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Effects of pollutants originating from plastics on ascidians: development and survival

PhD Thesis

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Abstract

Abstract

Marine pollution was defined as “the introduction by man of substances or energy into the marine environment, which results or is likely to result in deleterious effects such as harm to marine life” (UNCLOS, 1982). To develop management protocols for pollutants present in marine environment it is important to fill the gap of knowledge about their effects on environmental and human health. This work centers on the effects of different kinds of pollutants that originate from plastics on marine invertebrates. The first part of this study focused on toxic and teratogenic effects of a chemical produced by plastic degradation, Bisphenol A (BPA), particularly considering the effects on nervous system and sensory organs of ascidians. The second part is centered on the impact of microplastics (MPs), on ascidians and sea urchin larvae.

BPA is an organic compound used in the manufacturing of polycarbonate plastics and epoxy resins. After the degradation of food and beverage containers made of BPA, it can be released in food and water and be ingested, or reach the environment. Many different negative effects have been reported on human health and on wildlife. In this study, the effects of different concentrations of BPA have been analyzed on ontogenetic processes, embryogenesis, nervous system development and sensory organ differentiation in different ascidian species, namely *Ciona robusta*, *Ciona intestinalis* and *Phallusia mammillata*. The most sensitive developmental step was the first cell division both in *C. robusta* and *P. mammillata*. The teratogenic effect of BPA was confirmed in ascidians and the main alterations reported involved GABAergic and dopaminergic neural populations and pigment cells differentiation. Specific alterations of pigmented organs were reported, namely abnormal deposition of pigments, absence of one or both pigmented organs or presence of supernumerary ones. Since similar effects on nervous system and sensory organs were already reported in different vertebrate species, ascidian species confirmed their value as invertebrate animal model for preliminary screenings of effects of pollutants.

In *C. intestinalis*, exposure to BPA altered genes expression of transcription factors involved in central nervous system and pigment cells specification, as *Ci-Six3/6* and *Ci-Pax6*. Similar alteration were previously described as a consequence of perturbation of FGF and Wnt signaling pathways leading to hypothesize that BPA act at some point of the cascade activated by these pathways.

At last, the co-exposure to BPA and an inverse agonist of estrogen related receptor (ERR), 4-hydroxytamoxifen (4-OHT), during embryogenesis produced a rescue of normal pigmented organs phenotype in *P. mammillata*. In zebrafish, it was previously demonstrated that BPA

exposure determines otolith malformations through its binding to ERRγ. Our results support the idea that also in ascidians the effects of BPA were mediated by ERR.

Plastic debris that reach the marine environment can become a threat to wildlife not only because they release chemicals compound in the seawater, but also because the fragmentation and degradation of these products produce small particles named microplastics (MPs).
In this study, the effects of polystyrene micro particles of 1 and 10 µm of diameter were analyzed on embryonic development of ascidians and sea urchins and on metamorphosis and survival rate of ascidian juveniles. Ascidian lecitotrophic larvae were not affected by the presence of microsphere in culture medium. Filter feeders ascidian juveniles and suspension feeders plutei were both able to ingest MPs. Exposure during the metamorphosis produced a delay of growth in ascidians, since the presence of MPs in the digestive system reduced the food intake. Moreover, the effects on metamorphosis were more strong in presence of 10 µm beads than of 1 µm ones, probably because smallest ones can be more easily expelled with feces. Body growth was altered also in sea urchin. When MPs are present at low concentrations (0.125 and 1.25 µg/ml) juveniles were able to expel them in 24 hours and plutei in 6 hours. However at higher concentrations (12.5 and 25 µg/ml), MPs persisted longer in the digestive system. Moreover, comparing the intake efficiency of the ascidian and plutei, the sessile filtering organisms appeared more vulnerable to MPs effects than pelagic suspension feeders.
At last, 1 µm MPs were able to translocate from digestive system of ascidian juveniles. Histological analysis revealed that some particles crossed the stomach wall, and some were phagocytized by blood cells. These data highlight that MPs not only negatively impact the feeding ability of marine species, but probably further affect the physiology of organisms after translocation.

The wide range of alterations and the possible subsequent negative effects of BPA and MPs make clear that a monitoring of concentrations and distributions of these pollutants in natural environment is required. Moreover, a reduction of the use of plastic products and new policies on plastic wastes are desirable to avoid the increase of distribution of these new pollutants that could negatively impact marine wildlife.

Riassunto

L'inquinamento marino è stato definito come "l'introduzione da parte dell'uomo di sostanze o energia nell'ambiente marino, che porta o può portare effetti deleteri, come danni agli organismi marini" (UNCLOS, 1982). Per sviluppare protocolli di gestione per gli inquinanti presenti in

ambiente marino è importante raccogliere informazioni relative agli effetti sull'ambiente stesso e sull'uomo. Questo lavoro si concentra sull'effetto di diversi tipi di inquinanti che derivano dai prodotti di plastica su invertebrati marini. La prima parte dello studio è dedicata agli effetti tossici e teratogeni di una molecola rilasciata dalla degradazione della plastica, il bisfenolo A (BPA), analizzando principalmente gli effetti sul sistema nervoso e sullo sviluppo degli organi di senso nelle ascidie. La seconda parte si concentra sull'impatto delle microplastiche (MPs) su ascidie e ricci di mare.

