The Ascidian Embryo Teratogenicity assay in *Ciona intestinalis* as a new teratological screening to test the mixture effect of the co-exposure to ethanol and fluconazole

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ABSTRACT

The aim of this work was to evaluate the Ascidian Embryo Teratogenicity assay (AET) as new alternative invertebrate model to test the developmental effects of the co-exposure to ethanol and fluconazole. *Ciona intestinalis* embryos were exposed to the azolic fungicide fluconazole, (FLUCO, 7.8–250 µM), to ethanol (Eth, 0.01–0.5%) and to their mixture (0.01% Eth + FLUCO 7.8–250 µM) from neurula to larval stage. At the end of the exposure period, larvae were morphologically evaluated and benchmark analysis performed by using the PROAST modelling software. Both compounds were teratogenic in a concentration-related manner, particularly affecting the pigmented organs. The co-exposure to Eth enhanced the effects of FLUCO, the additive hypothesis was not rejected by the modelling. The results demonstrated that AET could be considered a good vertebrate-free alternative model for toxicological investigation in embryos.

1. Introduction

Various animal models have been used to study teratogenic effects on embryos (Randall et al., 1977; Brown et al., 1979; Streissguth et al., 1980; Sulik et al., 1981; Cartwright and Smith, 1995). Despite the use of the *in vitro* culture of mammalian embryos (Brown et al., 1979) is probably the best experimental system for studying direct teratogenic effects on embryos, many difficulties and inconveniences involved with the technical complexity of culturing mammalian embryos still remain. Moreover, the use of vertebrates in scientific procedures is subjected to law and guidelines that limit the number of animals that can be employed.

Recently, ascidian embryos have been proposed as an excellent alternative experimental system for investigating the mechanisms underlying the development of chordates, and therefore of vertebrates (Passamaneck and Di Gregorio, 2005; Sasakura et al., 2012). Ascidians are widespread marine sessile, filter-feeding chordate organisms belonging to the Subphylum *Urochordata*, which has been recognized as the sister group of vertebrates (Delsuc et al., 2006).

Ascidian embryos display striking similarities to vertebrate ones as they develop through a swimming, tadpole like larva, which represents a simple prototype of the chordate body plan (Passamaneck and Di Gregorio, 2005), comprising a hollow neural tube lying dorsal to a rod-like notochord (Satoh, 1994). The central nervous system is composed by a sensory vesicle and a visceral ganglion, localized in the trunk, and by a nerve cord localized in the tail. The sensory vesicle contains two pigmented sensory organs, the otolith and the ocellus. Gametes can be obtained by *in vitro* fertilization. Moreover, ascidian embryos develop quickly, taking ~18 h from fertilization to the development of a free-swimming larva at 18 °C, allowing observing the effects of treatments within a day. All these characteristics offer several advantages for toxicological studies. The tadpole larva of *Ciona intestinalis*, one of the most studied species, consists of only ~2600 cells. Moreover, the release of its sequenced genome, which consists of ~16.000 genes (Dehal, 2002), has favoured the possibility to study toxicant effects on gene expression. Particularly, ascidian *Ciona intestinalis* larvae are used as model organisms for marine pollution monitoring (Bellas et al., 2004), in developmental and evolutionary biology (Satoh, 1995; Di Gregorio and Levine, 1998; Simmen et al., 1998; Corbo et al., 2001; Dehal, 2002), cardiac development (Davidson, 2007), central nervous system regeneration (Dahlberg et al., 2009), endocrine disruptor toxicity (Cima et al., 1996; Cangialosi et al., 2013) and for applying embryo-toxicity tests related to xenobiotic exposure (Cima et al., 1996; Pennati et al., 2006; Groppelli et al., 2007; Zega et al., 2009;

Matsushima et al., 2013).

The aim of this work to use *Ciona intestinalis* as new invertebrate alternative teratological screening test (AET, Ascidian Embryo Teratogenicity assay) to evaluate the effects of the co-exposure to fluconazole, an azole compound, and ethanol.

Evaluating the toxicity of mixtures of multiple chemicals is one of the major objectives of today's toxicology since humans and all other organisms are exposed to multi-component chemical mixtures. The huge number of combinations of chemicals, the paucity of efficient test strategies for the risk assessment of mixtures, and the increasing societal need to reduce animal testing make the study of mixtures a very complex issue (EPA, 2002).

Azole compounds, classified into triazoles and imidazoles, are widely used as antifungal agents in human and veterinary pharmaceuticals, and some are also prescribed in cancer therapy (Kahle et al., 2008; Zarn et al., 2002). Their biological activity is based on the inhibition of fungal lanosterol-14R-demethylase (a cytochrome P-450 enzyme, encoded by the CYP51 gene) (Vanden Bossche, 1985; Zarn et al., 2002). Some authors, by *in vitro* and *in vivo* teratological studies in rat, as well as in patients with acute promyelocytic leukemia, also demonstrated the inhibitory potency of azole derivatives on CYP26, a P-450 enzyme that mediates the catabolism of retinoic acid (RA) (Van Wauwe et al., 1990; Schwartz et al., 1995; Vanier et al., 2003).

Among azoles, fluconazole (FLUCO), a bis-triazole derivative, is a clinically used fungicides commonly dosed for treating a variety of mycoses and infections (Debruyne, 1997). The use of FLUCO for prophylaxis and treatment of mycotic infections is also widespread among pregnant women. It is known that triazole derivatives closely mimic the morphological effects induced by excess of RA in mammals (Menegola et al., 2004, 2003) and in frogs (Groppelli et al., 2005). FLUCO causes specific teratogenic effects at the level of the branchial apparatus in mouse (Tiboni, 1993) and in rat embryos (Menegola et al., 2004), that are very similar to those obtained after RA exposure. The observed malformations are dose and stage dependent and consist in the perturbation of the hindbrain and of the branchial region from which craniofacial structures originate.

