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Short communication

Strong lethality and teratogenicity of strobilurins on *Xenopus tropicalis* embryos: Basing on ten agricultural fungicides

Dan Li [a], Mengyun Liu [a], Yongsheng Yang [a], Huahong Shi [b], Junliang Zhou [b], Defu He [a, c, *]

[a] Lab of Toxicology, School of Ecological and Environmental Sciences, East China Normal University, Shanghai 200241, China
[b] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200062, China
[c] Shanghai Key Lab for Urban Ecological Processes and Eco-Restoration, Shanghai 200241, China

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ABSTRACT

Agricultural chemical inputs have been considered as a risk factor for the global declines in amphibian populations, yet the application of agricultural fungicides has increased dramatically in recent years. Currently little is known about the potential toxicity of fungicides on the embryos of amphibians. We studied the effects of ten commonly used fungicides (four strobilurins, two SDHIs, two triazoles, fludioxonil and folpet) on *Xenopus tropicalis* embryos. Lethal and teratogenic effects were respectively examined after 48 h exposure. The median lethal concentrations (LC50s) and the median teratogenic concentrations (TC50s) were determined in line with actual exposure concentrations. These fungicides except two triazoles showed obvious lethal effects on embryos; however LC50s of four strobilurins were the lowest and in the range of 6.81–196.59 μg/L. Strobilurins, SDHIs and fludioxonil induced severe malformations in embryos. Among the ten fungicides, the lowest TC50s were observed for four strobilurins in the range of 0.61–84.13 μg/L. The teratogenicity shared similar dose–effect relationship and consistent phenotypes mainly including microcephaly, hypopigmentation, somite segmentation and narrow fins. The findings indicate that the developmental toxicity of currently-used fungicides involved with ecologic risks on amphibians. Especially strobilurins are highly toxic to amphibian embryos at μg/L level, which is close to environmentally relevant concentrations.

1. Introduction

Global decline in populations of amphibians is one example of the most critical threats to the biodiversity (Brühl et al., 2013). The assessment showed that 32% of the world's amphibian species were unequivocally threatened with extinction (Stuart et al., 2004). Amphibians are sensitive to both aquatic and terrestrial environmental factors; so they are deemed to be more susceptible to environmental risks than other organisms (Quaranta et al., 2009). Infectious diseases, habitat destruction, over-exploitation, chemical pollution, alien species, ultraviolet-B radiation and climatic change are discussed as potential causes of amphibian declines (Sodhi et al., 2008). Chemical pollution, especially agricultural chemical inputs is receiving much attention as a major risk. Many malformed amphibians have been reported to occur in agricultural areas where pesticides are applied extensively (Mann et al., 2009). As a major class of pesticides, fungicides are mostly employed to combat fungal diseases and prevent the outbreak of plant diseases. Agricultural fungicides are a major class of pesticides, which are mostly used to kill or inhibit fungi and fungal spores. More than 3600 fungicides have been globally registered (Reilly et al., 2012). Application of fungicides, especially new fungicides such as strobilurins and other bio-fungicides, have dramatically increased over the past decade. For instance, the global sales of strobilurins in values have ranked first among fungicides (Yang, 2014). Despite the likely ecological risks, fungicides have received relatively little attention in comparison with other types of pesticides, such as insecticides and herbicides (Wightwick et al., 2012). There is also limited data on the effects of fungicides, especially new fungicides such as strobilurin and succinate dehydrogenase inhibitor (SDHI) fungicides, on amphibians (Belden et al., 2010; Di Renzo et al., 2010; Hooser et al., 2012).

The widespread use of agricultural fungicides can pose a potential risk to aquatic ecosystems, particularly if residues persist in

* Corresponding author. Lab of Toxicology, School of Ecological and Environmental Sciences, East China Normal University, 500# DongChuan RD, Shanghai 200241, China.
E-mail address: dfhe@des.ecnu.edu.cn (D. He).

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the soil or migrate off-site and enter waterways (Berenzen et al., 2005; Reilly et al., 2012). Actually fungicides were frequently detected in aquatic habitats (Battaglin et al., 2011; Wightwick et al., 2012; Smalling et al., 2013). Recent reports showed the occurrence of strobilurin fungicides in aquatic ecosystems; however the environmental concentration levels varied largely (Reilly et al., 2012; Lefrancq et al., 2013; Rodrigues et al., 2013). As non-target organisms, embryos of amphibians may actually remain at risk for exposure to residue fungicides in aquatic ecosystems (Belden et al., 2010). In the process of pesticide registration, risk evaluation on fish, rodents and mammals is requested from EU authorities (Olsvik et al., 2010; Wang et al., 2012); but amphibians are relatively less utilized in the ecological risk assessment (Reilly et al., 2012). Recent studies have clearly indicated that herbicide and fungicide formulations, although not targeted towards animals, can be acutely toxic to amphibians (Relyea and Jones, 2009; Belden et al., 2010; Jason et al., 2010; Hooser et al., 2012). These fungicides have the potential to affect amphibian populations directly by causing mortality, or influence growth and development at environmentally relevant concentrations (Belden et al., 2010; Hooser et al., 2012). However, previous assays were mostly checked on tadpole or adult frogs; little is known about the toxicity of agricultural fungicides on amphibian embryos.

