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Graphical Abstract

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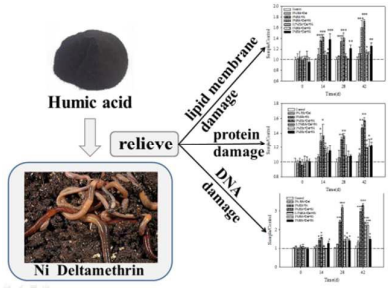

Environmental impact

Ni and deltamethrin is potentially detrimental to human and animal health because of its high toxicity and mobility in soil, especially in cultivated soils. Humic acids (HAs), one of the most important components in soil, are also common used to improve the soil structure. This study provide evidences to figure out whether HAs can influence the bio-availability of Ni and deltamethrin due to its variation of molecular structures which can provide adsorption sites, thus relieve the toxic effect. The results show that HAs could alleviate toxicity caused by Ni and deltamenthrin (like Gene toxicity and Biochemical toxicity).

Environmental Science Process & Impacts

ARTICLE

Could humic acid relieve the biochemical toxicities and DNA damage caused by nickel and deltamethrin in earthworms (*Eisenia foetida*)?

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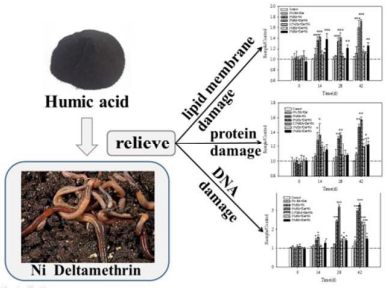
Humic acid alleviated nickel and deltamethrin toxicity in earthworms (*Eisenia foetida*), preventing (in decreasing order of effectiveness) damage to DNA, proteins, and lipid membranes.



The aim of the study was to determine whether humic acid (HA) prevented gene and biochemical toxic effects in earthworms (*Eisenia foetida*) exposed to nickel and deltamethrin (at 100 and 1 mg kg^{-1}, respectively) in soil. Cellular- and molecular-level toxic effects of nickel and deltamethrin in earthworms were evaluated by measuring damage to lipid membranes and DNA and the production of protein carbonyls over 42 days of exposure. Nickel and deltamethrin induced significant levels of oxidative stress in the earthworms, increasing the production of peroxidation products (malondialdehyde and protein carbonyls) and increasing the comet assay tail DNA% (determined by single-cell gel electrophoresis). DNA damage was the most sensitive of the three indices because it gave a higher sample/control ratio than did the other indices. The presence of HA alleviated (in decreasing order of effectiveness) damage to DNA, proteins, and lipid membranes caused by nickel and deltamethrin. A low HA dose (0.5–1% HA in soil) prevented a great deal of lipid membrane damage, but the highest HA dose (3% HA in soil) prevented still more DNA damage. However, the malondialdehyde concentrations in the earthworms were higher at the highest HA dose than at the lower HA doses. The amounts of protein carbonyls produced at different HA doses were not significantly different. The toxic effects to earthworms caused by increased oxidizable nickel concentrations could be relieved by adding HA.

1. Introduction

Rapid industrial development and the widespread use of pesticides has caused serious problems with heavy metal pollution[1] and pesticide pollution[2] in agricultural environments. Ni and deltamethrin are two of the main contaminants in agricultural soils. A survey of soil contamination in China in 2014 showed that 19.4% of cultivated land is polluted, and that 4.8% of land has Ni concentrations higher than the acceptable limit.[3] Approximately 40% of vegetable fields in the Pearl River Delta region were found to contain heavy metal concentrations higher than the acceptable limits. Of the heavy metals, Ni (for which the acceptable limit is 50 mg kg^{-1}) was found to be the main pollutant.[4] Ni concentrations of 139–1099 mg kg^{-1} have been found in soil on which wheat is grown in northwest China.[5] Deltamethrin, which is the most toxic pyrethroid,[6] has been widely used in Chinese agricultural areas. Deltamethrin has been found to have a half-life in soil of between 14 and 291 d, depending on the soil properties,[7] but it is likely to have accumulated in many soils because it has been repeatedly applied. Deltamethrin concentrations of 0.03–1.16 mg kg^{-1} have recently been found in Chinese agricultural soils.[8]

