Chemosphere 168 (2017) 1194–1202

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Responses of earthworms and microbial communities in their guts to Triclosan

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HIGHLIGHTS

- Triclosan was more abundant in the intestine than epidermis of *E. fetida.*
- Triclosan induced a dose-dependent increase in antioxidant enzymes of *E. fetida.*
- Triclosan altered bacterial and eukaryotic community in *E. fetida* intestine.
- Nine intestine bacterial taxa were more sensitive than enzymes to Triclosan.

ARTICLE INFO

Article history:
Received 19 August 2016
Received in revised form
19 October 2016
Accepted 21 October 2016
Available online 31 October 2016

Handling Editor: Shane Snyder

Keywords:
Ecotoxicological assessment
Indicator
Invertebrate
Toxicity
Genomics
Next generation sequencing
Antimicrobial

ABSTRACT

Responses of the earthworm (*Eisenia fetida*) and compositions of associated microbial communities were determined after exposure to various concentrations of Triclosan (TCS) for 7 d. Concentrations of TCS were greater in intestines than in epidermis of earthworms, which suggested that earthworms accumulate TCS mainly by ingestion rather than by epidermic penetration. Exposure to TCS caused a concentration-dependent increase in activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) and in malondialdehyde (MDA) in *E. fetida*. Analyses of both the bacterial and eukaryotic community by next generation sequencing (NGS), demonstrated that TCS caused a concentration-dependent decrease in sensitive genera. While relative abundances of *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* were increased. Nine susceptible microbial groups were more sensitive to exposure to TCS, than were activities of enzymes in earthworms. Thus, rapid genomic measures of gut flora can be used as indicators to assess adverse effects of chemicals on earthworms.

1. Introduction

Larger metazoans and their associated microbial communities interact and cooperate to perform functions. The microbiomes of both vertebrates and invertebrates have recently been associated with various functions including nutritional, immunological and even neuro-behavioral (Devkota and Chang, 2013; Flint, 2012; Ghosh et al., 2014; Kau et al., 2011; Kelly and Mulder, 2012; Martínez et al., 2013; Wang and Kasper, 2014). Conventional assessments of effects of chemicals or organisms have been conducted at the individual level or have focused on molecular or cellular aspects of model species. However, effects on intestinal microbial communities, which play essential roles in physiological process and long-term health host organisms, have been neglected (Xie et al., 2016).

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, TCS) is a broad-spectrum antibacterial and antifungal compound that has been widely used since the 1970s in pharmaceutical personal care

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http://dx.doi.org/10.1016/j.chemosphere.2016.10.079
0045-6535/

products, textiles, cleaning supplies, toys, medical disinfectants, and computer equipment (Chen et al., 2012). Approximately 96% of TCS originating from consumer products is discarded in residential drains, leading to measurable concentrations of TCS in water, sediments, sewage sludge, and agricultural soils amended with biosolids (Cha and Cupples, 2009; Reiss et al., 2009; USEPA, 2000). Although TCS has a moderate water solubility of 12 mg L^{-1} and is a lipophilic compound with log Kow of 4.8, it is relatively persistent in soil and thus has the potential to bioaccumulate in soil-dwelling organisms (Ying et al., 2007). TCS has a half-life in aerobic and biosolids-amended soils of between 18 and 107 d, but can persist in anaerobic soils or sterile aerobic conditions (Ying et al., 2007). Significant bioaccumulation of TCS has been observed in earthworms collected from soils to which biosolids have been applied (Higgins et al., 2011; Kinney et al., 2008).

The earthworm (*Eisenia fetida*) is a well-recognized and prevalent bioindicator in soil and commonly serves as an ecotoxicological test species in evaluations of effects of chemicals in soils. While conventional ecotoxicological endpoints on earthworms are typically limited to mortality, growth and reproduction, cellular oxidative stress responses are often used as an early indicator of adverse effects of chemical stressors. The anti-oxidative defense system of all aerobic cells includes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). These enzymes confer cellular protection to reactive oxygen species, together with synthesis of stress proteins in *E. fetida*, and can therefore provide sensitive indicators of potential effects of pollutants in soils (Lin et al., 2010). Lipid peroxidation is a well-studied mechanism of injury in both plants and animals. Measurement of formation of malondialdehyde (MDA), a product of lipid peroxidation, can be used as a proxy for direct measures of oxidative stress in cells and tissues. TCS-induced cytotoxic effects in the form of oxidative stress and damage were recently demonstrated in the earthworm (Lin et al., 2010), but whether these biochemical responses also involve the microorganisms prevalent in its intestine was unclear.

The gut of the earthworm is a transient habitat for soil microflora and it hosts a broad range of microorganisms, including soil prokaryotes and eukaryotes with diverse phylogenies and functions. Earlier studies of these microorganisms were based on classical cultivation and microscopy methods, while recent molecular studies have yielded insights into both cultivatable and non-cultivatable species in intestines of earthworms (Hong et al., 2011; Procházková et al., 2013). The microbiome of the earthworm and its response to a gradient of the metalloid, arsenic has recently been characterized by pyrosequencing of 16S ribosomal RNA (rRNA) (Pass et al., 2015). Microbiomes of intestines of earthworms were related to the community in the surrounding environment, which might be resulted from both filtering and the altered environment associated with the gut micro-habitat (neutral pH, anoxia and increased carbon substrates). The gut micirobiota was significantly modified along an arsenic and iron contamination gradient at both the family- and species-level. Because the earthworm presents an ecologically important taxon in terrestrial ecosystems, this observation suggested potential effects on both the host and microbiome that could affect ecological functions in the global environment (Pass, 2015). However, eukaryotic microbes associated with earthworms have not been characterized. The previous study focused on effects of metals, while the current study focused on effects of an organic contaminant that is known to have direct effects on microbial communities.

The objectives of this study were: (i) to examine effects of TCS on the earthworm's anti-oxidant enzyme system and the potential relationship between activities of these enzymes and relative proportions of several predominant intestinal microorganisms, and (ii) to investigate responses of bacterial and eukaryotic microorganisms in microbiomes of intestines of earthworms, including taxonomic structure, diversity, richness of resistant and sensitive species richness, determined by use of next generation sequencing.

2. Materials and methods

2.1. Chemicals and reagents

TCS (CAS nos. 3380-34-5, purity >97%) was purchased from Sigma (MO, USA). All other reagents used to study bioaccumulation or determination of activities of enzymes, were purchased from Ameresco (OH, USA) and were of analytical grade, with a chemical purity of 97–99%. The following DNA isolation and PCR amplification kits were used: MoBio Power Soil DNA kit (MoBio Laboratories, CA, USA), Platinum Taq polymerase (Life Technologies, CA, USA), MinElute gel extraction kit (Qiagen, CA, USA), and Qubi dsDNA HS assay kits (Invitrogen, CA, USA). Personal Genome Machine (PGM) sequencing was carried out using the Ion Xpress Plus region library kit and the Ion OneTouch 200 template kit v.2 DL (both from Life Technologies).

2.2. Spiking of soil with TCS

Yellow-brown soil at a depth of 5–20 cm was obtained from Nanjing, Jiangsu Province in China. The soil was air-dried and passed through a 2-mm sieve to remove gravel and roots. The soil had an organic matter content of 15.3 ± 0.81 g kg^{-1} and a pH of 6.12 ± 0.21. The clay, sand, and silt contents were 24.7%, 13.4%, and 61.9%, respectively. Spiking of the soil with TCS was achieved as follows: TCS dissolved in acetone was added drop-wise to 50 g of soil placed on a piece of tinfoil, followed by shaking to obtain an even distribution then acetone was evaporated by leaving the sample undisturbed in the dark for 2 h in a fume hood, after which an additional 450 g of soil was added and the sample was shaken well again. This procedure achieved concentrations of TCS of 0 (solvent control: SC), 10, 50, 200, and 1000 mg kg^{-1} soil (coded as treatments T10–T1000, respectively) (OECD, 1984). The greatest nominal concentration (1000 mg kg^{-1} dm) was based on reports of the predicted no-effect and half-maximal lethal concentrations of TCS for earthworms, which is 1026 mg TCS kg^{-1} soil dry mass (dm) (Fuchsman et al., 2010; Wuethrich, 1990) and 866 mg TCS kg^{-1} soil dm (Mónica et al., 2010). A natural soil control without solvent (NC) was also included. Samples of spiked soils were placed in 2-L glass beakers and moisture content was adjusted to 25% of the final soil mass. The beakers were then stored in darkness overnight to allow full penetration of water into soil. The next day, ten mature worms were added to each soil sample. Four replicates were prepared for each treatment and all exposures were conducted in darkness at 20 ± 1 °C for 7 d.

2.3. Exposure of earthworms to TCS

Adult earthworms with mean mass 300 ± 50 mg and with fully well-developed clitella were obtained from the Jurong Special Animal Factory in Zhenjiang city, China. They were bred with cattle feces for 2 weeks in an incubator maintained at 20 ± 1 °C and 75% humidity. After 7 d of incubation, earthworms were removed from beakers, rinsed with sterile distilled water, and placed for 24 h on sterile moist filter paper on a sterile operating board to allow collection of their evacuated gut contents (OECD, 1984). These were stored at −80 °C until used in the analysis of intestinal microorganisms (Ley et al., 2008a, 2008b). In addition, three earthworms were dissected using a scalpel and wax cylinders, separating the epidermis and intestine. Each sample was mixed with 3 g of

anhydrous sodium sulfate and subsequently crushed using a mortar and pestle. The ground earthworm tissues were placed in 20-mL glass bottles for extraction and analysis.

