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Toxicity of marine pollutants on the ascidian oocyte physiology: an electrophysiological approach

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Summary

In marine animals with external fertilization, gametes are released into seawater where fertilization and embryo development occur. Consequently, pollutants introduced into the marine environment by human activities may affect gametes and embryos. These xenobiotics can alter cell physiology with consequent reduction of fertilization success. Here the adverse effects on the reproductive processes of the marine invertebrate *Ciona intestinalis* (ascidian) of different xenobiotics: lead, zinc, an organic tin compound and a phenylurea herbicide were evaluated. By using the electrophysiological technique of whole-cell voltage clamping, the effects of these compounds on the mature oocyte plasma membrane electrical properties and the electrical events of fertilization were tested by calculating the concentration that induced 50% normal larval formation (EC_{50}). The results demonstrated that sodium currents in mature oocytes were reduced in a concentration-dependent manner by all tested xenobiotics, with the lowest EC_{50} value for lead. In contrast, fertilization current frequencies were differently affected by zinc and organic tin compound. Toxicity tests on gametes demonstrated that sperm fertilizing capability and fertilization oocyte competence were not altered by xenobiotics, whereas fertilization was inhibited in zinc solution and underwent a reduction in organic tin compound solution (EC_{50} value of 1.7 μM). Furthermore, fertilized oocytes resulted in a low percentage of normal larvae with an EC_{50} value of 0.90 μM. This study shows that reproductive processes of ascidians are highly sensitive to xenobiotics suggesting that they may be considered a reliable biomarker and that ascidians are suitable model organisms to assess marine environmental quality.

Keywords: Ascidian, Fertilization, Heavy metal, Ion current, Oocyte, Organic tin, Phenylurea herbicide

Introduction

Over the last decades, diverse substances have been released into the marine environment due to agricultural and industrial processes. These chemicals include heavy metals, organic tin compounds, herbicides and pesticides, which can cause endocrine disruption and reproductive disorders in animals and affect the survival of aquatic wildlife (Danzo, 1997, 1998; Henson & Chedrese, 2004; Wurl & Obbard, 2004; Pennati *et al*., 2006; Zega *et al*., 2009a). Animal reproduction is a step-by-step process that starts with the production and maturation of the gametes (spermatogenesis and oogenesis), and ends with fertilization. This process is a highly specialized mixture of cell interaction and signal transduction that gives rise to a new individual of the same species. Among the events underling reproduction, a key role is played by the reciprocal activation of the gametes, which include consecutive steps such as recognition, binding and fusion between oocyte and sperm (Tosti & Ménézo, 2016). At the time of their interaction, the oocyte undergoes three main types of modifications: electrical, morphological, and metabolic (Dale, 1994).

Gametes are excitable cells that, during the processes of maturation and fertilization, undergo transient modifications of their electrical properties due to the activity of ion channels present on their plasma mem-

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brane. These channels participate in the regulation of gamete physiology, and ion current activities have been demonstrated widely in oocytes and spermatozoa of all animals studied (Tosti & Boni, 2004; Tosti *et al*., 2011; Gallo & Tosti, 2015b). Ascidians (Chordata, Ascidiacea), or sea squirt, constitute a widely studied species in reproductive and developmental biology (Dale, 1989; Satoh, 1994). As sessile filter-feeding organisms, ascidians are able to accumulate and concentrate substances present in seawater in their tissues.

By using electrophysiological techniques, ion current patterns were studied from the immature stage up to early embryo development in the ascidian *Ciona intestinalis* (Cuomo *et al*., 2006; Silvestre *et al*., 2009). In particular, a role for sodium (Na^+) current was highlighted, underlying the mature oocyte stage and the interaction with the spermatozoon. In *C. intestinalis*, the first electrical modification of the oocyte at fertilization is the generation of an inward ion current [i.e. fertilization current (FC)] due to gating of non-selective channels (Dale & De Felice, 1984; Dale, 1994). This step is followed by depolarization of the resting potential and contraction of the cell body. The latter step is generated by a calcium wave that crosses the oocyte from the vegetal pole to the animal pole (Brownlee & Dale, 1990). A very quick developmental cycle gives rise within 24 h to a swimming larva that promptly settles to a substrate and undergoes dramatic metamorphosis that generates the adult individual. As all these processes occur in the seawater after gamete spawning, it is evident that they are influenced by the quality of the environment.