Xenopus tropicalis is an emerging model animal used in developmental toxicology (Guo et al., 2010; Hu et al., 2015). In the present study, we studied the effects of ten commonly used fungicides on *X. tropicalis* embryos. These representative fungicides were four strobilurins (pyraclostrobin, trifloxystrobin, picoxystrobin and azoxystrobin, two SDHIs (isopyrazam and bixafen), two triazoles (tebuconazole and myclobutanil), fludioxonil and folpet. The lethal effects and concentrations were respectively examined. Their teratogenicity and malformed phenotypes were systematically distinguished. Teratogenic concentrations were respectively determined in line with actual exposure concentrations. Our aim was to identify the developmental toxicity of these fungicides and evaluate their ecological risks on amphibian embryos.

2. Materials and methods

2.1. Chemicals

Ten commonly used fungicides were investigated in this study (Table 1). These fungicides, dimethyl sulphoxide (DMSO), 3-aminobenzoic acid ethyl ester (MS-222) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All chemicals used in this study were of analytical grade.

2.2. X. tropicalis

Adult *X. tropicalis* were purchased from Nasco (Fort Atkinson, WI, USA). Mature frogs paired were separately maintained in aquariums with dechlorinated tap-water at a 26 ± 0.5 °C, alternating 12 h light/dark cycles and fed a semi synthetic diet (Zhejiang, China) three times a week. Breeding was induced by subcutaneous injection of human chorionic gonadotrophin (hCG) (Zhejiang, China) in six pairs of adult frogs. Each male or female was injected with 20 IU hCG, and 36 h later each animal was injected with 100 IU hCG. The use of live organisms was conducted in accordance with protocols approved by Science and Technology Commission of Shanghai Municipality, which ensures that the experimental procedures adhere to national guidelines for the protection of human subjects and animal welfare.

2.3. Exposure experiments

Exposure experiments were conducted following the frog embryo teratogenesis assay-*Xenopus* (FETAX) with some modifications (ASTM, 1998; Fort et al., 2000). On the second morning after the injections, adults were removed, and embryos were harvested without removing the jelly coats. Embryos from paired frogs were chosen for exposure experiments. Test fungicide solutions were dissolved in DMSO (<0.1%) and prepared just prior to the exposure. One FETAX medium control and one DMSO control were simultaneously run. Four replicate dishes were used in each group. The dishes were incubated at 26 ± 0.5 °C with 24 h dark; and the media were renewed at 24 h intervals. Exposure was carried out for 48 h, in which the dead embryos were removed from the dish at 12 h intervals.

2.4. Assay for actual concentrations

Depending on preliminary experiments, five appropriate nominal concentrations were designed for each tested fungicide. Actual concentrations of exposed fungicides in Ringer's solution were measured at the beginning of the treatment. Three replicate samples were used for measurements in each exposed group, and each sample was tested in triplicates. The actual concentrations were determined using high-performance liquid chromatography (HPLC) (Agilent 1260 fitted with a photodiode array detector, Palo Alto, CA, USA), with a ZORBAX Eclipse XDB-C18 reverse phase column. The mobile phase was a mixture of acetonitrile and Millipore water with 0.01 M formic acid (75:25, v/v), and the flow rate was 1.0 ml/min. The optimum wavelengths were 254 nm (PY), 250 nm (TR), 245 nm (PI), 254 nm (AZ), 270 nm (FL), 225 nm (FO), 254 nm (IS) and 250 nm (BI), 220 nm (TE), 223 nm (TE),

Table 1
Properties of ten fungicides used in the present study.

Chemical Names	Abbr.	Fungicide class	CAS number	Launching Year	Water Solubility (Avg, mg/L) [a]	Soil Adsorption Coefficient (K_{oc})[a]	Hydrolysis Half-life (Avg, Days) [a]
Pyraclostrobin	PY	Strobilurin	175013-18-0	2002	20.00	9300	30.0
Trifloxystrobin	TR	Strobilurin	141517-21-7	2010	0.61	2377	40.0
Picoxystrobin	PI	Strobilurin	117428-22-5	2001	3.10	898	180.0
Azoxystrobin	AZ	Strobilurin	131860-33-8	1996	6.70	581	8.7
Fludioxonil	FL	Phenylpyrrole	131341-86-1	1997	1.80	1610	30.0
Folpet	FO	Thiophthalimide	133-07-3	1952	0.80	304	4.7
Isopyrazam	IS	SDHI	881685-58-1	2010	0.55	2416	54.3
Bixafen	BI	SDHI	581809-46-3	2011	0.49	2914	0.9
Tebuconazole	TE	Triazole	107534-96-3	1988	32.0	1000	28.0
Myclobutanil	MY	Triazole	88671-89-0	1998	132.0	518	15.0

[a] Data from IUPAC Pesticide Properties Database, The Pesticide Action Network (PAN) Pesticide Database, and http://www.farmchemicalsinternational.com.

respectively.

