Enantiomerization and stereoselectivity in bioaccumulation of furalaxyl in *Tenebrio molitor* larvae

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ABSTRACT

Furalaxyl is a chiral pesticide and widely used in modern agriculture as racemate mixture. The enantiomerization and enantioselective bioaccumulation by a single dose of furalaxyl to *Tenebrio molitor* larvae under laboratory conditions were studied using a high-performance liquid chromatography tandem mass spectroscopy method based on a ChiralPAK IC column. Our results showed that a significant enantiomerization (interconversion between R-enantiomer and S-enantiomer) was observed in *Tenebrio molitor* larvae under R- or S-furalaxyl exposure. Though the two furalaxyl enantiomers exhibited low-capacity of bioaccumulation in *Tenebrio molitor* larvae, bioaccumulation of *rac*-furalaxyl was enantioselective with a preferential accumulation of S-furalaxyl at 10 mg/kg dosage exposure. In addition, enantiomerization and enantioselective degradation of the two enantiomers was not observed in wheat bran. These results showed that enantioselectivity of furalaxyl enantiomers was an important process combined with degradation, metabolism and enantiomerization in organisms.

1. Introduction

About 40% of currently registered pesticides are chiral and the proportion is expected to increase as compounds with more complex structures are introduced (Lewis et al., 1999; Garrison, 2006; Ye et al., 2010). Chiral pesticides have different bioavailability, distribution, metabolic and excretion behavior due to enantioselectivity of enantiomers (Maier et al., 2001). Consequently, attention has recently been focused on the enantioselective behavior of chiral pesticides in the environment (Müller and Kohler, 2004; Genualdi et al., 2009; Xu et al., 2009). However, most of the chiral pesticides have been manufactured and applied to agro-ecosystems as racemate mixture, whereas considering only the racemates does not sufficiently describe its actual environmental fate and ecological risks. In order to decrease application rates and thus release smaller amounts of chemicals into the environment, adopting biologically active enantiopure products in place of racemic products was proposed by green chemistry advocates.

Some chiral pesticides are configurationally unstable and undergo enantiomerization (see Table S1) (Liu et al., 2005; Qin and Gan, 2007; Nillos et al., 2009; Li et al., 2011; Sun et al., 2013; Gao et al., 2013, 2014; Wang et al., 2014, 2017). It is important to obtain the information on the stability of individual enantiomers of a chiral pesticide when

assessing the possibility of developing a single enantiomer as a commercial pesticide (Diao et al., 2009).

Furalaxyl is a systemic acetanilide fungicide that is used for prevention and treatment of plant diseases caused by pathogens of the *Oomycota* (Cabras et al., 1985; Price and Fox, 1986; Rouchaud et al., 1989; Zhang et al., 2012). The compound consists of a single pair of enantiomers with R- and S-configuration (Fig. 1) and is marketed as a racemate mixture. Although furalaxyl has been widely used in modern agriculture, enantioselective behavior of the furalaxyl enantiomers in organisms has not been documented, especially in insects.

Insects play a vital role in the food chain and the global eco-system of the planet (Wiegert and Evans, 1967). Pesticides would be indirectly transferred to higher trophic levels through predation insects and food chain while these chemicals are widely abused in environment. *Tenebrio molitor* (*T. molitor*) is an important economic pest in stored grain products. Their larvae are used as a model pest in the fields of environmental toxicology, genetics and so on (Kostaropoulos et al., 2001; Prabhakar et al., 2007). In our previous studies, enantioselective processes have been observed in *T. molitor* under chiral pesticides exposure such as metalaxyl, benalaxyl, diniconazole and epoxiconazole. To further understand the enantioselectivity of chiral pesticides in organisms, it is necessary to investigate the effect of the furalaxyl enantiomers on *T.*

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Fig. 1. Structures of furalaxyl enantiomers.

molitor comprehensively.

In this study, an effective method for stereoselective determination of furalaxyl residues in *T. molitor* larvae and wheat bran using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was developed. Enantiopure furalaxyl enantiomers were employed to elucidate potential enantiomerization and enantioselective bioaccumulation in *T. molitor*. The main goal of the present study was to emphasize the importance of enantiomerization in the environmental fate and behavior of chiral pesticides and to fill up the data gaps to accurately evaluate the potential effects of the furalaxyl enantiomers in organisms.

