below). All experiments were performed in triplicate.

Elimination Experiment. To study the elimination kinetics of ^{14}C-4-NP$_{111}$ in the earthworm bodies, six earthworms were exposed to ^{14}C-4-NP$_{111}$ in 150 g soil as in the uptake experiment described above. After 20 days of exposure, the earthworms were transferred to a new flask containing 100 g fresh soil lacking ^{14}C-4-NP$_{111}$. The flask was incubated in the dark at 20 ± 1 °C. At 0, 1, 3, 6, 10, and 16 days, one earthworm individual were removed from the flask, treated, and analyzed as in the uptake experiments. All experiments were performed in triplicate.

Extractable and Bound Radioactivity in Earthworms. The freeze-dried earthworm samples were cut into small pieces (<3 mm) with a scissor and ground with a mortar and pestle. Based on our preliminary experiments and on studies in the literature (*10, 11, 34*), ethyl acetate and methanol were chosen as the extraction solvents; these solvents provided a good recovery and a low content of earthworm cell matrices in the extract. The samples were extracted with ethyl acetate three times (each 10 mL) and methanol once (10 mL) by repeated ultrasonic resuspension (0.09 kW, 20 kHz) and centrifugation (8000*g*, 10 min). The supernatants were combined, and radioactivity was determined by LSC (see below). Using this extraction procedure, the recovery of ^{14}C-4-NP$_{111}$ spiked into the earthworm tissues prior and after freeze-drying was 95 ± 2% and 90 ± 1% (n = 3), respectively. This extracted radioactivity was defined as extractable radioactivity. The radioactivity remaining in the earthworm residues was defined as bound radioactivity. The sum of the extractable and the bound radioactivity was defined as the total radioactivity accumulated in the earthworms.

Determination of Conjugates and Metabolites of ^{14}C-Compounds in Earthworms. To determine the conjugates of ^{14}C-4-NP$_{111}$ and its metabolites, five earthworm individuals were exposed to ^{14}C-4-NP$_{111}$ (12.3 mg kg^{-1} and 3.67 MBq kg^{-1} dry soil) in 150 g soil under the same conditions as in the uptake experiments described above. After an exposure of 20 days, the earthworms were ground and extracted with ethyl acetate (three times, each 50 mL) and methanol (once, 50 mL) to yield the extractable radioactivity. The extracts were combined and concentrated to 5 mL using a rotary evaporator. Aliquots (1.0 mL, approximately 360 Bq) of the concentrate were evaporated to dryness, resuspended in sodium acetate buffer (3.0 mL, pH 5), and incubated with 10 μL β-glucuronidase (116.3 U) at 37 °C overnight to release possible glucuronide conjugate metabolites. The suspension was extracted three times with n-hexane (total recovery 90% ± 3%, n = 3), and the extract was concentrated to 0.5 mL and analyzed by autoradiography (see below).

For autoradiography, the extracts before and after the enzyme treatment were separated by thin layer chromatography (TLC) on silica gel 60 with a fluorescence indicator (Sil G-25 UV254, 0.25 mm; Macherey-Nagel, Düren, Germany) using a mixture of n-hexane:ethyl acetate:formic acid (100:20:1.2 by vol.) as eluent. ^{14}C-4-NP$_{111}$ was used as the reference. The developed TLC plate was autoradiographed using an imaging scanner (Typhoon Trio$^+$, GE Healthcare, U.S.) and quantitatively analyzed using the ImageQuant software supplied with the scanner.

Determination of Radioactivity. Radioactivity was quantitatively counted in a liquid scintillation counter (LS6500; Beckman Coulter; U.S.). For organic extracts, 0.2 mL extract was mixed with 2 mL of scintillation cocktail (Lumasafe Plus; Lumac LSC, Groningen, The Netherlands), and for radioactivity of $^{14}CO_2$ in 1 M NaOH, 0.5 mL of the alkaline solution was mixed with 2 mL of scintillation cocktail. For bound radioactivity in the earthworm residues, 0.2−0.4 g of the extracted earthworm tissues was combusted to CO_2 with a biological oxidizer (OX-500; Zinsser Analytic, Germany). The CO_2 was absorbed by 15 mL Oxysolve C-400 (Zinsser Analytic, Germany) and then counted by LSC.

