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Comparing the sensitivity of two cogeneric ascidian species to two plastic additives: Bisphenol A and the flame retardant tris(chloro-propyl)phosphate

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Abstract

Although sensitivity to pollutants is well known to be species specific, closely related species are often assumed to respond similarly to them. We tested this assumption, comparing the sensitivity of *Ciona intestinalis* and *Ciona robusta* to two common marine pollutants: Bisphenol A (BPA) and tris(1-chloro-2-propyl)phosphate (TCPP). In particular, we focused on ascidian embryonic development and determined whether *C. intestinalis* and *C. robusta* displayed different responses. Our results demonstrate that closely related species can display either similar or very different sensitivity based on the tested contaminant. *Ciona intestinalis* and *C. robusta* had similar sensitivity to BPA, as their median effective concentration (EC₅₀) and median lethal concentration (LC₅₀) values were comparable. In contrast, TCPP showed very different teratogenic potential in the two analyzed species. *Ciona robusta* proved more vulnerable to this flame retardant as its teratogenic index was more than twice that calculated for *C. intestinalis*. Chemical modes of action as well as genetic differences could determine the diverse responses to environmental stressors. These results underline the presence of species-specific differences in embryonic sensitivity to contaminants and point out the importance of evaluating chemicals' teratogenic profile in several species.

Keywords: BPA TCPP, *Ciona intestinalis* and *Ciona robusta*, closely related species, contaminant

1. Introduction

Every year tons of pollutants reach the marine environment (Zandaryaa & Frank-Kamenetsky 2021). Most of these materials and chemicals originate from land-based sources (Vikas & Dwarakish 2015) and negatively affect the marine communities (Lionetto et al. 2021). For instance, plastic additives are released into the marine environment via industrial and municipal wastewater, river transport or in loco plastic degradation, and accumulate mainly along the coastline (Hermabessiere et al. 2017; Hahladakis et al. 2018; Wang et al. 2020). Their potential health risks have been demonstrated for a variety of marine organisms, including invertebrates such as mussels, shrimps, sea urchins and ascidians (Arslan et al. 2007; Arslan & Parlak 2008; Oehlmann et al. 2009; Messinetti et al. 2018, 2019; Darin 2021; Mercurio

et al. 2021; Miglioli et al. 2021; Naveira et al. 2021). Comprehensive understanding and assessment of pollutants in the marine environment are crucial to define their toxicity and prevent any irreparable damage to the ecosystems (Zandaryaa & Frank-Kamenetsky 2021). It is well known that sensitivity to pollutants can differ among species (Bellas et al. 2005; Bao et al. 2011; Mdaini et al. 2021). For example, the median lethal concentration (LC₅₀) of Bisphenol A (BPA) varied from 3.5 mg/L for *Poecilia vivipara* to 107.2 mg/L for *Artemia salina* (Naveira et al. 2021), while the median effective concentration (EC₅₀) of tributyltin (TBT) was 0.3 µg/L for *Paracentrotus lividus* and 7.1 µg/L for *Ciona intestinalis* (Bellas et al. 2005).

Even if chemical tolerance has been largely demonstrated to be species specific, closely related species have often been assumed to respond similarly to

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pollutants. However, a few rare studies have suggested this is not the case (Rocha-Olivares et al. 2004; Feckler et al. 2012; Monteiro et al. 2018). It has been reported that cryptic *Gammarus fossarum* lineages are characterized by different environmental stress tolerance (Feckler et al. 2012), and, similarly, cryptic species of nematodes substantially differ in their response to heavy metal contaminants (Monteiro et al. 2018). The different levels of sensitivity to pollutants exhibited by two marine copepods, *Cleotampus fourchensis* and *C. stimpson*, have even led scientists to consider them one of the main causes of the ongoing losses of genetic diversity (Rocha-Olivares et al. 2004).

Ascidians are key members of coastal benthic communities, able to colonize both natural and artificial substrates. Adults are sessile filter-feeding animals while larvae are free-swimming (Shenkar & Swalla 2011). These latter display the typical chordate body plan, comprising a notochord and a dorsal tubular nervous system, as they belong to the tunicates, the sister group of vertebrates (Delsuc et al. 2006). Recently, detailed morphological and molecular analyses recognized the ascidians *Ciona intestinalis* and *Ciona robusta* as two separate entities. These species were formerly part of a complex of cryptic species under the name *Ciona intestinalis* (Brunetti et al. 2015). Despite their morphological similarity, members of these species are highly genetically divergent (Brunetti et al. 2015; Pennati et al. 2015) and show different tolerance to ecological factors such as temperature (Sato et al. 2015).

