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Release of tetrabromobisphenol A (TBBPA)-derived non-extractable residues in oxic soil and the effects of the TBBPA-degrading bacterium *Ochrobactrum* sp. strain T

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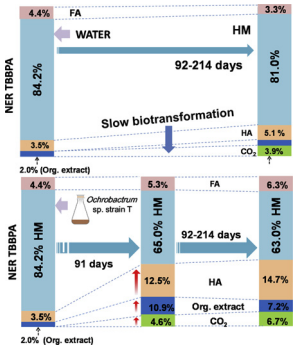
GRAPHICAL ABSTRACT



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ABSTRACT

Tetrabromobisphenol A (TBBPA) forms large amount of non-extractable residues (NER) in soil. However, the stability of TBBPA-NER with TBBPA degrader in soil had not been determined. In this study, a ^{14}C-tracer was used to follow the release and alteration of TBBPA-derived NER during 214 days of incubation in oxic soil and in the presence or absence of the TBBPA-degrading bacterium *Ochrobatrum* sp. strain T. In the absence of strain T, 1.89% of the TBBPA and its metabolites were slowly released from the NER, with TBBPA as the predominant component, accompanied by 2.47% mineralization by day 91 of the incubation. The addition of active cells strongly stimulated the release and mineralization of NER (10.93% and 4.64%, respectively), reduced the amount of the ester-linked fraction, and transformed NER from humin-bound to HA-bound forms. Cells added to the soil in sterilized form had much smaller effects on the stability and internal alterations of NER. Among the ester-linked compounds, 47.4% consisted of TBBPA; two metabolites were so detected. These results provide new information on the stability and internal transformation of TBBPA-NER in soil during its long-term

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incubation and underlines the importance of microbial TBBPA degraders in determining the composition of NER in soil.

1. Introduction

Tetrabromobisphenol A (TBBPA) [4,4′-isopropylidenebis (2,6-dibbromophenol)] is a brominated flame retardant widely used in consumer products, especially in electrical equipment, computers and other electronic devices [1–3]. As a very large amount of e-waste is disposed of without detoxification, TBBPA has become ubiquitous in the air as well as in soil, rivers, marine sediments, and dust [4–8]. Previous studies have shown that TBBPA is an endocrine disruptor, with thyroid hormone activity in animals, and causes oxidative stress both to plants and to soil organisms [4,5,9–11,41]. Thus, the fate and biotransformation of TBBPA and its metabolism in different environmental matrices are of global concern [39,40].

In oxic, anoxic, or submerged soils, TBBPA and its metabolites form large quantities of non-extractable residues (NER). In fact, NER account for 28–80% of the initially applied amount of TBBPA [6,12–15]. A previous study showed that pesticide-derived NER can enter the food chain, such that long-term environmental risk assessments of these compounds were recommended [26]. The formation of xenobiotics-derived NER in soil may involve physical entrapment, chemical covalent bonding, including ester or ether linkages, and incorporation into biomass [6,12,16,17]. Given the low bioavailability of NER and their recalcitrance with respect to biotransformation, the formation of these compounds is usually considered to be a route of detoxification for organic pollutants in soil [18–21]. However, changes in the soil conditions, including pH, redox potential, humidity, aeration, temperature, carbon source, microbial activity, and the humification of soil organic matter, may affect the stability of NER in soil [13,20,22,23]. For example, following a change in the soil from anoxic to oxic, 55% of TBBPA-derived NER (TBBPA-NER) were released as TBBPA and its metabolites [13]. By contrast, in oxic soils, amendment with rice root exudates did not effectively alter the mineralization, release, and distribution of NER [15].

Bacteria are able to stimulate the release of NER derived from various organic pollutants in soil, such as parathion and atrazine [23–25]. The long-term release of TBBPA and its metabolites from TBBPA-NER by TBBPA degraders may result in toxicity and thus pose a risk to the environment [16,23,26]. However, few studies have examined this process and its potential environmental impact. *Ochrobatrum* sp. strain T (*O*. sp. strain T), a gram-negative bacterium isolated from the sludge of an e-waste recycling site, degrades and mineralizes TBBPA under oxic conditions [27]. To understand the long-term impact of *O*. sp. strain T on the behavior of TBBPA-NER in soil and thus the potentially adverse environmental consequences, in this study we investigated: (i) the long-term stability of TBBPA-NER in an oxic silty clay soil and the mechanism of TBBPA-NER release, (ii) the impact of TBBPA-degrading *O*. sp. strain T on the stability of TBBPA-NER in soil, (iii) the biotransformation of released TBBPA-NER and its metabolites during a long-term incubation. Our results provide further insights into the stability, mechanism of release, and biotransformation of TBBPA-NER in the presence or absence of TBBPA-degrading *O*. sp. strain T.