Data Analysis. The data from the uptake experiments were fitted to a two-compartment first-order kinetics model (eq 1 (*35*)), in which the decline of the bioavailable ^{14}C-4-NP$_{111}$ in the soil as a result of aging is considered:

$$\frac{dC_t}{dt} = k_s C_s e^{-\lambda t} - k_d C_t \quad (1)$$

where C_t is the concentration of the total radioactivity in the earthworm at uptake time t [Bq (g lipid)$^{-1}$], C_s is the initial radioactivity of ^{14}C-4-NP$_{111}$ in the soil [Bq (g soil TOC)$^{-1}$], k_s is the uptake rate constant of ^{14}C-4-NP$_{111}$ from the soil by the earthworm [g soil TOC (g lipid)$^{-1}$ days^{-1}], k_d is the depuration rate constant of the ^{14}C-4-NP$_{111}$ residues from the earthworm (days^{-1}), and λ is the rate constant for ^{14}C-4-NP$_{111}$ to become unavailable in the soil (days^{-1}).

The integrated form of eq 1 is

$$C_t = [k_s C_s (e^{-\lambda t} - e^{-k_d t})] / (k_d - \lambda) \quad (2)$$

The value of λ for ^{14}C-4-NP$_{111}$ in the rice paddy soil was 0.0191 days^{-1}, which was determined by incubating ^{14}C-4-NP$_{111}$ in this soil (12 mg NP kg^{-1} soil dry weight) and measuring the extractable radioactivity ($C_{extract}$) at various incubation times. The extraction procedure (three times with methanol and once with ethyl acetate, with a ratio of 4:1/solvent:soil) yielded a recovery of 93%. The data were fitted to the first-order

decay kinetic model ($C_{extract} = C_{extract\ at\ time\ 0}e^{-\lambda t}$), which delivered the kinetic constant λ.

The data from the elimination experiments were fitted to an availability-adjusted first-order decay kinetic model (eq 3)

$$\frac{dC_t}{dt} = k_e C_{ew} e^{-at} \quad (3)$$

with the integrated form (eq 4)

$$C_t = C_{ew} e^{-k_e(1-e^{-at})/a} \quad (4)$$

where, C_{ew} and C_t [Bq (g lipid)$^{-1}$] is the concentration of ^{14}C-4-NP$_{111}$ residues (i.e., total residues, extractable residues, or bound residues) in the earthworm at elimination time 0 and t (days), k_e is the elimination rate constant (days^{-1}), and a is the unavailability constant of the residues in the earthworm (days^{-1}), similar to the constant λ for NP in the soil. For total ^{14}C-4-NP$_{111}$ residues, k_e is the same as k_d in eq 2, except that the constants are calculated from different models. The half-life of the residues in the earthworm can be calculated using eq 5:

$$T_{1/2} = -\frac{1}{a}\ln\left(1 - \frac{0.693a}{k_e}\right) \quad (5)$$

Data were fitted to the bioaccumulation and the elimination kinetics models using iterative nonlinear regression and the software Sigma Plot 11.0. Significance was analyzed using the software SPSS 13.0; a statistical probability of $P < 0.05$ was considered significant.

The extent of the bioaccumulation of NP and its residues in the earthworm was expressed with a biota-soil accumulation factor (BSAF), which was normalized for soil organic carbon and earthworm lipid contents and calculated according to eq 6:

$$BSAF = \frac{C_{ew}/f_{lipid}}{C_s/f_{oc}} \quad (6)$$

where C_{ew} and C_s are the concentration of total radioactivity in the earthworm [Bq (g dry earthworm)$^{-1}$] and the soil [Bq (g dry soil)$^{-1}$], respectively, f_{lipid} is the lipid content of the earthworm (%), and f_{oc} is the organic carbon content of the soil (%).

Results

All *M. guillelmi* earthworms survived the accumulation and elimination experiments. The lipid content of the earthworms was 7.1 ± 0.4% (n = 3) of the dry body weight. Less than 0.3% of the initial radioactivity was found in the NaOH trap at the end of the accumulation kinetic experiment, which indicated that mineralization of ^{14}C-4-NP$_{111}$ in the soil within 20 days was negligible.