2.5. Observations and measurements of embryos

The embryos were observed under a Carl Zeiss Discovery V8 Stereovmicroscope (MicroImaging GmbH, Göttingen, Germany), and images were taken using an AxioCam digital camera. Developmental stages were determined following Nieuwkop and Faber (1956). Phenotypes of malformations were distinguished; and percentages of total malformations were evaluated. All of 20 types of common malformed phenotypes of each embryo were evaluated, and an exact score in the range of 0–5 was assigned to each phenotype following Hu et al. (2015). Briefly, normal anatomic structures were assigned a score of 0; and scores of 1–5 signified abnormalities of increasing severity (1–2 = mild abnormality, 3 = moderate abnormality, and 4–5 = severe abnormality).

2.6. Statistical analysis

All data were expressed as mean ± Standard Error of Mean (SEM). Mean differences between treated groups and controls were determined by one-way analysis of variance (ANOVA), followed by Dunnett's test. A *P*-value of less than 0.05 was considered significant. Probit analysis was used to determine 10% lethal concentration (LC10), the median lethal concentration (LC50), 10% teratogenic concentration (TC10), and the median teratogenic concentration (TC50). A teratogenic index (TI = LC50/TC50) of each fungicide was calculated.

3. Results and discussion

3.1. Exposure concentrations

The actual exposure concentrations of each fungicide were determined when exposure experiments were conducted. Planned nominal and actual concentrations of the exposure groups for ten fungicides were shown in Supplementary data (Table S1). Actual concentrations were then used to analyze effective concentrations for these fungicides.

3.2. Lethal effects of fungicides on X. tropicalis embryos

No significant differences were found in survival percentage, the body length, and embryo morphology between the FETAX medium controls and DMSO controls. Therefore, only the DMSO control group was used for the statistical analysis. Of tested fungicides, four strobilurins showed obvious lethal toxicity at μg/L level (Fig. 1). Exposure to higher than 5.15 μg/L pyraclostrobin, 37.32 μg/L trifloxystrobin, higher than 26.27 μg/L picoxystrobin or higher than 73.81 μg/L azoxystrobin resulted in significant decrease of the percentage of survival of *X. tropicalis* embryos in comparison of the control (Fig. 1A–D). Otherwise, 0.56 mg/L fludioxonil, 0.47 mg/L folpet, higher than 0.96 mg/L isopyrazam, or high than 1.07 mg/L bixafen could induce significant lethal effects on embryos (Fig. 1E–H). However, we did not observe any lethal effects on embryos after exposed to tebuconazole or myclobutanil (data not shown). Results showed that LC10 of four strobilurin fungicides were in range of 2.47–82.46 μg/L, while their LC50 not more than 196.59 μg/L (Table 2). But the LC10 and LC50 of other six fungicides were respectively higher than 0.27 mg/L and 1.57 mg/L (Table 2). We also observed growth inhibition effects of all fungicides on *X. tropicalis* embryos to different degrees (Supplementary data, Table S2).

The first strobilurin fungicide was introduced in 1996, and now the strobilurins become the most important fungicide group with 23.8% of the total fungicide market share (Hua, 2013). Some common strobilurins are azoxystrobin, picoxystrobin, kresoxim-methyl, fluoxastrobin, oryzastrobin, dimoxystrobin, pyraclostrobin and trifloxystrobin (Balba, 2007; Warming et al., 2009). Our results indicate strong lethality of strobilurins on amphibian embryos.

