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Short communication

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

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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

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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.