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Plant growth may be stunted and invertebrates such as earthworms, nematodes, and springtails can be threatened because of Ni and deltamethrin accumulated in soil. Ni is a calcium-channel blocker,[9] and can enter cells and cause free radicals to be produced,[10] inducing oxidative stress and both double- and single-stranded DNA damage.[11] Deltamethrin has been found to be neurotoxic, acting by activating voltage-sensitive sodium channels[12] and affecting the activities of antioxidant enzymes.[13] Earthworms, which are some of the most widespread invertebrates in soil ecosystems, play important roles in improving soil structure and fertility.[14] The direct contact between earthworms and soil and the way in which earthworms feed make them important model organisms in environmental monitoring studies. The physiological, cellular, and molecular characteristics of earthworms change significantly when the earthworms are stressed because of the presence of contaminants.[15] These changes have been used for a number of years to study the sub-acute toxic effects of soil contaminants in earthworms caused through oxidative stress.[16,17] Contaminants entering an earthworm may cause the earthworm to accumulate reactive oxygen species,[18] which may cause the oxidation and anti-oxidation systems to become imbalanced. Malondialdehyde (MDA) is one of the main products of the peroxidation of polyunsaturated fatty acids, and it can be used as an indicator of lipid peroxidation.[19] Protein carbonyls are chemically stable oxidized groups on protein chains. It is relatively easy to store protein samples and to detect protein carbonyls, making protein carbonyls convenient markers of damage.[20] Exposure to pollutants can cause DNA chains to become broken, negatively affecting gene replication and expression. These effects can be detected using the comet assay, which is a single-cell gel electrophoresis method.[21] The actual toxic effects of a pollutant in soil cannot be determined from the total concentration of the pollutant in the soil because the pollutant could become modified or the availability of the pollutant could change over time (because of the processes of complexation, metabolism, precipitation, solubilization, and sorption).

Humic acid (HA), one of the most important components of soil, is a relatively stable group of organic compounds that have a range of structural features (including alkylaromatic, carbonyl, carboxyl, phenyl, and quinoid moieties)[22] that can act as adsorption sites. It has been found that HA can mitigate the toxicities of contaminants by affecting the forms of the contaminants that are present.[23] The soil structure can also be improved by HA, and this can make the soil a better environment for earthworms to grow in. However, adding HA can have negative effects, such as decreasing the soil pH, which can increase the availabilities (and therefore the acute and chronic toxicities) of certain heavy metals.[24] It is still not clear if adding HA to soil will decrease the toxicities of Ni and deltamethrin in the soil. The effects on earthworms of adding HA to soil are also unclear.

The earthworm *Eisenia foetida* was used as a model terrestrial organism in the study presented here. The aim of the study was to determine whether HA could decrease the combined toxic effects of Ni and deltamethrin on earthworms. The MDA and protein carbonyl concentrations and the comet assay Tail DNA% were used to indicate the cellular- and molecular-level toxic responses.

2. Experimental

2.1 Materials and chemicals

The *E. foetida* used in the study were supplied by an earthworm breeding farm in Suzhou, Jiangsu Province, China. Healthy earthworms aged 60 d, each weighing 200–300 mg and having an obvious clitellum, were kept under the experimental conditions for 14 d before the exposure experiments were started. Surface soil (0–20 cm depth) was collected from an agricultural field in Hangzhou, China. The main physical and chemical properties of the test soil were: pH 6.51; organic matter content 23.54 g kg^{-1}; cation exchange capacity 17.5 cmol kg^{-1}; texture 28.2% clay, 40.3% silt, and 31.5% sand. The As, Cd, Cr, Cu, Hg, Pb, and Zn concentrations in the soil were 3.86±0.04, 0.16±0.01, 28.2±0.8, 18.6±0.64, 0.20±0.01, 11.8±0.37, and 46.8±1.01 mg kg^{-1}, respectively. Deltamethrin was not detected in the soil. The soil samples were dried in air, then passed through a sieve with a 2 mm mesh before use.

Deltamethrin (>99.5%) was obtained from the Shanghai Jingchun Biochemical Technology Co. (Shanghai, China), and $Ni(NO_3)_2$ (>98%) was obtained from the Shanghai Lingfeng Chemical Reagent Co. (Shanghai, China). The HA that was used (fulvic acid >90%) was purchased from the Nanjing Chemical Reagent Co. (Nanjing, China).

2.2 Exposure of the earthworms to Ni and deltamethrin

The Ni and deltamethrin test concentrations were set at 100 and 1 mg kg^{-1}, respectively, because similar concentrations have been found in real soil samples. We also used similar concentrations in toxicity tests performed before the exposure experiments were performed. Ni was dissolved in deionized water and spiked into the soil to give a final concentration of 100 mg kg^{-1}. Water was then added to the contaminated soil to bring the soil to 70% of its water-holding capacity. The soil was then allowed to equilibrate for 2 weeks. Deltamethrin was dissolved in a small amount of acetone, and the solution was sprayed onto the soil to give a final concentration of 1 mg kg^{-1} once the soil had equilibrated. The soil was then placed in a fume hood until the acetone had completely evaporated. HA was then added directly to the soil samples to give final HA contents of 0%, 0.5%, 1%, and 3% (these samples are called THA-0, THA-0.5, THA-1, and THA-3, respectively, later), then the soil samples were mixed thoroughly. Soil with only Ni added (called TNi) and only deltamethrin added (called TDel), and no HA added, were prepared for use in single-contaminant tests. Soil without any Ni, deltamethrin, or HA added was prepared for use in clean soil control tests. Each treatment was performed in triplicate. Earthworms (with an average total wet weight of 0.30–0.50 g) were then added to each test sample after 2 days. The gut contents of the earthworms were voided before the earthworms were used in the experiments. Each test sample contained 80 earthworms. Each sample container was