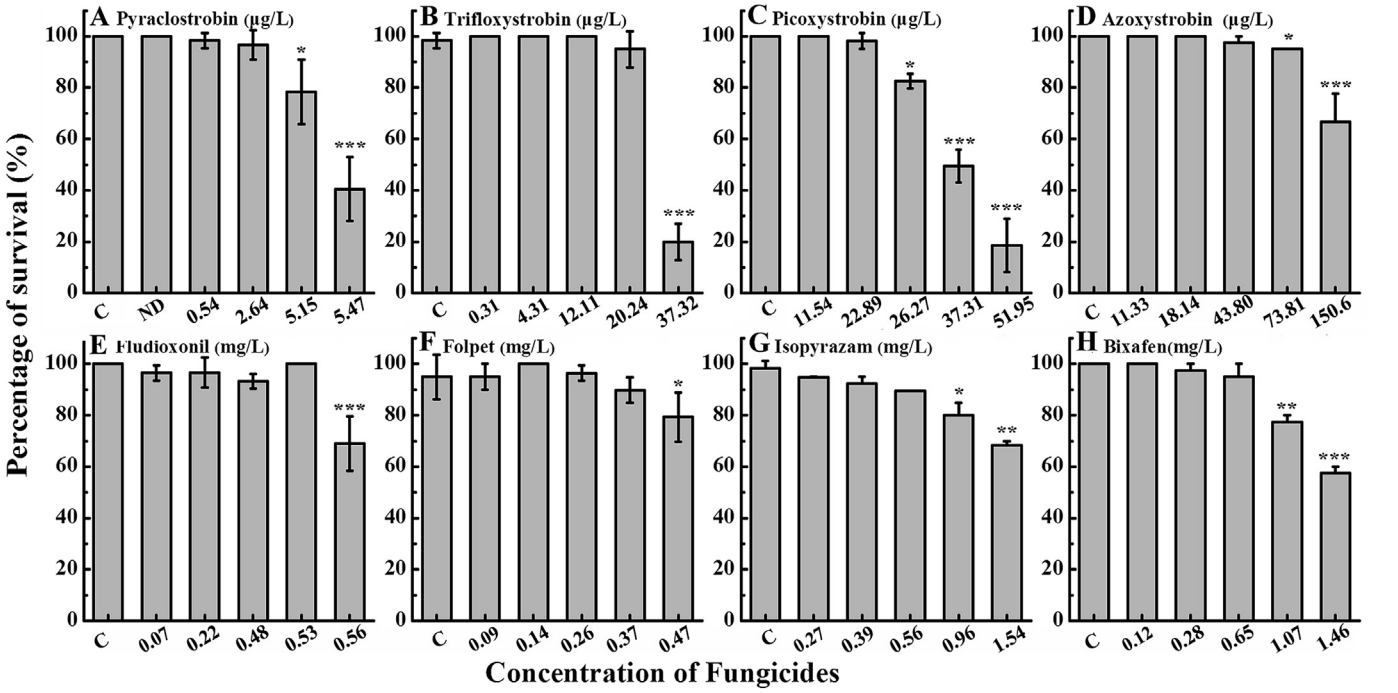

Fig. 1. Survival percentages of *X. tropicalis* embryos after exposed to pyraclostrobin, trifloxystrobin, picoxystrobin, azoxystrobin, fludioxonil, folpet, isopyrazam and bixafen for 48 h. Data were expressed as mean ± SEM of four replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the DMSO control group.

Table 2
Comparison between the effective concentrations and environmental concentrations of ten fungicides.

Fungicides	Effective values in this study					Environmental concentrations (natural water)		
	LC10	LC50	TC10	TC50	TI[a]	Average	Maximal	References
Pyraclostrobin	2.47 μg/L	6.81 μg/L	0.37 μg/L	0.61 μg/L	11.2	0.05 μg/L	0.10 μg/L	Wightwick et al., 2012
Trifloxystrobin	16.21 μg/L	30.37 μg/L	0.39 μg/L	2.28 μg/L	13.3	0.08 μg/L	0.73 μg/L	Wightwick et al., 2012
Picoxystrobin	25.29 μg/L	37.45 μg/L	22.29 μg/L	27.27 μg/L	1.4	ND	ND	
Azoxystrobin	82.46 μg/L	196.59 μg/L	8.47 μg/L	84.13 μg/L	2.3	3.03 μg/L	29.7 μg/L	Berenzen et al., 2005
Fludioxonil	0.27 mg/L	1.57 mg/L	0.08 mg/L	0.18 mg/L	8.7	<0.01 μg/L	<0.01 μg/L	GPEI, 2015
Folpet	0.36 mg/L	0.75 mg/L	0.13 mg/L	0.31 mg/L	2.4	ND	0.05 μg/L	Konstantinou et al., 2006
Isopyrazam	0.49 mg/L	2.87 mg/L	0.24 mg/L	0.31 mg/L	9.3	ND	ND	
Bixafen	0.68 mg/L	1.84 mg/L	0.10 mg/L	0.14 mg/L	13.1	ND	ND	
Tebuconazole	>7.29 mg/L	>7.29 mg/L	1.11 mg/L	2.76 mg/L	ND	0.053 μg/L	0.115 μg/L	Battaglin et al., 2011
Myclobutanil	>11.97 mg/L	>7.29 mg/L	2.73 mg/L	8.98 mg/L	ND	0.24 μg/L	2.9 μg/L	Wightwick et al., 2012

[a] Teratogenic Index = LC50/TC50. ND: No data.

