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Tissue distribution and metabolism of triadimefon and triadimenol enantiomers in Chinese lizards (*Eremias argus*)

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ABSTRACT

Triadimefon (TF, S-(+)-TF, R-(-)-TF) and its metabolite triadimenol (TN, TN-A1, A2 and TN-B1, B2) are two systemic fungicides and both of them are chiral pharmaceuticals which are widely used in agricultural industry. Many researches focused on the toxicity effects of triadimefon on mammals, while the ecotoxicological data of tiradimefon on reptiles is limited. In order to understand the toxicity mechanism of triadimefon in reptiles, the current study administrated S-(+)-TF or R-(-)-TF traidimefon (50 mg/kg^{bw}) to Chinese lizards (*Eremias argus*) respectively, the absorption, distribution of triadimefon and the formation of triadimenol were analysed at different sampling times. The metabolic pathways were demonstrated through relative gene expression using quantitative real-time PCR reaction. During the experiment time, triadimefon was quickly peaked to the maximum concentration within 12 h in liver, brain, kidney, and plasma, eliminated slowly. The biotransformation in kidney was the lowest and fat possessed the worst degradation ability among others. The metabolite, triadimenol was detected in blood in 2 h and reached to a plateau at about 12 h in most organs (fat excepted), while the process of metabolism is stereoselective. The mainly metabolite in R-(-)-TF treated group was TN-B1, and TN-A2 in S-(+)-TF group which showed the selective metabolism to other species caused by environmental conditions, differences in the animal models and concentration of TF. The related gene expression of *cyp1a1*, *cyp3a1* and *hsd11β* mRNA level in lizards showed different metabolic pathways in the liver and brain. Both P450s enzymes and *11β*-hydroxysteroid dehydrogenase participated in metabolic reaction in liver, while no *11β*-hydroxysteroid dehydrogenase pathway observed in brain. This diversity in liver and brain may cause different degradation rate and ecotoxicological effect in different organs.

1. Introduction

Triazoles are a class of systemic fungicides that contain the 1, 2, 4-triazole moiety and they are excellent in antifungal activity and resistance risk (Manclus et al., 2008; Sanagi et al., 2004). In molecular structure, a large number of triazole fungicides have chiral centres, and their metabolic products may also be chiral in various of environmental compartments (Li et al., 2012).

The agrochemical systemic fungicide triadimefon (TF, Fig. 1.) has one chiral carbon centre. It is a triazole-derived molecule extensively used to control powdery mildews, rusts and other fungal pests on cereals, fruits, vegetables, turf, shrubs and trees (Kenneke et al., 2009). Triadimefon may be transformed in plants, soils, and fungi via enzymatic action by reduction of a carbonyl group to its corresponding alcohol, triadimenol (TN, Fig. 1). Triadimenol is also registered separately as a systemic fungicide and has greater fungicidal activity than triadimefon (Deas et al., 1986; Liang et al., 2013). Triadimenol possessed 2 chiral centres, and consisted of 4 stereoisomers (including 2 pairs of diastereomers), TN-A [(1R,2S) and (1S,2R) enantiomers] and TN-B [(1R,2R) and (1S,2S) enantiomers] (Kenneke et al., 2008). The two fungicides are commercialized as the racemate agrochemicals and released into environment as a mixture of enantiomers. However, there were researches verified the enantiomers of the same compound may perform differently in environmental fate and ecological risk (Diao et al., 2010; Zhang et al., 2012). And Deas et al. (1986) pointed out the (1S, 2R)-TN isomer metabolized from S-(+)-TF was 1000 times more active than the other three.

Triadimefon and many other triazoles have been used in agriculture to inhibit the lanosterol-14α-demethylase and subsequent disruption of ergosterol biosynthesis, because of its critical role in fungal cell membrane formation. Both triadimefon and triadimeol are classified as possible human carcinogens and more and more research groups showed triazoles may cause hepatotoxic, carcinogenic, neurological, reproductive and endocrinological effects in vivo or in vitro rodent models (Di Renzo et al., 2007; Menegola et al., 2000; Peffer et al., 2007). Their toxicity had been attributed to the activation of a series of

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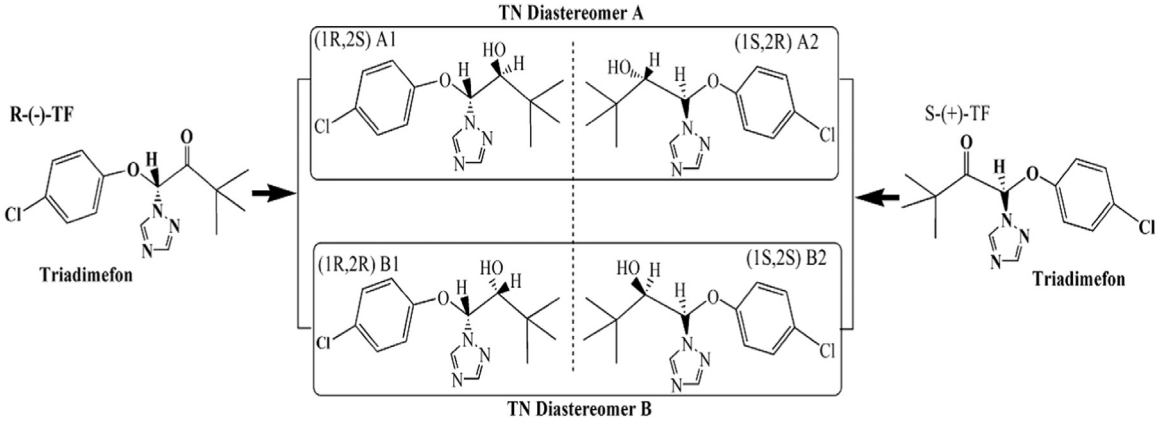

Fig. 1. The metabolic transformation of triadimefon to triadimenol and the molecular structure of enantiomers of triadimefon and triadimenol.

nuclear receptors (CAR, PXR, and PPAR) and the induction of xenobiotic metabolizing enzymes (e.g., cytochrome P450 monooxygenases) (Allen et al., 2006; Kenneke et al., 2008).