Bioaccumulation of Radioactivity in Earthworms. The accumulation of total, bound, and extractable radioactivity in the earthworms during 20 days of exposure to ^{14}C-4-NP$_{111}$ in the rice paddy soil is shown in Figure 1. Bound-residue formation in the earthworms was rapid, accounting for 60% of the total accumulated radioactivity already at the beginning (1 day) of the exposure period and almost reached the stable value (about 77%) after about 3 days of exposure (Figure 2). The bioaccumulation of ^{14}C-4-NP$_{111}$ in the earthworms (BSAF) fitted well to the two-compartment accumulation model (eq 2, $R^2 > 0.97$) (Figure 2), which yields the earthworm uptake rate (k_s = 17.8 ± 2.4 (g soil TOC (g lipid)$^{-1}$ days^{-1})) of ^{14}C-4-NP$_{111}$ from the soil and the depuration rate (k_d = 0.10 ± 0.02 days^{-1}) of the total residues from the earthworms. The model shows that the bioaccumulation of the ^{14}C-4-NP$_{111}$ residues in the earthworm *M. guillelmi* almost reached the steady state (99% of the asymptote value) after about 17.5 days of exposure, with a BSAF value of 120 (Figure 2).

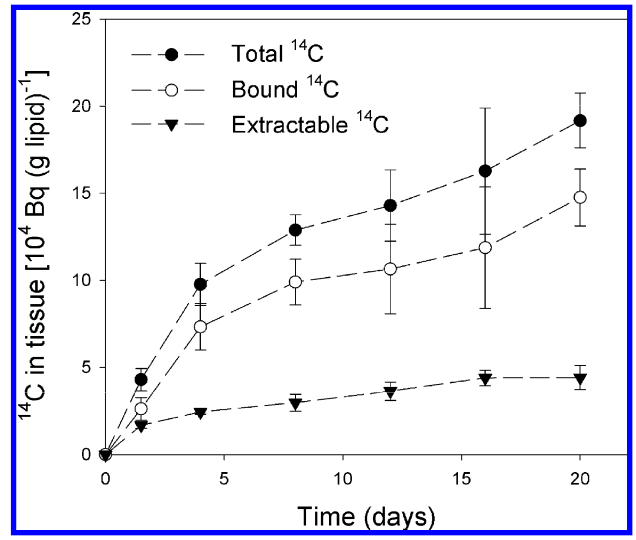

FIGURE 1. Accumulation of total, bound, and extractable radioactivity in the earthworm *M. guillelmi* exposed to ^{14}C-4-NP$_{111}$ in rice paddy soil. Values represent means with standard deviations of three individual experiments, and are expressed as per gram lipid content of the earthworm.

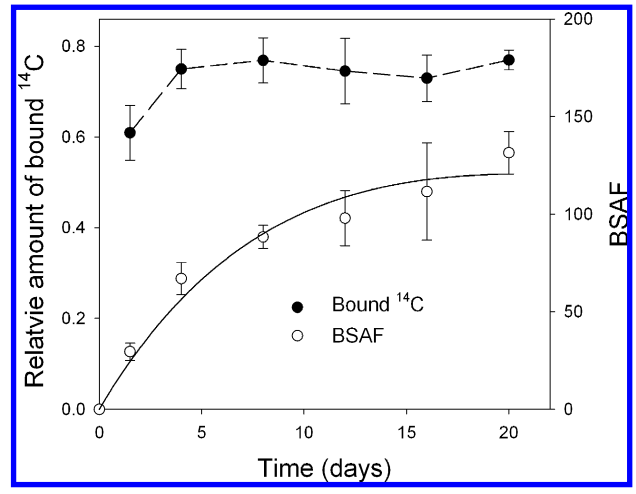

FIGURE 2. Amount of bound radioactivity relative to the total radioactivity (left axis) and biota-soil accumulation factor (BSAF, eq 6) of the total radioactivity (right axis) in *M. guillelmi* during the uptake experiment. The solid line represents the regression curve for the BSAF, determined according to the two-compartment accumulation model as described in eq 1. Values represent means with standard deviations of three individual experiments.

