SHORT RESEARCH AND DISCUSSION ARTICLE

New approaches on the use of tunicates (*Ciona robusta*) for toxicity assessments

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Abstract

After the accidental release of crude oil in marine environment, dispersants are applied on sea surface transferring oil into the water column where it can be broken down by biodegradation, thereby reducing potential pollution to coastal areas. Before they can be used in the wild, the ecotoxicity of such dispersants is usually evaluated with toxicity assays using algae, crustacean and fishes. Nowadays, there is a need to find alternative species to reduce the use of vertebrates both for ethical considerations and for reducing the cost of bioassays. *Ciona robusta* is a solitary ascidian that inhabits shallow waters and marine coastal areas. This species has been recently adopted as valuable biological model for ecotoxicity studies, thanks to its rapid embryonic and larval development, resemblance to vertebrates, and low risk of ethical issues. On this ground, the lethal and sublethal toxicity of two dispersants has been evaluated on *Ciona* juveniles. At this stage, the organisms become filter-feeders and the morphological alterations of the organs can be easily observed. The median lethal concentrations at 96 h ($96hLC_{50}$) for Dispersant 1 (non-ionic surfactant) and for Dispersant 2 (mixture of non-ionic surfactants and anionic surfactants) are 41.6 mg/L (38.6–44.9) and 92.5 mg/L (87.7–97.5), respectively. The *Ciona* juvenile model was compared to *Dicentrarchus labrax* fish juveniles test, and it showed increased sensitivity for *Ciona* to these compounds. These results suggest that 96 h mortality test bioassay could be a good alternative method to 96 h mortality assay with *D. labrax*, limiting the use of vertebrates for dispersant toxicity.

Keywords Oil spill management · Dispersants · Toxicity · Alternative biological pattern · Ascidian

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Introduction

Dispersant products mainly used during oil spill response in marine environment are a combination of surfactants and solvents. The surfactants are characterized by a mix of hydrophilic and oleophilic components that facilitates the separation between oil and water in case of oil spill events (Wise and Wise 2011). The solvents (water, water miscible hydroxy compounds or hydrocarbons) dissolve surfactants and favor their solubility in the oil (IMO/UNEP 2011). Then, the oil droplets, formed by the dispersant action, are naturally dispersed by the wave and/or wind and degraded by bacteria.

Dispersants, alone or in combination with hydrocarbons, may induce toxic effects on marine organisms. For this reason, it is important to know the chemical and ecotoxicological characteristics of these products to assess their environmental suitability.

Despite few exceptions, in many European countries, the dispersant use is regulated by national policies. In particular,

in Italy, dispersant ecotoxicity has to be assessed during approval procedure before their use at sea. Three toxicity assays are required: algal growth inhibition test and mortality tests with crustaceans and fish.

The need for alternative approaches to the use of vertebrates has become of growing significance for ethical considerations and for reducing the cost of ecotoxicological bioassays. As a matter of fact, considerable advances have been made in this field over the last few decades, as reviewed in Lillicrap et al. (2016). However, fish tests are still required in some countries for regulatory purposes, including dispersant approval procedures. However, there is a need for new and alternative protocols to Reduce, Replace, and Refine fish tests according to 3R strategy (Halder et al. 2014), which have to be also Reproducible, ecologically Relevant, and Regulatory acceptable (additional "3Rs").

In this regard, ascidians such as *Ciona robusta*, could be taken into consideration as biological models for alternative methods in dispersant ecotoxicity testing because (a) they are a reliable and sensitive model system for ecotoxicology studies, (b) they are invertebrates, (c) their genome seems to lack genes related to pain (Okamura et al. 2005), and (d) the small size of *Ciona* larvae and/or juveniles (stage used for fish tests, OECD/203 1992) requires only small volumes of test water.