2.4. Bioavailability extraction and analysis

Each sample of soil (3.0 g) and ground earthworm sample was extracted for 10 min in 10 mL of acetone: hexane (1:1, v/v) via ultrasonic vibration and subsequent centrifugation. The supernatant was collected in a glass flask. The precipitate was then re-extracted with another 10 mL of 1:1 (v/v) acetone: hexane, centrifuged, and the resulting supernatant was collected in the same flask. This process was repeated four times. Pooled supernatants were dehydrated through an anhydrous Na_2SO_4 column and the residue was eluted with 1:1 (v/v) acetone: hexane. Supernatants were dried via rotary evaporation and the resulting residue was dissolved in 5 mL of methanol. Before analysis by use of HPLC, the solution was passed through a 0.22-μm Teflon filter to remove particulate matter. TCS was analyzed at 30 °C on an HPLC system (Shimadzu LC-20AT HPLC, Japan) equipped with a UV/visible detector (LC-20AT, SPD-20A/20AV, Japan) and a reverse-phase C18 column (4.6 mm × 250 mm, Japan) (Lubarsky et al., 2012). Methanol (100% HPLC grade; Sigma) was used as the mobile phase at a flow rate of 1.0 mL min^{-1}. TCS was detected at 280 nm using a 20-μL injection volume.

2.5. Determination of enzyme activity and MDA content

Earthworms were placed in a pre-chilled mortar and pestle to which ice-cold 50 mM Tris–HCl buffer (1:9, w/v, pH 7.5) containing 250 mM sucrose, and 1 mM EDTA was added. The homogenate was centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was used to assay enzyme activities and MDA content according to previously described procedures. SOD activity was determined by measuring its inhibition of the photochemical reduction of nitro blue tetrazolium (Dhindsa et al., 1980). CAT activity was assayed based on changes in substrate required to consume 1 μmol H_2O_2 per min at 25 °C (Saint-Denis et al., 1998). POD activity was estimated by monitoring the increase in absorbance at 470 nm for 1 min at 25 °C (Argandona et al., 2001). MDA activity was determined by the formation of thiobarbituric acid reactive substances (Livingstone et al., 1990).

2.6. Isolation of DNA and PCR amplification

DNA was extracted from a 0.25-g aliquot of homogenized intestinal contents using the MoBio Power soil DNA kit (MoBio Laboratories). Extracted DNA was quantified and checked for purity based on absorbance at A260/280 nm using a Take3 microplate in a Synergy H4 hybrid multi-mode microplate reader (BioTek, VT, USA) and then stored at −80 °C. Microbial 16s rRNA V4 fragments were amplified using the 563F and V4R primer cocktail, and 18s rRNA V9 fragments using 1380F and 1510R primers (Table 1). Each PCR amplification was conducted in a final reaction volume of 20 μL and using Platinum Taq polymerase (Life Technologies). Amplification was conducted in a SureCycler 8800 thermal cycler (Agilent Technologies, CA, USA) under the following conditions: initial denaturation at 94 °C for 2 min, 28 cycles at 94 °C for 15 s, annealing at 68 °C for 30 s, and a final extension at 68 °C for 7 min. Triplicate PCRs for each sample were performed to minimize potential PCR bias. Products of amplified were checked for size and specificity by electrophoresis on a 2% (w/v) agarose gel and purified using the MinElute gel extraction kit (Qiagen). Purified products were quantified using Qubit dsDNA HS assay kits (Invitrogen) and adjusted to a concentration of 10 ngμL^{-1} using molecular grade water. All purified PCR products were pooled for subsequent sequencing.

2.7. PGM sequencing

Sequencing adaptors were ligated to purified PCR products by use of the Ion Xpress Plus region library kit (Life Technologies). Prior to high-throughput sequencing, all amplicons were assessed on a Bioanalyzer 2100 (Agilent Technologies) for region size distribution and concentration. Samples were adjusted to a final concentration of 26 pM and then attached to surfaces of Ion Sphere particles (ISPs) using the Ion OneTouch 200 template kit v.2 DL (Life Technologies) according to the manufacturer's instructions. ISPs were enriched on the Ion OneTouch enrichment system (Life). ISPs with template sequenced on “318” micro-chips using the Ion PGM Sequencing 300 kit and the Ion Torrent PGM (Life Technologies) for 130 cycles (520 flows).

2.8. Bioinformatics

All PGM quality filtered sequence data were exported as fastq files and PGM's adaptor sequences were automatically trimmed using the default parameter of the ION server (version 3.6.2). Data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010). Reads containing ambiguous ‘N’, homopolymers, and read lengths <150 bp were discarded. Operational taxonomic units (OTUs) were selected using the UPARSE pipeline with a sequence similarity cut-off of 97% (use arch software v.7) (Edgar, 2010), removing chimeric sequences. Clusters with a size smaller than 2 were also removed (Edgar et al., 2011). A representative sequence was chosen as the most abundant “clean” read. Aligned sequences that did not align in the appropriate zone were removed. The sequences of each OTU were assigned to representative taxa using QIIME's parallel wrappers for the Ribosomal Database Project classifier (Wang et al., 2007). The

Table 1
Primer sets used in PCRs of the microbial community.

Target	Primer	Sequence	Length	Ta	Reference
16s rRNA V4	563F	5′-xxx-AYTGGGYDTAAAGNG-3′[a] (*E. coli* positions 563–578)	~245 bp	50 °C	
	V4R cocktail[b]	5′-TACCRGGGTHTCTAATCC-3′ 5′-TACCAGAGTATCTAATTC-3′ 5′-CTACDSRGGTMTCTAATC-3′ 5-TACNVGGGTATCTAATC-3′ (*Escherichia coli* positions 785–802)			
18s rRNA V9	1380F[c]	5′-xxx-GT-CCCTGCCHTTTGTACACAC-3′	~190 bp	57 °C	
	1510R	5′-CCTTCYGCAGGTTCACCTAC-3′			

Ta, Annealing temperature.
[a] A 6-base barcode was added to primer 5′ 563F.
[b] A cocktail of four equally mixed reverse primers was used as the reverse primer.
[c] Add unique error correcting Golay barcode and GT′ spacer to primer 5′ 1380F.

probability of correctly assigning OTUs to taxa was >0.50. For V4, a small fraction of unexpected archaeal sequences as well as chloroplast sequences were removed. For V9, only eukaryotic OTUs were retained; a small fraction containing OTUs of metazoan phyla was removed. Alpha rarefaction was performed using Phylogenetic Diversity, Chao1, and observed species metrics. All samples were rarefied at an equal sequencing depth to reduce related biases. The V4 and V9 library size of each sample was normalized to 7298 and 8538 sequences, respectively.

2.9. Statistical analysis of biochemical indexes

Statistical analyses of communities in sediment were performed using R Statistical Language (https://cran.r-project.org/). The probability threshold used for the statistical significance was $P < 0.05$. Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. The nonparametric Kruskal-Wallis (KW) test was conducted to detect significant differential features on the groups, for example, enzyme activities and relative abundances of microbial genus. If the effects of treatment to features were determined to be significant, pairwise comparisons for features were made using Tukey's post-hoc HSD test (Parks et al., 2014). Clustering of community structures was assessed and visualized by principal component analysis (PCA). Within 19 bacterial genera and one eukaryotic genus which relative abundances were significantly different among treatment groups, abundant genera (mean relative abundance > 1%) were selected to determine the dose-response relationships, as well as enzyme activities. To compare the dose-response relationships between significantly different genera and enzyme activities, the features were normalized using the dissimilarity distance $d_i^{'}$ (Eq. (1)). After the normalization, the changing direct of stimulated features were transformed as that of inhibited features. The dissimilarity distance was imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) and fitted to a four-parameter logistic model.

$$d_i^{'} = \begin{cases} \dfrac{d_i - \min(d)}{\max(d) - \min(d)} \times 100 & \textit{Stimulation} \\ \dfrac{\max(d) - d_i}{\max(d) - \min(d)} \times 100 & \textit{Inhibition} \end{cases} \quad (1)$$

2.10. Nucleotide sequence accession numbers

Sequence data were deposited in the NCBI Sequence Read Archive under accession number PRJNA271366.

3. Results and discussion

3.1. TCS concentration in soil at the end of 7 d exposure

After 7 d of exposure, concentrations of TCS in soil decreased to 31.2%, 55.0%, 65.4%, and 62.3% of the nominally spiked concentrations of 10, 50, 200, and 1000 mg kg^{-1} dm, respectively (Fig. S1). In the pre-experiment spiked soil was analyzed at the beginning of the test and achieved a 90% recovery of TCS from both the 10 and the 1000 mg kg^{-1} dm soil samples (standard deviation 14% and 4%, respectively, confirming the homogeneous distribution of TCS at both lesser and greater concentrations). Although TCS has strong antimicrobial properties, the loss of extractable chemical might still have been due to its biodegradation and mineralization by large numbers of microorganisms, especially bacteria, stimulated by earthworm activities in soil (Cha and Cupples, 2010). In another study, half-life for loss of TCS applied directly to a loam soil under laboratory conditions was 18 d (Ying et al., 2007).