2. Materials and methods

2.1. Chemicals

Uniformly ^{14}C-ring-labeled TBBPA (^{14}C-TBBPA) with a radiochemical purity of 99% was synthesized in our laboratory from uniformly ^{14}C-ring-labled phenol [13]. Other chemicals of chromatographic and analytical grade were purchased from Sigma-Aldrich (China, Shanghai) and Nanjing Chemical Reagent (China, Shanghai), respectively.

2.2. Soil

TBBPA-NER-containing silty clay soil was obtained from pervious TBBPA degradation study [15] sampled from Jiangning, Nanjing, China. The soil composition was 6.7% organic matter, 41.9% clay, 7.0% sand, and 51.1% silt. The soil pH was 7.4 (0.01 M $CaCl_2$), and the cation exchange capacity 46.2 cmol (+) kg^{-1}. The fresh soil was air dried, sieved through a 2-mm sieve, and stored at room temperature before use.

2.3. Preparation of TBBPA-NER

The TBBPA-NER containing soil prepared as follows: The 35.0 g soil containing both extractable and unextractable TBBPA extracted by methanol (105 mL) 3 times, evaporated at room temperature (for > 24 h) and then stored at −20 °C. Before experiment, the methanol-extracted soil was air-dried at room temperature, and 250 mg of the soil subsample was determined by oxidizer, the remaining radioactivity in soil was defined as the TBBPA-NER [23]. The TBBPA-NER sample (34.65 g) was thoroughly mixed with fresh soil (28.35 g) at the rate 55:45 to recover the microbial activity, and the final radioactivity of TBBPA-NER was 2.1 kBq g^{-1}.

2.4. Bacterium Ochrobactrum sp. strain t

Ochrobactrum sp. T is a gram-negative bacterium able to degrade and mineralize TBBPA under oxic conditions. For this study, the bacterium was cultivated for 24 h at 25 °C in Luria-Bertani (LB) medium, pH 6.5, as reported previously [27]. The cells were harvested when they had reached the exponential growth phase, washed three times with distilled water, and resuspended in distilled water at the concentrations stated below. Sterilized cells were prepared by autoclaving 20 mL of the suspension at 120 °C for 30 min.

2.5. Incubation of TBBPA-NER in soil in the presence and absence of Ochrobactrum sp. Strain T

1.0 g TBBPA-NER containing soil (dry weight, dw) was distributed in 50-mL flasks. After the soil moisture was adjusted to 60% of its maximum water-holding capacity (MWHC) by the addition of distilled water, the flasks were divided into three groups: active cell treatment, sterilized cell treatment, and control. For the active and sterilized treatments, 400 µL (13.7 mg dw) of an active or sterilized suspension of strain T was added to the soil in the flasks while controlling for the moisture content. For the control treatment, distilled water instead of the cell suspension was added to the soil. The soil in the flasks was mixed thoroughly and three flasks from each group were immediately sacrificed for soil extraction and NER fractionation (see below). Each flask was closed with a polytetrafluoroethylene-wrapped rubber stopper and incubated at 25 ± 1 °C in the dark. The $^{14}CO_2$ released from the NER was trapped in a 6-mL vial suspended from the stopper and containing 1 mL of 1 M NaOH. Throughout the incubation, all flasks were opened to the air for 10 min every day to ensure oxic conditions. On days 18, 42, 65, 91, 127, and 214, three flasks from each treatment were sacrificed for soil extraction and NER fractionation (see below). The soil moisture in the remaining flasks was adjusted to 60% MWHC. On days 16, 42, 65, and 91, suspensions of active and sterilized strain T cells with a biomass of 2.5, 4.7, 2.1, and 2.0 mg were newly added to the remaining flasks of the corresponding active and sterilized cell

treatments. The control treatment was amended with an equivalent amount of water.

2.6. Soil extraction and NER fractionation

Soil samples (1.0 g) collected from the incubations were freeze-dried, extracted three times with 5 mL of methanol by repeated shaking (200 rpm for 2 h), and then centrifuged (4000 *g*, 10 min). The three supernatants obtained from the triplicate samples were pooled and their radioactivity was measured by liquid scintillation counting (LSC, see SI), with the amount expressed as the released amount of TBBPA-NER released from the soil. This extraction method recovered 99.5 ± 3.6% of the TBBPA from the silty clay soil [15]. The organic extracts were evaporated to dryness using a rotary evaporator at 40 °C, resuspended in 200 µL of acetonitrile, filtered through a 0.45-µm membrane, and analyzed by high-performance liquid chromatography coupled to an online LSC radioactivity detector (HPLC-^{14}C-LSC; see below) to determine the amount of TBBPA and its metabolites in the extracts [15].