then covered with plastic wrap to limit the loss of water. As suggested by Lin et al.,[25] 5 g of dry cow dung was added to the surface of each soil sample each week to feed the earthworms. Dry cow dung was considered to be an appropriate food because it will not have contained any chlortetracycline or other medication that could have adversely affected the earthworms during the tests. The sample containers were stored under controlled conditions (at 20±1 °C, with a 16 h: 8 h light: dark regime) for 48 d. None of the earthworms died during the test period. After the exposure period, 15 live earthworms were collected from each test sample for analysis. The earthworms from each sample were carefully washed in normal saline and then placed in a glass culture dish containing damp filter paper overnight, to void their gut contents, then the earthworms were rinsed with normal saline three times before being analysed.

2.3 Lipid peroxidation

Six live earthworms that had been used in a test were mixed with iced phosphate-buffered saline (PBS) (at pH 7.5) to give an earthworm weight: buffer volume ratio of 1:9. The worms were homogenized in the buffer, then the mixture was centrifuged at 3500 rpm (at 4 °C) for 10 min. The supernatant was kept at below 4 °C for the remainder of the analysis, which was performed on the same day. The MDA content of the extract was determined using the thiobarbituric acid technique.[26] Briefly, the extract was incubated with 1% thiobarbituric acid in an acetate buffer, then the mixture was heated in a boiling water bath for 1 h. The mixture was then cooled and centrifuged at 3500 rpm for 15 min, then the absorbance at 532 nm was measured.

2.4 Oxidative damage to proteins

The total protein content of each extract was determined using the method described by Dalle et al.,[18] and the protein carbonyl concentration was determined using the 2,4-dinitrophenylhydrazine colourimetry method.[27] A 2,4-dinitrophenylhydrazine solution (10 mmol L^{-1} 2,4-dinitrophenylhydrazine and 2 mol L^{-1} HCl) was added to a protein pellet produced from a sample, and only HCl (2 mol L^{-1}) was added to a reagent blank sample. Each mixture was then kept in the dark for 1 h and vortexed every 10 min. The mixture was then centrifuged, the supernatant discarded, and the protein pellet was washed with 1 mL of a 1:1 (v/v) mixture of ethanol and ethylacetate three times. The sample was then resuspended in guanidine hydrochloride at 37 °C for 15 min before the absorbance at 370 nm was measured.

2.5 DNA damage

Earthworm coelomocytes were collected using the method described by Dong et al.[28] Three earthworms were washed with physiological saline, then they were subjected to an irritating chilled extraction using a mixture of 5% ethanol and 95% saline containing 2.5 mg mL^{-1} EDTA and 10 mg mL^{-1} guaiacol glyceryl ether (at pH 7.3) for 3 min. The extrusion medium was then centrifuged (at 4 °C) at 9000 rpm for 10 min. The coelomocytes were washed with PBS three times, centrifuging between washes. All of the steps were conducted at 4 °C. More than 90% of the cells were required to remain viable throughout the procedure.

The comet assay described by Dong et al.[19] was used with slight modifications. Briefly, a clear glass slide was coated with 100 µL of 0.8% normal-melting agar in PBS. A 25 µL aliquot of a cell extract, as described above, was suspended in 75 µL of warm (37 °C) 0.8% low-melting agar in PBS. A 100 µL aliquot of this mixture was added to the glass slide covered with normal-melting agar. Once the sample had solidified, the slide was immersed in a lysis solution (100 mmol L^{-1}, 2.5 mol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, 1% Trion X-100, and 10% dimethyl sulfoxide, at pH 10) for 2 h. The slide was then placed in an electrophoresis tank containing iced electrophoresis buffer (300 mmol L^{-1} NaOH and 1 mmol L^{-1} Na_2EDTA) for 30 min, to despiralize the DNA, then electrophoresis was performed for 20 min at 25 V and 300 mA. The slide was then immersed in buffer (0.4 mol L^{-1} Tris-HCl, at pH 7.5) for 15 min, and then stained by adding 40 µL ethidium bromide (2 µg mL^{-1}) before being analysed by fluorescence microscopy.