Elimination of 4-NP$_{111}$ Residues from Earthworms. The elimination of the ^{14}C-4-NP$_{111}$ residues from the earthworms was observed for 16 days (Figure 3). All the experimental data fitted well to the availability-adjusted first-order decay model (eq 4) ($R^2 > 0.95$), with parameters (k_e and a) given in Table 1. The extractable radioactivity had the highest elimination rate constant (k_e = 0.71 days^{-1}) with a short half-life ($T_{1/2}$) of 1.9 days; however, the elimination of the total radioactivity in the earthworms ($T_{1/2}$ = 25.1 days) was mainly controlled by the elimination rate of the bound residues ($T_{1/2}$ = 22.6 days) (Table 1). The elimination rate of the extractable radioactivity became very low after 3 days of elimination and was controlled by the availability of the radioactivity released from the bound residues, which was in agreement with its high unavailability constant (a = 0.80). The k_e value

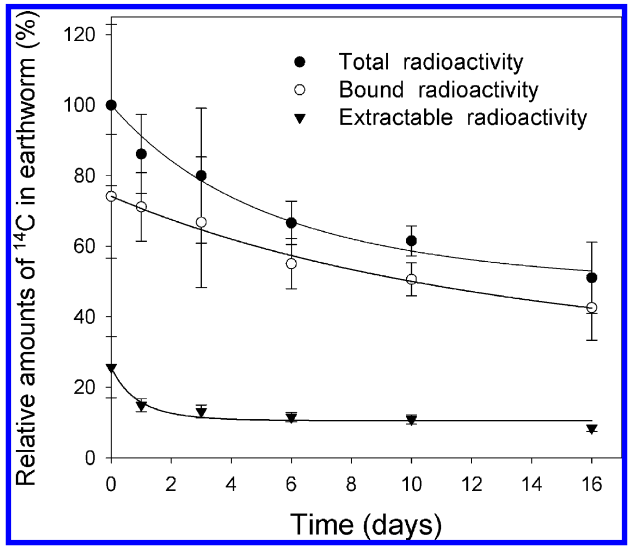

FIGURE 3. Elimination of total, bound, and extractable radioactivity of the ^{14}C-4-NP$_{111}$ residues from *M. guillelmi*. Solid lines represent regression curves of the elimination kinetics of the various radioactive fractions, determined according to the availability-adjusted first-order decay model (eq 4). Values are means with standard deviation of three individual experiments.

(0.10 days^{-1}) of the total radioactivity is equal to the k_d (0.10 days^{-1}) based on the accumulation model (eq 1 and 2), which indicated that the models used in our study were appropriate to describe the bioaccumulation and elimination processes.

Metabolites and Conjugates of 4-NP$_{111}$ in Earthworms. Analyses of the ethyl acetate/methanol extracts of the earthworms by TLC coupled to autoradiography showed that the extracts consisted of large amounts of polar metabolites of 4-NP$_{111}$ (as indicated by the start line on the TLC plates), one less-polar metabolite (M1, $R_f = 0.75$), and free 4-NP$_{111}$ ($R_f = 0.40$), accounting for 49.7, 36.7, and 8.7% of the extractable radioactivity, respectively (Table 2). When the extracts were treated with β-glucuronidase, the relative radioactivity of the metabolite M1 and free 4-NP$_{111}$ increased significantly ($P < 0.05$) to 42.9 and 13.0%, respectively, whereas the radioactivity of the polar component decreased to 37.6% (Table 2), which indicated that the polar conjugates were transformed into metabolite M1 and free 4-NP$_{111}$.

Discussion

Bioaccumulation and Elimination. At the end of the exposure time, the BSAF value (120) of the total residues of 4-NP$_{111}$ in the earthworms was higher than that of 4-NP previously found in the aquatic worm *Lumbriculus variegatus* and in amphipods (BSAF = 14−55) (*15, 16, 36*). This difference can probably be ascribed to the tissue chloragocyte in terrestrial oligochaetes, which is thought to immobilize more micropollutants in the body than tissues of aquatic oligochaetes (*37, 38*).