The ascidian *Ciona* is a marine sessile invertebrate that belongs to the Subphylum of *Urochordates*, which have been recognized as the closest living relatives of vertebrates (Delsuc et al. 2006). The *Ciona* lifespan is relatively short and includes embryonic, larval, juvenile, and adult phases (Satoh 1994). Fertilized eggs develop, in 20–24 h, into swimming tadpole larvae showing typical chordate characteristics,

like a rigid notochord and a dorsal neural tube (Sasakura et al. 2012). The larval stage of this species is thus very useful for testing the effects of environmental stressors on the embryonic development of a "simplified chordate ancestor." After swimming for a few hours, the non-feeding larva attaches to a substrate and starts the metamorphosis process to become a juvenile. According to Chiba et al. (2004), 4 days after fertilization, *Ciona* juveniles reach the stage 4 in which all organs are formed: the heart, which is specified by known gene regulatory networks (GRNs) (Anderson and Christiaen 2016), the digestive tract (esophagus, stomach and intestine), the nervous system, the gonad rudiment, the gill slits, and the oral and atrial siphons. At this stage, the juveniles become filter-feeders and they can accumulate any toxicant present in the water. Notably, the juveniles have a transparent tunic, thus permitting to see all the internal organs, under the microscope, and visualize any eventual morphological alteration under stress conditions (Chiba et al. 2004; Sato et al. 1997; Willey 1893a, 1893b; Yamamoto and Okada 1999). Juvenile development takes about 10 days, then the young adults become reproductive, grow isometrically, and die in 12–18 months (Berrill 1947; Dybern 1965; Millar 1953; Petersen et al. 1995). Here too, *Ciona* adults, as filter-feeders, represent an important sentinel for marine environmental monitoring because they tend to accumulate and therefore sequester trace elements.

After being used for more a century for embryological and more recently in evo-devo studies, ascidians such as *Ciona robusta* are currently attracting growing interest for toxicological analyses. This model system allows evaluating different endpoints besides the death rate of juveniles. A brief survey of

Table 1 Experimental conditions for 96 h mortality tests of *C. robusta* and *D. labrax*

	C. robusta	D. labrax
Test type	Semi-static, solutions renewed daily	Semi-static, solutions renewed daily
Exposure time	96 h	96 h
Endpoint	Mortality	Mortality
Sublethal endpoint	morphological abnormalities	swimming behavior
Concentrations nr	4 + control	4 + control
Replicates	3	3
Test volume	6 ml	5 l
Test chambers	6 well microplates	Plastic vessels
Organisms	Juveniles (stage 4)	Juveniles
No. of organisms for each replicate	30	7
Test medium	natural seawater (38‰)	Synthetic seawater (with Instant Ocean® salt mixture) (21‰)
Test conditions (photoperiod, T, agitation)	Dark, 18 °C	16 h light, 20 °C
Reference	Messinetti et al. (2017), modified	OECD/203 (1992) modified
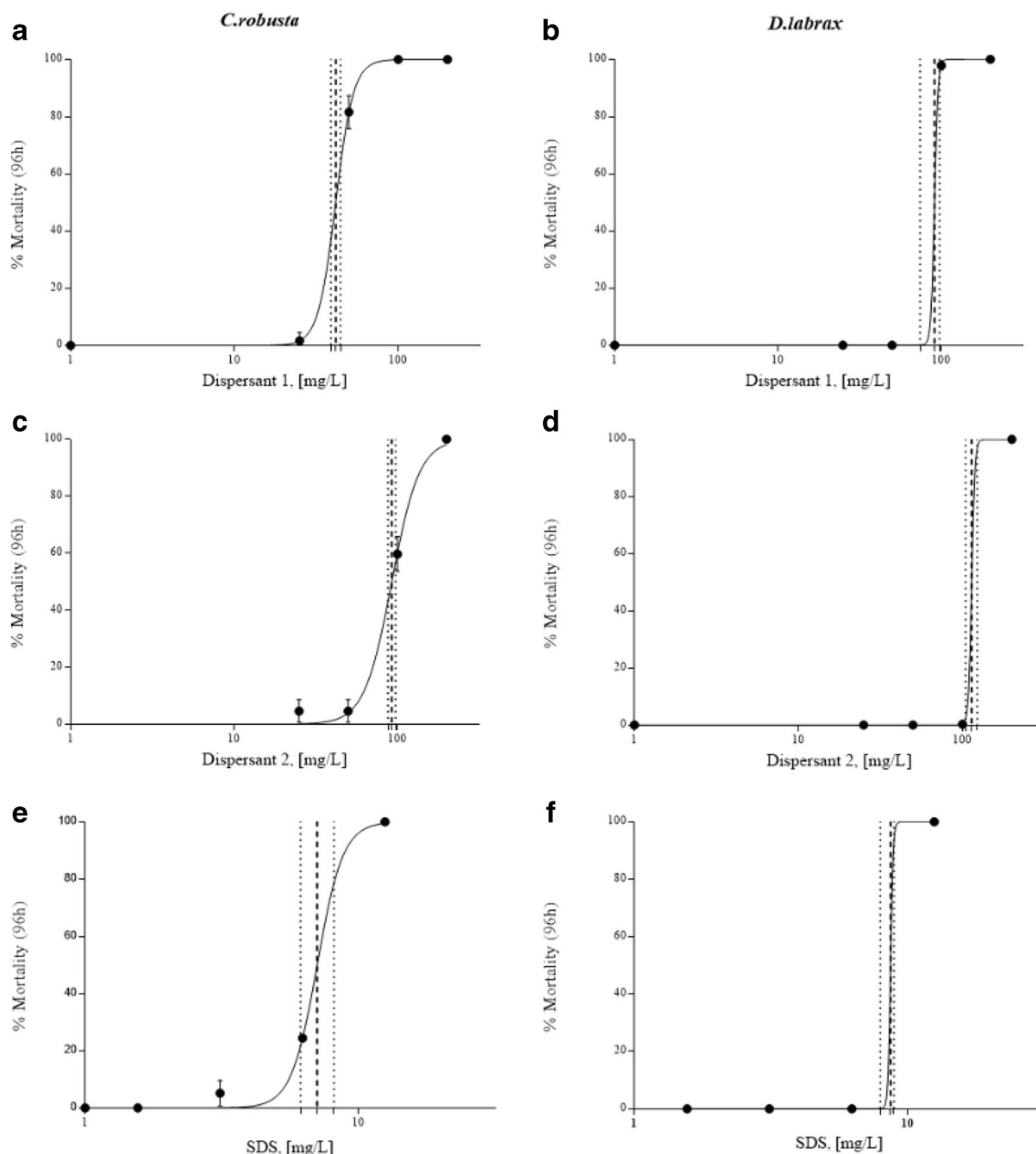
Fig. 1 Mortality rate of *Ciona robusta* and *Dicentrarchus labrax* after 96 h of exposure to different concentrations of dispersant D1 (**a**, **b**), dispersant D2 (**c**, **d**) and sodium dodecyl sulfate SDS (**e**, **f**). Curves represent the nonlinear regression of mortality data (sigmoidal) with the best fit for LC_{50} values (dashed lines) as well as the relative 95% CIs (dotted lines). Error bars represent standard deviation

