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Dottorato in Scienze e tecnologie ambientali, geologiche e polari

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"Ciona robusta (formerly Ciona intestinalis type A) as model system for ecotoxicological studies"

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ABSTRACT

The invertebrate urochordate *Ciona robusta* (formerly *Ciona intestinalis* type A) is a sessile marine benthic organism distributed worldwide that attaches to the surfaces of both natural and artificial substrates, from shallow water to the deep sea. Based on its phylogenetic position as basal chordate, from more than a century *Ciona* represented an excellent model system for studying developmental biology, thanks to the rapid embryonic and larval development, resemblance to vertebrates, ease of management, low cost, transparent body, low risk of ethical issues and a number of techniques and genomic resources developed in the course of the years. Recently, in light of such interesting features, *Ciona* has been used to evaluate the embriotoxicity of legacy pollutants (e.g. heavy metals, pesticides, organic compounds). Notably, the genetic, genomic and molecular tools available for *Ciona* allow a deeper investigation of the molecular mechanisms affected by pollutants and could provide insights on their mode of actions (MoA). Within this thesis, the effects of two classes of contaminants of emerging concerns (CECs) have been tested on the embryogenesis of *C. robusta*. CECs are a group of natural and synthetic chemicals including nanoscale particles and transformation products, which have been increasingly found at low levels in surface waters. These compounds may pose a risk to aquatic life and, thus, it is fundamental to assess their potential effects on marine organisms. In detail, the effects of two dispersants named as A and B, used for cleaning up the petroleum hydrocarbon contamination in case of accidental oil spills at sea, and polystyrene nanoparticles (PS NPs), as proxy for nanoplastics have been investigated on *C. robusta* larval development. The four chapters of the thesis report findings on the effects of the two dispersants and PS NPs bearing different surface charges by using two approaches: 1) embryotoxicity, by looking at adverse effects in developing embryos and sub-lethal biological responses on functional proteins and enzymes; 2) mechanisms of action (MoA) at molecular level using different techniques as Real-Time PCR, RNA sequencing and bioinformatics. We demonstrated how embriotoxicity in *C. robusta* could represent a useful tool to evaluate the impact of dispersants on marine species. The data obtained indicated a different toxicity between dispersants A and B, confirmed also by phenotype alterations. Moreover, the evaluation of the expression of selected genes involved in stress response (SODa, SODb, MnSOD, GPx, HSP60, HSP70), detoxification (Cyp450, GST, GluR) and cell survival (p38, Cas8) indicated dispersant B as teratogen while dispersant A having less impact on *C. robusta* larvae. Regarding nanoplastic, surface charges seem to play a significant role in the observed embryotoxicity of the amino-modified PS NPs (PS-NH_2) in agreement with their behavior in exposure media. No effects were found for carboxyl-modified PS NPs (PS-COOH) on *C. robusta* embryo development while from mild to severe

phenotype alterations were observed upon exposure to PS-NH_2, including behavioral traits (e.g. swimming performances). Among those mostly evident, embryos resulted unable to hatch and several abnormal phenotypes were found. In addition, induction of oxidative stress linked to an increase of ROS production and the down-regulation of some representative genes involved in stress response (HSP70, HSP60, MnSOD, cytochrome b, p-38 mapk and caspase 8) were observed. The analysis of transcriptome, through differential RNA-seq, allowed to identify altered pathways affected by PS-NH_2. Several genes resulted dysregulated upon the exposure to PS-NH_2, while the GO analysis, which classified genes in three different subclasses, revealed that the number of genes affected, belonging to different subclasses, have a dose-response relationship with the concentration tested. Going deeply into the bioinformatic analysis, "glutathione synthesis and recycling pathway", "neurotransmitter clearance pathway", "passive transport by aquaporins" and "fructose and mannose metabolism", "starch and sucrose metabolism" and "glycolysis" pathways resulted affected. The alteration of these pathways could be related to the hypoxic microenvironment due to the dense coating of PS-NH_2 around the egg envelopes of *Ciona* embryos. Similar findings in terms of embryotoxicity and phenotype alterations have been observed in another ascidian species, *Phallusia mammillata*, exposed in similar conditions to amino-modified PS NPs (PS-NH_2). Furthermore, the quantitative analyses of *Phallusia* phenotype using the software Toxicosis8, revealed the affection of both central and peripheral nervous system. The use of *C. robusta* embryos as a model to study the effects of dispersants and PS NPs as proxy for nanoplastics proved to be instrumental in shedding light on different aspects of developmental toxicity exerted by those CECs. Moreover, these results will hopefully provide important information useful for higher and more complex chordates.

ABBREVIATIONS

List of the abbreviations used in the thesis, in alphabetical order:

AChE: Acetylcholinesterase

A-P: Antero-Posterior

BCF: Bioconcentration Factor

bp: base pairs

cas8: caspase 8

CECs: Contaminants of Emerging Concerns

ChE: Cholinesterase

CNS: Central Nervous System

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/Caspase9

Cyp450: Cytochrome P450

CytB: Cytochrome B

dbESTs: database of Expressed Sequence Tags

DCFH-DA: 2',7'–dichlorodihydrofluorescein diacetate

DDBJ: DNA Data Bank of Japan

DEGs: Differentially Expressed Genes

DLS: Dynamic Light Scattering

D-V: Dorsal-Ventral

EC_{50}: Half maximal effective concentration

EMBL: European Molecular Biology Laboratory

EN: European Standards

ESTs: Expressed Sequence Tags

EU/EFTA MS: Europe/European Free Trade Association Member State

GluR: glutathione reductase

GO: Gene Ontology

GPx: Glutathione Peroxidase

GST: Glutathione S-transferase,

HLB: Hydrophilic-Lipophilic Balance

hpf: hours post fertilization

HSP: Heat Shock Protein

IRSA/CNR: Water Research Institute of the National Research Council

ISO: International Organization for Standardization

ISPRA: Istituto Superiore per la Protezione e Ricerca Ambientale

ISS: Istituto Superiore di Sanità

L-R: Left-Right

MnSOD: Manganese Superoxide Dismutase

MOA: Mode of Action

mQW: milli-Q water

NOM: Natural Organic Matter

NPs: Nanoparticles

NCBI: National Center of Biotechnology Information

NSW: Natural Sea Water

OECD: Organization for Economic Co-operation and Development

p38: p38 mitogen-activated protein kinases

PBS: Phosphate Buffered Saline

PDI: Polydispersity Index

PFA: Paraformaldehyde

PS: Polystyrene

PS NPs: Polystyrene Nanoparticles

PS-COOH: carboxylated polystyrene

PS-NH_2: amino-modified polystyrene

RNA-seq: RNA sequencing

ROS: Reactive Oxygen species

RT q-PCR: Real Time quantitative PCR

SD: standard deviation

SDS: Sodium Dodecyl Sulfate

SE: Standard Error

SODa: Cu,Zn Superoxide Dismutases isoform a

SODb: Cu,Zn Superoxide Dismutases isoform b

TALENs: Transcription Activator-Like Effector Nucleases

ThOD: Theoretical oxygen demand

TLCs: Trunk Lateral Cells

TVCs: Trunk Ventral Cells

UNI: Italian National Unification

WWTP : Waste Water Treatment Plant

Structure of the thesis

This PhD thesis is formed by a general introduction with a detailed description of *Ciona robusta*, the model organism used in this study. In detail, the phylogenetic position, the life cycle, the embryogenesis, and all the features which have ensured that this organism has been used for centuries for molecular and developmental biology studies, and more recently for ecotoxicological studies. Indeed, there is a section which encloses the studies to evaluate the effects of different contaminants on *C. robusta*. Afterwards, the main objectives of this study and the description of the content of each chapter are introduced.

The thesis is composed of 4 chapter. Each chapter contains an Introduction which describes in detail the two different types of contaminants of emerging concern (CECs) used and the different approaches to study the effects on the embryogenesis of *C. robusta*, taking full advantages of all the characteristics that this organism offers: from the fast embryonic development to the simplicity of larval morphology, up to the full sequenced genome. Then, the section Materials and Methods is included, with an in-depth description of all the methodologies used. In the part of Results and Discussion, the results obtained are explained and interpreted, while the section Conclusions summarizes the main results.
The end of the thesis is composed by Supplementary Material, which encloses a work done in collaboration with ISPRA and a special section of Acknowledgements to thank my supervisors and my colleagues.

The chapter 2 has been published as manuscript:

- Eliso, M.C., E. Bergami, L. Manfra, A. Spagnuolo, I. Corsi. 2020. "Disclose nanoplastic toxicity on the embryogenesis of the ascidian *Ciona robusta* (Phylum Chordata)", Nanotoxicology, DOI: 10.1080/17435390.2020.1838650.

INTRODUCTION

I. The model system Ciona robusta: phylogeny, life cycle and embryogenesis

Ciona robusta (formerly *Ciona intestinalis type A*) is a marine sessile invertebrate, which inhabits shallow waters present in harbours or semi-enclosed basins in extensive communities, playing an important role as filter-feeder. *Ciona* has a transparent or translucent ochre-yellow body, through which some internal organs are visible, provided with an atrial siphon with six lobes and an oral siphon with eight lobes. *Ciona* belongs to the subphylum of *Urochordata* or *Tunicata*, that together with *Cephalochordate* (amphioxus) and *Vertebrate* subphyla, represents the *Chordate* phylum (Figure IB) (Delsuc et al. 2006).

The *Tunicate* subphylum takes its name from the cellulose-containing tunic that covers the body of the diverse organisms belonging to it. Traditionally, this subphylum is divided into three classes: ascidians (sea squirts), thaliaceans (salps) and appendicularians (larvaceans).

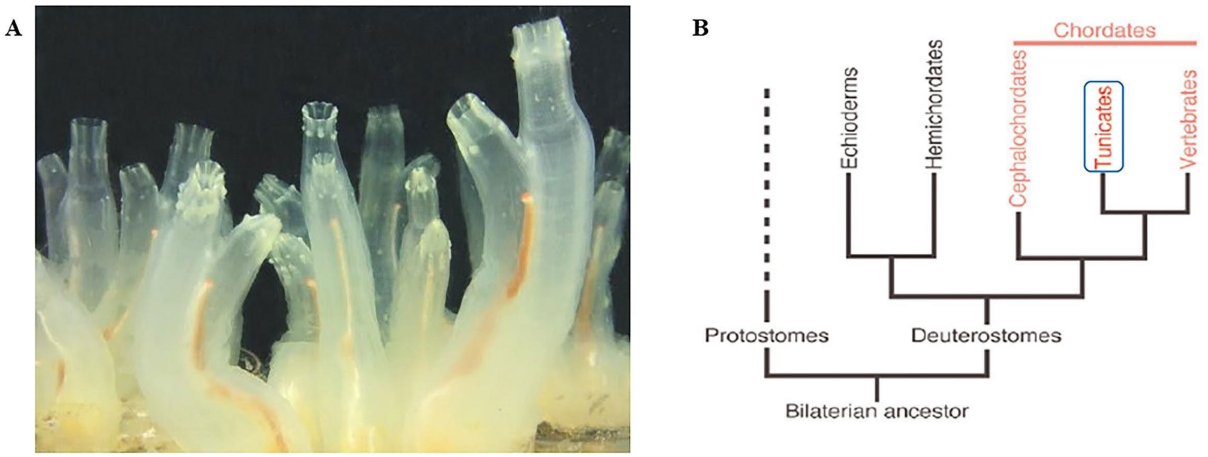

Figure I. (A) Adult of *Ciona robusta*. Images uploaded on the web by Rie Kusakabe; (B) Phylogenetic relationship of deuterostomes. Ascidians belong to the Tunicate *subphylum* (blue circle) which together with Cephalochordates and Vertebrates are part of the Chordates *phylum* (from Sasakura et al., 2007)

With a 12 to 18 months life span, *C. robusta* life cycle is relatively short and includes embryonic, larval, juvenile and adult phases (Figure II) (Satoh 1994). Individuals growth and reproduce from late spring through early fall, then entering a state of dormancy in winter (Carver et al., 2003).

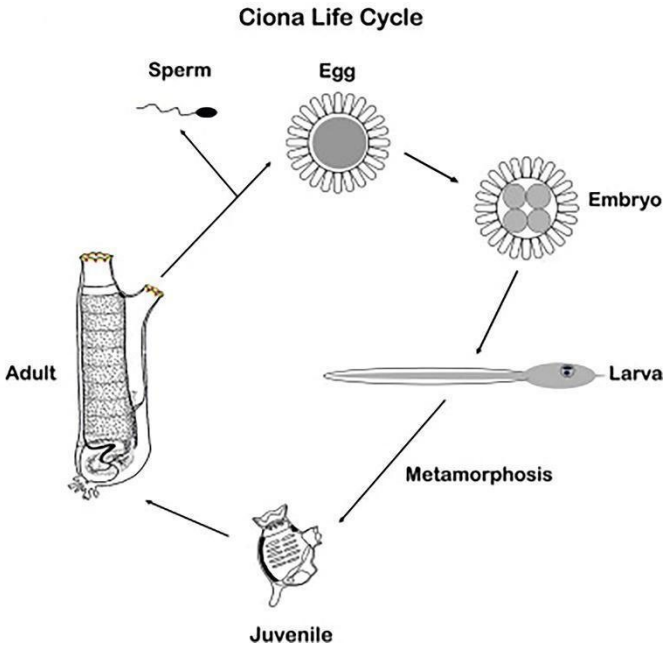

Figure II. *Ciona robusta* life cycle is characterized by an embryonic phase of ~ 18 hours, reaching the plantonic phase characterized by a larva stage, until settlement and metamorphosis phase before giving rise to the adult stage (adapted from Jeffery, 2018).

Being a hermaphrodite broadcast spawner, *Ciona* produces an enormous number of gametes that are released in the seawater where fertilization occurs. *Ciona* embryogenesis, finely described by Hotta et al. (2007) has been considered a typical example of mosaic development, in which each blastomere, since the 8-cell stage, inherits specific localized maternal determinants able to drive specific developmental fates (Conklin 1905a, b). Actually, once the pre-pattern for the zygotic program is established by maternal factors, cell-cell interactions become critical to induce the differentiation programs and accomplish a correct embryo development (Satou and Imai 2015). The first cleavage divides the embryo into left and right halves, while the second cleavage is perpendicular to the first one leading to the formation of 4 blastomeres. After the third cell division, the A–P (anterior–posterior), D–V (dorsal–ventral), and L–R (left–right) axes become evident. The nomenclature for the cell lineage consists of two letters for the entire cell lineage, A for the anterior half and B for the posterior half of the embryo. Lower case letters are used to identify cells in the animal hemisphere, while capital letters denote the vegetal cells. The letters are followed by numbers: the first digit denotes cell generation, which increases by one at each division, and the second digit (which is first used at the 8-cell stage) gives the cell number in quadrants of the embryo, which doubles at each division (Conklin 1905a).

The bilaterally symmetrical cell division continues, and after the 32-cell stage, the cleavage pattern becomes asynchronous in different parts of the embryo; at the 110-cell stage gastrulation starts. At this stage, most of the blastomeres are already fated to give rise to one tissue (Imai et al. 2004; Munro et al. 2006) (Figure III).

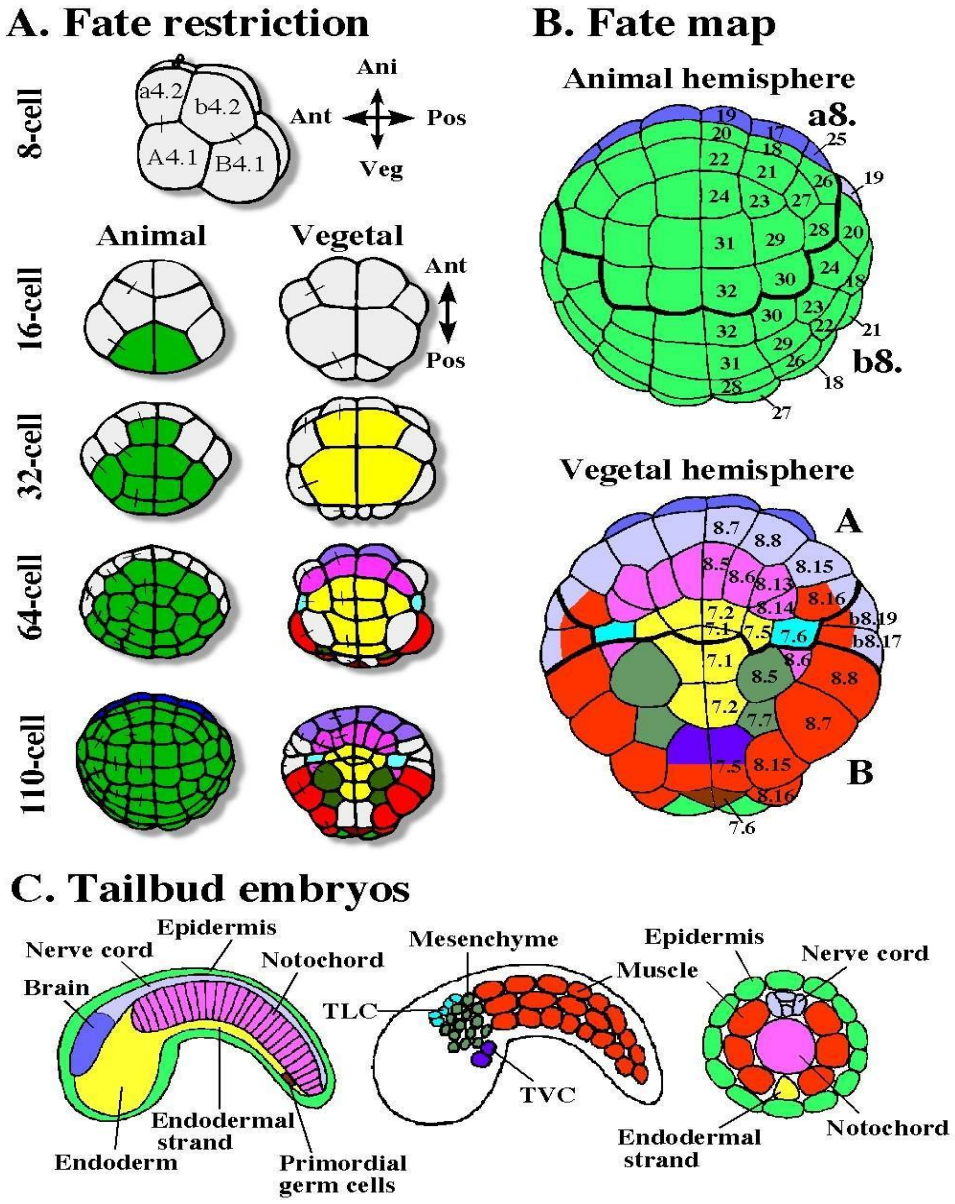

Figure III. Developmental fates of blastomeres of the ascidian embryo. A: Fate restriction during cleavage stages. Blastomeres are coloured when the fate of the blastomere is restricted to give rise to a single kind of tissue cell. The colors correspond to the colours of larval tissues indicated in C. Fate restriction in ascidian embryos proceeds quickly in the early embryo. Sister blastomeres of the previous cleavage are connected by bars. B: Fate map of the 110-cell stage. Animal and vegetal hemispheres. Names of blastomeres are indicated as 'a8.19' etc. C. Organization of tailbud embryos. Mid-sagittal planes, sagittal planes, and transverse sections of the tail (from Nishida, 2005).

Following neurulation, morphogenetic movements of presumptive notochord and muscle cells bring about formation of the tail. The occurrence of these events is very rapid, leading, in ~18 hpf at 18°C, to a swimming larva which shares a chordate body plan with more complex organisms, justifying its close phylogenetic relationship with vertebrates. In fact, in the tail, *Ciona* larva shows typical chordate characteristics, as a dorsal neural tube and a central notochord, which is flanked by segmented muscles, laterally, and by an endodermal strand, ventrally. The major larval tissues are: epidermis, nervous system, notochord, mesenchyme that includes trunk lateral and trunk ventral cells (TLCs and TVCs), endoderm, and muscle (Figure IV A). During this developmental stage, the larvae are part of the meroplankton and swim a few hours before settling onto a substrate to start metamorphosis, during which they reorganize completely their body plan to reach the adult stage (Figure IV B).

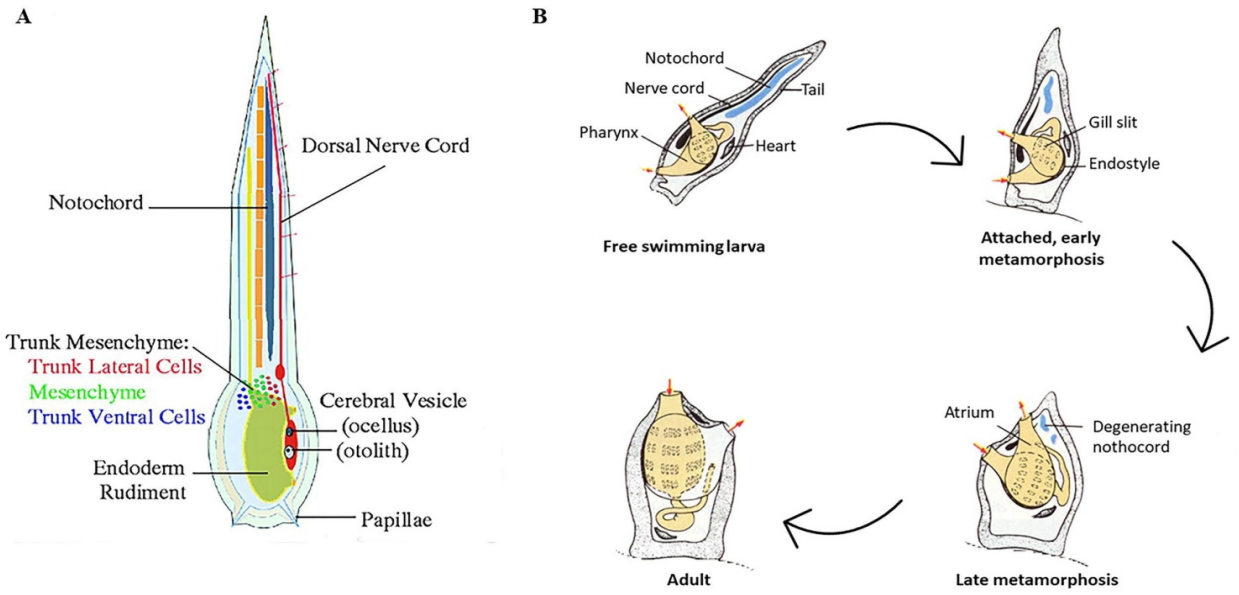

Figure IV. (A) Schematic overview of the main tissues in *Ciona robusta* tadpole larva; (B) Schematic representation of ascidian metamorphosis: after a brief free-swimming period, larva attaches to a substrate due to the adhesive papillae. The attachment is followed by a sequence of events such as tail retraction, destruction of larval tissues and formation of adult organs which complete the metamorphosis ((A) adapted from Davidson and Swalla, 2002; (B) adapted from Hickman et al., 1993).

Also the adult ascidian possesses some typical chordate features as, for example, the endostyle that is considered the precursor of the vertebrate thyroid gland, sequestering iodine and producing thyroid hormones or the feeding basket containing gill slits that appear to share a common origin with the gill slits of other chordates (Figure V) (Eales 1997; Aros and Virágh 1969).

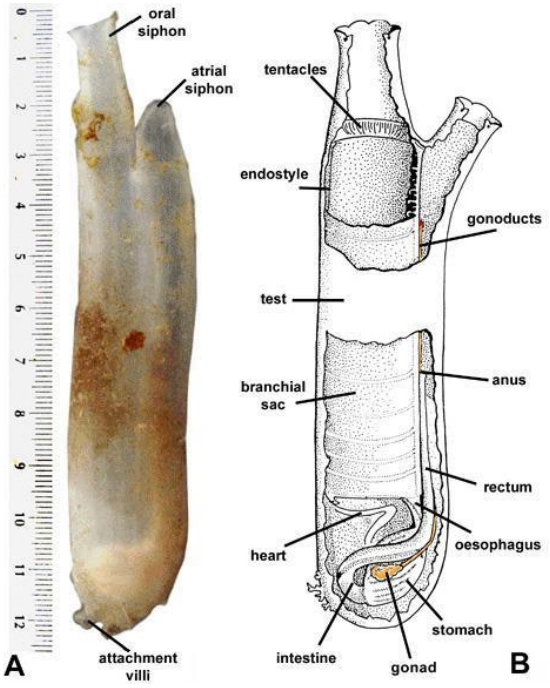

Figure V. (A) Lateral view of living adult *Ciona intestinalis;*(B) a schematic dissection, obtained by removing most of the left side test, shows the main anatomical structures of an adult. Gonads and gonoducts are coloured.

For over a century, *Ciona* represent a model organism for developmental and molecular biology studies. The fast embryogenesis and the invariant cleavage programs of embryo development are some of the characteristics behind its success. Moreover, *Ciona* larva is very simple, being composed of only 2600 cells, amongst which the notochord includes only 40 cells, the muscles 36 cells and the CNS 350 cells, of which 100 are neurons (Nicol and Meinertzhagen 1991; Satoh 2001).

Ciona robusta genome has been fully sequenced and published by Dehal and colleagues in 2002. Its size is ~160 Mb and it encodes almost 16000 genes (Dehal et al. 2002; Satou et al. 2008). Up to now, 68% of the genome sequences have been associated with specific chromosomes (Shoguchi et al. 2006; Satou et al. 2008). Actually, there are three annotated assemblies of the genome: 1) GCF_000224145.3 from RefSeq; 2) GCF_000224145.1 from ENSEMBL; 3) KHGene.2012 from Aniseed. A total of 1,205,674 expressed sequence tags (ESTs), at different developmental stages, have been registered in the National Center for Biotechnology Information (NCBI) database of expressed sequence tag (dbEST) database, and over 6700 full-length cDNA sequences are available in the DDBJ/GenBank/EMBL databases.

Ciona is one of the animals for which a large quantity of cDNA and EST (Expressed Sequence Tag) has been accumulated. A total of 1,205,674 ESTs, expressed at different developmental stages, have been registered in the National Center for Biotechnology Information (NCBI) database of expressed sequence tags (dbEST). Over 6700 full-length cDNA sequences are available in the DDBJ/GenBank/EMBL databases and 13,464 unique cDNA clones have been obtained. *Ciona* genes are organized in the genome more compactly than those of the protostomes (except *Caenorhabditis elegans*), deuterostomes, and vertebrates and most of the promoters are relatively short and usually located in close proximity of the transcription start site of the genes, usually within the first 1.5 kb upstream (Corbo et al. 1997; Fanelli et al. 2003; Alfano et al. 2007; Squarzoni et al. 2011). In addition, the genome of *Ciona savignyi*, a closely related species of *C. robusta*, has also been sequenced and comparisons between complementary *C. robusta* and *C. savignyi* sequences have indicated that these two species are at sufficient evolutionary distance to permit efficient identification of conserved regulatory sequence information (Bertrand et al. 2003; Johnson et al. 2004; Squarzoni et al. 2011). Transgenesis experiment by electroporation permits the transformation of hundreds or even thousands of embryos simultaneously (Corbo et al. 1997), thus favoring studies of transcriptional regulation of the genes of interest. This technique allows also to create "knock-out" phenotypes, by expressing dominant negative forms of particular genes using identified lineage-specific enhancers. Interference with gene function, initially relying on morpholino oligonucleotides, now includes the use of TALENs and CRISPR/cas9 strategies (Treen et al. 2014; Stolfi et al. 2014; Sasaki et al. 2014). Thanks to all these features *Ciona* has emerged as an intriguing model system to reconstruct the developmental patterning, even at single cell level, of tissues, organs, systems shared with higher chordates (Sharma et al. 2019; Ilsley et al. 2020; Horie et al. 2018).

II. Ciona robusta as new model in ecotoxicology

In the last years, the interest in ascidians as ecotoxicological models has increased since they offer several advantages (Bellas et al. 2003; Mansueto et al. 1993). The ease management in the laboratory, the numerous gametes that can be obtained and the rapid development made them suitable candidates for both *in vitro* and *in vivo* studies. Furthermore, different endpoints can analyzed as fertilization rate, embryogenesis, larval development-hatching-settlement, progression of metamorphosis and whole adults for biomarkers. Table I summarized main contributions on using *Ciona* as model organism. Heavy metals have been largely tested (e.g. Cd, Cu, Hg) organometallic ones as well as organic compounds as pesticides and antifoulants. *Ciona* embryos sensitivity towards exposure to legacy and emergent pollutants has been often correlated to that of bivalves as for instance *Mytilus*

galloprovincialis and sea urchin *Paracentrotus lividus*. As it can be argued from Table 1, the order of magnitude is almost comparable amongst these species in most cases. In this regard, *Ciona* assays could support the classical tests, used so far, with the aim of giving useful and additional information about the effects exerted by chemical substances, taking also into account the developmental stage used for the test. For example, alterations of egg/sperm function can provide information for reproductive toxicity, larval hatching and settlement assays can be used for marine water quality monitoring, while *Ciona* juveniles and adult stages can be used for bioaccumulation studies and chronic exposure assays.

Table I. Comparison of sensitivity towards inorganic and organic compounds among the three marine invertebrates *Ciona spp, Paracentrotus spp* and *Mytilus spp*.