Heavy metals are recognized as major pollutants of the marine environment and have toxic effects on marine organisms (Depledge *et al*., 1994). Metals are classified as essential and non-essential elements based on the fact that they may participate in some physiological processes (Falchuk & Montorzi, 2001) or they have no role in biological systems, although may be tolerated at low concentrations. Metals may exert a negative effect on reproductive processes such as gamete functionality, embryogenesis, embryo development, metamorphosis and even on growth and survival of different marine organisms (Martin *et al*., 1981; Wong *et al*., 1993; Itow *et al*., 1998; Au & Chiang, 2000; Fernández & Beiras, 2001; Beiras & Albentosa, 2004; Popek *et al*., 2005; Ferrer *et al*., 2006; Porte *et al*., 2006; Wang *et al*., 2009).

Organic tin compounds comprise a group of organometallic moieties characterized by a Sn atom covalently bound to one or more organic substituents largely used in industrial and agricultural applications. The growing use of organic tin compounds as fungicides, glass coatings, catalysts, PVC thermostabilizers, and biocides in antifouling paints represents the major sources of these contaminants in the marine environment. Organic tin compounds can be degraded (by exposure to sunlight and by bacteria) into inorganic tin compounds. The chemical, physical, and biochemical properties of inorganic tin compounds differ dramatically from those of organic tin compounds. Inorganic tin compounds are of low toxicological risk due largely to their low solubility, poor absorption, low accumulations in tissues, and rapid excretion. In contrast, organic tin compounds produce a variety of harmful effects but their toxicological pattern is not yet fully elucidated. The biological effects of these substances depend on the nature, number and type of organic groups. In recent years, several research groups have shown that organic tin compounds caused several adverse effects, including reproductive disorders in different marine species (Alzieu, 2000).

Phenylurea derivatives have been widely used as active ingredients in herbicidal preparations, so they are frequently found in the marine environment. Recent monitoring studies have reported the presence of several phenylurea derivatives such as isoproturon, diuron, linuron, monolinuron, metoxuron, and chlortoluron in seawater around several European countries. However, their fate and toxicity in the marine environment are still poorly understood, creating a concern for the adverse environmental effects on marine organisms (Voulvoulis *et al*., 1999; Yamada, 2007).

In this paper, we report the effects of two metals (zinc an essential element and lead a non-essential element), plus an organic tin compound and a phenylurea herbicide on the specific reproductive mechanisms of the marine invertebrate *C. intestinalis*. In particular, the effects of exposure to xenobiotics on oocyte physiology, FC and morphological (cell contraction) events induced at the time of sperm–oocyte interaction were investigated. Furthermore, the long-term effects of ion currents or FC impairment on embryo development and larval formation and morphology were evaluated.

These data provide a solid ground that confirms previous studies (Papadopoulou & Kanias, 1977; Zega *et al*., 2009b) on the possible use of ascidians as bioindicators of the marine environment, indicating the suitability of this species to test the effects of environmental stressors on marine animal reproductive physiology.

Materials and methods

Animals and gametes

Adults of *C. intestinalis* were collected from the Gulf of Naples (Italy) and maintained in laboratory aquaria with running seawater at 18°C. For the experiments, adults were anaesthetised in ice and mature oocytes

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were collected from the oviduct and kept in Petri dishes containing natural seawater (NSW) until use. Spermatozoa were collected from the sperm duct of other animals and then used for inseminating oocytes at a concentration of 10^6/ml in NSW.

Experimental solutions

Lead ($PbCl_2$) and zinc ($ZnCl_2$) stock solutions were prepared by dissolving chloride salts of 99+% purity (Sigma-Aldrich, Milan, Italy) in double-distilled water and then diluted in NSW at the desired final concentrations. In each experiment filtered (Millipore 0.22 μm; MilliQ, Medford, MA, USA) NSW was used as the control solution. In order to evaluate the effects of these metals on ion currents, FC and embryo and larval development, the oocytes were incubated for 30 min and/or fertilized in metal solutions at the following metal concentrations: 0.04, 0.05, 0.07, 0.4, 0.7, 1.1 μM of lead; and 7, 183, 367, 550 μM of zinc. The starting concentration of lead (0.04 μM) was taken from the maximum standard value established from Italian legislation for seawater quality (D.lgs. 152/06). In contrast for zinc there are no limits we referred to the concentrations tested by Gopalakrishnan *et al*. (2007).