Il BPA è un composto organico utilizzato nella produzione di plastiche di policarbonato e di resine epossidiche. In seguito alla degradazione dei contenitori di cibi e bevande che contengono BPA, esso può essere rilasciato nel cibo e nell'acqua ed essere ingerito dall'uomo o essere disperso nell'ambiente. Sono stati riportati numerosi effetti negativi sulla salute umana e sugli organismi animali. In questo studio sono stati analizzati gli effetti di diverse concentrazioni di BPA sui processi ontogenetici, sull'embriogenesi e sullo sviluppo del sistema nervoso e degli organi di senso in diverse specie di ascidia, *Ciona robusta*, *Ciona intestinalis* e *Phallusia mammillata.* La fase di sviluppo più sensibile è risultata essere la prima divisione cellulare sia in *C. robusta* che in *P. mammillata*. L'effetto teratogeno del BPA è stato confermato nelle ascidie e le principali alterazioni riscontrate riguardavano le popolazioni neuronali GABAergiche e dopaminergiche, e le cellule pigmentate degli organi di senso. Le alterazioni osservate negli organi pigmentati erano caratterizzate dalla deposizione anormale del pigmento, dall'assenza di uno e entrambi gli organi pigmentati o dalla presenza di organi pigmentati in sovrannumero. Poiché effetti simili sul sistema nervoso e sugli organi di senso erano già stati riportati nei vertebrati, le ascidie si sono confermate come un buon modello animale invertebrato per test preliminari sugli effetti degli inquinanti.

Nell'ascidia *C. intestinalis*, l'esposizione al BPA ha alterato l'espressione genica di alcuni fattori di trascrizione, come *Ci-Six3/6* e *Ci-Pax6*, coinvolti nella specificazione del sistema nervoso anteriore e delle cellule pigmentate. Alterazioni simili erano state descritte in letteratura come conseguenza della perturbazione delle due vie di segnalazione di FGF e Wnt, portando a ipotizzare che il BPA agisca sulla cascata attivata da queste vie di segnalazione.

Infine, in *P. mammillata*, la co-esposizione durante l'embriogenesi a BPA e 4-idrossitamoxifene (4-OHT), un agonista inverso dei recettori simili ai recettori degli estrogeni (ERR), ha prodotto larve che recuperavano un fenotipo normale degli organi pigmentati. In Zebrafish, è stato dimostrato che l'esposizione a BPA altera lo sviluppo degli otoliti in seguito al suo legame con

gli ERRγ. Questi risultati sostengono l'ipotesi che anche nelle ascidie il BPA agisca tramite il legame con questi recettori.

I rifiuti di plastica che raggiungono l'ambiente marino possono essere una minaccia per la fauna, non solo perché rilasciano sostanze chimiche ma anche perché, in seguito alla frammentazione e alla degradazione, sono trasformati in particelle sempre più piccole che vengono chiamate microplastiche (MPs).
In questo studio sono state utilizzate particelle di polistirene dal diametro di 1 o 10 µm e ne sono stati testati gli effetti sullo sviluppo embrionale delle ascidie *C. robusta* e *C. intestinalis* e del riccio di mare *Paracentrotus lividus* e sulla metamorfosi e tasso di sopravvivenza di giovanili di ascidia. Le larve di ascidia, che sono lecitotrofiche, non sono state influenzate dalla presenza di particelle nell'acqua di mare. Sia i giovanili di ascidia, che sono filtratori sessili, che i plutei, che sono sospensivori liberamente natanti, sono stati in grado di ingerire le microplastiche. L'esposizione durante la metamorfosi ha prodotto un ritardo nello sviluppo dei giovanili, poiché la presenza di microplastiche nell'apparato digerente riduce l'assunzione di cibo. Inoltre gli effetti sulla metamorfosi sono stati più intensi in presenza delle particelle da 10 µm rispetto a quelle da 1 µm, probabilmente perché le sfere più piccole potevano essere espulse tramite pellet fecali più facilmente. La crescita corporea è risultata alterata anche nel riccio di mare. Quando le microplastiche sono presenti a basse concentrazioni (0,125 e 1,25 µg/ml) i giovanili sono in grado di espellerle in 24 ore e i plutei in 6 ore. Tuttavia, alle più alte concentrazioni (12,5 e 25 µg/ml), le microplastiche persistono più a lungo nell'apparato digerente. Inoltre, l'efficienza di assunzione delle particelle nei giovanili è più elevata che nei plutei, quindi gli organismi sessili e filtratori potrebbero essere più vulnerabili dei sospensivori agli effetti delle microplastiche.
Infine le microplastiche da 1 µm sono in grado di uscire dall'apparato digerente dei giovanili. Un'analisi microscopica ha rivelato che alcune particelle avevano attraversato le pareti dello stomaco mentre altre sono state fagocitate da cellule del sangue. Questi dati evidenziano che le microplastiche non hanno impatti negativi solo sulla capacità di assunzione del cibo nelle specie marine, ma che essendo in grado di spostarsi in diversi tessuti probabilmente possono causare ulteriori effetti deleteri.

L'ampio spettro di alterazioni e di possibili effetti negativi del BPA e delle microplastiche rendono evidente la necessità di monitorare la concentrazione e la distribuzione di questi inquinanti negli ambienti naturali. Inoltre sono desiderabili sia la riduzione nell'uso dei prodotti di plastica che nuove leggi sui rifiuti plastici per evitare un aumento della quantità di inquinanti che possono avere effetti negativi sulla vita marina.

Chapter 1 – General introduction

1.1. Aims

The United Nations Convention on the Law of the Sea (UNCLOS) is an international agreement defined after a long series of conferences between 1973 and 1982, and it came into force in 1994. It defines the marine pollution as "the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of the sea water and reduction of amenities". Even if pollutants are released in different environmental compartments such as air, land or water, they tend to end up in the oceans (Islam & Tanaka 2004). The main sources of pollution are human activities such as industry, transport, agriculture, urbanization, tourism infrastructural development and construction (Gavrilescu et al 2015, Islam & Tanaka 2004). In the last 70 years the production of anthropogenic chemicals increased from 1 million to 400 million tons per each year and over 50% of chemicals produced between 2002 and 2011 were environmentally harmful compounds (Gavrilescu et al 2015).

