Title

Acute toxicity, bioconcentration, elimination, action mode and detoxification metabolism of broflanilide in zebrafish, *Danio rerio*

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ABSTRACT

Broflanilide, a novel meta-diamide insecticide, shows high insecticidal activity against agricultural pests and is scheduled to be launched onto the market in 2020. However, little information about its potential toxicological effects on fish has been reported. In this study, broflanilide showed low toxicity to the zebrafish, *Danio rerio*, with LC_{50} > 10 mg L^{-1} at 96 h and also did not inhibit GABA-induced currents of the heteromeric Drα1β2Sγ2 GABA receptor. Broflanilide showed medium bioconcentration level with a bioconcentration factor at steady state (BCF_{ss}) of 10.02 and 69.40 in *D. rerio* at 2.00 mg L^{-1} and 0.20 mg L^{-1}, respectively. In the elimination process, the concentration of broflanilide rapidly decreased within two days and slowly dropped below the limit of quantification after ten days. In the 2.00 mg L^{-1} broflanilide treatment, CYP450 activity was significantly increased up to 3.11-fold during eight days. Glutathione-*S*- transferase (GST) activity significantly increased by 91.44% within four days. In conclusion, the acute toxicity of broflanilide was low, but it might induce chronic toxicity, affecting metabolism. To our knowledge, this is the first report of the toxicological effects of broflanilide on an aquatic organism, which has the potential to guide the use of broflanilide in the field.

***Keywords*:** Bioconcentration factor; CYP450; Glutathione-*S*-transferase; MCI-8007; Metabolism.

Abbreviations

a.i., active ingredient; BCF, bioconcentration factor; BCFss, BCF at steady state; BSA, bovine serum albumin; CarE, carboxylesterase; cRNA, capped RNA; CYP450, cytochrome P450; DTW, dechlorinated tap water; EC_{50}, median effective concentration; GABAR, γ-aminobutyric acid receptor; GST, glutathione-S-transferase; IRAC, Insecticide Resistance Action Committee; LOQ, limit of quantification; MCPA, 2-methyl-4-chloro-phenoxyacetic acid; NaCl, sodium chloride; PBS, phosphate buffered solution; PMSF, phenylmethylsulfonyl fluoride; PSA,

primary/secondary amine; RSD, relative standard deviation; RT, room temperature; SECP, Standard for Environmental Safety Evaluation of Chemical Pesticides; SOS, standard oocyte saline; SC, suspension concentrate; TC, technical material.

1. Introduction

Emergence and rapid evolution of insecticidal resistance is a worldwide problem, and exploring novel insecticides with unique modes of action is always expected in agricultural production (Casida 2009). Broflanilide (**Fig. A.1A**) is a novel meta-diamide insecticide derived from flubendiamide (Katsuta et al., 2019). It shows highly insecticidal activity against agricultural pests, such as Lepidopteran, Thysanopteran and Coleopteran (El Qacemi et al., 2019; Qi et al., 2017; Wang et al., 2018a; Wang et al., 2018b; Xu et al., 2019), and is therefore of considerable interest (Casida 2015; Casida and Durkin 2015). For example, broflanilide has good activity on the rice stem borer, *Chilo suppressalis* (Walker), and the rice leaf folder, *Cnaphalocrocis medinalis* Guenée, in either indoor or field assays. In addition, the LD_{50} values of broflanilide against *C. suppressalis* and *Cn. medinalis* were 1.49- and 1.61-fold higher than those of flubendiamide, respectively (Xu et al., 2019). Compared with chlorantraniliprole (LC_{50}, 0.056 μg mL^{-1}), broflanilide has the same level of activity (LC_{50}, 0.042 μg mL^{-1}) on the diamond back moth, *Plutella xylostella* (Linnaeus) (Qi et al., 2017). In the field, broflanilide shows hardly any side-effects on natural enemies including spiders (e.g. *Erigonidium graminicolum*, *Pirata subpiraticus*, *Singa pygmaea*, and *Theridion octomaculatum*) and the plant bug, *Cyrtorhinus lividipennis* Reuter (Xu et al., 2019). For instance, after 3-7 days' treatment with a dose of 18.75-30.00 g hm^{-2} paddy, MCI-8007, which is the 5% suspension concentrate (5% SC) of the formulated product of broflanilide, may inhibit the occurrence of spiders with a corrected population decline rate less than 38.10%, whilst having little effect on the occurrence of *C. lividipennis*. However, the densities of spiders and *C. lividipennis* were able to return to normal levels after 14 days (Xu et al., 2019). Moreover, broflanilide leaves no residue in rice and very low levels of residue in soil, rice plants and rice husks in paddy fields due to its short half-life period (Xu 2018). Therefore, broflanilide has been scheduled to be launched onto the market in 2020 by Mitsui Chemicals Agro, Inc. (MCAG, Tokyo, Japan) and BASF (Florham Park, NJ) (Katsuta et al., 2019) and. billions of market could be clearly foreseeable.