The tested phenylurea was 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and its stock solutions were prepared in dimethyl sulfoxide (DMSO). A stock solution of the organic tin compound, i.e. tributyltin, was prepared in ethanol. Test solutions were obtained by diluting the stock solution in NSW at the following concentration: 1, 2, 3, 4, 5, and 10 μM from reference to the literature on the *C. intestinalis* and other ascidian species (Cima *et al*., 1996; Franchet *et al*., 1999; Patricolo *et al*., 2001). As ethanol and DMSO were used to prepare stock solutions, negative controls were made with equivalent volumes at a final concentration of 0.1% (that is, the higher per cent in the test solutions) and results showed no observable effects on gametes and fertilization.

Three replicates of each concentration were tested. Each experiment was performed 10 times.

Toxicity tests

Spermatozoa

Freshly collected spermatozoa were diluted in NSW at final concentration of 10^6/ml. Aliquots of this dilution were added to vials containing 1 ml of test solutions and incubated for 30 min at room temperature. Following exposure, spermatozoa were added to 10 ml of NSW containing about 200 oocytes. As a control, an untreated aliquot of the sperm dilution was used to fertilize oocytes from the same batch (*ca.* 200). The dishes were incubated in a culture chamber at 18°C and, after 50 min, fertilization rate was determined by the occurrence of the first cleavage in the control. Then the dishes were incubated again for 24 h and the percentage of abnormal larvae was calculated.

Oocytes

Mature oocytes (*ca.* 200 oocytes), collected from the same animal, were transferred into Petri dishes containing 5 ml of test solutions and incubated for 30 min at room temperature. Oocytes were then rinsed with NSW, transferred to Petri dishes containing 10 ml of NSW and then fertilized. As a control, untreated oocytes (*ca.* 200) from the same batch were fertilized. The dishes were incubated in a culture chamber at 18°C. Fertilization rate and the percentage of abnormal larvae were determined as previously described.

Fertilization

Mature oocytes (*ca.* 200 oocytes) and spermatozoa were added to Petri dishes containing 10 ml of tested solutions. As a control, gametes (*ca.* 200 oocytes) were added to Petri dishes containing NSW. The dishes were incubated in a culture chamber at 18°C. Fertilization rate and the percentage of normal larvae were determined as above.

Electrophysiological techniques

Electrophysiological recordings were performed using whole-cell configuration of the patch-clamp technique. The chorion and follicle cells surrounding the mature oocytes were removed manually using steel needles, naked oocytes were then transferred to a recording chamber containing NSW. Recordings were performed using a List EPC-7 patch-clamp amplifier (HEKA Electronics, Cologne, Germany), filtered at 3 kHz and digitized with a Digidata 1322A. Ion currents were acquired and analyzed using pClamp9 software (Axon Instruments, Union City, CA, USA).

Patch pipettes were made from borosilicate glass capillaries (Warner Instruments, Hamden, CT, USA) and pulled using a Sutter P-87 (Sutter Instrument, Novato, CA, USA) with a 1–2 μm tip in diameter showing a resistance of 3–5 megaOhms when filled with an intracellular-like solution (200 mM K_2SO_4; 20 mM NaCl; 200 mM sucrose; 10 mM EGTA; 10 mM HEPES, pH adjusted to 7.5).

Electrophysiological recordings were performed as follows: after formation of a cell-attached configuration, a light negative pressure was applied to induce the rupture of the membrane patch allowing the access to oocyte cytoplasm. In this configuration, from a holding potential of −80 mV, voltage-dependent Na^+ currents were elicited by applying depolarizing ramps of 10 mV to the test potential from −70 mV to +20 mV. These currents were maximally activated by voltage step to −40 mV. The FC was generated by adding spermatozoa to the recording bath containing an oocyte voltage

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clamped at −80 mV. The effect of tested xenobiotics on Na^+ currents and FC was evaluated by incubating the oocytes for 30 min in test solutions before the electrophysiological recording.

Statistical analysis

Data were reported as mean ± standard deviation (SD). In order to test significant differences between the control group and test concentrations and among the test concentration groups, one-way analysis of variance (ANOVA) was performed followed by Fisher's least significant difference (LSD) test. For values expressed as percentages, data were analyzed after arcsine transformation to achieve normality. Significance level was always set at $\alpha = 0.05$.

The Probit method was used to calculate the EC_{50}.

Results

Toxicity tests

Gametes

Pre-exposure of fertilizing spermatozoa to all the tested xenobiotics had no significant effect on fertilization rate and embryo and larval development. Similarly, no significant effects on fertilization rate and percentage of normal larvae were observed after fertilization of treated oocytes with untreated spermatozoa even at the highest concentrations used (data not shown).