2.6 Total Ni and deltamethrin contents of the soil samples

A 0.1 g aliquot of each air-dried soil sample that had been passed through a sieve with a 0.149 mm mesh was digested in a 5:3 mixture of HNO_3 and HF using a microwave accelerated digestion system (MARS 5; CEM, Matthews, NC, USA). The Ni concentration in the digested solution was determined by graphite furnace atomic absorption spectrophotometry (ZEEnit 700 p; Analytik Jena, Jena, Germany). Blanks and reference material (Chinese National Standard Soil Reference Material GBW07417; Chinese CRM/RM Information Center, Beijing, China) samples were included to allow the quality of the results to be assessed. The measured concentrations were never more than 10% different from the nominal concentrations in the spiked soil samples or the certified concentrations in the reference materials.

The deltamethrin concentrations in the soil samples were determined using the method described by You et al.[29] The soil samples were extracted, and the deltamethrin concentrations in the extracts were determined using a gas chromatograph equipped with an electron capture detector (GC-ECD 7890A; Agilent Technologies, Santa Clara, CA, USA). The gas chromatograph was fitted with an HP-5 (30 m long, 0.25 mm internal diameter, 0.5 µm film thickness) capillary column (Agilent Technologies). The oven temperature program started at 65 °C (held for 1 min), and increased at 20 °C min^{-1} to 280 °C (held for 2 min). A 1 µL aliquot of each extract was injected, and split injection mode (with a split ratio of 1: 10) was used. The injector and detector temperatures were 250 and 300 °C, respectively. A deltamethrin recovery test was performed in each batch of samples. The deltamethrin recovery was determined by analysing triplicate dried soil samples that had been spiked with a deltamethrin standard. The deltamethrin recovery was always within the range 90–110%.

2.7 Nickel fractions in the soil

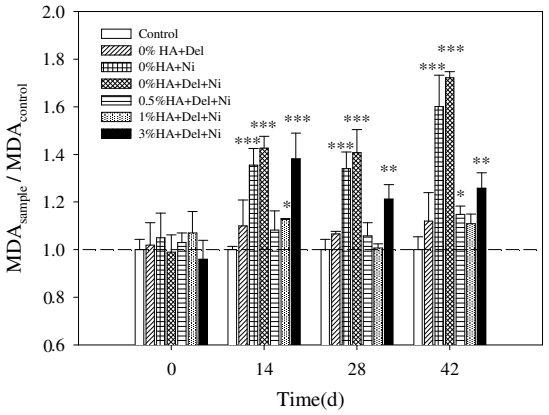

Fig. 1 Effects of the presence of humic acid on the peroxidation of membrane lipids in *Eisenia foetida* exposed to Ni and deltamethrin. The ratios of malondialdehyde concentration in samples and controls different exposure times are shown. 0%HA+Del means the TDel samples, 0%HA+Ni means the TNi samples, 0%HA+Del+Ni means the THA-0 samples, 0.5%HA+Del+Ni means the THA-0.5 samples, 1%HA+Del+Ni means the THA-1 samples, and 3%HA+Del+Ni means the THA-3 samples. The means are shown, and the bars show the standard deviations (n=3). Values significantly different from the controls are indicated with asterisks, * = P<0.05, ** = P<0.01, and *** = P<0.001.

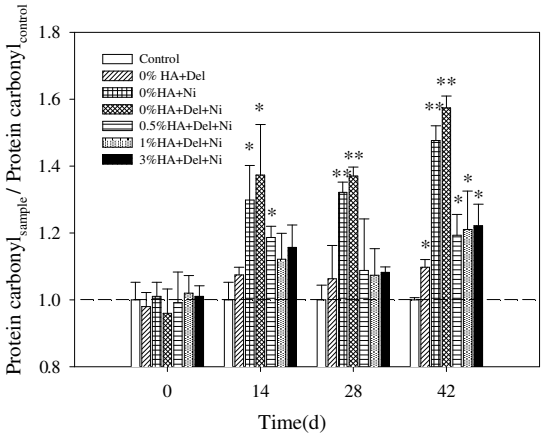

Fig. 2 Effects of the presence of humic acid on protein damage in *Eisenia foetida* exposed to Ni and deltamethrin. The ratios of protein carbonyl concentration in samples and controls after different exposure times are shown. 0%HA+Del = TDel, 0%HA+Ni = TNi, 0%HA+Del+Ni = THA-0, 0.5%HA+Del+Ni = THA-0.5, 1%HA+Del+Ni = THA-1, and 3%HA+Del+Ni = THA-3. The means are shown, and the bars show the standard deviations (n=3). Values significantly different from the controls are indicated with asterisks, * = P<0.05 and ** = P<0.01.