It is worth noting that the use of some pesticides, such as fipronil, has been limited due to its high risk to aquatic organisms (Agriculture 2017). However, the potential risk of broflanilide to fish has yet to be determined in detail. Due to its easy availability, short life-cycle, sensitivity to chemicals, genetically high similarity to human and well annotated metabolites, the zebrafish *Danio rerio* (Hamilton), a tropical freshwater fish, has become a useful model for developmental and toxicological studies (Howe et al., 2013; Lawrence 2007; Okuda et al., 2008). For example, *D. rerio* has been used in toxicological studies of many insecticides, including chlorpyrifos, fluralaner and imidacloprid (Gómez-Canela et al., 2017; Ge et al., 2015; Jia et al., 2018). Meanwhile, the exposure of insecticides can affect the activities of detoxification enzymes, such as cytochrome P450 (CYP450), glutathione-*S*-transferase (GST) and carboxylesterase (CarE), which play an important role in the metabolism of exogenous substances in many organisms including *D. rerio* (Glisic et al., 2015; Jia et al., 2018; Jones et al., 2010) and are considered as biomarkers for exposure to exogenous compounds, (e.g. insecticides, chemicals) (Sinaei and Rahmanpour 2013; Velki et al., 2019; Velki et al., 2017). Therefore, in the present study the acute toxicity, bioconcentration, elimination, and detoxification responses to broflanilide in *D. rerio* were investigated in order to evaluate its ecological effects on aquatic organisms.

Broflanilide acts on a unique binding site of the insect γ-aminobutyric acid receptor (GABAR) (Casida and Durkin 2015) and is defined as a member of the 30th group (GABA-gated chloride channel allosteric modulator) insecticide by the Insecticide Resistance Action Committee (IRAC 2019). Broflanilide shows little or no cross-resistance with other marketed insecticides, including fipronil, avermectin, α-endosulfan, which also act on the GABAR (Casida and Durkin 2015; Chen et al., 2006). Oocytes of the African clawed frog *Xenopus laevis* (Daudin) are a well-established heterologous expression system often used for assaying the actions of candidate ligands on ionotropic neurotransmitter receptors, including GABARs (Choi et al., 2003; Kuang et al., 2005; Rahman et al., 2006). Therefore, two-electrode voltage-clamp electrophysiology

was applied to *X. laevis* oocytes to characterize the actions of broflanilide on the *D. rerio* GABAR.

2. Methodology

2.1. Ethical statement

For the protection of animal welfare, both *D. rerio* and *X. laevis* were reared and performed following the ethical requirements of the Laboratory Animal Guideline for Ethical Review of Animal Welfare from China (GB/T 35892-2018).

2.2. Tested animals and chemicals

According to the requirements of the Standard for Environmental Safety Evaluation of Chemical Pesticides (SECP) – Part 7: Bioconcentration Test (GB/T 31270.7-2014), the *D. rerio* (length 2.0 ± 1.0 cm) were purchased from a local Aquarium Breeding Center at Confucius Temple (Nanjing, Jiangsu province, China) and acclimated in dechlorinated tap water (DTW) containing dissolved oxygen at a level of 6.8 ± 0.5 mg L^{-1} for at least 7 days. In order to guarantee health and quality, *D. rerio* was fed with artificial dry food every day and the total mortality must be less than 5% during the acclimation period. In addition, feeding of all fish was stopped 24 h before experiments began. *Xenopus laevis* were provided by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Broflanilide (technical material (TC), purity ≥ 98.67%) and MCI-8007 were produced by Mitsui Chemicals Agro, Inc.. For the bioconcentration assay, acetonitrile (HPLC grade) was purchased from J.T. Baker® (Avantor Performance Materials, Inc., Center Valley, PA). Bondesil Cleanert primary/secondary amine (PSA) with diameter of 40-60 μm was supplied by Agela Technologies (Tianjin, China). Deionized water was obtained from the Milli-Q SP Reagent Water system (Millipore, Bedford, MA). Sodium chloride (NaCl) and other reagents as

analytical reagent grade were purchased from Sinopharm Chemical (Beijing, China). For electrophysiological assays, the expression vector pGH19 used for heteromeric DrGABAR expression in *X. laevis* oocytes was provided by Professor Ze-Wen Liu laboratory (Nanjing Agricultural University). Bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate, phosphate buffered solution (PBS, consisting of 8 mM Na_2HPO_4, 136 mM NaCl, 2 mM KH_2PO_4, and 2.6 mM KCl, pH 7.2-7.4) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Paranitroanisole was purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai). The kits for detection of GST and CarE activities were purchased from Nanjing Jiancheng Bioengineerion Institute (Nanjing).