2. Material and methods

2.1. Chemicals and reagents

Standard *rac*-furalaxyl (> 98.0% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Enantiopure enantiomers standard of R- and S-furalaxyl were prepared via chiral synthesis methods. Methanol (HPLC grade), n-hexane (HPLC grade), acetonitrile (HPLC grade), alumina-N-solid-phase extraction (alumina-N-SPE) cartridge and filter membrane (pore size, aperture: 0.22 μ m, diameter: 13 mm) were purchased from Dikma Company (Beijing, China). Pyridine, trifluoromethanesulfonic anhydride, methyl (S)-(-)-lactate, methyl (R)-(+)-lactate, 2, 6-dimethyl aniline, 2-furoyl chloride, sodium bicarbonate, toluene, dichloromethane(CH_2Cl_2), ether, ethyl acetate, acetone, acetonitrile (analytical grade) and anhydrous sodium sulfate (Na_2SO_4) were purchased from Beijing Chemical Reagent Co. Ltd (Beijing, China).

2.2. HPLC-MS/MS conditions

HPLC was performed using Thermo ACCELA series (Thermo Electron Corporation, Hopkinson, MA) equipped with ACCELA Autosampler. Enantiomers were separated on a ChiralPAK IC [cellulose-tris-(3, 5-dichlorophenyl)-carbamate]. The mobile phase was a mixture of acetonitrile/water (80/20, v/v) with a flow rate of 0.4 mL/min. Chromatographic separation was conducted at 20 $^{\circ}\text{C}$ and the injection volume was 1 μ L.

TSQ QUANTUM ACCESS MAX was used for LC-MS/MS analysis (Thermo Electron Corporation, Hopkinson, MA). The optimized major working parameters were provided in support information (Table S2). For furalaxyl, transition m/z 302 $\rightarrow$ 95 was used for confirmation, m/z 302 $\rightarrow$ 242 was used for quantification, and collision energies were 30 eV and 16 eV, respectively. The signals were received and processed with Thermo Xcalibur 2.2 SP1.48 software.

2.3. Chiral synthesis of the two enantiomers of furalaxyl

Given the difference activity between R- and S-furalaxyl in biochemical effects, obtaining enantiopure enantiomers standards was necessary. The two enantiomers of furalaxyl were prepared via modified chiral synthesis methods according general procedure (Effenberger

et al., 1986; Palla et al., 2001).

Briefly, pyridine (1.42 mL, 17.6 mmol, 1.1 equiv) in 30 mL of CH_2Cl_2 was cooled to 0 $^{\circ}\text{C}$, trifluoromethanesulfonic anhydride (2.8 mL, 16.2 mmol, 1.05 equiv) was added, and the resulting mixture was stirred over 10 min. Methyl (S)-(-)-lactate (1.53 mL, 16.0 mmol, 1 equiv) was added dropwise. The reaction mixture was stirred at 0 $^{\circ}\text{C}$ for 30 min and then warmed to room temperature. The mixture was filtered, and the filter cake was washed with ether. The filtrate was evaporated in vacuo (< 25 $^{\circ}\text{C}$), and the residue was triturated with ether and filtered. After evaporating the solvent, the residue was dissolved in 20 mL of dichloromethane and 2, 6-dimethyl aniline (1.97 mL, 16.0 mmol, 1 equiv) was added. The reaction solution was stirred at room temperature overnight. Removal of the solvent in vacuo followed by flash chromatography gave the light yellow oil which N-xylyl-D-methyl alaninate (2.76 g).

Sodium bicarbonate (0.5 g, 6 mmol, 1.1 equiv) was added to a solution of N-xylyl-D-methyl alaninate (1.12 g, 5.5 mmol, 1 equiv) in 30 mL of toluene, cooled to a temperature ranging from 5 to 10 $^{\circ}\text{C}$, and subsequently 2-furoyl chloride (0.93 g, 6 mmol, 1.1 equiv) was slowly added dropwise. After 4 h at room temperature, the above solution was washed with saturated sodium chloride and the organic phase was evaporated in vacuo. The obtained crude product was crystallized with hexane to give 1.2 g of white crystalline solid alaninate with an enantiomeric R/S ratio = 97 (yield 68%).