In the present work, we took advantage of the accessibility of these closely related ascidian species to compare their sensitivity to two pollutants. We tested the effects of BPA and tris(1-chloro-2-propyl)phosphate (TCPP) on embryonic development and determined the responses of *C. intestinalis* and *C. robusta* to these pollutants. BPA and TCPP are common contaminants in marine ecosystems, whose adverse effects have been already characterized for one of the two species (Messinetti et al. 2019; Mercurio et al. 2021). BPA is a monomer of polycarbonate also used as stabilizer. It is one of the most commonly produced chemicals worldwide and, despite its activity as endocrine disruptor, it is still used in drink and food packaging. In the marine environment, BPA concentrations have been reported to vary from traces to 2.6 µg/L in UK estuaries (Hermabessiere et al. 2017). In ascidians, BPA was shown to affect embryogenesis mainly at the level of the nervous system, interfering with the development of dopaminergic and GABAergic systems as well as with sensory organ formation at concentrations close to those recorded in marine polluted areas (Messinetti et al. 2019). TCPP is an organophosphorus flame retardant, highly present in seawater; it is generally

added to rigid and spray polyurethane and can be released into the environment even by direct contact (Truong et al. 2017). Flame retardants are highly detected in seawater, ranging from a few ng/L to more than 15 ng/L (Hermabessiere et al. 2017). In *C. intestinalis*, TCPP was observed to specifically alter myogenesis, while no effects were found on neural differentiation. Even if these effects were reported at concentrations far from the environmental ones, the ability of the pollutant to accumulate in animal tissues and its potential additive toxicity make these findings noteworthy (Mercurio et al. 2021). Overall, this study increases our knowledge about the toxicological profiles of these common pollutants in the marine environment, and contributes to improving science-based policy and environmental management.

2. Materials and methods

2.1. Animals and chemicals

Adults of *Ciona robusta* were collected from natural populations in Chioggia (Italy) while *Ciona intestinalis* was collected by the fishing service of the station Biologique de Roscoff (France). Animals were maintained in 50 L aquaria filled with artificial seawater (ASW, Instant Ocean®, Aquarium System) and equipped with mechanical, chemical and biological filters. The temperature was fixed at $18 \pm 1^\circ\text{C}$ and constant light conditions were applied to avoid gamete release.

All the experimental procedures were performed at $18 \pm 1^\circ\text{C}$. For each experiment, three animals were sacrificed. Gametes were obtained by dissection, and cross-fertilization was performed in glass Petri dishes (Ø 4 cm).

TCPP (MW = 327.57) was purchased from Sigma (Milan, Italy). A stock solution of 100 mg/mL was made in dimethyl sulfoxide (DMSO; Sigma, Milan, Italy) and then diluted in filtered artificial sea water with 1 M HEPES pH 8.0 (ASWH) to reach the final test concentrations (0.1, 1.25, 12.5, 25, 50, 75 and 100 µg/mL). A solution of 0.1% DMSO in ASWH was used as a solvent control each time. BPA (MW = 228.29) was purchased from Sigma (Milan, Italy). A stock solution of 100 mM BPA was made in DMSO and then diluted in ASWH to reach the final test concentrations (0.1, 0.5, 1, 5, 10 and 20 µM). As a solvent control, a solution of 0.02% DMSO in ASWH was used. Fresh solutions were prepared each time. For both TCPP and BPA experiments, concentrations were chosen based on previous works (Messinetti et al. 2019; Mercurio et al. 2021). Preliminary trials were performed starting from

concentrations close to environmental ones to define effective and lethal doses.

2.2. Exposure during ascidian embryogenesis

About 50 embryos at the two-cell stage were transferred to Petri dishes filled with 10 mL of the various test solutions and reared until the larva stage (~18 hours post fertilization (hpf)). Experiments were performed in triplicate and considered reliable only if at least 80% of control embryos hatched. When control embryos reached the larval stage, all samples were fixed in 4% paraformaldehyde, 0.5 M NaCl and 0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS fixative; pH 7.5) for 90 min, washed in Phosphate Buffered Saline (PBS) and examined under a microscope. The numbers of normal, malformed and dead larvae were noted, and the corresponding percentages were calculated.

Ciona intestinalis samples were exposed to BPA while *C. robusta* was used to test TCPP. Data about BPA in *C. robusta* have been partially published in Messinetti et al. (2019). To compare the two species, previous data were further analyzed. The effects of TCPP on *C. intestinalis* were already reported by Mercurio et al. (2021).

2.3. Statistical analysis

Analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test,

was performed to assess the effects of TCPP/BPA on larval development. Prior to analyses, we verified the homogeneity and normality of the variances. Probit analysis was performed following the simple least squares regression method to calculate LC_{50} and EC_{50} . All the analyses were performed in the R 3.6.3 environment (Team 2019).