Among these substances it is possible to find the so called Emerging Pollutants (EPs): "synthetic or naturally occurring chemicals that are not commonly monitored in the environment but which have the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects" (Geissen et al 2015). More than 700 substances have been categorized in 20 classes related to their origin (http://www.norman-network.net), and the most significant are pharmaceuticals, pesticides, disinfection by-products, wood preservation and industrial chemicals, cosmetics, personal and household care products (Gavrilescu et al 2015, Geissen et al 2015). Moreover, most of these new pollutants can act as endocrine disrupting chemicals (EDCs). EDCs are compounds, natural or synthetic, that can affect endocrine system both mimicking endogenous hormones and/or inhibiting normal hormone activities and metabolism (Roepke et al 2005). They can act through numerous mechanisms, for example binding to different type of receptors (nuclear and non-nuclear steroid receptors, non-steroid receptors, orphan receptors) or altering enzymatic pathways for steroid biosynthesis and/or metabolism. A complete inventory of these contaminants is not available and insufficient information exists regarding their occurrence, fate and impact in the environment. Most of them have pharmaceutical, personal care and household uses (i.e. hormones, glucocorticoids, analgesics, additives in drugs and cosmetics, and household cleansers), while other kinds of EDCs are fire-

retardants, heavy metals, widely used industrials chemicals (Bisphenol A) and some pesticides (Caliman & Gavrilescu 2009, Cook et al 2012, Gavrilescu 2009, Preda et al 2012).

To develop control and management protocols for these pollutants it is important to fill the gap of knowledge on their effects on environmental and human health.

This thesis aims to analyze the effects of one of these pollutants, Bisphenol A, on the development and survival of ascidians, animals evolutionarily close to vertebrates (Delsuc et al 2006) and important components of benthic assemblages worldwide (Zega et al 2009).

The threats to oceans and marine life do not come only from chemicals products; in fact one of the major human impact is due to pollution by plastic debris (Fig. 1).

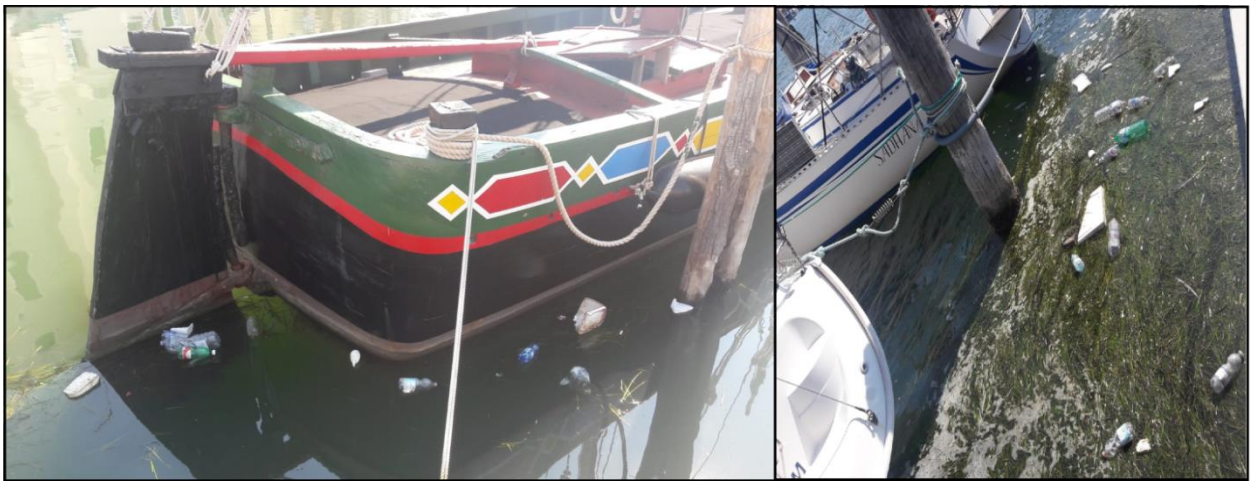

Figure 1: Plastic debris in the port of Chioggia, August 2018.

Plastics are synthetic organic polymers with high versatility, being lightweight, strong, durable and cheap (Laist 1987). Huge volumes of plastics are dumped in beaches, lakes, navigation channels and other forms of water masses. About 77% of debris present in the north-western Mediterranean are constituted by plastic (Goldberg 1995). Survey on beaches were conducted in Japan and Russia (Kusui & Noda 2003), Australia (Frost & Cullen 1997, Whiting 1998), South Georgia (Walker et al 1997) and South Caribbean (Debrot et al 1999), always reporting high percentage of plastic in the litter deposits. Other than an aesthetic problem, plastics can be hazardous for the environment, in fact buoyant debris can be dispersed over long distances from the source point and when they reach the sediment, they may persist for centuries. Starting from the ‘80s the problem of marine plastic pollution began to be taken in consideration, with a continuously growing increase of studies on debris distribution and effects on marine wildlife (Stefatos et al 1999). Particularly the main threat for organisms is given by the ingestion of debris (Stefatos et al 1999), followed by entanglement and ghost fishing (Gregory 1999). Once

the plastic debris accumulate organic coatings which adsorb shells, sand and other debris, they sink to the sea floor, covering it and preventing transfer of nutrients and gases between water and sediments (Islam & Tanaka 2004). Moreover, from the degradation of plastic debris, small plastic particles, at the micro-level scale, can originate (Browne et al 2011; Davidson 2012; O'Brine & Thompson 2010). Biomonitoring studies revealed the widespread ingestion of microplastic particles by different marine organisms. Nowadays, microplastics represent one of the main threats to marine ecosystems, being able to affect organisms at different stages of their life cycle and at different levels of the food web.

In this work, the effects of exposure to microplastics of different size have been tested on ascidians and sea urchins in order to evaluate their ability to uptake particles and the effects on development.