The radioactivity retained in the methanol-extracted soil samples represented the residual TBBPA-NER in the soil. These residual NER were fractionated as follows into fulvic acids (FA), humic acids (HA), and humin-bound residues according to their solubility in alkaline solution [28]: The extracted soil samples were air-dried at room temperature and aliquots of the air-dried pellets (0.3 g) were extracted with 0.1 M oxygen-free NaOH (1.2 mL) for 16 h with horizontal shaking at 200 rpm. After centrifugation (16,000 *g*, 30 min), the supernatants, comprising the alkaline-soluble humic substances, were separated and acidified to pH 1 with 6 M HCl to precipitate HA, which were separated from FA (in the supernatants) by centrifugation (10,000 *g*, 30 min). The radioactivity in the alkaline soluble humic substances and the FA was determined by LSC (see SI), and that in the humin fraction by combustion followed by LSC (see SI).

2.7. Cleavage of the ester linkages in NER

The ester linkages in the TBBPA-NER were cleaved by alkaline hydrolysis, as previously reported [12,29]. Briefly, aliquots of the air-dried, methanol-extracted soil samples (0.4 g) were suspended in 1.6 mL NaOH (1 M) and shaken on a reciprocal shaker for 4 h at 90 °C. After the samples had cooled to room temperature, they were centrifuged (5000 *g*, 15 min) and the resulting precipitate was washed with distilled water (1.6 mL) whereas the supernatants (hydrolysates), containing NER bound to soil matrices via ester linkages, were pooled and their radioactivity was determined by LSC (see SI).

2.8. HPLC analysis

The HPLC system consisted of an Eclipse XBD C18 column (250 mm × 4.6 mm, 5 µm particles size; Agilent Technologies, USA) at 30 °C coupled to an Agilent HPLC 1100 series system equipped with an autoinjector, a degasser, and a diode array detector. Fifty µL of each sample dissolved in acetonitrile was injected repeatedly into the HPLC system, and fractions of the eluents were collected according to the retention times of TBBPA and its metabolites. The mobile phase consisted of an acetonitrile/distilled H_2O gradient running at a flow rate of 1 mL min^{-1} [15]. The gradient program was as follows: 80% acetonitrile for 10 min; increased to 90% acetonitrile within 5 min and maintained for 10 min; decreased to 80% acetonitrile within 5 min and then maintained for 2 min. TBBPA and its metabolites were quantified by LSC (see SI).

2.9. Data analysis

The mineralization of TBBPA and its NER in soil was fitted to Eq. (1)