Chemical	**EC_{50} *Ciona spp* (20h)**	**EC_{50} *Paracentrotus spp* (48h)**	**EC_{50} *Mytilus spp* (48h)**	**References**
Copper	0.72 µM	1.8 µM	0.05 µM	Bellas et al. (2001); Fernández & Beiras (2001); Gharred et al. (2016); His et al. (1999)
Mercury	0.27 µM	0.04 µM	0.61 µM	
Cadmium	7.46 µM	3.1 µM	19.6 µM	
Tributyltin	7.1 µg/L (0.022 µM)	0.309 µg/L (0.009 µM)	0.377 µg/L (0.0012 µM)	Bellas et al. (2005)
Lindane	4412 µg/L (15.2 µM)	>91,000 µg/L (>313.5 µM)	1992 µg/L (6.8 µM)	Beiras et al. (2008); Bellas et al. (2005)
Chlorpyrifos	5666 µg/L (15.7 µM)	300 µg/L (0.83 µM)	154 µg/L (0.44 µM)	
Sodium Dodecil Sulphate	5145 µg/L (17.8 µM)	4100 µg/L (14.18 µM)	2353 µg/L (8.2 µM)	
Naphthalene	1.9 µg/L (15 nM)	4.72 µg/L (37.3 nM)	6.55 µg/L (51.7 nM)	Bellas et al. (2008)
Phenanthrene	>2400 nM	>2400 nM	809 nM	
Pyrene	>640 nM	>640 nM	>640 nM	
Fluoranthene	>1250	>1250	>1250	

Chlorothalonil	123 nM	25 nM	33 nM	Bellas (2006)
Sea-Nine 211	372 nM	43 nM	38 nM	
Dichlofluanid	846 nM	1881 nM	244 nM	
Tolylfluanid	625 nM	1165 nM	213 nM	
Irgarol 1051	8346 nM	15871 nM	6076 nM	
Bisphenol A	168 µg/L (0.74 µM)	710 µg/L (5.7 µM)	3.68 µg/L (0.016 µM)	Fabbri et al. (2014); Matsushima et al. (2013); Özlem et al. (2008)
Paclitaxel	3 µM (MTC)	10 µM	-	Mizotani et al. (2015); Semenova et al. (2006)

As shown in Table II, the endpoint mainly investigated up to now is the percentage of normal hatched larvae (or embryogenesis). In fact, the simple body plan of the larva allows a fast screening of malformed specimens. In particular, the *Ciona* larval tadpole has a stereotyped chordate body plan with a long tail with a central 40-cell notochord and 36 flanking muscle cells. Anterior to the tail, the trunk contains the larval brain (or sensory vesicle), as well as the precursors of the circulatory and digestive systems (undifferentiated mesenchyme and endoderm) (Figure VI). The larval brain contains two pigmented organs, the otolith and the ocellus, involved in gravity and light perception, respectively. The larval brain continues towards the posterior end forming the neck, the visceral ganglion, containing the motor ganglion, and the tail nerve cord running above the notochord (Imai and Meinertzhagen 2007). Anteriorly three adhesive papillae or palps, containing sensory neurons, allow the larvae to choose a substrate to start the metamorphosis (Groppelli et al. 2003; Pennati et al. 2007). This minimal and simplified chordate body structure is thus instrumental for assessing toxic effects and looking at any modified phenotype possibly resulting from altered developmental programs. Moreover, thanks to the full sequenced genome, a transcriptomic approach can be used to better understand the mode of action (MoA) of chemicals and stressors. In fact, transcriptomic data together with bioinformatic studies, like Gene Ontology or Pathway analyses, can help in shedding light on the physiological and/or developmental processes affected by the selected compounds, thus providing important information also for more complex organisms.

Table II. List of all the different endpoints investigated using the ascidian *Ciona robusta*.

Compounds	Endpoint	Time of Exposure	EC_{50}	References
Stage of treatment: Gametes				
Chlorothalonil	Spermiotoxicity Oocyte toxicity Fertilization toxicity	30 min 30 min 24 hpf	23.9 µg/L (90 nM) 11.2 µg/L (42 nM) 2.3 µg/L (8.5 nM)	Gallo et al. (2015)
Stage of treatment: Embryos 2-4 cell stage				
Copper	Larval hatching		46 µg/L (0.18 µM)	Bellas et al. (2001)
Mercury			54 µg/L (0.27 µM)	
Cadmium			838 µg/L (7.46 µM)	
Tributyltin			7.1 µg/L (0.022 µM)	Bellas (2005)
Lindane			4412 µg/L (15.2 µM)	
Chlorpyrifos			5666 µg/L (15.7 µM)	
Sodium Dodecil Sulphate (SDS)			5145 µg/L (17.8 µM)	
Naphthalene	Embryogenesis		1.9 µg/L (15 nM)	Bellas et al. (2008)
Chlorothalonil	Larval hatching		0.12 µM	Bellas (2006)
Sea-Nine 211			0.37 µM	
Dichlofluanid			0.85 µM	
Tolylfluanid			0.62 µM	
Irgarol 1051			8.34 µM	
Microplastics			NC	Messinetti et al. (2017)
Bisphenol A	Larval Phenotype Abnormalities		168 µg/L (0.74 µM)	Matsushima et al. (2013)
Stage of treatment: Larvae				
Copper	Larval settlement	48 h	34 µg/L (0.54 µM)	Bellas et al. (2001)
Mercury			35 µg/L (0.18 µM)	
Cadmium			>146 µM	
Chlorothalonil			0.16 µM	Bellas (2006)
Sea-Nine 211			0.15 µM	
Dichlofluanid			0.39 µM	
Tolylfluanid			0.28 µM	
Irgarol 1051			>25.60 µM	
Microplastics	Metamorphosis	4 days	NC	Messinetti et al. (2017)

Vinblastine	Developmental indicators	From 2 to 4 dpf	3 µM (MTC)	Mizotani et al. (2015)
Doxorubicin			30 µM (MTC)	
Cisplatin			30 µM (MTC)	
Rotenone			0.3 µM (MTC)	
Stage of treatment: Juveniles				
Microplastics	Survival Juveniles	8 days	NC	Messinetti et al. (2017)
Tributyltin	Morphological abnormalities	1 h	-	Mansueto et al. (2011)
Bisphenol A			-	
Dispersant 1 (non-ionic surfactant)	Survival Juveniles	96 h	41.6 mg/L	Eliso et al. (2020)
Dispersant 2 (mixture of non-ionic surfactants and anionic surfactants)	Survival Juveniles		92.5 mg/L	

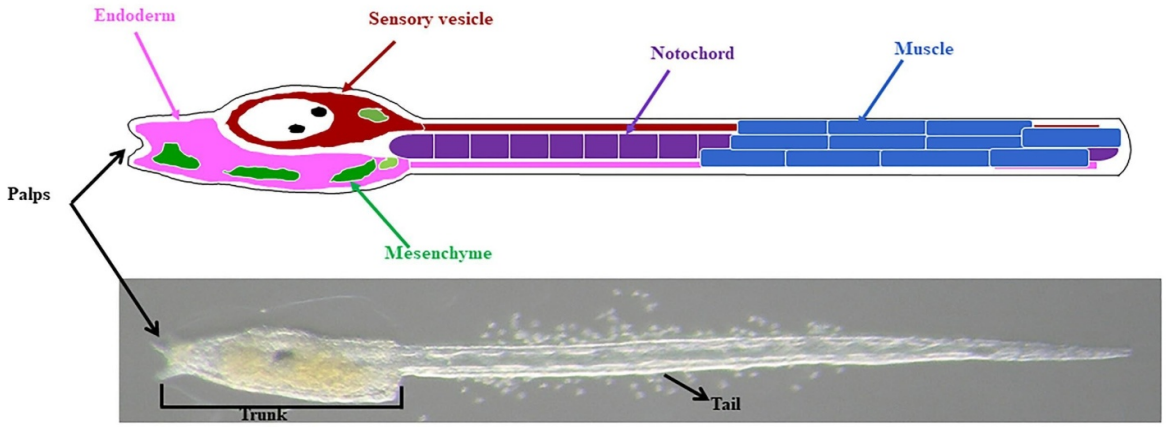

Figure VI. Schematic representation of *Ciona* larva.

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Objectives of the PhD thesis

Considering all the advantages offered by *C. robusta* as model organism in different fields, including the *in vivo* assays aimed at inspecting the chemicals toxicodynamics, this PhD project has been focused on the investigation of the effects exerted by two different types of contaminants of emerging concern (CECs) on *Ciona* embryogenesis up to the larval stage.

In details, the two types of CECs are:

- Two dispersants, named A and B, which are chemicals used for cleaning up the petroleum hydrocarbon contamination in case of accidental oil spills at sea.
- Two surface charged PS NPs, negatively charged PS-COOH and positively charged PS-NH_2 with nominal size range of 40- 60 nm.

They are not currently covered by existing water-quality regulations, are thought to be potential threats to environmental ecosystems and human health and only recently their effects have been studied on classical model organisms to evaluate their impact on aquatic environment.

The objectives of the present thesis are:

1) Evaluate the sensitivity upon the exposure to these two types of contaminants during *Ciona* embryogenesis.

2) Investigate developmental alterations in *Ciona* larvae.

3) Measure toxicity through the use of biomarkers.

4) Study the toxicodynamic behind the effects observed in the exposed organisms through molecular analyses.

Here, the following chapters are presented to address the objectives of the thesis:

In Chapter 1, the effects of two dispersants, named A and B, have been evaluated on *Ciona* development up to the larval stage, looking at any morphological alteration and analysing the possible variations in the expression of typical stress response genes.

In Chapter 2, the effects of PS NPs on *Ciona* embryogenesis have been investigated in terms of morphological aberrations in larvae phenotype, ROS production, cholinergic function (i.e. AChE activity) and developmental genes. The study included the characterization of PS NPs suspensions, in order to evaluate their behaviour under environmental relevant conditions in exposure media as natural sea water (NSW).

The effects exerted by PS-NH_2 on *Ciona* embryogenesis prompted us to inspect, as illustrated in Chapter 3, the transcriptomic profiles of *Ciona* tadpoles upon 13 h of exposure to two different concentrations of PS-NH_2, with the aim of getting insights in the toxicodynamic of these NPs.

In Chapter 4, a comparative study between *Ciona robusta* and the ascidian species, *Phallusia mammillata*, has been performed in order to explore any "common phenotypic malformation" exerted by PS-NH_2 during their embryonic development, in order to figure out the ontogeny of the phenotypes.

CHAPTER 1

Effects of two selected dispersants on the embryogenesis of the ascidian *Ciona robusta:* alterations in development and gene expression analysis.

ABSTRACT

In this chapter, the effects of two chemicals used after an oil spill event and named dispersant A and dispersant B were evaluated on the embryogenesis of *C. robusta* after 22 hours of exposure. Moreover, the expression of genes involved in in stress response (SODa, SODb, MnSOD, GPx, HSP60, HSP70), detoxification (Cyp450, GST, GluR) and cell survival (p38, Cas8) were measured at the selected endpoint (22 hpf). Both dispersants caused a dose-response effect on the development of ascidian embryos, but dispersant B resulted more toxic compared to dispersant A with an EC_{50} value of 44.30 μg mL^{-1} and 160 μg mL^{-1}, respectively. Also the degrees of malformations (*mild, severe* and *not developed*) were different for the two dispersants, since the percentage of embryos with *severe* malformation (problem at trunk and tail level) and *not developed* was higher for dispersant B at lower concentrations compared to dispersant A. The exposure to dispersant A induced a significant up-regulation of GST, Cyp450 (167 and 250 μg mL^{-1}), and HSP70 (250 μg mL^{-1}), while the exposure to dispersant B resulted into a significant down-regulation of Cas8 (50 μg mL^{-1}), HSP70 and p38 (100 μg mL^{-1}). These findings highlight 1) the different toxicity of the two selected dispersants, suggesting the teratogenic effects of dispersant B compared to dispersant A, and 2) support the use of *Ciona* embryotoxicity test as a valuable tool for determining the impact of dispersants on the biological performance of marine species.

1.1 Introduction

Oil spill in offshore marine environments require a serious consideration to mitigate ecological impacts and human health. There are various clean-up methods for oil spill response which include: 1) mechanical methods (skimmers, absorption felt, and booms); 2) chemical methods (chemical dispersant) and 3) biological method (biodegradation). Chemical dispersants are known to be an efficient tool used to clean and disperse crude oil into the water column at very low concentration. This accelerates the process of degradation of oil and can significantly reduce the impact on shorelines and habitats (Lessard & Demarco, 2000). Dispersants are made of a mixture of three ingredients: surfactants (surface active agents), solvents and additives. The surfactants are the main components, in fact they contain both oil-compatible (lipophilic) and water-compatible (hydrophilic) groups. The lipophilic group can easily attach to the oil phase and the hydrophilic group attach to the water phase. In this way there is a reduction of the interfacial surface tension between water and oil, which contributes to the oil breaking into small oil droplets and dissolving into the water column. The formation of these very small dispersed oil droplets increases the surface area of the oil tremendously, thus favouring enhanced aerobic microbial degradation promoting removal from the environment by natural processes. The microorganisms biodegrade oil as a carbon source (Figure 1.1). The solvent components are primarily used to dilute the oil and facilitate the penetration of the surfactants into an oil slick. The role of additives is to increase the stability of the dispersed oil.

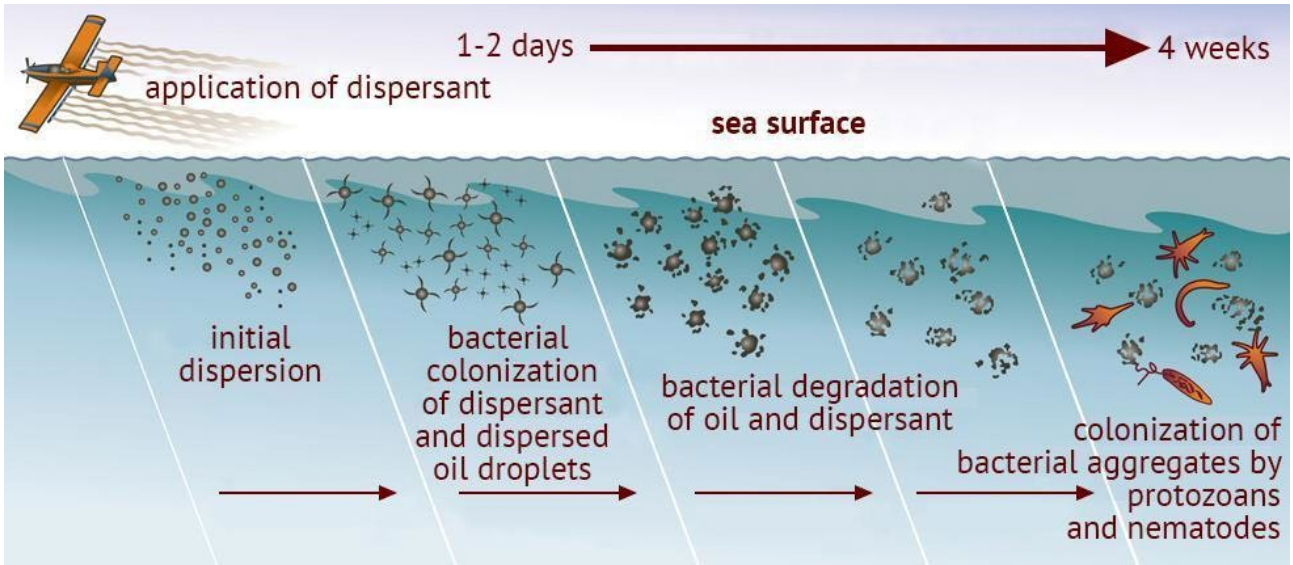

Figure 1.1. Oil slicks on surface waters were treated with dispersants to break down large surface slicks and to form small droplets. In this way, microorganisms in the upper ocean (pelagic) can easily degrade dissolved hydrocarbons in the contaminated water column. Image Credit: Medscape

The parameter used to characterize them is the hydrophilic-lipophilic balance (HLB). HLB is determined using theoretical equations that relate the length of the water-soluble portion of the surfactant to the oil-soluble portion of the surfactant. A surfactant with an HLB between 1 and 8 promotes the formation of oil-in-water emulsions and one with an HLB between 12 and 20 promotes the formation of water-in-oil emulsions. A surfactant with an HLB between 8 and 12 may promote either type of emulsion, but generally promotes oil-in-water emulsions. Dispersants have an HLB with a range between 9 and 12. Some dispersants contain ionic surfactants in small proportion, yielding an average HLB more toward 15 than 10. In fact, ionic surfactants are strong water-in-oil emulsifiers, very soluble in water, and relatively insoluble in oil. Such products disappear rapidly in the water column and are not effective on oil. Because they are readily available at a reasonable price, many ionic surfactants are proposed for use as dispersants. These agents are better classified as surface-washing agents. However, a typical dispersant formulation consists of a pair of non-ionic surfactants in proportions to yield an average HLB of 10 and some proportion of ionic surfactants (Fingas and Ka'aihue., 2005). With this chemical composition, when applied to a film of oil, surfactants diffuse to the oil/water interface. In this way, the lipophilic portion can attach the oil, while the hydrophilic portion extends into the water phase with the formation of the small droplets and the biodegradation by bacteria (Lubchenco et al., 2012).

The appropriate conditions for using dispersants include optimum water column depth and distance from the shore that ensure the correct dilution and water column mixing (National Research Council, 2005). Although the use of dispersants is considered an effective tool, in the United States dispersants have been used ~20 times since 1969 (National Oceanic and Atmospheric Administration 2018). After the massive use of dispersants (~1.84 million gallons, primarily Corexit 9500A) during the *Deepwater Horizon* oil spill in the Gulf of Mexico, many scientists and the public raised concerns about the toxicity of these substances to water column organisms. In fact, it is generally well accepted that the drivers of toxicity after the usage of dispersants are the dissolved fractions of the treated oil (National Research Council, 2005) and studies conducted in the laboratory demonstrated the less toxicity of dispersants than the tested crude oils (Hemmer et al. 2011; Barron et al. 2013; Claireaux et al. 2013; McConville et al. 2018). In general, the acute toxicity of dispersants is attributed to the disruption of biological membrane integrity, a process that leads to different issues as electrolytic imbalance, loss of cell osmotic permeability and cell lysis (National Research Council 1989, 2005; Singer et al. 1991, 1996).

Right after the Deepwater Horizon incident in 2010, dispersant usage has been a topic of renewed interest for the European national administrations in recent years. Among the twenty-five coastal

EU/EFTA Member States (MS), twenty-three countries consider dispersant usage in their pollution response strategy (as primary, secondary or last response option), alongside the mechanical recovery of oil or only under very specific conditions. Only two countries (Bulgaria and Slovenia) do not use dispersants in an oil spill situation at all.

The testing and approval procedures have been developed only in six countries (France, Greece, Italy, Norway, Spain and the United Kingdom). Of the remaining nineteen EU/EFTA countries that don't have their own testing and approval procedures, at least seven of these allow the use of dispersants approved in a neighbouring country or within the Bonn Agreement region. Approval procedures include tests for the effectiveness and their toxicity. Other additional assays include biodegradation, bioaccumulation, and similar toxicological tests, and in some cases other criteria (e.g. physical criteria such as maximum viscosity).

Even if all countries share the same concerns and objectives when approving dispersants, they just approach these in different ways and with different tests requirements and procedures. One of the main difference is that in France, Greece, Italy, Norway, Spain the ecotoxicological characterization is carried out on dispersant product, while in the United Kingdom dispersant toxicity is tested on a mixture of chemically dispersed oil and it is compared to that of mechanically dispersed oil (EMSA, 2016).

In Italy, a standard approval scheme has been defined by the Italian Ministry for Environment and Territory and Sea in February 2011. These procedures have been prepared by a group of experts belonging to ISPRA (Institute for Environmental Protection and Research), ISS (Istituto Superiore di Sanità) and IRSA-CNR (Water Research Institute of the National Research Council). According to the Decree Law 2/25/2011, the effectiveness, stability, toxicity, biodegradability and bioaccumulation of depolluting products with dispersant or absorbent action have to be assessed.

The effectiveness of the dispersant is determined in terms of suspended and emulsified oil after the addition of the product in standardized conditions of shaking. Total hydrocarbons are measured according to the method UNI EN ISO 9377-2-2002 water quality-determination of hydrocarbon oil index-method using solvent extraction and gas chromatography. To pass the effectiveness test, the product must disperse at least 60% of oil.

The stability of dispersants is intended as how much petroleum product is retained by the absorbent product and for this kind of assay there is not a threshold.

The biodegradability test includes the "Closed bottle" method described in the OECD n. 306 (17 July 1992). All the dispersant components shall be biodegradable with an oxygen depletion greater than 60% of the ThOD.

Regarding the bioaccumulation potential, the Bioconcentration Factor (BCF) must be provided for each substance present in the product. If this is not available, the log Kow value of each substance making up the product, determined experimentally, using standardized methods, must be provided in alternative. For all the organic components of the product, the *log Kow* must be ≤ 3, while the BCF < 500. If neither the BCF nor the *log Kow* can be determined, the technical impossibility of carrying out the tests or the irrelevance of the bioaccumulation potential must be justified on the basis of adequate documentation.

The toxicity assessment requires the acute toxicity assay (≤ 96 h) with three trophic levels (algae, crustaceans and fish) and all EC_{50} values have to be higher than 10 mg/L. The algae that can be used are the diatoms *Skeletonema costatum and Phaeodactylum tricornutum* according to UNI EN ISO 10253:2016; for the test with crustaceans, at least one of these species has to be used: *Acartia tonsa, Artemia franciscana, Amphibalanus Amphitrite, Corophium orientale, Tigriopus fulvus* according to UNICHIM pr MU 2365 (2012), APAT-IRSA-CNR 8060 (2003), UNICHIM pr MU 2245 (2012), UNICHIM pr MU 2246 (2012), UNICHIM pr MU 2396 (2014), respectively, and the batch sensitivity is checked by testing it with a reference toxicant as Sodium Dodecyl Sulfate (SDS) or Potassium dichromate ($K_2Cr_2O_7$).Ffinally, the toxicity test performed using or the fish *Dicentrarchus labrax* or *Sparus aurata* is conducted following the OECD n. 203 (1992) and using as reference toxicant SDS. According to the Italian approval procedures, all the effect concentration (EC_{50}) values have to be greater than 10 mg L^{-1} for all the three trophic levels exposed to dispersants. Thus, the ecotoxicological characterization requires the evaluation of the inhibition of growth for algae, the evaluation of immobilization of neonates for copepods, and survival for fishes. None of these take into account the impact of these chemicals on the embryonic development of marine species, which represent the most sensitive stage of the organisms to environmental stress. In fact, several external factors may result in embryotoxicity and teratogenicity in exposed aquatic organisms, contributing to the decline of aquatic populations worldwide (Pašková et al. 2011). In this regard, the embryotoxicity test using *Ciona robusta* could work as a greater support to the toxicity assessment provided for the Italian national law. As mentioned in the main introduction, the advantages in using this marine invertebrate for this kind of assay are the fast embryo development (22 h) and the simplicity of the larval structure which could give valuable insights on the identification of those dispersants that represent a major threat to marine species. Moreover, due to its close relationship to vertebrates, this information could be useful for higher and more complex chordates.

In this chapter, the effects of two selected dispersants on the embryonic development of *C. robusta* are presented. Besides the effects on the normal larval development and the analysis of the phenotypes, the molecular mechanisms caused by both dispersants through the expression of genes involved in stress response, detoxification and cell survival as Cu,Zn superoxide dismutases (SODa, SODb), manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx), Heat Shock Proteins (HSP60, HSP70), Cytochrome P450 (Cyp450), glutathione S-transferase (GST), glutathione reductase (GluR), p38 mitogen-activated protein kinases (p38), and caspase 8 (Cas8) have been investigated.

1.2 Material and Methods

1.2.1 Preparation of Dispersants

The two dispersant products given for this study were sent by ISPRA and as in Manfra and co-workers (2017) have been named dispersant A (Finasol OSR 52) and dispersant B (Cleanîng ECO 85). Dispersant A is a mixture of anionic surfactant (20-25%), hydrocarbons (C11-C14), n-alkanes, isoalkanes, cyclics and aromatics (15-20%), (2-methoxymethylethoxy)propanol (15-20%) and 2-aminoethanol (0-1%). The *log Kow* is not available, while the degradability is satisfactorily maintained above the 80%. Dispersant B is a mixture of non-ionic surfactant (5-15%), Anionic surfactant (10-30%) and 2-butoxyethanol (<5%), with a *log Kow* < 3 and a biodegradability ≥ 80% for 14 days. Both dispersants have been provided in dark glass bottles and the working solutions (1 mg mL^{-1}) have been prepared in satural sea water of the Gulf of Naples (NSW, filtered 0.22 μm). To identify the concentration-range for the embryo-toxicological assays, preliminary range-finding tests for each dispersant have been conducted.

1.2.2 Animal and gametes collection and in vitro fertilization

Adults specimens of the ascidian *C. robusta* were collected in the Gulf of Taranto (Italy) by local fishermen between November 2018-March 2019. The organisms were transported in cool boxes, in plastic bags filled with NSW (salinity 40‰, pH 8) within a few hours to the aquarium facility of the Zoological Station Anton Dohrn of Naples (Italy). Before the exposure experiments, animals were acclimated for 7 days and they were maintained in flow-through circulating aquarium in NSW (filtered 0.45 μm) with a temperature of 18 ± 1°C, salinity of 40 ± 1 ‰, dissolved O_2 of 7 mg L^{-1} and pH of 8.1 under constant aeration and continuous light to stimulate gametes maturation and to avoid

spawning (Lambert & Brandt, 1967). They were fed with a mixed marine algae every 48 h *ad libitum* (Shellfish Diet 1800®). Gametes were obtained from each individual by dissecting the gonoducts with a scalpel. To avoid the self-fertilization, oocytes and sperms were collected by distinct individuals. The oocytes were rinsed twice in 0.22 µm filtered NSW while dry sperm was pooled and stored on ice until fertilization. Before fertilization, sperm was diluted 100X in NSW and then added to the egg's suspension. After 10 min of incubation on a rotating shaker, the fertilized eggs were transferred to tissue culture plates and further rinsed in 0.22 µm filtered NSW.

1.2.3 Embryotoxicity

Embryotoxicity assay was carried out according to Bellas et al. (2003). 60 embryos (~two-cell stage, about 1 h post-fertilization (hpf)) were added to 6-well plates and exposed to increasing concentrations of both dispersants in NSW as follows: Dispersant A (50-100-167-250 µg mL^{-1}) and Disperdant B (20-33.3-50-100 µg mL^{-1}). Embryos were incubated under dark static conditions at 18°C until the free swimming larval stage was reached (22 hpf).

The impact of both dispersants was evaluated as percentage of normal hatched larvae and morphological alterations at 22 hpf compared to controls, as described below. Larvae were first fixed in 4% paraformaldehyde and then washed twice in 1X PBS. A larva was recorded as *normal* when presented a good general embryo morphology, with proper trunk and palps formation, as well as tail elongation following the Four-dimensional Ascidian body Atlas Ver. 2 (https://www.bpni.bio.keio.ac.jp/chordate/faba2/top.html). Larvae phenotypes were examined by using the microscope Zeiss Axioscope and classified for simplicity in *mild*, *severe* and *not developed.* The assay was run at least three times and considered valid when controls (only in NSW) showed a percentage of normal hatched larvae ≥ 80% at 22 hpf.

1.2.4 RNA extraction and cDNA synthesis

At 22 hpf, about 180 swimming larvae treated with dispersant A (0-167-250 µg mL^{-1}) and dispersant B (0-50-100 µg mL^{-1}) were collected by centrifugation at 3000 rcf for 3 min. The remaining NSW was removed and the collected larvae were frozen in liquid nitrogen and kept at -80°C. Total RNA was extracted using RNAqueous-micro kit (Ambion) according to the manufacturer's instructions. The RNA extracted was quantified measuring the absorbance at 260 nm (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA) and the integrity was evaluated by agarose gel electrophoresis. For each sample, 1 µg of total RNA was retrotranscribed using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instruction.

1.2.5 Gene expression by Real Time-quantitative PCR (RT-qPCR)

The variation of expression of stress response genes for each condition was analysed by RT-qPCR. The entire coding sequence of the selected 12 genes was obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and Aniseed (https://www.aniseed.cnrs.fr/). Specific primers were designed with the help of Primer 3 software (Table 1.1) and then the selected amplicon were amplified. The reaction mix contained 1X Fast Start SYBR Green Master Mix (Roche), 1 µL of cDNA template (1:100 dilution) and 0.7 pmol μL^{-1} for each primer. PCR amplifications were performed in MicroAmp Optical 384-Well reaction plate with Optical Adhesive Covers (Applied Biosystems) in a ViiA™ 7 Real Time PCR System (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s, 1 cycle for melting curve analysis (from 60 to 95 °C, reading every 0.5 °C) to verify the presence of a single product. All the reactions were carried out in triplicate and each assay included three negative controls with no template for each primer pair. Expression levels of target genes were normalized using as reference gene cytoskeletal actin (GenBank ID: NM_001032502.1, Fujikawa et al., 2010). Actin-specific primers were: sense primer, 5′-CCCAAATCATGTTCGAAACC-3′; antisense primer, 5′-ACACCATCACCACTGTCGAA-3′. Fluorescence was analyzed with ViiA™ 7 Real-Time PCR software (Life Technologies) and then quantified according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on Ct values of each gene, and the Ct average of the selected reference gene, in order to calculate the relative mRNA expression level. The expression levels of the selected genes were evaluated in number-fold increase relative to the control condition that has been assigned as “1”.

Table 1.1- Accession number and/or Gene Model ID, sequences and length of PCR fragments are listed for the analysed genes.

Gene Name	Acronym	Accession Name and or Gene Model ID	Primer	Sequence (5′ → 3′)	Amplicon length (bp)
Superoxide dismutases _a	SODa	XM_002121064.4 KH2012:KH.S1012.1	CiSODa_F	CCACAAAATATAG ACGAAGGCGAC	95
			CiSODa_R	GACAACGCACTAT TCAACGGG	
Superoxide dismutases_b	SODb	XM_002122490.5 KH2012:KH.L50.19	CiSODb_F	AAGTGAGAGTGTG AGTGGGAC	89
			CiSODb_R	CAGCCAACCCTGT AAGCGA	
Glutathione peroxidase	GPx	NM_001190351.1 KH2012:KH.C9.698	CiGPx7_F	TTGAGAAGAAGCA CGGGGCAA	93
			CiGPx7_R	GGATGGCCCCCAC TCATTT	
Gluthatione S-transferase	GST	XM_002128135 KH2012:KH.C2.852	CiGST_F	CCAAGCGATGCTA ATGCGAG	94
			CiGST_R	CGGCGGGATTGAG GTATGT	
Gluthatione Reductase	GluR	XM_002119519.5 KH2012:KH.C7.514	CiGRed_F	AGCACTTCTTACAC CAGTTGC	130
			CiGRed_R	CCCAATGGGTGGA TGACTGA	
Cytochrome p450	Cyp450	NM_001113557.2	CiCyP450_F	CCCAACCAGATGT TCCTGTCC	156

		KH2012:KH.C12.176	CiCyP450_R	CTGCCTGTCGGGGAATGTAG	
Heat shock protein 60 kDa	HSP60	XM_018812296.2 KH2012:KH.C6.85	CiHSP60_1F	AGACGAACAAGTTGGGGTTG	196
			CiHSP60_1R	GACTTTCGTTGGGTCCAGAA	
Heat Shock Protein 70 kDa	HSP70	NM_001033834.1 KH2012:KH.L46.6	CiHSP70_2F	TCTCTGTGCTCACCATCGAC	241
			CiHSP70_2F	GCTTTTTCGACAGGGTCAAG	
Superoxide dismutase, mitochondrial	MnSOD	XM_002128454.4 KH2012:KH.C12.466	CiMSOD_2F	TCAAGGTTCTGGCTGGAGTT	175
			CiMSOD_1R	GTAATCTGGCCGCACATTTT	
p38 mitogen-activated protein kinases	p38	NM_001078490.1 KH2012:KH.C11.76	CiP38_1F	GACGACCACGTCCAGTTTTT	172
			CiP38_1R	CGGTCATTTGGTCATCTGTG	
Cytochrome b	CytB	ENSCINT00000037367.1	CiCytb_1F	TTATCGGTTACCGGCTCCTA	151
			CiCytb_1R	ATGGTTCACAGAAGAAAAAGCTA	
Caspase 8	Cas8	XM_002122812.4 KH2012:KH.C8.550	CiCas8_2F	GGTCATGTCTCATGGGCTCT	184
			CiCas8_2R	ATCAACTGGAGCGGAATGTC	

1.2.6 Statistical analyses

All the statistical analyses were performed using Graphpad Prism 6. All data were expressed as mean ± standard deviation (SD). The median effective concentration (EC_{50}), corresponding to a 50% reduction of normal hatched larvae, was calculated using a sigmoidal dose−response model according to the equation: $y=b+(a–b) / 1+10^{(\text{Log EC50} – x)}$ where y is response, b response minimum, a response maximum, x the logarithm of effect concentration and EC_{50} the concentration of effect giving 50% of maximum effect. Data were normalized to the control mean percentage of larval abnormality using Abbot's formula:

$$P= (P_e\text{-}P_c/100\text{-}P_c)\cdot 100$$

Where P_c and P_e are the control and the experimental percentages of response, respectively.