1.1.1. Bisphenol A

Bisphenol A (BPA, 2,2-bis-(4-hydroxyphenyl)-propane; CAS Registry No. 80-05-7; Fig. 2) is an organic compound used in the manufacturing of polycarbonate plastic and epoxy resins, the former employed in the realization of food and beverage containers, the latter in dental materials, for the lining of food and beverage containers and water supply pipes (NTP 2008). Moreover BPA is used in the production of polyester resins, polysulfone resins, polyacrylate resins and flame retardants and in the recycling of thermal paper (NTP 2008).

BPA was synthesized for the first time in 1891 by the Russian chemist A. P. Dianin. In the early 1930s the function of BPA as an artificial estrogen was tested, but it resulted 37'000 times less effective than estradiol (Dodds & Lawson 1936, Dodds & Lawson 1938) and was replaced by diethylstilbestrol (DES). BPA started to be used as a plasticizer in 1950s. World production capacity of BPA was 1 million tons in the 1980s, and in 2013, the global consumption of BPA exceeded 5 million tons (Lin et al 2017). In 2015, BPA was one of the highest volume chemicals produced worldwide (more than 3 million tons/year; Gao et al 2015). The plastic identification code utilizes number from 1 to 7 to indicate the plastic resin out of which the product is made. Some plastics that are marked with the Code 7 may contain BPA, and also Code 3 PVC plastics include BPA as an antioxidant (Fiege et al 2000).

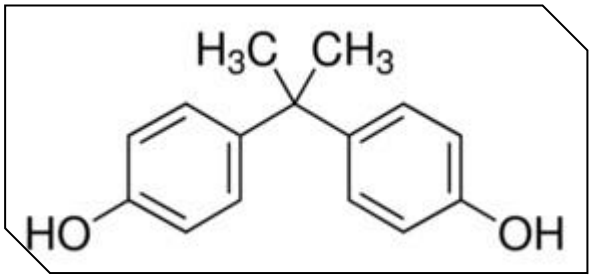

Figure 2: Bisphenol A molecule.

BPA can be released into the environment from degradation of BPA-based products, from sewage treatment effluents and landfill leachates (Crain et al 2007). It is also discharged from wastewater treatments in fresh and marine waters. Indeed, it was estimated that the amount of BPA released into the environment reaches 500 tons per years (Mileva et al 2014). The concentration of BPA was measured in different environmental compartments (Table 1).

Once BPA reaches the environment, it can negatively interfere with animal physiology and development. In fact, the possibility of adverse effects after low-dose BPA exposure in laboratory animals was first proposed starting from 1997 (Erickson 2008). Now it is known that BPA effects on living organisms are particularly numerous and variable, as it can act both as an endocrine disruptor by binding to different nuclear receptors (Rubin 2011) and as a teratogenic molecule (Iwamuro et al 2003, Kiyomoto et al 2006, Rubin 2011, Sone et al 2004, Yang & Chan 2015). Most of the research on BPA focused on reproductive toxicity in aquatic vertebrates such as fish and amphibians, in which it mainly causes reduction of sperm quality, alteration of sex

steroid levels, embryonic deformities and intersex condition as reviewed in Flint et al (2012). Studies on invertebrates showed the absence of reproductive effects at BPA environmental concentrations in cnidarians, nematodes and crustaceans (Hoshi et al 2003, Marcial et al 2003, Pascoe et al 2002, Tominaga et al 2003). However, chronic exposure altered or suppressed crustaceans development and growth (Andersen et al 1999, Mariager 2001), altered nematodes feeding behavior (Kohra et al 2002) and caused morphological deformities in sponges (Hill et al 2002). In their review Crain et al (2007) concluded that the comparison of measured BPA environmental concentrations with chronic values suggests that no significant margin of safety exists for the protection of aquatic communities against the toxicity of BPA.

There is still a big ongoing debate regarding the effects of BPA on human health. In fact the European Food Safety Authority (EFSA) states that "BPA poses no health risk to consumers of any age group (including unborn children, infants and adolescents) at current exposure levels" (EFSA 2015), while the European Chemical Agency (ECHA) supports the inclusion of BPA in the list of substances of very high concern (ECHA 2017).

In the United States, the Food and Drug Administration (FDA) banned BPA from baby bottles but maintained it in other products that hold food. At last, the Endocrine society stated that, on the basis of the precautionary principle, BPA should continue to be assessed and tightly regulated (Gore et al 2015). Even if some agencies considered BPA of low concern, the scientific literature is rich of works that correlate exposure to BPA with a series of pathologies: diabetes, cardiovascular diseases, altered liver enzymes (Lang et al 2008), miscarriage (Sugiura-Ogasawara et al 2005), premature deliveries (Cantonwine et al 2010), decreased semen quality and sperm DNA damage (Meeker et al 2010), aggression and hyperactivity (Ejaredar et al 2017), obesity (Bhandari et al 2013), neurological effects (Jones & Miller 2008), thyroid function (Boas et al 2009, Zoeller 2007), cancer (Soto & Sonnenschein 2010), asthma (Midoro-Horiuti et al 2010). The main human route of exposure to BPA is ingestion of contaminated food and water that accumulated this pollutant from leachates of plastic containers.

The EFSA has evaluated BPA toxicity several times proposing a temporary Tolerable Daily Intake (t-TDI) of 4 μg/kg bw per day, but this value will be reconsidered in 2018 (EFSA 2017).

To make possible a correct evaluation of risks for animals, humans and environment correlated to BPA exposure, it is necessary to increase the data available on its effects and mechanism of action.