ecotoxicological studies using *Ciona robusta* has been reported in the Supplementary section. Although there are still few studies, the filter-feeding *Ciona* juveniles have been used to monitor the potential toxicity of two endocrine disruptors (EDCs), bisphenol A (BPA) and tributyltin (TBT) (Mansueto et al. 2011), and to investigate the effect of polystyrene microplastics (Messinetti et al. 2017).

In this study, the lethal and sublethal toxicity of two dispersants has been tested on *Ciona* juveniles and compared to toxicity response of fish juveniles. The aim was to explore the possibility to use the ascidian *C. robusta* as alternative

biological model to the vertebrate *Dicentrarchus labrax* in dispersant approval procedures.

Materials and methods

Animal collection

Adults of *C. robusta* were collected from natural habitat (Taranto, Italy) and transported within few hours into the aquarium of the Zoological Station Anton Dohrn of Naples

Table 2 96hLC$_{50}$ values and confidential limits (mg/L) for *Ciona robusta* and *Dicentrarchus labrax* exposed to sodium dodecyl sulfate (SDS) and dispersants (D1 and D2)

Chemical	*C. robusta*	*D. labrax*
SDS	7.0 (5.4–9.1)	8.6 (8.0–9.1)
D1	41.6 (38.6–44.9)	86.2 (75.2–98.8)
D2	92.5 (87.7–97.5)	136.8 (128.3–145.9)

(Italy). The animals were fed daily with a mixed algal diet and maintained for a week in flow-through circulating aquarium at 18 ± 1 °C under continuous light to promote gamete production and to avoid spawning (Lambert and Brandt 1967).