Lethal concentrations (LC10 and LC50) of four strobilurins were all at μg/L level. In horticultural catchments in Australia, pyraclostrobin, trifloxystrobin and azoxystrobin were respectively detected at maximal concentrations of 0.10, 0.73 and 0.03 μg/L in surface waters of natural rivers (Wightwick et al., 2012). A report about 29 streams of the USA showed that azoxystrobin was frequently detected with maximum concentration of 1.13 μg/L (Battaglin et al., 2011). Additionally, maximum concentration of azoxystrobin arrived at 29.7 μg/L in 18 small lowland streams (Berenzen et al., 2005). However, maximum concentration of pyraclostrobin was predicted at 150 μg/L level in wetlands (Hooser et al., 2012). Although there are no published data of accurate value of environmental concentrations about picoxystrobin, most fungicides including pyraclostrobin were demonstrated to occur in edge-of-field runoff at higher than 1 μg/L concentrations (Deb et al., 2010). Despite discrepant in previous studies, concentration levels of strobilurin fungicides were frequently detected at μg/L level, which is close to the lethal concentrations observed in the present study. Therefore, lethal risks of four strobilurins on amphibian embryos at environmentally relevant concentrations are likely to occur. Comparatively, LC50 of six non-strobilurin fungicides were mostly at mg/L level or far higher than environmental concentrations (Table 2), which indicates low lethal risks on amphibian embryos at currently environmental concentrations.

3.3. Teratogenic effects of fungicides on X. tropicalis embryos

The tested fungicides induced multiple malformations in dose dependent manners (Fig. 2). Four strobilurins resulted in obvious malformations on *X. tropicalis* embryos at μg/L level (Fig. 2A–D). Especially after exposure to 5.47 μg/L pyraclostrobin, 37.32 μg/L trifloxystrobin, 51.59 μg/L picoxystrobin or 150.60 μg/L azoxystrobin, embryos presented severe malformations (Fig. 2A3, B3, C3, D3). The major phenotypes of malformation included microcephaly, hypopigmentation, somite segmentation, narrow fin, stunted body and enlarged proctodaeum (Fig. 2). These results indicate the strong teratogenicity of strobilurins to amphibian embryos. Previous study showed that 2% of frogs and toads had physical abnormalities affecting the skeleton and eyes (Sodhi et al., 2008). Malformed morphology in somite, eye, head and body shape