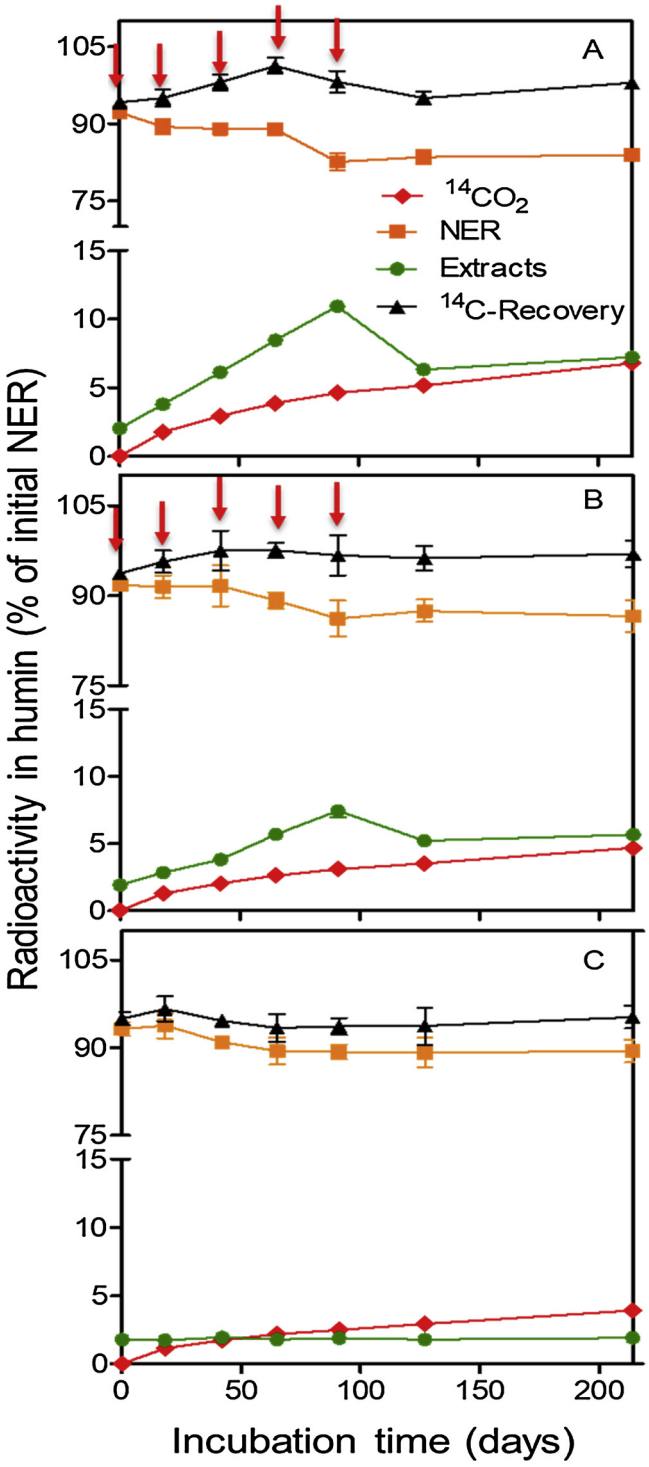

Fig. 1. Transformation of ^{14}C-TBBPA-derived NER into extractable NER residues and CO_2 during a long-term incubation in oxic soil amended with active (A) and sterilized (B) cells of *Ochrobactrum* sp. strain T and in the unamended control (C). Bacterial cells were added to the soil on days 0, 18, 42, 65 and 91 (indicated by the arrows). The data are the means of three individual experiments; the error bars indicate the standard deviations. Error bars smaller than the symbols are not shown.

$$C_{Mineralization,t} = C_0(1 - e^{-k_m t}) \quad (1)$$

where $C_{\text{Mineralization},t}$ is the cumulative $^{14}CO_2$ concentration (dpm kg^{-1} soil dw) at time t (day), C_0 is the initial concentration of TBBPA or NER (dpm kg^{-1} soil dw), and k_m is the mineralization rate of TBBPA or

NER (day^{-1}).

The data were fitted to the equation using the software SigmaPlot 12.0 (Systat Software Inc., USA). Significance was analyzed using an ANOVA or Student's *t*-test. A P value < 0.05 was considered to indicate statistical significance.

3. Results and discussion

3.1. Release of TBBPA-derived NERs in oxic soil

During 214 days of incubation in soil, for all soil treatments the ^{14}C recovered from CO_2, the organic extract, and the residual NER was in the range of 93.7–101.2%, indicating good ^{14}C recovery in the experimental system (Fig. 1). At the beginning of the incubation, 1.8% of the initial ^{14}C was detected in the organic extract (Fig. 1C), probably owing to the disturbance of the NER during the initial mixing with fresh soil, which may have resulted in NER release [15] and the establishment of a release/incorporation equilibrium between NER and soil matrices [30]. However, this value did not significantly change during the incubation. By contrast, mineralization increased continually and after 214 days accounted for 3.9 ± 0.1% of the initial radioactivity, which was much higher than the amount of extractable ^{14}C in the soil (Fig. 1C). NER mineralization was likely due to the rate-limiting step in which the residues were released from their binding sites [31]. The low amounts of mineralization and extractable radioactivity after 214 days of incubation indicated that NER are quite stable in soil under oxic conditions. However, the continuous mineralization indicated the slow but continuous release of TBBPA-NER in the oxic soil. Since the amount of extractable residues in the soil remained almost constant throughout the incubation, then either 1) mineralization was a direct process in which the NER themselves were mineralized, without the accumulation of released residues in extractable form or 2) the released (mineralizable) NER underwent rapid mineralization.

The addition of *O.* sp. strain T to the soil significantly increased the proportion of extractable residues in soil on day 91 of the incubation: to 10.9 ± 0.7% in the active cell treatments and 7.4 ± 0.5% in the sterilized cell treatments (Fig. 1A-B) vs. 1.89 ± 0.17% in control ($p < 0.01$). The increased release of extractable residues in the sterilized cell treatments was probably due to the stimulation of indigenous soil microorganisms by the carbon and nutrient sources contained in the added sterilized cells [32]. Previous studies have shown that the addition of easily degradable organic matter (e.g., cow manure, glucose, and citrate) stimulates bacterial activity [23,24] and that active bacteria are an important factor in the release [23,33] and degradation [33,34] of NER in soil.

The mineralization of TBBPA-NER was also stimulated by the addition of active and sterilized cells, by 4.6 ± 0.1% and 3.1 ± 0.2% on day 91, and 6.8 ± 0.4% and 4.7 ± 0.2% on day 214, respectively (Fig. 1A-B). The corresponding mineralization rate constants (k_m) were (3.1 ± 0.4) × 10^{-4} day^{-1} and (2.1 ± 0. 4) × 10^{-4} day^{-1} (Table 1) and were significantly ($p < 0.05$) higher than the control rate of (1.6 ± 0. 2) × 10^{-4} day^{-1}. The greater mineralization of NER in the soil amended with active cells can be attributed to the ability of strain T to degrade and mineralize TBBPA and its metabolites. The release of NER suggests the breakdown of the residues from the soil matrices by the bacteria, which then used the degradation products as a carbon source. These results show that while a large change in the redox state of the soil can potently induce the release of TBBPA-NER [13], commonly occurring TBBPA-degraders [27] may play a more important role in the biotransformation of TBBPA-NER under moderate conditions and may thus be a major determinant of TBBPA-NER release into the environment, especially from oxic soil.