The nickel fractions in the soil were determined using a revised Bureau Communautaire de Référence extraction procedure.[30] Briefly, 40 mL of acetic acid was added to a 1 g aliquot of an air-dried soil sample, then the mixture was shaken overnight to remove the acid-extractable Ni. A 40 mL aliquot of hydroxylammonium chloride (0.5 mol L^{-1}, at pH 1.5) was then added to the residue to remove the reducible Ni. The residue was then washed twice with hydrogen peroxide (8.8 mol L^{-1}) and then dried. A 50 mL aliquot of nitric acid (pH 2) was then added to the residue to extract the oxidizable Ni. The residual Ni was then determined by digesting the residue as described in subsection 2.6, using the MARS 5 microwave accelerated extraction system.

3. Results and discussion

3.1 Effects of HA on the peroxidation of membrane lipids in *E. foetida* exposed to Ni and deltamethrin

The ratios between the MDA concentrations in the samples and controls were used to evaluate whether HA prevented the earthworm cell membrane lipids being damaged by peroxidation caused by Ni and deltamethrin. Both Ni and deltamethrin were found to cause toxic effects in the earthworms, but Ni was found to be the most toxic, as is shown in Fig. 1. Ni caused significant (P<0.01) oxidative damage to the lipid membranes, and the presence of Ni caused the MDA concentration to increase. The MDA sample/control ratio was 1.10 in the TDel samples but 1.35 in the TNi samples on day 14. Ni and deltamethrin appeared to have simple additive effects on the lipid membranes. Divalent heavy metals have been found to make the membranes of lysosomes in earthworm coelomocytes unstable and to cause lipid peroxidation in a number of studies.[31,32] Deltamethrin can cause oxidative stress by affecting the activities of antioxidant enzymes.[33] Less peroxidation of membranes was found to be caused by Ni and deltamethrin when HA was present, but more membrane peroxidation occurred when the HA concentration was high than when the HA concentration was low. The highest MDA concentration in the THA-0 samples was found on day 28 (when the sample/control ratio was 1.41), but the THA-0.5 and THA-1 samples had sample/control ratios of only 1.05 and 1.06, respectively, on day 28. The sample/control ratio for the THA-3 samples was significantly higher (P<0.01), at 1.21, on day 28 than the ratios in the THA-0.5 and THA-1 samples. Deltamethrin is degraded slowly in soil, but the toxic effects of Ni and deltamethrin increased with time. Adding HA was found to decrease the damage caused to the lipid membranes by Ni and deltamethrin, but oxidative stress has been found to be induced by high HA concentrations. The superoxide dismutase (an enzyme that destroys oxygen free radicals) activity has been found to be increased by the presence of HA.[34] However, high HA concentrations have been found to damage lipid membranes by inhibiting glutathione activity.[35,36] The results of the studies just mentioned explain why HA prevented Ni and delta deltamethrin damaging the lipid membranes less effectively at high HA concentrations than at lower HA concentrations.

3.2 Effects of HA on protein damage in *E. foetida* exposed to Ni and deltamethrin

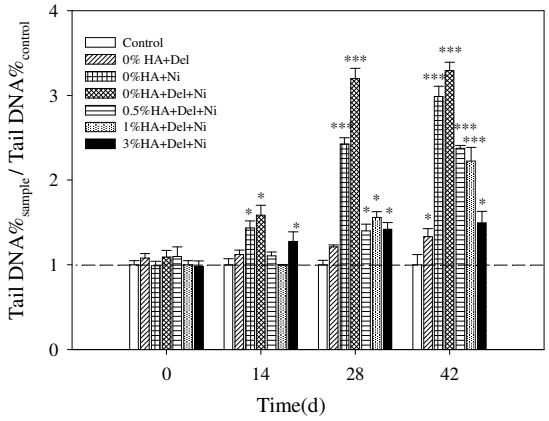

Fig. 3 Effects of the presence of humic acid on DNA damage in *Eisenia foetida* exposed to Ni and deltamethrin. The ratios of comet assay tail DNA% in samples and controls after different exposure times are shown. 0%HA+Del = TDel, 0%HA+Ni = TNi, 0%HA+Del+Ni = THA-0, 0.5%HA+Del+Ni = THA-0.5, 1%HA+Del+Ni = THA-1, and 3%HA+Del+Ni = THA-3. The means are shown, and the bars show the standard deviations (n=50). Values significantly different from the controls are indicated with asterisks, * = P<0.05, ** = P<0.01, and *** = P<0.001.

Ni and deltamethrin. The protein carbonyl concentrations were significantly higher (P<0.05) in the TNi and THA-0 samples than in the TDel samples on day 14. The protein carbonyl concentration sample/control ratios in the TDel, TNi, and THA-0 samples were 1.07, 1.29, and 1.37, respectively, on day 14. HA was found to prevent some of the protein damage caused by Ni and deltamethrin. The sample/control ratios in the THA-0.5, THA-1, and THA-3 samples were only 1.18, 1.12, and 1.23, respectively.