Data from the embryotoxicity assay and for the analyses of the morphological alterations are representative of at least three independent experiments and were analyzed using the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.

RT-qPCR data are presented as mean ± standard error and the significance of the relative $2^{-\Delta\Delta Ct}$ of each group (biological replicates, N = 3), compared to the controls, was determined using 'unpaired parametric t-test'.

1.3 Results and Discussion

The toxic effects of dispersants have been widely studied in different organisms ranging from zooplankton to rats (Wise and Wise, 2011). The effects exerted by these compounds depend on test-species, exposure time, physical-chemical parameters (especially temperature and salinity) and dispersant composition (George-Ares and Clark, 2000; De Lorenzo et al., 2017). In fact, dispersant formulations may contain one or more non-ionic surfactants, an anionic surfactant, and one or more solvents (National Research Council, 1989). In different EU countries, the toxicity assessment of dispersants is required before their approval procedure; in Italy this characterization involves the use of organisms belonging to three different trophic levels, by evaluating the inhibition of growth for algae, the immobilization of crustacean neonates and fish survival (Decree Law 2/25/2011). These analyses do not include embryotoxicological evaluation, although embryo development represents the most sensitive life stage of the organism to marine stressors and induction of changes in its life cycle, sex ratios, growth or varying degrees of anatomical deformities could lead to a diminished

biological performance of populations (Pineda et al., 2012). On these grounds our studies aimed at investigating the effects of two dispersants on the embryogenesis of the ascidian *C. robusta.*, at both morphological and molecular levels, in order to collect new ed important information useful for this type of assays.

1.3.1 Embryotoxicity

In this group of experiments, we tested the effects of two dispersants named dispersant A (Finasol OSR52) and dispersant B (Cleaning ECO85). Dispersant A is one of the main product used after an oil spill event thanks to its effectiveness (Steffek et al., 2016), while less is known about dispersant B. Both dispersants resulted toxic in a dose-dependent manner, but the EC_{50} values were completely different: 160 µg mL^{-1} (150.4 to 171.2) for dispersant A and 44.30 µg mL^{-1} (39.92 to 49.15) for dispersant B, thus indicating a higher toxicity exerted by dispersant B compared to dispersant A on *Ciona* larval development (Figure 1.2).

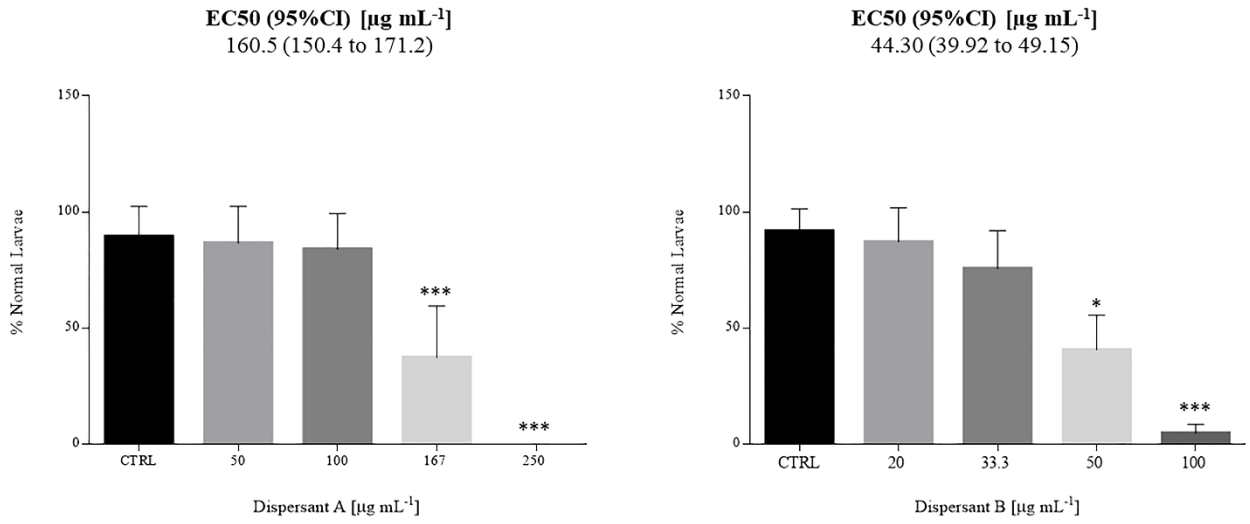

Figure 1.2. Percentage (%) of normal hatched larvae of *C. robusta* upon exposure to dispersant A (left) and dispersant B (right) in NSW for 22h. Bars represent mean ± SD (dispersant A n=2700; dispersant B n=2700). Asterisks indicate values that are significantly different compared to the control (Kruskal-Wallis test, Dunn's post hoc test, **p<0.01, ***p<0.001). EC_{50} values have been shown.

Previously, Manfra and collaborators (2017) tested the same dispersants on other marine organisms, looking at different endpoints. Two crustaceans, *T. fulvus* and *A. franciscana*, were used to evaluate the percentage of nauplii immobilization, the alga *P. tricornutum* to assess the percentage of growth inhibition, and the fish *D. labrax* to establish the percentage of juvenile survival. Unlike *Ciona*, in

these experiments dispersant A resulted more toxic than dispersant B, for all the tested species. Concerning dispersant B, the E(L)C_{50} values were comparable to those of *Ciona* (Table 1.2).

Table 1.2. E(L)C_{50} values (μg mL^{-1}) calculated for dispersant A and B.

	Dispersant A **E(L)C_{50}**	**Dispersant B** **E(L)C_{50}**
Phaeodactylum tricornutum **(72 h)**	6.60 (4.40–8.80)	82.70 (63.40–102.00)
Tigriopus fulvus **(96 h)**	5.89 (4.86–7.04)	49.66 (40.36–62.42)
Artemia franciscana **(96 h)**	1.60 (1.20–1.97)	–
Dicentrarchus labrax **(96 h)**	16.19 (13.75–19.07)	71.18 (65.34–77.55)
Ciona robusta **(22 h)**	160 (150.4 to 171.2)	44.30 (39.92 to 49.15)

The discrepancies about the toxicity exerted by the two dispersants on *Ciona*, compared to the other organisms, could be related to different factors, including the species itself, the endpoints used for the assays, the stability of dispersants and their biodegradability in the course of the years, during their storage (Leal et al., 1991; Fingas et al., 2005). Not to mention that *Ciona* embryos are surrounded by the egg envelopes formed by an acellular layer named vitelline coat (or chorion), and two populations of maternally-supplied cells, the follicle cells and the test cells. This complex structure protects *Ciona* embryos from external insults and may provide a stronger shielding role towards some chemicals present in dispersant A mixture.

The different impact on *C. robusta* embryogenesis, exerted by the two dispersants, was better defined by observations of larval phenotypes under the microscope Zeiss Imager.M1. For simplicity we classified the abnormalities as follow (Figure 1.3 A):

- *Mild*: larvae with a shorter trunk and normal tail;
- *Severe*: larvae with a shorter, kinked and disorganized tail and malformed trunk;
- *Not developed*: embryos stopped in development.

For both dispersants there was a bell-shaped curve for the *mild* abnormalities, with an increased percentage of embryos with problems just at the trunk level only at the concentration near the EC_{50} value and with a greater incidence of dispersant A compared with dispersant B (46% vs 26%). Concerning the *severe* phenotype, dispersant A caused an increase in the percentage of embryos showing abnormalities, both at the trunk and tail levels, only at the highest concentration tested (250 $\mu g\ mL^{-1}$), while for dispersant B a dose response relationship was detected. Interestingly, dispersant B, at the highest concentration (100 $\mu g\ mL^{-1}$), induced a block in development of around 50% of embryos, while dispersant A, at the highest concentration (250 $\mu g\ mL^{-1}$), resulted, almost completely, in malformed larvae showing a severe phenotype (around 85%). Thus, both the embryo-toxicological and morphological evaluations indicate the highest toxicity and teratogenicity of dispersant B versus dispersant A (Figure 1.3 B-C).

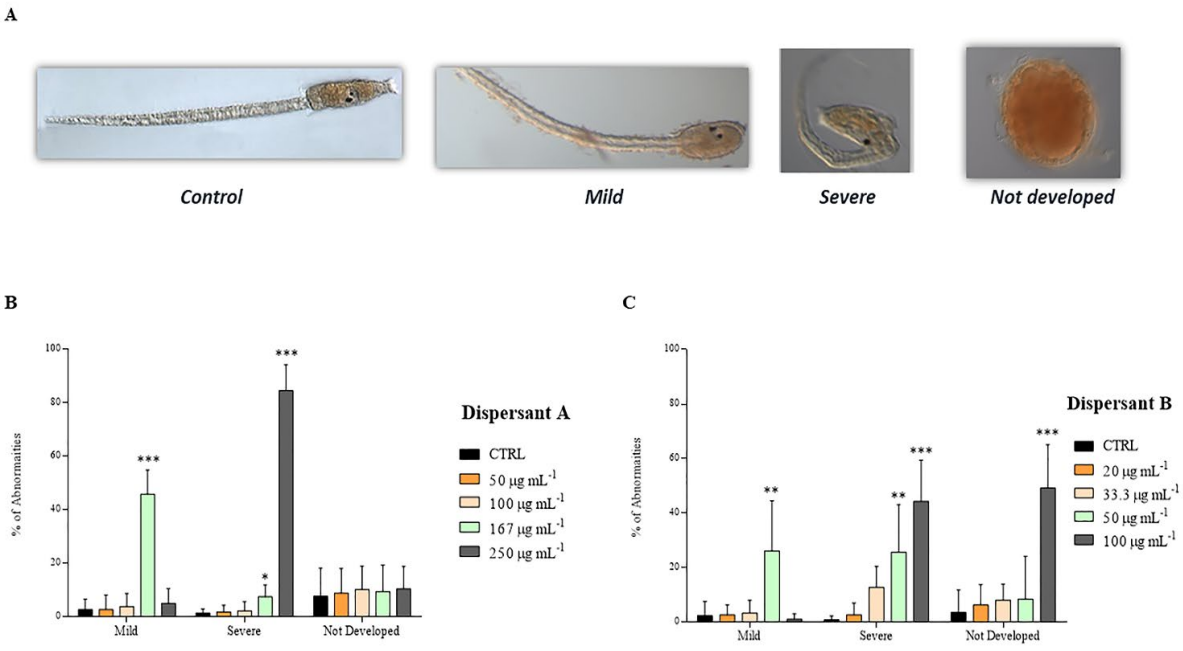

Figure 1.3. (A) Light microscopy images of the different phenotypes obtained upon exposure to dispersant A and dispersant B. from left to right: control larva; mild phenotype; severe phenotype and not developed embryo**;** (B) Percentages (%) of total abnormalities identified upon exposure to dispersant A; (C) Percentages (%) of total abnormalities identified upon exposure to dispersant B. Bars represent mean ±SD (n=1080). Asterisks indicate values that are significantly different compared to controls (Kruskal-Wallis test, Dunn's post hoc test, *=p<0.05; **p<0.01; ***=p<0.001).

1.3.2 RT-qPCR on Stress Response Genes

Since the exposure to environmental pollutants could lead to an increment of Reactive Oxygen Species (ROS) and the activation of the Antioxidant Defense System (ADS) (Regoli and Giuliani 2014), our studies were focused on the analysis of expression of genes involved in stress response (SODa, SODb, MnSOD, GPx, HSP60, HSP70), detoxification (Cyp450, GST, GluR) and cell survival (p38, Cas8) in larvae exposed to 167-250 µg mL^{-1} for dispersant A, and 50-100 µg mL^{-1} for dispersant B.

In particular, SOD and GPx act as the first line of antioxidants defense: SOD can convert O^{2-} to O_2 and H_2O_2, which is subsequently transformed into H_2O by CAT and GPx (Ighodaro and Akinloye, 2017). *Ciona* genome contains two SOD genes, MnSOD and two isoforms of Cu,Zn SOD, named SODa and SODb (Ferro et al., 2013).

Heat shock proteins (HSPs) are a large group of proteins, highly conserved, involved in chaperoning the folding and/or degradation of proteins (Kiang and Tsokos, 1998; Young et al., 2001). They play

an important role in cell protection against oxidative stress at several levels. In fact, some HSPs, mainly members of the HSP70 family, play a crucial role in protein sorting and quality control by selecting and directing aberrant proteins to the proteasome or lysosomes for degradation (Mayer and Bukau, 2005). On the other hand, some HSPs can negatively regulate apoptosis by binding and inhibiting members of the apoptotic cascade, intervening to rescue the cells from dying (Kalmar and Greensmith, 2009).

Cyp450, GST and GluR are involved in the detoxification mechanisms of xenobiotics; in particular, Cyp450 is involved in xenobiotics oxidation during phase I (Nebert and Dalton, 2006), followed by phase II with the conjugation, catalyzed by enzymes as GST and GluR, of the modified xenobiotic to small polar moieties (Chen, 2012).

Concerning the p38 MAPK, it is a member of a well-studied pathway that plays essential roles in the signalling events associated to many cellular processes, as survival response, apoptosis, autophagy, development, cell cycle regulation and differentiation (Bradham & McClay, 2006; Coulthard et al., 2009).

Cas8 is a specific initiator of apoptosis. It takes part in the extrinsic pathway, or death receptor pathway of apoptosis, in which the ligation of death receptors on the cell surface leads to caspase activation (Tummers and Green, 2017).

Larvae treated with dispersant A showed a significant up-regulation of GST and Cyp450 at both 167 and 250 µg mL^{-1}, compared to the controls, and a significant increment of the expression of HSP70 (20.5-fold) at 250 µg mL^{-1} (Figure 1.4 A).

Dispersant B showed a different regulation of the selected genes; in fact at 50 µg mL^{-1} only Cas8 resulted significantly down-regulated (0.39-fold), whereas HSP70 and p38 were down-regulated (0.14- and 0.005-fold, respectively) at 100 µg mL^{-1} (Figure 1.4 B).

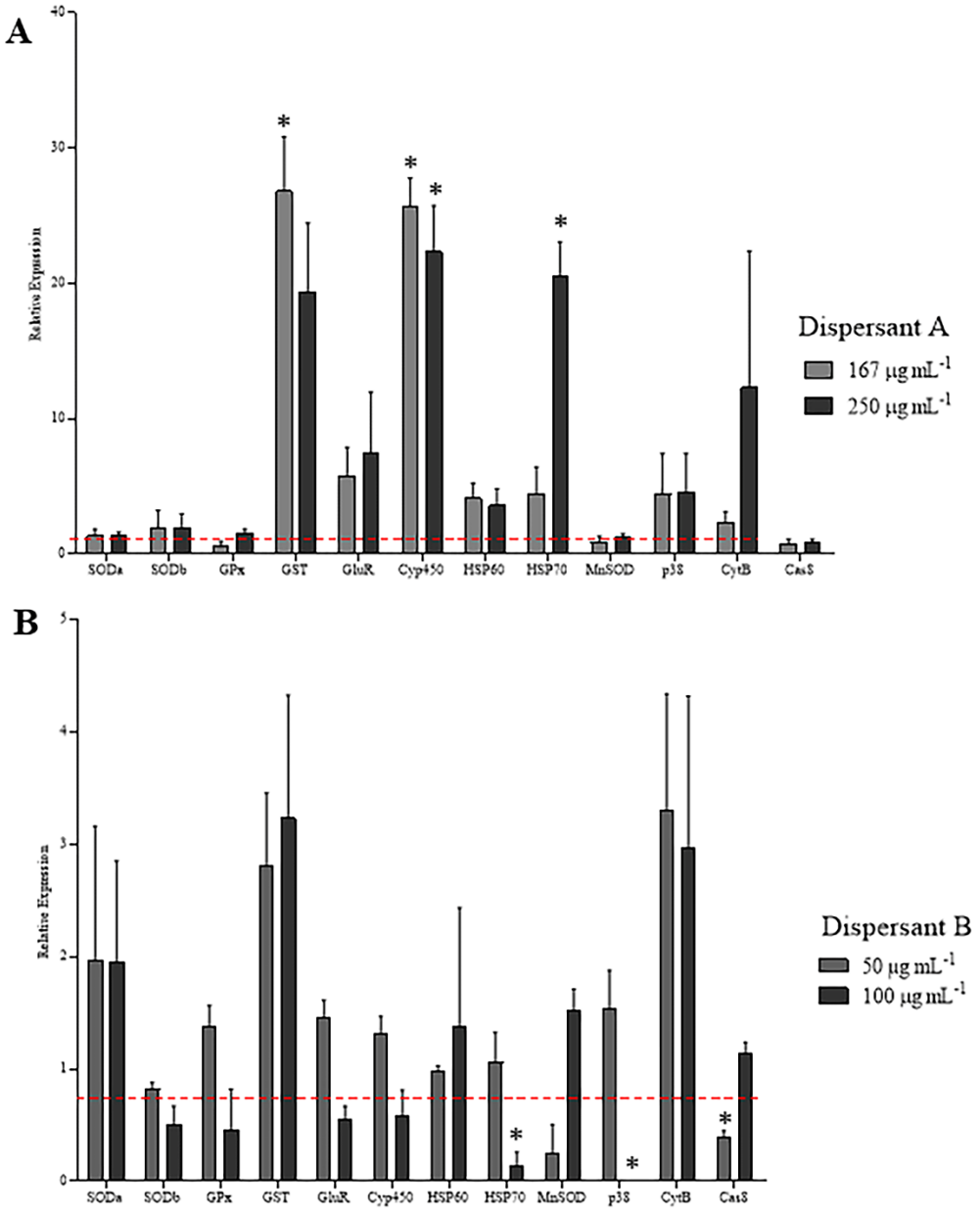

Figure 1.4. Expression of SODa, SODb, GPx, GST, GluR, Cyp450, hsp60, hsp70, MnSOD, cytB, p-38 mapk and cas8 genes by comparative RT q-PCR with total RNA isolated from control and exposed embryos to dispersant A (A) and dispersant B (B) after 22 hpf. Results are expressed as fold increase compared to controls assumed as 1, using cytoskeletal actin as reference gene for normalization. Each bar represents the mean of three independent experiments ± SEM. * indicates a significant difference (t-test, $p < 0.05$) respect to controls.

The RT-qPCR analyses of stress gene expression further confirmed the different toxicity exerted by these chemicals on *Ciona* embryogenesis. Among the genes analysed, the exposure to dispersant A caused a significant up-regulation of the genes GST, at 167 µg mL^{-1}, HSP70, at 250 µg mL^{-1}, and

Cyp450, at both the tested concentrations. These results seem to suggest that the embryos, exposed for 22 h to these two different concentrations of dispersant A, try to counteract the toxicity induced by this chemical by increasing the transcription of typical stress genes. One can suppose that this defence mechanism, while being able to counteract the block of development, as actually we detect, cannot be able to prevent the phenotypical alterations, since the percentage of larvae, showing a mild and/or severe phenotype, rises sharply at the highest dispersant A concentrations. By contrast, in embryos exposed to dispersant B, no gene turned out to be upregulated; rather we observed a significant downregulation of HSP70 and p38, at 100 μg mL^{-1}, and cas8, at 50 μg mL^{-1}. Although we cannot exclude that other enzymatic mechanisms are elicited by dispersant B, these results suggest that dispersant B somehow blocks the activation of the defence mechanisms acting through the enzymes we analysed. As a consequence, the phenotype we detected, consisting in the block of development at the highest dispersant B concentrations, could be just related to the inefficient detoxification mechanisms. Overall, these data suggest the higher teratogenic power of dispersant B compared to dispersant A.

1.4 Conclusion

For the first time, the effects of two CECs (dispersant A and dispersant B), previously characterized according to the Italian rules, have been studied on the embryogenesis of the marine invertebrate *C. robusta.* The data showed a dose-response relationship for both dispersants, but dispersant B resulted more toxic (44.30 μg mL^{-1}) compared to dispersant A (160 μg mL^{-1}).

The analysis of the phenotype revealed the higher toxicity of dispersant B compared to dispersant A, with an increased % of embryos with *severe* malformation (problem at trunk and tail level) and *not developed* for dispersant B.

The expression of genes involved in stress response (SODa, SODb, MnSOD, GPx, HSP60, HSP70), detoxification (Cyp450, GST, GluR) and cell survival (p38, Cas8) have been investigated for both dispersants, underlying the teratogenic power of dispersant B and the less toxic effects of dispersant A on the embryogenesis of *Ciona*.

Taking together all the results, the use of *Ciona* embryotoxicity assay could be identified as a valuable tool for determining the impact of dispersants on the biological performance of marine species.

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CHAPTER 2

Discolse nanoplastic toxicity on the embryogenesis of the ascidian *Ciona robusta* (Phylum Chordata)

ABSTRACT

Nanoplastics are considered contaminants of emerging concern at the global scale. The recent evidence of their occurrence in seawater from the Mediterranean Sea calls for a thorough evaluation of their impact on marine life and in particular on vulnerable life stages such as planktonic embryos. In this chapter the impact of increasing nominal concentrations of 50 nm amino-modified (PS-NH_2) and 60 nm carboxy-modified (PS-COOH) polystyrene nanoparticles (PS NPs) on the embryonic development of the ascidian *Ciona robusta* (phylum Chordata), a common benthic invertebrate living in Mediterranean coastal areas with the peculiarity of being an early chordate developmental model. A strong agglomeration of PS-COOH (approx. 1 μm) was observed in natural sea water (NSW) already at time 0, while PS-NH_2 resulted still monodispersed (approx. 130 nm) but largely aggregated after 22 h with a microscale dimension similar to those negatively charged. However, their effect on *C. robusta* embryos development largely differed at 22 h: PS-COOH did not affect larvae phenotypes nor their development, while PS-NH_2 caused a dose-dependent effect (EC_{50} (22 h) of 7.52 μg mL^{-1}) with various degrees of phenotype malformations (from mild to severe) and impairment of larval swimming. Embryos (up to 30%) exposed to 15 μg mL^{-1} PS-NH_2 resulted not developed and the majority was unable to hatch. Calculated PS-NH_2 EC_{50} resulted higher than those available for other marine invertebrate species, suggesting a protective role of the egg envelopes surrounding *C. robusta* embryos towards nanoplastics exposure.

2.1 Introduction

Plastics have become an inseparable and integral part of our lives. These synthetic organic polymers are used in a wide range of fields as hygiene, medicine encapsulations, food preservation and clean water systems. The factors behind their massive usage into different fields are low density, strength, user-friendly designs, fabrication capabilities, long life, light weight, and low cost (Andrady and Neal, 2009; Zalasiewicz et al., 2016).

For these reasons, global plastic production has moved from 1.5 million tons in the 1950s to 335 million tons in 2016 (Li et al., 2016; Lebreton et al., 2017; Statista, 2019).

Due to the rapid growing production and their persistent nature, numerous plastics are released in the environment and will persist in the environment for up to a century (Cole et al., 2010). In 1970s, some researchers revealed for the first time the presence of plastic debris in marine environments (Carpenter & Smiths, 1972; Moore, 2015; Ryan, 2015). The major sources of plastic debris in the aquatic environment are wastewater treatment plants, domestic swages, urban pollution, industry activities, and storms (Koelmans et al. 2019). Remaining sources are litters generated from ships/boats through recreational activities, discharge of litters, fishing nets etc. (Mattsson et al., 2015). According to the World Economic Forum (2016), at least 8 million tons of plastic will enter the world's oceans each year since plastic production is expected to double or quadruple over the next 20 years. Most of the plastics start the process of degradation with the formation of smaller fragments, which have in turn a higher degradation rate due to their surface-to volume ratio (Gewert et al., 2015). These smaller fragments are called Microplastics (MPs< 5 mm) and they were found widespread in the oceans and accumulated in the pelagic zone and sedimentary habitats for the first time by Thompson and colleagues (2004). The smallest group of plastics is formed by the so called Nanoplastics (NPs) and are defined as plastic materials with size below 0.1 μm (Hartmann et al., 2019).

MPs and NPs are classified into primary and secondary according to their sources. Primary particles are intentionally manufactured in small sizes for different applications, including cosmetic, personal care and cleaning products, and pre-production pellets for fabrication of other plastic goods (Arthur et al., 2009; Roex et al 2013; Ryan et al., 2009; Storck et al., 2015; Thompson et al., 2004). These particles are discharged into the aquatic environment through Waste Water Treatment Plant (WWTP) facilities (Roex et al., 2013). Secondary particles may originate from the breakdown of larger plastic pieces due to weathering processes such as photodegradation, thermo-oxidation, hydrolysis and biodegradation occurring in marine waters (Figure 2.1; Andrady, 2011; Lambert et al., 2014).

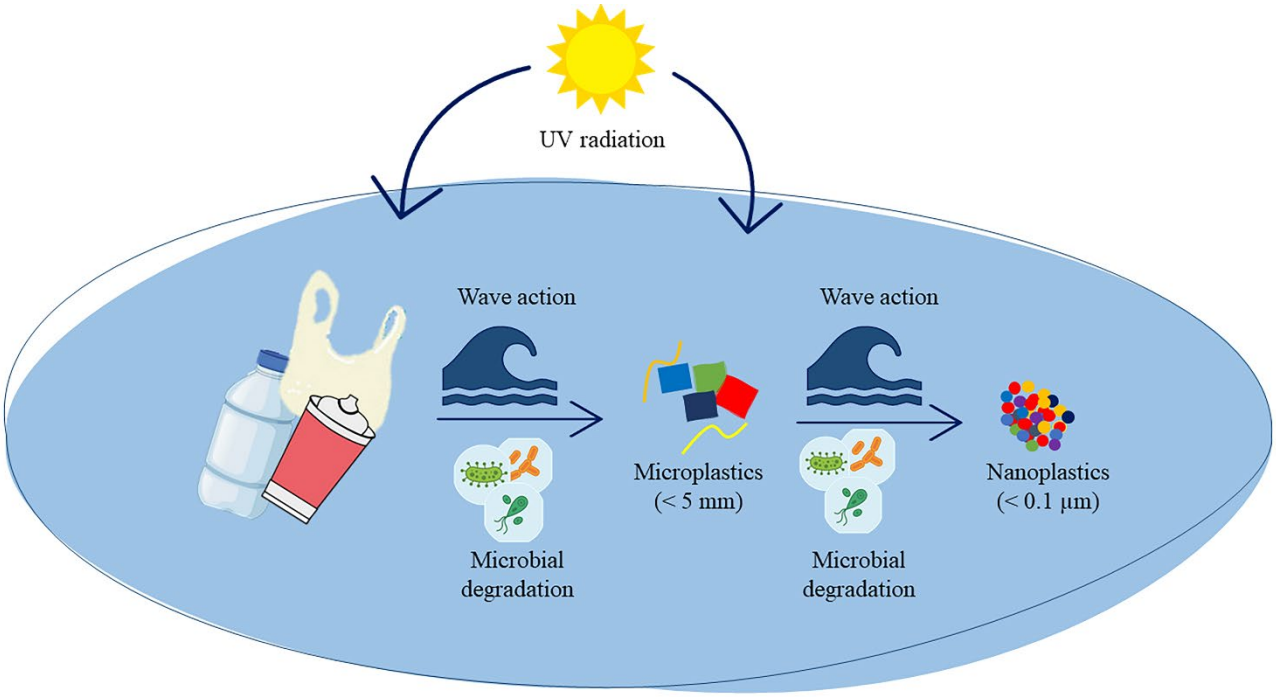


Figure 2.1. Schematic representation of the fragmentation of larger plastics (or macroplastics) into microplastics and nanoplastics. Weathering processes as UV radiation, wave action and microbial degradation are involved in the breakdown of plastics in marine waters.

In recent years, much attention has been paid to nano-sized plastics. Such particles have a different impact on aquatic life compared to larger plastics due to their unique physicochemical nanoscale properties. Their small size allows them to pass biological barriers, to penetrate tissue and to accumulate in organs (Kashiwada, 2006; von Moos et al., 2012; Rossi et al 2014). The increased surface-volume-ratio causes the possible sorption of hydrophobic substances from the marine environment, leading to an increased exposure to these toxicants (Velzeboer et al., 2014; Rochman 2013a, 2013b; Holmes et al. 2014). Moreover, due to their intrinsic (e.g. size, shape, crystallinity, surface chemistry), NPs have the tendency to aggregate with other particles, Natural Organic Matter (NOM) and colloids content (Galloway et al., 2017). The aggregation of other particles leads to the formation of homo-aggregates (aggregation of the same particles) or hetero-aggregates (aggregation between different nanoparticles or nanomaterials). The interaction of NPs with biomolecules dissolved in sea water leads to the formation of a biomolecular coating named *eco-corona*, which influences the biological activity of NPs in terms of biodistribution, cellular uptake and toxicity (Corsi et al., 2020).

One of the most commonly used plastic is polystyrene (PS) made by linking together large numbers of styrene molecules. CDs, toys, cup covers, and styrofoam, a material used for food containers, are made of PS. Furthermore, this polymer is also used in nanotechnology applications for the production of biosensors and photonics (Velev and Kaler, 1999), and in nanomedicine to provide insights into different cellular and molecular mechanisms (reviewed in Corsi et al., 2020).