Juveniles of the marine fish *D. labrax* (size: 4.1 ± 0.1 cm and 0.7 ± 0.1 g) were obtained directly from a fish farm (Rovigo, Italy) and transported within few hours into the aquarium of the Regional Agency for Environmental Protection in Emilia-Romagna of Ferrara (Italy). The animals were fed with commercial marine fish food (2% of body weight), until 24 h before beginning toxicity testing. The fish were acclimated for 7 days, and no mortality was recorded in this period.

Gamete collection, in vitro fertilization, and juvenile collection of *C. robusta*

Gametes of *C. robusta* were obtained by dissecting the gonoducts with a scalpel. Different specimens were used to collect oocytes and sperm. Pooled oocytes were suspended in filtered natural sea water (0.2 μm) and washed twice. Fertilization was performed by adding a dilution (1:100 in FNSW) of pooled sperm to the egg suspension. After an incubation of 10 min on a rotating shaker, the fertilized eggs were washed, transferred in tissue culture plates, and grown until the desired stage of development. Stage 4 (4 days after hatching, size: 2 mm) was chosen for toxicity testing exposure, since at this stage, all the organs are present and the individuals can feed and contract their siphons (Chiba et al. 2004).

Toxicant exposures

Juveniles of *C. robusta* and *D. labrax* were exposed for 96 h at five concentrations (0–25–50–100–200 mg/L) of two dispersants, called D1 and D2. D1 is a non-ionic surfactant (10–20%) in alkaline aqueous solution; it is soluble in water and its bioaccumulation is very low in the environment (log Kow < 3). D2 is a mixture of non-ionic surfactants (> 24%) and anionic surfactants (12–24%); it contains hydrocarbons (C11–C14), *n*-alkanes, isoalkanes, cyclic, and aromatics. D2 is soluble in water, and its bioaccumulation is very low in the environment (log Kow < 3). Sodium dodecyl sulfate (SDS) is an anionic surfactant and contains sodium salt and dodecyl sulfate, with moderate solubility in water (15 g/100 mL at 20 °C) and low bioaccumulation (log Kow < 3). It was used as reference toxicant (positive control) and tested at five concentrations (0–1.56-3.12-6.25-12.5 mg/L). SDS was selected as a reference toxicant because (a) it is a surfactant as the dispersants; (b) it is the reference toxicant in dispersant fish tests according to Italian law (D.D. 02/25/2011), and (c) there are many marine toxicity data for it in literature as reported in Manfra et al. (2017, 2019). The toxicant concentrations were prepared by dissolving SDS or dispersant in seawater. All the assays were performed with daily change of toxicants, because SDS can be easily aerobically degraded in nonsterilized aqueous solution (Scott and Jones 2000), and dispersant degradability was unknown. The SDS concentrations were chosen based on literature data (Conti et al. 2015; Mariani et al. 2006), while a wider range of concentrations was preferred for dispersants, given the lack of information about their toxicity. A control sample (seawater without toxicant) was tested in all experiments, and 3 replicates were done for each concentration. Thirty and 7 individuals were exposed for each replicate for *C. robusta* and *D. labrax*, respectively. All bioassays were performed at the experimental conditions reported in Table 1. The experiments were carried out according to Messinetti et al. (2017) with some modifications (no feeding, 96 h

Fig. 2 - Control juveniles of *Ciona robusta* (**a**) and *Dicentrarchus labrax* (**b**) raised in seawater without toxicant. Scale bar: **a** 100 μm; **b** 1 cm

