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Toxic effects of two pesticides, Imazalil and Triadimefon, on the early development of the ascidian *Phallusia mammillata* (Chordata, Ascidiacea)

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Abstract

Azole compounds are fungicides used in agriculture and in clinical area and are suspected to produce craniofacial malformations in vertebrates. Toxicity tests on sperm viability, fertilization and embryogenesis of the solitary ascidian *Phallusia mammillata* were performed to evaluate the effects of two azole derivatives, Imazalil and Triadimefon. Ascidian (Chordata, Ascidiacea) embryos and larvae could provide biological criteria for seawater quality standards because the larvae are lecitotrophic and have a short pelagic period, allowing to run the larval toxicity tests over a short period of time. Imazalil and Triadimefon proved to have strong consequences on *P. mammillata*. They could influence the reproductive rate of the animal exerting their effects at different levels: acting as spermiotoxic agents, inhibiting fertilization and impairing embryological development. Fertilization rate significantly decreased after 30 min exposure of sperm to 25 μM Imazalil ($P < 0.0001$) and after exposure of both gametes to 50 μM Imazalil ($P < 0.05$) and 1 mM Triadimefon ($P < 0.0001$) as compared to controls. Malformations caused by exposure of embryos to both substances were dose dependent. Imazalil median teratogenic concentration (TC_{50} concentration, the concentration that resulted in 50% malformed larvae) value was 0.67 μM and median lethal concentration (LC_{50}, the concentration that resulted in 50% embryos dead before completing the development) value was 10.23 μM while for Triadimefon TC_{50} value was 29.56 and LC_{50} value was 173.7 μM. Larvae developed from embryos treated with Imazalil and Triadimefon showed alterations of the anterior structures of the trunk: papillary nerves and the anterior central nervous system failed to correctly differentiate, as showed by immunostaining with anti-β-tubulin antibody. Comparing the anomalies caused by retinoic acid, reported in a previous study, it was possible to hypothesize that malformations induced by Imazalil and Triadimefon could be due to a perturbation of the endogenous retinoid content, as it has been proposed for mammals. Ascidians proved to be good models to study the toxic effects of pesticides since they offered both the convenience of working with an invertebrate species and the tissue sensitivity to chemical compound comparable to vertebrates.

Keywords: Azole derivatives; Pesticides; Embryotoxicity; *Phallusia mammillata*; Marine invertebrate; Retinoic acid

1. Introduction

Fungicides, insecticides and other industrial compounds can be detected at relatively high concentration in the water, the sediments and the biota. Their uncontrolled use may cause profound effects and a long-term environmental impact on natural aquatic environments (Bellas et al., 2004). Imazalil (IMA: 1-[2-(2,4-dichlorophenyl)-2(2-propenyloxy)ethyl]-1H-imidazole) an imidazole fungicide and Triadimefon (FON: 1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone), a triazole derivative, are both used in agriculture to control a wide range of fungi on fruit and vegetables (Ortelli et al., 2005; Menegola et al., 2000). These compounds interfere with the cellular permeability of pathogenic fungi and, as other azole derivatives, they belong to the demethylation inhibitor group of fungicides, which inhibit a cytochrome P450 dependent enzyme, involved in fungine cell wall synthesis (Vanden Bossche et al., 1989). Azole derivatives have been reported to exert teratogenic effects on vertebrates, inducing craniofacial malformations and affecting specifically the morphogenesis of the branchial apparatus (Menegola et al., 2005, 2006; Groppelli et al., 2005). The aim of the present work was to test the effects of azole derivatives on the early developmental stages of an aquatic invertebrate. IMA and FON, which are intensively used in agronomic field and

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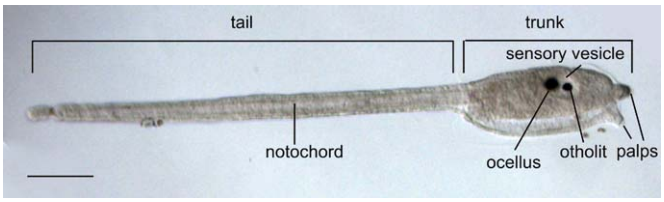

Fig. 1. Swimming larva of *Phallusia mammillata*. Scale bar: 100 μm.