Environmental compartments	BPA concentrations	References
Soil	0.24-147 µg/kg d.w.	(Gibson et al 2010, Kinney et al 2008, Staples et al 2010, USEPA 2010, Xu et al 2008)
Sediments	0.24-492 µg/kg d.w.	(Fu et al 2007, Gorga et al 2014, Gorga et al 2015, Heemken et al 2001, Kawahata et al 2004, Michałowicz 2014, Pojana et al 2007, Stewart et al 2014, USEPA 2010, Vethaak et al 2005, Wu et al 2013)
Groundwater	0-2550 mg/m^3	(Félix-Cañedo et al 2013, Godejohann et al 2009, Lacorte et al 2002, Latorre et al 2003, Loos et al 2010, Luo et al 2014, Michałowicz 2014, Stuart et al 2011, USEPA 2010)
Surface water	0.0001-92 mg/m^3	(Azevedo et al 2001, Basheer et al 2004, Cespedes et al 2005, Félix-Cañedo et al 2013, Fu et al 2007, Gorga et al 2015, Heemken et al 2001, Kawahata et al 2004, Loos et al 2007, Luo et al 2014, Melo & Brito 2014, Michałowicz 2014, Patrolecco et al 2006, Pojana et al 2007, USEPA 2010, Vethaak et al 2005, Vousta et al 2006, Wu et al 2013, Xu et al 2014, Yoon et al 2010, Zhang et al 2014)
Landfill leacheates	17200 µg/L	(Yamamoto et al 2001)
Waste water	140 ng/L	(Kolpin et al 2002)
Air in house/office	2-3 ng/m^3	(Rudel et al 2001)
Air outdoor	0.02–1.92 ng/m^3	(Matsumoto et al 2005)
Air in plastic processing plants	208 ng/m^3	(Rudel et al 2001)
Dust house/office	0.25-0.48 µg/g	(Rudel et al 2001)

Table 1: BPA concentrations in different environmental compartments.

1.1.2. Microplastics

Microplastics (MPs) are defined as plastic particles with a diameter comprised between 1 μm and 1 mm (Andrady 2015). These particles can be primary MPs, specifically produced at the micro-level scale, such as sandblasting media, virgin pellet and cosmetics (Fendall & Sewell 2009), or secondary MPs, originated from the degradation of bigger plastic debris, such as polyester fibers, polyethylene plastic bags and polystyrene particles from buoys and floats (Browne et al 2011, Davidson 2012, O'Brine & Thompson 2010). Recent studies have reported the presence of MPs pollution in different environmental compartments all around the world. According to Eriksen et al (2014), there are over 5 trillion microplastics floating in the oceans and, in the marine ecosystem, they are present in surface water, water column, beaches and sediments (Table 2 from Wright et al (2013)).

Moreover, their presence has been reported also in freshwater bodies and estuaries (Lima et al 2014). Areas of particular concentration of MPs, named "hotspots", can be located in the oceanic gyres (Goldstein et al 2012), close to industrial coastal areas (Norén & Naustvoll 2010) or densely populated coastal areas (Browne et al 2011). Most of the data on MPs abundance and distribution come from sampling performed with plankton nets, with a mesh size between 80 and 330 μm, producing an underestimation of the real number of particles present in the environment (Andrady 2015). Moreover, the influence of tide, wind, wave action and oceans currents determines a high variability of spatial and temporal distribution of particles, making extremely difficult a real quantification of MPs abundance. Currently, the most widely used synthetic plastics are low- and high-density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET). Altogether, these plastics represent ~90% of the total world production (Andrady & Neal 2009) and it is plausible that the plastic pollution of coastal and marine environment it is due to these materials (Andrady 2011, Engler 2012).

Location	Maximum observed concentration	References
Coastal waters, Sweden	102 000 particles m^3	(Norén & Naustvoll 2010)
Coastal Waters, California	3 particles m^3	(Doyle et al 2011)
Coastal waters, New England	3 particles m^3	(Carpenter et al 1972)
Open ocean, North West Atlantic	67 000 particles km^2	(Colton et al 1974)
Northwest Mediterranean Sea	1 particle m^2	(Collignon et al 2012)
Beach, Malta	>1000 particles m^2	(Turner & Holmes 2011)
Beach, UK	8 particles kg^{-1}	(Thompson et al 2004)
Estuarine sediment, UK	31 particles kg^{-1}	(Thompson et al 2004)
Subtidal sediment, UK	86 particles kg^{-1}	(Thompson et al 2004)
Subtidal sediment, Florida	214 particles l^{-1}	(Graham & Thompson 2009)
Subtidal sediment, Maine	105 particles l^{-1}	(Graham & Thompson 2009)
Harbour sediment, Sweden	50 particles l^{-1}	(Norén 2008)
Industrial harbour sediment, Sweden	3320 particles l^{-1}	(Norén 2008)
Industrial coast sediment, Sweden	340 l^{-1}	(Norén 2008)
Ship-breaking yard sediment, India	89 mg kg^{-1}	(Reddy et al 2006)
Harbour sediment, Belgium	7 mg kg^{-1}	(Claessens et al 2011)
Continental shelf sediment, Belgium	1 mg kg^{-1}	(Claessens et al 2011)
Beach, Belgium	1 mg kg^{-1}	(Claessens et al 2011)
Beach, Portugal	6 particles m^2	(Martins & Sobral 2011)
Beach, East Frisian Islands, Germany	621 particles 10 g^{-1}	(Liebezeit & Dubaish 2012)

Table 2: The spatial distribution and abundance of microplastics, as summarized from a selection of reports from Wright et al (2013).

When MPs reach the marine environment, they can interact with a wide range of organisms (Barnes et al 2009). The main physical threats for marine organisms that ingest MPs are damaging and blocking of the feeding appendages and digestive system and limiting of the food intake (Derraik 2002, Laist 1997, Murray & Cowie 2011). MPs ingestion can also produce toxic effects after the leaching of monomers and plastic additives, that are able to cause carcinogenesis and endocrine disruption and with the transferring of pollutants in living organisms (Mato et al 2001, Oehlmann et al 2009, Talsness et al 2009, Teuten et al 2009). Indeed, thanks to the large surface area to volume ratio of MPs particles, they can become contaminated with persistent

organic pollutants (POPs) up to six orders of magnitude greater than seawater (Hirai et al 2011, Mato et al 2001).