Fertilization and embryo development

Lead had no toxic effects on fertilization rate and embryo development at concentration up to 0.4 µM, whereas zinc inhibited fertilization at all the tested concentrations.

The organic tin compound reduced the fertilization rate with an EC_{50} value of 1.7 µM and normal larvae percentage with an EC_{50} of 0.90 µM (Fig. 1). Furthermore, at concentrations higher than 1 µM, embryo development was affected resulting in morphologically abnormal larvae especially for tail malformations (Fig. 2). Even if phenylurea did not affect the fertilization rate, a significant decrease in the percentage of normal larvae was detected with an EC_{50} value of 2.8 µM (Fig. 1).

Effects on ion currents

Na^+ currents

As illustrated in the concentration–response curves (Fig. 3), all tested xenobiotics reduced the amplitude of Na^+ currents, following typical sigmoidal pattern. The lowest EC_{50} was obtained for lead (0.4 µM) followed by organic tin compound (1.7 µM), phenylurea (4.3 µM) and zinc (106 µM). The four xenobiotics tested were ranked consistently in the following order from highest to lowest toxicity: lead > organic tin compound > phenylurea > zinc.

Fertilization current

In oocytes incubated with lead and phenylurea and then fertilized under voltage clamp conditions, the frequency of FC showed no significant differences between the control and the test concentration groups. In contrast, oocytes incubated with all tested concentrations of zinc did not generate FC, whereas FC frequency significantly decreased in a concentration-dependent manner in oocytes incubated with organic tin compound with an EC_{50} value of 3.2 µM (Fig. 4).

Discussion

In this study, the adverse effects exerted by two inorganic and two organic xenobiotics on the reproductive processes of the ascidian *C. intestinalis* and, in particular, on oocyte physiology and fertilization were shown.

Modulation of electrical properties of oocyte plasma membrane represents a fundamental step in gamete maturation and the fertilization process. In *C. intestinalis* oocytes, the presence and modification of voltage-dependent currents were well described starting from the immature stages. In particular, a key role of Na^+ currents was highlighted, as their amplitude is high at the MI stage and in the zygote and decreases in the following developmental stages (Cuomo *et al.*, 2006; Silvestre *et al.*, 2009; Tosti *et al.*, 2011). All xenobiotics tested in the present study, although different for their chemistry and mode of action, significantly reduced the amplitude of Na^+ currents, but exerted different selective actions on other events such as the FC frequency, fertilization rate and larval development.

A dose–response relationship was found between xenobiotic concentration and Na^+ current amplitude. The four tested xenobiotics could be ranked in the following order from highest to lowest toxicity on Na^+ currents of *C. intestinalis* mature oocytes: lead > organic tin compound > phenylurea > zinc. Na^+ currents were also shown to be sensitive to xenobiotic effects in our recent study (Gallo & Tosti, 2015c). Taken together, all these data suggest that Na^+ currents can be a suitable biomarker in the risk assessment of aquatic ecosystems giving key insight into pollutant exposure and effects and providing an early warning to potential damage in marine organisms.

Lead was the most toxic compound for ion currents of *C. intestinalis* mature oocytes, while zinc was the least toxic xenobiotic tested. Moreover, zinc and organic tin