3. Results

3.1. TCPP effects on *C. robusta* development

TCPP exposure affected *C. robusta* embryonic development (Figure 1). Control and DMSO larvae appeared normally developed and motile (Figure 1(a)). The larval trunk was elongated with three adhesive papillae at the anteriormost region (Figure 1(b)) and the tail was long and straight (Figure 1(c)). Larvae exposed to concentrations higher than 25 $\mu\text{g}/\text{mL}$ were usually unable to swim. Their trunks were roundish and tails were variously bent (Figure 1(d,e)). At the bend point, a large cell was always observed. This ovoid cell was about 20 μm long and localized among muscle cells (Figure 1(f,g)). Dead larvae showed a completely disrupted morphology and remained inside the chorion membrane (data not shown).

A significant increase in the percentage of malformed larvae was observed from 25 $\mu\text{g}/\text{mL}$ TCPP (Figure 1(h); ANOVA: $F = 9.7465$, $p < 0.0001$; Tukey's post hoc test: controls vs 25 $\mu\text{g}/\text{mL}$, $p < 0.05$; controls vs 50 $\mu\text{g}/\text{mL}$, $p < 0.0001$). The percentage of dead samples proved to be significantly higher than that of controls

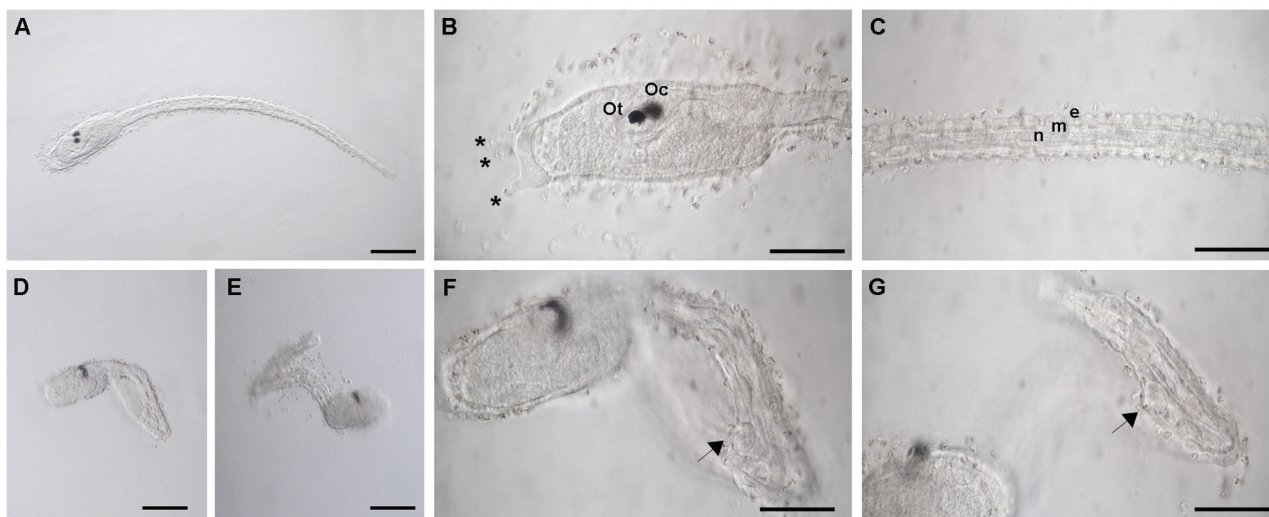


Figure 1. Morphological evaluation of TCPP's effects on *Ciona robusta* development. Morphology of control (A–C) and treated (D–G) larvae. (A) Control larva developed in in 0.1% DMSO in ASWH. (B) Magnification of the trunk of a control larva in which the otolith (Ot) and the ocellus (Oc) are observable as well as the three anterior papillae (*). (C) magnification of the tail of a control in which epidermis (e), muscle (m) and notochord (n) are visible; (D, E) malformed larva developed in 25 $\mu\text{g}/\text{mL}$ TCPP; (F, G) magnification of malformed larvae displaying a large ovoid cell (arrow) at tail bend point. Scale bars: A, D, E = 100 μm ; B, C, F, G = 50 μm .

at 75 and 100 µg/mL TCPP (Figure 1(b); ANOVA: $F = 67.24, p < 0.0001$; Tukey's post hoc test: controls vs 75 µg/mL, $p < 0.0001$; controls vs 100 µg/mL, $p < 0.0001$).