2.3. Acute toxicity experiment of broflanilide

The acute toxicity experiment was conducted according to the SECP-Part 12: Fish Acute Toxicity Test (GB/T 31270.12-2014). Twenty *D. rerio* were exposed to 10 L working solution of broflanilide (TC) and MCI-8007, respectively, with concentration of 10.0 mg L^{-1} (active ingredient, a.i.) based on its solubility and pre-experiments (data not shown). DTW with/without 0.1 mL L^{-1} dimethyl sulfoxide (DMSO) and 0.1 mL L^{-1} Tween-80 were used as controls. Each test concentration was replicated three times. During the experiment, *Danio rerio* were not fed, and dead ones were removed. The water-quality parameters, including pH, dissolved oxygen and temperature were monitored daily.

2.4. Heterologous expression and electrophysiological recording of heteromeric DrGABAR

In order to examine the actions of broflanilide on heteromeric DrGABAR, the subunits of α1, β2S and γ2 were selected and heterologously expressed in *X. laevis* oocytes to generate the heteromeric Drα1β2Sγ2 GABAR *in vitro*. Briefly, the extraction of total RNA, cloning of DrGABAR subunits (α1, β2S and γ2), transcription of capped RNAs (cRNAs) and expression of cRNAs in *X. laevis* oocytes were performed as previously described (Huang et al., 2019). The injection ratio of α1: β2S: γ2 cRNAs was 2: 2: 1. Electrophysiological assays were performed at

a holding potential of -60 mV with a pipette puller resistance of 0.5-3 MΩ at 20 ºC. The oocytes were placed in a recording chamber using standard oocyte saline (SOS) medium with perfusion speed at 8-10 mL min^{-1}. The agonist, GABA, was dissolved in SOS medium and used to stimulate the oocytes for 5 s at intervals of 85 s. The GABA-induced current was recorded by the Axon Digidata 1440A Data Acquisition System (Molecular Devices). Broflanilide (TC) solution prepared with DMSO was added to perfusate after successive control applications of the median effective concentration (EC_{50}) of GABA, and then applied consecutively for the remainder of experiments for 5 s at 85 s intervals during perfusion. For each concentration of agonist and antagonist, more than four oocytes from two *X. laevis* were used.

To obtain the EC_{50} value, which is the concentration of GABA that evokes half maximal current, the data were nonlinear fitted using the least square method (**Eq. 1**) in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

$$I/I_{norm} = I_{max}/(1 + 10^{(LogEC_{50} - Log[GABA]) \times nH}) \quad \textbf{Eq. 1}$$

where I is the current evoked by GABA, I_{max} is the maximal current, and nH is the Hill coefficient.

The scatter plot was performed using GraphPad Prism 6 (GraphPad Software, Inc.) to detect the inhibition of GABA-induced currents in heteromeric Drα1β2Sγ2 GABAR by broflanilide.

2.5. Bioconcentration and elimination experiments of broflanilide

In the recovery assay, several concentrations of broflanilide (TC) (0.1, 0.5, 1, 5, 10, 50 and 100 mg L^{-1}) were established, with 0.025 and 0.05 mg L^{-1} not being detected by HPLC. The broflanilide solutions were dissolved in acetonitrile and were mixed with *D. rerio* and water samples. Subsequently, broflanilide was then extracted as previously described (Jia et al., 2018) and detected by HPLC using a diode array detector at 254 nm (Xu et al., 2018). Each concentration was repeated three times. In brief, 800 μL DTW sample was mixed with 900 μL acetonitrile and 100 mg NaCl. The mixture was subsequently whirled at 1,500 rpm for 1.5 min using a Silence Shake HYQ-3110 vortex (Crystal Technology & Industries, Inc., Addison, TX),

centrifuged at 6,100 g for 5 min, and the supernatant was collected before being filtrated by a 0.22 μm nylon filter (Jinlong Material Co. Ltd., Tianjin, China). The whole *D. rerio* body was cut into small pieces with stainless steel scissors, and 2.00 g *D. rerio* were used for each treatment. One gram sample was collected into 2 mL Eppendorf tubes containing two zirconium beads (diameter 0.5 cm) and 0.5 mL acetonitrile, and homogenized at room temperature (RT) by a MM 400 grinding mill (Verder Shanghai Instruments and Equipment Co., Ltd., Shanghai). The homogenate was then placed into a 15 mL plastic tube together with another 7 mL acetonitrile and handled using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method as previously described (Jia et al., 2018). Finally, the filtrated supernatant of water and *D. rerio* sample was detected on an Agilent 1260 Infinity LC (Agilent Technologies, Santa Clara, CA) fitted with a quaternary pump. Ten microliter filtrated supernatant was injected and separated in a ZORBAX Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm) (Agilent Technologies) at 25 ^{o}C for 15 min using the optimized mobile phase A (acetonitrile, 90%) and B (water, 10%) at a flow rate of 0.5 mL min^{-1}. The amount of broflanilide was calculated with an external standard calibration curve.