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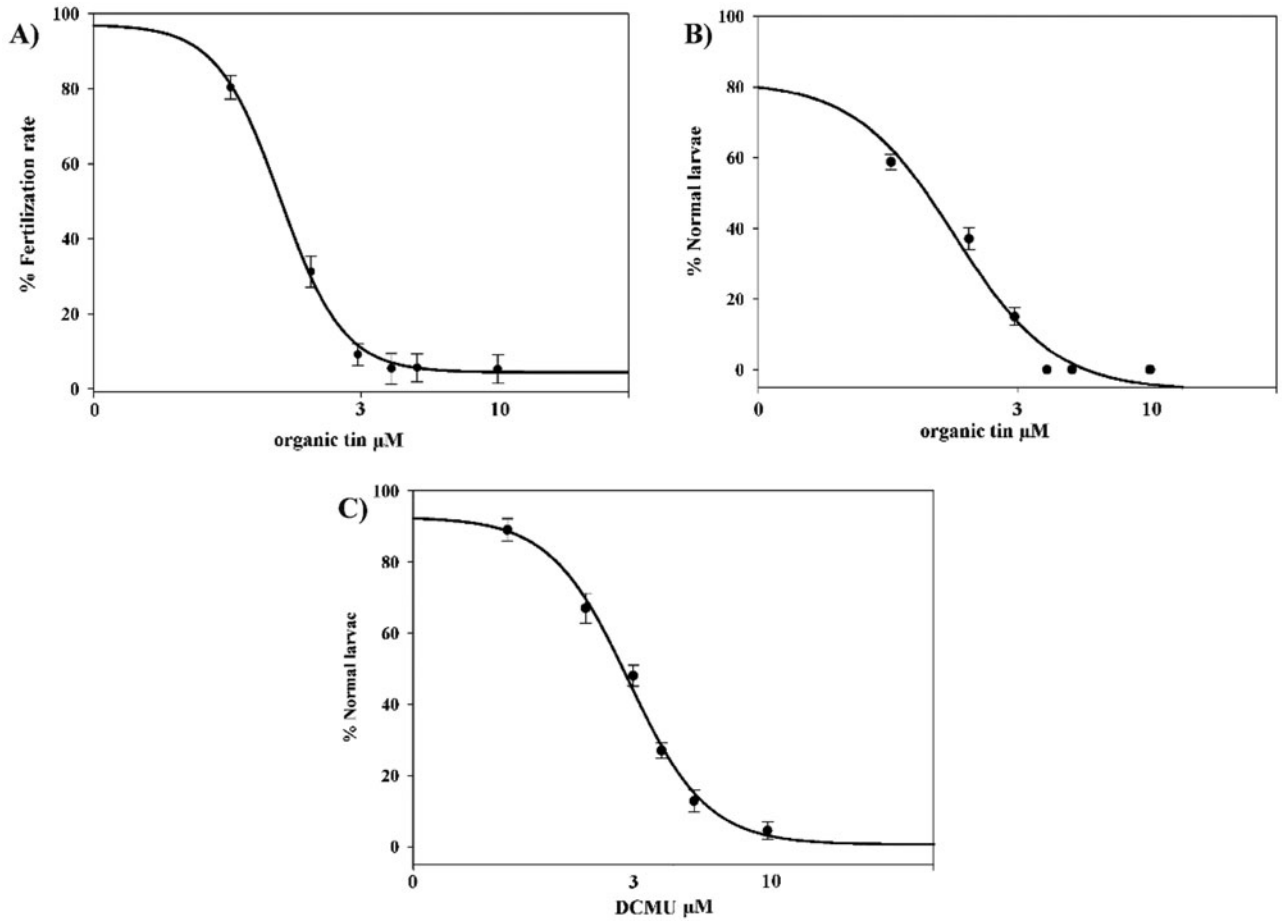

Figure 1 Organic tin compound and phenylurea effects on *C. intestinalis* fertilization. Fertilization rate (*A*) and percentage of normal larvae (*B*, *C*) obtained after fertilization in organic tin compound and phenylurea DCMU solutions, respectively. Solid circles represent the mean of 10 experiments, and the solid line indicates the fit of the experimental data to the logistic model. Error bars indicate the standard deviation (SD).

compound, but not lead and phenylurea, interfered with FC suggesting a different affinity of these compounds with channels that generate FC (Gallo & Tosti, 2013). Xenobiotic pre-exposure of *C. intestinalis* spermatozoa did not affect their fertilization ability and offspring quality in agreement with previous data that reported no effects of other metals and tributyltin on the fertilizing capacity of spermatozoa of either *C. intestinalis* or other ascidians (Franchet *et al.*, 1999; Bellas *et al.*, 2001), although one study demonstrated that the fertilization ability of spermatozoa of the ascidian *Ascidia malaca* was influenced by organotin compounds (Villa *et al.*, 2003). Furthermore, treatment of oocytes with each of the four xenobiotics did not prevent fertilization nor alter embryo development.

When *C. intestinalis* oocytes were fertilized in toxic solutions, the tested xenobiotics showed none (lead and DCMU) or major (zinc and organic tin compound) effects on the fertilization rate. These results correlated with the observed reduction of FC frequency, suggesting a new possible mechanism of action of these compounds on ion channel activity and highlighting the role of ion currents in the fertilization process.

Moreover, organic tin compounds exert a long-term effect on larval development and morphology, inducing various degrees of larval malformations such as coiled tail and irregular head. This finding is crucial as tail retraction is a key event in the initial stage of metamorphosis and their malformation may impede the correct transformation of the larva into an adult organism.