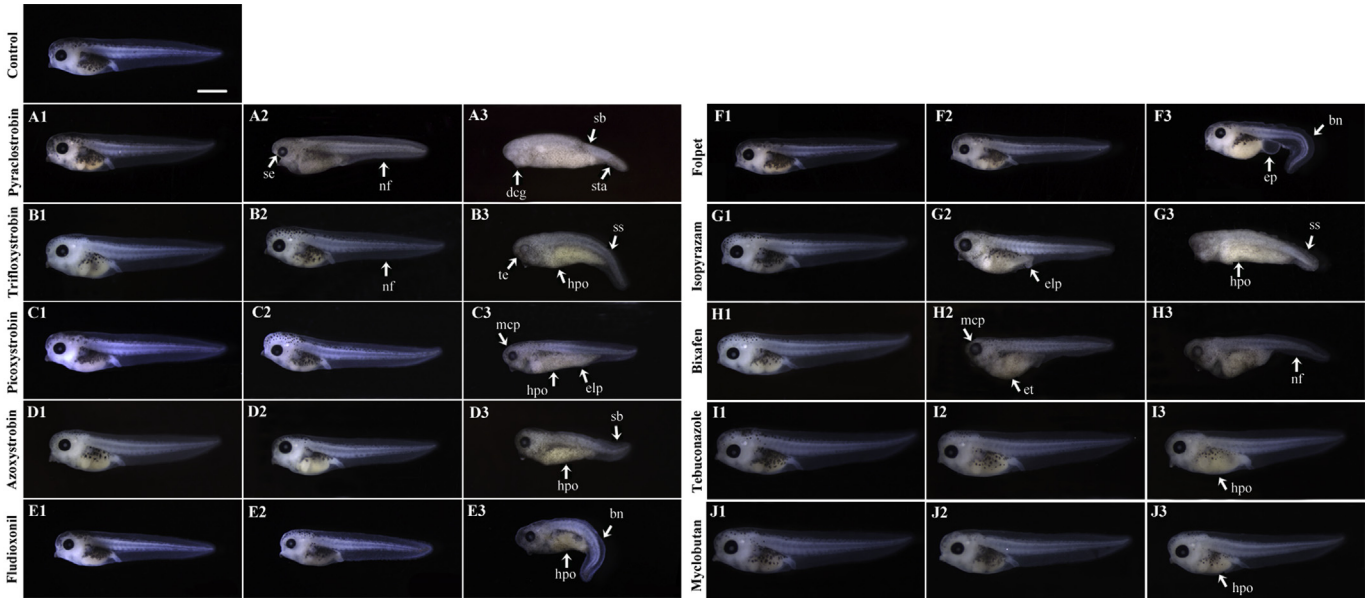

Fig. 2. Teratogenic effects of pyraclostrobin (A1, A2, A3: ND, 2.64, 5.47 μg/L), trifloxystrobin (B1–B3: 0.31, 12.1, 37.32 μg/L), picoxystrobin (C1–C3: 11.54, 26.27, 51.95 μg/L), azoxystrobin (D1–D3: 11.33, 43.80, 150.60 μg/L), fludioxonil (E1–E3: 0.07, 0.48, 0.56 mg/L), folpet (F1–F3: 0.09, 0.26, 0.47 mg/L), isopyrazam (G1–G3: 0.27, 0.56, 1.54 mg/L), bixafen (H1–H3: 0.12, 0.65, 1.46 mg/L), tebuconazole (I1–I3: 0.85, 4.11, 7.29 mg/L) and myclobutanil (J1–J3: 1.15, 6.41, 11.97 mg/L) on *Xenopus tropicalis* embryos after 48 h exposure. Abbreviations: bn, bent notochord; dcg, displaced cement gland; ep, edema in proctodaeum; elp, enlarged proctodaeum; et, enlarged trunk; se, small eye; mcp, microcephaly; nf, narrow fin; hpo, hypopigmentation; sta, short tail axis; ss, somite segmentation; sb, stunted body; te, turbid lens of eyes. Bar = 0.5 mm suitable for all images.

in this study may be associated with malformations of adult frogs (Sodhi et al., 2008; Mann et al., 2009). It indcates that agricultural fungicide-induced teratogenesis occurs in embryo, a susceptive stage of amphibians. Previous studies have shown high toxicity of trifloxystrobin, picoxystrobin and azoxystrobin to non-target aquatic organisms (Belden et al., 2010; Junges et al., 2012; Rodrigues et al., 2013). For example, toxic effects of trifloxystrobin on *Bufo cognatus* tadpoles were observed at 40 μg/L (Belden et al., 2010). For the first time, we found strong lethal and teratogenic effects of strobilurin fungicides to *X. tropicalis* embryos at μg/L level. It indicates that strobilurin fungicides might have profound effects on the survival and adaptation of amphibians, which implicate the ecological risks of the new generation fungicides.

The strobilurins have replaced triazoles and became most largely used fungicide group. In 2011, global market sales for strobilurins were $3.944 billion, which had increased by 6 folds since 1998 (Yang, 2014). In addition, new types of strobilurins were continually introduced to the market. The broad-spectrum fungicide azoxystrobin has become the best-selling fungicide with sales of more than $1 billion (Yang, 2014; Hua, 2013; Rodrigues et al., 2013). Pyraclostrobin was adopted in 2002 and now becomes the widely used fungicide. Picoxystrobin was firstly used in Europe in 2001, and registered both in China and US in 2012 (Hua, 2013). Belden et al. (2010) showed that pyraclostrobin caused acute toxicity on *Bufo* at 15–150 μg/L levels. EC50 of pyraclostrobin exposed to fresh water mussels were in the range of 30–80 μg/L (Bringolf et al., 2007). Chronic effects of pyraclostrobin on *B.*