Fig. 3 Morphological abnormalities in *Ciona robusta* exposed at increasing concentrations of sodium dodecyl sulfate SDS (**a–d**), dispersant D1 (**e–h**) and dispersant D2 (**i, l, m, n**). All the treatments (except **a**) caused a reduction in size, internal organ disorganization and presence of necrotic tissues (see **b, c, e, f, i, l, m**); the dead juveniles were shown in the images **d, g, h** and **n**. The percentage of surviving juveniles is depicted on the pictures
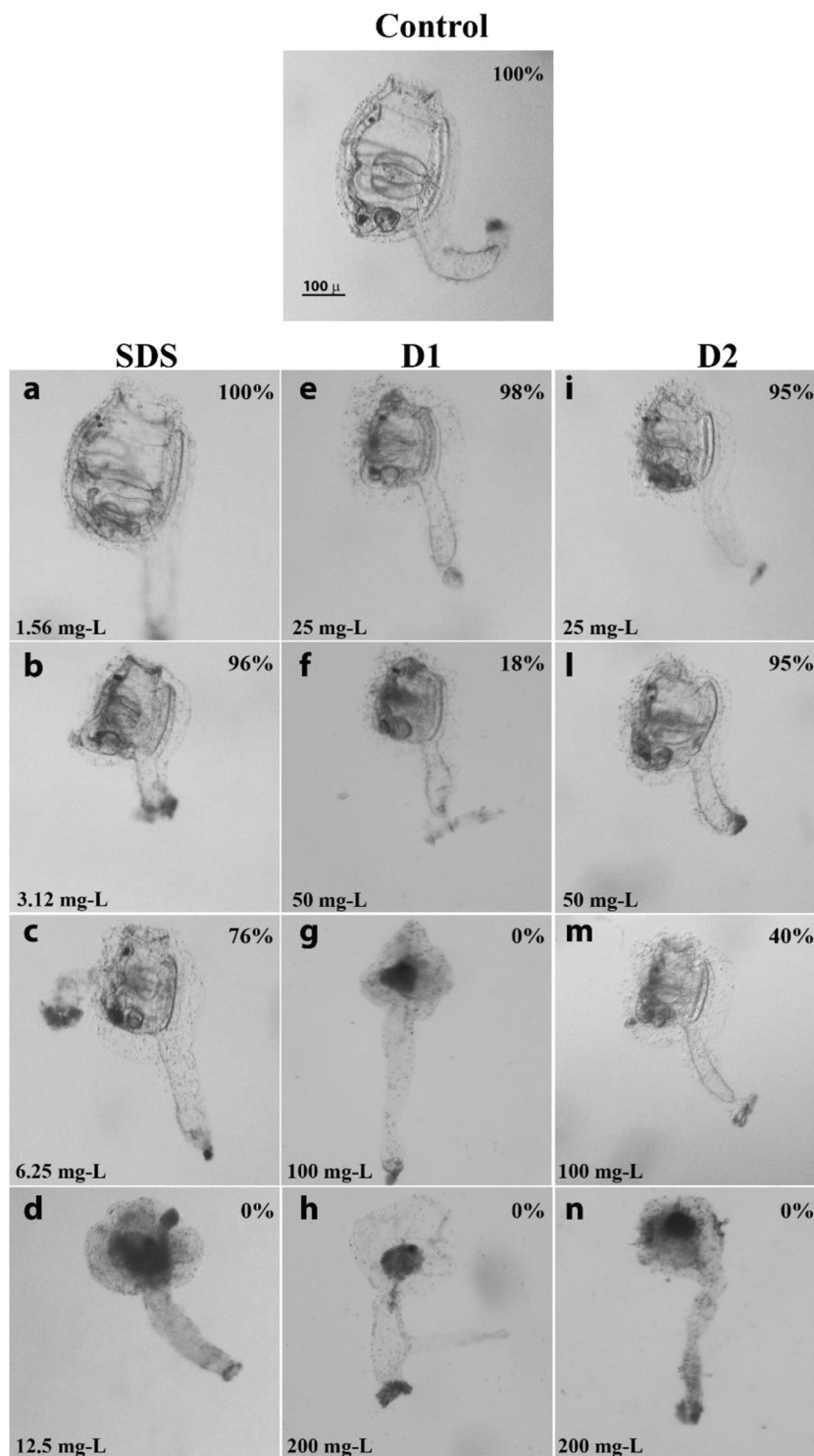
exposure) and OECD/203 (1992.) for *C. robusta* and *D. labrax*, respectively. Morphological and behavioral endpoints were considered as qualitative sublethal endpoints. Tunic thickening, internal organ disorganization, and slowdown in growth were observed daily using the stereomicroscope Zeiss Stemi 2000 for *C. robusta*, while *D. labrax* swimming was recorded for 2 min.

Statistical analyses

After 96 h of exposure, the mortality rate of juveniles was evaluated and $96hLC_{50}$ values were calculated by using the GraphPad Prism 6 and ToxStat software for ascidians and fish, respectively. All bioassays were considered acceptable when the control mortality percentage was equal or lower than 10%.