Teratogenesis caused by the maternal consumption of ethanol (Eth) has become a major concern to investigators in the field of health sciences given the widespread use and abuse of this substance also during pregnancy (Nakatsuji, 1983). Jones and Smith (1973) used the term "foetal alcohol syndrome" (FAS) to describe characteristic malformations observed in offspring of chronic alcoholic mothers: microcephaly, flat midface with short palpebral fissures, low nasal bridge with short nose and long smooth or flat phyltrum (de Sanctis et al., 2011; Joya et al., 2012; Memo et al., 2013). In studies on postimplantation rat embryos exposed *in vitro* to Eth, the reported malformations are mostly neural tube defects, rotation and cardiac abnormalities and hypoplasia of the first branchial arch (Giavini et al., 1992; Deltour et al., 1996; Duester, 1998; Kot-Leibovich and Fainsod, 2009).

Considering the increasing consumption of ethanol also in pregnant women, the co-exposure of ethanol and FLUCO and their possible interactions are matter of concern.

Since *Ciona intestinalis* larvae are sensible to the teratogenic action of exogenous RA (Nagatomo and Fujiwara, 2003) and azole compounds, we decide to use the AET to evaluate the existence of mixture effect by co-exposure to FLUCO and Eth.

2. Materials and methods

2.1. Animals and treatments

Adults of *C. intestinalis* were collected by the fishing service of the Roscoff Biological Station (France). Animals were maintained in aquaria filled with artificial seawater (Instant Ocean, salinity 32‰) at 16 °C and provided with circulation system as well as mechanical, chemical and biological filters. Constant light condition was preferred

to promote gamete production.

Gametes collected from dissected gonoducts of at least three adults were transferred in Petri glass dishes containing Artificial Sea Water with Hepes (ASWH; pH 8) for *in vitro* cross-fertilization and maintained at 16 °C (Hotta et al., 2007). Seven hours post fertilization (hpf), early neurula stage embryos were collected for treatments. They were exposed for 15 h to increasing concentrations of Eth (0.01, 0.05, 0.1, 0.25, 0.5% corresponding to 1.7, 8.5, 17, 42.5, 85 mM), to increasing concentrations of FLUCO (0, 7.8, 15.75, 31.5, 250 µM) and to mixtures of 0.01% Eth + FLUCO (0, 7.8, 15.75, 31.5, 250 µM) dissolved in 10 mL of ASWH, without renewing the solution. FLUCO concentrations were chosen based on previous works (Groppelli et al., 2007) plus a high dose as positive control. Control embryos were maintained in ASWH. All chemicals were of reagent grade. FLUCO and Eth absolute were purchased from Sigma, Italy. FLUCO stock solution (250 µM) was prepared in ASWH to reach the final treatment concentrations. All solutions were freshly prepared.

Approximately 100 embryos, randomized between those derived from the three adults, were used for each treatment and each treatment was replicated three times (n ≈ 300 per experimental group). When controls reached swimming larva stage (22 hpf), all specimens were fixed with 4% paraformaldehyde in PBS. After fixation, larvae of each experimental group were counted and morphologically examined under a dissecting microscope. For each experimental group, the percentage of dead and malformed larvae was recorded. Larval abnormalities were classified in four malformed phenotypes:

- trunk abnormalities, in which trunk appeared round in shape and the anterior side was flat, due to impairment of adhesive papillae development (Fig. 1C and D);
- pigmented organ abnormalities, in which otolith and ocellus appeared fused in a single spot and/or displaced on the dorsal portion of the sensory vesicle (Fig. 1E and F). In the samples displaying sensory vesicle protrusion on the trunk dorsal side, the pigmented organs were exposed to the surface;
- tail abnormalities, in which the larval tail appeared coiled, flexed or reduced in length (Fig. 1G);
- severe malformations, in which plurimalformed larvae were characterized by absence of sensory vesicle cavity, presence of a short, bent tail, round trunk with not elongated papillae. This group includes also larvae that failed the hatching event (Fig. 1H).

2.2. Statistical analysis

We used generalized linear models (GLM) to test the significance of differences in the incidence of malformations between each experimental group. The number of individuals with/without malformations per each experimental group was the dependent variable, while experimental groups were considered as fixed factors. GLMs showed overdispersion, as residual deviance was larger than the residual degrees of freedom, therefore we used a quasi-binomial error structure, and we tested significance using a F-test (Crawley, 2007). If substance exposures were significant, we performed Tukey's *post-hoc* tests (significant at $P < 0.05$) using the multcomp package in R (Hothorn et al., 2008), in order to identify specific effects of each concentration on larvae development.