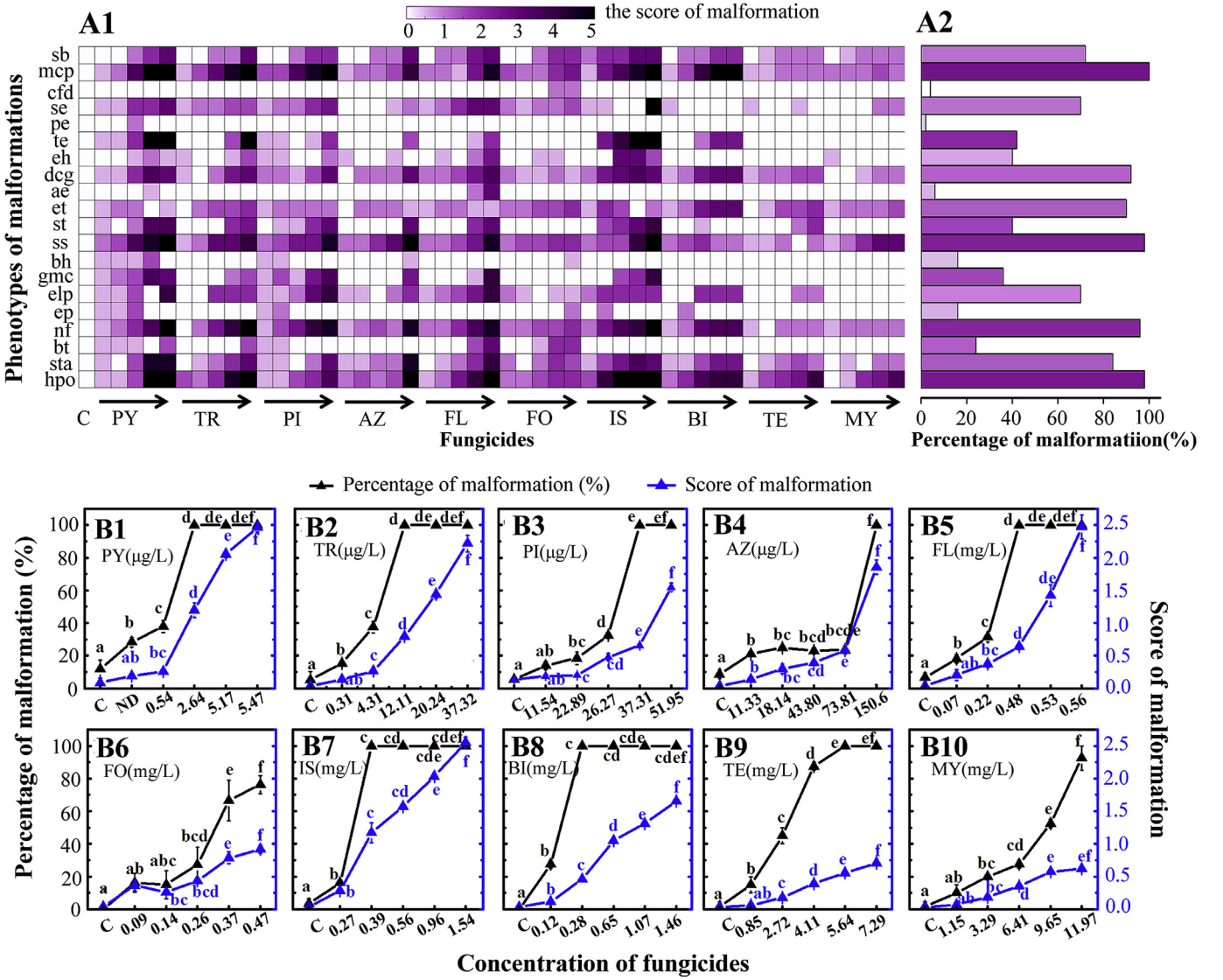

Fig. 3. Malformed phenotypes and percentages of malformation in *X. tropicalis* embryos after exposed to ten fungicides for 48 h. A1: 0–5 scoring evaluation of 20 malformed phenotypes in *X. tropicalis* embryos induced by increasing concentrations of ten fungicides. Concentrations were same as in B1–B10. Arrows indicate increasing exposure concentrations of fungicides. Abbreviations: ae, abdominal edema; bn, bent notochord; bt, bent tail; cfd, craniofacial edema; dcg, displaced cement gland; eh, edema in heart; ep, edema in proctodaeum; elp, enlarged proctodaeum; et, enlarged trunk; se, small eye; gmc, gut miscoiling; mcp, microcephaly; nf, narrow fin; hpo, hypopigmentation; pe, protruding eye; sta, short tail axis; ss, somite segmentation; st, stretched trunk; sb, stunted body; te, turbid lens of eyes. A2: 0–5 scoring evaluation of 20 malformed phenotypes and their percentages of average values for ten fungicides. B1–B10: Percentages (black) and scores (blue) of total malformation in *X. tropicalis* after exposed to pyraclostrobin (PY) (B1), trifloxystrobin (TR) (B2), picoxystrobin (PI) (B3), azoxystrobin (AZ) (B4), fludioxonil (FL) (B5), folpet (FO) (B6), isopyrazam (IS) (B7), bixafen (BI) (B8), tebuconazole (TE) (B9), and myclobutanil (MY) (B10) for 48 h. One-way analysis of variance (ANOVA) and multiple comparisons were used to calculate the significant differences. The letters around the bars indicate significant differences ($P < 0.05$). If two exposure groups of each test fungicide have the same letter, they were not significantly different. Each value was expressed as mean ± SEM of four replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cognatus were also observed at 1.7 μg/L level (Hartman et al., 2014). Combined with previous studies, the results suggest that strobilurin fungicides present strong ecological risks. Developmental toxicity of strobilurins might occur in non-target amphibians at environmentally relevant concentrations.

Additionally, our results showed that 0.56 mg/L fludioxonil induced bent notochord, hypopigmentation and enlarged proctodaeum (Fig. 2E3). Folpet and two triazole fungicides (tebuconazole and myclobutanil) also showed slight teratogenicity (Fig. 2F, I, J). However, we found strong teratogenic toxicity of two SDHI fungicides on *X. tropicalis* embryos (Fig. 2G, H). 1.54 mg/L isopyrazam or 1.46 mg/L bixafen induced microcephaly, hypopigmentation, enlarged proctodaeum and narrow fin of embryos (Fig. 2G3, H3). Isopyrazam and bixafen are new pyrazole carboxamides and commonly used as succinate dehydrogenase inhibitor (SDHI) fungicides. SDHI fungicides have a single site-specific mode of action, inhibiting succinate dehydrogenase of the fungal respiratory chain (Veloukas et al., 2013). For the first time, we observed that isopyrazam and bixafen emerged obvious teratogenic toxicity to *X. tropicalis* embryos. Despite that there are no reports about environmental concentrations until now; our results indicate potential ecologic risks of the SDHI fungicides on amphibian embryos, especially under local pollution involved in these fungicides.