The bioavailability of MPs depends on their size, density, abundance and colour (Wright et al 2013). Moreover some biological factors can increase MPs availability, as the formation of marine aggregates, and expulsion of fecal matters that concentrate particles, making them available for ingestion from suspension feeders and detritivores (Wright et al 2013). Even if many studies arose on MPs, major questions still need to be answered about the risks to marine organisms and ecosystems, and to food safety and public health (Law & Thompson 2014). According to Law & Thompson (2014) "more information must be collected on the behavior of different polymers in the environment, including fragmentation, chemical release, degradation, transport, and accumulation; the rate at which organisms encounter microplastics, based on particle size and degradation time; and the physical, chemical, and interactive risks to organisms from these encounters, including possible magnification with increasing trophic level". At last, since the removal of MPs from the environment is not easily feasible, the best way to protect the environment is the identification and removal of the major inputs.

1.2. Model organisms

1.2.1. Ascidians

Ascidians, or sea squirts, are marine invertebrates belonging to the Subphylum Urochordata or Tunicata and to the Phylum Chordata that also comprises Cephalocordata and Vertebrata. They are filter-feeders and can live as solitary individuals or as colonies. About 3000 species of Tunicate have been described, with 2150 living species. Ascidians have been widely used for years as model organisms for developmental biology (Satoh 1994) and recently they have been proposed also as models for ecotoxicological bioassay (Dumollard et al 2017, Gallo & Tosti 2015, Zega et al 2009). In fact, hermaphrodite adults can be easily collected from natural environments and handled in laboratory. They produce a high number of gametes that, after fertilization, develop synchronously and reach the larval stage in 18-22 hours at 18°C. Since the release of gametes, fertilization and embryogenesis occur naturally in the marine water, these processes can be affected by the presence of aquatic pollutants, making really important to test the effects of toxicants at different endpoints. Moreover, juvenile and adults are filter feeding organisms, and filter huge amount of water, with all the substances dissolved or suspended in the water itself. Another important characteristic is their worldwide distribution, usually in shallow water. At last, the phylogenetic position of Tunicates as sister group of Vertebrate (Delsuc et al 2006, Putnam et al 2008) makes these invertebrate model animals useful for preliminary screenings of effects of pollutants on vertebrates.

Adult ascidians have a sacciform shape, are sessile and are covered by a tunic made of the polysaccharide cellulose from which they get the name Tunicate. They filter sea water from the oral siphon, than the water is conveyed through the pharynx, passes through mucus-covered gill slits and reaches the atrium. At last, it exits through the atrial siphon. Some organs and tissues are present only in adults and juveniles. The chordate features recognizable in the adults are the gill slits and the endostyle. Gill slits are contained in the feeding basket and share a common origin with the gill slits of other chordate (Aros & Viragh 1969). The endostyle is a longitudinal ciliated fold, located in the ventral pharyngeal wall, which produces mucus used to trap food particles and to pass them along the digestive tract. In ascidians it also sequesters iodine and produces thyroid hormone, suggesting it is the homologue of vertebrate thyroid gland (Eales 1997). Adults also possess a heart that is a V-shaped tube composed by a single layer of unicellular, striated muscle cells and is positioned in the proximal region of the body close to the holdfast (Damas 1899, Willey 1893).

Ascidians blood contains at least six type of cells: stem cells, amoebocytes, signet ring cells, morula cells, compartment cells, and orange cells (Rowley 1981). Between these, hyaline and granular amoebocytes are capable of phagocytic activity (Rowley 1981).

They also present sensory structures in the oral and atrial siphons (Passamaneck & Di Gregorio 2005). Each individual is hermaphrodite, presenting gonads with gonoducts that release the gametes from the atrial siphon (Fig. 3).

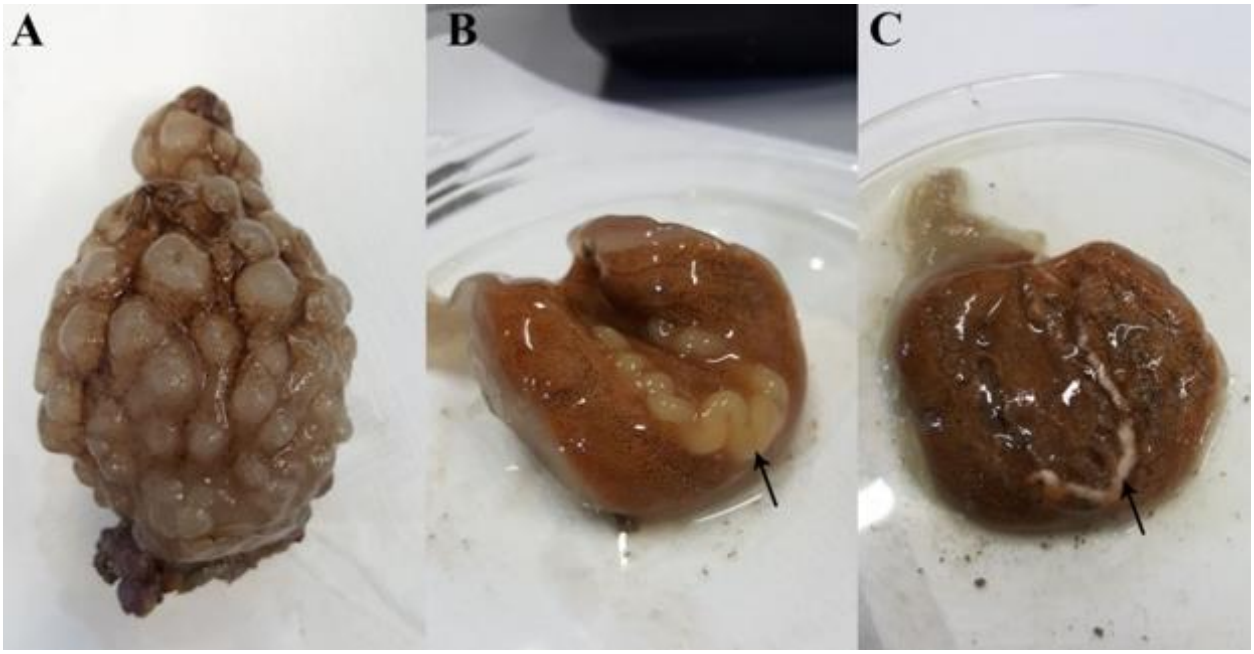

Figure 3: A) Adult of ascidian *Phallusia mammillata*. B) Adults of *P. mammillata* after the removal of tunic, arrow indicate the oviduct with eggs. C) Adult of *P. mammillata* after the collection of eggs, arrow indicates the spermiduct with sperm.

