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REVIEW

Solitary ascidians embryos (Chordata, Tunicata) as model organisms for testing coastal pollutant toxicity

G Zega, R Pennati, S Candiani, M Pestarino, F De Bernardi

Department of Biology, University of Milan, Milan, Italy

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Abstract

Marine coastal communities are daily exposed to several chemical compounds commonly used in agriculture and industrial activities. Therefore, toxicological studies evaluating the effects of these compounds on marine organisms are of primary importance for marine environment preservation. Different model organisms are used to perform toxicity tests with potential pollutants, under laboratory conditions. In last decades, solitary ascidians have been selected as valuable model organisms to run bioassays with embryos and larvae. In fact, by in vitro fertilization, it is easy to obtain thousands of embryos, rapidly developing and therefore allowing a fast screen of pollutant toxicity.

The aim of this review was to summarize results from toxicity tests, run with heavy metals, organo-metal and organic compounds, on solitary ascidian development and settlement to evidence that these animals offer several advantages as models to perform these kind of studies. First of all, they have a sensitiveness directly comparable to that of other marine model organisms. Moreover, the effects of toxicants on exposed embryos and larvae could be studied using different approaches, from ultrastructure to genetic analysis. Finally, since ascidians are chordates morphological and gene expression analyses could provide data for comparative studies with vertebrates.

Key words: heavy-metals; antifoulants; pesticides; development; Tunicates; ascidians

Introduction

Marine environment pollution is a concrete risk along densely populated coastal regions, where urban and industrial development could facilitate the dispersal of several chemical agents. Therefore, marine coastal ecosystems could be endangered by pollutants, such as heavy metals, pesticides and antifoulants that could be easily detected in the water column or in the sediment of harbours and estuaries (Castillo *et al*., 2006; Antizar-Ladislao, 2008; Bellas *et al*., 2008). These areas, often very rich in nutrients, host filter-feeders communities encompassing bivalves, serpulids and ascidians. Marine mussels have been selected early for the study of coastal pollution impact on marine life. More recently, ascidians have been selected as potential model organisms for testing pollutants toxicity as they offer several advantages for these studies (Mansueto *et al*., 1993; Cooper *et al*., 1995; Cima *et al*., 1996, 2008; Bellas *et al*., 2003).

Correspondig author:
Fiorenza De Bernardi
Department of Biology
University of Milan
via Celoria 26, 20133 Milan, Italy
E-mail: fiorenza.debernardi@unimi.it

Solitary ascidians (Chordata, Tunicata) are marine benthic filter-feeders that occur in dense populations along eutrophic coastal habitats, and therefore they could be easily sampled. They are hermaphrodite organisms that reproduce sexually by the simultaneous emission of eggs and sperm. Fertilized eggs develop in the water column in about a day into a planktonic tadpole larva that shows some chordate characters, a dorsal hollow neural tube and a notochord flanked by muscle cells. Adult solitary ascidians of *Ciona, Phallusia* and *Styela* genus are world wide distributed and fertile almost all year round. Gametes can be easily obtained by gonoduct dissection and, from in vitro fertilization, it is possible to obtain thousands of synchronously dividing embryos . Under laboratory conditions, development is completed in about 16-24 hours in a range of decreasing temperature from 22 to 16°C. For these characteristics, solitary ascidians are valuable and reliable organisms to run toxicity tests on gametes and embryos, for the high number of specimen easy available every time and the rapid development.

In last decades, several studies have been made to test the effect of different pollutants on ascidian development that is evaluating the percentage of

Table 1 List of compounds whose toxicity has been tested on solitary ascidians embryos to determine median effective (EC_{50}) concentration on development and settlement. When available the environmental concentration is also listed in bold, together with its reference