2.3. Benchmark approach

Benchmark approach was applied only on data regarding target structures (pigmented organs) both for Eth and for FLUCO. Data on pigmented organ abnormalities were modelled by using PROAST 62.3 software in order to characterize the single dose response curves, obtain the relative potency factor (RPF) of Eth versus FLUCO and express Eth concentrations in FLUCO equivalents. Dose-additivity hypothesis was finally evaluated using the best model with covariates (Hill model, m3-
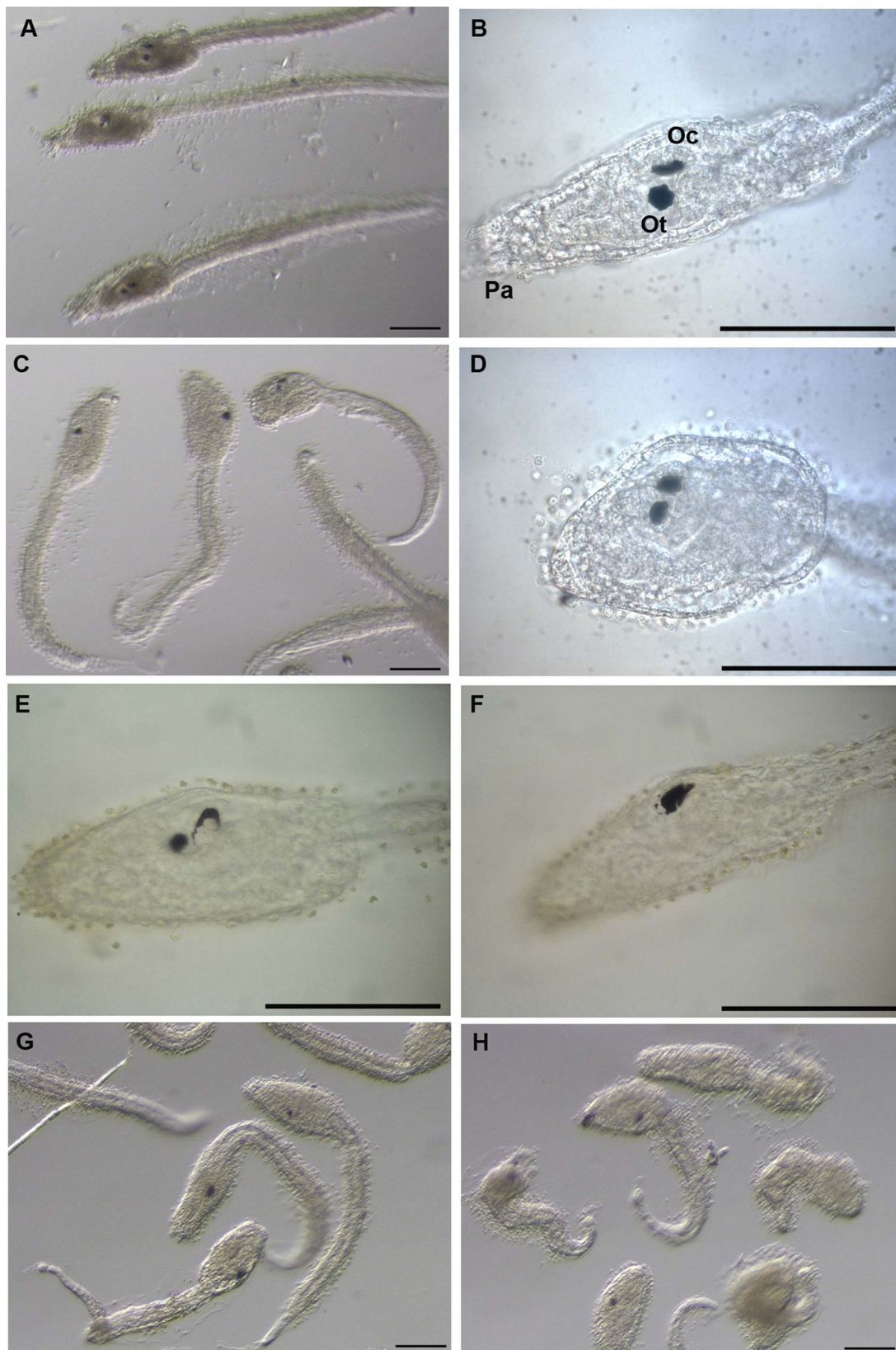
Fig. 1. Morphological analysis of *C. intestinalis* larvae. A and B) Normally developed larvae. The lateral view of the trunk (B) allows to distinguish the otolith (Ot) and the ocellus (Oc), separated and well differentiated. Adhesive papillae (Pa) are also visible at the anterior end. C and D) Larvae displaying trunk abnormalities. The trunk appears round in shape with impairment of adhesive papillae development. D–F) Larvae displaying malformed pigmented organs, abnormal in shape (D and E) or fused (F). G) Larvae with coiled or flexed tail. H) Plurimalformed larvae displaying severe malformations to almost all organs. Magnification: 60X (A, C, G, H), 120X (B, D, E, F). Scale bar: 100 μm.

Table 1

Frequency ± standard error (%) and distribution of the observed abnormalities in larvae exposed to increasing Eth concentration.

	0 (N = 290)	1.7 (N = 300)	8.5 (N = 289)	17 (N = 290)	42.5 (N = 300)	85 (N = 300)
Tail	11.30 ± 1.48	15.00 ± 7.56	21.72 ± 5.91	29.11 ± 2.19	22 ± 3.52	20.67 ± 4.41
Pigmented organs	5.63 ± 1.63	10.00 ± 3.22	20.45 ± 4.70 [*,**]	22.26 ± 2.26 [*,**,***]	31.00 ± 1.73 [*,**,***,****]	35.33 ± 1.67 [*,**,***,****,*****]
Trunk	0 ± 0.00	2.33 ± 1.45	4.83 ± 0.60	5.85 ± 0.60	3.33 ± 1.33	9.67 ± 3.39 [*,**]
Severe malformations	4.59 ± 1.70	5.67 ± 3.48	5.47 ± 1.82	9.70 ± 1.87	7.33 ± 2.61	9.67 ± 3.18
Tot. Malformed	21.52 ± 2.04	31.33 ± 12.21	49.39 ± 5.71 [*]	56.96 ± 5.29 [*,**]	53.00 ± 6.03 [A]	61.00 ± 2.89 [*,**]

* p < 0.01 *vs*. ETH 0.

** p < 0.01 *vs*. ETH 1.7 mM.

*** p < 0.01 *vs*. ETH 8.5 mM.

**** p < 0.01 *vs*. ETH 17 mM.

***** p < 0.01 *vs*. ETH 42.5 mM.

Table 2

Frequency ± standard error (%) and distribution of the observed abnormalities in larvae exposed to increasing FLUCO concentrations (µM).

	0 (N = 300)	7.8 (N = 251)	15.75 (N = 300)	31.5 (N = 300)	250 (N = 264)
Tail	8.33 ± 1.86	6.26 ± 1.13	3.33 ± 1.20	14.33 ± 6.97	7.62 ± 1.79
Pigmented organs	3.33 ± 2.03	9.14 ± 0.78	16.33 ± 3.85 [*]	21.66 ± 4.41 [*]	27.18 ± 2.52 [*,**,***]
Trunk	7.00 ± 1.53	11.76 ± 3.45	21.66 ± 3.18 [*]	41.33 ± 7.70 [*,**]	40.39 ± 3.87 [*,**]
Severe malformations	6.00 ± 3.06	1.29 ± 0.66	3.00 ± 1.16	4.33 ± 1.86	9.72 ± 2.57
Tot. Malformed	24.33 ± 2.85	27.18 ± 5.30	41.67 ± 3.93 [*]	64.00 ± 5.14 [*,**]	62.98 ± 3.46 [*,**]

* p < 0.01 *vs*. FLUCO 0 µM.