3.2. Acute contact toxicity of TCS to earthworms

Numbers of earthworms and their masses decreased gradually during 7 d of exposure to TCS (Fig. 1 A). There was no statistically significant ($P < 0.05$) difference in the numbers or masses of living earthworms between the NC, SC, and least concentration of TCS (10 mg kg^{-1} dm). However, the numbers of surviving earthworms was inversely proportional to concentration of TCS 10–1000 mg kg^{-1} dm. Growth of earthworms was inversely proportional to concentration of TCS. Inhibition of growth relative to that of controls was 7.0% at 10 mg TCS kg^{-1} and 48.0% at 1000 mg TCS kg^{-1} soil ($P < 0.05$). There were also observable effects of TCS on behavior of the worms. In the presence of the latter concentration, earthworms wriggled only on the soil surface and crept towards the wall of beaker whereas at lesser concentrations of TCS they burrowed into the middle layers, where they stirred and sieved the soil.

3.3. The effect of bioavailability of TCS on earthworms

Earthworms can accumulate pollutants via two pathways (Jager et al., 2000): (1) passive epidermal uptake, also referred to as the equilibrium assignment model, in which compounds are usually dissolved in pore-water and/or weakly associated with the surfaces of particulate matter (Jager et al., 2000) and (2) active intestinal assimilation, in which soil organic matter is swallowed, ground in the gizzard, and then digested by the gut microbiota (Jager et al., 2000). The latter pathway leads to the desorption and transformation of pollutants tightly adsorbed on soil particles into forms that are readily absorbed by the earthworm's internal organs. Additionally, secretions released from the earthworm's digestive tract not only decrease the polarity of liquid-phase materials but also aid in the accumulation of pollutants within visceral fat. TCS was not detected in earthworms in the NC and SC samples, but it was detected in all worms exposed to soils spiked with TCS. Concentrations of TCS increased from 11.1 to 75.1 mg kg^{-1} in epidermis and from 39.3 to 162.0 mg kg^{-1} in intestine when concentrations in soil were 10–1000 mg TCS kg^{-1} dm (Fig. 1B). Greater concentrations of TCS in intestines than in epidermis suggested that active assimilation from ingested materials was more important than adsorption through the epidermis. The fact that earthworm tissue concentrations of TCS were less than those in soil is probably due to sorption and rapid degradation of TCS in soil.

3.4. Oxidative stress responses in TCS exposed earthworms

Concentration-dependent increases in antioxidant activities (SOD, CAT and POD activities) and concentrations of MDA were observed in earthworms exposed to TCS (Fig. 1C–F). While activities of SOD and CAT were elevated only at greater concentrations of TCS, both POD activity and concentrations of MDA in worms exposed to all concentrations of TCS differed significantly from those of controls and reached their maxima (3.59-fold and 3.9-fold greater for POD and MDA, respectively, $P < 0.05$) at the greatest concentration of TCS. These results were consistent with those of a previous report of stimulated SOD and CAT activities and an increase in concentrations of MDA in earthworms exposed to 50 mg TCS kg^{-1} dm (Lin et al., 2010). The concentration-dependent expression of antioxidant enzymes and concentrations of MDA suggests that reactive oxygen species and lipid-peroxidation-induced cellular oxidative injury were primary effects of TCS on *E. fetida*.

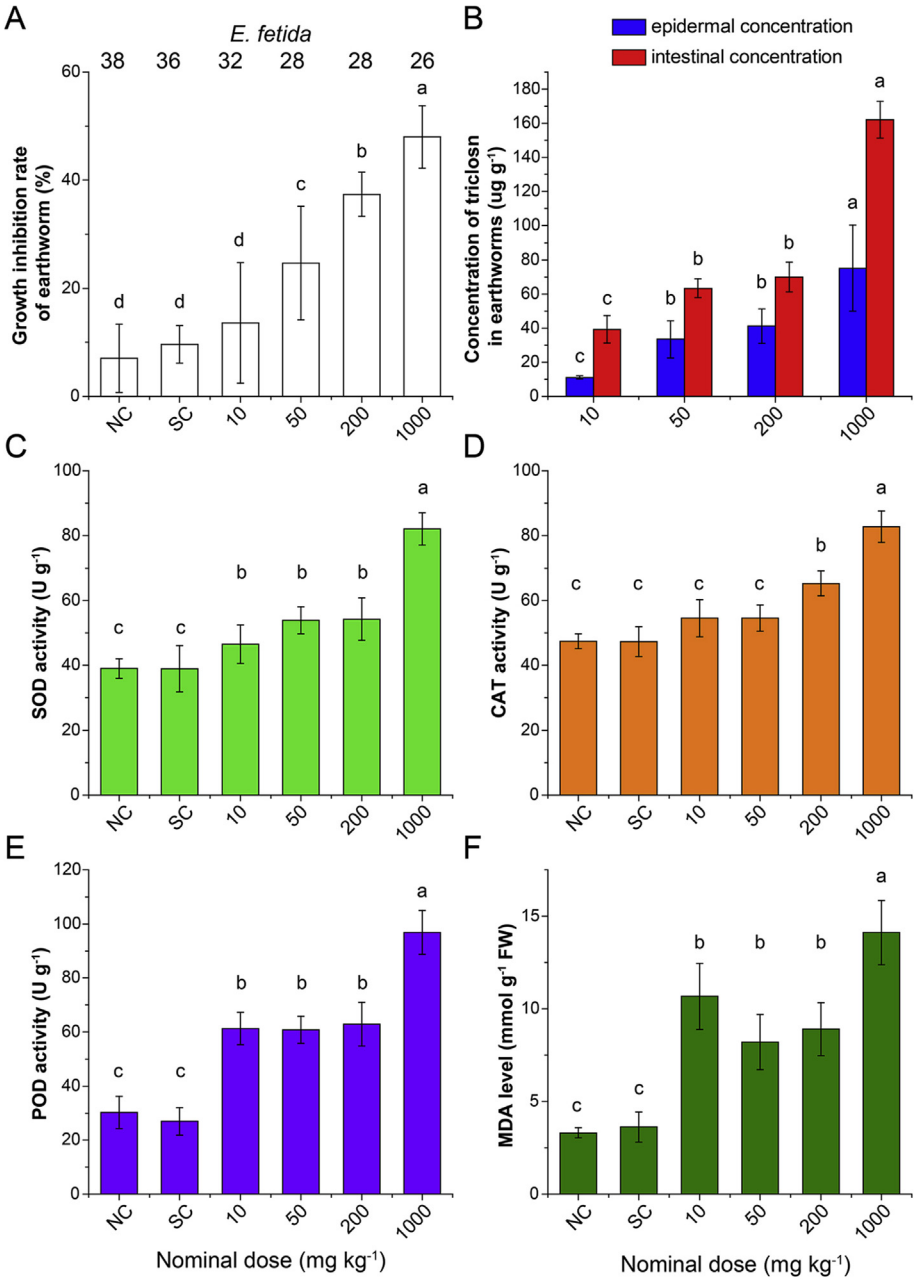

Fig. 1. Acute toxicity and bioavailability of triclosan to earthworms after 7 d of exposure. (A) Inhibition of growth of *E. fetida*. (B) Epidermal and intestinal concentrations of triclosan in *E. fetida*. (C) activity of superoxide dismutase (SOD). (D) activity of catalase (CAT). (E) activity of peroxidase (POD). (F) malondialdehyde (MDA) level. A P value < 0.05 indicated a significant difference from the control.

3.5. Effects of TCS on intestinal microorganisms of earthworms

3.5.1. Composition of the bacterial community

For the 16s rRNA V4 region, 293,808 clean sequences were obtained. Sequence libraries ranged from 12 to 23,839 sequences and the number of OTUs ranged from 4 to 1556 (Table S1). NC1, T1000.4, and T200.4 were filtered out because of low sequencing throughputs. The patterns of the Choa 1 and PD values for the whole tree were consistent with the number of OTUs obtained (Fig. S2).

Bacterial communities were dominated by Proteobacteria (43.1–89.3%, mean 65.2%), Actinobacteria (3.6–20.5%, mean 10.9%), and Bacteroidetes (0.2–27.3%, mean 8.6%) (Fig. S3), accounting for 74.7–94.7% of all phyla, which were consistent with results of previous studies (Pass et al., 2015; Singh et al., 2015). The phylum Bacteroidetes was present in control worms and those exposed to lesser concentrations of TCS, but was not present in samples from earthworms exposed to greater concentrations of TCS. This result indicates the sensitivities of these species to TCS. TCS not only inhibits fatty acid (FA) synthesis, with subsequent perturbation of the bacterial membrane (Lubarsky et al., 2012), but also interferes with the quorum-sensing signaling of gram-negative bacteria (Dobretsov et al., 2007). Species of *Bacillus* and some bacteria associated with the gut wall (*Bradyrhizobium*, *Mycobacterium*, and *Streptomyces*) were detected in every sample. This result is in agreement with previous reports of these species in various earthworm species under various conditions (Thakuria et al., 2010).