P450 monooxygenases are mainly distributed in the endoplasmic reticulum and mitochondrial membrane which are widely existed in vertebrate species. They are responsible for the metabolism of endogenous (e.g., steroids) and exogenous compounds (e.g., drugs and fungicides) (Shityakov et al., 2014). The induction of P450 , which may significantly alter the curative effect of medications and so as to alter endogenous biochemical pathways. And this phenomenon may result in elevating human health risks. Triadimefon has been reported to induce both the 1 A and the 3 A P450 subfamilies in rats, mices and aquatic organisms (Sun et al., 2007; Sun and Lotan, 2002). And *Cyp3a* isoforms may metabolize as much as 50–60% of all therapeutic compounds (Riley et al., 2001). Besides, triadimefon not only metabolized via the oxidative P450 mediated pathway, but also be mediated by *11β*-hydroxysteroid dehydrogenase type 1 (*11β-hsd1*) (Atanasov and Odermatt, 2007). *11β-hsd1* catalyzed the carbonyl reduction of several endogenous oxidized sterols and played a pivotal role in the regulation of energy metabolism through the activation of endogenous glucocorticoids in tissues such as liver, adipose and skeletal muscle (Kenneke et al., 2008; Mazur et al., 2009; Meyer et al., 2013).

Due to the wide residue of triadimefon and its metabolite in soils, sediments, natural waters and agricultural products, many studies have pointed out the potential threat to mammal and aquatic animals (Li et al., 2014, 2011). However, little had been known about the toxicokinetic effects of triadimefon on reptiles. Reptiles are essential part for keeping ecological balance and comprise 28% of known vertebrate species (Gervais, 2011). The environment contamination is supposed to be a reason for population decline in reptiles, especially intensification and expansion of agriculture accelerated exposure risk to wildlife (Wagner et al., 2015).

Lizards were the most suitable model among reptiles to assess the toxicological effects of fungicides because they could easily contact with soil and more sensitive to environmental contamination. In addition, lizards played an important role in some food webs and within terrestrial food chain (Campbell and Campbell, 2000). Thus, it is an urgency issue to make clear about the toxicity effect of triadimefon on lizards. In this study, we evaluated the absorption, distribution of triaidmefon, and the formation of triadimnol in lizards. The metabolic pathways were also analysed through the related gene expression of *cyp1a1*, *cyp3a1* and *hsd11β* mRNA level in *Eremias argus*, a most abundant lizard specie in Asia (Kim et al., 2010).

2. Materials and methods

2.1. Chemicals and reagents

Analytical standard of triadimefon and triadimenol (99.8% purity) were kindly provided by College of science, China Agricultural University (Beijing, China). The stock solutions of triadimefon and triadimenol were prepared in methanol. The two triadimefon enantiomers were prepared by normal chiral PAK IC Column [cellulosetris-(3, 5-dichlorophenyl-carbamate)] (4.6 cm i.d. × 25 cm long) on an Agilent 1260 high performance liquid chromatograph (HPLC) system under the UV detection wavelength 225 nm. The mobile phase fractions corresponding to the purer enantiomers were collected manually by observing their UV signals. The purity of each separated stereoisomer was 96%, which was checked by chiral HPLC using the same system. All the chemicals and solvents were analytical grade and purchased from commercial sources (Table S1).

2.2. Animals husbandry

Adult lizards (about 2 years old) were obtained from Abag Banner, Inner Mongolia (China), which has no history of chemical application, and maintained in our laboratory for more than 4 years. The lizards were kept in 5×1.2×0.4 m solid bottom indoor aquarium covered with 15 cm mollisol and fallen leaves. The temperature and humidity were maintained at 25–30 °C and 25–30%. Daylight lamps (100 W) were set to a 14-h light: 10-h dark photoperiod to provide enough light. The lizards were fed with mealworms once a day and the water was sprayed twice a day. The excreta of lizards were cleaned every two days.

2.3. Ethical permissions

All the test lizards were obtained from Abag Banner, Inner Mongolia (China), which has no history of chemical application, and maintained in our laboratory for more than 4 years and the lizards used in our experiment received humanitarian concern. As seen from both historically and currently, avians have been used as surrogates for reptile ecological risk assessment (ERA) (Weir et al., 2015). So, in our present study, we also used toxicity test of avian for the experiment and the dosing method was set up in steps (Suski et al., 2008).

2.4. Degradation studies

Treated groups were assigned with R-(-)-TF or S-(+)-TF dissolved in corn oil respectively and administered orally at 50 mg/kg^{bw}. Combined avian toxicity test of triadimefon (LD_{50} > 2000 mg/kg^{bw}) published by U.S Environmental Protection Agency (U.S. EPA) (Extoxnet, 2010) and the previous study by Wang et al. (2014), 2.5% of the LD 50 was chosen as the dosing concentration. The control group received only corn oil orally and the dosing method was set up in steps (Suski et al., 2008). Before dosing, triadimefon and corn oil lactescence were first mixed continually with a magnetic stirring apparatus. The lizards in treated groups (63 in each group) were euthanized at 0, 2, 6, 12, 24, 72, 120 and 168 h after lizards were dosed once. The lizards in treated and control groups were analysed in triplicate. To ensure the deglutition of triadimefon by lizards, we pushed the lavage needle very slowly in order to give them enough time to assimilate the pharmaceutical, and kept their mouths closed for a few minutes after dosing. In the previous research, a preliminary experiment at 90 mg/kg^{bw} was conducted which no difference clinical signs were found between the control and treated groups (Wang et al., 2014). So, in present study, the control group was only sacrificed at 168 h. The organs including liver, brain, kidney and fat in each group were weighted and collected in heparinized tubes separately. The blood in each sampling point was collected together in order to meet the minimum sample volume. All the samples were kept at −20 °C.

2.5. Chemical analysis

In order to identify the residue of TF and its metabolite in tissues and blood, the analytical method was developed for the treatments. The samples were analysed on liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) by chiral column (CHIRALPAK IC).