Synthesis of S-furalaxyl with Methyl (R)-(+)-lactate as a starting material followed the same experimental procedure. The structures of R- and S-furalaxyl were confirmed by comparing with mass spectrum of furalaxyl standard. The optical purities of R-enantiomer and S-enantiomer were 98.0% and 98.4%, respectively.

2.4. *T. molitor* larvae

T. molitor larvae were purchased from Beijing Jie Matt Biological Technology Co., LTD and were reared in ventilated terraria (25 $\times$ 15 $\times$ 20 cm). A layer of wheat bran covered the terrarium floor. The population was kept at 25 $^{\circ}\text{C}$, 80% humidity and the light cycle adopted was 16 h light and 8 h dark.

2.5. Wheat bran formulation and *T. molitor* larvae exposure

To ensure that the *rac*-furalaxyl was dispersed uniformly in the 100 g dry weight of wheat bran, we did the procedure in steps (dilution spike). First, *rac*-furalaxyl was dissolved in acetonitrile to make a stock solution at a concentration of 1000 mg/L. Next, 1 mL of stock (1000 mg/L) was dissolved into 50 mL of acetone. Thereafter, 100 g of wheat bran was soaked in the 50 mL of acetone solution of *rac*-furalaxyl, followed by drying in fuming cupboard overnight. The final concentration of *rac*-furalaxyl in wheat bran was 10 mg/kg. The wheat bran fortified with the enantiopure R-furalaxyl and S-furalaxyl followed the same experimental procedure.

In this experiment, the *T. molitor* was used with the larval stage (0.1 g mean body weight). Moulting or pupating larvae were excluded from the experiment. Before the larvae were introduced, they were fed with unfortified wheat bran for 1 week to acclimate. A piece of paper resting on top of the bran provided cover for the worms. Thirty grams of *T. molitor* larvae were exposed to the chemical in each ventilated terrarium containing 7 g of contaminated wheat bran. For exposure experiment, each ventilated terrarium containing 7 g wheat bran which were contaminated by R-, S- or *rac*-furalaxyl separately exposed to 30 g wet weight of *T. molitor* larvae, respectively. Food was renewed once a week. All of the plastic terraria were placed in the dark in an environmental chamber to keep consistent environmental condition.

For the uptake experiment, 1 g of *T. molitor* larvae (approximately 8 to 10 worms) and 0.5 g of wheat bran were collected at 1, 3, 9, 24, 72, 168, 336 and 504 h exposure, and three parallel samples were prepared at each time point. Wheat bran on the surface of worms was removed by

Fig. 2. Representative HPLC-MS/MS chromatogram of the expections of *rac*-furalaxyl enantiomers in (a) *T. molitor* larvae and (b) wheat bran after 21 days of exposure. Flow rate = 0.4 mL/min, ultrapure water: acetonitrile = 20: 80 (v/v).

a sieve, and the worms were frozen at -20°C in 50 mL of polypropylene centrifuge tube. The samples of wheat bran collected from uptake experiment were analyzed the enantioselective degradation of *rac*-furalaxyl in wheat bran, simultaneously. For the degradation assay of *rac*-furalaxyl in *T. molitor* larvae, the method was carried out as following. The *T. molitor* larvae were partially transferred to uncontaminated wheat bran and collected after 0, 0.5, 1, 1.5, 2 days. Three parallel samples were prepared at each time point. Wheat bran samples from each plastic terrarium were also stored at -20°C . All experiments were performed at $25 \pm 0.5^{\circ}\text{C}$.