Species of *Bacillus* might facilitate mineralization of phosphate and reduction of nitrogenous compounds by earthworms (Judy and Wong, 2004); *Bradyrhizobium* species colonize the gut of many soil-dwelling animals, including earthworms (Citernesi et al., 1977); mycobacteria make use of humic and fulvic acids in soils (Richard et al., 1999); and species of *Streptomyces* possess glucose isomerase activities. Thus, the combined presence of these bacteria might be functionally significant in terms of metabolism of both C and N. In addition to the dominant groups, other major phyla detected were Acidobacteria, Verrucomicrobia, Firmicutes, and Chloroflexi (mean abundance > 1%). Within the Proteobacteria, α- and δ-Proteobacteria accounted for only a small proportion of identified OTUs, whereas both β- and γ-Proteobacteria were abundant. Dominance of β-Proteobacteria suggests that degradation is a major process occurring in the gut of *E. fetida* (Bowman and Mccuaig, 2003). The results reported here are consistent with those of a previous report that demonstrated specific sensitivities of α- and γ-Proteobacteria as well as Cytophagia, from the phylum Bacteroidetes, to TCS.

3.5.2. Composition of the microbial eukaryotic community

For the 18s rRNA V9 region, 538,659 "denoised" sequences were generated. Sequence libraries ranged from 3643 sequences (T10.4) to 128,654 sequences (T50.1) (Supporting Information Table S1). The V9 library size of each sample was normalized to 8538 sequences to retain as many samples and sequences as possible. The normalized samples contained 312–1236 OTUs. In alpha rarefaction curves, the alpha diversity richness of T10, T200, and T1000 was relatively low (Fig. S3). Opisthokonta; fungi (2.4–65.8%, mean: 24.2%) and Amoebozoa; Discosea (3.2–23.9%, mean: 11.4%) dominated eukaryotic communities across all samples. However, a large fraction of clean sequences of V9 (14.1–81%, mean:46.6%) regions could not be assigned to any taxa, which indicated the extent of the novel sequences observed in this study.

3.5.3. Similarity analysis

Structures of communities of intestinal microorganisms were significantly altered by exposure to TCS for 7 d (PERMANOVA test with 9999 permutations: bacterial communities, $P < 0.001$, eukaryotic communities, $P < 0.001$). Relationships shared between communities of bacteri from the various treatments were demonstrated using PCA plots (Fig. 2A). The first two principal components explained 89.5% of the variance among communities of bacteria in intestines of *E. fetida*. NC and SC grouped together. Distributions of T200 and T1000 were similar, whereas T10 was similar to controls. Unlike communities of bacteria, those of eukaryotic microorganisms from various treatments could be grouped into three large clusters (Fig. 2B). The first two principal components explained 71.8% of the variance. NC, SC, and T10 grouped together, as did T50, T200, and T1000.

3.5.4. Treatment-related differences in diversity of microbial communities

Bacteria were found to be more susceptible to adverse effects of TCS than eukaryotic microbes in the microbial community of the earthworm intestine. Totally, there were 19 bacterial genera and one eukaryotic genus relative proportions of which varied significantly among the various treatments (Fig. 3). Genera sensitive to TCS, such as *Bacillus*, *Balneimonas*, *Cupriavidus*, *Hylemonella*, *Kaistobacter*, *Lysobacter*, *Thermomonas*, *Flavihumibacter*, *Flavisolibacter*, and *Flamella* (amoebozoa) were inhibited at 10 mg TCS kg^{-1} dm. Genera *Janthinobacterium*, *Arthrobacter*, *Phenylobacterium*, and *Streptomyces* were inhibited at 200 mg TCS kg^{-1} dm. These observations were consistent with the mechanism of action of TCS, in that it inhibits the enoyl-acyl carrier protein reductase step of

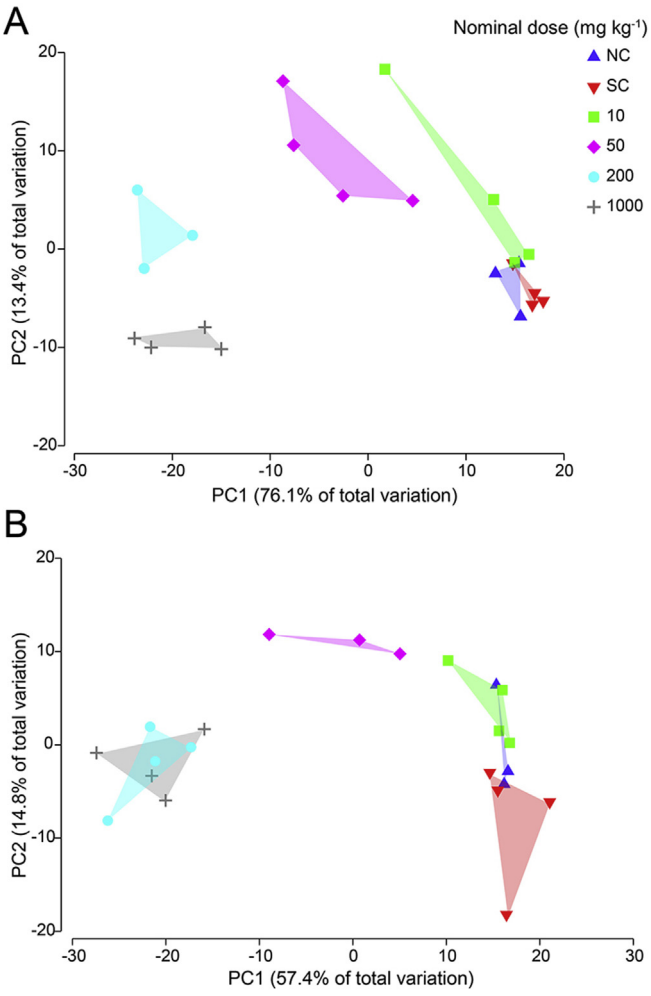

Fig. 2. Community clusters at the family level (including unclassified sequences) determined using a principal components analysis. (A) bacterial communities; (B) eukaryotic microbial communities.

bacterial FA synthesis that proceeds via type II FA synthase (Butler et al., 2012; Heath et al., 1999), while eukaryotic microbes, including fungi, use a FA synthesis pathway that is controlled by type I FA synthase (Butler et al., 2012). Although TCS is a broad-spectrum antimicrobial, populations of intestinal microbes of the genera *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* were enhanced by exposure to TCS. *Pseudomonas putida*, isolated from soil, was previously shown to be resistant to TCS by producing degrading enzymes (Dixit et al., 2010). *Escherichia coli* and *Pseudomonas* cells acquire resistance to TCS from missense mutations in the *fabI* gene (Escalada et al., 2005). Exposure to TCS might select for mutants od *Stenotrophomonas maltophilia* that overexpress the multidrug resistance pump SmeDEF, which would allow the bacterium to actively pump TCS out of cells (Matl et al., 2009), or for strains in which transcription of other detoxifying genes (e.g., *micF*, *acrAB*, *marA*, *bcsA*, and *bcsE*) are up-regulated (Tabak et al., 2007). Some strains of *S. maltophilia* are able to transfer resistance genes to bacteria such as *P. aeruginosa*, Enterobacteriaceae, and *Proteus mirabilis* (Yazdankhah et al., 2006). Thus, tolerance to TCS might be achieved through effective detoxification mechanisms, such as active efflux from the cell, the ability to biodegrade/inactivate TCS, such as expression of TCS-degrading enzymes, or development of resistance to TCS, via mutations in enoyl reductase. Extensive use of TCS in household, industrial, and clinical settings results in its