For bioconcentration and elimination tests, the quantitative determination of broflanilide in water and *D. rerio* were performed according to the requirement of SECP – Part 7: Bioconcentration Test (GB/T 31270.7-2014) and our previous study (Jia et al., 2018). Briefly, 0.20 mg L^{-1} and 2.00 mg L^{-1} (a.i.) MCI-8007 working solution were selected and three glass aquaria for each concentration were set. One hundred *D. rerio* were held in each glass aquarium with 60 L working solution. At the specific time points (0, 0.5, 1, 2, 4, 6, 8, 12, 14, 16, 18, 20 and 22 day(s)), six *D. rerio* and 2.0 mL water from each aquarium were harvested, frozen and stored at -20 ^{o}C before detection. The working solutions were renewed every 2 days to maintain constant concentration of broflanilide (**Fig. A.2**). The remaining *D. rerio* were transferred from the working solution into DTW at 14 day for the elimination test.

2.6. Bioconcentration factor (BCF) of broflanilide

The uptake rate constant (k_1), depuration rate constant (k_2) and BCFss of broflanilide in *D. rerio* were calculated according to OECD 305 (http://www.oecd.org/env/ehs/testing/E305_Fish%20Bioaccumulation.pdf) and the State Standard of the People's Republic of China (GB/T 21858-2008):

In this study, the bioconcentration curve reached a plateau and become approximately asymptotic to the time axis, therefore , the BCF at steady state (BCF_{ss}) was calculated as **Eq. 2**:

$$BCF_{ss} = C_{f(ss)} \ / C_{w(ss)} \qquad \textbf{Eq. 2}$$

Where $C_{f(ss)}$ and $C_{w(ss)}$, represented the mean concentration of broflanilide in zebrafish at the steady state.

In addition, graphical methods could be used to calculate the k_1 and k_2, which is the constants of chemical uptake and depuration, respectively (Gobas and Zhang 1992; Mackay and Fraser 2000). The concentration of the test substance found in each fish sample were plotted against sampling time on semi-log. The slope of the line is k_2. t_1 and t_2 represent the time points of C_{f2} and C_{f1}, respectively.

$$k_2 = ln(C_{f1}/C_{f2})/(t_2 - t_1) \qquad \textbf{Eq. 3}$$

Given k_2, k_1 was calculated as follows:

$$k_1 = (C_f \times k_2)/(C_w \times (1 - e^{-k_2 \times t})) \qquad \textbf{Eq.4}$$

The value of C_f is read from the midpoint of the smooth uptake curve produced by the data when log concentration is plotted versus time (on an arithmetical scale). The software OriginPro v.9.2 (OriginLab, Northampton, MA) was used for kinetic calculations and curve generation of bioconcentration and elimination in *D. rerio* and water. Data were shown as mean ± standard error (SE).

2.7. Biochemical analysis of detoxification enzymes

Adult *D. rerio* were exposed to sub-lethal concentration of MCI-8007 (2.00 mg L^{-1}, a.i.) and DTW control. The working solution was renewed every 2 days. Triplicates were conducted for each treatment. Three *D. rerio* were collected after 0.5, 1, 2, 4, 6, and 8 days from each aquaria

247 before being dried with filter paper, frozen in liquid nitrogen, and stored at -80 ºC for further
248 use.
249
250 The *D. rerio* samples were homogenized in cold mortar and transferred into 50 mL tubes,
251 together with 15 mL cold PBS and 150 μL PMSF (100 mM). The mixture was ice-bathed for 30
252 min and then centrifuged at 12,000 g for 20 min at 4 ºC. The supernatant was removed into
253 another clean tube for biochemical analysis. Total protein concentration of supernatant was
254 measured according to the Bradford method (Bradford 1976) using BSA as the standard with a
255 Molecular Devices Spectra Max M5 (Molecular Devices). CYP450 activity was measured with
256 paranitroanisole as the substrate at 405 nm using a Synergy H1 Microplate Reader (BioTek
257 Instruments, Inc., Winooski, VT) (Wang et al., 2017). GST and CarE activities were examined
258 using a Glutathione-*S*-transferase (GSH-ST) kit (Colorimetric method) (Wang et al., 2015) and
259 a Carboxylesterase (CarE) kit (Jia et al., 2018), respectively, with a Molecular Devices Spectra
260 Max M5 (Molecular Devices).
261
262 Graphs of biochemical analyses were generated using GraphPad Prism 6 (GraphPad Software)
263 and data were showed as mean ± standard error (SE). Significant difference was calculated by
264 one-way ANOVA with a post-hoc Tukey's LSD test using IBM SPSS Statistics 22 (International
265 Business Machines Corporation, Armonk, NY), and values were considered statistically
266 significant if $P < 0.05$.