2.6. Analysis of Furalaxyl Residue

Wheat bran pretreatment: 15 mL of acetonitrile was added to a 50 mL polypropylene centrifuge tube containing 0.5 g wet weight of incubated wheat bran sample. Next, the tube was shaken for 3 min on a vortex mixer, exposed to ultrasonic vibration for 10 min and then centrifuged at 3000 r/min for 5 min. The solvent extract was transferred to a new tube. The remaining material was extracted again and the two extracts combined. The combined extract was filtered through anhydrous sodium sulfate (5 g) into a pear-shaped flask and evaporated to dryness by vacuum rotary evaporator at 35°C . A alumina-N-SPE cartridge was used to clean up other interfering substances. The cartridge was preconditioned by rinsing with 5 mL of ethyl acetate followed by 5 mL of n-hexane and equilibrated with 5 mL of ethyl acetate/n-hexane (1:4). The dry extract was dissolved in 2 mL of 20% ethyl acetate in n-hexane, and then, the solution was passed through the alumina-N-SPE cartridge. The cartridge was eluted with an additional 8 mL of 1:3 ethyl acetate/n-hexane. The combined 10 mL of eluate was collected in a pear-shaped flask and evaporated to dryness in vacuo at 35°C and the dry extract was redissolved in 1 mL acetonitrile and passed through a filter membrane.

The pretreatment method for the *T. molitor* larvae is essentially the same as the wheat bran with a few minor changes. The wet weight of *T. molitor* larvae sample was one gram. For cleanup (fat destruction), 3×10 mL of n-hexane was added for liquid-liquid partition to extract most of the lipid. The upper layer of n-hexane was discarded, and the layer of acetonitrile was evaporated to dryness by vacuum rotary evaporator. An alumina-N-SPE cartridge was used to clean up other interfering substances. The cartridge was preconditioned by rinsing with 5 mL of ethyl acetate followed by 5 mL of n-hexane and equilibrated with 10 mL of 1:5 ethyl acetate: n-hexane. The sample of dry extract was dissolved in 2 mL of 25% ethyl acetate in n-hexane, and then, the solution was passed through the alumina-N-SPE cartridge. The cartridge was eluted with additional 8 mL of 1:3 ethyl acetate: n-hexane.

2.7. Data analysis

The degradation rate constant of the furalaxyl enantiomers in wheat bran and *T. molitor* larvae was estimated using the pseudo-first-order kinetic expression (1):

$$\ln\left(\frac{C}{C_0}\right) = -kt \quad (1)$$

The half-life ($t_{1/2}$) was determined using regression plots of $\ln(C/C_0)$ versus time (t):

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

Wherein C_0 is the initial concentration of enantiomer (mg/kg), C is the concentration (mg/kg) at time t (hours), and k is the degradation rate constant.

The enantiomeric fraction (EF) was employed to measure the enantioselective degradation or bioaccumulation of *rac*-furalaxyl in wheat bran and *T. molitor* larvae. The EF values defined range from 0 to 1, with $\text{EF} = 0.5$ representing the racemic mixture (Geus et al., 2000). EF was expressed as follows:

$$\text{EF} = \frac{[S]}{[R] + [S]} \quad (3)$$

Bioaccumulation factor (BAF) was employed to further illustrate the enantioselective bioaccumulation of the furalaxyl enantiomers. BAF is a function of relative sorptive capacities of organism versus the surrounding food, and it is defined as:

$$\text{BAF} = \frac{C_T}{C_{WB}} \quad (4)$$

C_T is the concentration of compound in *T. molitor* larvae tissue and C_{WB} is the concentration of compound in wheat bran.

3. Results and discussion

3.1. Assay validation

The analysis results of furalaxyl in *T. molitor* larvae and wheat bran showed that R- and S-furalaxyl were well separated with no interfering peaks through validated HPLC-MS/MS method (Fig. 2). Calibration curves were linear between 0.01 and 1.0 mg/L, with correlation coefficients (r^2) for R- and S-furalaxyl of 0.998 and 0.999, respectively. Uncontaminated *T. molitor* larvae wheat bran samples were fortified with 0.1, 0.5, and 1 mg/kg of the two furalaxyl enantiomers. The

Fig. 3. Enantiomerization of enantiopure R-furalaxyl (a) and enantiopure S-furalaxyl (b) in *T. molitor* larvae. Values are represented as mean $\pm$ SD ($n = 3$ at each time point).

Fig. 4. Representative HPLC-MS chromatogram of the expectations of (a) enantiopure R-furalaxyl in wheat bran and (b) enantiopure S-furalaxyl in wheat bran after 21 days of exposure. Flow rate = 0.4 mL/min, ultrapure water: acetonitrile = 20:80 (v/v).