Also the uptake pathways for micropollutants in terrestrial earthworms and benthic organisms may differ. In most of the studies on the bioaccumulation of 4-NP$_1$ or ^{14}C-ethinyl estradiol (EE$_2$) by *L. variegatus* (*15, 16, 39*), no steady-state phase was reached at the end of the exposure period (35−56 days). In contrast, the uptake of ^{14}C-4-NP$_{111}$ by *M. guillelmi* was fast within the first 4 days of the exposure and then slowly reached a steady-state phase (Figure 2). Considering the low water content (20%) and the limited aqueous concentration of 4-NP owing to its high sorption to soil particles (*6*), an uptake of 4-NP$_{111}$ by the earthworms from soil pore water was probably negligible. The predominant pathway for the uptake was therefore probably from 4-NP$_{111}$ sorbed on soil particles and desorbed into the earthworm gut fluid. This desorption can be enhanced by the surfactant-like substances in the gut fluid of the animals (*23*) and by physical grinding of soil particles in the gizzard and the gut of the earthworms (*40*). This ingestion pathway is significant for the bioaccumulation of organic chemicals with high K_{ow} values (*19, 41*). Recently, Qi and Chen (*23*) proposed that enhanced uptake of desorption-resistant pollutants by the epigeic earthworm *Eisenia fetida* from ingested soil particles may be important for the bioaccumulation of the pollutants.

In contrast to the bioaccumulation, the elimination of the residues of 4-NP$_{111}$ from the earthworms was slow. After living in rice paddy soil lacking ^{14}C-4-NP$_{111}$ for 16 days, the earthworms still contained 51% of the initially accumulated total residues and 57% of the initial bound residues (Figure 3). Since the bound forms of 4-NP$_{111}$ residues were the predominant forms in the earthworms, the elimination rate of these bound forms determined the total elimination of the 4-NP$_{111}$ residues. During elimination, the bound residues should first be released to form extractable residues. Because the extractable residues had a much higher elimination rate ($k_e = 0.71$ days^{-1}, $T_{1/2} = 1.9$ days) than the bound residues ($k_e = 0.05$ days^{-1}, $T_{1/2} = 22.6$ days) (Table 1), the limiting step for the 4-NP$_{111}$ elimination in the earthworms may be the transformation of the residues from bound to extractable forms. This would explain why the amount of extractable residues remained almost the same after 6 days of elimination, while the bound residues decreased continually (Figure 3). The mechanisms for the binding of the residues to the earthworm tissues and their release again as extractable forms require further studies. Roles of the binding of 4-NP$_{111}$ or its metabolites via the phenolic group to macromolecules in the earthworm tissues during formation of the bound residue cannot be ruled out, even though the amounts of extractable β-glucuronide conjugates were low (Table 2).

Transformation of NP in Earthworms. The formation of conjugates and bound residues are two pathways for the detoxification of xenobiotics in organisms (*26*). Our results indicated that the formation of bound residues, and not of conjugates, is the detoxification pathway mainly used by the earthworm *M. guillelmi*.

The relative amounts of bound 4-NP$_{111}$ residues in the earthworm increased rapidly during the early stages of exposure (Figure 2), which indicated a fast incorporation of 4-NP$_{111}$ or its metabolites into cellular constituents of the earthworms after uptake. At the end of the exposure, the bound residues were the predominant radioactive form in the earthworms, accounting for 77% of the total radioactivity (Figure 2). Large amounts of the less-polar metabolite (M1, 36%) and the polar metabolites of 4-NP$_{111}$ were found in the extract of the earthworms (Table 2), whereas only <9% of the free form of 4-NP$_{111}$ was detected, which suggested that 4-NP$_{111}$ and its metabolites were largely transformed in the earthworm body. Further work is needed to elucidate whether the metabolite M1 is only produced by the earthworms or first produced in the soil and then taken up by the earthworms. In aquatic fish and invertebrates, metabolites of technical NP and another branched isomer, 4-NP$_{112}$, have also been observed, but these metabolites are more polar than their parent compounds (*14*).