Finally, probit analysis revealed that LC_{50} was 63.48 µg/mL (95% Confidence interval (CI) for the coefficient estimates [0.02, 0.11]) while EC_{50} was 21.19 µg/mL (95% CI for the coefficient estimates [0.03, 0.08]) (Figure 1(c)). The TCPP teratogenic index ($TI = LC_{50}/EC_{50}$) was 2.99.

3.2. BPA effects on the development of *C. robusta* and *C. intestinalis*

We analyzed the larval general morphology to determine BPA's effects on ascidian embryogenesis (Figure 2). Control and DMSO larvae of both species developed normally, displaying an elongated trunk and

a long, straight tail (Figure 2(a,g)). Low concentrations of BPA did not affect larval development (Figure 2(b–e, h–j)). Larvae exposed to concentrations higher than 10 µM showed malformations, mainly consisting in a roundish trunk and/or a curved tail (Figure 2(f,k)). These phenotypes increased significantly from 10 µM concentration in both *C. intestinalis* (Figure 3A and Figure 4A; ANOVA: $F = 28.02, p < 0.0001$; Tukey's post hoc test: controls vs 10 µM, $p < 0.0001$) and *C. robusta* (Messinetti et al. 2018; ANOVA: $F = 15.806, p = .00428$; Tukey's post hoc test: control vs 10 µM, $p < 0.0001$). At 20 µM BPA, most of the larvae did not hatch and were considered dead (*C. intestinalis*: ANOVA: $F = 25.69, p < 0.0001$; Tukey's post hoc test: controls vs 20 µM, $p < 0.0001$; *C. robusta*: ANOVA: $F = 14.538, p < 0.001$; Tukey's post hoc test: controls vs 20 µM, $p < 0.001$) (Figures 2(l) and 3(a)).