Compounds	Chemical classification	EC_{50} (µM) Embryos	EC_{50} (µM) Larval settlement	Environmental concentration (µM)	
Hg	Heavy metal	0.22	0.39	**0.002**	Bellas *et al*., 2004; OSPAR Commission, 2000
Cu	Heavy metal	0.58	1.61	**5.67**	″
Cd	Heavy metal	6.42	6.7	**0.23**	″
Cr	Heavy metal	226	289	**-**	″
TBT Tributyltin	Organometallic anti-foulant	0.02	-	**0.01**	Bellas *et al*., 2005
Zinc pyrithione (Zpt) Zinc 1-oxidopyridin-1-ium-2-thiolate	Organometallic bactericide, anti-foulant	0.23	0.11	-	Bellas, 2005
Lindane 1,2,3,4,5,6-hexachlorocyclohexane	Organochloride insecticide	15.20	-	**0.004**	Bellas *et al*., 2005; OSPAR Commission, 2000
Chlorpyrifos Diethoxy-sulfanylidene-(3,5,6-trichloropyridin-2-yl)oxyphosphorane	Organo-phosphorus pesticide	15.70	-	-	″
Diuron 3-(3,4-dichlorophenyl)-1,1-dimethylurea	Urea derived herbicide	17.80	-	-	″
Chlorothalonil 2,4,5,6-tetrachlorobenzene-1,3-dicarbonitrile	Organochloride fungicide, anti-foulant	0.12	0.16	**0.005**	Bellas, 2006
Sea-Nine 211 (Kathon 930) 4,5-dichloro-2-octyl-1,2-thiazol-3-one	Organochloride anti-foulant	0.37	0.15	**0.013**	″ ″
Dichlofluanid N-(dichloro-fluoromethyl)sulfanyl-N-(dimethylsulfamoyl)aniline	Organochloride anti-foulant	0.85	0.39	**0.017**	″ ″
Tolylfluanid N-(dichloro-fluoromethyl)sulfanyl-N-(dimethylsulfamoyl)-4-methylaniline	Organochloride anti-foulant	0.62	0.28	-	″
Irgarol 1051 N-tert-butyl-N'-cyclopropyl-6-methylsulfanyl-1,3,5-triazine-2,4-diamine	Anti-foulant	8.34	>25.60	**0.016**	″ ″
Imazalil 1-[2-(2,4-dichlorophenyl)-2-prop-2-enoxyethyl]imidazole	Organochloride triazole-imidazole fungicide	0.67	-	**0.47**	Pennati *et al*., 2006; FAO, 2001
Triadimefon 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)butan-2-one	Organochloride triazole fungicide	29.56	-	-	″
Fluconazole 2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl)propan-2-ol	Organofluoride triazole fungicide	74.70	-	-	Groppelli *et al*., 2007

normal larvae hatching from different treatments. In fact, larvae have a simple body plan that allows the rapid screening of malformed specimen. The tadpole larva body is formed by a trunk and a tail. The trunk bears main sensory organs: the three adhesive papillae or palps, situated at its anterior end, and one or two pigmented organs, situated in the sensory vesicle. The palps contain sensory neurons, through which the larva is able to choose a substratum where to settle, and mucus secreting cells to perform permanent attachment (Groppelli *et al*., 2003, Pennati *et al*., 2007). Commonly, larvae possess two pigmented organs, the ocellus and the otolith respectively a photo- and gravity-receptor, but some larvae could have only one, usually called the photolith, as it perceives both kind of stimuli.

The sensory vesicle is the anterior portion of the central nervous system that continues towards the posterior end as a dorsal hollow tube, divided in three portions, the neck, the visceral ganglion and the tail nerve cord. The neck, devoid of neurons, connects the sensory vesicle to the visceral ganglion. This latter contains neurons with ascending projections and motor neurons with descending projections to tail muscle cells (Imai and Meinertzhagen, 2007).

Interestingly, tunicates are considered the vertebrate sister group (Delsuc *et al*., 2006) and their embryos and larvae share basic homologies with vertebrates also at the level of the expression of developmental regulatory genes (Meinertzaghen *et al.*, 2004). In particular, the availability of *Ciona intestinalis* genome sequences (Dehal *et al.*, 2002) could favour the study of toxicant effects on gene expression. For example, a chip for cDNA microarray analysis has been developed to investigate gene expression profiles in TBT (Table 1) exposed ascidians (Azumi *et al*., 2004).

In this review, results from toxicity tests run with heavy metals, organometals and organic compounds, such as pesticides and anti-foulants, on ascidian development will be reported. This overview has the aim of evidencing how it is possible to take advantage of solitary ascidians to perform toxicity studies, with several approaches.

Effects of heavy metals, organometallic and organic compounds on ascidian development and settlement

Among pollutants, heavy metals and organo-metallic compounds showed the highest toxic effects on ascidian development and settlement. Exposure to Hg, Cu, Cd and Cr of *C. intestinalis* embryos for 20 h severely reduced percentage of hatching of normal larvae and of settlement. The EC_{50} (median effective concentration that determines larval malformation) values of Hg, Cu and Cd were very low indicating that these metals could effectively impair development and consequently larval attachment (Table 1). Moreover, *C. intestinalis* sensitiveness to such pollutants resulted comparable to what previously reported for other marine organisms commonly used in toxicity test, such as the bivalve *Mytilus galloprovincialis* and the sea-urchin *Paracentrotus lividus* (Bellas *et al.*, 2004).