After the fertilization, ascidian embryos develop with invariant cleavage program and the fate of every cell has been accurately described in several species (Lemaire 2009, Nakamura et al 2012, Nishida 1987, Nishida 2005). The most studied species, *C. intestinalis* passes through 26 developmental stages from one cell zygote to hatching larva (Fig. 4; Hotta et al 2007).

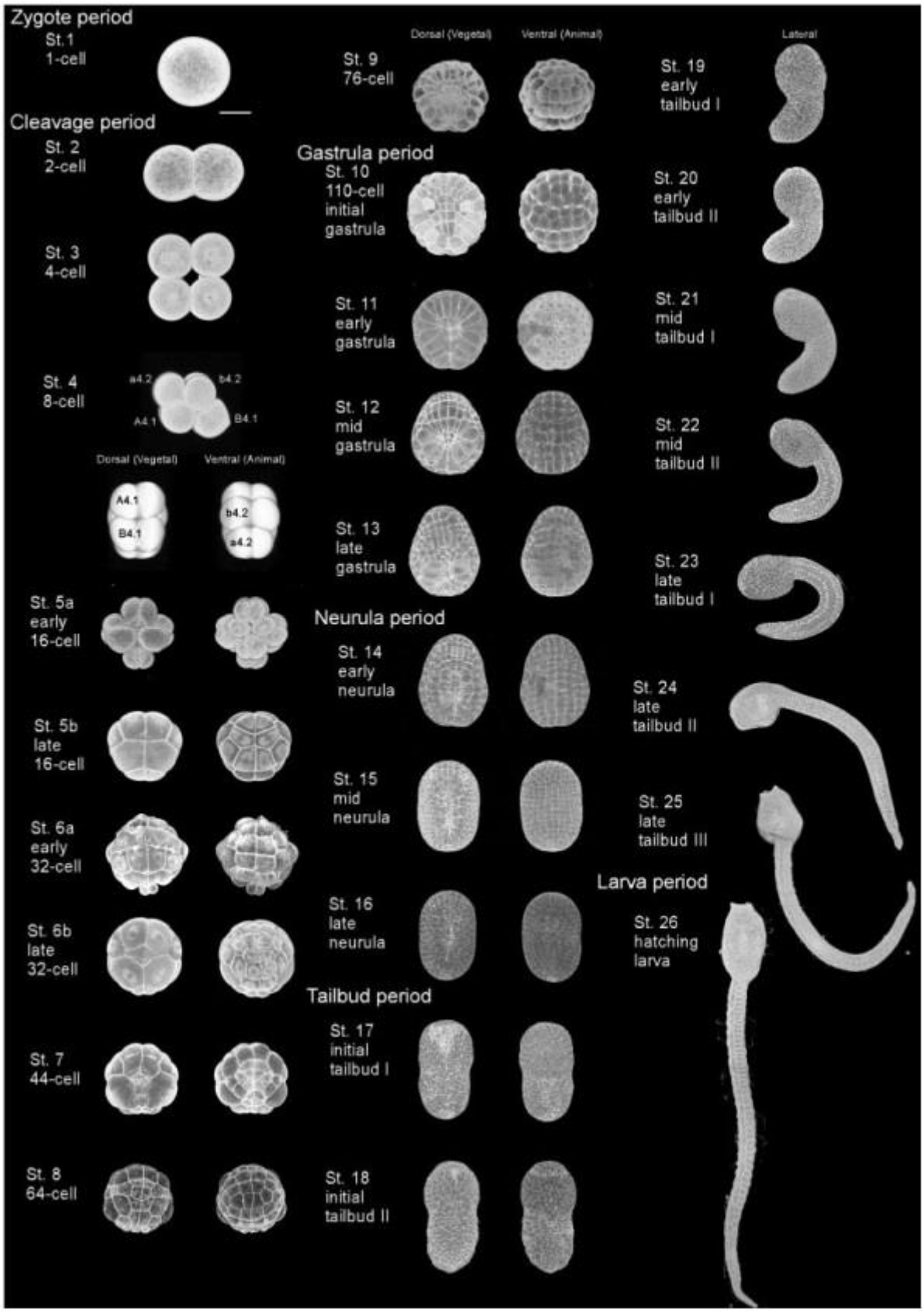

Figure 4: Developmental stages of *Ciona intestinalis* embryos (Hotta et al., 2007).