The amount of glucuronide conjugates of 4-NP$_{111}$ and the metabolite M1 in the earthworms was only 4.3 and 6.2% (difference between values after and before the β-glucuronidase treatment, Table 2), respectively. Therefore, glucuronide conjugates probably play a less important role in the transformation of ^{14}C-4-NP$_{111}$ and its metabolites in the earthworms. Conjugation of NP with glucuronic acid seems to be more significant in aquatic organisms than in the earthworms, e.g., 18% of ^{14}C-4-NP$_{112}$ in tissue extracts of the snail *Lymnaea stagnalis* was recovered in glucuronide conjugates (*14*) and 84% of the synthetic phenolic steroid

TABLE 1. Fitting Parameters of the Availability-Adjusted First-Order Decay Model (eq 4) for the Elimination of the Various Fractions of the ^{14}C-4-NP$_{111}$ Residues in the Earthworm *M. guillelmi* in Rice Paddy Soil (see Figure 3)

	parameters of the model[a]			
fraction	k_e(days^{-1})	a (days^{-1})	R^2	$T_{1/2}$ (days)[b]
total radioactivity	0.10 ± 0.02	0.14 ± 0.04	0.98	25.1
bound radioactivity	0.05 ± 0.01	0.05 ± 0.02	0.99	22.6
extractable radioactivity	0.71 ± 0.20	0.80 ± 0.26	0.95	1.9

[a] Values are regression results from three individual experiments. [b] Half-life of the fraction in the earthworms, calculated from the model.

TABLE 2. Relative Distribution of the Radioactivity (%) between Various Components within the Extractable ^{14}C-4-NP$_{111}$ Residues in the Earthworms before and after the Treatment with β-Glucuronidase, As Determined by TLC-Autoradiography

		relative amounts (%)[b]	
component	R_f[a]	prior to enzymatic treatment	after enzymatic treatment
free NP$_{111}$	0.40	8.7 ± 0.5	13.0 ± 0.5
metabolite M1	0.75	36.7 ± 0.7	42.9 ± 1.2
others		4.9 ± 0.4	6.2 ± 0.2
start line	0	49.7 ± 0.4	37.6 ± 0.9

[a] Retention factors (R_f) of the components separated on the TLC plates. [b] Values are averages with standard deviations of three analyses.

hormone ethinyl estradiol in the body of the aquatic worm *L. variegatus* was present as glucuronide conjugates (*39*).

Why the bioaccumulation intensity and the metabolites of NP isomers of terrestrial earthworms and aquatic organisms differ is not known, but is probably related to their feeding and digestion characteristics. The storage and sequestration of micropollutants in the chloragocyte tissues of the terrestrial oligochaetes (including earthworms) might reduce the sensitivity of the organisms to micropollutants in the soil (*15*). Sequestration of the free form and metabolites of NP in the chloragocyte tissues may be one possible pathway for the bound-residue formation in the earthworm bodies. Another mechanism for the formation of the bound residues might be an oxidative coupling, that is, covalent binding, of NP to the cellular components of the earthworm. Indeed, peroxidases are present in earthworms at high concentrations (*42*). Using eq 4 and the kinetic constants k_e and a in Table 1, we can predict that 36.7% of the bound residues could be unremovable (irreversible binding), and this might be attributed to covalent binding of the residues to the earthworm tissues.

Environmental Implication. To our knowledge, this study provides the first results of the kinetics of bioaccumulation, elimination, and bound-residue formation of NP residues in a terrestrial organism. NP, as well as other endocrine-disrupting compounds, pharmaceuticals, and pesticides, can be transferred from biosolids to soil-dwelling earthworms in agricultural soil amended with the biosolids (*12*). In an earlier study, the bioaccumulation factor of 4-NP in earthworms in field soil amended with biosolids was determined to be 2.1, based on the free concentration of 4-NP in the earthworms and in the soil (*12*). We, in contrast, used ^{14}C-labeling and showed that *M. guillelmi* accumulated large amounts of the 4-NP$_{111}$ residues, including free 4-NP$_{111}$ and conjugated residues, and that most of the accumulated 4-NP$_{111}$ residues (77% of the total accumulation) were present as the bound, nonextractable forms in the earthworm tissues (Figures 1 and 2). Therefore, the bioaccumulation and risk assessment based on the concentrations of only free NP in earthworms are probably significantly underestimated. Identification of the metabolites in the earthworms will lead to a better understanding of the metabolism and toxicity of NP in terrestrial organisms.

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