The potential release of PS NPs in the marine environment is associated not only to primary sources, but also to the slow breakdown of larger PS plastics due to mechanical (Zhang et al., 2012; Kuo et al., 2014), weathering agents (e.g., UV radiation and high temperature) (Lambert and Wagner, 2016), and biota (Davidson, 2012). Recent evidence of PS NPs originating from larger pieces has been provided for in bench-scale studies (Gigault et al., 2016; Lambert et al., 2016; Hernandez et al., 2019; Zhu et al., 2019) and its occurrence has been reported in the field (Ter Halle et al., 2016, 2017). Also the Mediterranean Sea, historically considered a sink of chemical pollutants, is strongly affected by plastic contamination, in particular its coastal areas which receive outflows from large rivers and sewage treatment plants which all end up into the sea (Cózar et al., 2015; van Sebille et al., 2015; Suaria et al., 2016), and recent studies showed the presence of PS NPs in estuarine and coastal waters in the West Mediterranean (Schirinzi et al., 2019). Based on the most recent estimates, marine species within these coastal areas are at higher risk to plastic exposure (Compa et al., 2019; Guerrini et al., 2019; Macias et al., 2019), in particular for small debris including micro- and nanoplastics (Missawi et al., 2020).

PS NPs have widely been used as proxy for nanoplastic in environmental risk assessment, with several injuries documented in aquatic species belonging to different trophic levels and life-stages, raising concerns on their ecological impact on marine ecosystems (Corsi et al., 2020). Species sensitivity distribution based on polymeric NPs ecotoxicity studies revealed that 50 nm PS NPs with positive surface charge can be more harmful for marine species than negatively charged ones and other polymers (e.g., PMMA) (Venâncio et al., 2019). The surface charge has been recognized as the main factor responsible for the observed toxicity in developing embryos and larvae of model organisms such as mussels, oysters, sea urchins and rotifers. For these species, the EC_{50} (48h) values of amino-modified PS NPs (PS-NH_2) are ranging from 0.14-0.16 μg mL^{-1} in the mussel *M. galloprovincialis* (Balbi et al., 2017) and oyster *Crassostrea gigas* (Tallec et al., 2018), to 2.61 μg mL^{-1} in the sea urchin *P. lividus* (Della Torre et al., 2014) and 6.62 μg mL^{-1} in the rotifer *Brachionus plicatilis* (Manfra et al., 2017). In brine shrimp (*Artemia spp*.) larvae, cationic PS-NH_2 have been found to increase moulting in short-term acute exposure (48 h) (Bergami et al., 2016) and cause

mortality after prolonged exposure (14 days) (Bergami et al., 2017; Varó et al., 2019), in contrast to a negligible impact of anionic carboxy-modified (PS-COOH) NPs. Due to their planktonic nature, these organisms could be impacted by nanoplastics which could affect their developmental success and pose a serious risk for the survival of the entire species. Whether in marine coastal areas or in deep oceans, larvae are exposed to a variety of environmental stressors which alter their physiology and development as well as subsequent stages of their life cycle. Understanding the sensitivity of these developmental stages to anthropogenic pollutants, including those of emerging concerns such as nanoplastics, is therefore urgently needed in order to predict how the plankton biodiversity responds to current and predicted future levels of pollution (Corsi and Marques-Santos 2018).

Since the embryonic development is the most sensitive life stage to environmental perturbations that could influence marine biodiversity and ecosystem functioning (Pandori et al., 2018), we studied the effects of PS-COOH and PS-NH_2 on the embryogenesis of the ascidian *C. robusta*, up to the free swimming larval stage at 22 h post-fertilization (hpf). At the end of the exposure, the morphological alterations, cholinergic function, the oxidative stress levels and the behavioural changes induced by these functionalized PS NPs were determined.

2.2 Materials and Methods

2.2.1 Characterization of polystyrene nanoparticles suspensions

Unlabeled 50 nm PS-NH_2 and 60 nm PS-COOH NPs, bearing positive and negative surface charge respectively, were purchased from Bangs Laboratories Inc. (Fishers, IN, USA) and received as stocks suspensions in deionised water (at 100 mg mL^{-1}). Unlabelled PS-NH_2 stock (PA02N, lot: 12839) was supplied at 10% solids without any surfactant. PS-COOH stock (PC02N, lot: 11652) was supplied at 10.1% solids containing 0.1% of sodium dodecyl sulfate (SDS) and 0.05% of sodium azide (NaN_3). These stabilizers were not removed from PS-COOH stock to avoid unknown changes in NP dispersion. At the concentrations tested, unlabelled PS-COOH suspensions contained some residues of stabilizers ranging from 0.05 to 1 µg mL^{-1} of SDS and from 0.025 to 0.5 µg mL^{-1} of NaN_3, at 5 and 100 µg mL^{-1} of PS-COOH (i.e., min and max concentrations tested), respectively. Any effect on NP toxicity related to residues of SDS and NaN_3 in PS-COOH working suspensions was considered negligible, based on their EC_{50} values reported for marine model species (Table 2.1) and our previous experience showing the lack of acute toxicity to PS-COOH NPs-containing NaN_3 on marine invertebrate species (see Bergami et al. 2020). Recent studies (Pikuda et al., 2018; Heinlaan et al., 2020) highlighted that stabilisers and preservatives, such as NaN_3, commonly present in commercial

formulations of PS NPs, provoke acute toxicity in aquatic organisms and confound the effects of nanoplastics. However, these studies are limited to freshwater species, while data on marine organisms show that NaN_3 toxicity significantly diminishes at higher ionic strength (Anderson et al., 1995), in agreement with this current study showing no evident toxicity was associated to PS-COOH.

Table 2.1. E(L)C_{50} values and related exposure time (h) for SDS and NaN_3 in relevant marine model species, from bacteria to zooplankton/microcrustaceans, based on available literature. E(L)C_{50} values are reported as mean ± SD or (95% CI).

Reagent	Model organism	E(L)C_{50} (µg/mL)	Time (h)	Salinity (‰)	Reference
SDS	*Vibrio fischeri*	2.62 ± 0.90	0.25	30	(Mariani et al., 2006)
	Artemia spp.	23.20 ± 6.50	24	n.a.	(Libralato et al., 2016)
NaN_3	*Vibrio fischeri*	> 100	0.5	20	(Heinlaan et al., 2020)
	Artemia spp.	84 (76-92)	24	0	(Sleet and Brendel, 1985)
	Atherinops affinis	43.31 (40.1-46.7)	168	34	(Anderson et al., 1995)

After a brief sonication, by following the protocols described in Varó et al. (2019), PS NPs intermediate suspensions (at 10 mg mL^{-1}) were prepared in 0.22 µm milli-Q water (mQW) and stored in sterile vials at 4°C until use. For the embryotoxicity tests (see section Embryotoxicity), PS NPs working suspensions were prepared in Natural Sea Water as exposure media (NSW, salinity 40‰, pH 8) without further sonication.

Size-related parameters (Z-average and polydispersity index, PDI) and ζ-potential in of PS NPs working suspensions (20 µg mL^{-1}) in mQW and NSW were determined by dynamic light scattering (DLS) and electrophoretic mobility (EM), respectively, using a Zetasizer Nano ZS90 (Malvern) equipped with the Zetasizer Nano Series software (Ver. 7.02). Time-dependent variations in PS NPs key parameters were investigated both in mQW and NSW at 0, 6 and 22 h after suspension, following the time-points of the embryotoxicity test. Considering the behaviour observed for PS-NH_2 suspensions in NSW at the time-points, their aggregation kinetic trend within 1 h after suspension

was also determined by DLS analysis, measuring Z-average and PDI at 5 min intervals. DLS and EM data are referred to at least three independent measurements.

2.2.2 Animal collection, gametes emission and in vitro fertilization

Adults of the ascidian *C. robusta* were collected in the Gulf of Taranto (Italy) by local fishermen between November 2018-March 2019. The organisms were placed in plastic bags filled with NSW (salinity 40‰, pH 8) and transported in cool boxes, within few hours, to the aquarium facility of the Zoological Station Anton Dohrn of Naples (Italy). After an acclimation of 7 days, ascidians were maintained in flow-through circulating aquarium in NSW (filtered 0.45 μm) at the following conditions: temperature 18 ± 1°C; salinity 40 ± 1 ‰, dissolved O_2 7 mg L^{-1}; pH 8.1 under constant aeration and continuous light to stimulate gametes maturation and to avoid spawning (Lambert & Brandt, 1967). They were fed with a mixed marine algae diet every 48 h *ad libitum* (Shellfish Diet 1800®).

Gametes were obtained from each individual by dissecting the gonoducts with a scalpel. To avoid the self-fertilization, oocytes and sperms were collected by distinct individuals. The oocytes were rinsed twice in 0.22 μm filtered NSW while dry sperm was pooled and stored on ice until fertilization. Fertilization was performed by adding diluted sperm (1:100 in NSW) to the eggs suspension. After 10 min of incubation on a rotating shaker, the fertilized eggs were transferred to tissue culture plates and further rinsed in 0.22 μm filtered NSW.

2.2.3 Embryotoxicity

Embryotoxicity assay was carried out according to Bellas et al. (2003). About 1 h post-fertilization (hpf), 60 embryos (~two-cell stage) were added to 6-well plates containing PS NPs suspensions in NSW and incubated under dark static conditions at 18°C until the free swimming larva stage was reached (22 hpf). During the incubation, embryos were exposed to increasing nominal concentrations of both PS NPs as follows: PS-COOH (5-25-100 μg mL^{-1}) and PS-NH_2 (2-5-7.5-10-15 μg mL^{-1}).

The impact of PS NPs was evaluated as percentage of normal hatched larvae and morphological alterations at 22 hpf compared to controls, as described below. Larvae were first fixed in 4% paraformaldehyde and then washed twice in 1X PBS. Each larva was recorded as *normal* according to the general description: good general embryo morphology, with proper trunk and palps formation, as well as tail elongation following the Tunicate Anatomical and Developmental Ontology (TUNICANATO; https://www.bpni.bio.keio.ac.jp/tunicanato/3.0/developmental_table.html; Hotta

et al., 2020). Larvae phenotypes were examined by using the stereomicroscope Zeiss Axio Imager M1 and classified for simplicity in *mild*, *severe* and *not developed.* The assay was run at least three times and considered valid when controls (only in NSW) showed a percentage of normal hatched larvae ≥ 80% at 22 hpf.

Based on the results obtained after the embryotoxicological evaluation and from the analysis of the phenotypes, additional incubations were performed only for PS-NH_2 to collect the samples and evaluate the sub-lethal endpoints.

2.2.4 Sub-lethal effects

2.2.4.1 Swimming behaviour

At the end of the exposure period (22 hpf), a qualitative observation of the swimming behavior of ascidian larvae was performed. Several larvae from each experimental group (control and PS NPs treated) were carefully transferred in glass petri dishes with NSW and imaged using the stereomicroscope Zeiss Stemi 2000-C supplied with Canon Power Shot A640.

2.2.4.2 Neurotoxicity

C. robusta larvae were also tested for Cholinesterases (ChE) activity. At 22 hpf, embryos (1000 for each condition) were centrifuged at 600 *x g* for 15 min. The pellet was resuspended in 200 µL of homogenization buffer (20 mM Tris, 5 mM $MgCl_2$, 0,1 mg mL^{-1} Bacitracin, 8 x 10^{-3} TIU mL^{-1} Aprotinin, 1% Triton X-100, pH 7.4) sonicated for 1 min at 6 cycles (power 10%) and then centrifuged at 8,400 *x g* (4°C) for 20 min, according to the method of Corsi et al. (2007) with slight modifications as reported below. The supernatant was used for ChE enzymes activity assay according to the method of Ellman et al. (1961) adapted to microplate readers. Two thiocholine esters were used as substrates to gain information about the ChE type: acetylthiocholine iodide (ASCh) and butyrylthiocholine iodide (BSCh) as specific diagnostic substrates for Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) enzymes. Initial assay conditions in the reaction mixture (final volume 300 mL) were as follows: 0.1 M Na_2PO_4 (pH 7.2), 0.5 mM DTNB, 1 mM ChE substrates, embryos homogenate. The increase in absorbance at 405 nm was followed for 5 min at 20°C using a 550 Model microplate reader (Bio-Rad). ChE enzymes activity was expressed in nmol per min per mg of protein: nmol min^{-1} mg $protein^{-1}$. A selective AChE enzyme inhibitor, BW284c51, was tested at various concentrations (from 3.3 $\cdot 10^{-10}$ to 3.3 $\cdot 10^{-4}$ M) and incubated for 15 min with embryos

homogenates and residual ChE vs ASCh activities and fifty percent inhibition concentration (IC_{50}) values were determined. In order to evaluate the potential direct interaction of PS-NH_2 with ChE vs ASCh activity, embryos extracts were also incubated with PS-NH_2 suspensions at the following concentrations (2-7.5-10-15 µg mL^{-1}) for 15 min, ASCh was then added to start the reaction and the activity was measured for 5 min following the same procedure described above. Total proteins were measured in embryos homogenates according to Bradford (1976) and values were expressed as mg protein mL^{-1} supernatant.

2.2.4.3 Reactive oxygen species

The intracellular Reactive Oxygen Species (ROS) were determined according to Kang et al. (2013) with some modifications reported below. Control and PS NPs exposed embryos (22 hpf) were rinsed twice with filtered NSW and incubated with 10 µM of the fluorescent dye 2',7'–dichlorodihydrofluorescin diacetate (DCFH-DA) for 2 h in dark conditions. Control samples were incubated with DMSO. After incubation, half of embryos were directly observed at the optical microscope (Zeiss Axio Imager M1), for qualitative analyses, and half embryos were rinsed twice with NSW, centrifuged at 3000 *x g* at +4°C for 10 min and stored at -80°C for quantitative analyses of intracellular ROS.

Frozen embryos were resuspended in 0.5 mL of 40 mM Tris–HCl buffer (pH 7) and sonicated for 15 sec (3 cycles; 30% amplitude). The homogenate was then centrifuged for 10 min at 7800 *x g* at +4°C and supernatant was used for the fluorescence measurement at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a spectrofluorometer (Tecan). Fluorescence values were normalized by subtracting the autofluorescence of unlabeled extracts (DMSO). Results were expressed as arbitrary units of fluorescence (a.u.) referred to 60 embryos.

2.2.5 RNA extraction and gene expression analysis

For gene expression analysis, 180 embryos at two cell-stage were exposed to PS-NH_2 (7.5-10-15 µg mL^{-1}) and embryos collected at 22 hpf from three independent experiments and then centrifuged at 3000 rcf for 3 min. The excess of media was removed, and the collected larvae were frozen in liquid nitrogen and kept at -80°C until their usage. Total RNA was extracted using RNAqueous-micro kit (Ambion) according to the manufacturer's instructions. RNA concentrations were measured using measuring the absorbance at 260 nm (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA) and the integrity was evaluated by agarose gel electrophoresis. For each

sample 1 µg of total RNA was retrotranscribed using QuantiTect Reverse Transcription and quality confirmed by 1% agarose gel showing discrete 18 S and 28 S rRNA bands. The total RNA extracted was quantified Kit (Qiagen) following manufacturer's instruction.

Specific genes related to stress response (HSP60, HSP70, MnSOD, Cas8, CytB, p38) were initially investigated to try to understand the effects exerted by these NPs.

RT q-PCR was performed using a MicroAmp Optical 384-Well reaction plate with Optical Adhesive Covers (Applied Biosystems) in a ViiA^{TM} 7 Real Time PCR System (Applied Biosystems, Monza, Italy) thermal cycler. The reaction mix contained 1X Fast Start SYBR Green Master Mix (Roche), 1 µL of cDNA template (1:100 dilution) and 0.7 pmol μL^{-1} for each primer. The cycling conditions were: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s, 1 cycle for melting curve analysis (from 60 to 95 °C, reading every 0.5 °C) to verify the presence of a single product.
The complete sequences of the selected genes were taken from NCBI (https://www.ncbi.nlm.nih.gov/) and Aniseed (https://www.aniseed.cnrs.fr/). Specific primers were designed with the help of Primer 3 software (list in table) and then the selected amplicon were amplified. Cytoskeletal actin (GenBank ID: NM_001032502.1, Fujikawa et al., 2010) was used as reference gene to normalize the expression levels of target genes (Table 2.2). Fluorescence was analyzed with ViiA™ 7 Real-Time PCR software (Life Technologies) and then quantified according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on Ct values of each gene, and the Ct average of the selected reference gene, in order to calculate the relative mRNA expression level. The expression levels of the selected genes were evaluated in number-fold increase relative to the control condition, that has been assigned as "1".

Table 2.2. Accession number and/or Gene Model ID, sequences and length of PCR fragments are listed for the analysed genes.

Gene Name	Acronym	Accession Name and or Gene Model ID	Primer	Sequence (5′ → 3′)	Amplicon length (bp)
Cytoskeletal actin	Act	NM_001032502.1	Act_Fw	CCCAAATCATGTTCGAAACC	88
			Act_Rv	ACACCATCACCACTGTCGAA	
Heat shock protein 60 kDa	HSP60	XM_018812296.2 KH2012:KH.C6.85	CiHSP60_1F	AGACGAACAAGTTGGGGTTG	196
			CiHSP60_1R	GACTTTCGTTGGGTCCAGAA	
Heat Shock Protein 70 kDa	HSP70	NM_001033834.1 KH2012:KH.L46.6	CiHSP70_2F	TCTCTGTGCTCACCATCGAC	241
			CiHSP70_2F	GCTTTTTCGACAGGGTCAAG	
Superoxide dismutase, mitochondrial	MnSOD	XM_002128454.4 KH2012:KH.C12.466	CiMSOD_2F	TCAAGGTTCTGGCTGGAGTT	175
			CiMSOD_1R	GTAATCTGGCCGCACATTTT	
p38 mitogen-activated protein kinases	p38	NM_001078490.1 KH2012:KH.C11.76	CiP38_1F	GACGACCACGTCCAGTTTTT	172
			CiP38_1R	CGGTCATTTGGTCATCTGTG	
Cytochrome b	CytB	ENSCINT00000037367.1	CiCytb_1F	TTATCGGTTACCGGCTCCTA	151
			CiCytb_1R	ATGGTTCACAGAAGAAAAAGCTA	

2.2.6 Statistical analysis

All the statistical analyses were performed using Graphpad Prism 6. All data were expressed as mean ± standard deviation (SD). The median effective concentration (EC_{50}), corresponding to a 50% reduction of normal hatched larvae, was calculated using a sigmoidal dose−response model according to the equation: $y=b+(a–b) / 1+10^{(\text{Log EC50} - x)}$ where y is response, b response minimum, a response maximum, x the logarithm of effect concentration and EC_{50} the concentration of effect giving 50% of maximum effect. Data were normalized to the control mean percentage of larval abnormality using Abbot's formula:

$$P= (P_e\text{-}P_c/100\text{-}P_c)\cdot 100$$

Where P_c and P_e are the control and the experimental percentages of response, respectively.

Data from the embryotoxicity assay and for the analyses of the morphological alterations are representative of at least three independent experiments and were analyzed using the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test. Intracellular levels of ROS, ChE vs ASCh activities were analyzed by One-Way ANOVA followed by Bonferroni's post hoc Test. Each analysis was performed three to five times.

RT-qPCR data are presented as mean ± standard error and the significance of the relative $2^{-\Delta\Delta Ct}$ of each group (biological replicates, N = 3), compared to the controls, was determined using 'unpaired parametric t-test'.

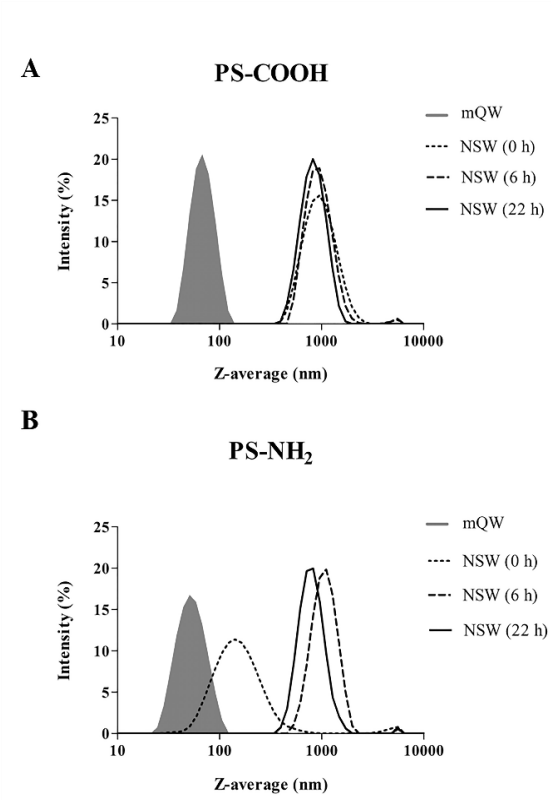
2.3 Results and Discussion

2.3.1 Behavior of PS NPs suspensions in NSW

Nanoplastics are contaminants of emerging global concern, whose presence along the water column calls for a thorough evaluation of their impact on aquatic life and in particular to vulnerable life stages, such as planktonic embryos and larvae. Here, we investigated for the first time the impact of two functionalized PS NPs on the embryo development of the ascidian *C. robusta*, a benthic invertebrate living in marine coastal areas with a key ecological role, with the peculiarity of being an early chordate developmental model (Delsuc et al., 2006). In order to achieve this aim, we used PS NPs as model nanoplastics with different surface functionalization (PS-COOH vs PS-NH_2) and thus surface charge (negative vs positive), recently recognized as a predictor of their behavior in high ionic strength media, such as seawater, and consequently toxicity for marine phyto- and zooplankton (Della

Torre et al., 2014; Bergami et al., 2016, 2017; Manfra et al., 2017; Varò et al., 2019; Bellingeri et al., 2020; Grassi et al., 2020; Corsi et al., 2020). At the cellular level, studies conducted using mammalian models showed different mechanisms of actions and cell recognition driven by PS NPs surface charged, which are able to activate specific signaling cascade and pathways of toxicity (reviewed in Yong et al., 2020).

The results obtained from DLS and EM of PS NPs suspensions in NSW (ascidian exposure medium) are reported in Figure 2.2.



Medium	Time (h)	Z-Average (nm)	PDI	ζ-potential (mV)
mQW	0	64.46 ± 0.59	0.061 ± 0.021	-59.60 ± 2.61
	6	61.08 ± 2.94	0.136 ± 0.011	-62.10 ± 0.46
	22	65.97 ± 1.10	0.114 ± 0.032	-57.00 ± 4.52
NSW	0	921.10 ± 49.02	0.159 ± 0.078	-13.10 ± 1.61
	6	951.40 ± 89.80	0.186 ± 0.054	-12.40 ± 0.83
	22	926.70 ± 89.85	0.266 ± 0.004	-14.50 ± 1.49

Medium	Time (h)	Z-Average (nm)	PDI	ζ-potential (mV)
mQW	0	46.76 ± 0.63	0.169 ± 0.009	+47.50 ± 0
	6	51.89 ± 3.26	0.198 ± 0.009	+27.30 ± 0.49
	22	50.12 ± 0.25	0.175 ± 0.044	+31.80 ± 0.49
NSW	0	130.00 ± 10.93	0.290 ± 0.024	+12.80 ± 1.71
	6	1104 ± 94.65	0.265 ± 0.053	+7.66 ± 1.25
	22	999.70 ± 54.19	> 0.400	+7.19 ± 3.28

Figure 2.2. Physico-chemical characterization of PS-COOH (A) and PS-NH2 (B) in milli-Q water (mQW) and natural sea water (NSW) (ascidian exposure medium) (Gulf of Naples, 0.20 μm filtered, salinity 40‰, pH 7.95) used for the embryotoxicity tests, at 0, 6 and 22 h. Intensity-weighted size distributions (mQW shown as a reference) are reported in the left panel and relative values of Z-average (nm), polydispersity index (PDI) and ζ-potential (mV) are shown in the tables, right panel. Data are referred to a PS NP concentration of 20 μg mL^{-1} and values are the mean ± SD of 3 measurements.

The analyses confirmed the primary size of PS-COOH and PS-NH_2 of 60 and 50 nm, respectively, as indicated by their average hydrodynamic diameters and narrow PDI values in mQW. Likewise, ζ-potential values (average of -59.6 mV for PS-COOH and + 47.5 mV for PS-NH_2) show their negative and positive surface charges in this medium. The optimal dispersion in mQW was observed at

different exposure time-points (between 0 and 22 h), reaching a Z-Average of 66 ± 1 nm and PDI 0.114 ± 0.032 for PS-COOH, and a Z-Average of 50 ± 0.25 nm and PDI 175 ± 0.04 for PS-NH_2. On the opposite, their behavior in NSW largely differed from the one in mQW already at 0 h. Soon after dispersion in NSW, PS-COOH were found as large agglomerates (Z-Average of 921.1 ± 49.02 nm) at 0 h and after 6 and 22 h (Z-Average of 926.7 ± 89.85 nm). A different trend was observed for PS-NH_2, which were initially well dispersed in NSW as small nanoscale objects (Z-Average of 130 ± 10.93 nm), but quickly agglomerated within 1 h of suspension in NSW to about 850 nm (Figure 2.3), reaching large agglomerates already after 6 h (Z-Average of 1104 ± 94.65 nm). After 22 h, the agglomeration of PS-NH_2 (Z-Average of 999.7 ± 54.19 nm) was comparable to that of PS-COOH, with broader PDI values (> 0.400).

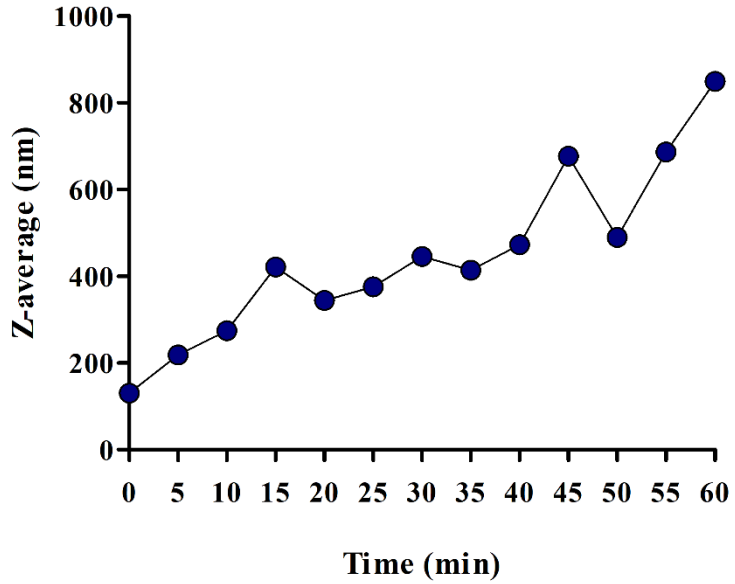


Figure 2.3. Aggregation trend of PS-NH_2 NPs (at 20 µg mL^{-1}) in natural sea water (NSW) (Gulf of Naples, 0.20 µm filtered, salinity 40‰, pH 7.95) within 1 h of suspension. As an indication of NP hydrodynamic diameter, Z-average values (nm) at 5 min intervals measured by DLS analysis are shown.

The absolute decrease in the ζ-potential values (average of -14.50 mV for PS-COOH and + 7.19 for PS-NH_2) showed the instability of PS NPs in this high ionic strength medium. These results are in agreement with Varó et al. (2019) and Tallec (2019) and suggest a dynamic agglomeration state of surface charged PS NPs over time. The peculiar behaviour of surface charged PS NPs has been linked to electrostatic and hydrophobic interactions occurring with ionic species and natural organic matter present in NSW (reviewed in Corsi et al., 2020).

2.3.2 Embryotoxicity

PS NPs effect on *C. robusta* embryos development largely differ at 22 h. As shown in Figure 2.4, PS-COOH did not cause any relevant effect on *C. robusta* embryos development up to 100 µg mL^{-1}. Conversely, PS-NH_2 significantly affected embryos hatching success and development in a dose-dependent manner with a EC_{50} of 7.52 µg mL^{-1} with 95% confidence interval (CI) from 6.87 to 8.24 µg mL^{-1} (Figure 2.4) and with various degrees of phenotype malformations (*mild to severe*) and *not developed* embryos (up to 30%) at the highest concentration tested (15 µg mL^{-1}). PS-NH_2 of similar size range (50 nm) and exposure concentrations (from 0.1 to 50 µg mL^{-1}) have already been reported to affect embryo development in other marine species, such as the sea urchin *P. lividus* and the bivalves *M. galloprovincialis* and *C. gigas* (Della Torre et al., 2014; Balbi et al., 2017; Tallec et al., 2018). In particular, concentrations between 2.5 and 5 µg mL^{-1} caused various alterations in developing sea urchin embryos, as incomplete or absent skeletal rods, fractured ectoderm, reduced length of the arms, with the involvement of some molecular targets responsible of stress response and embryo development (Della Torre et al., 2014; Pinsino et al., 2017). Furthermore, PS-NH_2 (0.15 µg mL^{-1}) also affected shell formation of D-larvae in both bivalves, interfering with molecular mechanisms involved in the mineralization processes (Balbi et al., 2017; Tallec et al., 2018). In both cases, the toxicity of PS-NH_2 has been related to their positive charge and their nanoscale dispersion compared to PS-COOH, that allows them to be internalized by cells through endocytosis or to disrupt cell membrane and induce oxidative stress (Liu et al., 2011; Wang et al., 2013a, 2013b; Bexiga et al., 2013).

As a result of the embryotoxicity caused by PS-NH_2, the calculated EC_{50} value of 7.52 µg mL^{-1} (22 hpf) resulted higher than those available for the oyster (0.15 µg mL^{-1}, 48 hpf) and mussel (0.14 µg mL^{-1}, 48 hpf), as well as the sea urchin (2.61 µg mL^{-1}, 48 hpf) although at the same order of magnitude (Della Torre et al., 2014; Balbi et al., 2017; Tallec et al., 2018). Such differences in embryos sensitivity towards PS-NH_2 exposure could be therefore species-specific. According to Bellas and co-workers (2001, 2005), ascidian embryos have been documented to be less sensitive, compared to sea urchin and mussel embryos, to inorganic (i.e., metals) and organic contaminants (e.g., pesticides, herbicides, surfactants). Such low sensitivity could be due to the presence of the egg envelopes surrounding *Ciona* embryos, formed by two populations of maternally-supplied cells, one external (follicle cells) and one internal (test cells), separated by an acellular layer named vitelline coat (or chorion). The egg envelopes remain associated with the oocyte following its release into the water column until the hatching of the embryo at the larval stage (De Santis et al., 1980; Cotelli et al., 1981;

Sato & Morisawa, 1999; Thompson & Shimeld, 2015). This complex structure differs significantly from the simple vitelline coat, a thin lamina that envelops the eggs of sea urchin and bivalves (Mazzini et al., 1984), thus leaving the embryos potentially more exposed to chemicals and nanomaterials. Given the great difference in the external structure of the coats, the nano-bio interactions and mode of action of PS-NH_2 could be different between ascidian and sea urchin and bivalve embryos, resulting in diverse impact on embryos development. The egg envelopes, during embryogenesis, may act a physico-chemical barrier against pollutants, as shown in zebrafish embryos in which the protective effect of embryonic chorion has been identified by the enhancement of sensitivity to exogenous compounds after chorion removal (van Pomeren et al., 2017; Liegertová et al., 2018; Vranic et al., 2019). It is conceivable to envisage a similar shielding role of *Ciona* egg envelopes towards PS-NH_2 exposure that could motivate the observed differences in sensitivity of ascidian embryos compared to other marine invertebrate species.