For analysis of the tissues and plasma, the samples were transferred to polypropylene centrifuge tubes and balanced to room temperature. After the tissues were mixed well, 10 mL of acetonitrile was added to the tubes and stirred for 3 min on a vortex mixer, then had an ultrasound for 20 min. The mixture was centrifuged at 8000 r/min for 5 min. The supernatants were transferred to a separatory funnel and the residue was re-extracted in the same way. After that, the extraction combined the extraction together and 20 mL n-hexane was added into separatory funnels for liquid-liquid partition to extract most of lipid. The upper layer of n-hexane was discarded, and the layer of acetonitrile was collected and evaporated to near dryness with a vacuum rotary evaporator at 35 °C. Until the extraction was dried under a gentle nitrogen stream, the residue was redissolved in 1 mL of acetonitrile/water (70:30) and filtered through a 0.22 μm filter into a sample vial for HPLC-MS/MS analysis.

HPLC was performed using Thermo ACCELA series (Thermo Electron Corporation, Hopkinson, MA) equipped with ACCELA Autosampler, ACCELA 600 pump, a 20 μL injection loop and a 2 μL flow cell. The mobile phase was a mixture of 70% acetonitrile and 30% water at the flow rate of 0.5 mL/min. TSQ QUANTUM ACCESS MAX was used for HPLC-MS/MS analysis (Thermo Electron Corporation, Hopkinson, MA). The signals were collected and processed with Thermo Xcalibur 2.2SP1.48 software. The analyses were performed in the positive mode with a 3500 V spray voltage, 250 °C vaporizer temperature, 32 psi sheath gas pressure and a 350 °C capillary temperature.

MS analyses were performed in the multiple reactions monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of triadimefon and its metabolites. After investigation of several dwell times in the 20–100 ms range, a dwell time of 20 ms per ion pair was used to maintain the high sensitivity of the analysis, and a number of data points across the chromatographic peak were required. The precursor and product ions of TF and TN with corresponding declustering potentials and collision energies are summarized in Supporting information (Table S2).

2.6. RNA-isolation and reverse transcription

54 lizards in treated and control groups (18 in each group) were euthanized at 168 h in triplicate and separated by gender for the analyse of relative gene expression. Liver and brain were collected and kept in RNA sample store solution (purchased from TIANGEN Biotech, Beijing). The total RNA was isolated from different tissues (liver, brain) of adult lizards using the Trizol reagent (TIANGEN Biotech, Beijing China) and dissolved in ribonuclease-free water. The samples were stored at −80 °C for reverse-transcriptase polymerase chain reaction.

The reverse transcription reaction mixtures with 10 μL of total RNA, 2 μL (0.05 μg/μL) of Oligo (dT) primers, and diethyl pyrocarbonate-treated water (a total of 50 μL). Then the samples were heated to 70 °C for 5 min and quickly chilled on ice (5 min). After cooling, 40 units of RNAsin (RNAase inhibitor; TIANGEN Biotech) were added to the mixture. The total mixtures were incubated for 50 min at 42 °C and heated to 95 °C for 5 min to inactivate the reverse transcription.

2.7. Quantitative real-time PCR

Real-time PCR was performed in a MX3005 P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 20 μL, consisting of the SYBR Green QPCR master mix, 500 nM forward primer and 500 nM reverse primer. The forward and reverse primers used in experiment and PCR products are listed in Supporting information (Table S3). The thermal cycle parameters used were: 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 40 s 54 °C and 30 s at 72 °C. All the samples were analysed in triplicate and the mean value of these triplicate measurements were used for calculations of mRNA expressions. Results were analysed according to delta-delta Ct method. The *cyp1a1*, *cyp3a1* and *hsd11β* mRNA expression was normalized for *β-actin* mRNA expression. Dissociation curve analysis was performed for each gene to check the amplification of untargeted fragments. Only one peak was observed for each amplification, indicative for the amplification of the target gene only. Gene expression data are presented as fold change relative to control group within the same treatment period.

2.8. Data analysis

Tissue index expressed as tissue weighting factor elsewhere was calculated according to the equation [tissue index = (tissues mass/lizard mass)× 100%]. The liver, brain, kidney, and fat were weighted after sampled.

All experimental data were presented as the mean ± SD and statistically analysed with SPSS 21.0 software. A *t*-test was used to check the variance and all data met the assumptions of equal variance. Significant differences between the control and treated groups were evaluated using one-way ANOVA at 95% confidence limits, *$p < 0.05$, **$p < 0.01$.

3. Results and discussion

3.1. Assay validation

The recovery assays of R-(-)-TF and S-(+)-TF in all samples were carried out to investigate the accuracy and precision of the method (Table 1). Spiked tissue samples were added with 0.5, 2 and 5 mg L^{-1} of enantiomers (R-(-)-TF or S-(+)-TF) and analysed according to the described methodology in three duplicates to check the recoveries (Table 1). After analysing the samples, recoveries were identified by computing the measured concentrations with the fortified concentrations. An external standard calibration curve was used to calculate the amount of TF enantiomers. The liner regression equations of each stereoisomer were constructed by plotting the peak area ratios against

Table 1
Summary of method recovery for triadimefon and triadimenol enantiomers from fortified lizard tissues and plasma.