According to several studies, ascidians play a relevant role in the environment, representing species to be protected as cosmopolitan components of the marine ecosystems. Although these species themselves are fouling organisms, evidence has accumulated over the last decade that indicated that they are particularly sensitive to environmental pollution (Franchet *et al.*,

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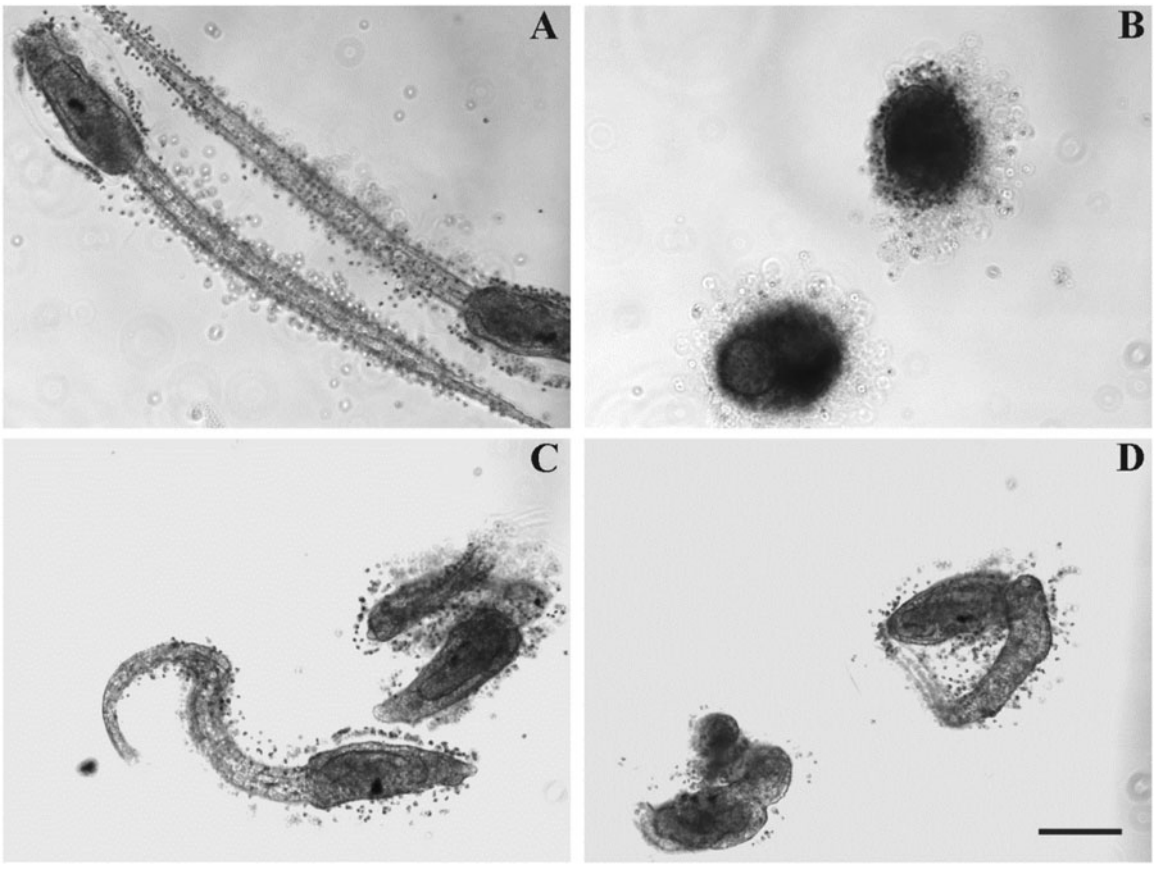

Figure 2 Organic tin compound and phenylurea effects on *C. intestinalis* larval development. (*A*) Normal larvae at 24 h post fertilization developed from oocytes fertilized in NSW. (*B*) Embryo developed in organic tin solution arrested at different developmental stages. Abnormal larvae developed from oocytes fertilized in organic tin (*C*) and the phenylurea DCMU (*D*) solution at different concentrations showing various degrees of malformations such as abnormal heads and stunted and coiled tails. Bar represents 100 μm.

1999; Bellas *et al*., 2001; Bellas, 2005, 2006; Pennati *et al*., 2006) and this characteristic makes them a suitable model for toxicity evaluation of marine contaminants and for the seawater quality assessment (Gallo & Tosti, 2015a).