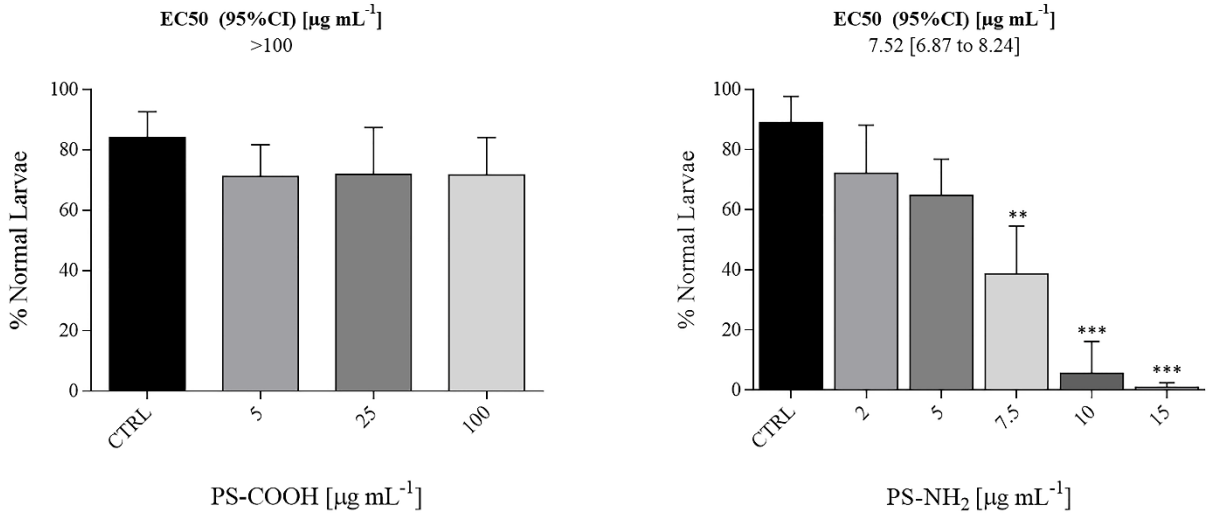


Figure 2.4. Percentage (%) of normal hatched larvae of *C. robusta* upon exposure to PS-COOH (left) and PS-NH_2 (right) in NSW for 22h. Bars represent mean ± SD (PS-COOH n=720; PS-NH_2 n=1080). Asterisks indicate values that are significantly different compared to the control (Kruskal-Wallis test, Dunn's post hoc test, **p<0.01, ***p<0.001). When available, EC_{50} values have been shown.

Besides the hatching success, we also evaluated any phenotypic alterations in developing *C. robusta* embryos exposed to PS-NH_2. Observation under a light microscope indicated that the morphological defects were mainly related to the development of the trunk. Immediately after the hatching, the trunk is round and, as the development proceeds, it adopts an increasingly square shape with the elongation

of the palps (https://www.bpni.bio.keio.ac.jp/chordate/faba/1.4/developmental_table_3D.html and https://www.bpni.bio.keio.ac.jp/chordate/faba2/top.html) (Figure 2.5 A,A').

As shown in Figure 2.5, the shape of the trunk was normal in the 5 µg mL^{-1} treated larvae (Figure 2.5 B,B'), rectangular but not well elongated in the 7.5 µg mL^{-1} treated larvae (Figure 2.5 C,C') and became progressively shorter, round and not elongated by increasing PS-NH_2 concentrations (Figure 2.5 D,D' and 2.5 E,E'). In most cases, the malformed larvae failed to hatch upon exposure to 15 µg mL^{-1} PS-NH_2. For the sake of simplicity, we thus classified the abnormalities as *mild*, *severe* (Figure 2.5) and *not developed* (not shown).

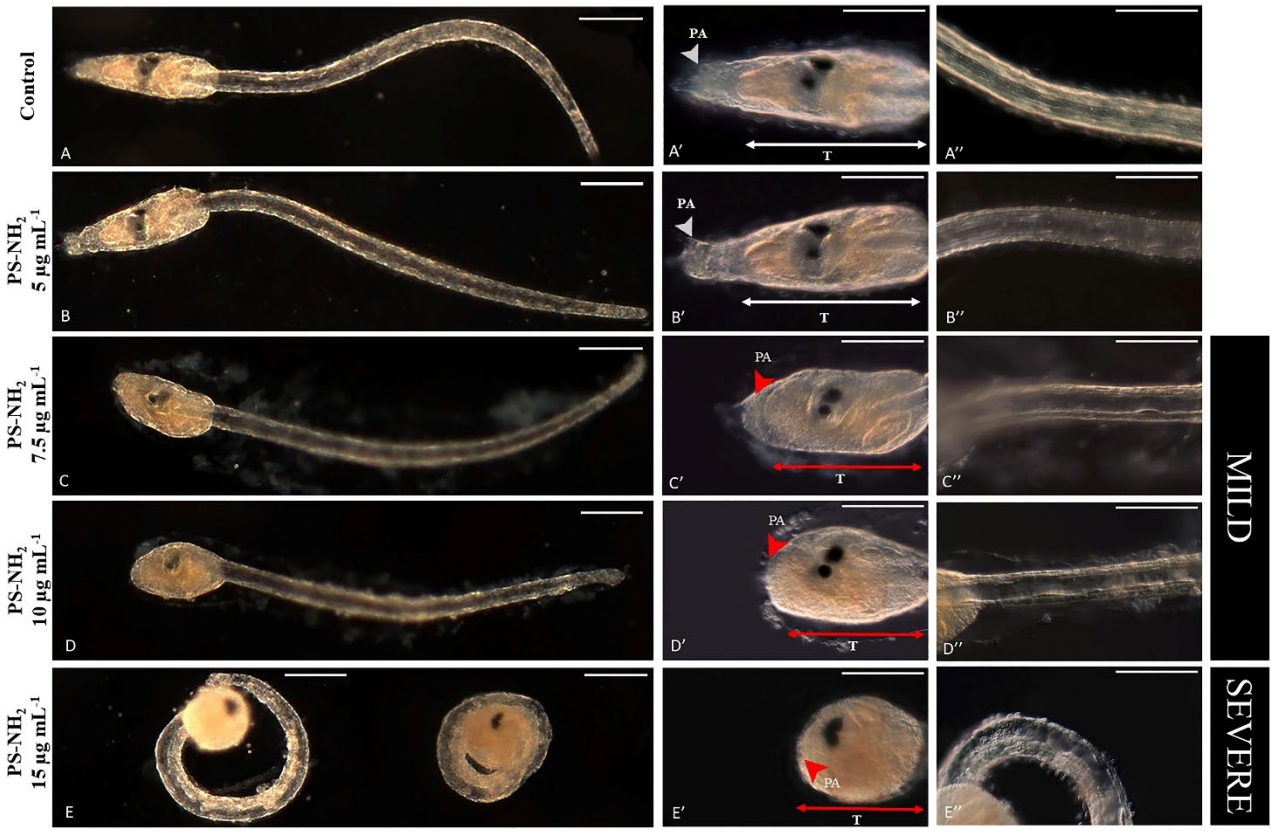


Figure 2.5. Light microscopy images of *C. robusta* embryos exposed for 22 h to PS-NH_2: (A-A'') Control; (B-B'') 5 µg mL^{-1}; (C-C'') 7.5 µg mL^{-1}; (D-D'') 10 µg mL^{-1}; (E-E'') 15 µg mL^{-1}. White arrows (PA) indicate normal development of palps; white arrows (T) indicate a proper elongation of the trunk; red arrow heads (PA) indicate absence of palp; red arrows (T) indicate a not well elongated trunk. Scale bar: 100 µm

Conversely, the development of the tail appeared not strongly affected by PS-NH_2 if compared to control larvae, as shown in Fig. 2.5 A''-E''. Tails, in fact, resulted elongated and notochord vacuolation seemed well accomplished even at the highest PS-NH_2 concentration of 15 µg mL^{-1} (https://www.bpni.bio.keio.ac.jp/chordate/faba/1.4/developmental_table_3D.html).

Noteworthy, the incidence of the *severe* phenotype was PS-NH_2 concentration-dependent up to 15 µg mL^{-1}. Almost 40% of larvae exhibited the *mild* phenotype at both 7.5 and 10 µg mL^{-1}. However, the *severe* phenotype was present in around 40% of 10 µg mL^{-1} treated embryos and only in less than 20% of larvae exposed to 7.5µg mL^{-1}. A higher occurrence of the *severe* phenotype (more than 70%) was observed at the highest PS-NH_2 concentration (15 µg mL^{-1}) with most of the malformed larvae blocked within the egg envelopes, able to twitch weakly the tail but unable to hatch, while the remaining 30% did not develop (Figure 2.6).

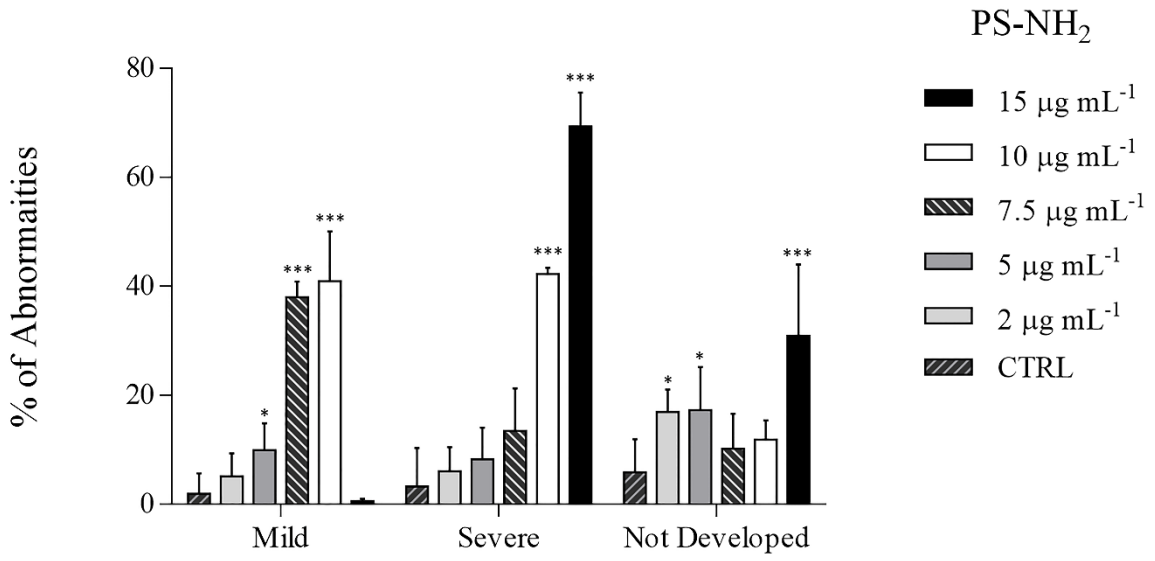


Figure 2.6. Percentages (%) of total abnormalities identified upon exposure to PS-NH_2. Bars represent mean ±SD (n=1080). Asterisks indicate values that are significantly different compared to controls (Kruskal-Wallis test, Dunn's post hoc test, *=$p<0.05$; ***=$p<0.001$).

Hatching impairment has been detected also in zebrafish embryos exposed to different types of NPs. For instance, hatch was delayed by exposure to 10 mg L^{-1} nCdSe and nAg at 72 hpf, and complete inhibition of hatch was observed in zebrafish embryos exposed to 10 and 100 mg L^{-1} nZnO sphere and leaf, respectively, and 100 mg L^{-1} nCdSe (Ong et al. 2014). The main mechanism associated with such hatch inhibition was likely through the interaction of these NPs with the zebrafish hatching enzyme. In another study, zebrafish hatching inhibition was observed after PS NPs exposure treatment (Duan et al 2020). The authors showed that PS NPs (100 nm) could form a dense coating around the chorion after 24 hpf, almost blocking their uptake, although the pores of the chorionic membrane have a diameter around 500 nm (Cheng et al., 2007). This covering layer led to a hypoxic microenvironment in the inner space of the chorion that in turn damaged the antioxidant system of

the organism. In our treatments, as for zebrafish embryos, we detected PS-NH_2 aggregates around the egg envelopes of *Ciona* embryos (Figure 2.7) that increased at higher PS NPs concentration. One can suppose that in *Ciona*, as in zebrafish, these aggregates might cause an internal hypoxic microenvironment for embryos, resulting in the inhibition of hatching and affecting also larval development. However, as previously mentioned, we cannot exclude that PS-NH_2 might be able to pass through the egg envelopes, thus directly inducing larval phenotypic malformations, or interact with *Ciona* hatching enzyme. This enzyme, which shows both trypsin-like activity and metalloproteinase activity, is localized in the papillae and in the tip of the tail, and its secretion allows the digestion of the envelopes surrounding the larvae at the time of hatching (D'Aniello et al., 1997; Scippa et al., 2006).

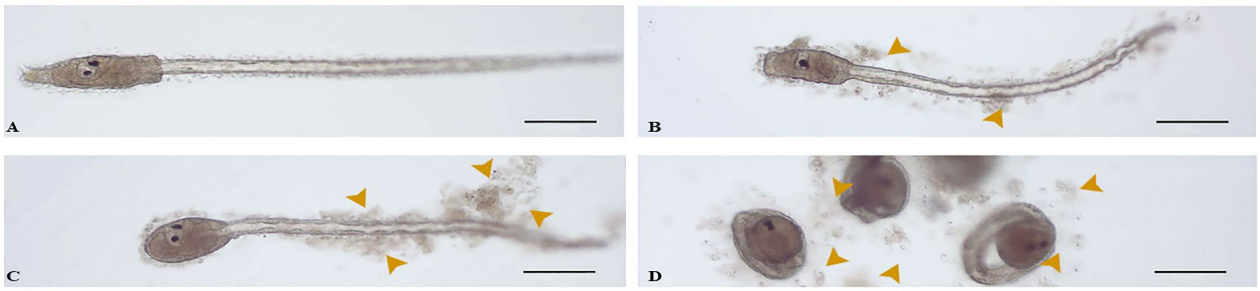


Figure 2.7. Optical microscope images of *C. robusta* embryos exposed for 22 h to different concentrations of PS-NH_2: (A) Control; (B) 7.5 µg mL^{-1}; (C) 10 µg mL^{-1}; (D) 15 µg mL^{-1}. Yellow arrows indicate PS-NH_2 NPs aggregates that adhere on embryo surface. Scale bar 100 µm.

Interestingly, the hatched larvae showing both *mild* and *severe* phenotypes, although viable and motile, moved slowly and were not very reactive, compared to the controls, suggesting a possible neuromuscular impairment.

2.3.3 Sub-lethal effects

Swimming behaviour and neurotoxicity

Besides the phenotypic malformations, the larvae also showed behavioral abnormalities. No differences in the swimming behavior of *C. robusta* embryos were observed between PS-COOH treatments and the control group. In contrast, larvae treated with 7.5 µg mL^{-1}, 10 µg mL^{-1} and, even more so, with 15 µg mL^{-1} PS-NH_2 NPs (in this case manually dechorionated) appeared alive but not very healthy, moving slowly and being not very reactive, compared to the controls. In fact, larvae were able to twitch their tails, allowing them to change direction, but they did not alternate the bending of the tail, thus being incapable of swimming. Moreover, embryos exposed to the highest

PS-NH_2 concentration (15 µg mL^{-1}) could just twitch the tail, but they were unable to hatch. Interestingly, exposure to PS NPs also affected zebrafish larvae locomotor activity, as shown by swimming hypoactivity in treated larvae (Chen et al. 2017; Pitt et al. 2018), related to oxidative stress and reduced AChE activity (Chen et al. 2017). We thus evaluated the biochemical response to the AChE activity in *Ciona* treated larvae, based also on the similarities of swimming control between ascidians and vertebrates. *Ciona* larval motor neurons innervating the tail express cholinergic promoters and genes (Takamura et al., 2002; Yoshida et al., 2004), neuromuscular junctions are cholinergic (Ohmori & Sasaki, 1977), while GABA is present in the visceral ganglion and it has been shown to modulate *Ciona* swimming, as in vertebrates (Brown et al., 2005).

Before running the assay, ChE activities towards specific substrates (i.e. ASCh and BSCh) were first characterized in *C. robusta* larvae. Results indicate that AChE enzyme was mainly responsible for ChE activities in *C. robusta* developing embryos, as confirmed by the use of its selective inhibitor BW284c51 (Figure 2.8).

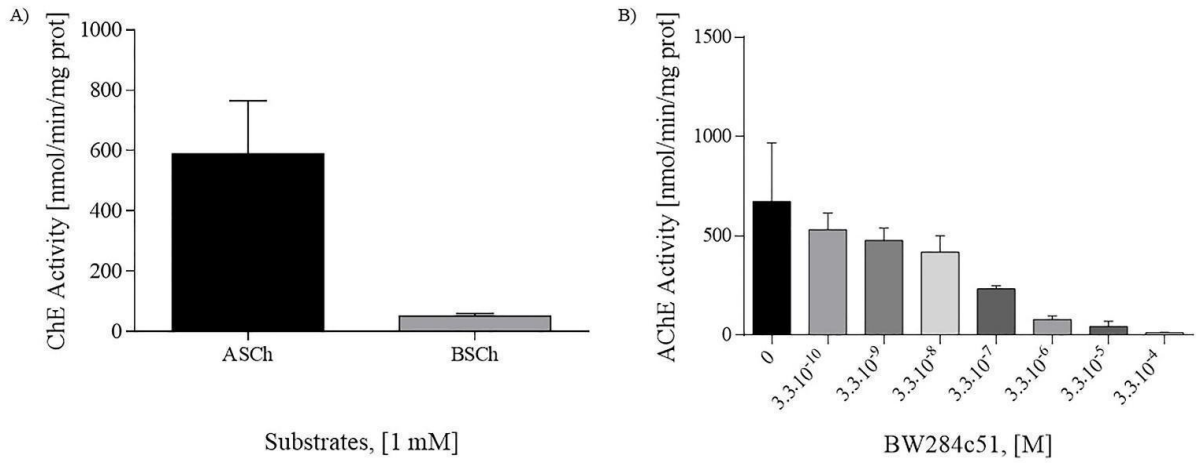


Figure 2.8. (A) Substrate specificity of esterase (ChE) activities in *Ciona* larvae homogenate. (B) AChE activity with the selective inhibitor BW284c51. Data were analysed by One-Way ANOVA followed by Bonferroni's Multiple Comparison Test. Bars represent mean ± SD.

On the other hand, *in vivo* exposure did not cause a significant effect on ACh vs ASCh activities in *C. robusta* embryos at all PS-NH_2 concentrations tested, even if there was a slight increase in the activities at the highest concentration (15 µg mL^{-1}) (Figure 2.9A). The results have been confirmed by the *in vitro* assays, exposing the embryo extracts to increasing concentrations of PS-NH_2 (Figure 2.9B).

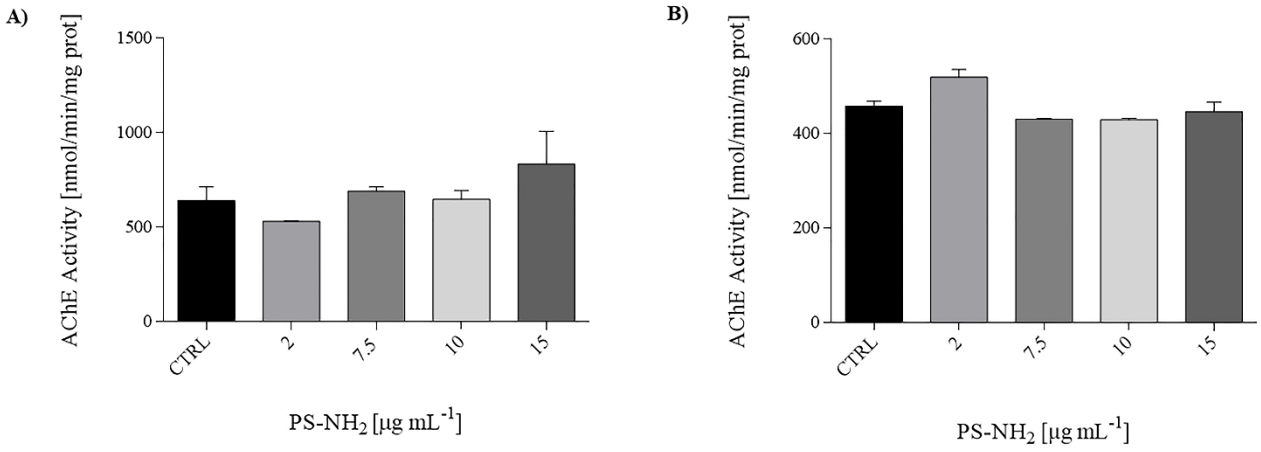


Figure 2.9. ChE vs ASCh activities (A) of *C. robusta* embryos in vivo exposed to PS-NH_2 after 22 hpf. (B) ChE vs ASCh activities of *C. robusta* embryos extract in vitro exposed to PS-NH_2. Bars represent mean ± SD (n=5000, One-Way ANOVA, Bonferroni's post hoc test, $p<0.05$).

Even if our results showed no alteration in AChE enzyme activities upon PS-NH_2 exposure, compared to the control groups, we cannot exclude that the hypoactivity is related to a general decreased organismal fitness of *Ciona* treated larvae or to enzymatic mechanisms, other than cholinergic, controlling the swimming, as, for example, the GABAergic/Glycinergic and/or Dopaminergic systems (Moret et al., 2005; Horie et al., 2010; Takamura et al., 2010).

Reactive Oxygen Species

Many ways can lead to the cellular uptake of NPs: passive diffusion, membrane disruption and non-specific or specific endocytosis (Foroozandeh et al., 2018). For example, it has been shown that PS NPs can easily permeate into lipid membrane (Rossi et al, 2014), while negatively and positively PS NPs are internalized by different mechanisms of endocytosis (Lunov et al., 2011; Smith et al, 2012; Bhattacharjee et al., 2013). Once in the cell, PS-NH_2 can cause disruption of cell membrane and generate oxidative stress in different territories (Liu et al., 2011; Wang et al., 2013a; Bexiga et al, 2013; Bhattacharjee et al., 2013). Notably, fluorescently labeled PS-NH_2 (20 nm) injected in zebrafish embryos were able to bioaccumulate and cause oxidative DNA damages in the brain tissues (Sökmen et al., 2019), while zebrafish larvae exposed to fluorescently labeled PS NPs showed significant fluorescence in diverse territories, included the head (Pitt et al. 2018). The results of the DCFDA assay in *C. robusta* embryos exposed to PS-NH_2 (5-7.5-10-15 µg mL^{-1}) are shown in Figure 2.10. A dose-dependent increase in fluorescence was observed with significant higher values at 10 and 15 µg

mL^{-1}, compared to controls. This data was further confirmed by a direct observation of exposed larvae under fluorescence microscope (Figure 2.11). *C. robusta* larvae exposed to 7.5 µg mL^{-1} PS-NH_2 showed a fluorescent signal in the brain vesicle (Figure 2.11B), mostly around the ocellus which was absent in larvae from the control group (Figure 2.11A). Interestingly, the fluorescence signal expanded in almost all CNS of the larval trunk (brain vesicle and visceral ganglion) as well as in the palps of larvae exposed to 10 µg mL^{-1} (Figure 2.11C) and almost in the whole trunk, including the endoderm and the mesenchyme, at 15 µg mL^{-1} PS NPs treatment (Figure 2.11D). Notably, oxidative stress territories and phenotypic morphological alterations were both localized mostly at the level of trunk structures. It is also possible that the increased level of ROS in the brain vesicle and visceral ganglion affected development of both territories and, consequently, the swimming behavior.

Thus, the presence of PS-NH_2 might cause oxidative damage in *Ciona* larvae, though further studies, by fine microscopical observations using fluorescent NPs, will help to monitor the localization and the possible uptake of NPs by the egg envelopes during embryogenesis, in order to get insights into the toxic mechanisms involved. The ultrastructure of *Ciona* chorion, including the pore size, also needs to be defined to better support such hypotheses and understand the protective role of this membrane.

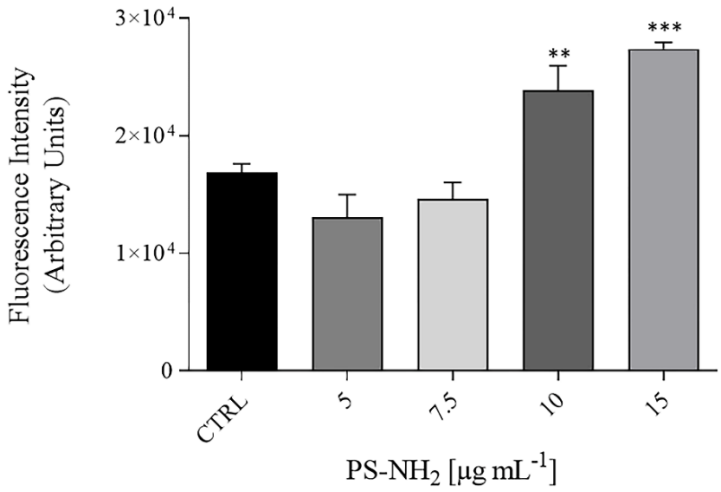


Figure 2.10. Quantitative analyses of intracellular ROS levels (fluorescence arbitrary Units) of *C. robusta* embryos exposed to PS-NH_2. Bars represent mean ± SD (n=900). Asterisks indicate values that are significantly different compared to the controls (One-Way ANOVA, Bonferroni's post hoc test, **=$p<0.01$ ***=$p<0.001$).

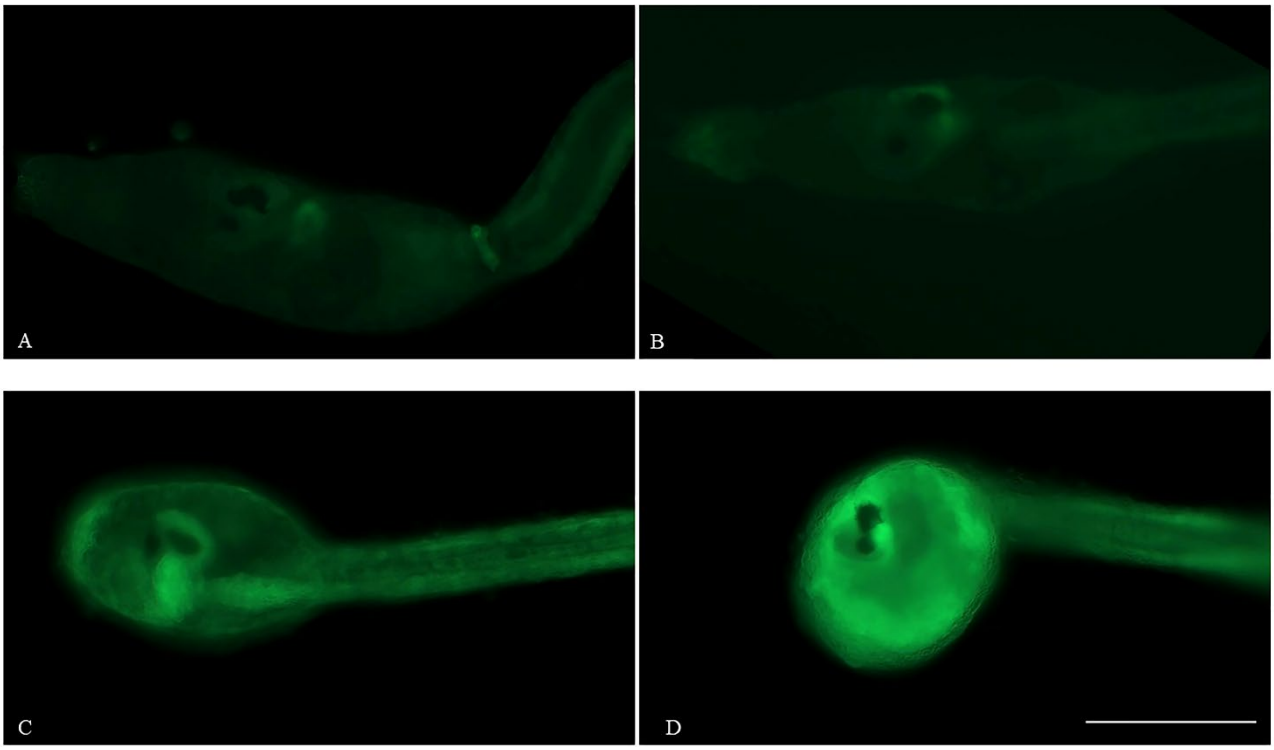


Figure 2.11. ROS production in *C. robusta* embryos (22 hpf) observed through optical fluorescence microscopy (GFP-filter ex 488 nm/em 525 nm): (A) control, (B) 7.5, (C) 10 and (D) 15 µg mL^{-1} of $PS\text{-}NH_2$. Scale bar: 100 µm.

2.3.4 Gene expression analysis

The increased levels of ROS prompted us to monitor the expression of typical genes related to stress response (HSP70, HSP60, MnSOD, cytochrome b, p-38 mapk and caspase 8) at three concentrations of $PS\text{-}NH_2$ (7.5-10-15 µg mL^{-1}) (Figure 2.12).

The expression of the selected genes involved in stress response has been previously studied in *P. lividus* embryos exposed to $PS\text{-}NH_2$, with a resulting up-regulation of the genes cas8, HSP70 and p38 mapk after 24 hpf exposure, and a non-modulation of the genes at the pluteus stage (48 hpf). Going deeply into the function of these gene, HSP60 and HSP70 have a fundamental role in stress response and protein metabolism (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005); MnSOD converts H_2O_2 in the mitochondrial matrix space, which is the main place producing ROS and the most sensitive position suffering from ROS effects (Murphy, 2009; Sies et al., 2017), cytB is responsible for the mitochondrial superoxide production (Zhu et al., 1999), cas8 and p38 mapk are associated with survival response, apoptosis and autophagy (Tummers and Green, 2017; Bradham & McClay, 2006; Coulthard et al., 2009). In our study, the level of expression of all these genes

resulted down-regulated. The concentration of PS-NH_2 which had a great impact on *Ciona* larvae was the highest one (15 µg mL^{-1}) with a significant altered expression of hsp60 (0.60-fold), hsp70 (0.23-fold), MnSOD (0.44-fold), cytB (0.51-fold), p-38 mapK (0.55-fold). Moreover, we found a down-regulation of MnSOD (0.30-fold) at 7.5 µg mL^{-1} PS-NH_2 and cas8 (0.36-fold) at 10 µg mL^{-1} PS-NH_2. These results are in line with the data obtained on ROS determination and the increased levels of ROS, at 15 µg mL^{-1}, could be strictly related to the downregulation of stress response genes. Thus, it appears that the antioxidant defence system of the embryos exposed to PS-NH_2 is not able to counteract the oxidative status. The resulting burst of ROS could, in turn, be responsible for the phenotypic morphological alterations we detect at the larval stage.