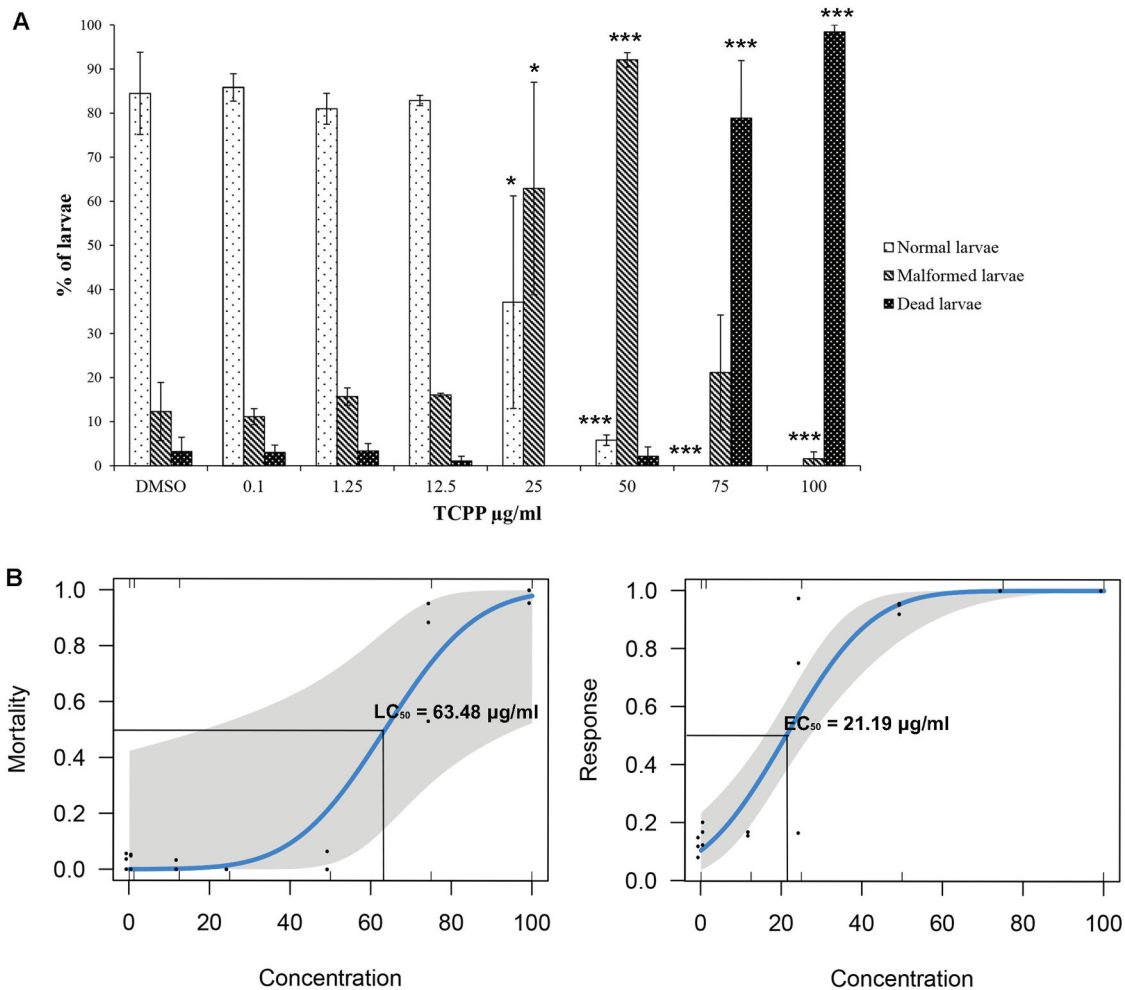


Figure 2. Effects of TCPP exposure on the embryonic development of *Ciona robusta*. (A) Percentages of normal, malformed and dead larvae of *C. robusta* exposed to TCPP. Differences from control; the number of asterisks indicates the level of significance: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. (B) TCPP dose–response curves for mortality and malformations in *C. robusta*. EC_{50} and LC_{50} values were calculated using probit models.

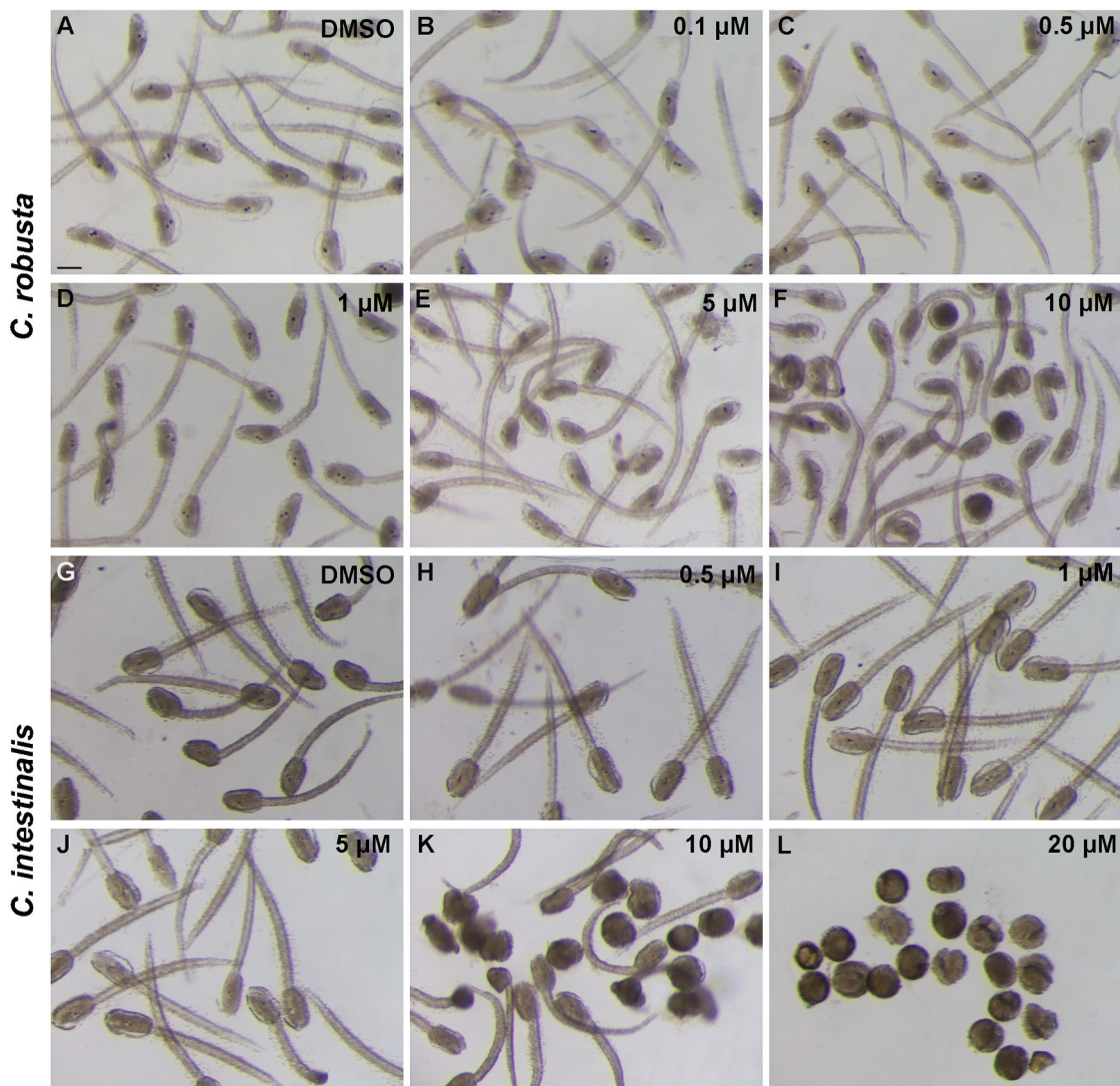


Figure 3. Morphological evaluation of the effects of BPA exposure on *Ciona robusta* (A–F) and *C. intestinalis* (G–L) development. (A, G) controls; (B–F) and (H–L) BPA. Scale bar = 100 μ m.