The effect of HA on the protein carbonyls was not strongly related to the dose (like the MDA response was). The protein carbonyl concentration increased with time when HA was not present but did not change to a significant degree (P>0.05) when HA was present. The sample/control ratio in the THA-0, THA-0.5, THA-1, and THA-3 samples had increased to 1.57, 1.19, 1.21, and 1.22, respectively, on day 42. This showed that protein carbonyls were accumulated when Ni and deltamethrin were present but that HA slowed the occurrence of these toxic effects.

Deltamethrin and Ni both tend to inhibit antioxidant enzyme activities, leading to reactive oxygen species being accumulated. This may have been responsible for the protein carbonyl concentrations increasing in our experiments. Proteins are not resistant to damage caused by transition metals[24] (such as Ni in our study), which can catalyse protein damage involving the formation of carbonyl groups.[37] Unlike damage to the lipid membranes, the amount of protein damage that occurred did not appear to be significantly different (P>0.05) at the different HA concentrations. The lowest dose of HA appeared to allow the proteins to withstand oxidative stress.

3.3 Effects of HA on DNA damage in *E. foetida* exposed to Ni and deltamethrin

As is shown in Fig. 2, the total protein carbonyl concentration increased significantly when the earthworms were exposed to

The amounts of DNA damage in *E. foetida* caused by exposure to Ni and deltamethrin for 14, 28, and 42 d were determined using the comet assay, and the results are shown in Fig. 3. Less DNA damage was found in the earthworms exposed to deltamethrin than in the earthworms exposed to Ni. Most of the DNA damage occurred at the beginning of the deltamethrin exposure tests, and further damage did not occur with time. In contrast, much more DNA damage was caused by Ni, and the amount of damage caused increased with time. For example, the tail DNA% sample/control ratios in the TDel and TNi samples were 1.33 and 2.99, respectively, on day 42. DNA is an important target of environmental stress in terrestrial organisms.[38] DNA damage can be directly caused by chemicals such as H_2O_2 and reactive oxygen species.[39] We found a positive response between the HA concentrations and the tail DNA% values. In previous studies, it has been found that many substances, such as free radicals and bases that are contaminant metabolites, can directly break DNA chains.[28] Ni can bind to enzymes that repair DNA and generate oxygen free radicals that can cause protein damage in situ.[17]. We found that adding HA decreased the amount of DNA damage that occurred, and adding more HA caused less DNA damage to occur. For example, the tail DNA% sample/control ratios in the THA-0, THA-0.5, THA-1, and THA-3 samples were 3.29, 2.37, 2.22, and 1.49, respectively, on day 42. The tail DNA% in the THA-0, THA-0.5, and THA-1 samples increased as the exposure time increased but varied little in the THA-3 samples.

Lipid peroxidation, protein damage, and DNA damage were analysed in this study, and DNA damage was found to be the more sensitive indicator of the toxic effects of Ni and deltamethrin. More lipid peroxidation occurred when the HA dose was high (in the THA-3 samples) than when lower HA doses were used (in the THA-0.5 and THA-1 samples). There is an optimum amount of HA that should be added to prevent as much protein damage as is possible. However, more DNA damage was prevented at the highest HA dose (THA-3) than at the lower doses. Lipid peroxidation, protein damage, and DNA damage have previously been used widely to indicate the ecotoxicities of contaminants. It has previously been found that Ni is a calcium-channel blocker[9] but that deltamethrin is neurotoxic[12] because it can activate voltage-sensitive sodium channels. With this in mind, we will choose biomarkers relevant to these effects to evaluate the toxic effects of Ni and deltamethrin in earthworms in future studies.

3.4 Effects of HA on the soil pH

The pH decreased as the HA dose increased and with time, as is shown in Table 1. The soil pH values in the control and THA-3 samples were 6.50 and 6.27, respectively, on day 0. The soil pH values in the control and THA-3 samples had decreased to 6.15 and 5.94, respectively, on day 42.

The components of HA contain a great range of structural moieties, such as carboxyl and hydroxyl groups, that can cause acid hydrolysis. Organic acids (such as carboxylic and carbonyl

Table 1. Soil pH during the exposure tests

Time (d)	Control	TNi	TDel	THA-0	THA-0.5	THA-1	THA-3
0	6.50±0.01	6.52±0.01	6.49±0.02	6.58±0.04	6.51±0.07	6.44±0.04	6.27±0.02*
14	6.28±0.01	6.22±0.01	6.21±0.04	6.22±0.01	6.28±0.07	6.21±0.03	5.95±0.02*
28	6.21±0.05	6.20±0.02	6.22±0.03	6.18±0.06	6.12±0.04	6.09±0.04*	5.93±0.02*
42	6.15±0.03	6.10±0.04	6.12±0.07	613±0.05	6.16±0.07	6.09±0.02	5.94±0.08*

Results are expressed as the mean ± the standard deviation (n=3). Statistical significance versus control group; *p < 0.05.