** p < 0.01 *vs*. FLUCO 7.8 µM.

*** p < 0.01 *vs*. FLUCO 15.75 µM.

ab).

3. Results

3.1. Morphological analysis of induced abnormalities

Nearly 80% of unexposed larvae (Tables 1–3) developed normally, displaying an elongated trunk with three elongated adhesive papillae, a clearly visible sensory vesicle and two pigmented organs (otolith and ocellus) well differentiated. Particularly, the ocellus, the dorsally located photoreceptor, and the otolith, the gravity-sensing organ, were normally located inside the sensory vesicle. The tail was straight and elongated (Fig. 1A and B). The comparison among control and treated larvae by stereomicroscope observation revealed that larval phenotype could be affected by Eth, by FLUCO and by their mixture (Fig. 1C–H). Frequency ± standard error (%) and distribution of the observed abnormalities in larvae of the different experimental groups are reported in Tables 1–3.

3.2. Eth

During the development of *C. intestinalis* embryos, exposure to Eth increased in a concentration-related manner the total number of malformed larvae (GLM: $F_{5,12} = 4.67$, $P = 0.013$) (Table 1). The lowest effective concentration was 85 mM (Tukey's *post-hoc*, all $P \leq 0.001$). The Eth lowest tested concentration (1.7 mM) did not cause a

significant increase of malformations at any district compared to the controls (*post-hoc*, $P = 0.632$) but showed value in trend with the effective concentrations. The main target for Eth were specifically the pigmented sensory organs ($F_{5,12} = 15.33$, $P < 0.0001$): otolith and ocellus appeared fused or protruded at any effective concentrations and showed value in trend with the effective concentrations at the concentration of 1.7 mM. A strict dose-relationship was observed and confirmed also by *post-hoc* tests. At the highest tested concentration (85 mM), Eth also determined a significant increase on trunk malformations ($F_{5,12} = 6.42$, $P \leq 0.001$) in less than 10% of larvae. The incidence of tail abnormalities was not significantly affected by Eth at any concentration level ($F_{5,12} = 1.58$, $P = 0.239$).

3.3. FLUCO

The incidence of total number of malformations induced by FLUCO during the development of *C. intestinalis* embryos was dose-dependent (GLM: $F_{4,20} = 12.003$, $P < 0.00001$): FLUCO concentrations ≥15.75 µM significantly increased the total number of malformations, compared to controls (*post-hoc*: all $P < 0.002$), while we did not detect significant differences between the controls and FLUCO 7.8 µM groups ($P = 0.170$) (Table 2). FLUCO specifically affected the pigmented organs and the trunk (respectively GLM: $F_{4,20} = 10.43$, $P < 0.00001$, GLM: $F_{4,20} = 16.34$, $P < 0.00001$). The lower FLUCO concentration (7.8 µM) did not cause a significant increase of these malformations compared to the controls (Tukey's *post-hoc*, pigmented organs:

Table 3

Frequency ± standard error (%) and distribution of the observed abnormalities in larvae co-exposed to increasing FLUCO concentration (µM) + Eth 1.7 mM.

	0 + ETH 1.7 mM (N = 286)	7.8 + ETH 1.7 mM (N = 300)	15.75 + ETH 1.7 mM (N = 300)	31.5 + ETH 1.7 mM (N = 299)	250 + ETH 1.7 mM (N = 275)
Tail	5.60 ± 2.03	9.33 ± 0.88	10.00 ± 3.47	9.69 ± 1.31	6.56 ± 1.45
Pigmented organs	8.22 ± 1.88	21.66 ± 8.85	27.00 ± 5.01	27.09 ± 3.78	51.44 ± 15.21 [*]
Trunk	7.55 ± 1.45	24.00 ± 5.51	37.33 ± 6.07 [*]	36.42 ± 6.68 [*]	40.56 ± 9.71 [*]
Severe malformations	3.00 ± 2.08	7.66 ± 2.73	12.33 ± 3.93 [*]	19.05 ± 5.02	15.77 ± 3.62
Tot. Malformed	22.32 ± 4.28	52.00 ± 8.09	64.00 ± 7.01	70.50 ± 10.66 [*,**]	78.80 ± 14.77 [*,**]

* p < 0.01 *vs*. FLUCO 0 + ETH 1.7 mM.

** p < 0.01 *vs*. FLUCO 7.8 + ETH 1.7 mM.
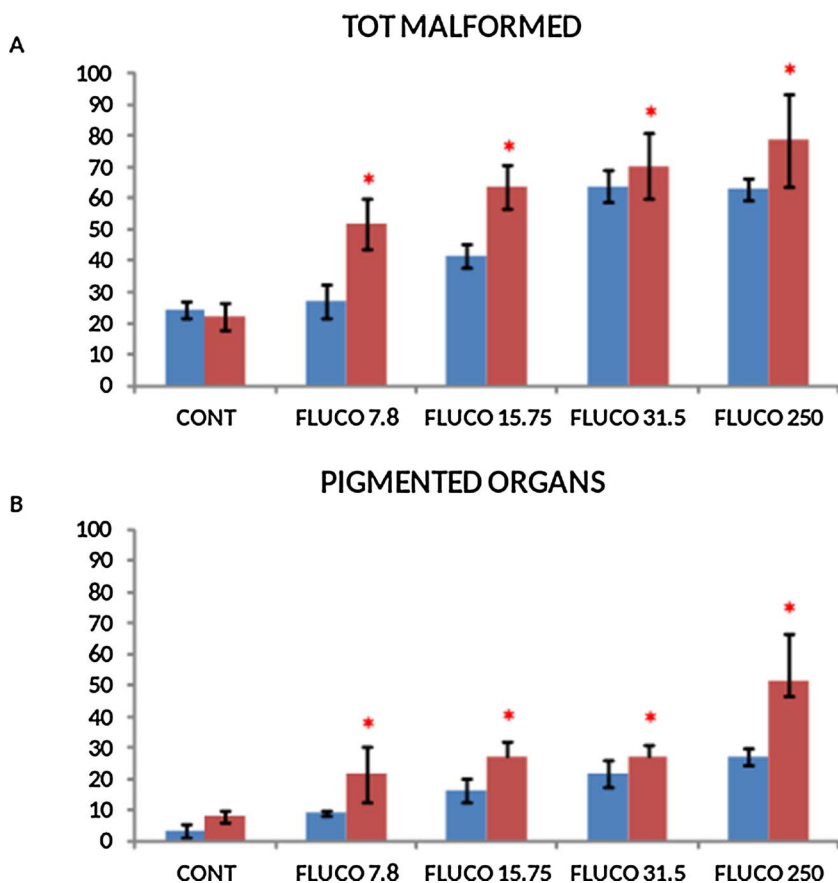
TOT MALFORMED

PIGMENTED ORGANS

Fig. 2. Histograms showing the percentage of total malformed larvae (A) and the percentage of larvae with pigmented organs abnormalities (B) in experimental groups exposed to increasing FLUCO concentrations (left bars) compared to groups co-exposed to FLUCO + Eth 1.7 mM (right bars).

* = The co-exposure to the sub-teratogenic concentration of Eth (1.7 mM) is statistically significant compared to FLUCO dose alone.
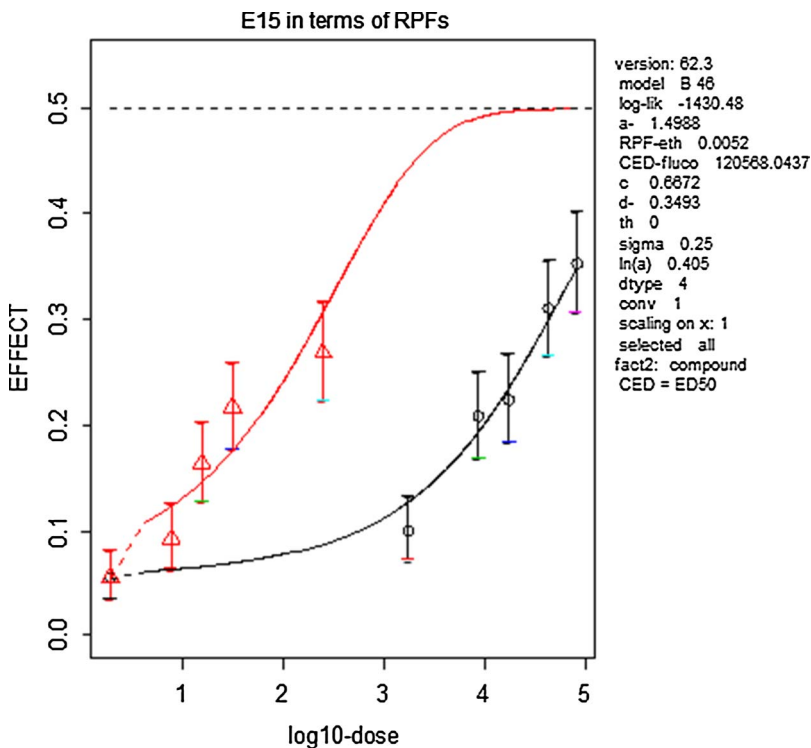

E15 in terms of RPFs

```
version: 62.3
model  B 46
log-lik  -1430.48
a-  1.4988
RPF-eth  0.0052
CED-fluco  120568.0437
c  0.6872
d-  0.3493
th  0
sigma  0.25
ln(a)  0.405
dtype  4
conv  1
scaling on x: 1
selected  all
fact2: compound
CED = ED50
```

Fig. 3. Comparison between FLUCO (line with triangles) and Eth (line with circles) dose-response curves, with Eth concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of Eth versus FLUCO resulted 0.0052 confirming the parallelism hypothesis.

$P = 0.15$, trunk: $P = 0.05$) but showed value in trend with the effective concentrations (≥ 15.75 µM, *post-hoc*, all $P \leq 0.001$). A clear dose-dependence was visible for these abnormalities. Tail abnormalities were not significantly affected by FLUCO ($F_{4,20} = 1.48$, $P = 0.245$) at any tested concentration. The total number of severe malformations was significantly affected by FLUCO ($F_{4,20} = 3.52$, $P = 0.025$), even though *post-hoc* tests did not detect significant differences between specific treatments (all $P > 0.2$). Results are shown in Table 2.

3.4. FLUCO + Eth

After co-exposure with Eth (1.7 mM), larvae exposed at embryotoxic concentrations of FLUCO (15.75–250 μM) showed abnormalities at the level of pigmented organs ($F_{4,10} = 4.48$, P = 0.02479), of the trunk ($F_{4,10} = 5.89$, P = 0.01058). Also the incidence of severely malformed larvae was significantly higher than in controls ($F_{4,10} = 3.25$, P = 0.05942) (Table 3). Comparing the larvae developed from embryos exposed to FLUCO alone and from embryos exposed to the FLUCO + 1.7 mM Eth mixture, a worsened picture was evident (Fig. 2) in the total number of malformed larvae (GLM: $F_{1,20} = 7.69$, P = 0.012) and the number of malformed pigmented organs (GLM: $F_{1,20} = 11.89$, P = 0.0025). By contrast, Eth was unable to increase the percentage of FLUCO-related trunk malformation (GLM: $F_{1,20} = 2.25$, P = 0.15) and tail development was not significantly affected by the co-exposure of FLUCO and Eth ($F_{4,20} = 0.02$, $P = 0.883$, Table 3).

3.5. Benchmark analysis

Dose-response curves were described by using a four-parameters exponential equation ($y = a[c-(c-1) \exp (-bx^d)]$), in which a is the background response, b the potency, c the maximum fold change in response, d the steepness. The comparison of FLUCO and Eth curve parameters confirmed the parallelism hypothesis and the RPF of Eth versus FLUCO was calculated and resulted 0.0052 (Fig. 3). The overall dose-response curve obtained fitting all concentrations in FLUCO equivalents showed the p value goodness of fit < 0.05 and indicated Hill family models as a good fit (Fig. 4). Finally, the hypothesis that the mixture effect is at least additive is not rejected after modelling FLUCO, Eth, mixture expressed in FLUCO equivalents. PROAST analysis, in fact, showed that the Benchmark doses 50 are 1763 (FLUCO), 1238 FLUCO equivalents (Eth) and 191 FLUCO equivalents (mixture) and the calculated RPFs of mixture are 4.3 and 4.4 respectively versus FLUCO and Eth (all expressed in FLUCO equivalents) (Figs. 5 and 6).
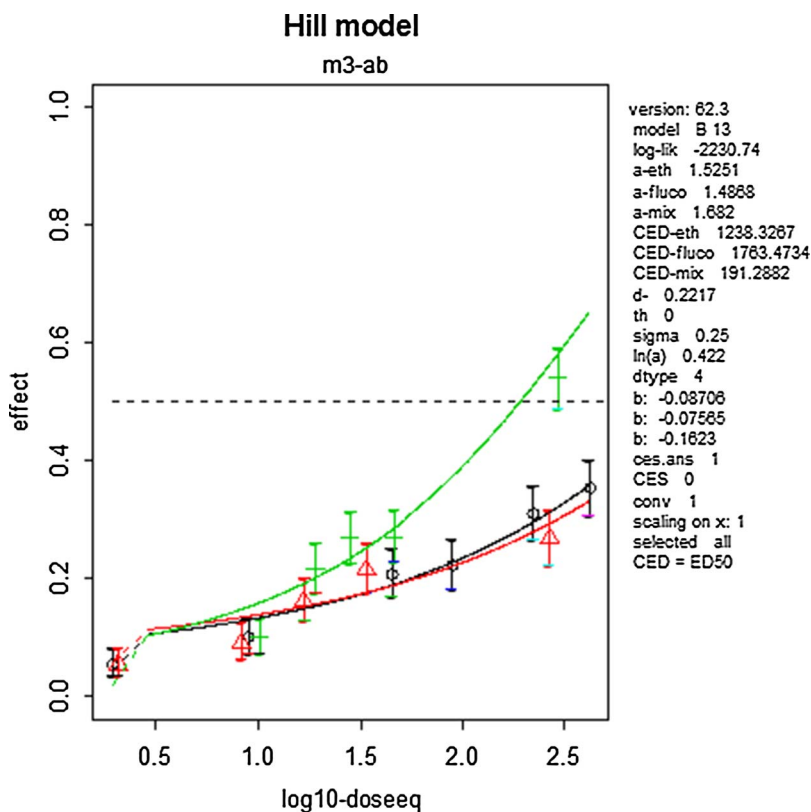
4. Discussion

Results from this study demonstrated that both FLUCO and Eth could influence embryonic development of the ascidian *C. intestinalis*.

The teratogenic potential of Eth had never been previously evaluated in ascidians, while teratogenic effects of FLUCO were previously observed in the ascidian *Phallusia mammillata* at concentrations comparable to those used in the present work (Groppelli et al., 2007). Similarly to results obtained in the present work, *P. mammillata* embryos exposed to FLUCO showed characteristic malformations of the adhesive papillae, the sensory vesicle and the associated nervous network (Pennati et al., 2006; Groppelli et al., 2007). The same alterations of the anterior structures were also observed in this study in *C. intestinalis* embryo exposed to FLUCO.

In vertebrates such as *X. laevis* (FETAX) and rats (WEC), exposure to azole derivatives during embryonic development led to anomalies to the hindbrain, accompanied by characteristic malformations of the branchial apparatus (fusion or reduction of the first and the second branchial arches) which could be considered the target organ of teratogenic activity of azoles (Table 4). As retinoic acid (RA) is implicated in the correct differentiation of the branchial apparatus along the antero-posterior axis, the disruption of the anterior structures in treated embryos suggested that these fungicides could alter RA levels (Groppelli et al., 2005; Menegola et al., 2006a; Menegola et al., 2006b; Di Renzo et al., 2011). In vertebrate embryos, it has been proposed that FLUCO could increase endogenous RA levels by inhibiting the cytochrome P-450 enzyme (CYP26), involved in RA catabolism. Perturbation of RA gradient during neurulation resulted in alteration of the anterior structures with consequent craniofacial malformations (Schwartz et al., 1995; Menegola et al., 2004, 2006a,b).

Since branchial arches are a vertebrate innovation, a direct comparison with ascidians is not possible. Nonetheless, some similarities are evident. In fact, azole derivatives caused malformations principally at the anterior structures of *C. intestinalis* larvae, in particular to the pigmented organs. Moreover, this study, as well as previous studies
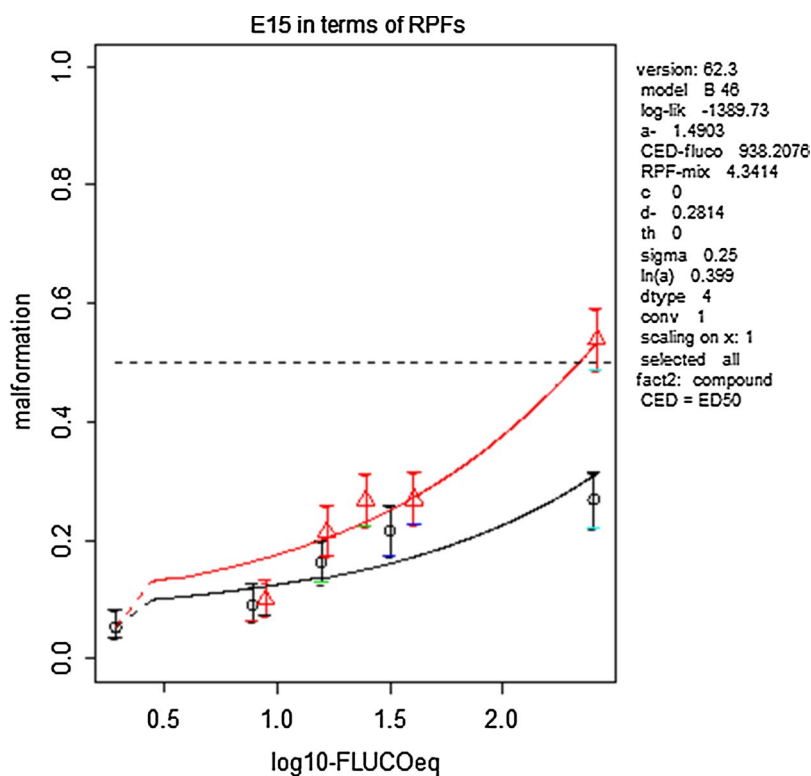