Tissues	Fortification (mg/L)	Recovery rate (%)					
		R- (-)-TF	S- (+)-TF	TN-A1	TN-A2	TN-B1	TN-B2
Liver	0.50	85.4 ± 0.17	95.5 ± 1.50	89.2 ± 1.31	96.9 ± 3.02	105 ± 2.35	95.6 ± 2.51
	2.00	93.3 ± 0.24	93.3 ± 2.47	94.4 ± 3.59	89.6 ± 1.45	97.3 ± 2.55	98.6 ± 2.72
	5.00	89.4 ± 0.52	104 ± 2.89	95.5 ± 1.36	92.7 ± 2.53	87.6 ± 1.62	99.5 ± 3.15
Brain	0.50	96.4 ± 0.26	87.5 ± 2.17	96.5 ± 2.81	94.4 ± 2.15	95.4 ± 2.58	104 ± 2.26
	2.00	104 ± 1.05	92.5 ± 1.42	99.5 ± 2.85	94.6 ± 1.47	97.6 ± 1.53	105 ± 1.35
	5.00	104 ± 2.15	95.8 ± 2.41	104 ± 3.17	98.5 ± 2.19	97.4 ± 1.36	95.4 ± 2.73
Kidney	0.50	98.4 ± 2.41	106 ± 3.29	103 ± 2.51	97.5 ± 1.49	104 ± 2.48	93.9 ± 1.82
	2.00	107 ± 1.52	108 ± 3.26	107 ± 2.82	93.5 ± 2.41	100 ± 1.05	97.6 ± 1.74
	5.00	102 ± 2.48	115 ± 4.51	107 ± 1.73	96.6 ± 1.82	95.3 ± 1.48	98.4 ± 1.71
Fat	0.50	89.4 ± 1.52	94.5 ± 2.48	108 ± 1.21	98.4 ± 2.47	104 ± 3.26	92.5 ± 2.65
	2.00	95.4 ± 1.58	97.4 ± 2.51	105 ± 2.47	95.5 ± 1.39	89.5 ± 2.06	88.6 ± 2.41
	5.00	99.4 ± 2.08	103 ± 2.57	98.7 ± 3.21	96.3 ± 1.45	90.6 ± 2.01	95.7 ± 1.48
Plasma	0.50	106 ± 2.47	92.5 ± 3.02	99.7 ± 2.83	99.1 ± 2.51	95.5 ± 1.36	96.6 ± 2.59
	2.00	108 ± 2.58	86.9 ± 2.15	101 ± 1.49	98.6 ± 2.17	95.4 ± 2.51	97.7 ± 1.72
	5.00	111 ± 3.26	95.6 ± 3.06	96.5 ± 2.18	95.4 ± 3.61	98.4 ± 2.26	107 ± 2.46

The data were showed as the mean ± SD.

its designed concentrations, respectively. Each triadimefon enantiomer showed excellent linearity, with correlation of greater than 0.99. The recoveries of triadimefon enantiomers were measured in ranged from 85% to 115%.

3.2. Mortality, body weight/length and tissue indices

Adult lizards were administered R-(-)-TF or S-(+)-TF once for 168 h, respectively. During the exposure time, the two enantiomers of triadimefon did not appear to influence the body weight and length ($p < 0.05$) of the lizards between the control and treated groups (Table 2). There was also no mortality or treatment-related clinical signs in either group of lizards, which might due to the short duration of experiment that did not allow for much growth.

The liver, brain, kidney, and fat indices were detected and could be seen from the Table 2. The tissue index was used to evaluate hyperplasia, swelling, or atrophy of tissues induced by TF exposure. No significant difference was observed in kidney and fat ($p < 0.05$). The brain index in both treated-groups were dramatic lower than control which indicate the brain was more sensitive than other tissues after administrating with triadimefon. This was in consistent with the study of Di Renzo et al. (2011), larvae revealed serious craniofacial defects, bent forebrain, and abnormal hindbrain segmentation after triadimefon exposure. The liver index in S-(+)-TF group was significantly lower than control while the R-(-)-TF treated-group was not. This phenomenon may indicate that S-(+)-TF may have larger toxicity than R-(-)-TF on liver. The different toxicity effect of enantiomers on tissues directly proved the dissimilarity in degradation and metabolism when two chemicals enter into organs. Previous study has been reported the stereoselectivity of racemic triadimefon in *Rainbow Trout* microsomes (Kenneke et al., 2010).

3.3. Degradation of triadimefon in tissues and plasma

Degradation of traidimefon enantiomers in lizards was investigated under the tissues: liver, brain, kidney, fat and plasma. The concentrations of R-(-)-TF and S-(+)-TF in different tissue samples were exhibited over time in Figs. 2 and 3. In our study, the lizards were given triadimefon by oral lavage. When the enantiomers enter into esophagus, they first reach to the stomach, and a part of the pharmaceutical will be absorbed by it. So the initial concentration of enantiomers in the other tissues will be lower than 50 mg/kg^{bw}. After administration of triadimefon enantiomers, a rapid distribution phase and a slower elimination phase were observed among the tissues (Figs. 2 and 3). In tissues except fat, R-(-)-TF and S-(+)-TF reached to the maximal concentration within 12 h, while 24 h in fat. However, the maximal concentrations were dissimilar in the following order: liver, plasma, brain, fat, kidney in both of the two treated group. The enantiomers were extensively and fastly absorbed by tissues, while the elimination of triadimefon was not as fast as the absorption process. After 168 h, all the tissues, especially fat, still had slight amount of triadimefon residue. In addition, no significant selectivity in chiral biotransformation was observed in both the enantiomers during the experiment time, which was similar to the study of Konwick et al. (2006) in rainbow trout.

The pharmaceutical reached to the maximal concentration (Figs. 2 and 3), 4.68 and 3.82 mg/L in R-(-)-TF and S-(+)-TF, respectively only after 2 h in plasma, which was faster than others. And this phenomenon is in consistent with the research of Crowell et al. (2011), which may because of the pharmaceutical could pass through the transmembrane transport and diffuse across lipid membranes, ultimately enter into the blood circulation system. After that, the pharmaceutical will be carried to different tissues via the passive diffusion of plasma. Although the enantiomers fastly distributed in plasma, the concentration was not the highest among other organs. The lower concentrations in plasma may

Table 2
Body weight, length and tissue indices of lizards after 168 h exposure to traidimefon enantiomers.

Group	Concentration (mg/kg^{bw})	Body weight (g)	Body length (cm)	Tissues indices (%)			
				Liver	Brain	Kidney	Fat
Con.	–	3.63 ± 0.42	4.70 ± 0.31	3.64 ± 0.25	0.72 ± 0.34	0.63 ± 0.36	2.67 ± 0.25
R-(-)-TF	50	3.71 ± 0.31	4.65 ± 0.35	3.67 ± 0.28	0.61* ± 0.41	0.59 ± 0.25	2.57 ± 0.28
S-(+)-TF	50	3.68 ± 0.71	4.69 ± 0.40	3.43* ± 0.24	0.57* ± 0.21	0.61 ± 0.19	2.59 ± 0.14

The data were showed as the mean ± SD. Asterisks indicate significant difference from the control (*$p < 0.05$).