The ascidian larva, named tadpole, is composed by approximately 2500 cells and can be divided in two regions: the trunk and the tail. The body is constituted by six tissue types: nervous system, notochord, muscle, ectoderm, endoderm and mesenchyme. The chordate characteristics showed by ascidian larvae are (i) the notochord; (ii) dorsal neural tube and (iii) post anal tail. Larvae are completely transparent, allowing to observe every cell and tissue. The notochord, composed by 40 cells, is an axial structure present only in the tail and provides support (Passamaneck & Di Gregorio 2005). The muscles are formed by 36 cells and are disposed in three rows bordering either sides of the notochord (Passamaneck & Di Gregorio 2005). Mesenchyme is constituted by approximately 900 cells situated in the trunk. These cells will give rise to most of adult tissues after the metamorphosis (Satoh 1994). The endodermal cells are around 500 and form a primordial, non functional pharynx in the trunk and a strand that runs ventrally to the notochord (Passamaneck & Di Gregorio 2005). The whole body of the larva is covered by a single layer of epidermal cells (Satoh 2003). At last, the central nervous system (CNS) is constituted by ~350 cells of which ~100 are neurons (Nicol & Meinertzhagen 1991, Satoh 1994). The whole CNS can be divided in four regions. The first portion present in the trunk is the sensory vesicle (SV) that contains the sensory pigmented organs: otolith and ocellus. The otolith is probably a gravity sensing organs that is located it the left antero-ventral wall of the vesicle and composed by a statocyte that protrudes into the sensory vesicle (Dilly 1962). The ocellus is a photoreceptive organ and it is situated in the right posterior wall of SV (Dilly 1964, Horie et al 2005, Sakurai et al 2004, Tsuda et al 2003b). It is composed by different cell types: three lens cells, one pigmented cup cell and about 30 photoreceptor cells (Tsuda et al 2003a). The neck is located posteriorly to the SV, followed by the visceral ganglion (VG), that receives inputs from different sensory structures and contains the somata of motor neurons with axons forming the ventrolateral nerve bundles that project into the tail (Meinertzhagen & Okamura 2001). The caudal nerve cord is composed by four longitudinal rows, one dorsal, one ventral, and two lateral, of ependymal cells enclosing a neural canal (Nicol & Meinertzhagen 1991). The peripheral nervous system (PNS) is formed by isolated receptor neurons distributed in the epidermis of both trunk and tail (Takamura 1998). In the trunk, apical-trunk epidermal neurons (ATEN) that extend from the visceral ganglion to overlay the sensory vesicle are present, while rostral-trunk epidermal neurons (RTEN) and papillary neurons extend anteriorly from the sensory vesicle (Takamura 1998). The caudal peripheral nervous system is composed by caudal epidermal neurons, (CEN) and is located at the dorsal and ventral midline of the caudal epidermis (Takamura 1998). The presence of a small number of neuron in ascidian larvae nervous system made possible to characterize cell types by a combination of their morphology

and of the identification of genes that are specifically expressed in a particular subtype of neurons. This allowed to reveal neuronal networks at single-cell resolution (Horie et al 2009, Imai & Meinertzhagen 2007). The analysis of genes expression was achievable because the whole genome of the ascidian *C. robusta* was sequenced and it contains ~16'000 genes (Dehal 2002).

After hatching, larvae actively swim, initially moving upwards in the direction of illuminated surface water and subsequently they swim downward to reach a substrate for the metamorphosis (Svane & Young 1989). The adhesion to the substrate occurs thought the adhesive papillae, three mucus secreting organs located at the end of the trunk (Zega et al 2005). The settlement can be influenced by different environmental factors as light intensity fluctuations, presence of conspecific or of prey species, bacterial cues or mineral composition of the substrate (Groppelli et al 2003, Rodriguez et al 1993, Svane & Dolmer 1995). During metamorphosis, the larva undergoes a series of drastic morphogenetic changes starting with release of adhesives or papillary eversion, tail resorption, phagocytosis of the transitory larval organs (TLO), rearrangement of larval–juvenile organs (LJO) and leading to the differentiation of prospective juvenile organs (PJO; Cloney 1982). These processes have been well studied in the species *C. intestinalis*. The rotation of the body axis occurs between 12 and 48 hour post hatching (Hph). The third day after hatching the juvenile enters the so called 1st ascidian stage (Berrill 1947); it possesses two protostigmata, the precursor of gill slits, and starts to feed thanks to the contraction of oral and atrial siphons and to the movement of cilia located on the protostigmata itself (Chiba et al 2004). The 1st ascidian stage lasts until 10-12 days after hatching, when the two pre-atrial siphon fuse together and the juvenile possess six row of protostigmata entering the 2nd ascidian stage (Berrill 1947). At the end, 14 days after hatching the juvenile enters the adult stage.

These characteristics highlight how ascidian embryos can provide biological criteria for seawater quality standards, taking into account the sensitiveness of a chordate and contributing to the detection of harmful chemicals with no marked effect on the species currently in use in seawater quality bioassays (Bellas et al 2001). In the following studies, embryos of *Ciona robusta, Ciona intestinalis* and *Phallusia mammillata* and juveniles of *C. robusta* and *C. intestinalis* were used.

1.2.1.1. Ciona robusta, Ciona intestinalis and *Phallusia mammillata*

Ciona robusta, Ciona intestinalis and *Phallusia mammillata* are the main species used for ecotoxicological studies.

The cosmopolitan ascidian *C. intestinalis* has been recorded in all oceans and from high to low latitudes. Molecular studies on specimens collected in different localities revealed that *C. intestinalis* constitutes a complex of two species named 'type A' and 'type B' (Caputi et al 2007, Iannelli et al 2007, Nydam & Harrison 2007, Sato et al 2012, Suzuki et al 2005, Zhan et al 2010).

A detailed morphological comparison between *C. intestinalis* specimens from different localities and between larvae of the two types, provided the diagnostic characters that permitted to unambiguously distinguish the two formerly known *Ciona intestinalis* types A and B as the two species *C. robusta* and *C. intestinalis*, respectively (Brunetti et al 2015, Pennati et al 2015).

Adults of *C. robusta* (Fig. 5A) are usually 10-12 cm long, with a maximum dimension of 21 cm (Hoshino & Nishikawa 1985, Hoshino & Tokioka 1967). They present a transparent or translucent tunic, soft and gelatinous. *C. robusta* is native of the north-west Pacific and it is now distributed in Mediterranean Sea, Australia, West Coast of North America, Atlantic South America, New Zealand, South Africa, and Pacific South America. It is a fouling organism in harbors in the warmer parts of the world.

C. intestinalis (Fig. 5B) has been found on both coasts of North Atlantic Ocean, as well as in the Bohai and Yellow Seas (China; