Accepted Manuscript

Title: 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.

Authors: Maria Battistoni, Silvia Mercurio, Gentile Francesco Ficetola, Francesca Cristiana Metruccio, Elena Menegola, Roberta Pennati

PII: S1382-6689(17)30320-4
DOI: https://doi.org/10.1016/j.etap.2017.11.014
Reference: ENVTOX 2918

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 15-9-2017
Revised date: 27-11-2017
Accepted date: 28-11-2017

Please cite this article as: Battistoni, Maria, Mercurio, Silvia, Ficetola, Gentile Francesco, Metruccio, Francesca Cristiana, Menegola, Elena, Pennati, Roberta, 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.Environmental Toxicology and Pharmacology https://doi.org/10.1016/j.etap.2017.11.014

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.

Maria Battistoni[1], Silvia Mercurio[1], Gentile Francesco Ficetola[1], Francesca Cristiana Metruccio[2], Elena Menegola[1] and Roberta Pennati[1]

[1] Department of Environmental Sciences and Policy, Università degli Studi di Milano, via Celoria 26, 20133 Milan, Italy

[2] International Centre for Pesticides and Health Risk Prevention (ICPS), University Hospital Luigi Sacco, via G.B. Grassi 74, 20157 Milan, Italy

Corresponding author: Prof. Elena Menegola

via Celoria, 26

20133 Milan, Italy

mail: **e**lena.menegola@unimi.it

phone: +39-02-50314804

fax: +39-02-50314802

Highlights

- *Ciona intestinalis* embryos as new 3R invertebrate alternative model for embryotoxicity
- the study is in line with the modern toxicological topics and approaches on combined effects
- a useful model and modelling approach to understand the mechanism of action of substances

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 intestinali*s 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.

Keywords: Alternative test, Ascidian, Fluconazole, Ethanol, Mixture, Embryotoxicity

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 hours 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 ~2,600 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 ($n \approx 300$ 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. 1 C 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. 1 E 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 1 G);

- 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. 1 H).

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-ab).

3. Results

3.1 Morphological analysis of induced abnormalities

Nearly 80% of unexposed larvae (Tab. 1; Tab. 2; Tab. 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. 1 A 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. C – H). Frequency ± standard error (%) and distribution of the observed abnormalities in larvae of the different experimental groups are reported in Table 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: $F_{5,12} = 4.67$, $P = 0.013$) (Tab. 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) (Tab. 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: 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 Tab. 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) (Tab. 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, Tab. 3).

3.2 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) (Fig. 5 and Fig.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 (Tab. 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., 2006; 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, 2006).

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 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, 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, 2006), 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.

Acknowledgements

This work was sustained by the Environmental Science PhD course founds of Università degli Studi di Milano.

Conflict of interest

The authors declare that they have no conflict of interest.

Figure captions

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.

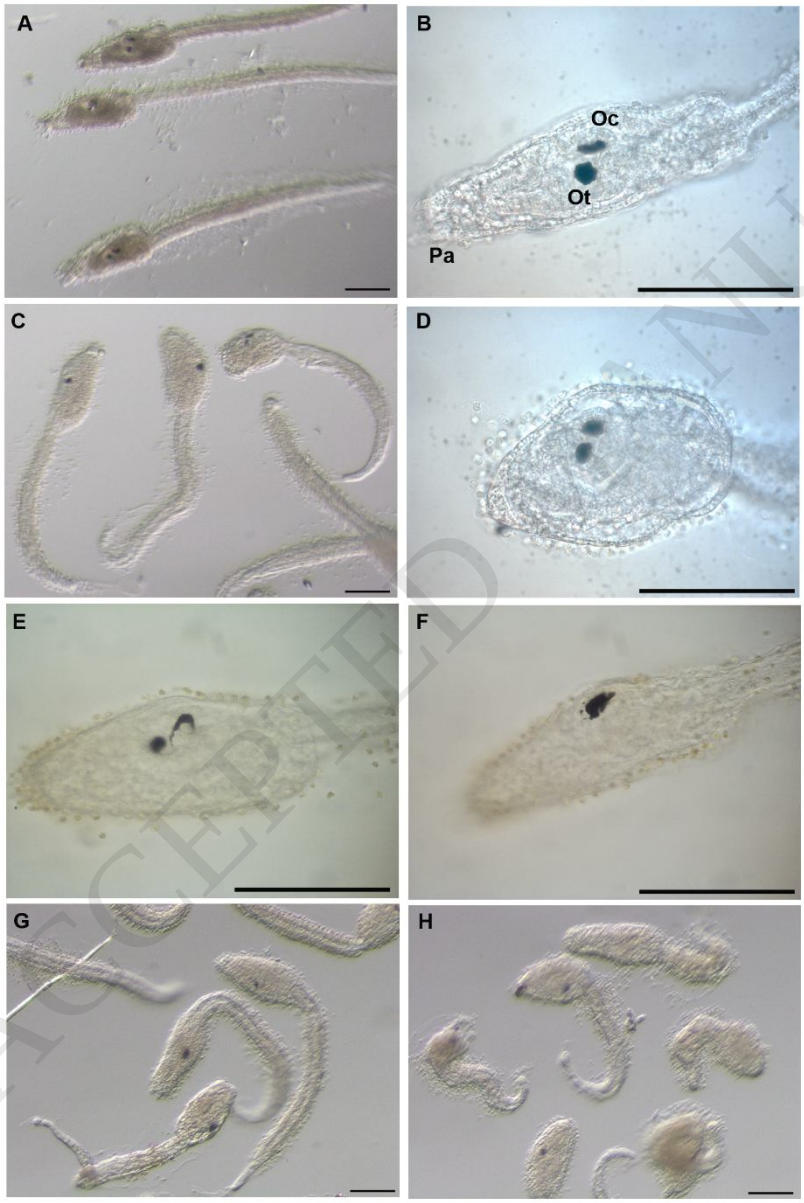


Fig. 2. Histograms showing the percentage of total malformed larvae (A) and the percentage of larvae with pigmented organs abnormalities (B) in experimental groups exposed to increasing FLUCO concentrations (blue bars) compared to groups co-exposed to FLUCO + Eth 1.7 mM (red bars).
* = The co-exposure to the sub-teratogenic concentration of Eth (1.7 mM) is statistically significant compared to FLUCO dose alone.

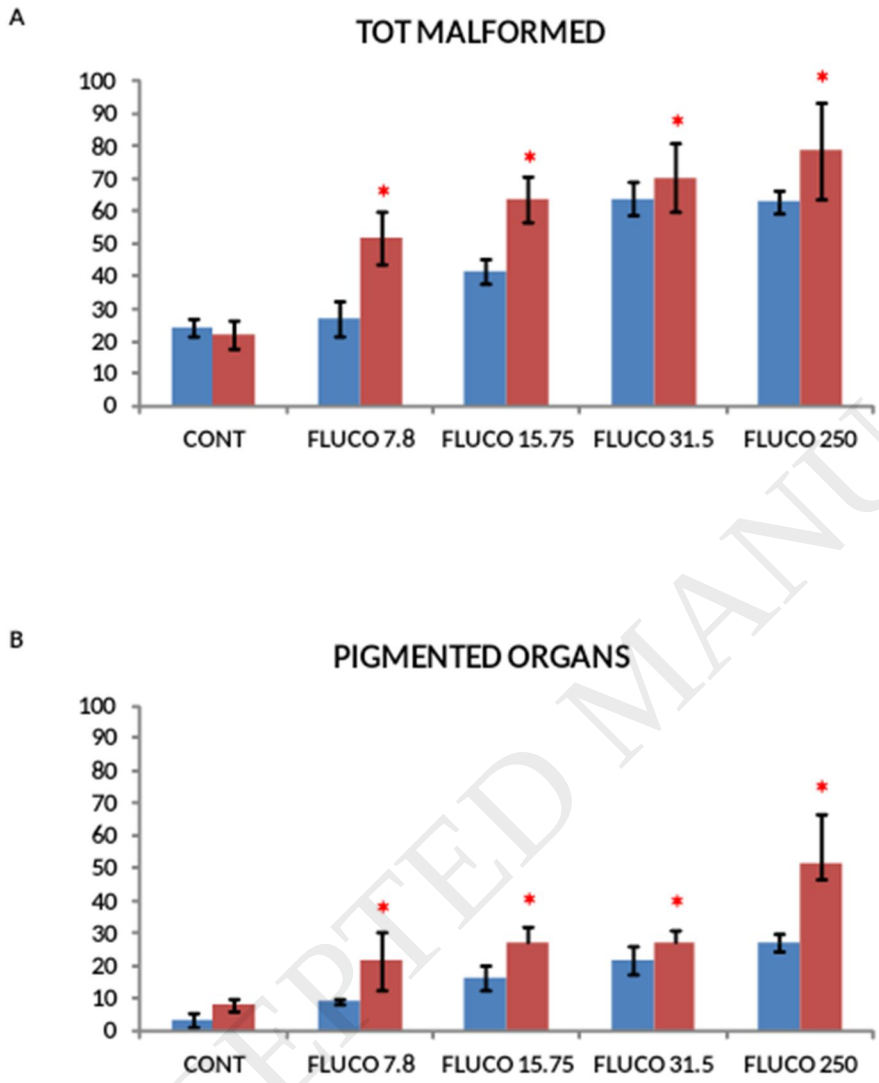


Fig. 3. Comparison between FLUCO (red) and Eth (black) dose-response curves, with Eth concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of Eth versus FLUCO resulted 0.0052 confirming the parallelism hypothesis.

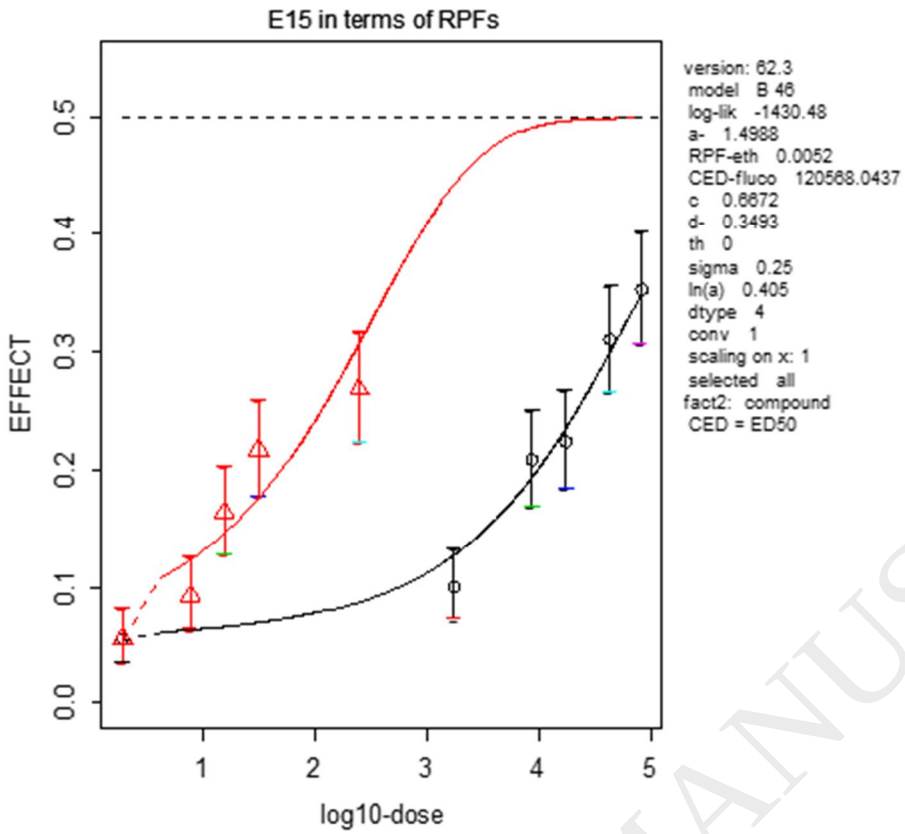


Fig. 4. Comparison between FLUCO (red), Eth (black) and mixture (green) dose-response curves, obtained fitting all concentrations in FLUCO equivalents. The overall dose-response curve showed the p value goodness of fit <0.05 and indicated Hill m3-ab as the best model with covariate. Finally, the hypothesis 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).

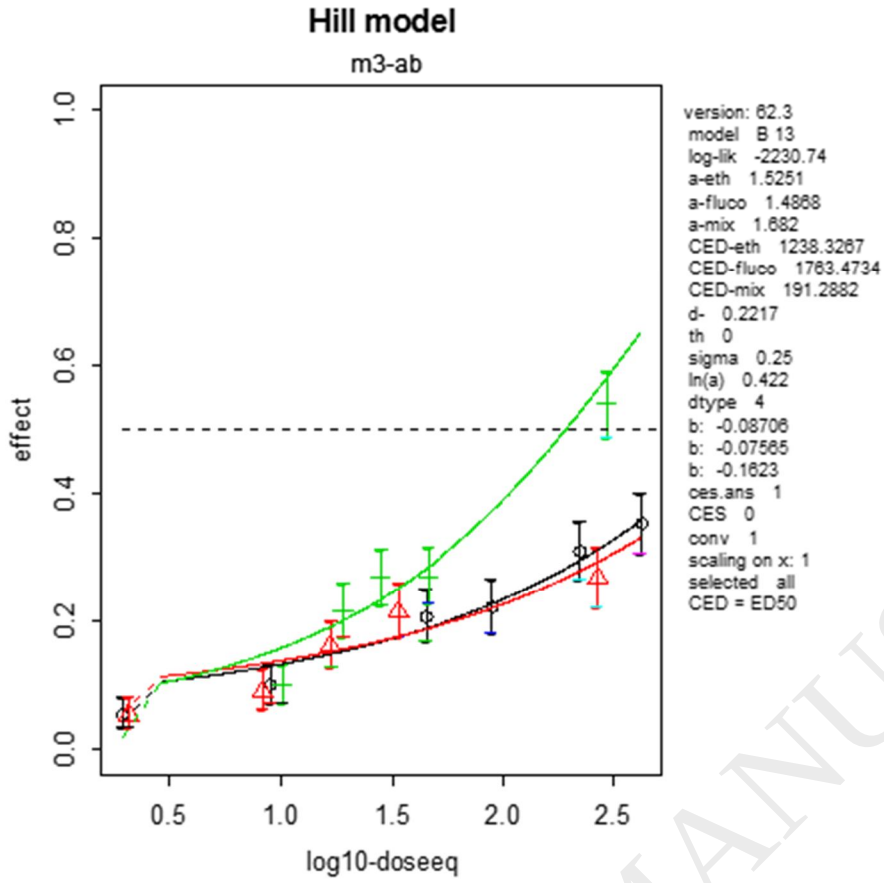


Fig. 5. Comparison between FLUCO (red) and mixture (black) dose-response curves, with mixture concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of mixture versus FLUCO resulted 4.3.

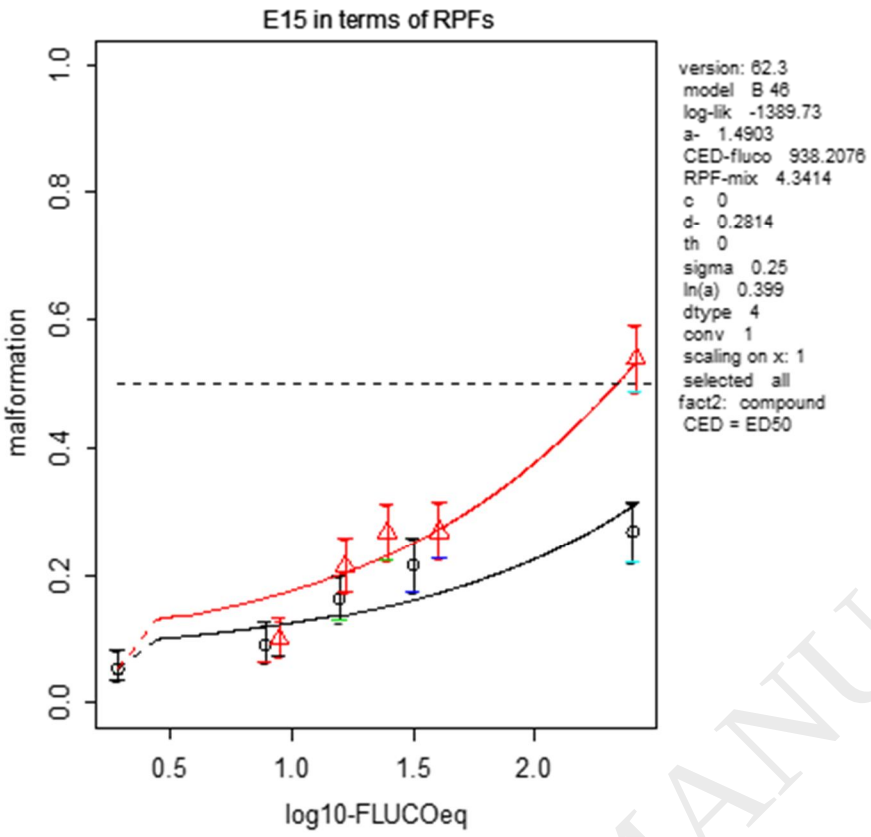
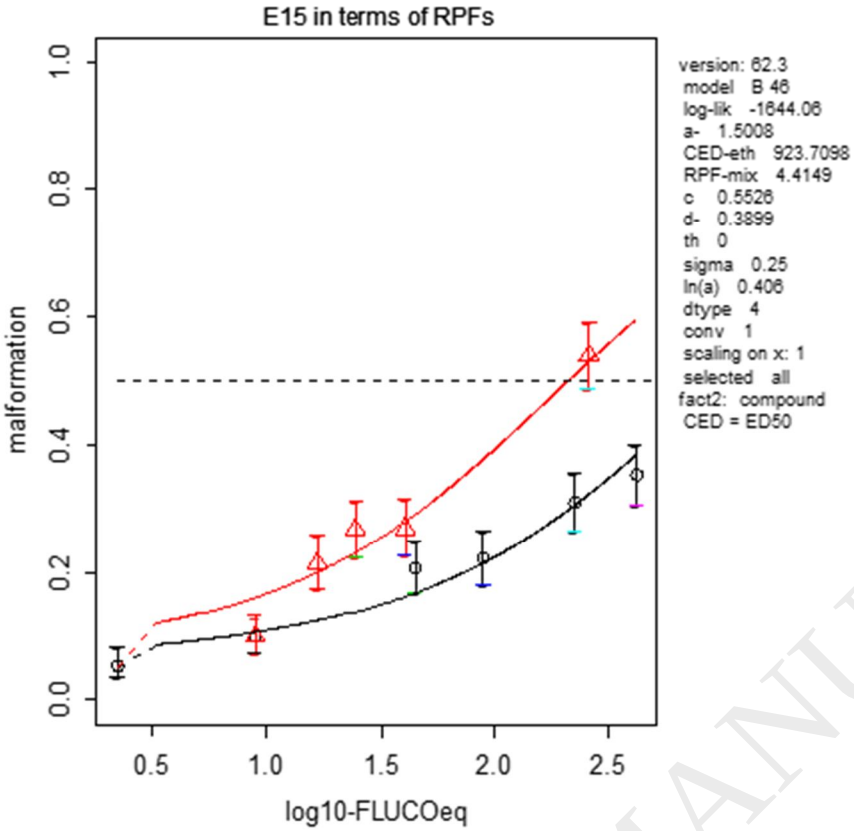


Fig. 6. Comparison between Eth (red) and mixture (black) dose-response curves, with all concentrations expressed in FLUCO equivalents. The relative potency factor (RPF) of mixture versus Eth resulted 4.4.



Tab. 1. Frequency ± standard error (%) and distribution of the observed abnormalities in larvae exposed to increasing Eth concentration. The pigmented organs and the total malformed larvae show statistically significant dose response.

Tab. 2. Frequency ± standard error (%) and distribution of the observed abnormalities in larvae exposed to increasing FLUCO concentrations (µM). The pigmented organs, the trunk and the total malformed larvae show statistically significant dose response.

Tab. 3. Frequency ± standard error (%) and distribution of the observed abnormalities in larvae co-exposed to increasing FLUCO concentration (µM) + Eth 1.7 mM The pigmented organs, the trunk and the total malformed larvae show statistically significant dose response.

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

References

Bellas, J., Beiras, R., V?zquez, E., 2004. Sublethal Effects of Trace Metals (Cd, Cr, Cu, Hg) on Embryogenesis and Larval Settlement of the Ascidian Ciona intestinalis. Arch. Environ. Contam. Toxicol. 46, 61–66. doi:10.1007/s00244-003-0238-7

Brown, N.A., Goulding, E.H., Fabro, S., 1979. Ethanol embryotoxicity: direct effects on mammalian embryos in vitro. Science 206, 573–575.

Cairns, T., Siegmund, E.G., Chiu, K.S., Nelson, R., 1989. Residue characterization of triadimefon in grapes by gas chromatography and mass spectrometry/mass spectrometry. Biol. Mass Spectrom. 18, 110–115. doi:10.1002/bms.1200180205

Cangialosi, M.V., Mansueto, V., Faqi, A.S., 2013. Bisphenol A (BPA) and atrazine inhibit the embryonic development of *Ciona intestinalis* (Ascidiacea, Urochordata). Caryologia 66, 97–102. doi:10.1080/00087114.2013.780438

Cartwright, M.M., Smith, S.M., 1995. Increased Cell Death and Reduced Neural Crest Cell Numbers in Ethanol-Exposed Embryos: Partial Basis for the Fetal Alcohol Syndrome Phenotype. Alcohol. Clin. Exp. Res. 19, 378–386. doi:10.1111/j.1530-0277.1995.tb01519.x

Chernoff, G.F., 1977. The fetal alcohol syndrome in mice: An animal model. Teratology 15, 223–229. doi:10.1002/tera.1420150303

Cima, F., Ballarin, L., Bressa, G., Martinucci, G., Burighel, P., 1996. Toxicity of Organotin Compounds on Embryos of a Marine Invertebrate (Styela plicata;Tunicata). Ecotoxicol. Environ. Saf. 35, 174–182. doi:10.1006/eesa.1996.0097

Climent, E., Pascual, M., Renau-Piqueras, J., Guerri, C., 2002. Ethanol exposure enhances cell death in the developing cerebral cortex: Role of brain-derived neurotrophic factor and its signaling pathways. J. Neurosci. Res. 68, 213–225. doi:10.1002/jnr.10208

Corbo, J.C., Di Gregorio, A., Levine, M., 2001. The Ascidian as a Model Organism in Developmental and Evolutionary Biology. Cell 106, 535–538. doi:10.1016/S0092-8674(01)00481-0

Crawley, M.J., 2007. The R Book. Chichester, UK.

Dahlberg, C., Auger, H., Dupont, S., Sasakura, Y., Thorndyke, M., Joly, J.-S., 2009. Refining the Ciona intestinalis Model of Central Nervous System Regeneration. PLoS ONE 4, e4458. doi:10.1371/journal.pone.0004458

Davidson, B., 2007. Ciona intestinalis as a model for cardiac development. Semin. Cell Dev. Biol. 18, 16–26. doi:10.1016/j.semcdb.2006.12.007

De Bernardi, F., Sotgia, C., Ortoloani, G., 1994. Retinoic acid treatment of ascidian embryos: effects on larvae and metamorphosis. Anim Biol 3, 75–81.

de Sanctis, L., Memo, L., Pichini, S., Tarani, L., Vagnarelli, F., 2011. Fetal alcohol syndrome: new perspectives for an ancient and underestimated problem. J. Matern. Fetal Neonatal Med. 24, 34–37. doi:10.3109/14767058.2011.607576

Debruyne, D., 1997. Clinical Pharmacokinetics of Fluconazole in Superficial and Systemic Mycoses: Clin. Pharmacokinet. 33, 52–77. doi:10.2165/00003088-199733010-00005

Dehal, P., 2002. The Draft Genome of Ciona intestinalis: Insights into Chordate and Vertebrate Origins. Science 298, 2157–2167. doi:10.1126/science.1080049

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439, 965–968. doi:10.1038/nature04336

Deltour, L., Ang, H.L., Duester, G., 1996. Ethanol inhibition of retinoic acid synthesis as a potential mechanism for fetal alcohol syndrome. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 10, 1050–1057.

Di Gregorio, A., Levine, M., 1998. Ascidian embryogenesis and the origins of the chordate body plan. Curr. Opin. Genet. Dev. 8, 457–463. doi:10.1016/S0959-437X(98)80118-4

Di Renzo, F., Corsini, E., Broccia, M.L., Marinovich, M., Galli, C.L., Giavini, E., Menegola, E., 2009. Molecular mechanism of teratogenic effects induced by the fungicide triadimefon: Study of the expression of TGF-β mRNA and TGF-β and CRABPI proteins during rat in vitro development. Toxicol. Appl. Pharmacol. 234, 107–116. doi:10.1016/j.taap.2008.09.025

Di Renzo, Bacchetta, R., Bizzo, A., Giavini, E., Menegola, E., 2011. Is the amphibian X. laevis WEC a good alternative method to rodent WEC teratogenicity assay? The example of the three triazole derivative fungicides Triadimefon, Tebuconazole, Cyproconazole. Reprod. Toxicol. 32, 220–226. doi:10.1016/j.reprotox.2011.05.001

Duester, G., 1998. Alcohol dehydrogenase as a critical mediator of retinoic acid synthesis from vitamin A in the mouse embryo. J. Nutr. 128, 459S–462S.

EPA, 2002. Guidance on cumulative risk assessment of pesticide chemicals that have a common mechanism of toxicity.

Flak, A.L., Su, S., Bertrand, J., Denny, C.H., Kesmodel, U.S., Cogswell, M.E., 2014. The Association of Mild, Moderate, and Binge Prenatal Alcohol Exposure and Child Neuropsychological Outcomes: A Meta-Analysis. Alcohol. Clin. Exp. Res. 38, 214–226. doi:10.1111/acer.12214

Giavini, E., Broccia, M.L., Prati, M., Bellomo, D., Menegola, E., 1992. Effects of ethanol and acetaldehyde on rat embryos developing in vitro. Vitro Cell. Dev. Biol. - Anim. 28, 205–210. doi:10.1007/BF02631093

Groppelli, S., Pennati, R., De Bernardi, F., Menegola, E., Giavini, E., Sotgia, C., 2005. Teratogenic effects of two antifungal triazoles, triadimefon and triadimenol, on Xenopus laevis development: Craniofacial defects. Aquat. Toxicol. 73, 370–381. doi:10.1016/j.aquatox.2005.04.004

Groppelli, S., Zega, G., Biggiogero, M., De Bernardi, F., Sotgia, C., Pennati, R., 2007. Fluconazole induces teratogenic effects in the tunicate Phallusia mammillata. Environ. Toxicol. Pharmacol. 23, 265–271. doi:10.1016/j.etap.2006.11.005

Hamey, P.Y., Harris, C.A., 1999. The Variation of Pesticide Residues in Fruits and Vegetables and the Associated Assessment of Risk. Regul. Toxicol. Pharmacol. 30, S34–S41. doi:10.1006/rtph.1999.1324

Hinman, V.F., Degnan, B.M., 1998. Retinoic acid disrupts anterior ectodermal and endodermal development in ascidian larvae and postlarvae. Dev. Genes Evol. 208, 336–345.

Hof, H., 2001. Critical Annotations to the Use of Azole Antifungals for Plant Protection. Antimicrob. Agents Chemother. 45, 2987–2990. doi:10.1128/AAC.45.11.2987-2990.2001

Holownia, A., Ledig, M., Ménez, J.-F., 1997. Ethanol-induced cell death in cultured rat astroglia. Neurotoxicol. Teratol. 19, 141–146. doi:10.1016/S0892-0362(96)00226-7

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. Biom. J. 50, 346–363. doi:10.1002/bimj.200810425

Hotta, K., Mitsuhara, K., Takahashi, H., Inaba, K., Oka, K., Gojobori, T., Ikeo, K., 2007. A web-based interactive developmental table for the ascidian *Ciona intestinalis* , including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. Dev. Dyn. 236, 1790–1805. doi:10.1002/dvdy.21188

Huang, Q., Yu, Y., Tang, C., Peng, X., 2010. Determination of commonly used azole antifungals in various waters and sewage sludge using ultra-high performance liquid chromatography?tandem mass spectrometry. J. Chromatogr. A 1217, 3481–3488. doi:10.1016/j.chroma.2010.03.022

Ikonomidou, C., 2000. Ethanol-Induced Apoptotic Neurodegeneration and Fetal Alcohol Syndrome. Science 287, 1056–1060. doi:10.1126/science.287.5455.1056

Jones, K., Smith, D., 1973. RECOGNITION OF THE FETAL ALCOHOL SYNDROME IN EARLY INFANCY. The Lancet 302, 999–1001. doi:10.1016/S0140-6736(73)91092-1

Joya, X., Friguls, B., Ortigosa, S., Papaseit, E., Martínez, S.E., Manich, A., Garcia-Algar, O., Pacifici, R., Vall, O., Pichini, S., 2012. Determination of maternal-fetal biomarkers of prenatal exposure to ethanol: A review. J. Pharm. Biomed. Anal. 69, 209–222. doi:10.1016/j.jpba.2012.01.006

Kahle, M., Buerge, I.J., Hauser, A., Müller, M.D., Poiger, T., 2008. Azole Fungicides: Occurrence and Fate in Wastewater and Surface Waters. Environ. Sci. Technol. 42, 7193–7200. doi:10.1021/es8009309

Kanda, M., Wada, H., Fujiwara, S., 2009. Epidermal expression of Hox1 is directly activated by retinoic acid in the Ciona intestinalis embryo. Dev. Biol. 335, 454–463. doi:10.1016/j.ydbio.2009.09.027

Kane, M.A., Folias, A.E., Wang, C., Napoli, J.L., 2010. Ethanol elevates physiological all-trans-retinoic acid levels in select loci through altering retinoid metabolism in multiple loci: a potential mechanism of ethanol toxicity. FASEB J. 24, 823–832. doi:10.1096/fj.09-141572

Kiecker, C., 2016. The chick embryo as a model for the effects of prenatal exposure to alcohol on craniofacial development. Dev. Biol. 415, 314–325. doi:10.1016/j.ydbio.2016.01.007

Kilburn, B.A., Chiang, P.J., Wang, J., Flentke, G.R., Smith, S.M., Armant, D.R., 2006. Rapid Induction of Apoptosis in Gastrulating Mouse Embryos by Ethanol and Its Prevention by HB-EGF. Alcohol. Clin. Exp. Res. 30, 127–134. doi:10.1111/j.1530-0277.2006.00008.x

Kotch, L.E., Chen, S.-Y., Sulik, K.K., 1995. Ethanol-induced teratogenesis: Free radical damage as a possible mechanism. Teratology 52, 128–136. doi:10.1002/tera.1420520304

Kotch, L.E., Sulik, K.K., 1992. Experimental fetal alcohol syndrome: Proposed pathogenic basis for a variety of associated facial and brain anomalies. Am. J. Med. Genet. 44, 168–176. doi:10.1002/ajmg.1320440210

Kot-Leibovich, H., Fainsod, A., 2009. Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation. Dis. Model. Mech. 2, 295–305. doi:10.1242/dmm.001420

Laverda, A.M., Ruga, E., Pagliaro, A., Pinello, M.L., Giaquinto, C., 1996. Intracranial hypertension and cryptococcal meningitis in a girl with AIDS. Brain Dev. 18, 330–331. doi:10.1016/0387-7604(96)00022-8

Light, K.., Belcher, S.., Pierce, D.., 2002. Time course and manner of Purkinje neuron death following a single ethanol exposure on postnatal day 4 in the developing rat. Neuroscience 114, 327–337. doi:10.1016/S0306-4522(02)00344-5

Marotta, F., Tiboni, G.M., 2010. Molecular aspects of azoles-induced teratogenesis. Expert Opin. Drug Metab. Toxicol. 6, 461–482. doi:10.1517/17425251003592111

Mastroiacovo, P., Mazzone, T., Botto, L.D., Serafini, M.A., Finardi, A., Caramelli, L., Fusco, D., 1996. Prospective assessment of pregnancy outcomes after first-trimester exposure to fluconazole. Am. J. Obstet. Gynecol. 175, 1645–1650. doi:10.1016/S0002-9378(96)70119-9

Matsushima, A., Ryan, K., Shimohigashi, Y., Meinertzhagen, I.A., 2013. An endocrine disruptor, bisphenol A, affects development in the protochordate Ciona intestinalis: Hatching rates and swimming behavior alter in a dose-dependent manner. Environ. Pollut. 173, 257–263. doi:10.1016/j.envpol.2012.10.015

Memo, L., Gnoato, E., Caminiti, S., Pichini, S., Tarani, L., 2013. Fetal alcohol spectrum disorders and fetal alcohol syndrome: the state of the art and new diagnostic tools. Early Hum. Dev. 89, S40–S43. doi:10.1016/S0378-3782(13)70013-6

Menegola, E., Broccia, M.L., Di Renzo, F., Giavini, E., 2006. Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. Reprod. Toxicol. 22, 186–195. doi:10.1016/j.reprotox.2006.04.008

Menegola, Broccia, M.L., Di Renzo, F., Giavini, E., 2003. Pathogenic pathways in fluconazole-induced branchial arch malformations. Birt. Defects Res. A. Clin. Mol. Teratol. 67, 116–124. doi:10.1002/bdra.10022

Menegola, Broccia, M.L., Di Renzo, F., Massa, V., Giavini, E., 2004. Relationship between hindbrain segmentation, neural crest cell migration and branchial arch abnormalities in rat embryos exposed to fluconazole and retinoic acid in vitro. Reprod. Toxicol. 18, 121–130. doi:10.1016/j.reprotox.2003.09.004

Menegola, Broccia, M.L., Renzo, F.D., Giavini, E., 2006. Dysmorphogenic effects of some fungicides derived from the imidazole on rat embryos cultured in vitro. Reprod. Toxicol. 21, 74–82. doi:10.1016/j.reprotox.2005.07.008

Miller, M.W., Kuhn, P.E., 1995. Cell Cycle Kinetics in Fetal Rat Cerebral Cortex: Effects of Prenatal Treatment with Ethanol Assessed by a Cumulative Labeling Technique with Flow Cytometry. Alcohol. Clin. Exp. Res. 19, 233–237. doi:10.1111/j.1530-0277.1995.tb01497.x

Nagatomo, K., Fujiwara, S., 2003. Expression of Raldh2, Cyp26 and Hox-1 in normal and retinoic acid-treated Ciona intestinalis embryos. Gene Expr. Patterns 3, 273–277. doi:10.1016/S1567-133X(03)00051-6

Nagatomo, K., Ishibashi, T., Satou, Y., Satoh, N., Fujiwara, S., 2003. Retinoic acid affects gene expression and morphogenesis without upregulating the retinoic acid receptor in the ascidian Ciona intestinalis. Mech. Dev. 120, 363–372. doi:10.1016/S0925-4773(02)00441-0

Nakatsuji, N., 1983. Craniofacial malformation in Xenopus laevis tadpoles caused by the exposure of early embryos to ethanol. Teratology 28, 299–305. doi:10.1002/tera.1420280220

Passamaneck, Y.J., Di Gregorio, A., 2005. Ciona intestinalis: Chordate development made simple. Dev. Dyn. 233, 1–19. doi:10.1002/dvdy.20300

Pennati, R., Groppelli, S., Zega, G., Biggiogero, M., De Bernardi, F., Sotgia, C., 2006. Toxic effects of two pesticides, Imazalil and Triadimefon, on the early development of the ascidian Phallusia mammillata (Chordata, Ascidiacea). Aquat. Toxicol. 79, 205–212. doi:10.1016/j.aquatox.2006.05.012

Randall, C.L., Taylor, W.J., Walker, D.W., 1977. Ethanol-Induced Malformations in Mice. Alcohol. Clin. Exp. Res. 1, 219–224. doi:10.1111/j.1530-0277.1977.tb05876.x

Santos, S.R.C.J., Campos, E.V., Sanches, C., Gomez, D.S., Ferreira, M.C., 2010. Fluconazole plasma concentration measurement by liquid chromatography for drug monitoring of burn patients. Clinics 65, 237–243. doi:10.1590/S1807-59322010000200017

Sasakura, Y., Mita, K., Ogura, Y., Horie, T., 2012. Ascidians as excellent chordate models for studying the development of the nervous system during embryogenesis and metamorphosis. Dev. Growth Differ. 54, 420–437. doi:10.1111/j.1440-169X.2012.01343.x

Satoh, 1994. Developmental Biology of Ascidians. Camb. Univ Press N. Y.