Results and discussion

Effects on survival

In control treatments (seawater without toxicant), all juveniles were alive and healthy. The analysis of the data by nonlinear regression of data obtained from the 96 h acute toxicity tests revealed a dose-response effect for *Ciona* juveniles whereas an all-or-none response for *Dicentrarchus* juveniles for all the compounds tested (Fig. 1).

The $96hLC_{50}$ values for SDS are 7.0 (5.4–9.1) and 8.6 mg/L (8.0–9.1) for *C. robusta* and *D. labrax*, respectively (Table 2). These values are comparable or lower than the LC_{50} of SDS published in the literature for *D. labrax* (Conti et al. 2015; Mariani et al. 2006), other fish species (Ribelles et al. 1995; Rosety et al. 2001), and invertebrates (Mariani et al. 2006; Rotini et al. 2015).

In D1 exposure, $96hLC_{50}$ of 41.6 (38.6–44.9) and 86.2 mg/L (75.2–98.8) were recorded for *C. robusta* and *D. labrax*, respectively (Table 2). In D2 exposure, $96hLC_{50}$ of 92.5 (87.7–97.5) and 136.8 mg/L (128.3–145.9) were recorded for *C. robusta* and *D. labrax*, respectively (Table 2). Sensitivity of organisms to SDS is similar in both ascidians and fish whereas the $96hLC_{50}$ of fishes were 2-fold and 1.5-fold higher than $96hLC_{50}$ of ascidians for D1 and D2 respectively, resulting in a slight different sensitivity of *Ciona* juveniles compared to *Dicentrarchus* juveniles.

Effects on morphology of ascidians and swimming behavior of fishes juveniles

Morphological abnormalities and swimming behavior were scored as sublethal endpoints in *C. robusta* and *D. labrax*, respectively. In control specimens, all *Ciona* juveniles had a beating heart located between the endostyle and the stomach, a tunic around the body, detectable gill slits I and IV, and the oral siphon open, while *D. labrax* juveniles were highly motile (Fig. 2a, b).

Surviving fish juveniles did not show gross morphological abnormalities. However, slow and uncoordinated swimming behavior was observed in surviving fishes at the higher concentrations of tested toxicants (6.25 mg SDS/L and 100 mg D1–D2/L), while no alteration was observed at the lower concentrations (data not shown). Concerning ascidians, surviving *Ciona* juveniles did not show gross morphological abnormalities at the lowest SDS concentration (1.56 mg/L), while intermediate concentrations (3.12 and 6.25 mg SDS/L) induced tunic thickening and internal organs disorganization, besides a slowdown in growth (compare Fig. 3a with b and c). At the highest SDS concentration (12.5 mg/L), no individual survived (Fig. 3d). Death of juveniles was also induced by D1 and D2 treatments (Fig. 3e–n), with D1 showing the strongest lethality (D1 at 50 mg/L resulted in 18% surviving juveniles whereas D2 at the same dose resulted in 95% survival) (Fig. 3f). It is intriguing to note that a darkening of internal organs was observed in almost all the surviving D1 and D2 treated individuals, probably due to necrotic tissue of unhealthy juveniles. These data clearly indicate that both D1 and D2 dispersants, already at the lower concentrations, strongly compromise the wellness of *Ciona* juveniles and consequently the viability of the surviving specimens.

Conclusion

In this study, we showed that the ascidian *Ciona robusta* juvenile model is a reliable and sensitive model system for ecotoxicology studies. *Ciona* tadpole larva, indeed, represents the basic and most simplified chordate ancestor while its juvenile and adult stages share many organs with higher chordates like a beating heart specified by known gene regulatory networks (GRNs), a digestive system and an endostyle. This, coupled with a number of computational tools, techniques, and genomic resources, makes *Ciona* a foundation to reveal the cellular/molecular processes in developing organisms, which could provide valuable information potentially useful for higher and more complex chordates. Moreover, being an invertebrate chordate and lacking in its genome most genes responsible for pain sensation, *Ciona* is less restricted than fishes by ethical and legal issues. Ninety-six hours mortality bioassay with *C. robusta* juveniles could thus be proposed as alternative method to 96 h mortality bioassay with *D. labrax* to reduce, refine, and replace (the 3Rs rule, 86/609/CEE) the use of vertebrates for dispersant toxicity testing.

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