In the group of tested organic compounds, organo-metallic ones resulted the most toxic for ascidians such as *Styela plicata* and *C. intestinalis.* Micromolar doses of organotin compounds (TBT, TPT, TCHT), blocked development of *S. plicata* embryos in a stage-dependent manner. In fact, earliest developmental stages, 2-4 cells to gastrula, were more sensitive (Cima *et al.*, 1996) (Table 2). The ultrastructural analysis of 1h exposed embryos of different stages revealed the presence of electron-dense precipitates in mithocondria, whose membrane were severely damaged. Moreover, blastomere shape and adhesion were also affected most probably because organotin compounds could interfere with cytoskeletal proteins. Similarly, *C. intestinalis* embryos exposed from neurula stage for 1 h showed malformed and disorganized blastomeres, lacking cytoskeletal elements. As a consequence, neurulation was blocked (Dolcemascolo *et al.*, 2005) (Table 2). The effect of TBT was studied also on late developmental stage of *C. intestinalis*. Pre-hatching and swimming larvae exposed for 1 h to 0.1µm TBT showed severe tail malformations. Muscle cells had an abnormal distribution along the tail and irregularly shaped nuclei. Moreover, the ultrastructure of sarcomeres and muscle mitochondria appeared completely compromised (Gianguzza *et al.*, 1996) (Table 2). When *C. intestinalis* embryos were exposed to TBT throughout development (about 20h) development was blocked and EC_{50} was 0.022µM (Bellas *et al.*, 2005). Another potent organometallic anti-foulant, zinc pyritione (Zpt) showed similar effect on *C. intestinalis* development and settlement (Table 1) (Bellas, 2005).

Pesticides and anti-foulants are the last group of compounds whose toxicity was investigated on ascidian development (Table 1). These substances have a broad-spectrum activity and their action on ascidian embryos were studied mainly evaluating dose-depending effects on development. For each compound the EC_{50} value was calculated (Table 1). For some organic pesticides and anti-foulants, such as Lindane, Chlorpyrifos, Diuron, Irgarol 1051, Triadimenfon and Fluconazole, EC_{50} values were quite high in terms of toxicity, corresponding to micro-molar concentrations. Organochloride anti-foulants (Chlorothalonil, Sea-Nine 211, Dichlofluanid, Tolylfluanid) instead resulted the most toxic substances, for their very low EC_{50} values. Moreover, among fungicides, Imazalil, that contains two chlorine atoms, showed a similar toxicity for ascidian embryos (Bellas *et al.*, 2005; Bellas, 2006; Pennati *et al*., 2006; Groppelli *et al*., 2007). Effects of the triazole fungicide was also evaluated in terms of teratogenicity as these substances induced specific malformations whose severity was dose-dependent. Therefore, larval phenotypes obtained after triazole exposure throughout development were classified using a dissection microscope and further characterized by means of histology and immunohistochemistry experiments. Triazole exposed larvae showed typical malformation: the trunk appeared shortened, the palps were fused or not completely differentiated, and the sensory vesicle was reduced with displaced pigmented organs (Fig. 1A, B). Moreover, the anterior nervous