267 **3. Results**

268 *3.1 Toxicity of broflanilide to D. rerio in vivo and in vitro*

269 In the acute toxicity experiment, no dead fish were found during the 96h test at 10 mg L^{-1} of
270 broflanilide (TC) and MCI-8007 (a.i.), which indicated that the LC_{50} values of broflanilide, either
271 TC or formulated, were higher than 10 mg L^{-1}. In addition, the *D. rerio* were observed every 12
272 h, and no abnormal behavior was apparent.
273

In *X. laevis* oocytes, Drα1β2Sγ2 successfully formed a functional heteromeric channel, where GABA stimulated inward currents (**Fig. 1A**) in a concentration-dependent manner with an EC_{50} value of 59.36 μM (95% confidence intervals of 52.63-66.95, n=7) (**Fig. 1B**). Broflanilide at 1 μM did not inhibit the GABA-induced current of heteromeric Drα1β2Sγ2 (**Fig. 1C**).

[FIG. 1 WAS INSERTED HERE]

3.2. Bioconcentration and elimination of broflanilide in D. rerio

As shown in **Fig. A.3**, the retention time of broflanilide was at 6.9-7.0 min and the related compound was verified by mass spectrum (**Fig. A.1B**). The calibration curve for broflanilide solution dissolved in acetonitrile was Y=18.037X - 0.635, R^2 = 1.0.

In the recovery assay, the fortified levels were 0.1, 0.5, 1.0, 2.5 and 5.0 mg L^{-1} for water and 0.5, 1.0, 2.5 and 5.0 mg kg^{-1} for *D. rerio* (**Table 1**). After many attempts of extraction and analytical methods, the optimal conditions for the limit of quantification (LOQ) for water and *D. rerio* were 0.1 mg L^{-1} and 0.5 mg kg^{-1}, respectively (**Fig. A.4**). The recovery rates were between 94.35% - 102.70% and 90.40% - 110.88%, with relative standard deviation (RSD) values $\leq$ 4.83% and $\leq$ 5.77 % in *D. rerio* and water, respectively (**Table 1**).

[TABLE 1 WAS INSERTED HERE]

Due to the renewal of solution every 2 days, the concentration of broflanilide in the water was maintained at a stable level, which kept more than 80% of 2.00 and 0.20 mg L^{-1}, respectively (**Fig. 2A**). The results showed that the concentrations of broflanilide in *D. rerio* increased rapidly to the highest levels at the 4^{th} and 10^{th} d with 19.11 $\pm$ 1.87 and 13.48 $\pm$ 0.51 mg kg^{-1}, respectively in 2.00 and 0.20 mg L^{-1} exposure and then kept at a relative steady state until the 14^{th} d, when the zebrafish were transferred into DTW (**Fig. 2B**). Subsequently, the concentration of broflanilide rapidly decreased within 2 days and then decreased gradually below the limit of detection (**Fig. 2B**).

[TABLE 2 WAS INSERTED HERE]

Table 2 shows the toxicokinetic values of broflanilide in *D. rerio* after exposure to 0.20 and 2.00 mg L^{-1}, including the values for concentrations in DTW (C_w), in *D, rerio* (C_f), as well as the bioconcentrate and eliminate rate constants (k_1, k_2), and the BCF value (BCF_{SS}). According

to the definition of SECP of China (GB/T 31270.7-2014): Low bioconcentration (BCF $\leq$ 10); medium bioconcentration (10 < BCF $\leq 10^3$) and high bioconcentration (BCF > 10^3), broflanilide showed medium bioconcentration level at the steady phase in exposure to 2.0 mg L^{-1} and 0.20 mg L^{-1} in *D. rerio*,. Notably, the BCF_{SS} in 0.20 mg L^{-1} was much higher than that in 2.00 mg L^{-1} (**Table 2**).

[FIG. 2 WAS INSERTED HERE]

3.3 Detoxification enzyme activity in D. rerio after exposure to broflanilide

Enzyme activities of CYP450, GST and CarE were measured for assessing the effect of broflanilide on *D. rerio* and the potential detoxification capacity of endogenous metabolic enzymes to break down broflanilide (**Fig. 2**). Compared to the control, CYP450 activity upon exposure to broflanilide significantly up-regulated to 1.64-fold within 0.5 days and continuously increased to 3.04-fold at the 4th day then maintained to the 8th day with the highest level of 3.11-fold (**Fig. 3A**). GST activity significantly increased by up to 91.44% within 4 days, and then reduced to a slightly higher level compared to that of the control (**Fig. 3B**). However, CarE activity did not occur at a constant change during the observed period except at the 2nd day, when CarE activity increased by 34.21% compared to that of the control (**Fig. 3C**).

[FIG. 3 WAS INSERTED HERE]

4. Discussion

As is well-known, broflanilide exhibits not only broad insecticidal spectrum (El Qacemi et al., 2019), but also no cross-resistance to conventional GABAR-targeting insecticides, such as dieldrin, fipronil and avermectin (Nakao and Banba 2016; Nakao et al., 2013). Its formulated product is forecasted to be sold in 2020 (Katsuta et al., 2019). However, the potential risk of it to aquatic life such as fish should be evaluated before entering into the market. To our knowledge, this is the first report measuring the toxicological effects of broflanilide on an aquatic organism. According to the SECP of China (GB/T 31270.12-2014) (high toxicity, LC_{50} <1.0 mg L^{-1}; medium toxicity, 1.0 mg $L^{-1} \leq LC_{50} <$ 10.0 mg L^{-1}; low toxicity, $LC_{50} \geqslant$10.0 mg L^{-1}), either broflanilide (TC) or MCI-8007 showed low toxicity to *D. rerio*.

As a novel pesticide, broflanilide showed equal or higher insecticidal activity to some important agricultural pests, such as *C. suppressalis*, *Cn. medinalis*, *P. xylostella*, compared to flubendiamide and chlorantraniliprole (Qi et al., 2017; Xu et al., 2019), which are widely-used insecticides in the control of Lepidopteran pests (Jeanguenat 2013; Lahm et al., 2009). It is worth noting that broflanilide is less harmful to non-target organisms than some commercial insecticides, such as fipronil, which was banned due to its high risk to wildlife (Agriculture 2017; Wu et al., 2014). For example, fipronil is highly toxic to *D. rerio* with LC_{50} of 0.22 mg L^{-1} at 24h (Wu et al., 2014). It has been demonstrated that electrophysiological assays could provide an informative platform for examining the toxic levels of pesticides to *D. rerio* (Huang et al., 2019). In the present study, GABA was found to induce concentration-dependent currents on the heteromeric Drα1β2Sγ2 channel (**Fig. 1A**) with an EC_{50} value of 59.36 μM (52.63-66.95) (**Fig. 1B**). Broflanilide showed no antagonistic actions on the GABA-induced current indicating that it has low toxicity probably due to its low level binding to the DrGABAR *in vivo* similar as *in vitro* to the heterologously expressed Drα1β2Sγ2 GABAR (**Fig. 1C**).

Even though its acute toxicity to *D. rerio* is low, it is worth noting that broflanilide could rapidly accumulate in *D. rerio* in sub-lethal concentrations after a short time exposure (**Fig. 2B**). Firstly, the "fast uptake and stable hold" trend of broflanilide was similar to those of others insecticides and chemicals with fast uptake and bioconcentration (Hou et al., 2009; Liang et al., 2007; Wang et al., 2015). For example, the concentration of δ-hexachloride and hexaconazole could rapidly increase in *D. rerio* to maximum levels at the 4^{th} and 6^{th} days, and then keep at a constant level during the observed period (Liang et al., 2007; Wang et al., 2015). However, the concentration of broflanilide in *D. rerio* still increased after the 4^{th} day and finally reached a stable level between the 10^{th} and 14^{th} day, which resembles atrazine (El-Amrani et al., 2012). Atrazine accumulated at high speed in *D. rerio* within 10 hours and then slowly to the maximum level (El-Amrani et al., 2012). Secondly, different concentrations of insecticides, such as diazinon, fluopicolide and fluralaner, have specific speeds of bioconcentration in *D. rerio* (Hou

et al., 2009; Jia et al., 2018; Lin et al., 2016). For example, the concentration of fluralaner in *D. rerio* accumulated to the maximum level within 2 days and 6 days in 2.00 mg L^{-1} and 0.20 mg L^{-1} fluralaner, respectively. Thirdly, the maximum bioconcentration level is not only dependent on exposure concentrations, but also on the chemical structure (Versteeg and Rawlings 2003). In the present study, the highest accumulated concentrations of broflanilide in *D. rerio* were 19.11 and 13.48 mg kg^{-1} after 2.00 and 0.20 mg L^{-1} exposure, respectively (**Fig. 2B**). Similarly, the maximum accumulated concentrations were 2.10 mg kg^{-1} and 1.55 mg kg^{-1} while exposed to 0.149 mg L^{-1} and 0.0149 mg L^{-1} fluopicolide in *D. rerio*, respectively (Lin et al., 2016). During the elimination process, the concentration of broflanilide in *D. rerio* gradually decreased below the LOQ at the 8^{th} day after being transferred into DTW (**Fig. 2B**). In fact, other pesticides, such as fluralaner, atrazine and chlorpyrifos (El-Amrani et al., 2012; Jia et al., 2018), follow a similar elimination trend and release rapidly to a very low-level-steady state in fish. Therefore, these results would support the potential suitability of this organism (zebrafish) to study the bioconcentration processes of broflanilide.