whose teratogenic effects are well described in vertebrates, were chosen as representatives of this class of molecules (Menegola et al., 2005, 2006). Bioassays on invertebrates have been proposed to test potential water pollutants. Early developmental stages of marine invertebrates have been shown to be more responsive to toxicants than adults (Ringwood, 1992) and have been used for assessing the biological quality of seawater and marine sediments. In particular, early developmental stages of sea urchins and bivalves have frequently been used to trace pollutions (Burovina et al., 1982; Warnau and Pagano, 1994; Warnau et al., 1996). However, data concerning toxicity of pesticides on fertilization and embryogenesis of marine chordates are scarce. Ascidians are marine sessile animals belonging to the subphylum Urochordata, phylum Chordata. They develop through a swimming lecitotrophic larva, which has a short pelagic period. The larva possesses the basic features of the chordate body plan with a simpler body organization than a vertebrate. A typical ascidian larva is formed by two regions: the trunk bearing three mucus secreting organs, the palps, and the tail supported by the notochord, flanked by muscle cells at both sides. The central nervous system is a hollow dorsal tube, which comprises an anterior sensory vesicle bearing two pigmented sensory organs, the otolith and the ocellus (Nicol and Meinertzhagen, 1991) (Fig. 1). Recently, ascidian embryos and larvae have been proposed as suitable models to evaluate the toxicity of organotin and metals compounds (Cima et al., 1996; Bellas et al., 2001, 2004, 2005).

In this work, the effects of Imazalil and Triadimefon on sperm viability, fertilization and embryogenesis of the solitary ascidian *Phallusia mammillata* were evaluated. This ascidian species is a suitable biological model for environmental toxicological studies because it is simple to keep in the laboratory and produces numerous transparent eggs that can be fertilized in vitro year round. Results from this work could increase our understanding of the action of azole derivatives on the development of a marine invertebrate and could shed light on the influence of agricultural activity on aquatic species.

2. Materials and methods

2.1. Animals and chemicals

Adults of *P. mammillata* were collected from a natural population in the Gulf of Lerici (La Spezia, Italy) and reared in aquarium at 16 °C with 12-h light cycle.

Gametes were obtained by dissection of gonoducts after the animals were anesthetized for 1 h with 0.25 g/l MS222 (3-aminobenzoic-acid-ethyl-ester methane sulfonate salt, Sigma, Italy). This treatment had no effects on gametes and on development, as earlier reported (Zega et al., 2005) and as confirmed by the high percentage of normal embryos and larvae obtained (Figs. 2 and 3). All procedures were carried out at 18 °C in a thermostatic chamber. Treatments were performed using 6-well plates (NUNC, Denmark). Imazalil (IMA) was purchased from Sigma, Italy and Triadimefon (FON) from Bayer, Italy. Stock solutions (100 μM IMA and 1 mM FON) were made in

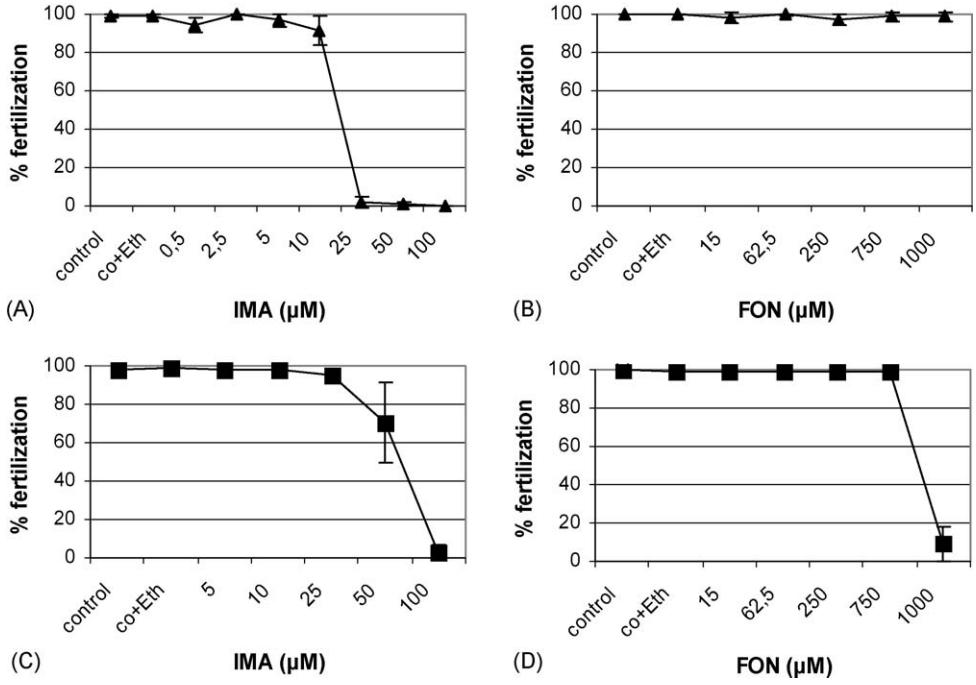

Fig. 2. Effects on sperm viability (A and B) and on fertilization (C and D) of IMA (A and C) and FON (C and D) at different concentrations. Mean values of three replicates and standard deviation are indicated.