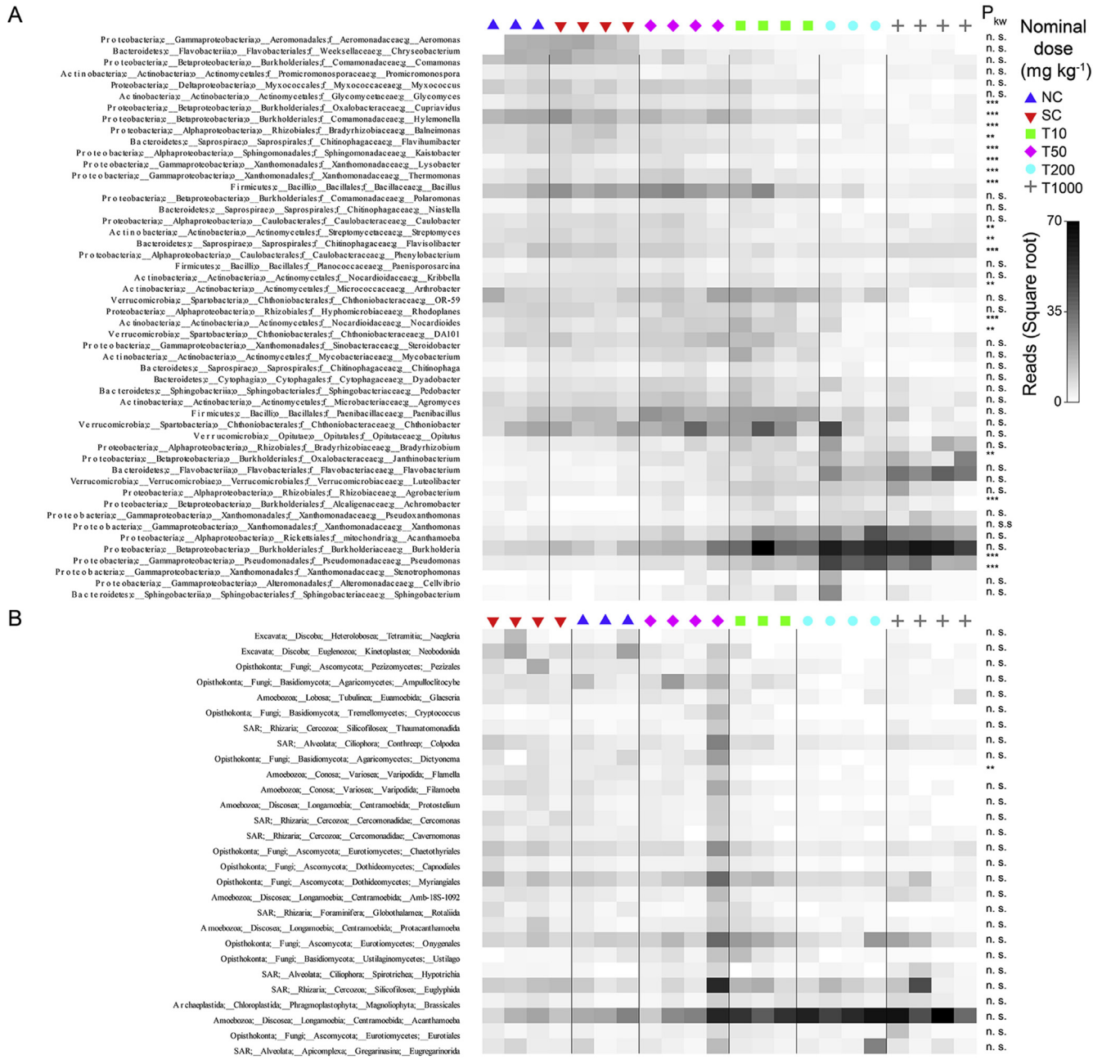

Fig. 3. Patterns of response of intestinal microbial communities to triclosan after 7 d of exposure. (A) bacterial communities; (B) eukaryotic microbial communities. P values for Kruskal-Wallis test (P_{KW}, adjusted with BH FDR approach) were present in column label. Significance of Mann-Whitney U tests was determined at P values < 0.001 (***), <0.01 (**), <0.05 (*) and non-significant (n. s.). Rare genera (reads # < 200) were not presented.

release to the environment, such that it is present in wastewater, environmental sediments, and aquatic biota (Drury et al., 2013; Novo et al., 2013). Environmental deposition of TCS might contribute to resistance to TCS and thus transfer of resistance genes to other bacteria.

It has been shown that differences in microbial communities of the midgut region of *Lumbricus terrestris* were related to food consumed by worms (Markus et al., 2004). 16S-rRNA gene-based clone library construction has been used to demonstrate the abundance of Actinobacteria, Bacteroidetes, Chloroflexi, Verrucomicrobia, Firmicutes, Planctomycetes, and Proteobacteria in guts of *E. fetida* exposed or not to ergovaline and fed a matrix of soil and plant detritus, an observation strongly congruent with our own results (Rogan et al., 2010).

3.5.5. Relationship between microbial indictors and activities of enzymes

Eleven indicators of microbial communities were fitted to the four-parameter logistic model (Fig. 4). Effects on the genera *Flavisolibacter*, *Hylemonella*, *Kaistobacter*, *Lysobacter*, *Flavihumibacter*, *Cupriavidus*, *Thermomonas*, *Bacillus*, and *Balneimonas* occurred earlier than to the genera *Flamella*, *Achromobacter*, *Pseudomonas*, and *Streptomyces*. This result suggests that the nine susceptible microbes could be used to monitor for several effects of TCS. Susceptible microbes were also more sensitive than the assayed enzyme activities (SOD, CAT, POD) or concentrations of MDA the

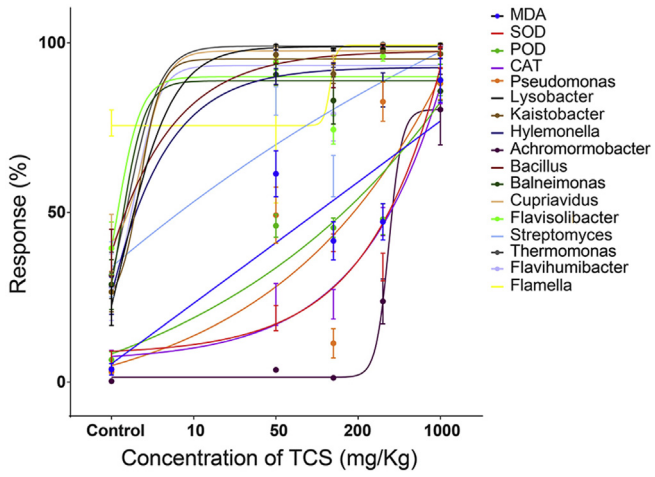

Fig. 4. Concentration-dependent response relationships of microbial indicators and enzyme activities. The significantly different abundant genera (mean relative abundance > 1%) and enzyme activities, the features were normalized using the dissimilarity distance d_i' (Eq. (1)). After the normalization, the changing direct of stimulated features were transformed as that of inhibited features. Concentration-dependent response was fitted using a four-parameter logistic model.

earthworm, caused by direct exposure to TCS. The four-parameter logistic model failed to identify a concentration-response relationship for the genera *Arthrobacter*, *Phenylobacterium*, *Janthinobacterium*, and *Stenotrophomonas*.

4. Conclusions

In summary, accumulation of TCS was greater in intestine than in epidermis of the earthworm. Toxicity-induced oxidative stress was indicated by significantly greater activities of antioxidant enzymes and concentrations of MDA, in response to medium-range TCS concentrations. In TCS-treated soils there was a significant shift in the structure of the microbial community of the earthworm gut, with lesser richness and lesser abundances of bacteria and Eukaryota in response to increasing concentrations of TCS. The observed differences in sensitivities of intestinal bacteria to TCS might have been due to mechanisms by which TCS inhibits bacterial FA synthesis. However, in the presence of TCS, abundances of the genera *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* were greater. These "resistant" intestinal bacteria might carry TCS-resistant or TCS-degrading enzymes; alternatively, they might have the capability to modify the permeability of their outer membranes. Nine susceptible intestinal microbes were more sensitive than the assayed enzyme activities and could be used as bioindicators of TCS contamination. Additionally, we demonstrated that an integrated approach would provide a more comprehensive assessment of the ecological effects of environmental chemicals on earthworms.

Acknowledgements

We thank the Environmental Protection Public Welfare Scientific Research Project of China (#201409040) and the Natural Science Foundation of Jiangsu Province (BK20130015 & BK20130100). X.Z. was supported by the Fundamental Research Funds for the Central Universities. The research is also supported by the Collaborative Innovation Center for Regional Environmental Quality. Prof. Giesy was supported by the Canada Research Chair program, the "High Level Foreign Experts" (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.10.079.