3.4. Characteristic malformations in X. tropicalis embryos induced by fungicides

According to morphology, the scores of the twenty common phenotypes were respectively evaluated (Fig. 3A1). Twenty phenotypes with six grades for each phenotype were mapped in order to explore the severity of the abnormality with increasing concentrations. Grayscale images showed the obvious dose–effect relationship of major phenotypes (Fig. 3A1). Analytical results of ten fungicides showed that the most common and severe phenotype was microcephaly, and then following hypopigmentation, somite segmentation and narrow fin (Fig. 3A2). Furthermore, teratogenetic degrees and effective concentrations were respectively analyzed for ten fungicides. Score of malformation of each exposed group was determined using the average value of the total scores for the twenty phenotypes. Ten fungicides exhibited different concentration-dependent relationships in the percentage of the malformation or the score of malformations (Fig. 3B1–B10). We found four strobilurins induced consistent malformed phenotypes and showed similar concentration–effect relationship in teratogenetic occurrences and degrees (Fig. 3A1, B1–B4). According to teratogenic index (TI), pyraclostrobin and trifloxystrobin presented stronger teratogenicity than picoxystrobin or azoxystrobin, which was consistent with the morphology (Fig. 2).

Strobilurin fungicides are the large group of QoI inhibitors, which act to inhibit the respiratory chain at Complex III. They induce a suppressive effect on other fungi for reducing competition for nutrients (Bartlett et al., 2002). Strobilurins inhibit electron transfer in mitochondria, disrupt metabolism and prevent growth of the target fungi (Balba, 2007). However, it is still unknown if the high toxicity of strobilurins is caused by blocking electron transfer in aquatic animals. In the present study, survival and malformation rates changed greatly within constricted range of exposure concentrations. Generally, the abrupt changes of toxicity in a narrow range of concentrations are caused by the quick absorption of the chemicals in the tested organisms (Wright and Westh, 2006). Some specific mode of action might also be related to this phenomenon (Relyea and Jones, 2009). According to previous studies, the phenotypes of malformations usually provide useful clues to reveal teratogenetic mechanisms (Bacchettaa et al., 2008; Wheeler and Brändli, 2009). In our study, consistent phenotypes and concentration-effect patterns indicate that strobilurins might share the same mechanism of teratogenesis.

Results showed that TC10s of four strobilurin fungicides were not more than 22.29 μg/L, while their TC50s not more than 84.13 μg/L (Table 2). But TC10s of six non-strobilurin fungicides were higher than 0.08 mg/L, while their TC50s higher than 0.14 mg/L (Table 2). Among the six fungicides, teratogenicity of SDHIs (isopyrazam and bixafen) and fludioxonil was relatively strong because of the high scores of malformations after exposure with increasing concentrations (Fig. 3B5–B10). Folpet and triazole fungicides (tebuconazole and myclobutanil) presented high frequency of malformation; however the scores of malformation were low, which indicated slight teratogenicity (Fig. 3B5–B10). Fludioxonil and folpet are two commonly used fungicides respectively belonging to phenylpyrrole and thiophthalimide (Konstantinou et al., 2006; Duan et al., 2013). Tebuconazole and myclobutanil are widely utilized as classic pesticides, triazole fungicides (Battaglin et al., 2011; Wightwick et al., 2012). According to previous studies, their environmental concentrations in surface water from rivers are far higher than the teratogenetic concentrations in this study (Table 2). So these non-strobilurin fungicides are relatively nontoxic to amphibian embryos at present environmentally concentrations.

4. Conclusions

Developmental toxicity of ten commonly used fungicides on *X. tropicalis* embryos was fully studied in this study. Strobilurin fungicides presented the lowest EC50s and TC50s, which are μg/L levels and close to the environmental concentrations. It suggests high risks of strobilurins on the survival and adaptation of amphibians. Fludioxonil and SDHI fungicides also possess lethal and teratogenic toxicity at relatively high concentration levels, which indicates potential risks on amphibian embryos. Otherwise, folpet and triazole fungicides showed slight teratogenicity on embryos at concentration levels which are far higher than environmental concentrations; so they are safe to amphibians.

Furthermore, we found strobilurins, fludioxonil and SDHI fungicides shared similar phenotypes of malformations and concentration–effect relationship. It hints these fungicides may affect the survival and adaptation of amphibians with a similar mode of actions. It requires further investigation to explore whether these agricultural fungicides produce joint or synergistic effects. Simultaneous exposure to multiple fungicides may enhance the ecologic risks on amphibian embryos. The global decline of populations and species in amphibians is the unequivocal threat for biodiversity. Our results suggest that the application of agricultural fungicides is associated with survival and teratogenesis of amphibians. Especially, strobilurin fungicides were highly toxic to amphibian embryos at environmentally relevant concentrations.

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Appendix. ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.010.

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