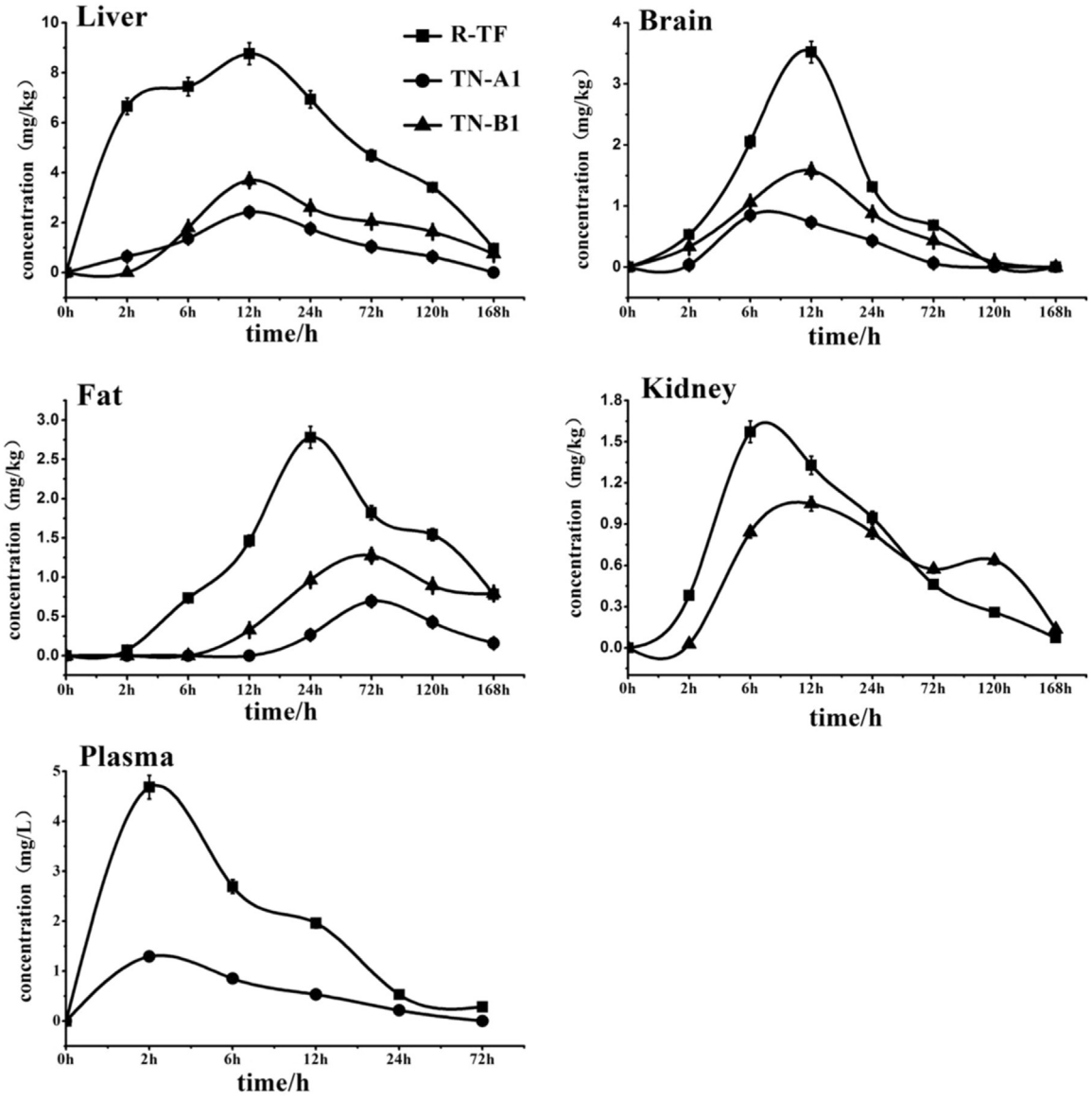

Fig. 2. Blood and tissues concentration-time profiles of R-(-)- triadimefon enantiomer and triadimenol following the administration of 50 mg/kg^{bw} in lizards (The data were showed as the mean ± SD).

be contributed to tissues absorption during the passive diffusion process and the small amount of plasma in lizards (less than 100 μL), which may lead to a limit ability for plasma to transport xenobiotic (Chen et al., 2016; Liu et al., 2010). In addition, the physicochemical properties of compounds such as lipophilicity and molecular weight may also effect the distribution in plasma (Lin, 2006).

Drugs exert their effects not only within plasma compartment, but in the defined target tissues. In our present study, the highest concentrations of the enantiomers were found in liver in both treated groups (8.77 mg/kg^{bw} and 6.78 mg/kg^{bw}, respectively), which might result from the extensive existing of metabolic enzymes in liver. The liver is better at oxidation, reduction and hydrolysis of the xenobiotic pollutants which can be easily excreted following further reaction. However, C_{max} time in S-(+)-TF (6 h) was faster than that in R-(-)-TF (12 h). Similar results were found in Kenneke et al. (2010) on rainbow trout and in Wang et al. (2014). Until the end of the experiment (7 days), there was still slight triadimefon residue (Figs. 2 and 3), which might indicate a slower elimination of triadimefon in liver. On the contrary, many of the triazoles (triadimefon included) were not detected past 2 days of depuration in aquatic organism which was much faster than lizards. This anomaly elimination process may due to the relatively simple monooxygenases enzyme systems and lower metabolic rate of lizards, and they could not be able to detoxify pesticides at the same high rate as warmblooded animals (Nagy et al., 1999). The high concentration and anomaly elimination process in liver may cause hepatocellular carcinoma through modes of action that are associated with the induction of liver enzyme activity, perturbed regulation of cell growth and death (Goetz and Dix, 2009). In addition, triazoles can reduce cholesterol concentration in liver which is regarded as an early sign of liver dysfunction that may be causally associated with the later pathological changes, and ultimately cell death, increased cell proliferation and tumors formation (Allen et al., 2006). As to seen above, liver may be the target organ for triadimefon enantiomers and seriously injured.