References

Argandona, V.H., Chaman, M., Cardemil, L., et al., 2001. Ethylene production and peroxidase activity in aphid-infested barley. J. Chem. Ecol. 27, 53–68.
Bowman, J.P., Mccuaig, R.D., 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within antarctic continental shelf sediment. Appl. Environ. Microbiol. 69 (5), 2463–2483.
Butler, E., Whelan, M.J., Ritz, K., et al., 2012. The effect of triclosan on microbial community structure in three soils. Chemosphere 89, 1–9.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336.
Cha, J., Cupples, A.M., 2009. Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. Water Res. 43, 2522–2530.
Cha, J., Cupples, A.M., 2010. Triclocarban and triclosan biodegradation at field concentrations and the resulting leaching potentials in three agricultural soils. Chemosphere 8, 494–499.
Chen, J.B., Zhou, X.F., Zhang, Y.L., et al., 2012. Binding of triclosan to human serum albumin: insight into the molecular toxicity of emerging contaminant. Environ. Sci. Pollut. Res. 19, 2528–2536.
Citernesi, U., Neglia, R., Seritti, A., et al., 1977. Nitrogen fixation in the gastro enteric cavity of soil animals. Soil Biol. Biochem. 9 (1), 71–72.
Devkota, S., Chang, E.B., 2013. Nutrition, microbiomes, and intestinal inflammation. Curr. Opin. Gastroenterol. 29 (6), 603–607.
Dhindsa, R.S., Dhinsa, P.P., Thorpe, T.A., 1980. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32, 127–132.
Dixit, V., Tewari, J., Obendorf, S.K., 2010. Fungal growth inhibition of regenerated cellulose nanofibrous membranes containing Quillaja saponin. Arch. Environ. Contam. Toxicol. 59 (3), 417–423.
Dobretsov, S., Dahms, H.U., Huang, Y.L., 2007. The effect of quorum-sensing blockers on the formation of marine microbial communities and larval attachment. FEMS Microbiol. Ecol. 60, 177–188.
Drury, B., Scott, J., Rosi-Marshall, E.J., et al., 2013. Triclosan exposure increases triclosan resistance and influences taxonomic composition of benthic bacterial communities. Environ. Sci. Technol. 47 (15), 8923–8930.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26 (19), 2460–2461.
Edgar, R.C., Haas, B.J., Clemente, J.C., et al., 2011. Improves sensitivity and speed of chimera detection. Bioinformatics 27 (16), 2194–2200.
Escalada, M.G., Harwood, J.L., Maillard, J.Y., et al., 2005. Triclosan inhibition of fatty acid synthesis and its effect on growth of Escherichia coli and Pseudomonas aeruginosa. J. Antimicrob. Chemother. 55, 879–882.
Flint, H.J., 2012. The impact of nutrition on the human microbiome. Nutr. Rev. 70, S10–S13.
Fuchsman, P., Lyndall, J., Bock, M., et al., 2010. Terrestrial ecological risk evaluation for triclosan in land-applied biosolids. Integr. Environ. Assess. 6, 405–418.
Ghosh, T.S., Gupta, S.S., Bhattacharya, T., et al., 2014. Gut microbiomes of indian children of varying nutritional status. PLoS One 9 (4) e95547–e95547.
Heath, R.J., Rubin, J.R., Holland, D.R., et al., 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. J. Biol. Chem. 274 (16), 11110–11114.
Higgins, C.P., Paesani, Z.J., Chalew, T.E.A., et al., 2011. Persistence of triclocarban and triclosan in soils after land application of biosolids and bioaccumulation in *Eisenia foetida*. Environ. Toxicol. Chem. 30, 556–563.
Hong, S.W., Lee, J.S., Chung, K.S., 2011. Effect of enzyme producing microorganisms on the biomass of epigeic earthworms (*Eisenia fetida*) in vermicompost. Bioresour. Technol. 102, 6344–6347.
Jager, T., Francisco, A., Sánchez, A., et al., 2000. Toxicokinetics of polycyclic aromatic hydrocarbons in *Eisenia andrei* (oligochaeta) using spiked soil. Environ. Toxicol. Chem. 19, 953–961.
Judy, H.C.W., Wong, M.H., 2004. Effects of earthworm activity and P-solubilizing bacteria on P availability in soil. J. Plant Nutr. Soil Sci. 167 (2), 209–213.
Kau, A.L., Ahern, P.P., Griffin, N.W., et al., 2011. Human nutrition, the gut microbiome and the immune system. Nature 474, 327–336.
Kelly, D., Mulder, I.E., 2012. Microbiome and immunological interactions. Nutr. Rev. 70, S18–S30.
Kinney, C.A., Furlong, E.T., Kolpin, D.W., et al., 2008. Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. Environ. Sci. Technol. 42, 1863–1870.
Ley, R.E., Hamady, M., Lozupone, C., et al., 2008a. Evolution of mammals and their gut microbes. Science 320 (5883), 1647–1651.
Ley, R.E., Lozupone, C.A., Hamady, M., et al., 2008b. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6 (10), 776–788.
Lin, D.S., Zhou, Q.X., Xie, X.J., et al., 2010. Potential biochemical and genetic toxicity

of triclosan as an emerging pollutant on earthworms (*Eisenia fetida*). Chemosphere 81, 1328–1333.
Livingstone, D.R., Garcia-Martinez, P., Michel, X., et al., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel Mytilus edulis and other molluscs. Funct. Ecol. 4, 415–424.
Lubarsky, H.V., Gerbersdorf, S.U., Hubas, C., et al., 2012. Impairment of the bacterial biofilm stability by triclosan. PLoS One 7 (4), e31183.
Markus, E., Sven, M., Bianca, W., et al., 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris*. (Oligochaeta: Lumbricidae). FEMS Microbiol. Ecol. 2 (48), 187–197.
Martínez, I., Lattimer, J.M., Hubach, K.L., et al., 2013. Gut microbiome composition is linked to whole grain-induced immunological improvements. ISME J. 7, 269–280.
Matl, F.D., Zlotnyk, J., Obermeier, A., et al., 2009. New anti-infective coatings of surgical sutures based on a combination of antiseptics and fatty acids. J. Biomater. Sci. Polym. Ed. 20 (10), 1439–1449.
Mónica, J.B.A., Eva, O., Amadeu, M.V.M., et al., 2010. Predicted No Effect Concentration (PNEC) for triclosan to terrestrial species (invertebrates and plants). Environ. Int. 36, 338–343.
Novo, A., André, S., Nunes, O.C., et al., 2013. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. 47 (5), 1875–1887.
OECD guideline for testing of chemicals, 1984. Earthworm, Acute Toxicity Tests, 207 Adopted, 4 April.
Parks, D.H., Tyson, G.W., Hugenholtz, P., et al., 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30 (21), 3123–3124.
Pass, D.A., 2015. The Earthworm Microbiome. Cardiff University.
Pass, D.A., Morgan, A.J., Read, D.S., et al., 2015. The effect of anthropogenic arsenic contamination on the earthworm microbiome. Environ. Microbiol. 17 (6), 1884–1896.
Procházková, P., Šustr, V., Dvořák, J., et al., 2013. Correlation between the activity of digestive enzymes and nonself recognition in the gut of *Eisenia andrei* earthworms. J. Invert. Pathol. 114, 217–221.
Reiss, R., Lewis, G., Griffin, J., 2009. An ecological risk assessment for triclosan in the terrestrial environment. Environ. Toxicol. Chem. 28, 1546–1556.
Richard, A., Kirschner, J., Parker, B.C., et al., 1999. Humic and fulvic acids stimulate the growth of Mycobacterium avium. Fems. Microbiol. Ecol. 30, 327–332.
Rogan, M.R., Sudeep, P., Forrest, S.A., et al., 2010. Microbiomic Comparison of the intestine of the earthworm *Eisenia fetida* fed ergovaline. Curr. Microbiol. 60, 229–235.
Saint-Denis, M., Labrot, F., Narbonne, J.F., et al., 1998. Glutathione, glutathione related enzymes and catalase activities in the worm *Eisenia fetida*. Arch. Environ. Contam. Toxicol. 35, 594–606.
Singh, A., Singh, D.P., Tiwari, R., et al., 2015. Taxonomic and functional annotation of gut bacterial communities of *Eisenia foetida* and *Perionyx excavatus*. Microbiol. Res. 175, 48–56.
Tabak, M., Scher, K., Hartog, E., et al., 2007. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms. FEMS Microbiol. Lett. 267, 200–206.
Thakuria, D., Schmidt, O., Finan, D., et al., 2010. Gut wall bacteria of earthworms: a natural selection process. ISME 4 (3), 357–366.
USEPA, EPA 832-F-00–064, 2000. Biosolids Technology Factor Sheet, Land Application of Biosolids. http://www.epa.gov/owm/mtb/land_application.pdf (Accessed 16 January 2004).
Wang, Y., Kasper, L.H., 2014. The role of microbiome in central nervous system disorders. Brain Behav. Immun. 38 (5), 1–12.
Wang, Q., Garrity, G.M., Tiedje, J.M., et al., 2007. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73 (16), 5261–5267.
Wuethrich, V., 1990. Acute Toxicity (LC50) Study of Triclosan (Code FAT 80'023/Q) to Earthworm.
Xie, Y.W., Xia, P., Wang, H., et al., 2016. Effects of captivity and artificial breeding on microbiota in feces of the red-crowned crane (Grus japonensis). Sci. Rep. 6, 33350.
Yazdankhah, S.P., Scheie, A.A., Høiby, E.A., et al., 2006. Triclosan and antimicrobial resistance in bacteria: an overview. Microb. Drug. Resist. 12 (2), 83–90.
Ying, G.G., Yu, X.Y., Kookana, R.S., 2007. Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling. Environ. Pollut. 150, 300–305.

Supporting Information

Responses of earthworms and microbial communities in their guts to Triclosan

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FIGURE LEGENDS

Fig. S1. Concentrations of TSC in soils to which *Eisenia fetida* were exposed for 7 days.

Fig. S2. Choa 1 and Phylogenetic Diversity alpha rarefaction curves for bacterial communities at cutoff levels of 3%.

Fig. S3. Choa 1 and Phylogenetic Diversity alpha rarefaction curves for eukaryotic communities at cutoff levels of 3%.

Fig. S4. Abundances of phyla and classes of Proteobacteriain in earthworm feces. Representative sequences were assigned to taxonomic levels from genus to phylum using the RDP Classifier at a 75% threshold. Large fractions of "denoised" sequences from the V4 and V9 regions could not be assigned to any taxa, indicating the extent of novel sequences obtained in this study. A: bacterial community structure; B: eukaryotic community structure.

Fig. S5. Individual concentration-response curves between microbial indicators and enzyme activities.