Probit analysis (Figure 4 B and C) confirmed these results: EC_{50} was 8.25 μ M (95% CI for the coefficient estimates [0.18, 0.31]) for *C. intestinalis* and 7.04 μ M (95% CI for the coefficient estimates [0.12, 0.28]) for *C. robusta*, while LC_{50} was 13.42 μ M (95% CI for the coefficient estimates [0.12, 0.19]) for *C. intestinalis* and 9.36 μ M (95% CI for the coefficient estimates [0.10, 0.15]) for *C. robusta*. The BPA TI was 1.63 for *C. intestinalis* and 1.32 for *C. robusta*.

4. Discussion

Ciona intestinalis and *C. robusta* are closely related species. They appear extremely similar at first glance and they were considered members of the same

species for decades. Nevertheless, their different ability to buffer water temperature was well known among researchers even before they were recognized as separate entities (Brunetti et al. 2015; Pennati et al. 2015; Sato et al. 2015).

Their sensitivity to pollutants was never compared, and probably assumed to be similar. However, a few studies have demonstrated that even closely related species can display very different sensitivities to chemicals (Rocha-Olivares et al. 2004; Feckler et al. 2012; Monteiro et al. 2018). Thus, in the present work, we compared the tolerance of *C. intestinalis* and *C. robusta* to two common marine pollutants, BPA and TCPP, demonstrating that their responses to environmental chemicals can differ according to the tested contaminant.