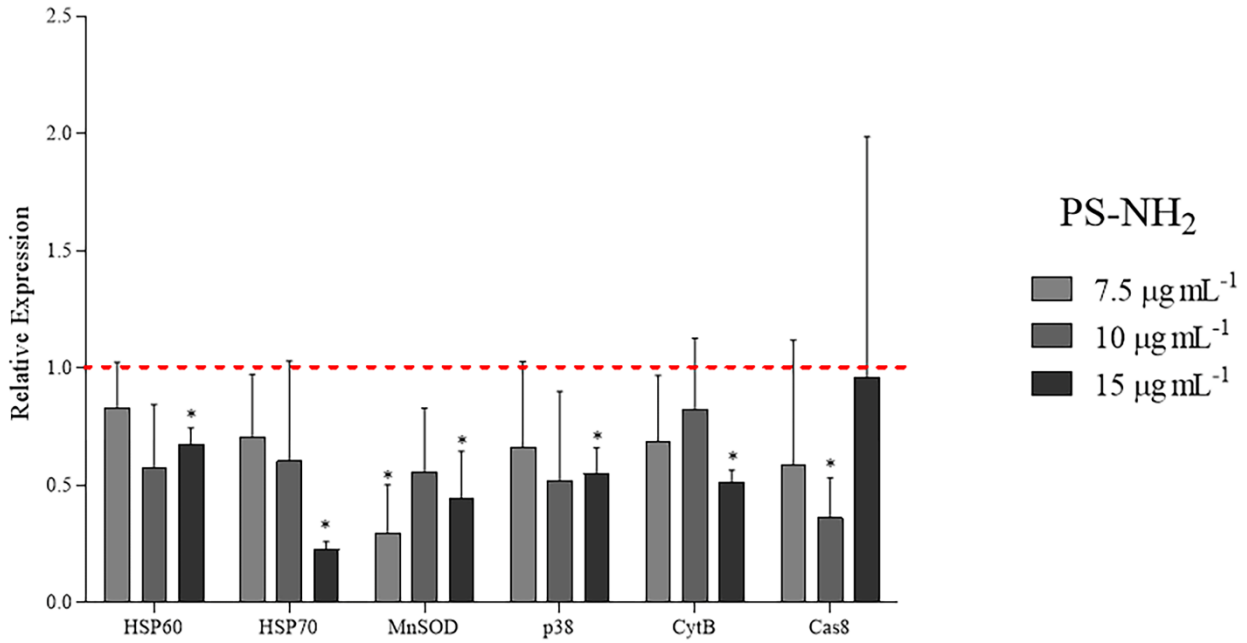


Figure 2.12. Expression of hsp60, hsp70, MnSOD, cytochrome b, p-38 mapk and caspase8 genes by comparative RT q-PCR with total RNA isolated from control and exposed embryos (7.5- 10-15 µg mL^{-1} of PS-NH_2) after 22 hpf. Results are expressed as fold increase compared to controls assumed as 1, using cytoskeletal actin as reference gene for normalization. Each bar represents the mean of three independent experiments ± SE (N=2160). * indicates significant difference (t-test, $p < 0.05$) respect to controls.

2.4 Conclusions

Our study showed the effects of surface charged PS NPs on the embryogenesis of *C. robusta*, an important member of a sister clade to the vertebrates. Overall, our findings clearly indicated that the development of *C. robusta* embryos is not affected by PS-COOH up to the concentration of 100 µg

mL^{-1}, while *Ciona* embryos are vulnerable to the positively charged PS-NH_2, affecting the normal morphology and behaviour of the embryo and inducing oxidative stress linked to an increase of ROS production and also to a dysregulation of some genes involved in stress response. Consequently, the compromised larval development could have an impact on species fitness and survival, leading to significant ecological implications also for other species sharing planktonic larval stages. These outcomes are of concern, due to increased sources of stress in the marine surface layer and coastal areas challenging rapid environmental changes and exposure to toxic contaminants. In the next chapter, I will discuss the data obtained through the RNA-seq of *Ciona* embryos at late tailbuld I stage to inquire into the entire biological response of the whole organism to PS-NH_2 exposure.

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Disclosure statement

The authors report no conflict of interest.

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CHAPTER 3

Transcriptomic profiling of *C. robusta* embryos upon exposure to amino-modified PS NPs (PS-NH_2)

ABSTRACT

The effects of nanoplastics have been widely investigated on the embryogenesis, survival, immune system and behavior of different aquatic organisms. Nowadays, there is a growing need to understand the mechanisms of action of nanoplastics, mainly using the transcriptomic approach. In this chapter, the global transcriptome analyses (RNA-seq) was performed on *C. robusta* embryos to elucidate the molecular responses upon the exposure of PS-NH_2. A total of 42 and 186 differentially expressed genes (DEGs) were detected in response to exposure to 10 and 15 µg mL^{-1} to PS-NH_2 for 13 h. The gene function and pathways were identified through GO and Pathways Analyses, with the resulting affection of the following pathways: (i) "glutathione synthesis and recycling", (ii) "neurotransmitter clearance" (iii) "passive transport by aquaporins", (iv) "fructose and mannose metabolism", (v) "starch and sucrose metabolism" (vi) "glycolysis". This work provides knowledge of the response upon PS-NH_2 exposure on *C. robusta* embryos, providing insights and useful information into the toxicodynamics nanoplastic proxies during the embryogenesis of a chordate model.

3.1 Introduction

In the chapter 2, the impact of PS-NH_2 on the development of the ascidian *C. robusta* has been studied. Our data demonstrated that PS-NH_2 affected the normal morphology and behaviour of *Ciona* larvae, induced oxidative stress, with an increase of ROS production, and led to a dysregulation of some genes involved in stress response and palps development.

Over the years, the effects of PS NPs on marine organisms has been widely characterized, and, nowadays, there is an increasing need to understand the mode of actions (MOAs) of such nanoparticles as proxy for nanoplastics. The omics technologies such as genomic-scale expression of messenger mRNA (transcriptomics), cell- and tissue-wide protein (proteomics), and metabolite profiling (metabolomics), coupled with bioinformatic methods and conventional toxicology are the approaches mainly used to study the response of a genome to hazardous substances (Sauer et al. 2017).

Until now, the transcriptomic approach is the most used in omics because the techniques for mRNA quantification and data interpretation are more developed, compared to proteomics or metabolomics. Methods for transcriptomics analysis are divided into methods for measuring global gene expression (DNA microarray and RNA sequencing) and methods for measuring the expression of distinctive genes that usually can be used as biomarkers for specific stressors (e.g. expression of genes involved in stress response). The first method is the most used, and the high volume of data are analysed through bioinformatic methods such as Gene Ontology (GO) and Pathway Analysis. GO is a major bioinformatic initiative (http://www.geneontology.org/) which allows to process the transcriptome data into structured ontologies that describe the gene products in terms of their associated biological processes, cellular components and molecular functions (Brinke and Buchinger 2017; Schirmer et al. 2010). This omics approach is providing a profound insight into the biochemistry and physiology of the cell and any change due to toxicant exposure.

DNA microarray and RNA sequencing (RNA seq) have been used to study the MoA of several man-made contaminants in zebrafish embryos (Yang et al. 2007), or to uncover those of Cu, Cd and Zn in Daphnia magna at sublethal concentrations (Poynton et al., 2007). Only recently, the RNA seq has been used to better understand the molecular mechanisms that are altered by 1 mg L^{-1} of PS NPs (71.18 ±6.03 nm) in the crustacean *Daphnia pulex*, showing the induction of oxidative stress, immune defence and glycometabolism disorder in neonates after a short-term exposure (48 h) and oxidative stress and sex change after long-term (21 days) exposure (Zhang et al. 2020; Liu et al. 2021).

The aim of the present study is to take advantage of the full sequenced genome of *Ciona* to shed light on the effect of amino-modified PS NP (PS-NH_2) at the transcriptomic level on ascidian embryos. The Differentially Expressed Genes (DEGs) together with GO analysis and Pathways analysis will give insights into the molecular responses and into the mechanisms of toxicity of PS-NH_2.

3.2 Material and Methods

3.2.1 Amino-modified PS NP (PS-NH_2)

Unlabeled and blue fluorescently labeled (358 nm excitation, 410 nm emission) 50 nm amino-modified PS NPs (PS-NH_2) were purchased from Bangs Laboratories and Sigma, respectively. Stock solutions (10 mg mL^{-1}) were made in deionised water without any surfactants and stored at 4°C until use. Details about PS secondary characterization in MqW and NSW exposure media by DLS are reported in paragraph 2.2.1 of Chapter 2.

3.2.2 Animal Collection and Exposure Experiments

Adults specimens of *C. robusta* were collected in the Gulf of Taranto (Italy) by local fishermen between November 2019-December 2019. Organisms were transported in plastic bags filled with Natural Sea Water (NSW, salinity 40 ‰, pH 8) within a few hours in the aquarium of the Zoological Station Anton Dohrn of Naples. After an acclimation of 7 days, as described in the paragraphs 1.2.2 and 2.2.2 of the chapters 1 and 2, mature organisms were used for the embryotoxicity assay. Gametes were obtained from each individual by dissecting the gonoducts with a scalpel. To avoid the self-fertilization, oocytes and sperms were collected by distinct individuals. The oocytes were rinsed twice in 0.22 µm filtered NSW while dry sperm was pooled and stored on ice until fertilization. Fertilization was performed by adding diluted sperm (1:100 in NSW) to the egg's suspension. After 10 min of incubation on a rotating shaker, the fertilized eggs were transferred to tissue culture plates and further rinsed in 0.22 µm filtered NSW. About 1000 embryos were exposed to two different concentrations of PS-NH_2 (10 and 15 µg mL^{-1}) until they reached the late tailbuld I stage (~13 hpf). NSW was used as control. For each of the three experimental conditions, a total of three biological replicates were used for RNA isolation and quantification. The exposure conditions were chosen based on the results obtained through the embryotoxicity assay of chapter 2. Fluorescent PS-NH_2 were used for the detection of the NPs.

3.2.3 Total RNA isolation and Transcriptomic Sequencing

Total RNA was extracted and isolated using RNAqueous-micro kit (Ambion) according to the manufacturer's instructions. The quality and quantitative control analysis of the samples was performed with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The samples were considered acceptable for the sequencing only with the 260/280 ratio (nucleic acid integrity) >2 and RNA Integrity Number (RIN) values >8.5 (Schroeder et al. 2006).

3.2.4 RNA-Seq Data Processing and Analysis

Next generation sequencing experiments were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). RNA concentration in each sample was assayed with a ND-1000 spectrophotometer (NanoDrop) and its quality assessed with the TapeStation 4200 (Agilent Technologies). Indexed libraries were prepared from 500 ng/ea purified RNA with TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 (Agilent Technologies) and a Qubit fluorometer (Invitrogen Co.), then pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 2nM. The pooled samples were subject to cluster generation and sequencing using an Illumina NextSeq 500 System (Illumina) in a 2x75 paired-end format at a final concentration of 1.8 pmol.

The raw sequence files generated (.fastq files) and a cleaning step was performed with Trimmomatic (Bolger et al., 2014), setting a minimum Phred Quality Score of 20 and a minimum reads length of 30; no adaptors were found. The quality check control was performed by FastQc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)

3.2.5 Mapping and Count

All samples were mapped on the *C. robusta* genome (downloaded from NCBI, version GCF_000224145.3) using the STAR aligner (default settings, (Dobin et al. 2013). The mapped reads were counted per mRNA using featureCount (Liao et al. 2014), allowing overlapping reads (-O option).

3.2.6 Analysis of differentially expressed genes (DEGs)

For each sample, the RPKM (Reads Per Kilobase per Million) counts per transcript were calculated using an in-house R script. DEGs calling was performed using two software, which implement two

different statistical approaches: DESeq2 (Love et al. 2014) and edgeR (Robinson et al. 2010). For each transcript, the mean of the log2 fold change values (Log2FC) obtained with the two software was calculated. The thresholds used for the DEGs calling were FDR ≤ 0.05 or P-adjusted ≤ 0.05. Heatmaps of the Log2 RPKM and Log2 FC for DEGs were created using an in-house R script.

3.2.7 Validation of transcriptomic data by qRT-PCR

To validate the transcriptomic data, 10 genes were selected from the list of DEGs. Total RNA extraction and cDNA synthesis were obtained as described in chapter 1 and 2. Specific primers were designed using the tool Pick Primers of NCBI and the product was verified using the In-silico tool of GenomeBrowser. The primer sequences for the selected genes are listed in Table 3.1. The qRT-PCR was performed in MicroAmp Optical 384-Well reaction plate with Optical Adhesive Covers (Applied Biosystems) in a ViiA^{TM}7 Real Time PCR System (Applied Biosystems, Monza, Italy) thermal cycler using the following thermal profile: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s, 1 cycle for melting curve analysis (from 60 to 95 °C, reading every 0.5 °C) to verify the presence of a single product. All the reactions were carried out in triplicate and each assay included three negative controls with no template for each primer pair. Expression levels of target genes were normalized using cytoskeletal actin as reference gene (GenBank ID: AJ297725, Fujikawa et al. 2010). Actin-specific primers were: sense primer, 5′-CCCAAATCATGTTCGAAACC-3′; antisense primer, 5′-ACACCATCACCACTGTCGAA-3′. Fluorescence was analyzed with ViiA™ 7 Real-Time PCR software (Life Technologies) and then quantified according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on Ct values of each gene, and the Ct average of the selected reference gene, in order to calculate the relative mRNA expression level. The expression levels of the selected genes were evaluated in number-fold increase relative to the control condition that has been assigned as “1”. The statistical analyses were performed using GraphpadPRISM 6 software. Significance of the relative $2^{-\Delta\Delta Ct}$ of each group (biological replicates, N = 3), compared to the controls, was determined using ‘unpaired parametric t-test’.

Table 3.1. Accession number and/or Gene Model ID, sequences and length of PCR fragments are listed for the analysed genes.

Gene Name	Acronym	Accession Name and or Gene Model ID	Primer	Sequence (5′ → 3′)	Amplicon length (bp)
transcription factor protein (atf4/5)	atf4/5	NM_001078191.1	atf4-5Fw	CCGCAAACAGCGCCAAGTAG	211
			atf4-5Rv	CGAAACTGCTGGCTGGGGAA	
transcription factor protein (foxi-b)	foxi-b	NM_001078244.1	foxi-bFw	CGAGGACGACCCGGGAAAAG	135
			foxi-bRv	TCCTCCGGTCTGTCCGGTTT	
transcription factor protein (xbpd)	xbpd	NM_001078383.1	xbpdF1	GCGTTGTCCCCAGCAATAACC	165
			xbpdR1	AGTGTTCGGACGACTCGGGG	
transcription factor protein (xbpb)	xbpb	NM_001078381.1	xbpbFw	CGCGAATCTGCCCAGAGAGC	235
			xbpbRv	ACCTGTCATCCTCGGCCTCA	
zinc finger protein (zf(c2h2)-24)	zf(cc2h2)-24	NM_001078398.1	ZF24Fw	AACGCGGTCGACGATACTGG	216
			ZF24Rv	GGTGCCCAGTGTGTTTCCGA	

integumentary mucin C.1	muc-c1	XM_002127826.4	Muc-c1Fw	CGTGCAGGAGAGT CAACCCA	86
			Muc-c1Rv	ACAACATCTCCAA CGCAGGCA	
mucin-5AC	muc5	XM_018812660.2	muc5F1	GAGCCGCAGGTTC AAACGTAG	86
			muc5R1	TCTACCAAGGCAA GATGCTGATGT	
alpha-tocopherol transfer protein-like	TTPA	XM_002124890.4	TTPAFw	CGGGAGGACCAA AACTGGGT	204
			TTPARv	AGCTTGGACGATG GAGACAACG	
SCO-spondin	sco-spondin	XM_018815902.2 XM_018815903.2 XM_018815905.2	ssponFw	GTGGGTTGGAGTG CTTGGGG	115
			ssponRv	AGCAGACATCTCC AGGACAACTGA	
acetylcholinesterase -like	AChE	XM_018813116.2	AChEFw	CCCGCCTATGCCA GTACAGC	220
			AChERv	GCATTGCTAGAGG TTTGCGTTGT	

3.2.8 Functional Enrichment

GO enrichment analysis of DEGs was performed with Ontologizer software (Bauer et al. 2008). The threshold used to identify significantly enriched functional terms was $p \leq 0.05$.

A functional annotation of the analysed transcripts was performed with the software InterProScan (version 5.33) (Jones et al. 2014), retrieving the metabolic pathways assignment.

3.3 Results and Discussion

In recent years, several studies, using PS NPs as proxy for nanoplastics have been conducted with end points as survival, embryogenesis, immune system and behaviour and using a wide range of organisms including algae (Bergami et al. 2017; Bellingeri et al. 2020), echinoderms (Della Torre et al. 2014; Pinsino et al. 2017), bivalves (Balbi et al. 2017; Tallec et al. 2018) and fishes (Mattsson et al. 2015; Pitt et al. 2018; Duan et al. 2020). Nowadays, there is an increasing need to understand MoA of PS NPs and, up to now, just one study focused the attention in exploring the molecular pathways affected by PS NPs on the neonates of the crustacean *Daphnia pulex* showing (Liu et al. 2021). Through the transcriptomic approach, the authors demonstrated that the exposure to 1 mg L^{-1} of PS NPs (71.18 ± 6.03 nm) induced oxidative stress, immune defence and glycometabolism disorders in these crustacean neonates.

Transcriptomic analysis through RNAseq is a useful tool to better understand the mechanisms of action of chemicals. It is based on the study of the entirety of messenger RNA (mRNA) which are a mirror of the genes that are actively expressed in a cell or an organism at a given time. This in turn allows one to deduce how organisms respond to changes in the external environment.

3.3.1. DEGs among N10vsCTRL and N15vsCTRL

As shown in Table 3.2, the raw reads obtained ranged from 13.197.023 to 17.343.874, while more than 11.000.000 clean reads were obtained removing low quality reads and those one with adapter and undetermined bases. The DEGs resulting from the comparison of N10 vs CTRL and N15 vs CTRL are listed in the table 3.3. A total of 225 and 1214 were identified for *Ciona* embryos for N10 vs CTRL and N15 vs CTRL, respectively. Those one with a log2FC≥ |1.5| were 42 and 186 for N10 vs CTRL and N15 vs CTRL, respectively. The genes up- or- down-regulated were visualized on a heatmap (Figure 3.1) which correlates all the conditions tested (N10vsCTRL, N15vsCTRL, N10vsN15).

Table 3.2. Number of raw, cleaned and uniquely mapped reads on the *C. robusta* embryos exposed to PS NPs as follows: PS-NH_2 (10 μgL^{-1}, **N10**), PS-NH_2 (15 μgL^{-1}, **N15**) and control (NSW, **CTRL**) and their replicates.

Sample code	Exposure group	Replicate	Pair	Raw reads	Cleaned reads	Uniquely
1_S19_R1	CTRL	1	1	15.061.953	13.113.435	11.856.408
1_S19_R2	CTRL	1	2			
4_S22_R1	CTRL	2	1	14.356.341	12.500.567	11.459.016
4_S22_R2	CTRL	2	2			
7_S25_R1	CTRL	3	1	13.197.023	11.175.404	10.191.555
7_S25_R2	CTRL	3	2			
2_S20_R1	N10	1	1	17.343.874	14.348.872	12.995.224
2_S20_R2	N10	1	2			
5_S23_R1	N10	2	1	13.068.352	11.304.485	10.411.836
5_S23_R2	N10	2	2			
8_S26_R1	N10	3	1	14.253.648	12.151.061	11.050.205
8_S26_R2	N10	3	2			
3_S21_R1	N15	1	1	14.026.431	12.185.159	11.140.173
3_S21_R2	N15	1	2			
6_S24_R1	N15	2	1	14.726.150	12.696.322	11.677.958
6_S24_R2	N15	2	2			
9_S27_R1	N15	3	1	14.245.688	12.471.090	11.358.762
9_S27_R2	N15	3	2			

Table 3.3. Number of DEGs per comparison.

	N10vsCTRL		N15vsCTRL	
	Trans.	Genes	Trans.	Genes
ALL FC	335	225	2076	1214
LOG2 FC >= |1.5|	59	42	256	186

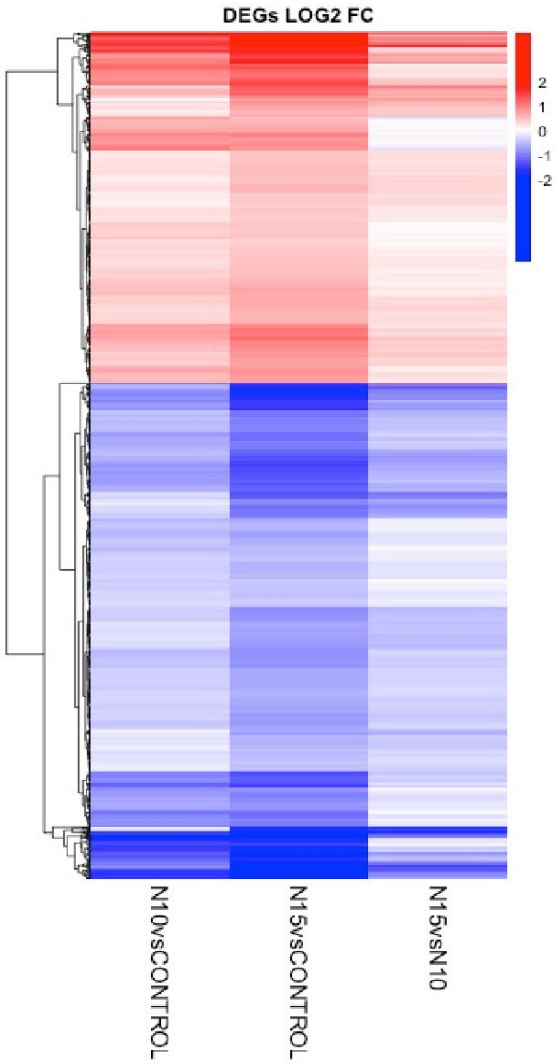

Figure 3.1. Heatmap of log2 FC of DEGs for all the conditions tested (N10vsCONTROL, N15vsCONTROL, N10vsN15). Red represents up-regulation and blue down-regulation.

3.3.2 Validation of transcriptomic results with qRT-PCR

Expression of atf4/5, foxi-b, xbpd, xbpb, zf(cc2h2)-24, integumentary mucin C.1 (muc-c1), muc5, TTPA, sco-spondin and AChE were tested by Real-Time qPCR. The data indicated an up-regulation of the genes atf4/5, foxi-b, xbpd, zf(cc2h2)-24, muc-c1, muc5, TTPA, while xbpb, sco-spondin and AChE resulted down-regulated (Figure 3.2B). These results are in line with the transcriptional response in *C. robusta* embryos indicated by RNA sequencing confirming the effects of PS-NH_2 (Figure 3.2 A).

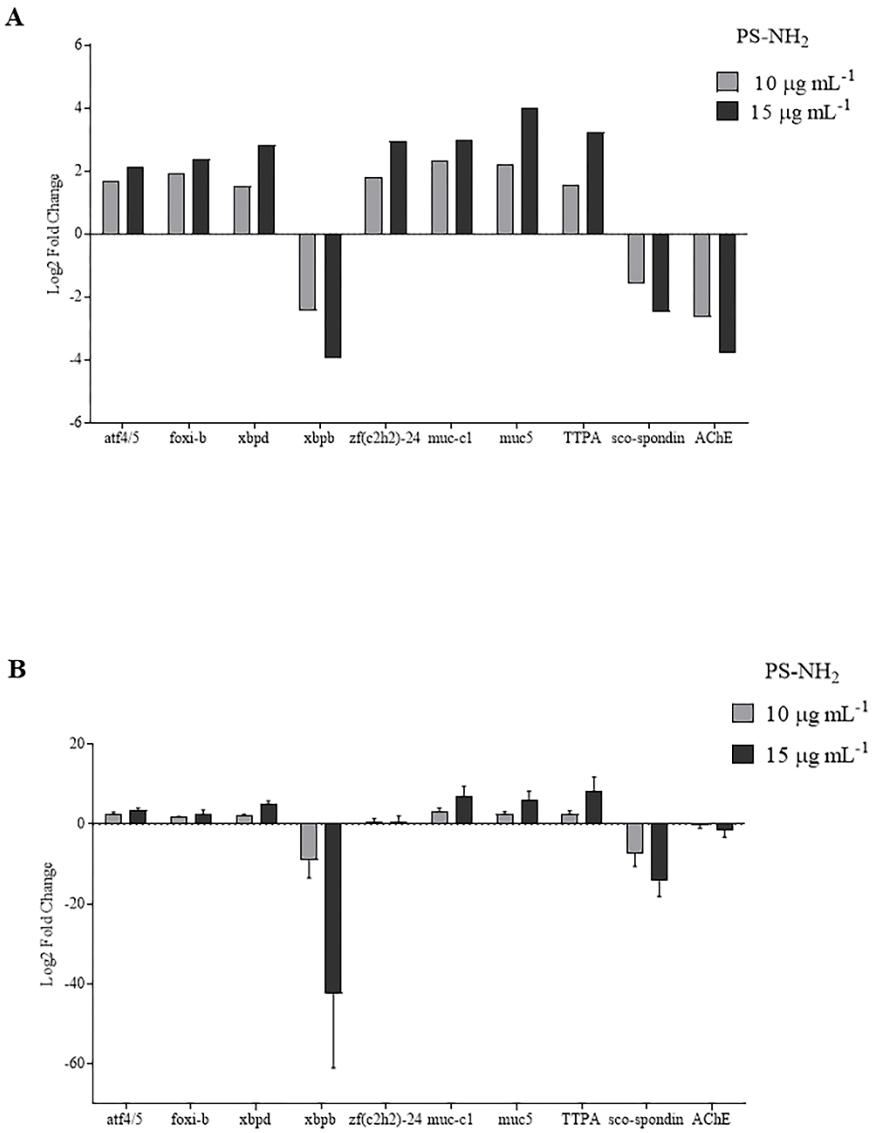

Figure 3.2. Log2 Fold Change of differentially expressed genes (DEGs) obtained by RNA sequencing (A) and Real-Time qPCR (B) from samples of *C. robusta* embryos exposed to 10 and 15 µg mL^{-1} of PS-NH_2. In A, all genes showed significant change with the threshold of $P < 0.05$ and fold change >1.5; in B, data are presented as mean± SEM.

3.3.3 Gene Functional Annotations

To process the transcriptome data into structured ontologies, all the DEGs were mapped into the three categories which describe the gene products in terms of their associated biological processes, cellular components, and molecular functions. Moreover, each category was divided into subclasses identifying, amongst them, 77 for biological processes, 34 for molecular functions and 4 for cellular components (Figure 3.3 and 3.4). The results of this analysis showed a dose-response relationship of the affected genes belonging to the different subclasses to increasing PS-NH_2 concentrations.

Among the biological processes affected in both conditions the following resulted up-regulated: (i) "biological regulation", (ii) "cellular biosynthetic pathway", (iii) "regulation of macromolecule biosynthetic process", (iv) "macromolecule biosynthetic process". On the contrary (i) "sulphur compound metabolic and biosynthetic processes", (ii) "glutathione metabolic and biosynthetic process" were down-regulated. Regarding molecular functions, (i) "organic cyclic compound binding", (ii) "heterocyclic compound binding", (iii) "DNA binding", (iv) "molecular function regulator" showed considerable up-regulation both in N10vsCTRL and N15vs CTRL, on the opposite, (i) "ligase activity" was down-regulated. As expected, the highest PS-NH_2 concentration (15 µg mL^{-1}) affected additional subclasses of genes for each category, thus resulting in a higher impact on embryo development. In particular, among the biological processes, the "metabolic processes of carbohydrate, monosaccharide, fructose" resulted up-regulated, while "response to stimulus", "cell communication and signal transduction" resulted down-regulated. In this condition, the cellular components mostly involved in counteracting PS-NH_2 treatment seem to be the "membrane" (up-regulated) and "cytoskeleton and myosin complex" (down-regulated).