Satoh, N., 1995. Chasing tails in ascidians: developmental insights into the origin and evolution of chordates. Trends Genet. 11, 354–359. doi:10.1016/S0168-9525(00)89106-4

Schwartz, E.L., Hallam, S., Gallagher, R.E., Wiernik, P.H., 1995. Inhibition of all-trans-retinoic acid metabolism by fluconazole in vitro and in patients with acute promyelocytic leukemia. Biochem. Pharmacol. 50, 923–928. doi:10.1016/0006-2952(95)00213-J

Simmen, M.W., Leitgeb, S., Clark, V.H., Jones, S.J., Bird, A., 1998. Gene number in an invertebrate chordate, Ciona intestinalis. Proc. Natl. Acad. Sci. U. S. A. 95, 4437–4440.

Smith, T.L., 1997. Regulation of glutamate uptake in astrocytes continuously exposed to ethanol. Life Sci. 61, 2499–2505. doi:10.1016/S0024-3205(97)00985-5

Streissguth, A., Landesman-Dwyer, S., Martin, J., Smith, D., 1980. Teratogenic effects of alcohol in humans and laboratory animals. Science 209, 353–361. doi:10.1126/science.6992275

Sulik, K., Johnston, M., Webb, M., 1981. Fetal alcohol syndrome: embryogenesis in a mouse model. Science 214, 936–938. doi:10.1126/science.6795717

Thomas, K.V., Hilton, M.J., 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Mar. Pollut. Bull. 49, 436–444. doi:10.1016/j.marpolbul.2004.02.028

Tiboni, G.M., 1993. Second branchial arch anomalies induced by fluconazole, a bis-triazole antifungal agent, in cultured mouse embryos. Res. Commun. Chem. Pathol. Pharmacol. 79, 381–384.

Van Wauwe, J.P., Coene, M.C., Goossens, J., Cools, W., Monbaliu, J., 1990. Effects of cytochrome P-450 inhibitors on the in vivo metabolism of all-trans-retinoic acid in rats. J. Pharmacol. Exp. Ther. 252, 365–369.

Vanden Bossche, H., 1985. Biochemical Targets for Antifungal Azole Derivatives: Hypothesis on the Mode of Action, in: McGinnis, M.R. (Ed.), Current Topics in Medical Mycology. Springer New York, New York, NY, pp. 313–351.

Vanier, K.L., Mattiussi, A.J., Johnston, D.L., 2003. Interaction of all-trans-retinoic acid with fluconazole in acute promyelocytic leukemia. J. Pediatr. Hematol. Oncol. 25, 403–404.

Wentzel, P., Eriksson, U.J., 2009. Altered gene expression in neural crest cells exposed to ethanol in vitro. Brain Res. 1305, S50–S60. doi:10.1016/j.brainres.2009.08.057

Zarn, J.A., Brüschweiler, B.J., Schlatter, J.R., 2002. Azole Fungicides Affect Mammalian Steroidogenesis by Inhibiting Sterol 14α-Demethylase and Aromatase. Environ. Health Perspect. 111, 255–261. doi:10.1289/ehp.5785

Zega, G., De Bernardi, F., Groppelli, S., Pennati, R., 2009. Effects of the azole fungicide Imazalil on the development of the ascidian Ciona intestinalis (Chordata, Tunicata): Morphological and molecular characterization of the induced phenotype. Aquat. Toxicol. 91, 255–261. doi:10.1016/j.aquatox.2008.11.015

Zhou, F.C., Zhao, Q., Liu, Y., Goodlett, C.R., Liang, T., McClintick, J.N., Edenberg, H.J., Li, L., 2011. Alteration of gene expression by alcohol exposure at early neurulation. BMC Genomics 12, 124. doi:10.1186/1471-2164-12-124

TAB. 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 **AB**	22.26 ± 2.26 **ABC**	31.00 ± 1.73 **ABCD**	35.33 ± 1.67 **ABCDE**
Trunk	0 ± 0.00	2.33 ± 1.45	4.83 ± 0.60	5.85 ± 0.60	3.33 ± 1.33	9.67 ± 3.39 **AB**
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 **A**	56.96 ± 5.29 **AB**	53.00 ± 6.03 **A**	61.00 ± 2.89 **AB**

A p<0.01 vs. ETH 0
B p<0.01 vs. ETH 1.7 mM
C p<0.01 vs. ETH 8.5 mM
D p<0.01 vs. ETH 17 mM
E p<0.01 vs. ETH 42.5 mM

TAB. 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 **A**	21.66 ± 4.41 **A**	27.18 ± 2.52 **ABC**
Trunk	7.00 ± 1.53	11.76 ± 3.45	21.66 ± 3.18 **A**	41.33 ± 7.70 **AB**	40.39 ± 3.87 **AB**
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 **A**	64.00 ± 5.14 **AB**	62.98 ± 3.46 **AB**

A p<0.01 vs. FLUCO 0 µM

B p<0.01 vs. FLUCO 7.8 µM

C p<0.01 vs. FLUCO 15.75 µM

TAB. 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 0.01% (*N* = 286)	7.8 + ETH 0.01% (*N* = 300)	15.75 + ETH 0.01% (*N* = 300)	31.5 + ETH 0.01% (*N* = 299)	250 + ETH 0.01% (*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 **A**
Trunk	7.55 ± 1.45	24.00 ± 5.51	37.33 ± 6.07 **A**	36.42 ± 6.68 **A**	40.56 ± 9.71 **A**
Severe malformations	3.00 ± 2.08	7.66 ± 2.73	12.33 ± 3.93 **A**	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 **AB**	78.80 ± 14.77 **AB**

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

B p<0.01 vs. FLUCO 7.8 + ETH 1.7 mM

C p<0.01 vs. FLUCO 17.75 + ETH 1.7 mM

TAB. 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)