When after day 91 active and sterilized cells of strain T were no longer added to the soils, the amount of extractable residues decreased sharply, to 7.3 ± 0.6% and 5.7 ± 0.2%, respectively, and during the next 87 days of incubation the release of TBBPA-NER was much slower

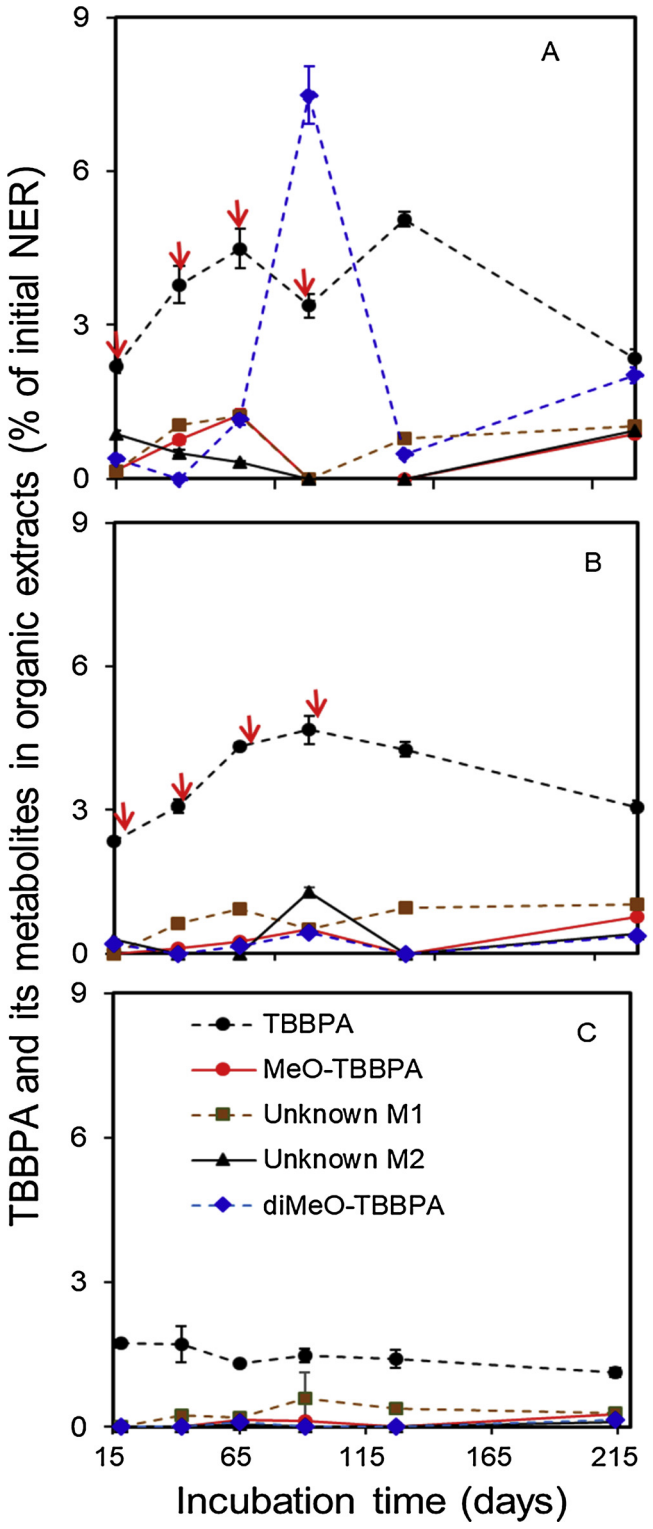

Fig. 2. Extractable TBBPA and its metabolites (MeO-TBBPA, diMeO-TBBPA, and unidentified M1 and M2) from ^{14}C-TBBPA-derived NER incubated in oxic soil amended with active (A) or sterilized (B) cells of strain T and in the unamended control (C). Bacterial cells were added to the soil on days 0, 18, 42, 65 and 91 (indicated by the arrows). The data points are the means of three individual experiments. The bars indicate the standard deviations; error bars smaller than the symbols are not shown.

(Fig. 1A–B). The decrease in extractable residues may be attributed to: 1) re-binding of the extractable residues to the soil matrix to regenerate NER or 2) incorporation of the extractable residues into bacterial

biomass [35,36].