TABLE LEGENDS

Table S1. Clean reads, total operational taxonomic units (OTUs), numbers of OTUs, and Chao 1 and Phylogenetic Diversity indices of each sample

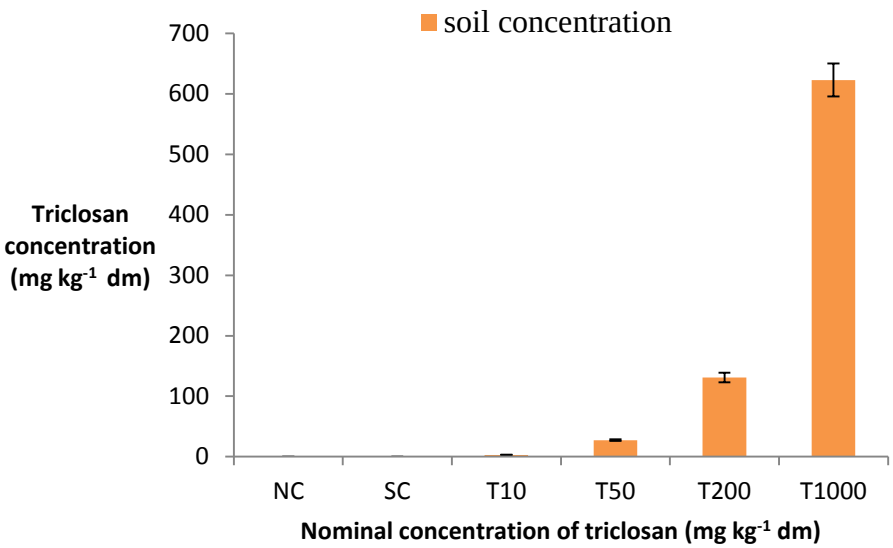


Fig. S1. Concentrations of TCS in soils to which *Eisenia fetida* were exposed for 7 days. SC stands for solvent control; NC represents a natural soil control without solvent.

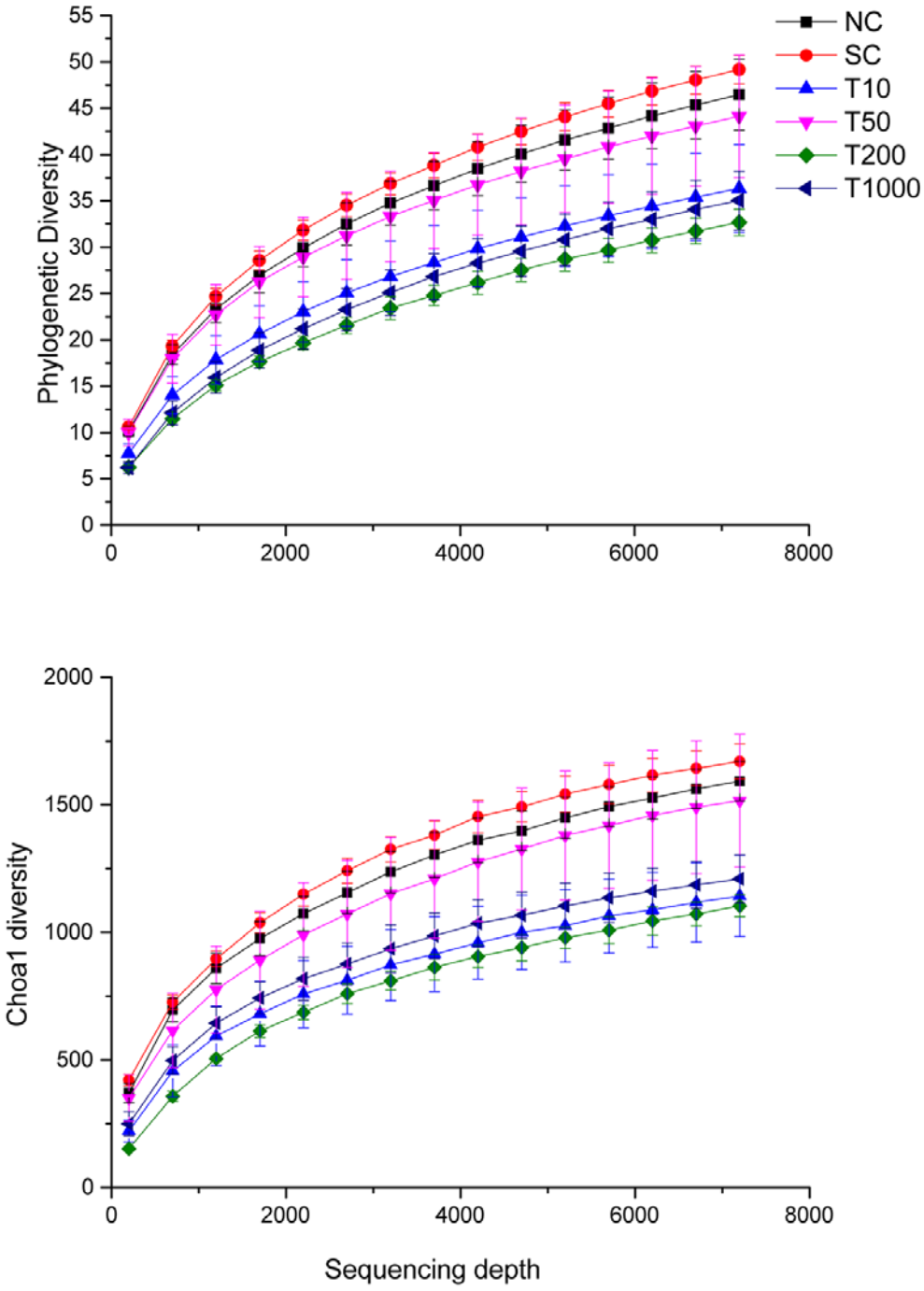


Fig. S2. Choa 1 and Phylogenetic Diversity alpha rarefaction curves of bacterial communities at cutoff of 3%.