Fig. 5. (a) Concentrations of *rac*-furalaxyl enantiomers in *T. molitor* larvae. (b) Enantiomeric fraction (EF) of *rac*-furalaxyl residue in *T. molitor* larvae and wheat bran. Values are represented as mean $\pm$ SD ($n = 3$ at each time point).

average recoveries for both enantiomers ranged between 86% and 94% in *T. molitor* larvae and between 90% and 95% in wheat bran. The relative standard deviations (RSDs) ranged from 2.6% to 7.2% in *T. molitor* larvae, and 2.1–3.7% in wheat bran. Based on the peak-to-peak signal to noise ratio of 3, the limit of detection of the method was 0.01 mg/kg both in *T. molitor* larvae and wheat bran.

3.2. Enantiomerization in *Tenebrio molitor* larvae

Wheat bran contaminated with enantiopure R-furalaxyl or S-furalaxyl was fed to larvae to evaluate the enantiomerization of furalaxyl enantiomers in *T. molitor* larvae. For the enantiopure R-furalaxyl, obvious enantiomerization from R to S was observed at every exposure time (Fig. 3a). The concentration of R-furalaxyl rapidly increased

Table 1
Calculated BAF for enantiomers of furalaxyl.

	Exposure time (hours)							
	1	3	9	24	72	168	336	504
BAF-S-furalaxyl	0.027 ± 0.002	0.039 ± 0.004	0.058 ± 0.011	0.049 ± 0.009	0.035 ± 0.014	0.045 ± 0.012	0.028 ± 0.007	0.029 ± 0.006
BAF-R-furalaxyl	0.021 ± 0.001	0.032 ± 0.007	0.042 ± 0.010	0.035 ± 0.015	0.028 ± 0.008	0.039 ± 0.011	0.025 ± 0.013	0.026 ± 0.009

during 9 h because contaminated wheat bran was uptake by *T. molitor* larvae. From 9 to 72 h, R-furalaxyl affected the larvae feeding ability resulting in the concentration rapidly decreased. Recovery of feeding capacity contributed to concentration increased again after 72 h exposure. The highest level (0.30 mg/kg) of R-furalaxyl was reached at the time of 9 h. The highest concentration of transformed S-furalaxyl reached 0.051 mg/kg, simultaneously. The concentrations of R-furalaxyl and S-furalaxyl decreased after 9 h exposure and reached a relatively steady-state level in 168–504 h.

Enantiomerization of enantiopure from S to R-furalaxyl in *T. molitor* larvae was also observed, and the trend of the concentration change of S-furalaxyl was similar to that of R-furalaxyl (Fig. 3b). Conversely, the highest concentration of transformed R-furalaxyl (0.076 mg/kg) appeared 24 h. These results suggest that furalaxyl enantiomers initially underwent significant enantiomerization at a fast rate in *T. molitor* larvae. In the later phase of exposure, the extent of enantiomerization was reduced due to the continuous uptake of major enantiomer and degradation of minor enantiomer. However, enantiomerization of the two furalaxyl enantiomers was not observed in the wheat bran during 21 days (Fig. 4).

Enzyme-mediated enantiomerization has been extensively studied for pharmaceuticals (Hutt and Caldwell, 1983; Testa et al., 1993), amino acids, and sugars (Friedman, 1999) with respect to its mechanism. The inversion of stereochemistry in a chiral chemical is generally catalyzed by racemases or epimerases through cleavage of the methane C–H bond adjacent to a carbonyl group followed by its reformation or successive oxidation-reduction at a chiral center (Tarrier et al., 1999). The chiral carbon of furalaxyl holds an electron-withdrawing carbonyl group and a conjugated acetanilide functional group (Fig. 1). Therefore, the proton on the chiral center is relatively acidic and is thus easily lost to create an intermediate carbanion. Under protic environment, reprotonation regenerates the parent compound from either the top or the bottom face of the anion, thus interconverts enantiomers.