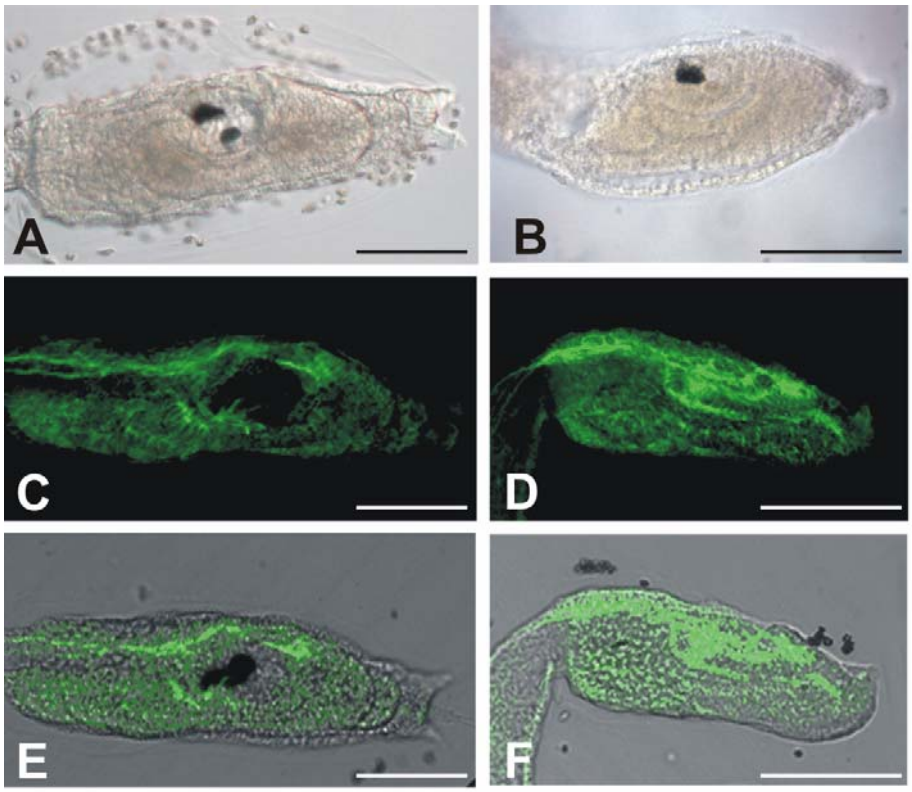

Fig. 1 Control larvae (A, C, E) and larvae developed from embryos exposed to 5 µm Imazalil (B, D, F) of the solitary ascidian *Ciona intestinalis*. Control (A) and malformed larva (B) showing the typical Imazalil induced phenotype. Immunohistochemical localization of β-tubulin in control (C, E) and malformed (D, F) larvae, whose anterior nervous network appeared disorganized. Bars = 100 µm.

network was compromised, as evidenced by immunolocalization of β-tubulin (Fig. 1C-F). In these studies, the teratogenic action of triazoles on ascidian development was directly compared with what known on vertebrate embryos, where these fungicides typically affect differentiation of the anterior structures, interfering with retinoic acid catabolism. The authors found evidences that also in ascidians the observed phenotypes could be due to an alteration of retinoic acid signalling (Pennati *et al.*, 2006; Groppelli *et al*., 2007).

Conclusions

Coastal pollution could stress marine communities determining a decrease in biodiversity for the disappearing of more sensitive species (Castillo *et al*., 2006; Bellas *et al*., 2008).

Marine benthic invertebrates are easily exposed to toxic compounds commonly used in agriculture, industrial and harbour activities, and have been selected as model organisms to evaluate effects of these substances on life processes.

Some of the listed inorganic and organic compounds were proved to impair ascidian development at very low doses, ranging from nano- to micro-molar concentrations. Even if the average environmental concentration of these compounds is lower than their EC_{50} values on ascidian development (Table 1), we believe that the chance of endangering coastal populations of sessile tunicates is a realistic risk. In fact, given the wide production of pesticides (Tilman *et al*., 2001), the possibility of local accumulation by accidental spills must be also considered. Similarly, the extensive uses of anti-foulants favour their accumulation in harbours shallow waters (Bellas, 2006). Moreover, among the substances considered in this review, Cu, TBT and Imazalil were detected in water or soils in concentrations directly comparable to their EC_{50} values on ascidian development (Table 1). From the conspicuous studies reviewed here, it is clear that, among benthic coastal invertebrates, solitary ascidians are valuable organisms to be considered among models to test toxicity of potential or known pollutants on their development and settlement ability. In fact, it is possible to run toxicity tests on a high number of embryos and to rapidly (1 day) screen the effects. Solitary ascidians embryos and larvae can be used in these laboratory studies with different approaches, from exposure bio-assays of different developmental stages to morphological analysis at different levels (ultrastructure, histology, immunohistochemistry). Results summarized here evidenced that several common pollutants strongly impaired ascidians development and consequently their dispersal and recruitment phases.

Table 2 Effects of different compounds on the development and/or morphology of larvae of three different ascidian species

	Effective concentrations (µM)	Developmental stage	Exposure time (h)	Target organs Effects	
Styela plicata					
TBT TPT TCHT	0.1/1 0.1 0.1	2-4 cells, morula, gastrula	1	Mitochondria, cytoskeleton Block of cleavage	Cima *et al*., 1996
Ciona intestinalis					
TBT	0.1	Pre-hatching larvae Swimming larvae	1	Tail muscle cells, mitochondria, cytoskeleton Block of hatching/swimming	Gianguzza *et al.*, 1996
TBT	0.1/10	Neurula	1	Mitochondria, cytoskeleton Block of neurulation	Dolcemascolo *et al*., 2005
Phallusia mammillata					
Imazalil Triadimefon	5 125	2 cells	10	Palps, central nervous system	Pennati *et al*., 2006
Fluconazole	125	2 cells	18	Palps, central nervous system	Groppelli *et al*., 2007

Recently, considering among other advantages, that ascidian have a sensitiveness, in terms of EC_{50}, directly comparable to that of other model organisms, such as bivalves or sea-urchins, a standardized protocol for ascidian embryo-larval bio-assays has been formulated (Bellas et al., 2003).

Finally, as *C. intestinalis* genome has been released (Dehal *et al*., 2002), it is also possible to investigate how expression of target genes could be altered by toxicants. For instance, Azumi and colleagues (2004) found, through microarray analysis of gene expression in *C. intestinalis* exposed to TBT, strong differential expression of more than 200 genes concerned with stress response, detoxification, oxidoreduction reaction, biosynthesis and catabolism. Considering basic vertebrate homologies of ascidians (Meinertzaghen *et al.*, 2004; Delsuc *et al.*, 2006), these genetic analysis could be powerful tools to forecast possible toxic effects on vertebrate organisms, including humans.

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