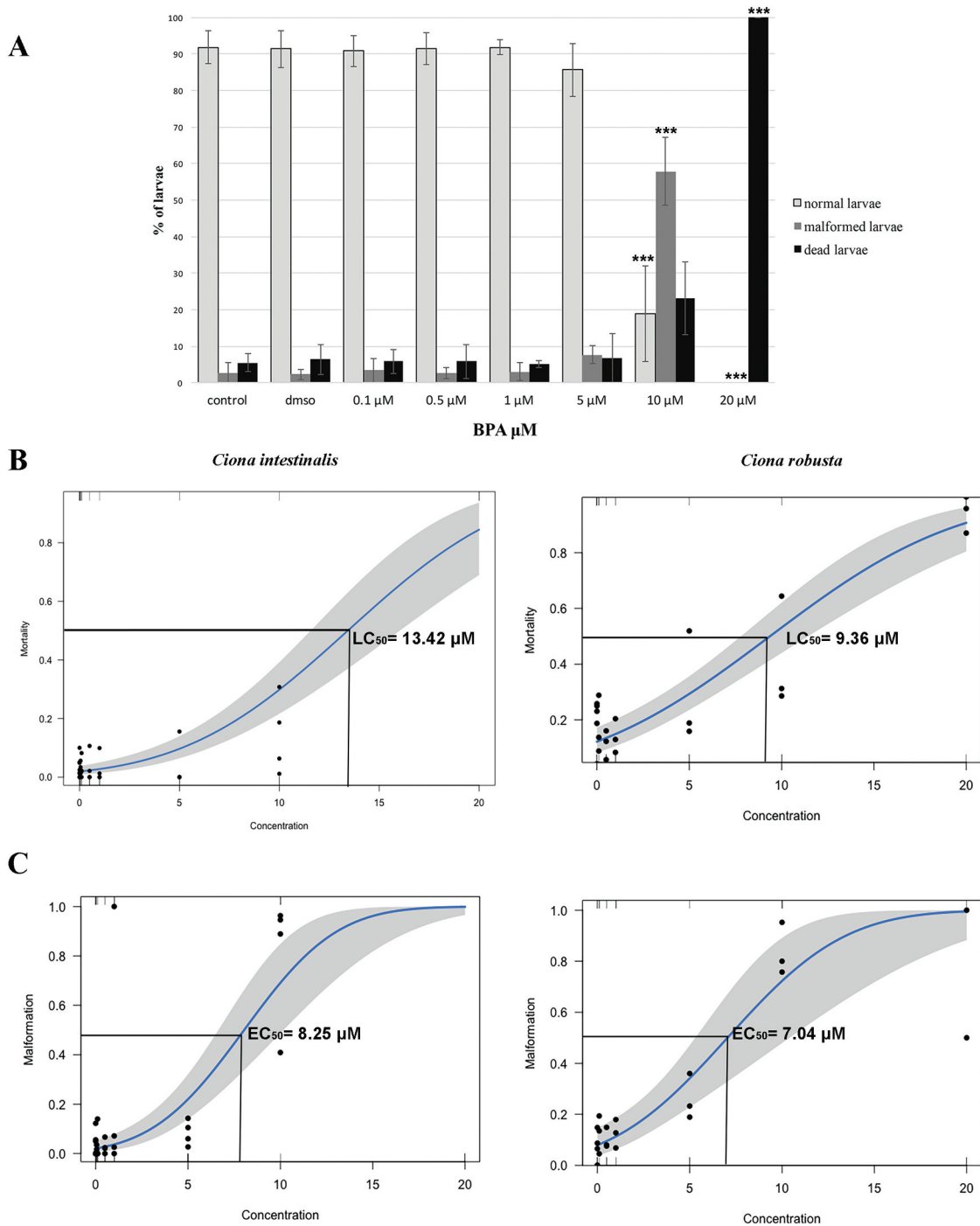


Figure 4. Effects of BPA exposure on embryonic development of *Ciona intestinalis* and *C. robusta*. (A) Percentages of normal, malformed and dead larvae exposed to different concentrations of BPA in *C. intestinalis*. Differences from control: *** $p < 0.001$. (B) Dose-response curves for mortality in *C. intestinalis* and *C. robusta*. (C) Dose-response curves for malformations in *C. intestinalis* and *C. robusta*. EC₅₀ and LC₅₀ values were calculated using probit models.

Ciona intestinalis and *C. robusta* displayed similar sensitivity to BPA. EC₅₀ values (8.25 μM for *C. intestinalis* and 7.04 μM for *C. robusta*) were comparable between the species while LC₅₀ values were slightly different (11.69 μM for *C. intestinalis* and 9.36 μM for

C. robusta). However, their TI values were comparable: 1.63 for *C. intestinalis* and 1.32 for *C. robusta*. Indeed, BPA induced the same type of malformations with similar incidences (Table I). In both species, this chemical caused the development of a roundish trunk and

Table I. Percentages of normal, malformed and dead larvae of *C. intestinalis* and *C. robusta* after Bisphenol-A exposure. Values are expressed as mean \pm standard error.

	<i>Ciona intestinalis</i>			<i>Ciona robusta</i>		
	Normal larvae	Malformed larvae	Dead larvae	Normal larvae	Malformed larvae	Dead larvae
Control	91.8 \pm 4.5	2.8 \pm 2.7	5.5 \pm 2.4	81.7 \pm 3.8	2.1 \pm 1.8	16.2 \pm 5.5
DMSO	91.3 \pm 5.1	2.3 \pm 1.3	6.3 \pm 4.0	76.1 \pm 5.9	6.5 \pm 2.9	17.4 \pm 5.7
0.1 μ M	90.8 \pm 4.2	3.4 \pm 4.2	5.8 \pm 3.2	72.5 \pm 5.2	10.4 \pm 3.4	17.1 \pm 5.2
0.5 μ M	91.8 \pm 4.5	2.7 \pm 1.4	6.9 \pm 4.6	79.7 \pm 3.9	8.9 \pm 1.5	11.3 \pm 2.6
1 μ M	91.8 \pm 2.1	3.0 \pm 2.4	5.1 \pm 0.9	75.5 \pm 5.0	10.5 \pm 2.0	13.9 \pm 3.0
5 μ M	85.6 \pm 7.2	7.7 \pm 2.4	6.7 \pm 2.4	53.7 \pm 10.0	17.4 \pm 0.8	28.9 \pm 9.9
10 μ M	18.9 \pm 13.0	57.8 \pm 9.2	23.1 \pm 10.0	10.9 \pm 4.0	47.7 \pm 6.1	41.4 \pm 9.9
20 μ M	0 \pm 0	0 \pm 0	100 \pm 0	0.7 \pm 0.6	5.0 \pm 3.5	94.3 \pm 3.3

DMSO: dimethyl sulfoxide.

a curved tail, in percentages significantly higher than in control samples starting from concentrations of 10 μ M. Previous research reported LC₅₀ values of 5.4 μ M (Matsushima et al. 2013) or 5.2 μ M (Messinetti et al. 2019) as probit analyses were performed with a logarithmic scale. In our analysis, we preferred not to convert data as results better fit the calculated percentages: at 10 μ M, *C. intestinalis* and *C. robusta* malformed larvae were 57.8 \pm 9.2% and 47.7 \pm 6.1, respectively, while at 5 μ M malformed larvae were 7.7 \pm 2.4% for *C. intestinalis* and 17.4 \pm 0.8% for *C. robusta*. A similar response to BPA was reported for another ascidian species, *Phallusia mammillata*. Here, BPA induced comparable anatomical malformations at the level of trunk and tail (Messinetti et al. 2018) with an EC₅₀ value (11.8 μ M; Gomes et al. 2019b) close to those we observed in *Ciona*. The calculated LC₅₀ was 21 μ M, suggesting that *P. mammillata* better tolerates this pollutant even if the concentration range did not vary consistently and remained far from environmental BPA levels (Hermabessiere et al. 2017).