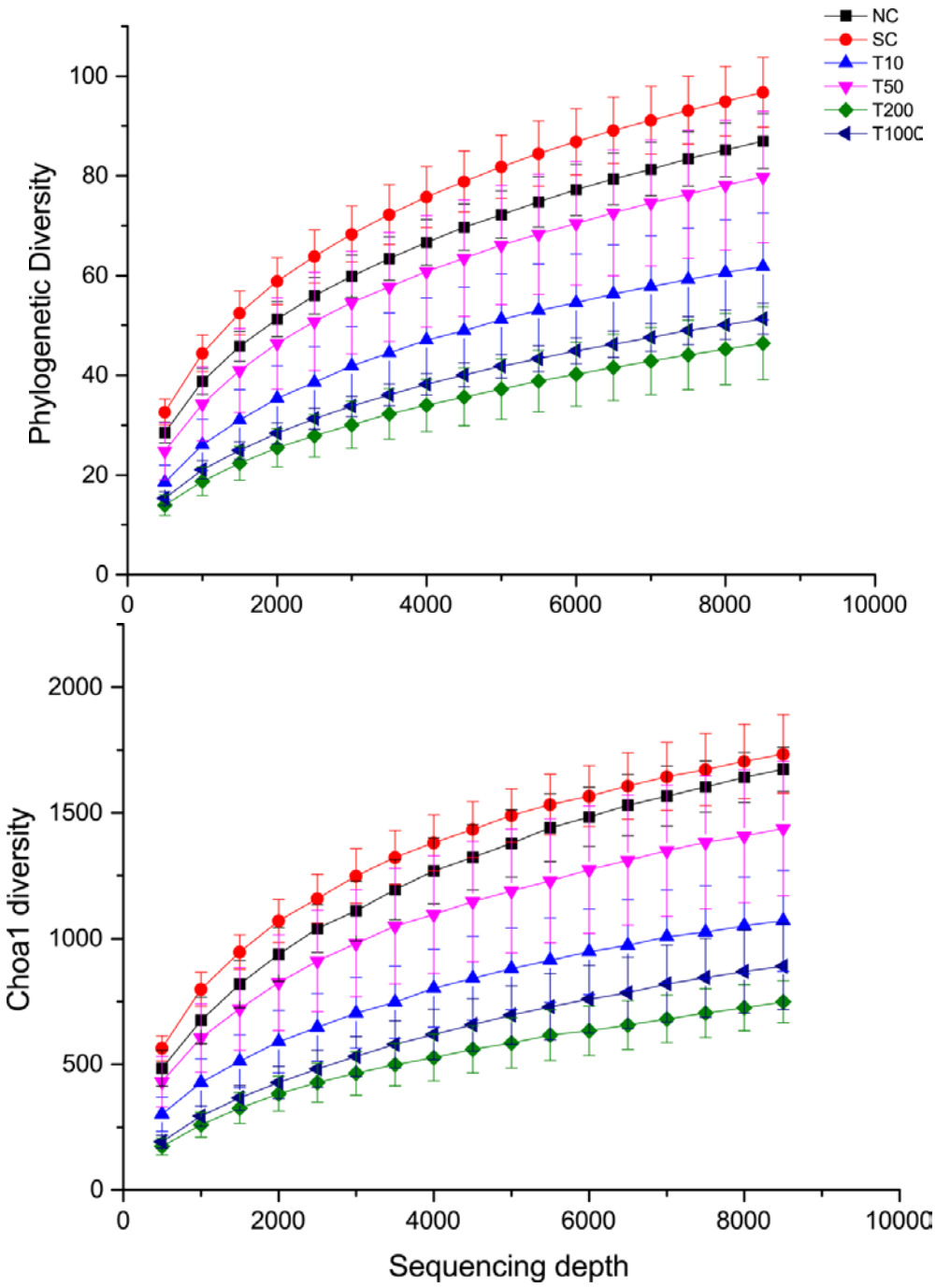
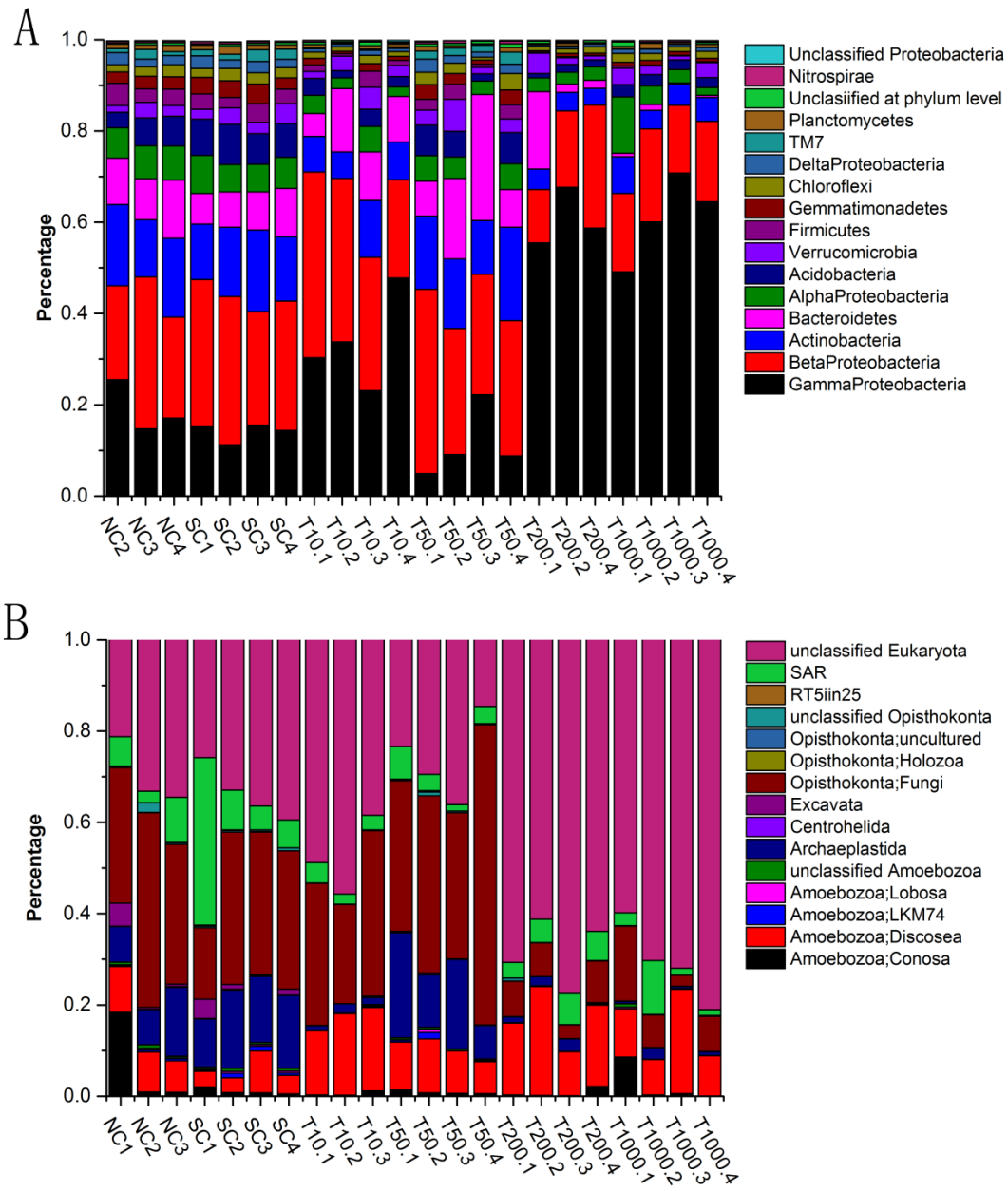


Fig. S3. Choa 1 and Phylogenetic Diversity alpha rarefaction curves of eukaryotic communities at cutoff of 3%.



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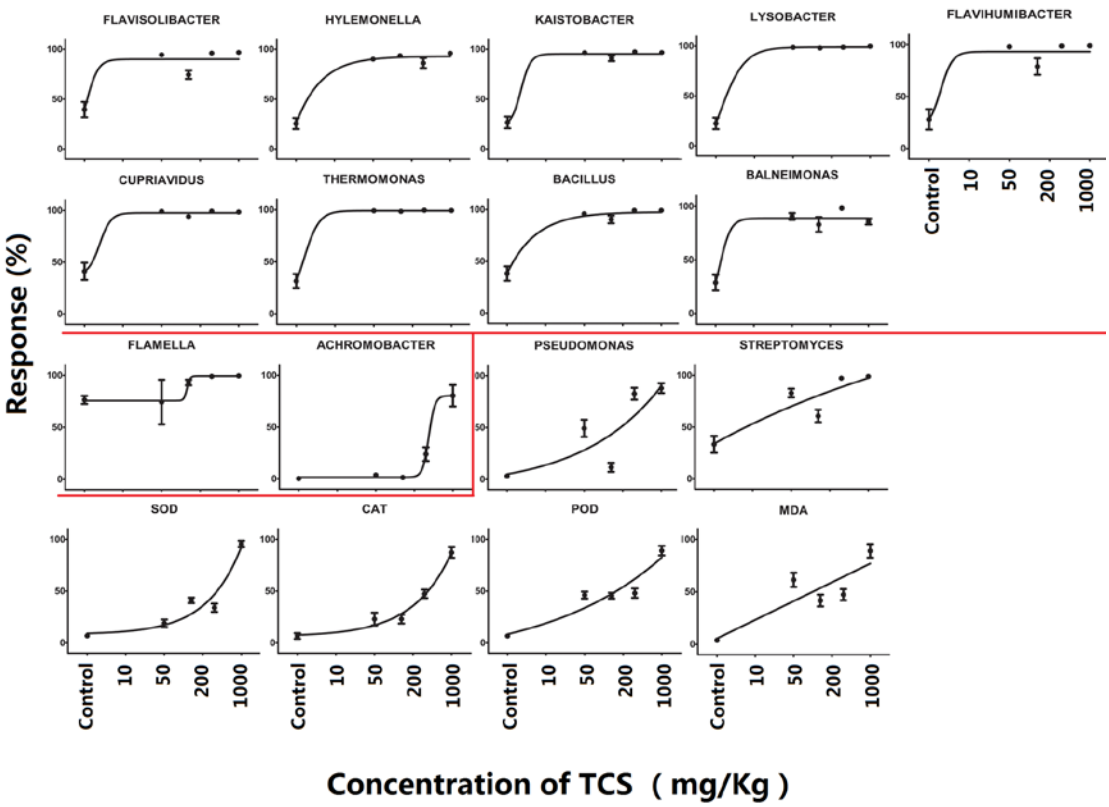

Fig. S5. Individual concentration-response curves for microbial indicators and enzyme activities

Table S1. Clean reads, total operational taxonomic units (OTUs), numbers of OTUs, and Chao 1 and phylogenetic diversity indices of each sample

Sample	Prokaryotic alpha diversity					Eukaryotic alpha diversity				
	Clean reads	Total OTUs	Sampling depth at 7200			Clean reads	Total OTUs	Sampling depth at 8500		
			Observed	Chao1	PD*			Observed	Chao1	PD
NC1	660	-	-	-	-	8538	850	849	1553	81
NC2	8984	1026	941	1488	41	10715	995	905	1704	86
NC3	15456	1420	1040	1674	50	13177	1345	1102	1764	94
NC4	7298	1105	1098	1615	48	7228	-	-	-	-
SC1	14350	1467	1106	1668	50	13037	1257	1028	1609	94
SC2	14957	1543	1129	1706	51	15334	1592	1236	1990	107
SC3	18226	1556	1099	1744	47	17234	1536	1124	1738	95
SC4	8862	1134	1039	1561	48	11634	1143	995	1602	91
T10.1	9110	796	701	1146	37	19206	934	634	1087	60
T10.2	10200	664	545	925	31	18265	650	443	817	49
T10.3	16000	1305	885	1375	44	20846	1169	754	1308	76
T10.4	23839	1110	614	1125	34	3643	-	-	-	-
T50.1	15600	1440	1087	1636	52	113237	2401	1060	1732	93
T50.2	10982	1150	945	1590	44	10913	1105	979	1611	89
T50.3	13883	827	604	1077	34	13534	727	583	1031	59
T50.4	15965	1502	1105	1759	47	20148	1199	769	1376	78
T200.1	17087	848	551	1148	32	22212	746	463	878	53
T200.2	10391	744	592	1117	31	12537	505	427	768	54
T200.3	12	-	-	-	-	21178	530	320	683	40
T200.4	11679	694	525	1045	35	22546	544	312	666	38
T1000.1	9386	895	773	1304	39	11457	489	422	840	52
T1000.2	14210	1016	722	1255	35	22810	783	480	1086	55
T1000.3	13426	834	590	1225	31	21097	550	332	634	47
T1000.4	13245	905	624	1054	35	44455	1049	454	996	57

PD, Phylogenetic Diversity index