Fig. 4. Comparison between FLUCO (line with triangles), Eth (line with circles) and mixture (line with dashes) dose-response curves, obtained fitting all concentrations in FLUCO equivalents. The overall dose-response curve showed the p value goodness of fit < 0.05 and indicated Hill m3-ab as the best model with covariate. Finally, the hypothesis that the mixture effect is additive is not rejected by PROAST analysis, being the benchmark doses 50 (in FLUCO equivalents) 1763 (FLUCO), 1238 (Eth) and 191 (mixture).

E15 in terms of RPFs

```
version: 62.3
model   B 46
log-lik  -1389.73
a-   1.4903
CED-fluco  938.2078
RPF-mix  4.3414
c    0
d-   0.2814
th   0
sigma  0.25
ln(a)  0.399
dtype  4
conv   1
scaling on x: 1
selected  all
fact2: compound
CED = ED50
```

Fig. 5. Comparison between FLUCO (line with triangles) and mixture (line with circles) dose-response curves, with mixture concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of mixture versus FLUCO resulted 4.3.

reported in literature, demonstrated that azole fungicides could influence the ascidian embryonic development with characteristic malformations resembling those elicited by exogenous RA exposure (Pennati et al., 2006; Groppelli et al., 2007; Kanda et al., 2009; Zega et al., 2009). RA-treated larvae showed dose-dependent malformations of the anterior trunk portion with severe anomalies of the nervous system, the same structure affected by FLUCO action. Considering that

the role of RA cascade and the expression of CYP26 in ascidians has been described and compared to the expression of CYP26 in vertebrate embryos (Nagatomo and Fujiwara, 2003), the specific alteration of anterior structure suggested that the teratogenic action of azole in ascidians could also depend on perturbation of the RA pathway in specific responsive embryonic tissues, similarly to what proposed in vertebrates (Menegola et al., 2003; Di Renzo et al., 2009; Marotta and Tiboni,
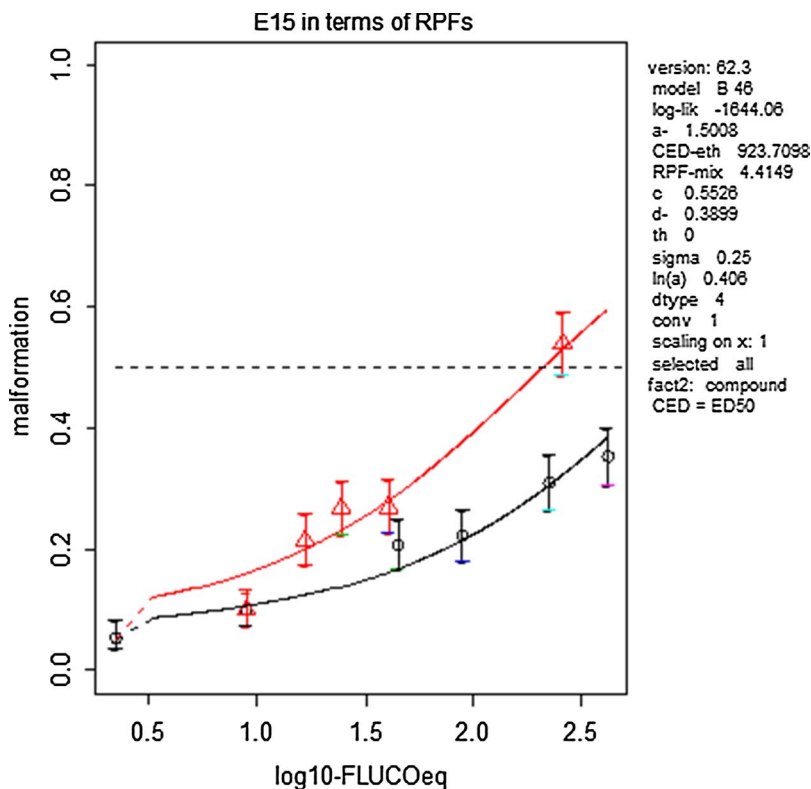
E15 in terms of RPFs