3.3. Enantioselective bioaccumulation in *Tenebrio molitor* larvae

Enantioselective bioaccumulation in *T. molitor* larvae was investigated with *rac*-furalaxyl. The concentrations of the two furalaxyl enantiomers in *T. molitor* larvae were determined. Initially, rapid uptake of the two furalaxyl enantiomers was observed in *T. molitor* larvae (Fig. 5a). Concentrations of the two enantiomers both reached the highest level in 9 h. A rapid decrease of concentrations was observed after 9 h. Finally, the concentrations tended to stability after 14 days. A significant difference was observed between the concentrations of two enantiomers in *T. molitor* larvae tissue at all sample points, with concentration of the S-furalaxyl higher than that of the R-furalaxyl (Fig. 5a). In addition, EF values deviated from 0.5 in the bioaccumulation experiment during 21 days of exposure and reached the highest level in 24 h and finally maintained approximately 0.53 (Fig. 5b). That is, the bioaccumulation of furalaxyl in *T. molitor* larvae was expected to be an enantioselective process with the preferential bioaccumulation of S-furalaxyl.

To further illustrate the bioaccumulation of the furalaxyl enantiomers, the BAF was employed. There was a significant difference in the BAF between the two enantiomers of furalaxyl (Table 1). The BAF

value of the S-enantiomer was larger than that of the R-enantiomer once more suggesting that the S-enantiomer is likely to be preferentially accumulated in *T. molitor* larvae. Both the furalaxyl enantiomers exhibited the low capacity of bioaccumulation in *T. molitor* larvae, which could be attributed to a small amount of furalaxyl taken up by *T. molitor* larvae, the low lipophilicity of furalaxyl and degradation at a high rate. Thus, enantiomerization could be considered as one of the most important causes which lead to enantioselective bioaccumulation of furalaxyl in *T. molitor* larvae. Given the enantioselective degradation and enantiomerization of furalaxyl in *T. molitor* larvae, the behavior of each enantiomer of racemate mixture should be ideally examined with enantiomerization to more correctly assess the safety in ecotoxicology on the most sensitive species. Enantioselectivity could make the environmental behavior and ecological effects of racemate mixture complicated (Diao et al., 2009).

3.4. Enantioselective degradation in wheat bran or *Tenebrio molitor* larvae

Contaminated wheat bran samples were evaluated the enantioselective degradation of *rac*-furalaxyl in wheat bran. Concentrations of R- and S-furalaxyl almost unchanged in wheat bran. The final concentrations of R- and S-furalaxyl were 4.78 ± 0.52 mg/kg and 4.77 ± 0.27 mg/kg after 504 h exposure. Because enantiopure R- and S-furalaxyl did not undergo enantiomerization in wheat bran, the decrease in the furalaxyl enantiomers must result from non-enzymatic reaction such as abiotic isomerism and volatilization. The EF values of two enantiomers in wheat bran were not obviously observed to deviate from 0.5 in 21 days (Fig. 5b). This result indicated that *rac*-furalaxyl did not enantioselective degrade in wheat bran. It has been demonstrated in other acetanilide fungicide such as metalaxyl and benalaxyl (Gao et al., 2013, 2014). In addition, the result of degradation assay of *rac*-furalaxyl indicated that R- and S-furalaxyl were faster degraded in *T. molitor* larvae (Table S3). The half-life ($T_{1/2}$) of R- and S-furalaxyl in *T. molitor* larvae were 1.47 day and 1.23 day, respectively. Although EF values of two enantiomers were slightly observed to deviate from 0.5 in *T. molitor* larvae, it had not been attributed to enantioselective degradation due to faster degradation and enantiomerization (Fig. S1). That is, *rac*-furalaxyl might not enantioselective degrade in *T. molitor* larvae also.

4. Conclusion

In our experiment, the enantiomerization and enantioselective bioaccumulation of furalaxyl enantiomers in *T. molitor* larvae from wheat bran were investigated by HPLC-MS/MS based on a ChiralPAK IC. The results indicated that exposure of enantiomers R-furalaxyl and S-furalaxyl in *T. molitor* larvae revealed significant enantiomerization with formation of the R enantiomer from the S enantiomer, and vice-versa. Enantiomerization was not observed in wheat bran during the period of 21 days. In addition, there was an obvious enantioselective bioaccumulation in the *T. molitor* larvae with a preferential accumulation of S-furalaxyl. This suggests that enantiomerization could make the biological effect of racemate mixture complicated with effective enantiomer transformed to the inactive enantiomer. Enantiomerization should be considered to be an important factor influencing the enantioselective behaviors of chiral pesticides in organisms in addition to

actual degradation and metabolism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2017.07.041>.

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