Biomonitoring programmes rely on the use of biomarkers and bioindicators that are processes or organisms, providing information on the environmental quality through biochemical, physiological or morphological reactions (Holt and Miller, 2011). In the past, many marine organisms have been proposed as bioindicators of environmental pollution such as echinoderms and molluscs (Bellas, 2007; Nacci *et al*., 1986). In particular sea urchin early embryo growth test and sperm cell toxicity test have been used for long time to evaluate toxicity of a closed environment and recently new modified bioassays have been found to be good alternatives to the traditional tests (Manzo, 2004; Matranga & Corsi, 2012; Gallo *et al*., 2018). At present, there is a need to identify new genotoxic and teratogenic tests to associate with environmental quality monitoring.

Some potential bioindicators for investigating pollutants effects that have recently been proposed are molluscs (Waykar & Deshmukh, 2012; Boni *et al*., 2016), zebrafish (Segner, 2009) or others (Zhou *et al*., 2008) with distinct advantages and disadvantages for each.

Ideal candidates for pollution biomonitoring should be sessile and filter-feeders organism. Sessile organisms have no possibility to avoid the pollution source, whereas a filter feeder processing large amounts of water is able to accumulate and concentrate toxicants. In either case, they may provide evidence of environmental changes and allow the identification of polluted areas.

Ascidians show a peculiar affinity for vertebrates (Delsuc *et al*., 2006), in particular *C. intestinalis* is a cosmopolitan ascidian used as a model for research on reproduction as it produces large numbers of synchronous gametes and has a short developmental

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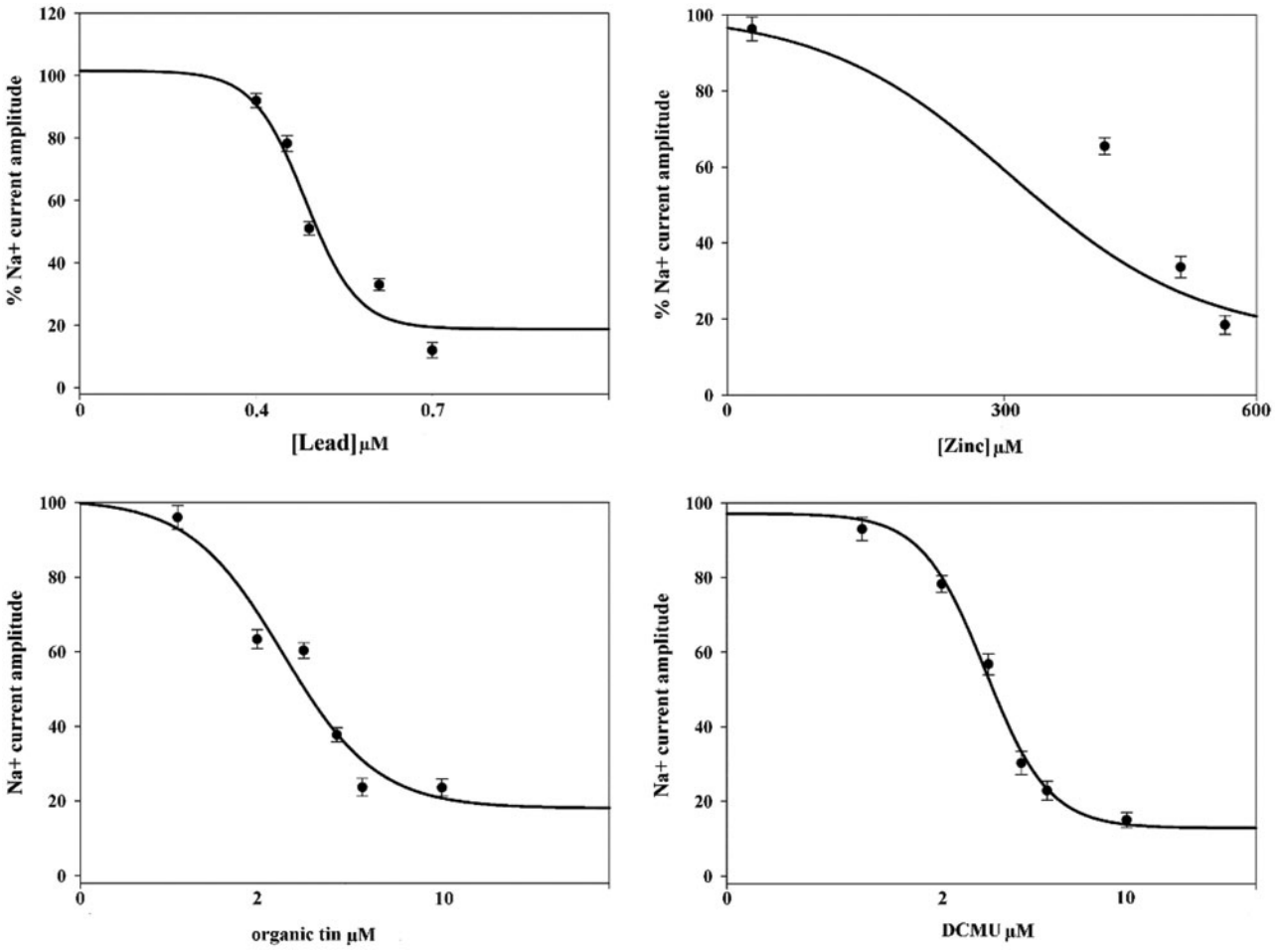