In our study, the concentrations in brain peaked to 3.52 mg/kg^{bw} in 12 h (R-(-)-TF) and 3.48 mg/kg^{bw} in 6 h (S-(+)-TF), then quickly dropped to 0.68 mg/kg^{bw} and 0.43 mg/kg^{bw} (Figs. 2 and 3), which was similar to the variety tracks in rats (Crowell et al., 2011). The lizards were observed to be restless with anxiety, hyperactivity in 6 h and 12 h after oral exposure. However, this phenomenon disappeared after 72 h, anorexia and lethargy instead. All the observation indicated the enantiomers might cause neurotoxicity to lizards and so as to brain

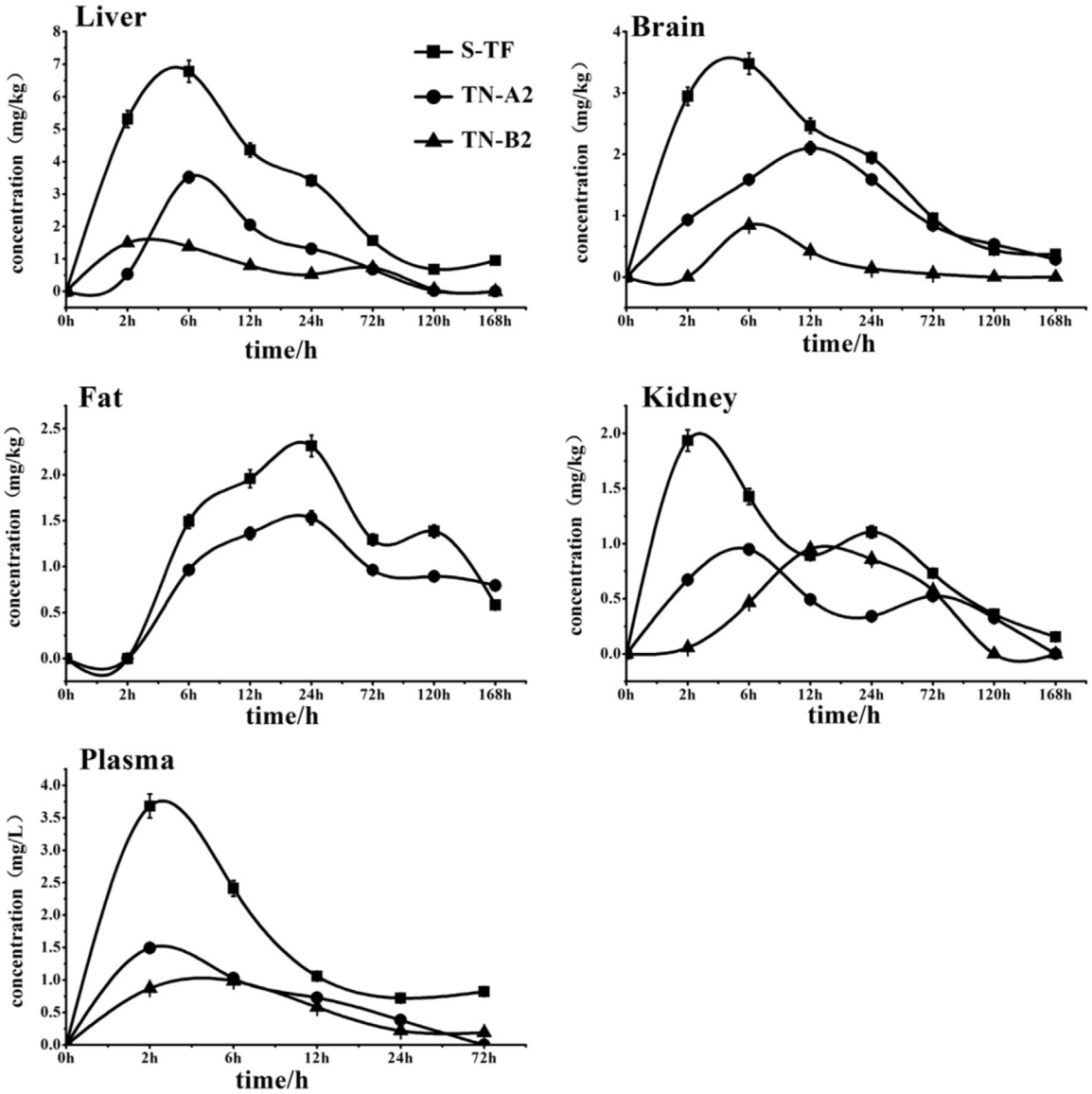

Fig. 3. Blood and tissues concentration-time profiles of S-(+)- triadimefon enantiomer and triadimenol following the administration of 50 mg/kg^{bw} in lizards (The data were showed as the mean ± SD).

was the target organ (Moser and Macphail, 1989). Our study was consisted with Crofton (1996), acute exposures to triadimefon affected the central nervous system catecholamines and induced a transient syndrome in rats that consists of hyperactivity and stereotyped behaviors. Another possibility has been pointed out by many reports that triazoles may do harm to the brain of organisms for it acts as an indirect dopamine (DA) agonist by binding to the dopamine transporter and increasing levels of synaptic DA (Reeves et al., 2004). The present study and all the researches enumerated above indicating the brain was seriously injured and was the mainly target tissue of triadimefon exposure.

The bioavailability in kidney (about 4%) was the lowest among the tissues in our study. Accumulating evidence has well documented that some pesticides are characterized by very low absolute oral bioavailability, but they can exert their pharmacological effects well. And this observation suggested that the distribution of their active ingredients in certain tissues or organs, particularly inside the active sites, may be contributory (Hao et al., 2007). Thus, to explain the low bioavailability, we hypothesis that the kidney has pharmacological effect after triadimefon exposure, nevertheless the active sites were less or be contributory by others. The fat possessed the worst degradation and elimination compare to others. The concentration in fat slowly increased from 0 h to 24 h and then slowly eliminated after 168 h. However, the final concentration in fat was the highest (about 0.6 mg/kg^{bw}) except liver. And it may be attributed to the high lipid content in fat and triadimefon is a kind of lipophilic compounds. What more Junnila et al. (2000) pointed out that high dose (115 mg/kg/day) triadimefon exposed to rats might be the indicative of increased conversion of choline to betaine in an attempt to cope with exposure, and beaine was typically though to act in a protective capacity in organs. According to this regulatory mechanism, the poor degradation and elimination in fat may due to the protective effect of betaine.