On the other hand, TCPP demonstrated a different teratogenic potential in the two analyzed species. Indeed, EC₅₀ values were 51.16 μ g/mL for *C. intestinalis* (Mercurio et al. 2021) and 21.19 μ g/mL for *C. robusta* (this paper). TI further highlighted the higher susceptibility of *C. robusta* to TCPP, as its TI value was more than twice that of *C. intestinalis* (2.99 for *C. robusta* and 1.29 for *C. intestinalis*). LC₅₀ values were, however, comparable between the two species: 66.18 μ g/mL for *C. intestinalis* (Mercurio et al. 2021) and 63.48 μ g/mL for *C. robusta*. In ascidians, different sensitivity to environmental stressors was reported also at the population level. In a *C. robusta* population sampled at the Fusaro Lagoon (Italy), three separate genetic clusters were found to respond differentially to environmental variables, such as salinity, temperature and oxygen availability. Moreover, these clusters appeared to differentially handle metal

pollution, suggesting that *C. robusta* is provided with great genetic pools, allowing a rapid adaptation to environmental changes (Caputi et al. 2019).

The different responses to BPA and TCPP observed in *C. intestinalis* and *C. robusta* could be explained by differences in pollutant mode of action. BPA is a well-known endocrine disruptor, which interferes with animal physiology mainly by binding nuclear receptors, such as estrogen-related-receptor γ (ERR γ) (Okada et al. 2008), thyroid receptor (TR) (Zoeller et al. 2005), pregnane X receptors and peroxisome proliferator-activated receptors (PPAR γ) (Khamphaya et al. 2021). In *P. mammillata* and *C. robusta*, several orthologues of vertebrate nuclear receptors have been identified (Gomes et al. 2019a) and, in particular, BPA interaction with *P. mammillata* ERR has been demonstrated (Messinetti et al. 2018; Gomes et al. 2019b). In ascidians, TCPP effects were mainly related to muscle development; disruption of the *Myogenic regulatory factor* (*Mrf*) gene network has been suggested (Mercurio et al. 2021), but the specific mechanism of action is still unknown. Furthermore, detoxification mechanisms of the two species can differ and may determine the diverse responses of the two species when facing the environmental stressors. In fact, the animals used in the present study were collected in nature, in two marine areas with different pollution profiles (Hermabessiere et al. 2017). Thus, it is conceivable that the exposure to different contaminants could have selected distinct tolerances to chemicals in animals prone to adapt quickly (Caputi et al. 2019). Moreover, ascidians are efficient filter-feeding organisms, which accumulate contaminants in their tissues. In particular, recent studies have underlined their ability to bioaccumulate microplastics and phthalates as well as heavy metals, enabling these animals to reflect the pollution levels of their environment (Tzafirri-Milo et al. 2019; Vered et al. 2019).

Data about levels of TCPP or other flame retardants in the marine environment are rare. In the northeast Atlantic and the Arctic Ocean, TCPP concentrations were found to range between 279 and 5773 pg/L (Li et al. 2017), while higher levels were detected in North Sea surface water (Bollmann et al. 2012). No precise data were found for the sampling areas of the species used in this work. Conversely, BPA was measured and found in traces (<0.001–0.145 µg/L) in the Venetian lagoon (Mediterranean Sea) (Pojana et al. 2007), close to our *C. robusta* sampling site. No study thus far has focused on BPA levels along the English Channel, but it was detected in fish from the northeast Atlantic Ocean (Barboza et al. 2020).

Overall, these results strongly underline the presence of species-specific differences in embryonic sensitivity to contaminants and point out the importance of evaluating chemicals' teratogenic profile in several species. Comprehensive toxicological analyses are necessary to make environmental management and science-based policy as inclusive as possible. Moreover, marine invertebrates are particularly threatened by environmental pollutants since both fertilization and embryonic development usually occur in the water column, in direct contact with a mixture of anthropogenic contaminants. Considering that the precise modes of action of most of these chemicals are still unknown, there is the possibility that they can interact with each other and induce additive effects, making ecotoxicological studies even more urgent.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure statement

No potential conflict of interest was reported by the authors.

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