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log-lik  -1644.06
a-   1.5008
CED-eth  923.7098
RPF-mix  4.4149
c    0.5526
d-   0.3899
th   0
sigma  0.25
ln(a)  0.406
dtype  4
conv   1
scaling on x: 1
selected  all
fact2: compound
CED = ED50
```

Fig. 6. Comparison between Eth (line with triangles) and mixture (line with circles) dose-response curves, with all concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of mixture versus Eth resulted 4.4.

Table 4
Published *in vitro* studies assessing the teratogenicity of azoles (fluconazole and triadimefon).

Azole derivative	Species	Dosage	LOAEL.	Results	Ref.
Fluconazole	Mouse	25–50–75 µg/mL	50 µM	Hypoplasia of the II branchial arch at doses ≥50 µM, abnormal shape of the II branchial arch at doses ≥75 µM	Tiboni (1993
	Rat	62.5–125 250 500 µM	125 µM	Reduction of the I branchial arch, agenesis of the II branchial arch, delay in neuropore, optic and otic vesicles development at doses ≥125 µM	Menegola et al. (2001)
	Rat	500 µM	–	Delay in the I branchial arch development, hypoplasia of branchial arches and fusion between the I and the II, severe changes in the distribution of the neural crest cells	Menegola et al. (2003)
	Rat	62.5 or 500 µM	62.5 µM	Hypoplasia of the I branchial arch at doses ≥62.5 µM, fusion between the I and the II branchial arches, fusion of V, VII, IX and X cranial nerve at doses of 500 µM	Menegola et al. (2004)
	Ascidian	30, 62.5, 125, 500, 1250, 2500, 5000 µM	125 µM	Malformations restricted to the anterior end (absence or reduction of the sensory vesicle cavity and reduction of the anterior nervous fibres at doses ≥125 µM	Groppelli et al. (2007)
	Ascidian	7.8, 15.75, 31.5, 250 µM	15.75 µM	Alterations of the anterior structures of the trunk, particularly at the pigmented organs, at doses ≥15.75 µM.	The present study
Triadimefon	Rat	12.5–250 µM	25 µM	Teratogenic effects at level of the I and II branchial arches at doses ≥25 µM	Menegola et al. (2000)
	Rat	250 µM	–	Fusion between the I and the II branchial arches, disorganization and fusion of V, VII, IX and X branchial nerves and VIII cranial nerve	Menegola et al. (2005)
	Frog	1, 2.5, 5, 10, 30, 62.5, 125, 250, 500 µM	2.5 µM	Defects in the mouth and in the anterior-dorsal part of the head at doses ≥250 µM	Groppelli et al. (2005)
	Ascidian	15, 62.5, 250, 750, 1000 µM	7.5 µM	Inhibition of fertilization at doses ≥800 µM, alterations of the anterior structures of the trunk at doses ≥7.5 µM	Pennati et al. (2006)

2010).

As concern Eth, results from the present study showed that Eth is teratogenic in the ascidian *C. intestinalis* as well and that Eth specifically is able to affect, like FLUCO, the pigmented organs in a dose-dependent manner.

The teratogenic effects of Eth in vertebrates have been deeply investigated, but the characterization of the mechanism by which ethanol exerts as teratogen is difficult due to the pleiotropic nature of its action. The teratogenic mechanism identified comprise impaired cell proliferation and enhanced cell death in target districts including the hindbrain and the branchial apparatus (Wentzel and Eriksson, 2009); metabolic stress (including oxidative stress) (Kotch et al., 1995) and impaired signalling by transcription factor or growth factor (Kotch and Sulik, 1992; Miller and Kuhn, 1995; Holownia et al., 1997; Smith, 1997; Ikonomidou, 2000; Climent et al., 2002; Light et al., 2002; Kilburn et al., 2006; Zhou et al., 2011).

One of the potential mechanisms, by which Eth could act as teratogen, is RA content impairment (Deltour et al., 1996; Kot-Leibovich and Fainsod, 2009; Kane et al., 2010). Early studies suggested that ethanol functions as a competitive inhibitor of the enzymatic conversion of retinol to RA, thereby lowering the overall levels of RA in the embryo (Duester, 1998). However, the exposure to ethanol could have also the opposite effect on RA level in the embryo: alcohol consumption is known to induce the enzymes involved in its catabolism and is related to the mobilisation of retinol stored in mother's liver, leading to increased retinoid exposure of the embryo (Kiecker, 2016). An effect of ethanol on the concentrations of RA likely would have a large impact on processes governed by vitamin A, including cranio-facial morphogenesis.

Due to the similarity of effects at the level of anterior structures in *C. intestinalis* (pigmented organs), in the present study we performed the co-exposure of increasing concentrations of FLUCO with the sub-teratogenic concentration of Eth (1.7 mM). Remarkably, the co-exposure significantly increased the incidence of malformed larvae at pigmented organs if compared to the single exposure to FLUCO alone, not rejecting the hypothesis of an additive effect, as evidenced by the Benchmark dose analysis. The mixture effect of the co-exposure to azole fungicides and Eth has never been investigated until now. This evidence, together with the notions that RA is implicated in the correct differentiation of the anterior structures, suggests that the alterations of the pigmented organs could be the result of a perturbation in RA content during early development (Schwartz et al., 1995; Menegola et al., 2004, 2006a,b), giving rise to the hypothesis that Eth and FLUCO could somehow act affecting the RA pathway.

Eth involvement in this process represents an intriguing but still highly speculative hypothesis also for vertebrates and further experiments to confirm azoles and ethanol interferences in RA pathways in mammals are needed.

This work shows that AET assay offers several advantages as teratological screening test for the study of mixture effects when compared to rodents, the more traditional vertebrate organisms used in developmental toxicity research. First, AET is a vertebrate-free approach. Ascidian are invertebrate animals which use is not regulated or restricted by ethical issues. Then, the test can be performed involving high numbers of embryos, allowing triplicates, within few days. The screening of malformations do not required particular expertise since the simple anatomy of the animals. Moreover, the phylogenetic proximity of ascidians to vertebrates can allow a genetic approach.

Concerning the issue of exposure to mixtures, the use of alternative models in toxicology has been strictly recommended to evaluate the potential effects of the co-exposure of chemicals in a mixture (EPA, 2002). Considering that ascidians are basal chordates recognized as belonging to the sister group of vertebrates (Delsuc et al., 2006), they could be considered good candidates for this purpose, and the AET assay could be considered indicative for deep toxicological investigation in mammal embryos.

5. Conclusions

AET assay demonstrated to be a good screening model for embryo xenobiotic exposure alone or in mixture and it is a useful tool to apply the Benchmark modelling.

The results highlight the potential mixture effect that could occur after exposure to azoles and Eth, suggesting a precautionary position in alcohol consumption during azoles exposure in pregnancy, and encourage the use of AET as a complementary alternative method for embryotoxicity studies.

The present work was performed using an invertebrate species not subjected to regulative guidelines.

Conflict of interest

The authors declare that they have no conflict of interest.

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