3.2. Release dynamics of TBBPA and its metabolites from NER

TBBPA and four metabolites were detected and quantified in the organic extracts of all treatments. Two of the metabolites were identified as TBBPA monomethyl (MeO-TBBA) and dimethyl (diMeO-TBBPA) ethers whereas two other metabolites (M1, M2) were unknown. These metabolites may have formed in the soil after the release of TBBPA into the soil, or they may have been released from soil matrices during the incubation. In the soil without added strain T cells, the majority of the ^{14}C released during the 214-day incubation was attributed to TBBPA (Fig. 2). Soil amendment with either sterilized or active strain T cells strongly stimulated the release of TBBPA and its metabolites. In the sterilized treatment, 62.8 ± 3.8% of the NER released after 91 days of incubation derived from TBBPA, accounting for 4.7 ± 0.3% of the initial NER. The addition of nutritionally rich dead cells stimulated the biotransformation of the TBBPA released into the soil and therefore the production of significant TBBPA methyl ethers (MeO-TBBPA and diMeO-TBBPA; Fig. 2B). However, when active cells of strain T were added to the soil, the proportion of TBBPA in the extract was less (Fig. 2A) than in either the sterilized or the control treatment, indicating the TBBPA-degrading activity of the added cells. TBBPA degradation by the active cells resulted in the production of large amounts of diMeO-TBBPA, accounting for 69.1 ± 4.7% of the extract (SI Figure S1) and 7.5 ± 0.6% of the initial NER after 91 days of incubation. Thereafter, the amount decreased as active cells were not further added to the soil.

3.3. Distribution of NER in humic fractions

The distribution of residual NER in the soil within the humic fractions (humin, HA, and FA) is shown in Fig. 3. In the control treatment, the distribution was almost constant during the 214 days of incubation (Fig. 3C) and most of the residual NER were in the humin fraction. However, when the soil was amended with either active or sterilized cells, the proportion of NER in the humin fraction (humin-bound residues) decreased rapidly within 91 days, from 84.0 ± 0.4% to 63.0 ± 2.1% and 66.9 ± 2.6%, respectively vs. to 81.0 ± 2.0% in the control soil. Thereafter, when no further cells were added, the amount decreased only slightly (Fig. 3). In parallels, the proportion of NER in HA increased rapidly in the soil amended with active or sterilized cells, from 3.7 ± 0.2% to 14.7 ± 1.3% and 13.1 ± 0.3%, respectively vs. to 3.3 ± 0.2% in the control soil after 214 days of incubation. By contrast, the proportion of the NER in the FA fraction did not change significantly (Fig. 3). The amendment of *O.* sp. strain T cells not only stimulated the release of NER, but it also altered the distribution of residual NER in soil organic matter, which in nature may impact the bioavailability and fate of NER in soil. A previous study showed that HA-bound pollutants can enter the groundwater, where they can be more readily used by plants, such as wheat and maize, as well as by microbial and aquatic organisms [37,38]. In our study, the increased proportion of radioactivity in the HA fraction after the addition of active cells suggests an increase in the amount of bioavailable NER in soil.

3.4. Alteration of ester-linked NER

Ester linkage is an important bonding mechanism in NER formation in soil [12,15,17,39]. In this study, ester-linked NER accounted for 53.5 ± 2.0% of the initial NER, with the amount in the control soil slowly decreasing to 46.1 ± 1.7% during 214 days of incubation (Fig. 4). The presence of active cells strongly stimulated a decrease in ester-linked NER, to 49.91%, after 18 days of incubation. Between day 91, when active cells were no longer added to the soil, and day 214, the amount of ester-linked NER (45.3 ± 1.4%) was similar to that in the control soil, while in the sterilized treatment there was only a slight stimulation (Fig. 4). The results indicated that the ester-linked NER of TBBPA were not stable during the long-term incubation, as also