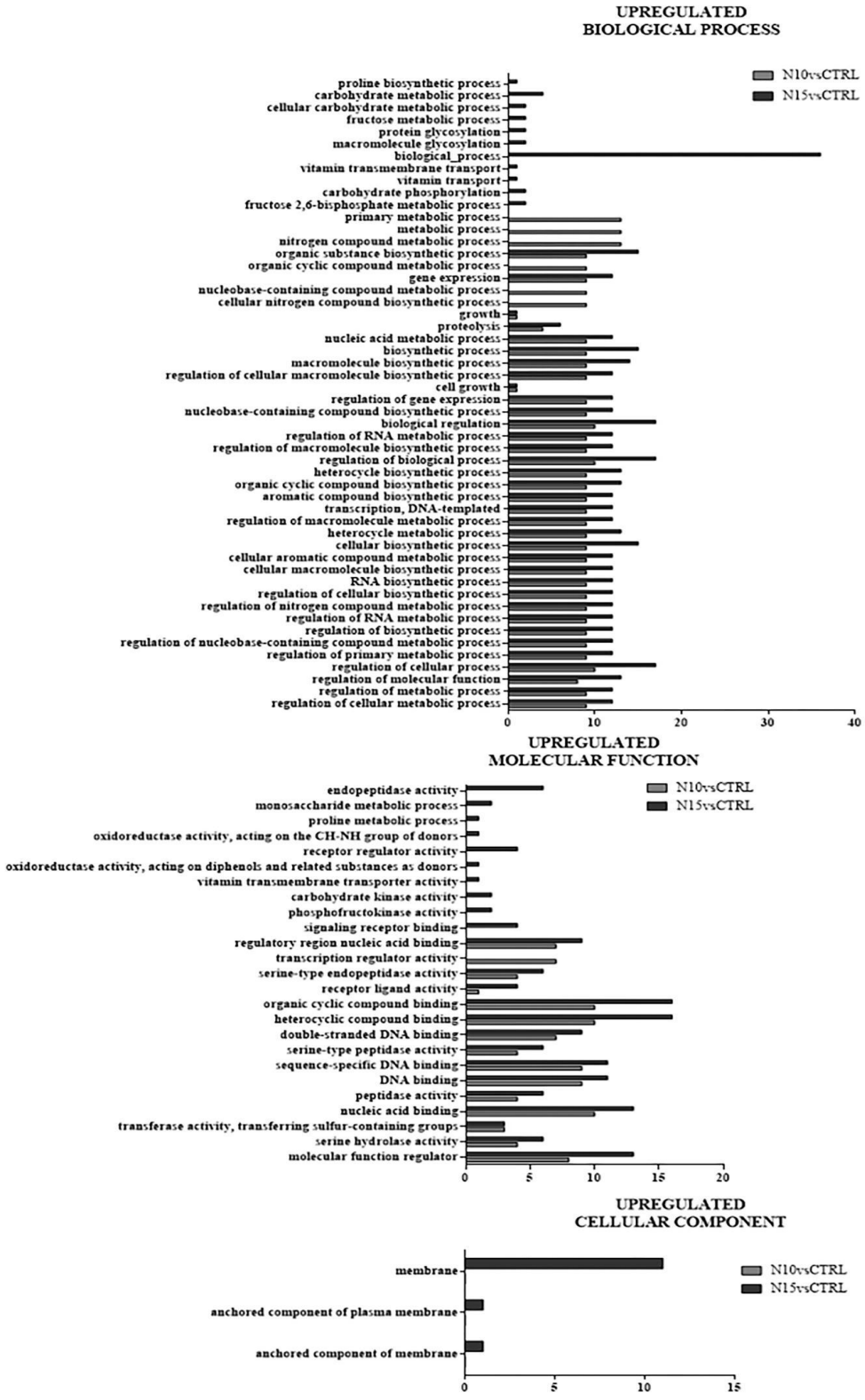

Figure 3.3. GO classification of DEGs late tailbuld embryos (13 hpf) of *C. robusta* exposed to PS-NH_2 (10 and 15 µg mL^{-1}). The overrepresented biological processes, molecular functions and cellular components are shown. Light grey and dark grey bars represent the number of up-regulated genes for each condition. The length of the bars is determined by the number of genes identified within each subcategory.

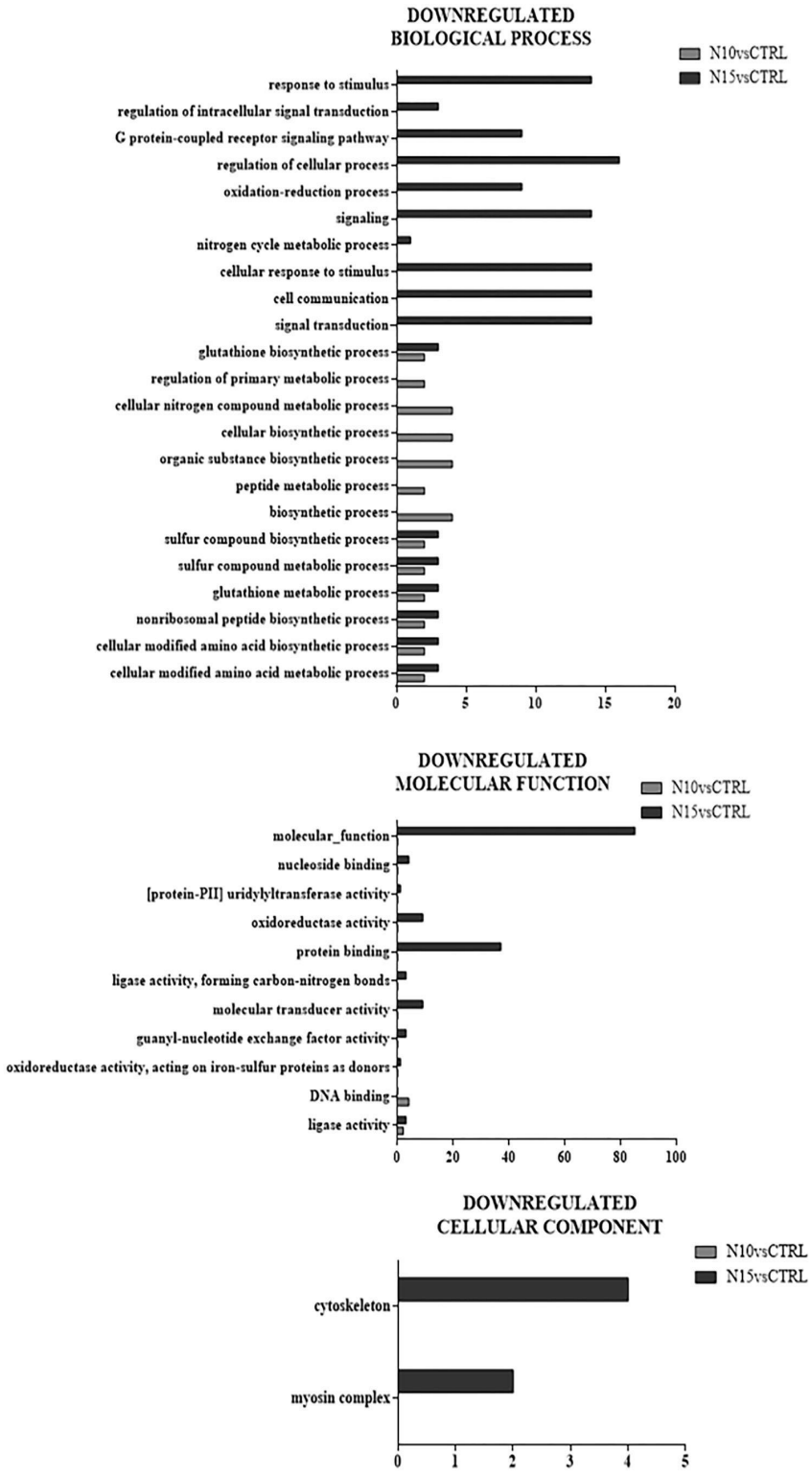

Figure 3.4. GO classification of DEGs late tailbuld embryos (13 hpf) of *C. robusta* exposed to PS-NH_2 (10 and 15 µg mL^{-1}). The underrepresented biological processes, molecular functions and cellular components are shown. Light grey and dark grey bars represent the number of down-regulated genes for each condition. The length of the bars is determined by the number of genes identified within each subcategory.

3.3.4 Pathways analysis

The analysis through the software InterProScan (version 5.33) indicated that the metabolic pathways significantly affected were "passive transport by aquaporins", "neurotransmitter clearance" and "glutathione synthesis and recycling" for both the N10vsCTRL and N15vsCTRL conditions (Table 3.4).

Table 3.4. List of the pathways affected by PS-NH_2 at both the concentrations tested (10 and 15 μgL^{-1}).

N10vsCTRL	N15vsCTRL
Passive transport by Aquaporins (REACTOME)	
Neurotransmitter clearance (REACTOME)	
Glutathione metabolism (KEGG)	

It is known that the exposure to PS NPs can cause the disruption of cell membrane and generate oxidative stress in different territories leading to increased levels of ROS (Liu et al. 2021; Wang et al. 2013; Bexiga et al. 2014; Bhattacharjee et al. 2013). Organisms and cells have evolved different ways to counteract the oxidative stress, such as modulation of ROS production, neutralization of produced ROS through free radical scavenging and repair or removal of the damaged structures (Davies 2000). This study revealed that the exposure of *Ciona* embryos to PS-NH_2 negatively alters the stress response pathway involved in glutathione metabolism, synthesis and recycling and drug metabolism, probably causing the disruption of the equilibrium between ROS production and defence. Glutathione pathway has a fundamental role in oxidative stress in marine organisms since it is the most abundant cytosolic scavenger, which directly neutralizes several reactive species through its oxidation to GSSG (Regoli and Giuliani 2014).

The oxidative stress condition could in turn affect the "neurotransmitter clearance" pathway, leading to the down-regulation of the Acetylcholinesterase (AChE) gene. In *Ciona*, the activity of AChE is present from unfertilized eggs to larval stage and it may play an important role for cell adhesiveness, proliferation, migration, differentiation, and secretion for the first developmental stages (Durante 1956; Mansueto et al. 2006) and later on neurotransmission (Meedel and Whittaker 1979; Takamura et al. 2010). Several studies reported that oxidative stress also plays a role in the gene regulation and

activity of AChE enzyme. For example, it has been reported that ethanol, which produces ROS, alters genetic expression and activity of AChE (Rico et al. 2007), while Schallreuter et al. (2004) identified activation/deactivation of human AChE by hydrogen peroxide (H_2O_2). One can suppose that the inability of the exposed embryos to counteract the oxidative stress condition, due to the impairment of the pathways involved in glutathione metabolism and drug metabolism, may have led to high levels of ROS which, in turn, could negatively regulate the transcription of AChE.

The involvement of "passive transports by aquaporins" pathway, in response to PS NPs treatments, appears very interesting. Aquaporins are a large family of membrane proteins with members in all kingdoms of life and are known as efficient water channels (Preston et al. 1992; Zardoya 2005). Up to now there is little information about the role of aquaporins in marine organisms during embryogenesis. Amaroli and colleagues (2013) studied the presence of these proteins on the vitelline layer and in the fertilization envelope of sea urchin *P. lividus* oocytes, suggesting their involvement in the appropriate homeostasis for embryo development (Amaroli et al. 2013). In our study the gene aquaporin-like resulted up-regulated at both N10vsCTRL and N15vsCTRL conditions and this dysregulation could be related to a need to restore the normal osmotic conditions under stress conditions.

The exposure of embryos to 15 μg mL^{-1} also affected additional pathways controlling the energy metabolism (Table 3.5). In particular, genes involved in fructose and mannose metabolism, starch and sucrose metabolism and glycolysis were up-regulated in *Ciona* embryos. This could be due to a growing need of energy to counteract the toxicity of PS-NH_2. Interestingly, the same metabolic pathways were affected in *Daphnia pulex* neonates exposed to 70 nm PS NPs, showing a glycometabolism change at the transcriptomic level (Liu et al. 2021).

Table 3.5. List of the pathways affected by 15 μg mL^{-1} of PS-NH_2.

N15vsCTRL
Glutathione metabolism (KEGG)
Citrate cycle (TCA cycle) (KEGG)
Fructose and mannose metabolism (KEGG); Glycolysis (REACTOME)
Starch and sucrose metabolism (KEGG)
Glutathione synthesis and recycling (REACTOME)

All the affected pathways appear to be related to a hypoxic condition caused by PS-NH_2 exposure. In fact during hypoxia, the production of free radicals at rates that exceed the scavenging capacity of antioxidant enzymes lead to a reduced activity of the antioxidant defence system (Giordano et al. 2008; Dong et al. 2013; Feng et al. 2016). Moreover, a hypoxic condition could also induce the activation of energy metabolism pathways to allow cell survival (Malthankar-Phatak et al., 2008; Goda and Kanai, 2012). The involvement of the aquaporin pathway seems to reinforce the hypothesis of a hypoxic condition since aquaporins are involved also in gas permeation (CO_2, NO and O_2) through the plasma membrane and their up-regulation has been already demonstrated under hypoxic condition in cell lines and animal tissues (Nakhoul et al. 1998; Herrera et al. 2006; Musa-Aziz et al. 2009; Echevarría et al. 2007; Abreu-Rodríguez et al. 2011). One can suppose that the hypoxic environment is caused by PS-NH_2 aggregates around the egg envelopes of *Ciona* embryos, as already highlighted in Chapter 2, thus inducing the alteration of the pathways described above. Actually, dense aggregates, localized all around the egg envelopes (Figure 3.5 C, D, E, F) surrounding *Ciona* embryos, have been detected using 50 nm fluorescent PS-NH_2 (10 and 15 μg mL^{-1}) with no visible fluorescence inside the embryos. However, these are preliminary results and further analyses are needed to better define if PS-NH_2 are able or not to pass through *Ciona* egg envelopes, in order to figure out their mode of action.

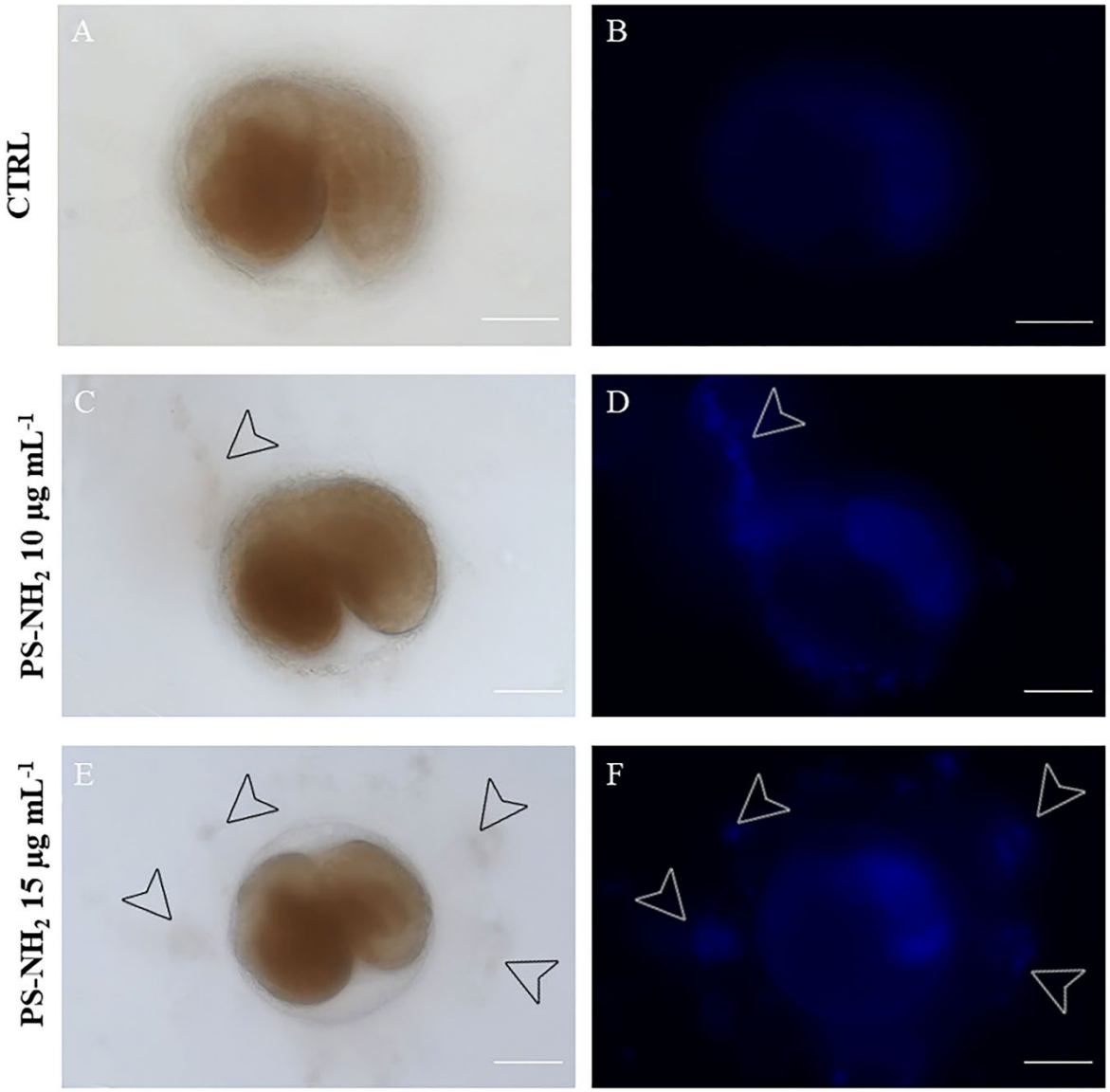

Figure 3.5. Optical fluorescence microscopy (Zeiss Axioscope; DAPI filter 365/445) showing the disposition on *Ciona* egg envelopes after an incubation of 13 h with fluorescent PS-NH_2. (A) Brightfield image of control; (B) Fluorescence image of control; (C) Brightfield image of embryo exposed to 10 µg mL^{-1}; (D) Fluorescence image of embryo exposed to 10 µg mL^{-1}; (E) Brightfield image of embryo exposed to 15 µg mL^{-1}; Fluorescence image of embryo exposed to 15 µg mL^{-1}. Scale bar= 50 µm.

In summary, the data obtained through the transcriptomic analysis of *Ciona* late tailbuld I exposed to PS-NH_2 showed an impairment of the pathways involved in different processes as "glutathione metabolism", "passive transport by aquaporins", "neurotransmitter clearance" and "energy

metabolism" probably due to indirect hypoxic conditions caused by the dense coating formed by PS-NH_2 surrounding *Ciona* egg envelopes as summarized in Figure 3.6.

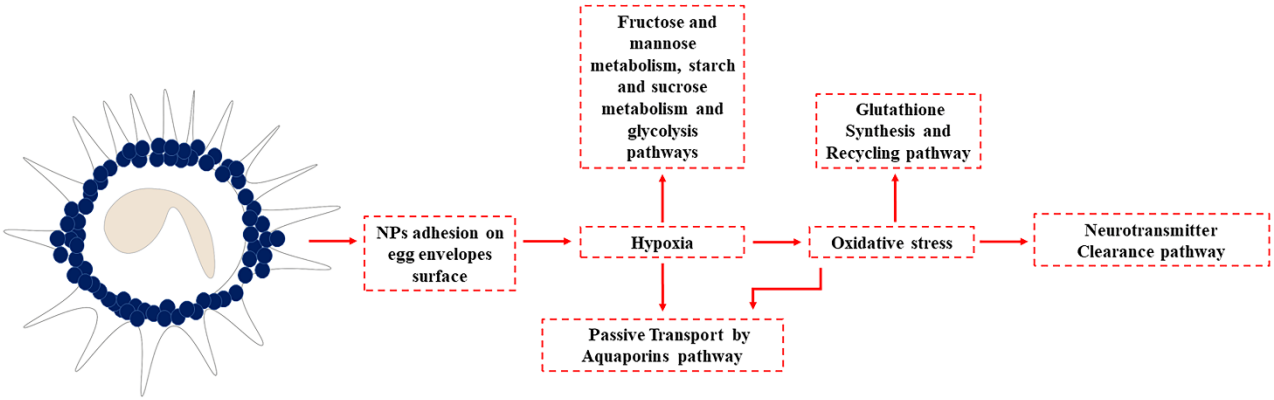

Figure 3.6. Biological responses of *C. robusta* late tailbuld I embryos exposed to PS-NH_2.The dense coating of PS NPs could have led to a hypoxic microenvironment probably altering the "passive transport by aquaporins" pathway. Moreover, the hypoxia conditions could have induced a high oxidative stress, altering "glutathione synthesis and recycling pathway", "neurotransmitter clearance pathway" and "fructose and mannose metabolism", "starch and sucrose metabolism" and "glycolysis" pathways.

3.4 Conclusions

This study investigated the effects of PS-NH_2 (10 and 15 µg mL^{-1}) at transcriptomic level on ascidian embryos, taking advantage of the full sequenced genome of *Ciona*.

Several genes resulted dysregulated upon the exposure to PS-NH_2, while the GO analysis, which classified genes in three different subclasses, revealed that the number of affected genes, belonging to different subclasses, have a dose-response relationship with the concentration tested. Going deeply into the bioinformatic analysis, "glutathione synthesis and recycling pathway", "neurotransmitter clearance pathway", "passive transport by aquaporins" and "fructose and mannose metabolism", "starch and sucrose metabolism" and "glycolysis" pathways resulted affected.

The alteration of these pathways could be related to the hypoxic microenvironment due to the dense coating of PS-NH_2 around the egg envelopes of *Ciona* embryos.

Overall, the transcriptomic approach is revealing very useful to shed light on the toxicodynamics of PS-NH_2.

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CHAPTER 4

Morphometric analysis of the teratogenic effects of amino-modified polystyrene nanoparticles, PS-NH_2, on embryos of the ascidian *Phallusia mammillata*

ABSTRACT

In this chapter, the effects of PS-NH_2 on the embryogenesis of the ascidian *Phallusia mammillata* were investigated, focusing the attention on the percentage of normal developed larvae and analyzing the phenotype. All the results were compared with those one achieved for *C. robusta*. Further, the use of the in-house software Toxicosis8 allowed the morphometric analysis of *P. mammillata* larval phenotypes and the quantification assessment of embryonic malformations. PS-NH_2 affected *P. mammillata* embryogenesis in dose-dependent manner, showing an EC_{50} value (22 h) of 6.26 µg mL^{-1}, also inducing phenotype malformations and hatching impairment. These preliminary findings are comparable between the two ascidian species, showing a similar sensitivity towards PS-NH_2 and similar morphological alterations with embryos showing round trunk and trapped into the egg envelopes. The morphometric analysis confirmed the lack of palps protrusion, the reduced length/width ratio (L/W) of the trunk, suggesting an impairment of both peripheral and central nervous system induced by PS-NH_2.

4.1 Introduction

During the third year of my PhD, from January 2020 to April 2020 I was a guest in the laboratory of Dr. Rémi Dumollard (Ascidian BioCell group) at the Institut de la mer de Villefranche (France). This collaboration aimed at studying the effects of PS-NH_2 NPs on the embryogenesis of the ascidian *P. mammillata* by exploring any "common phenotypic malformation" shared between the two ascidian species.

The ascidian *P. mammillata* (Figure 4.1) is found exclusively in the Northeast Atlantic and the Mediterranean. It is hermaphrodite although it is spawning from February to October, it is now possible to be maintained in the laboratory and collect eggs and sperms all year-round (Yasuo and McDougall 2018). It is an emerging ascidian model for developmental and molecular biology studies (i.e. live cell imaging) since eggs and embryos are completely transparent (Zalokar and Sardet 1984) and translate proteins from injected exogenous mRNAs (Prodon et al. 2010). In addition, genomic and transcriptomic resources are available thus facilitating functional gene studies (https://www.aniseed.cnrs.fr/).

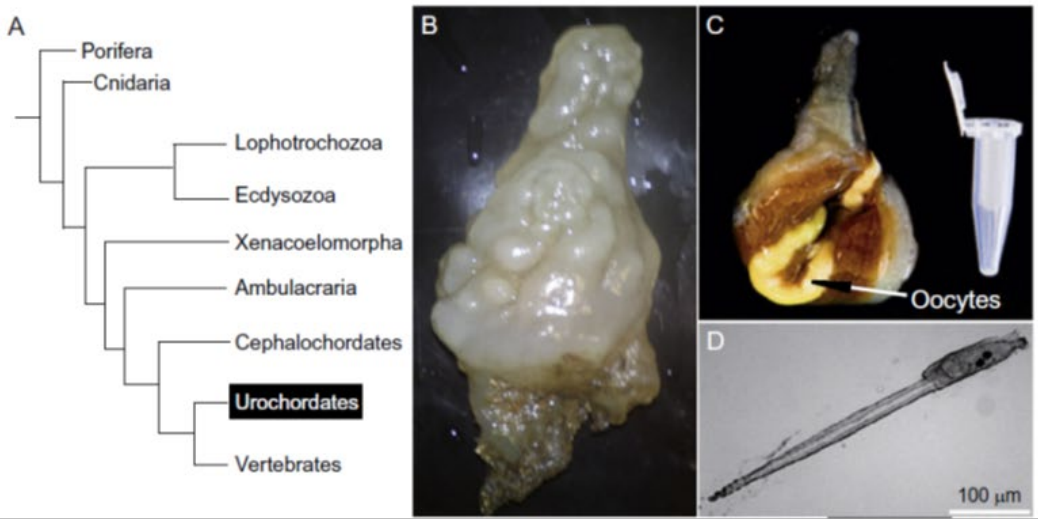

Figure 4.1. *Phallusia mammillata* model. (A) It is a nonvertebrate deuterostome and belongs to the urochordates or tunicates. (B) Adult form of *P. mammillata* surrounded by a tunic that provides protection. (C) Once the tunic is removed, the oocytes can be easily seen, while the sprmiduct is obscured by the oocytes in the oviduct. (D) Swimming larvae about 22 h after fertilization (adapted from McDougall et al., 2012).

More recently, *Phallusia* has been used in ecotoxicology for testing embryotoxicity of several CECs as Bisphenol A (BPA), Imazalil, Triadimefon and Fluconazole both at morphological and molecular levels (Pennati et al. 2006; Messinetti et al. 2016, 2017, 2018; Groppelli et al. 2007; Gomes et al. 2019).

The hosting team, and in particular Dr. Dumollard, has experience on different aspects of ascidians embryonic development for almost 20 years, from maternal to zygotic transition to, more recently, brain development (McDougall et al. 2012; Gomes et al. 2019), devising protocols for 3D time-lapse imaging and somatic transgenesis and for large-scale culture and semi-automated phenotyping, using an in-house software (Toxicosis8). This software has been developed to perform a morphometric analysis of larval phenotypes and to quantitatively assess embryonic malformations. It provides different morphological endpoints of ascidian larva, as the distance between Ocellus (Oc) and Otolith (Ot) (Oc/Ot distance) and the total area of pigment cells (PCs) (Oc+Ot area), in order to depict the development of the central nervous system. The presence/absence of palps is used as a marker of peripheral nervous system development. The trunk length to width ratio (trunk L/W) and tail length reflect general morphogenesis of the embryo (Figure 4.2; from Gazo et al, 2020 under revision in Aquatic Toxicology).

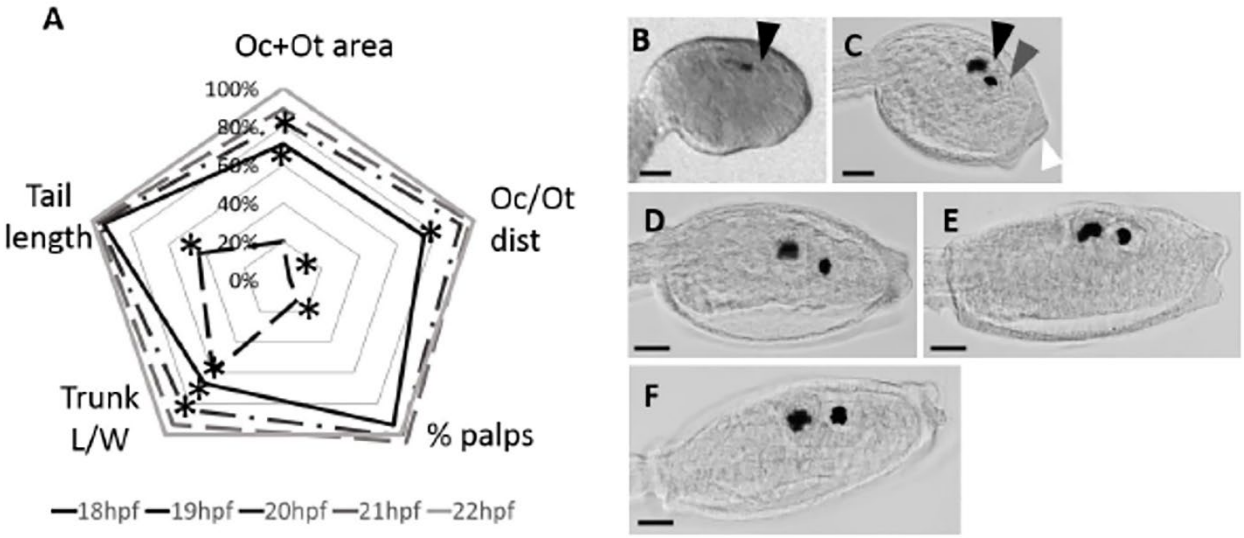

Figure 4.2. (A) Radar chart representing the 5 morphological endpoints during embryonic development (from stage 22 to stage 26). On the left the images of trunk development of (B) stage 22; (C) stage 23; (D) stage 24; (E) stage 25; (F) stage 26 (from Gazo et al, 2020 under revision in Aquatic Toxicology).

The aim of this study was to analyze the effects of by PS-NH_2 NPs on *Phallusia* embryogenesis and compare with those observed in *Ciona* as shown in Chapter 2. Moreover, *Phallusia* embryo malformations were quantified through the Toxicosis8 software to better characterize the phenotypes.

4.2 Materials and Methods

4.2.1 Animal and gametes collection

Adults of *P. mammillata* were collected in Sète (Hérault, France), and kept at 17±1ºC in circulating seawater aquaria. They were reared under constant light conditions to avoid uncontrolled spawning of eggs and sperm (Lambert and Brandt 1967). Gametes were obtained dissecting the gonoducts of several individuals. The sperm was stored at 4°C until use, while oocytes were collected in tissue culture plates and rinsed twice in 0.22 µm filtered NSW. Fertilization was performed adding diluted sperm (1:100 in NSW) to eggs suspension. After 10 min of incubation, eggs were rinsed 3 times with 0.22 µm filtered NSW.

4.2.2 Embryotoxicity

To perform the comparative analysis between the two ascidian species, *Phallusia* embryos were exposed to the same experimental conditions as those used for *Ciona* (chapter 2 paragraph 2.2.3). About 1 h 30 min post-fertilization (hpf), 60 embryos (~two-cell stage) were added to 6-well plates and exposed to PS-NH_2 NPs suspensions in NSW (2-5-7.5-10-15 µg mL^{-1}) and incubated in static conditions in the dark at 18°C until the free swimming larva stage about 22 hpf. Larvae were then fixed in 4% paraformaldehyde (4% PFA, 0.5 M NaCl, PBS; Sigma), washed 3 times in Phosphate Buffered Saline (PBS 1X) and imaged by transmitted light microscopy (Zeiss Axiovert 200) at 10x magnification. Each experiment was replicated 3 times. The percentage of normal hatched larvae was calculated following the protocol described in section 2.2.3. As described in Gomes and collaborators (2019), a *Phallusia* larva is considered normal when showing a good general embryo morphology, with proper trunk and palps formation, as well as tail elongation.

4.2.3 Embryo's morphology

Larvae phenotypes were imaged using Zeiss Axiovert 200 and classified for simplicity in *mild*, *severe* and *not developed*. For the morphometric analysis, bright-field images (Zeiss Axiovert 200) were analyzed with the in-house software Toxicosis8, developed in Dr. Dumollard laboratory (software is

deposited for protection by the SATT Lutech). At least 50 tadpoles were analyzed per each treatment. The five endpoints quantified were: the area of each PC (Oc/Ot area, μm^2), the distance between the two PCs (Oc/Ot distance, μm), the L/W ratio of the trunk and the length of the tail were measured. The resulting data was normalized to each respective control treatment (100%) and plotted in radar charts for a better comparison of the phenotypes between treatments.