3.4. Formation of triadimenol in tissues and plasma

The carbonyl group of triadimefon is reduced to an alcohol and the resulting metabolite, triadimenol is a considerably more potent fungicide than triadimefon. Triadimenol has two chiral centres and four stereoisomers. And the stereoisomers exist as two diastereomers, termed A and B, each of which consists of a pair of enantiomers (Fig. 1). In order to evaluate the intensity and duration action of triadimefon, we not only need to analyse the absorption and distribu-

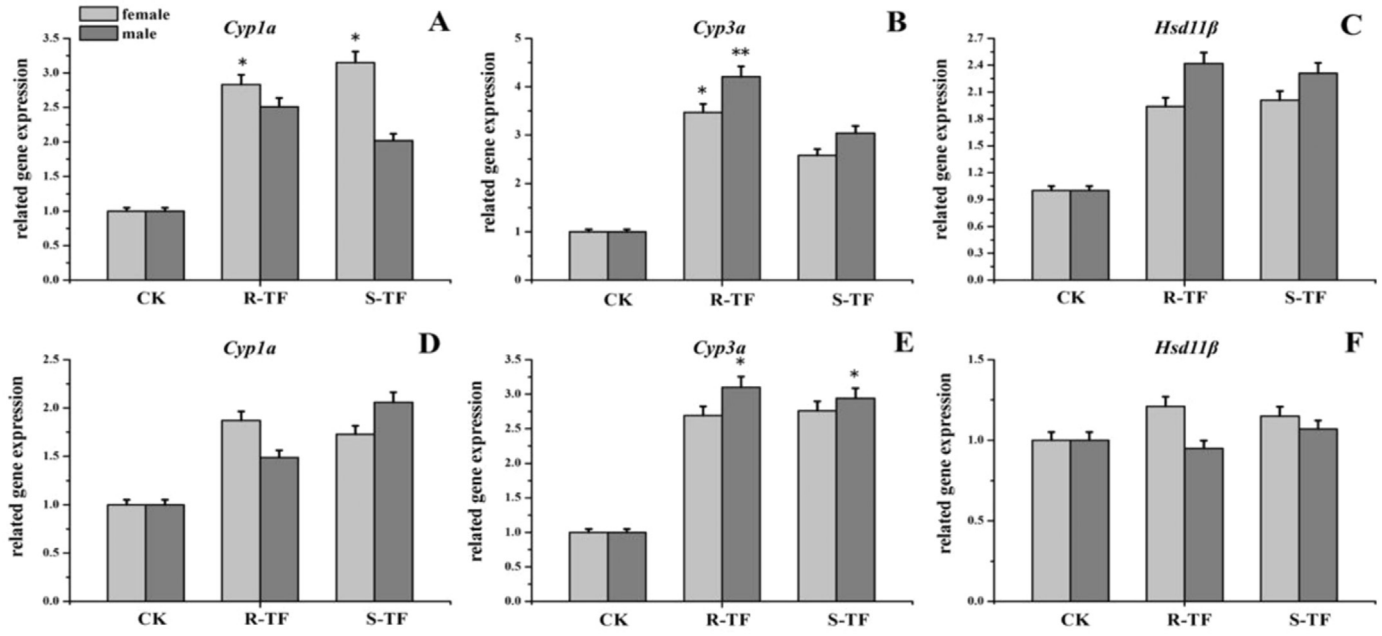

Fig. 4. Genes expression of *cyp1a*, *cyp3a*, and *hsd11β* in liver and brain following triadimefon enantiomers administration at 50 mg/kg^{bw} in lizards (A: *cyp1a* gene expression in liver; B: *cyp3a* gene expression in liver; C: *hsd11β* gene expression in liver; D: *cyp1a* gene expression in brain; E: *cyp3a* gene expression in brain; F: *hsd11β* gene expression in brain. The results were elevated as the relative ratio of the expression level of each mRNA to that of *β–actin*. The data were showed as the mean ± SD., *p < 0.05; **p < 0.01, relative to control.).

tion process in lizards, but also the formation of its metabolite. The decrease in the amount of parent compound was accompanied by the increase in the concentration of the metabolite. No other metabolites were analysed in present work.

After oral administration, R-(-)-TF was determined up to approximately 27.6%, 24.0%, and 25.0% transformed to TN-A1 in liver, brain, and fat (Table S4). There was no TN-A1 detected in kidney and plasma because of the lower concentration. The metabolites quickly appeared after 2-h exposure and reached to a plateau at about 12 h in most organs (fat excepted), then smoothly removed. The production of TN-B1 in R-(-)-TF were 42.1%, 61.8%, 45.8%, 49.2%, and 27.6% following the order of liver, brain, fat, kidney and plasma. The above data could indicate that, the main metabolite in R-(-)-TF was TN-B1 which processed a much higher proportion than TN-A1. This result was consistent with the study dominated by *Li, Y., et al., 2014* which apparently TN-A1 was the most preferentially produced isomer via the reduction reaction, and notably, which was more toxic than TN-B1 to *Dophnia magna* (Li et al., 2014).

In the S-(+)-TF treated group, the main production was TN-A2 which consisted about 40.0–70.0% of the metabolites and TN-B2 only about 20.0% in liver, brain, kidney, and plasma (Table S4). No TN-B2 was detected in fat because of the lower concentration. Similar discover was found in the study of Liang et al. (2013), the concentration of TN-A2 was much higher than TN-B2 and the former was the most toxic fungicide of the four stereoisomers. While a dissimilar pattern was observed earlier in studies of the stereoselective transformation of triadimefon to triadimenol in rainbow trout liver microsomes (Kenneke et al., 2010). After 480 min exposure of the microsomes to racemic triadimefon, isomer TN-B2 was at least twice as abundant as any of the other isomers, while TN-A2 was barely detectable. The conflicting results observed in aquatic organisms and reptiles may be associated with the differences in the animal models and dose of administration of TF used in the study. When triadimefon was treated in different media and even in different species of biota, each produced its own characteristic pattern of stereoisomer compositions of the productions of triadimenol (Li et al., 2014). There were reports about the large difference in metabolism and product stereoisomer relative abundances among higher level species (such as rats and human) (Mazur and Kenneke, 2008).