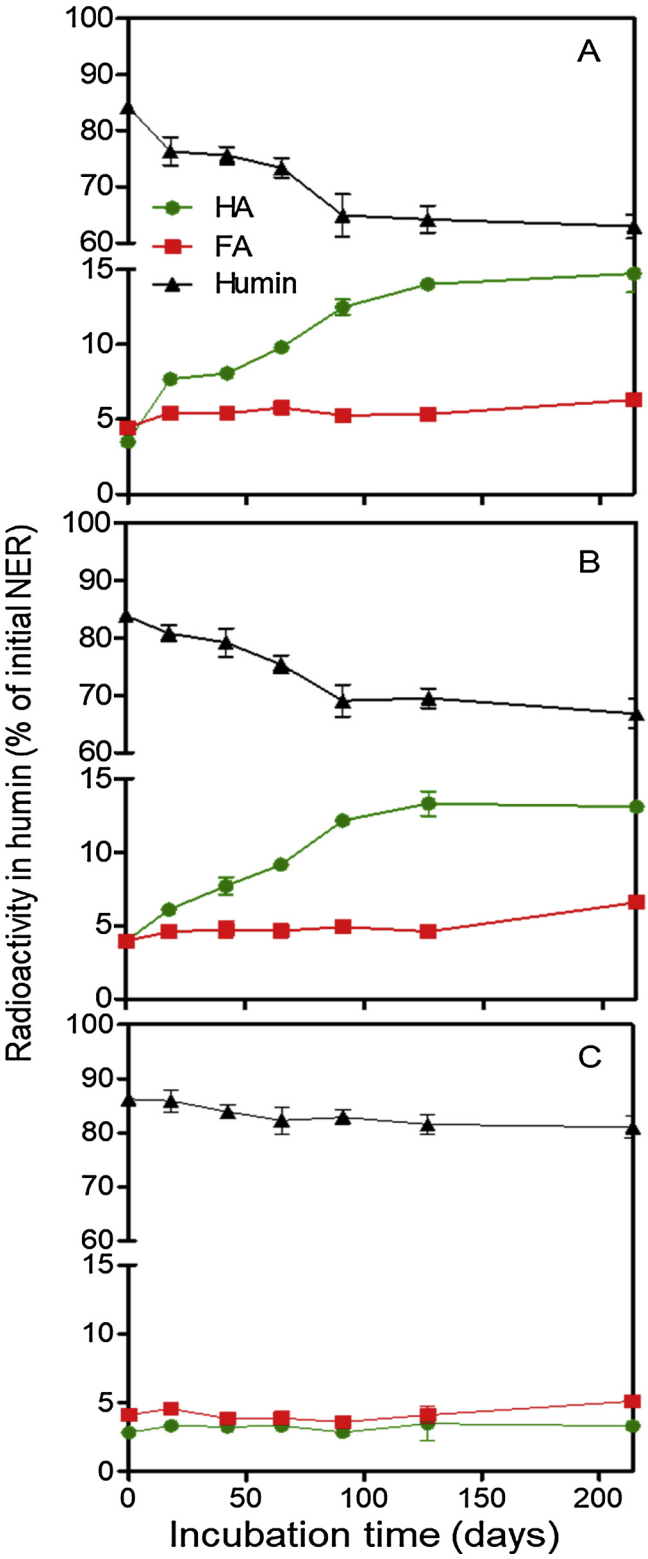

Fig. 3. Distribution of TBBPA-NER within humic fractions (FA, HA, and humin) during incubation in oxic soil amended with active (A) or sterilized (B) cells of strain T and in the unamended control (C). The cells were added to the soil on days 0, 18, 42, 65 and 91(indicated by the arrows). The data points are the means of three individual experiments. The error bars indicate the standard deviations; error bars smaller than the symbols are not shown.

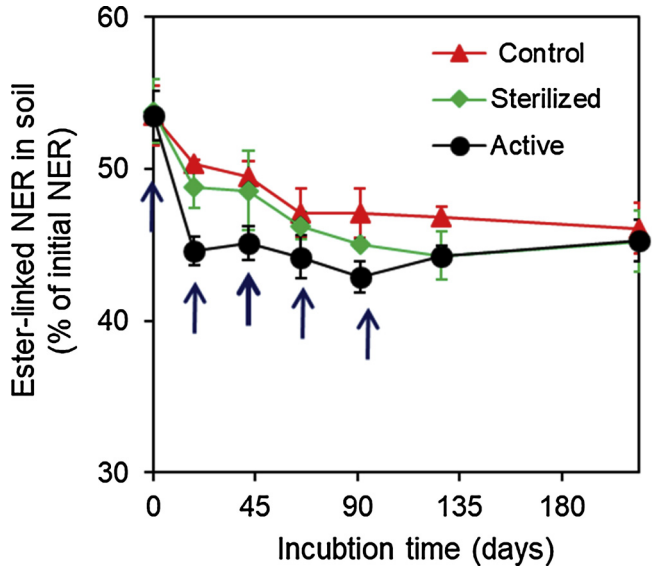

Fig. 4. Amount of ester-linked NER in soil during the incubation of TBBPA-NER under oxic conditions in the presence of active or sterilized cells of strain T (added to the soil on days 0, 18, 42, 65, and 91, as indicated with the arrows) and in the unamended control. The data points are the means of three individual experiments; the error bars indicate the standard deviations.

determined in a previous study [12]. The decreased amount of ester-linked NER in the three soil treatments suggests a conversion to extractable residues, CO_2, and/or HA-bound NER.

Further analysis of the released ester-linked NER by HPLC-^{14}C-LSC showed that they were mainly TBBPA, MeO-TBBPA, and M2 (Fig. 5), which initially accounted for 47.4 ± 1.8%, 4.9 ± 0.2%, and 1.3 ± 0.0% of the total NER, respectively (Fig. 5C), and 88.6 ± 0.4%, 9.1 ± 0.6%, and 2.3 ± 0.3% of the total ester-linked NER (SI Fig. 2). During the 214 days of incubation, the proportion of the three compounds did not significantly change in the control soil. However, in the active cell treatments, the amount of ester-linked TBBPA at day 91 was only 34.3 ± 0.8% of the initial amount of NER ($p < 0.01\%$) while the amount of ester-linked MeO-TBBPA increased to 7.6 ± 0.2% (Fig. 5A). The latter increase was consistent with the larger amount of MeO-TBBPA released into the soil during the incubation (Fig. 2). Thus, taken together, these results demonstrate that TBBPA and its metabolites initially bound to NER via ester linkages were released into the soil via the active disruption of these bonds by *O.* sp. strain T.