4.2.4 Statistical analysis

All the statistical analyses were performed using Graphpad Prism 6. All data were expressed as mean ± standard deviation (SD). The median effective concentration (EC_{50}), corresponding to a 50% reduction of normal hatched larvae, was calculated using a sigmoidal dose−response model according to the equation: $y=b+(a–b) / 1+10^{(Log\ EC50 – x)}$ where y is response, b response minimum, a response maximum, x the logarithm of effect concentration and EC_{50} the concentration of effect giving 50% of maximum effect. Data were normalized to the control mean percentage of larval abnormality using Abbot's formula:

$$P= (P_e\text{-}P_c/100\text{-}P_c)\cdot 100$$

Where P_c and P_e are the control and the experimental percentages of response, respectively.

Data from the embryotoxicity assay and for the analyses of the morphological alterations are representative of at least three independent experiments and were analyzed using the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test.

Data from the morphometric analyses were analyzed using the t-test and were representative of at least three independent experiments.

4.3. Results and Discussion

4.3.1 Embryotoxicity

As shown in figure 4.3, PS-NH_2 NPs significantly affected in a dose-dependent manner the normal development of *Phallusia* embryos with an EC_{50} value of 6.256 μg mL^{-1} with 95% Confidence Interval from 5.91 to 6.61 μg mL^{-1} (Figure 4.3 A). This data indicates that *P. mammillata* is slightly more sensitive than *C. robusta*, which showed an EC_{50} value of 7.52 μg mL^{-1} (Figure 4.3 B). As in the case of *Ciona,* also the ascidian *Phallusia* resulted less sensitive compared to other marine

invertebrates, as the sea urchin *P. lividus* (EC_{50} 2.61 µg mL^{-1}, Della Torre et al., 2014), the mussel *M. galloprovincialis* (0.14 µg mL^{-1}, Balbi et al., 2017) and the oyster *C. gigas* (0.16 µg mL^{-1}, Tallec et al., 2018).

Phallusia embryos, as *Ciona*, are enclosed in the egg envelopes formed by a rigid acellular envelope (vitelline coat), with vacuolated follicle cells attached to the outer surface and test cells to the inner surface (Honegger 1986). As largely discussed in Chapter 2 paragraph 2.3.2, this envelope could provide a great protection to developing embryos towards the exposure to PS-NH_2 NPs probably acting as physical barrier.

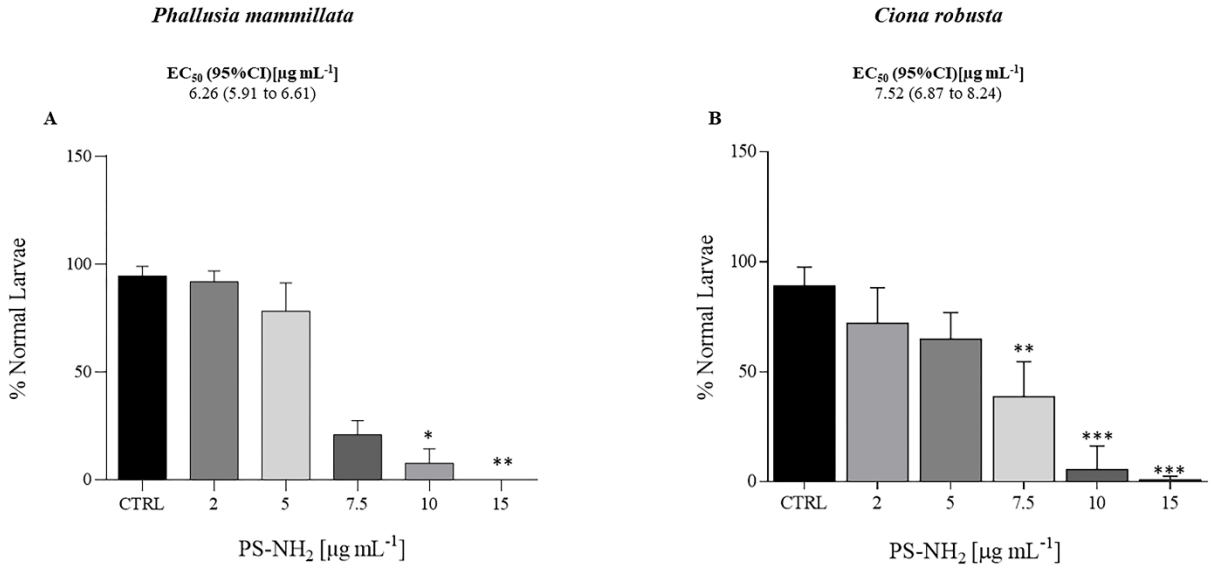

Figure 4.3. Percentage (%) of normal hatched larvae of *P. mammillata* (A) and *C. robusta* (B) upon exposure to PS-NH_2 in NSW for 22h. Bars represent mean ± SD. Asterisks indicate values that are significantly different compared to the control (Kruskal-Wallis test, Dunn's post hoc test, *p<0.05; **p<0.01, ***p<0.001). When available, EC_{50} values have been shown.

4.3.2 Embryo morphology analysis

Similarly to what observed for *Ciona,* alterated phenotypes was observed in *Phallusia* embryos. As shown in figure 4.4B, only embryos exposed to 5 µg mL^{-1} of PS-NH_2 showed morphology similar to controls (Figure 4.4 A and B). In those exposed to 7.5 µg mL^{-1}, the palps area started to be affected, with the lack of protrusion of the palps in agreement with what observed in *Ciona*. Upon the exposure to 10 and 15 µg mL^{-1} of PS-NH_2, the embryos failed to hatch, showing malformations at both trunk

and tail level. In fact, as shown in figure 4.4 D and E, the trunk resulted rounder and the tail shorter compared to the control (Figure 4.4 D-E).

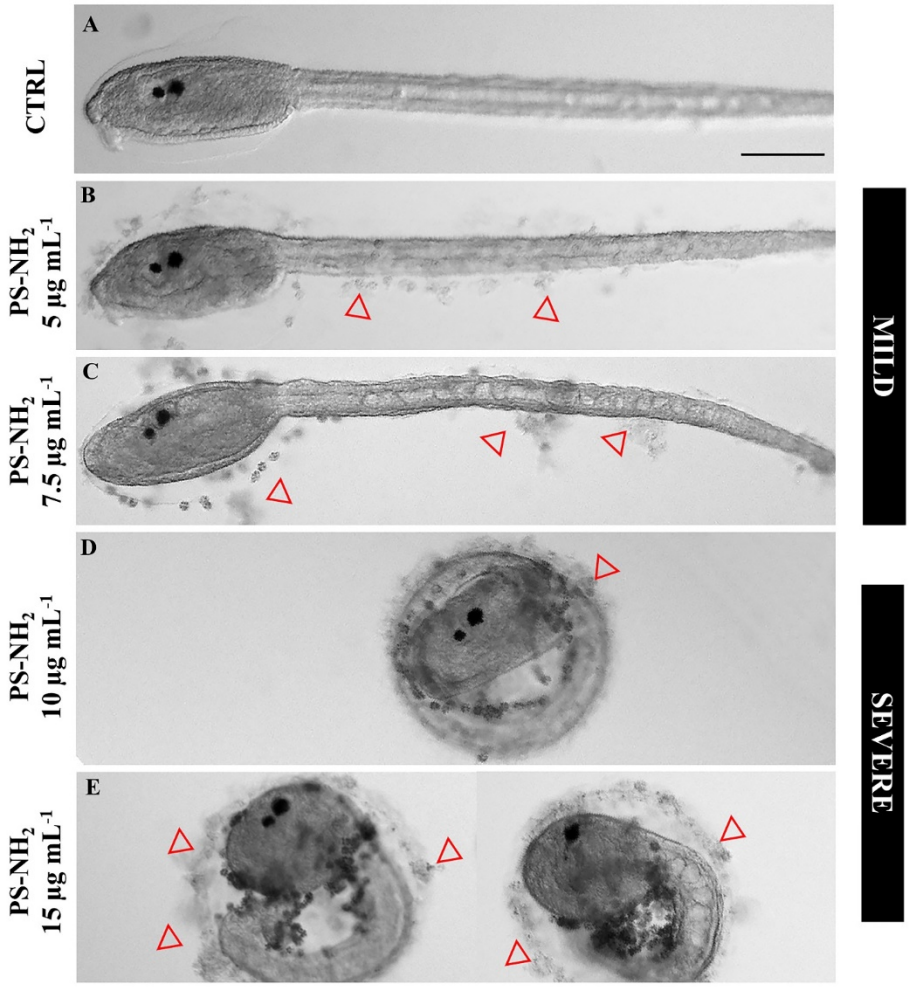

Figure 4.4. Light microscopy images of *P. mammillata* embryos exposed for 22 h to PS-NH_2: (A) Control; (B) 5 µg mL^{-1}; (C) 7.5 µg mL^{-1}; (D) 10 µg mL^{-1}; (E) 15 µg mL^{-1}. Red harrows indicate PS-NH_2 aggregates. Scale bar: 100 µm

The percentage of *mild*, *severe* and *not developed Phallusia* embryos has been characterized for each condition and compared to *Ciona* (Figure 4.5). Although phenotypes of both ascidians resulted similar, the percentage of *Phallusia* embryos showing the severe phenotype was higher compared to *Ciona,* thus indicating a greater impact on *Phallusia* embryogenesis than *Ciona* by PS-NH_2 NP exposure. In particular, at 5 and 7.5 µg mL^{-1}, 20% and 28% of embryos showed a *mild* phenotype, respectively, while the incidence of embryos showing the severe phenotype increased in a dose-

response manner, reaching 96% at 15 µg mL^{-1}, with embryos enclosed in the egg envelopes and unable to hatch (Figure 4.5).

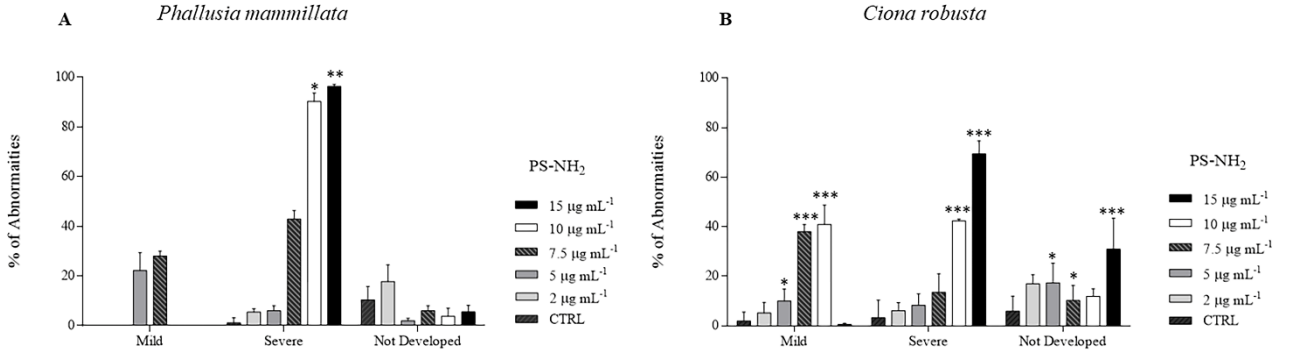

Figure 4.5. Percentage (%) of *mild*, *severe* and *not developed* of *P. mammillata* embryos upon exposure to PS-NH_2 (A). The results have been compared to the % of abnormalities analyzed in *Ciona* embryos (B) already reported in chapter 2, section 2.3.2.

The comparative analyses showed that PS-NH_2 NPs caused an inhibition of hatching in both ascidian species at the highest concentrations tested. As previously discussed in Chapter 2, hatching impairment has been observed also in zebrafish embryos exposed to 100 nm PS NPs. Authors showed that PS NPs (100 nm) could form a dense coating around the chorion after 24 hpf, almost blocking their uptake and leading to a hypoxic microenvironment in the inner space of the chorion that in turn damaged the antioxidant system of the organism (Duan et al., 2020). Indeed, in our study we observed the formation of dense aggregates around the egg envelopes in both acidians (Figure 4.4 E, F). This data suggests that, as for *Ciona*, PS-NH_2 aggregates might cause an internal hypoxic microenvironment for embryos, resulting in the inhibition of hatching and affecting also larval development. However, the possible uptake of PS-NH_2 NPs and their direct interaction with *Phallusia* developing embryos cannot be excluded.

Thanks to the software Toxicosis8 a quantitative analysis of five endpoints has been performed in *Phallusia* embryos. The pigment cell area (Oc/Ot area), the distance between pigment cells (Oc/Ot distance), the percentage (%) of embryos with palps, the length and width of the trunk (Trunk L/W ratio), and tail length have been measured and represented in a radar chart which shows simultaneously the five endpoints analyzed (Figure 4.6). The presence of palps, Oc/Ot distance and Oc/Ot area are three neurodevelopmental endpoints, while trunk L/W ratio and tail length represent two endpoints for general morphology, indicating non-specific toxicity probably associated with DNA aberration or a delay in development. This is an objective method to score the endpoints and

more reproducible compared to the observation under the microscope alone. Moreover, the shape of a radar chart, which reflects the most affected endpoints, could help in grouping different compounds based on the type of toxicity and targets (Gazo et al., 2020 in revision in Aquatic Toxicology).

In *Phallusia* embryos exposed to PS-NH_2, the Oc/Ot area was the only endpoint that was not altered, while all the other endpoints were significantly affected compared to controls. In particular, at the highest concentration tested (15 µg mL^{-1}), the percentage of embryos with palps was reduced to 1.8%, the Oc/Ot distance was reduced to 25%, the tail length was reduced to 51%, and the trunk L/W ratio was reduced to 67%. The results obtained through the morphometric analysis, and, in particular, the absence of palps and the reduced Oc/Ot distance seem to indicate an impairment of both peripheral and central nervous system, suggesting a neurodevelopmental toxicity exerted by PS-NH_2. These data are in line with those one present in literature, in fact PS NPs caused behavioral and neural damages at cholinergic and GABAergic neurons in *Caenorhabditis elegans* (Lei et al., 2020), and altered larval behavior, as evidenced by swimming hypoactivity in exposed zebrafish larvae (Pitt et al., 2018), probably caused by PS NPs uptake through tissues. However, due to the lack of knowledge on the potential neurotoxicity of PS NPs, further studies are needed to characterize their mechanisms of neurotoxicity.

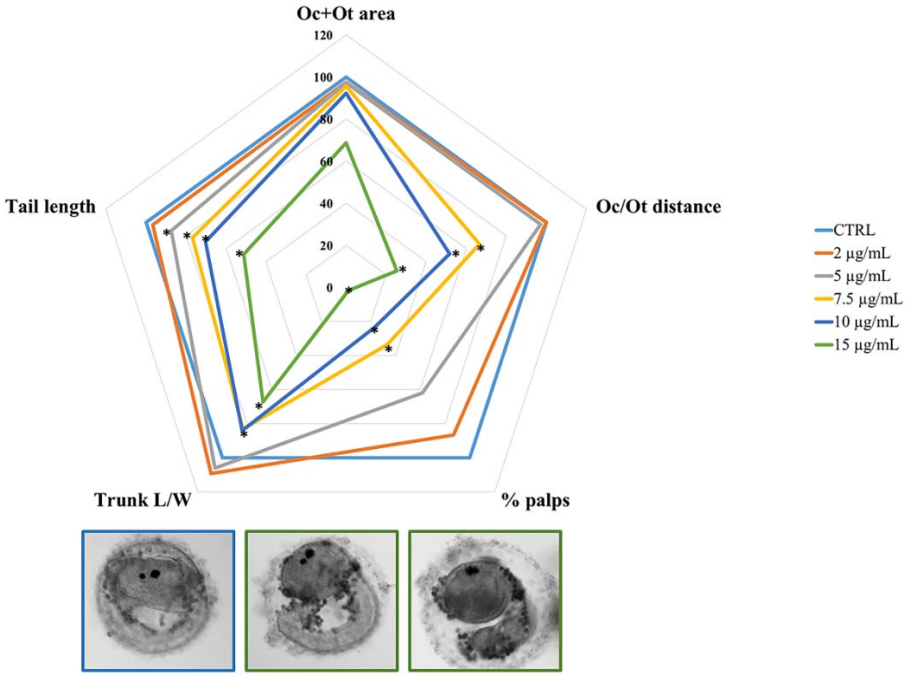

Figure 4.6. Quantitative analysis of morphological features of *Phallusia* embryos upon exposure to PS-NH_2. The following endpoints were measured: the PCs (Oc/Ot area), the distance between PCs (Oc/Ot distance), the percentage

(%) of embryos with palps, the length and width of the trunk (Trunk L/W ratio), and tail length. Graphs are normalized to the control. *p value<0.05.

4.4 Conclusions

This study was aimed at studying the effect exerted by PS-NH_2 NPs on the embryogenesis of the ascidian *P. mammillata* and exploring any "common phenotypic effect" shared with *Ciona* embryos. After 22 hpf, PS-NH_2 NPs affected the normal development of *Phallusia*, inducing alterations in larval morphology and inhibition of hatching. These results are in line and comparable with those obtained on *Ciona*, with embryos showing round trunk and trapped into the egg envelopes. In *Phallusia* embryos, the malformations have been quantified, confirming the lack of palps protrusion and the reduced L/W ratio. Based on the results obtained through the RNA-seq on *Ciona* tadpoles, the differentially expressed genes identified in *Ciona* will be searched for their orthologs in *Phallusia* databases. Future studies on the selected orthologous genes, by in Situ Hybridization and Real-Time qPCR experiments, will then permit to assess any "conservation" in the toxicodynamics exerted by PS-NH_2 during ascidian embryogenesis.

Acknowledgements

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Conclusions and Future Research Perspective

- The embryotoxicity assay performed using *C. robusta* indicated a different toxicity exerted by two dispersants, named A and B, as revealed by phenotypic and molecular analyses. The data showed the teratogenic power of dispersant B and the less toxic effects of dispersant A on the embryogenesis of *Ciona*.

- Future studies should include the use of *Ciona* embryotoxicity assay as a valuable tool for determining the impact of dispersants on the biological performance of marine species.

- Exposure to PS NPs revealed a different toxicity on the embryogenesis of *C. robusta* based on their different functionalization: PS-COOH NPs did not alter the normal larval development up to 100 µg mL^{-1}, while PS-NH_2 NPs strongly interfered with *C. robusta* embryogenesis, compromising the hatching and the normal development of the trunk of the larvae.

- PS-NH_2 were able to induce an increased production of ROS, localized at trunk structures (brain vesicle and visceral ganglion), and altered larvae swimming behaviour.

- At molecular level, PS-NH_2 induced the down-regulation of the typical genes involved in stress response (HSP60, HSP70, MnSOD, p38 mapk, caspase8), confirming that the increased level of ROS are due to the inability of the organism to counteract the stress condition.

- Exposure to PS-NH_2 during *C. robusta* embryogenesis induced the dysregulation of several genes and the alteration of the pathways involved in (i) “glutathione synthesis and recycling”, (ii) “neurotransmitter clearance” (iii) “passive transport by aquaporins”, (iv) “fructose and mannose metabolism”, (v) “starch and sucrose metabolism” (vi) “glycolysis”.

- The embryotoxicity assay performed using another ascidian species, *P. mammillata*, indicated that PS-NH_2 NPs induce similar morphological alterations during ascidian embryogenesis, with larvae of both species showing round trunk and unable to hatch.

- The use of the software Toxicosis8, developed for the morphometric analysis of *P. mammillata* phenotypes and for quantifying the alterations, confirmed the absence of the palps and a reduced Length/Width ratio of the trunk, two endpoints used for the characterization of peripheral and central nervous system development, suggesting a neurotoxicity effect exerted by PS-NH_2.

- Collectively, the data revealed interesting information on the toxycodinamics of PS-NH_2 during ascidian embryogenesis. Future studies, currently ongoing, on deep analysis of RNA-seq data, will hopefully contribute to give insights on the MoA of PS-NH_2.

- However, due to the lack of an effective method to quantify NPs, the environmental concentration is still uncertain. To date, environmental concentrations of NPs are estimated in the range between pg L^{-1} - µg L^{-1}, thus the concentrations tested in this study may be representative of a future worst-case scenario in Mediterranean coastal areas. Therefore, the results obtained in the PhD thesis are useful to understand the impact and the toxicological effects on aquatic organisms and to improve our knowledge for environmental risk assessment purposes.

Supplementary Material

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

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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 96h ($96hLC_{50}$) for Dispersant 1 (non-ionic surfactant) and for Dispersant 2 (mixture of non-ionic surfactants and anionic surfactants) is 41.6 (38.6-44.9) mg/L and 92.5 (87.7-97.5) mg/L

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 96h mortality test bioassay could be a good alternative method to 96h mortality assay with *D. labrax*, limiting the use of vertebrates for dispersants toxicity.

Key-words
Oil spill management, dispersants, toxicity, alternative biological pattern, ascidian

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 et al., 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), that have to be also Reproducible, ecological 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 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); 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 hours, 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 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), four 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 & 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 et al., 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 ecotoxicological studies using *Ciona robusta* has been reported in 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 (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 gametes production and to avoid spawning (Lambert, 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 hours 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 eggs suspension. After an incubation of 10 minutes 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 hours 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, cyclics and aromatics. D2 is

soluble in water and its bioaccumulation is very low in the environment (log Kow<3). Sodium Dodecil Sulphate (SDS) is an anionic surfactant and contains sodium salt and dodecyl sulfate, with moderate solubility in water (15g/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); c) there are many marine toxicity data for it in literature as reported in Manfra et al. (2017; 2018). 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 non-sterilized aqueous solution (Scott et al., 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. 30 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 exposure) and OECD/203 (1992.) for *C. robusta* and *D. labrax*, respectively. Morphological and behavioral endpoints were considered as qualitative sub-lethal 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 minutes.

Statistical analyses

After 96 hours 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 %.

Table 1 – Experimental conditions for 96 hours 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 hours	96 hours

Endpoint	Mortality	Mortality
Sub-lethal endpoint	morphological abnormalities	swimming behaviour
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
N. 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

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).

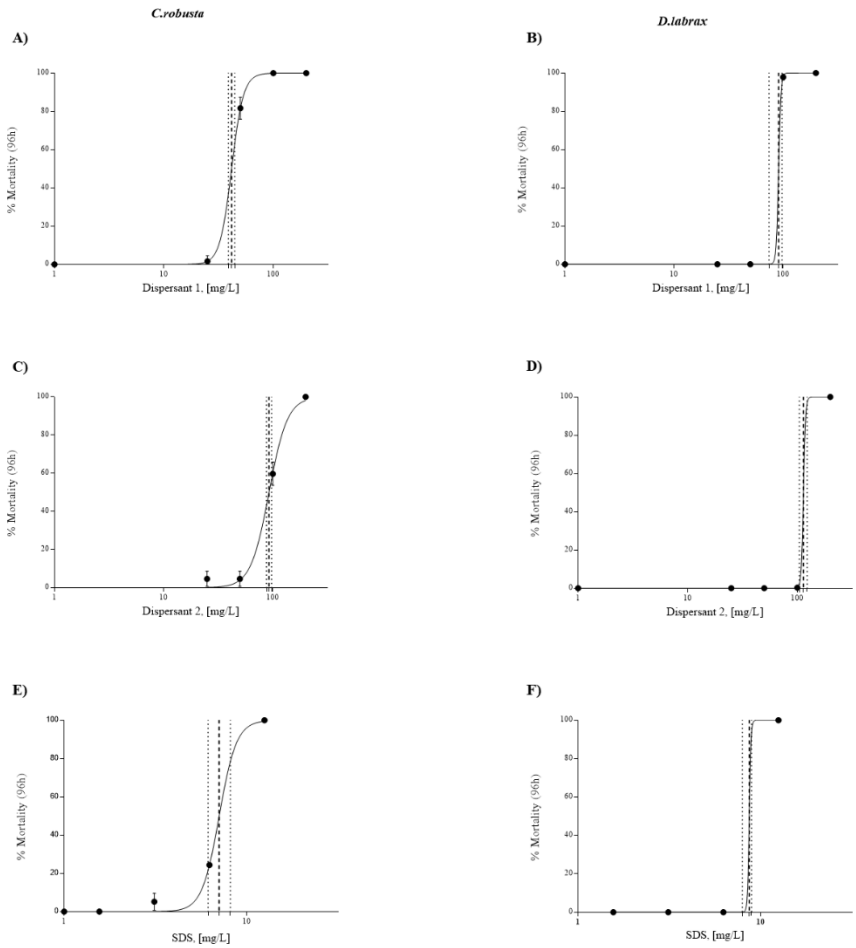

Figure 1 – Mortality rate of *Ciona robusta* and *Dicentrarchus labrax* after 96 h of exposure to different concentrations of dispersant D1 (a, b), dispersant D2 (c, d) and Sodium Dodecil Sulphate SDS (e, f). Curves represent the nonlinear regression of mortality data (sigmoidal) with the best fit for LC_{50} values (dashed lines) as well as the relative 95% CIs (dotted lines). Error bars represent standard deviation.

The 96hLC_{50} values for SDS are 7.0 (5.4- 9.1) and 8.6 (8.0-9.1) mg/L 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 (75.2-98.8) mg/L 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 (128.3-145.9) mg/L 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.

Table 2 - $96hLC_{50}$ values and confidential limits (mg/L) for *Ciona robusta* and *Dicentrarchus labrax* exposured at Sodium Dodecil Sulphate (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)

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. 2 a, b).

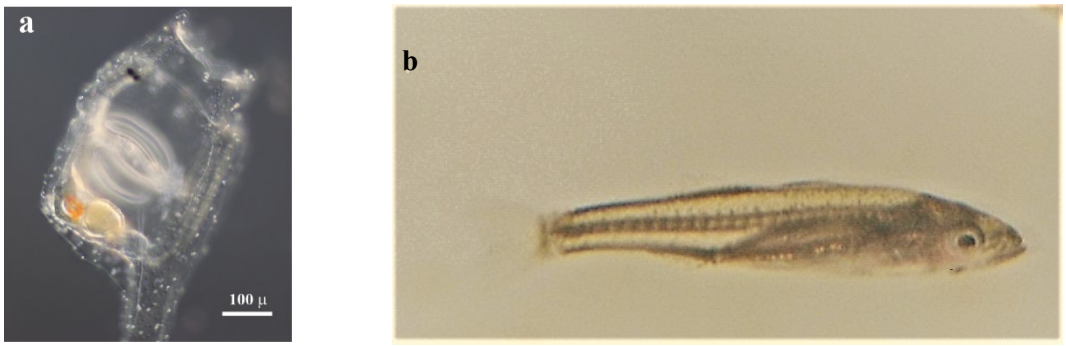

Figure 2 – Control juveniles of *Ciona robusta* (a) and *Dicentrarchus labrax* (b) raised in seawater without toxicant. Scale bar: a) 100 µm; b) 1 cm

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 slow-down in growth (compare fig. 3a with 3b and 3c). 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 3 e-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 3 f). 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.

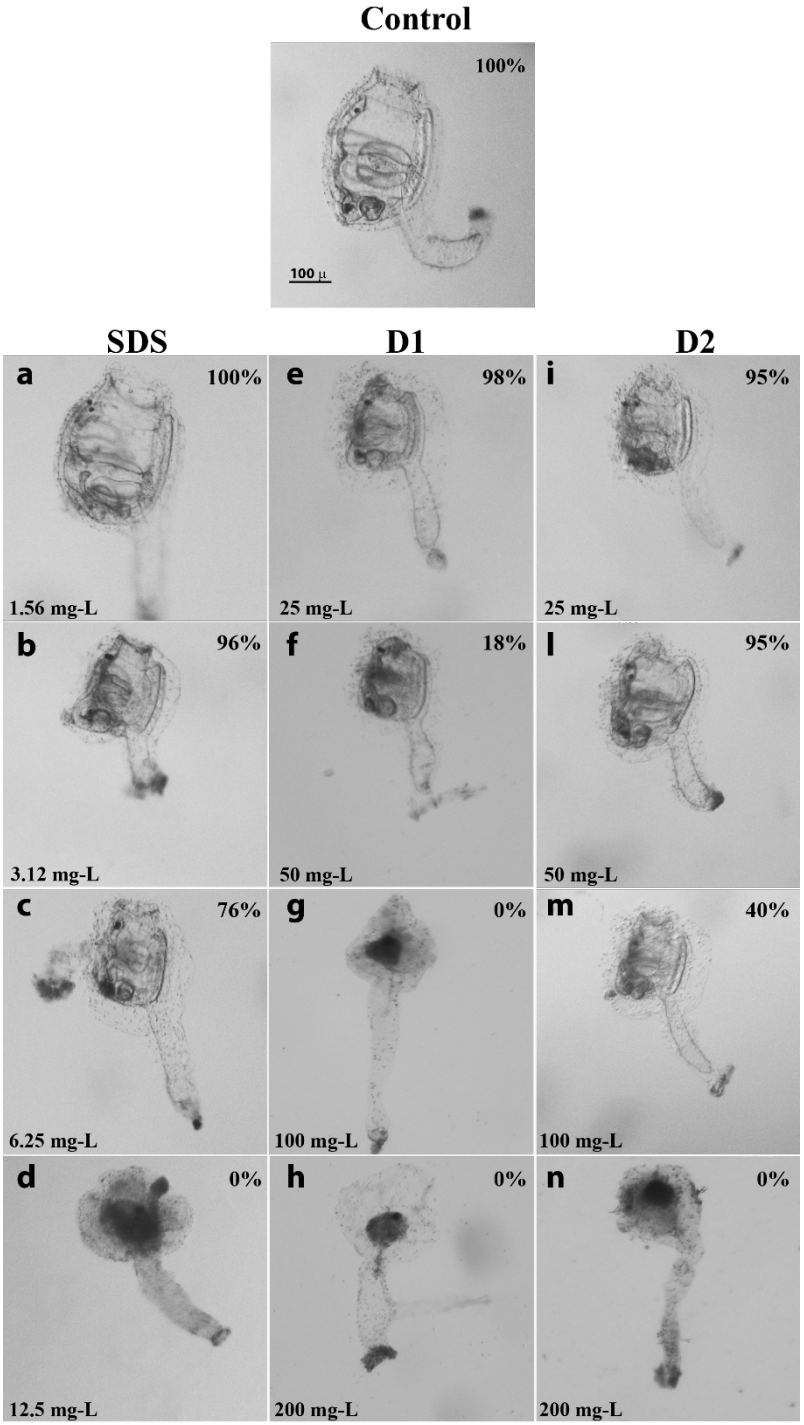

Figure 3 - Morphological abnormalities in *Ciona robusta* exposed at increasing concentrations of Sodium Dodecil Sulphate SDS (a, b, c, d), dispersant D1 (e, f, g, h) and dispersant D2 (i, l, m, n). All the treatments (except a) caused a reduction in size, internal organ disorganization and presence 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.

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. 96h mortality bioassay with *C. robusta* juveniles could thus be proposed as alternative method to 96h 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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