Figure 3 Effect of xenobiotics on *C. intestinalis* oocyte Na^+ current. Na^+ current amplitude after 30 min exposure of oocytes to different concentrations of lead (*A*), zinc (*B*), organic tin compound (*C*) and phenylurea DCMU (*D*). Solid circles represent the mean of replicates for all treatments, and the solid line indicates the fit of the experimental data to the logistic model. Error bars indicate the standard deviation (SD).

cell cycle that is easy to follow and a clear larval stage with a particular larval metamorphosis. These characteristics together with its sessile and filter-feeders behaviour promotes it for consideration as a new model for ecological risk assessment.

In previous studies (Gallo *et al.*, 2011; Gallo & Tosti, 2013, 2015c; Gallo *et al.*, 2016), we have accumulated much evidence that *C. intestinalis* may represent a suitable bioindicator as its selective cellular response to pollutants in the long term may induce changes in its normal reproductive physiology. Its sensitivity to pollutants may partially explain the progressive reduction of *C. intestinalis* populations observed over the last 2 decades along the Neapolitan coast.

Conclusion

There is no doubt that environmental pollutants are able to impair the reproductive processes of a wide range of wildlife species. Study of effects of invertebrate exposure to chemicals deserves special attention as these animals represent 97% of all ecosystems and therefore their reproductive disorders may lead to a reduction in such populations. In view of the ecological importance of invertebrates, there is an urgent need to conduct more research in this area.

Here, it is shown that, in the ascidian *C. intestinalis*, different reproductive parameters such as oocyte ion current, FC, larval development and morphology were seriously affected by exposure to diverse marine pollutants.

Due to the high representative value of *C. intestinalis* (Sasakura *et al.*, 2009) among the marine invertebrates, the availability of its complete genome and the close phylogenetic relationship between ascidians and vertebrates, the results reported here substantiate that ascidians may be considered a reliable model organism for reprotoxicological bioassays.

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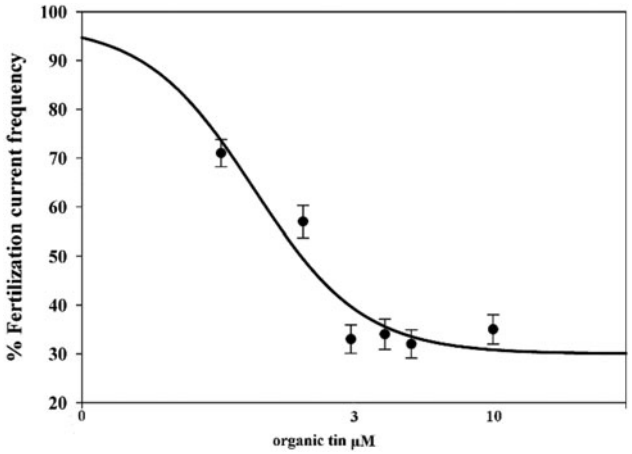

Figure 4 Organic tin compound effect on *C. intestinalis* fertilization current (FC). The frequency of FC recorded in oocytes after 30 min exposure to different concentrations of organic tin compound was reduced in a concentration-dependent manner. Solid circles represent the mean of replicates for all treatments, and the solid line indicates the fit of the experimental data to the logistic model. Error bars indicate the standard deviation (SD).

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Declaration of interest

The author declares that there is no conflict of interest.

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