As to the above observation, it is reasonable to understand that the toxicity of triadimefon formulations with different stereoisomer ratios would likewise differ with species. And the four triadimenol stereoisomers are independent entities with respect to many of their biological properties. Each isomer may differ in toxicity to a variety of species and may be transformed to environment by different rates, even to give unique products. Therefore, in order to obtain data on actual environment and potential human effect, it is critical to use appropriate triadimenol formulation (that is commercial product) in exposure experiments designed (Garrison et al., 2011).

3.5. Quantitation of cyp1a1, cyp3a1 and hsd11β mRNA in the liver and brain of lizards

The degradation, elimination of R-(-)-TF and S-(+)-TF is dissimilar, which was also appeared in the formation of triadimenol. This phenomenon may be caused by different metabolic pathways in tissues. What's more, triadimefon and triadimenol were considered to have hepatotoxicity, neurotoxicity, and tumorigenesis which might be due to the different toxicity modes in tissues (Hurley et al., 1998). Gene expression profiles induced by pharmaceuticals in tissues had been used to identify potential mechanisms of toxicity (McMillian et al., 2004). Carbonyl containing xenobiotics may be primarily susceptible to NADPH-dependent cytochrome P450 (P450), which is widely spread in living organism (Mazur et al., 2009). Carbonyl reduction is also an important process for the normal function of various endogenous substrates, and many pesticides use carbonyl moieties in their mode of action. Carbonyl-reducing enzymes are mainly attributed to *11β*-hydroxysteroid dehydrogenase (*hsd11β*), a short-chain dehydrogenase-reductase and considered the primary microsomal reductase for carbonyl-containing pesticides (Oppermann, 2007). This study demonstrated the value of gene expression profiling in confirming a mode of action and underlying toxicity mechanisms of triadimefon.

In the present study, we analysed both the two pathways that might be contributed to the metabolite of triadimefon in brain and liver (Fig. 4). And the expression of these genes in *E. argus* were shown in Supporting information (Table S5). After 168 h exposure, the related gene expression of *cyp1a1*, and *cyp3a1* were significantly upregulated in both of liver and brain (Fig. 4A, B, D, and E) which was consisted with

the previous study by Ward et al. (2006) in liver from mice. A 5-fold higher was observed in the *cyp3a1* expression in liver (Fig. 4B, $p < 0.01$) which indicated it was the most sensitive gene (Barton et al., 2006). The alteration of *cyp1a1, cyp3a1* illuminated that exposure to triadimenfon motivated P450s activities which would get involved in the metabolism process of triadimefon. And P450s have been reported to associate with hepatic hypertrophy and it may be a reason to explain the hepatotoxicity caused by triadimefon (Wei et al., 2000). Meanwhile, Ward et al. (2006), also pointed out the enrichment of *cyp1a1* and *cyp3a1* might influence the retinoic acid metabolism pathway, which was a metabolite of vitamin A. Vitamin A regulated numerous physiological processed in normal cells including cellular growth, differentiation, apoptosis, immune response, and embryonic development (Ward et al., 2006). As to be seen above, activated retinoic acid metabolism is considered to be a side-effect which may cause abnormal physiological function inside the cells during the period of traidimefon metabolism. Therefor, P450 enzymes induction may be a key event in conazole-induced tumorigenesis. This information is essential to evaluate the potential threat of conazoles to human health and other organism in ecological environment.

The gene expression of *hsd11β* significantly increased (about 2-fold, $p < 0.05$) in liver after 168 h exposure, such appearance was widely existed in rat microsomal activities for the inhibition of glycyrrhetinic acid (GA) and *hsd11β* played an important role in the regulation of energy metabolism through the activation of endogenous glucocorticoids in tissues such as liver (Atanasov and Odermatt, 2007). While similar phenomenon did not happen in brain which may evaluate there was no *hsd11β* metabolism pathways in brain of lizards. Meyer et al. (2013) have indicated the isoform specific difference expression of *hsd11β* in rats and human. As a results, triazoles can act both as inducers and inhibitors of P450s, the metabolic activities and levels of metabolites reflected both of these influences in vitro and in vivo. And the inhibition could not be obtained at current study suggesting a possible role of other enzymes such as *11β*-hydroxysteroid dehydrogenases (Labrie et al., 1992).

4. Conclusion

In this study, we exposured triadimefon enantiomers respectively to lizards for 168 h and analysed the degradation, absorption, elimination of the two chiral enantiomers in liver, brain, fat, kidney, and plasma after a single administration (50 mg/kg^{bw}). The enantiomers distributed quickly in plasma after 2 h and eliminated entirely in 72 h. Liver and brain were the target tissues for the higher concentration and slower elimination rate. Kidney possessed the worst biotransformation and fat had a resist action to the enantiomers. The mainly metabolites in R-(-)-TF is TN-B1 and in S-(+)-TF is TN-A2 which testified the selective metabolism during the process of triadimefon to triadimenol. The quantitation of *cyp1a1*, *cyp3a1* and *hsd11β* mRNA level in lizards showed different metabolic pathways in the liver and brain. The up-regulation of *cyp1a1*, *cyp3a1* in liver and brain indicated that P450 enzymes made mainly contribution to the metabolism of triadimefon accompanily with the side-effect of enrichment the metabolite in retinoic acid which may cause tumorigenesis. The differentiation of *hsd11β* mRNA level in liver and brain implied the dissimilarity of R-(-)-TF and S-(+)-TF in degradation and elimination, so as to the formation of triadimenol. These analyses revealed functional categories of triadimefon response genes that indicate mechanisms and provide direction for further research on triazoles mechanisms of action.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.04.035.

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