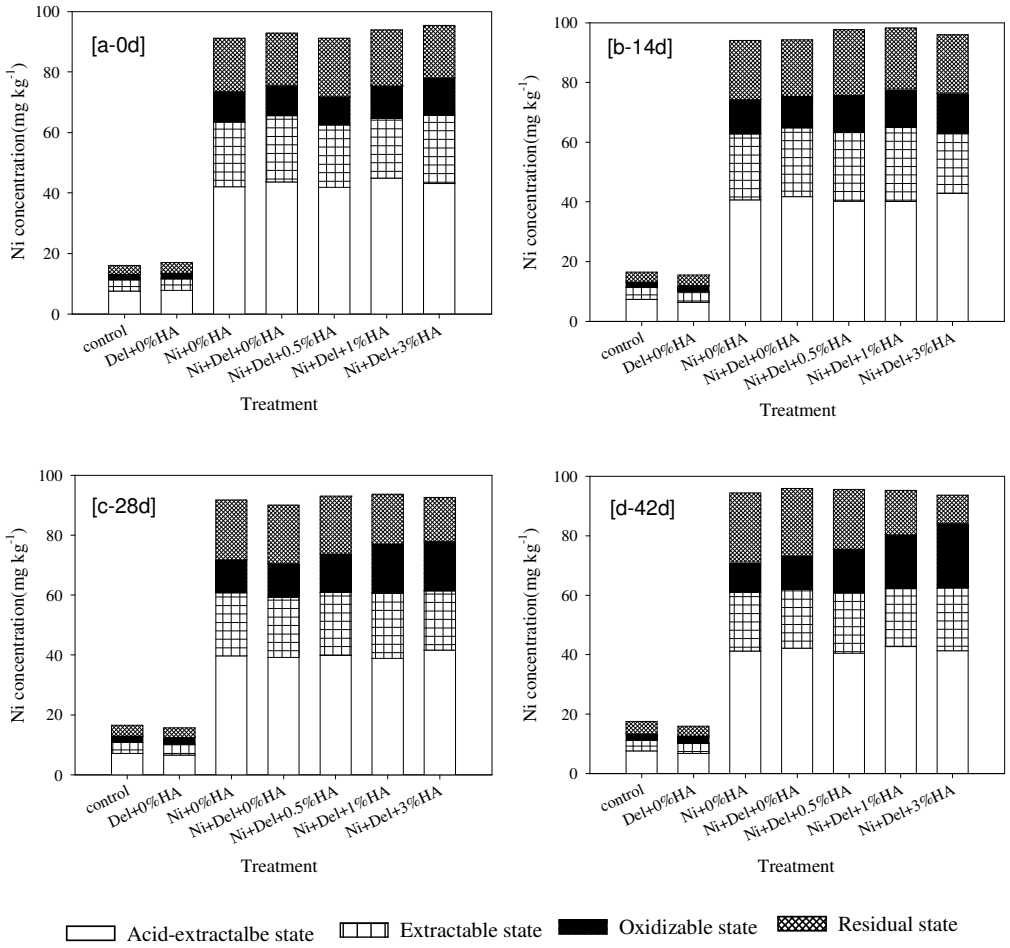

Fig. 4 Effects of adding humic acid on nickel fractionation in the soil

compounds) that are produced by earthworms (through their metabolic activities) could have decreased the control soil pH.[4] Toxicity benchmarks were found to increase in soils contaminated with pesticides and with relatively low pH values in a previous study,[41] and it was concluded that this was caused by chemical hydrolysis and degradation processes. It has been found that the EC_{50} values and bioavailabilities of heavy metals are significantly different (P<0.05) in soils with different pH values.[42,43]

3.5 Effects of HA on Ni fractionation

The fractionation of Ni is strongly linked to the mobility and bioavailability of Ni. As is shown in Fig. 4a, the amounts of Ni in the different soil fractions decreased in the order acid-