3.5. Conclusions

TBBPA-NER incubated in soil were to a large extent mineralized. The residues released in the soil over time were mostly TBBPA but also included MeO-TBBPA, diMeO-TBBPA, and two unknown metabolites. The release was consistent with the slow decrease in the amount of ester-linked NER, which mainly comprised TBBPA and MeO-TBBPA. The amendment of active cells of *O.* sp. strain T significantly enhanced the release and mineralization of NER in the soil. The distribution of NER in the humic fractions changed over the course of the incubation, as the proportion of humin-bound residues decreased, while that of HA-bound residues increased.

This study provided novel insights into the mechanisms of TBBPA-NER formation and the long-term stability of its NER, the nature of the released TBBPA-NER in soil, the alterations in ester-linked NER during long-term incubations. Importantly, it also showed that the enhanced microbial activity in soil arising from nutrient addition and the amendment of TBBPA-degrading microorganisms may increase the release of TBBPA-NER, resulting in their altered distribution in humic fractions and therefore possibly the bioavailability and environmental risk of these otherwise stable compounds in soil.

Acknowledgments

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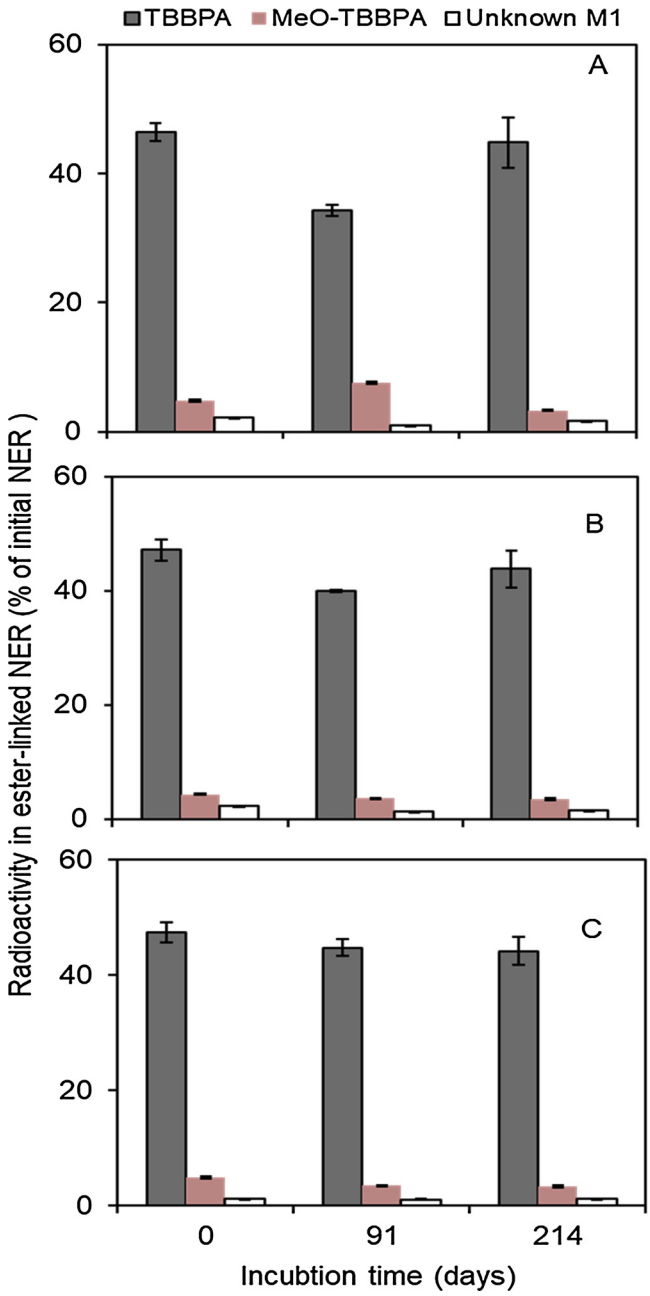

Fig. 5. Amount of ester-linked TBBPA, MeO-TBBPA, and unknown metabolite M1 in NER during 0, 91, and 214 days of incubation of TBBPA-NER under oxic conditions in soil amended with active (A) and sterilized (B) cells of strain T and in the unamended control (C). The cells were added to the soil on days 0, 18, 42, 65, and 91. The data points are the means of three individual experiments. The error bars indicate the standard deviations.

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