For a given pesticide, the accumulation rate mostly depends on both the exposure concentration and the exposure time (El-Amrani et al., 2012). Interestingly, there are inverse relationships between the BCFs and the exposure concentration with higher BCFs for the lower concentration level of some exposed chemicals and elements, including fluopicolide, dodecyl benzene sulfonate, 2-methyl-4-chloro-phenoxyacetic acid (MCPA), MCPA-isooctyl ester and tebuconazole (**Table A. 1**) (He et al., 2015; Lin et al., 2016; Liu et al., 2011; Versteeg and Rawlings 2003; Wu et al., 2017). The BCFs were 26.39 and 193.25 in *D. rerio* exposed to 0.149 and 0.0149 mg L^{-1} fluopicolide, respectively (Lin et al., 2016). Exposing *D. rerio* to fluralaner solutions exhibited median BCF levels of 21.3 and 12.1 in 0.20 mg L^{-1} and 2.00 mg L^{-1} (Jia et al., 2018), respectively. In the present study, a similar trend was observed in the broflanilide (**Table 2**). BCF_{SS} was much higher in 0.20 mg L^{-1} broflanilide than those in 2.00 mg L^{-1}. This phenomenon probably is due to regulatory processes, e.g. metabolization, excretion, or

384 saturation (Contardo-Jara et al., 2011). Therefore, the long-term ecological effects of broflanilide
385 on *D. rerio* should be considered in future studies.
386
387 Since broflanilide did not show obvious phenotypic effects in *D. rerio* and inhibition of
388 GABA-induced current in heteromeric Drα1β2Sγ2 GABAR, the metabolic abilities of
389 detoxification enzymes (CYP450, GST and CarE) from whole *D. rerio* were examined in 2.0
390 mg L^{-1} broflanilide (**Fig. 3**), which is consistent with the concentration used for the
391 bioconcentration test. Generally, changes of enzymatic activity could reflect the response of
392 organisms to xenobiotic stress at the biochemical level and provide early warning as a biomarker
393 (Wu et al., 2018). The CYP450 is a superfamily of heme protein enzymes that is responsible for
394 the phase I biotransformation of endogenous and exogenous compounds (Chang and Kam 1999),
395 such as aroclor, α-naphthoflavone, triazophos, imidacloprid, fipronil and atrazine (Dong et al.,
396 2009; Jones et al., 2010; Wu et al., 2014; Wu et al., 2018). In addition, CYP450 is considered as
397 the primary contributor responsible for the metabolism of pharmaceuticals in fish (Burkina et
398 al., 2015; Ribalta and Solé 2014). For example, the induction of CYP450-dependent *O*-
399 deethylation is extensively used as an indicator of exposure and response to organic pollutants
400 in *D. rerio* (Wu et al., 2014). Fipronil exposure was found to enhance 7-ethoxycoumarin *O*-
401 deethylase activity in the brain, gill, liver, and muscle tissues of *D. rerio* (Wu et al., 2014).
402 Atrazine, at several concentrations, could significantly enhance P450 activity after exposure for
403 10, 15 and 20 days in *D. rerio* (Dong et al., 2009). In the present study, the CYP450 activity
404 were significantly up-regulated compared to the control (**Fig. 3A**), which indicated that CYP450
405 may play an important role in the metabolism of broflanilide in *D. rerio*. Furthermore, the
406 synchronous change of CYP450 activity and the concentration of broflanilide in *D. rerio* (**Fig.**
407 **2B and 3A**) suggested that the increasing activity of CYP450 lead to a balance of
408 bioconcentration, and that CYP450 could be used as a biomarker for detection of broflanilide
409 effects in *D. rerio*. In addition, these results might, to some extent, also explain the significant
410 bioconcentration of broflanilide in *D. rerio*, even at environmentally relevant concentrations.
411

GST could mediate phase II of cellular detoxification and protect organisms from contaminants by mediating the conjugation of GSH with contaminants to form lower or non-toxic substances (Glisic et al., 2015; Richardson et al., 2008). In this study, exposure to broflanilide resulted in increased activity of GSTs within 2 days before a sharp decrease (**Fig. 3B**). This is similar to findings that sulfamethoxazole and sulfadiazine exposure lead to a dramatic decrease of GST activity after an initial increase in *D. rerio* during the first 3 days (Lin et al., 2014). Lin et al. (2014) pointed out that some xenobiotics could induce a defense response to GST in order to protect against oxidation in *D. rerio* (Lin et al., 2014). Our results suggest that GST could possibly participate in the detoxification of broflanilide in *D. rerio*, especially in the first 4 days. However, the limited detoxification capacity of GST would lead to chemical accumulation in the fish body with GST activity effectively inactivated resulting in the disappearance of the stress response. In line with this, the GST levels declined dramatically during the later stages of sulfonamide exposure as a result of inactivation in *D. rerio* (Lin et al., 2014).