extractable Ni > reducible Ni > residual Ni > oxidizable Ni after the pre-incubation period. The Ni fractions in the TNi samples were similar to the Ni fractions in the THA-0 samples, showing that deltamethrin did not affect Ni fractionation in the soil. The oxidizable Ni fraction increased in size and the residual Ni fraction decreased in size significantly ($P<0.05$) when HA was added. For example, on day 42, the oxidizable Ni fractions in the samples with HA contents of 0% and 3% were 10.17 and 21.74 mg kg^{-1}, respectively, and the residual Ni fractions were 23.21 and 9.58 mg kg^{-1}, respectively. The electronic structure of Ni and the total acidity, COOH content, and phenolic OH group content[23] are generally the main factors responsible for the binding of Ni to HA. These factors explain why the oxidizable Ni fraction increased in size as the HA content increased. The stability constants of metal complexes are lower at acid pH values than at neutral and alkaline pH values.[44] Adding HA caused the soil pH to decrease, as is shown in Table 1, and this slightly increased the size of the acid-extractable fraction over a short period and decreased the size of the residual Ni fraction. The acid-extractable Ni fractions in the THA-0 and THA-3 samples were 39.72 and 41.65 mg kg^{-1}, respectively, on day 28, and the pH values were 6.18 and 5.93, respectively.

In this study, Ni was found to be more toxic than deltamethrin to earthworms. Adding HA increased the size of the oxidizable Ni fraction, as is shown in Fig. 4. It has been shown that the ingestion of metals attached to Fe and Mn oxides is an important factor in determining the internal concentrations of metals in earthworms.[45] HA has been found to be poorly bioavailabile[34] and might made the Ni combined with Has to be hardly absorbed by earthworms, meaning that adding HA can decrease the toxicity of Ni to earthworms.

3.6 Effects of HA on the degradation of deltamethrin

The deltamethrin content decreased with time in all of the HA-treated soils. For example, the deltamethrin concentration decreased from 0.95 mg kg^{-1} on day 0 to 0.32 mg kg^{-1} on day 42 in the TDel samples, as is shown in Fig. 5. The deltamethrin degradation rates were similar to rates that were found in a previous study.[46] The half-life of deltamethrin in soil will depend on the soil type and the availability of oxygen. HA could decrease the rate at which deltamethrin is degraded. Adding HA caused the deltamethrin degradation rate to decrease slightly in our tests. The deltamethrin concentrations in the THA-0, THA-0.5, THA-1, and THA-3 samples on day 42 were 0.34±0.06, 0.51±0.01, 0.51±0.08, and 0.56±0.04 mg kg^{-1}, respectively. Deltamethrin may have been degraded less quickly in the presence than in the absence of HA because deltamethrin is stable at acid pH values but is more easily degraded at higher pH values.[47] As mentioned above, the soil pH decreased as the HA dose increased. It has previously been found that deltamethrin is more easily biodegraded at neutral and alkaline pH values than at acid pH values.[48]

Conclusions

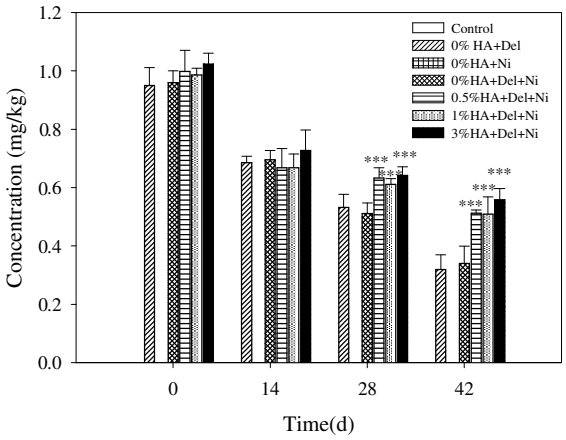

Fig. 5 Effects of the presence of humic acid on the degradation of deltamethrin in soil. 0%HA+Del = TDel, 0%HA+Ni = TNi, 0%HA+Del+Ni = THA-0, 0.5%HA+Del+Ni = THA-0.5, 1%HA+Del+Ni = THA-1, and 3%HA+Del+Ni = THA-3. The means are shown, and the bars show the standard deviations (n=3). Values significantly different from the controls are indicated with asterisks, * = $P<0.05$, ** = $P<0.01$, and *** = $P<0.001$.

Both Ni and deltamethrin were found to induce oxidative stress in earthworms, leading to damage to lipid membranes, proteins, and DNA.

Ni was found to be more toxic to earthworms and to cause more DNA damage than was deltamethrin. DNA damage was found to be a more sensitive indicator of Ni and deltamethrin exposure than were lipid membrane damage and protein damage. HA was found to prevent some of the toxic effects, including DNA damage, caused by Ni and deltamethrin exposure. Lipid membrane damage was prevented most effectively at a HA content of 0.5% or 1%, but DNA damage was prevented most effectively at a HA content of 3%. However, HA negatively affected the lipid membranes in the earthworms at a HA content of 3%. The degrees of protein damage prevented at the different HA contents that were tested were not significantly different. The oxidizable Ni fraction increased as more HA was added, and the residual Ni fraction decreased as more HA was added. The deltamethrin degradation rate decreased as the HA content increased.

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