CarE is a lipolysis enzyme capable of hydrolyzing ester- and amide- chemicals (Mentlein and Heymann 1984). Thus, we speculated that CarE could possibly metabolize broflanilide *in vivo* through amide linkages (**Fig. A.1**). In a previous study, sub-lethal concentrations of broflanilide enhanced the CarE activity from 24.51% to 26.77% in *P. xylostella* at the 3rd day (Qi et al., 2017). Similarly, we found that in *D. rerio* CarE activity increased to about 34.21% after exposure to 2 mg L^{-1} broflanilide at the 2nd day (**Fig. 3C**). However, increased activity of CarE was not detected at other time points indicating that CarE may be involve in the metabolism of broflanilide in *D. rerio* to a lesser degree than GSTs and CYP450.

5. Conclusion

Our results demonstrate that broflanilide has low toxicity towards *D. rerio*. However, it showed medium bioconcentration ability with a “fast uptake and stable hold” trend. Moreover, the long-term exposure to broflanilide could lead to high bioconcentration risks in fish, even at low

environmental concentrations. Biochemical assays indicated that CYP450 and GST could play important roles in detoxifying and eliminating broflanilide from *D. rerio*. Therefore, more attention should be given to the effects of broflanilide residue on fish, as well as its long-term ecological effects. It is concluded that studying the relationships between bioconcentration, elimination, and detoxification enzymes could enrich the understanding of potential toxicological effect of broflanilide on aquatic life.

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CRediT authorship contribution statement

Zhong-Qiang Jia: Investigation, Software, Writing- Original draft preparation, Formal analysis. **Yi-Chi Zhang**: Data Curation, Investigation, Validation. **Qiu-Tang Huang**: Investigation, Validation. **Andrew Jones**: Writing- Reviewing and Editing. **Zhao-Jun Han**: Funding acquisition, Writing- Reviewing and Editing. **Chun-Qing Zhao:** Conceptualization, Supervision, Project administration, Writing- Original draft preparation, Writing- Reviewing and Editing.

457 **Figure captions**

458 **Fig. 1.** Current traces (A) and electrophysiological responses of heteromeric Drα1β2Sγ2
459 GABAR to GABA (B), and inhibition of broflanilide to GABA (EC_{50}) –induced response in
460 heteromeric *Dr*α1β2Sγ2 GABAR (C). Note, data are obtained from four to seven oocytes from
461 two to three frogs and shown as mean ± SE (B) and scatter plots of individual replicates (C).
462
463 **Fig. 2.** Concentrations of broflanilide in water (A) and *D. rerio* (B) during exposure to 2.00 and
464 0.20 mg L^{-1}. Note, the left and right Y-axis indicate the concentration of broflanilide in water (A)
465 in 0.2 and 2.0 mg L^{-1}. The error bars represent the means ± SE of three replicates.
466
467 **Fig. 3.** Enzyme activities of CYP450 (A), GST (B) and CarE (C) in *D. rerio* after exposure to
468 2.0 mg L^{-1} broflanilide. Values are shown as mean $\pm$ SE and asterisk (s) indicate values that are
469 significantly different ($P < 0.05$) compared to those of the control.
470
471
472

Tables

Table 1. Recovery assay of broflanilide in whole *D. rerio* and water samples

Source	Concentration	Recovery (%)			Mean recovery (%)	RSD (%)
Water ($mg\ L^{-1}$)	0.1	103.01	101.00	95.93	99.98	3.65
	0.5	106.34	111.96	114.34	110.88	3.70
	1.0	103.47	105.18	95.81	101.49	4.92
	2.5	91.84	86.86	92.51	90.40	3.41
	5.0	97.52	109.47	103.57	103.52	5.77
Zebrafish ($mg\ kg^{-1}$)	0.5	92.02	96.16	94.86	94.35	2.24
	1.0	96.76	106.44	100.39	101.20	4.83
	2.5	98.46	93.07	96.13	95.89	2.82
	5.0	102.80	102.03	103.28	102.70	0.61

Table 2. Toxicokinetic parameters and bioconcentration factors obtained from experimental data analysis.

Exposure concentration ($mg\ L^{-1}$)	C_f ($mg\ kg^{-1}$)	C_w ($mg\ L^{-1}$)	k_1 (d^{-1})	k_2 (d^{-1})	BCF_{ss}
0.20	3.60	0.19	19.49	0.40	69.40
2.00	4.34	2.03	1.25	0.56	10.02

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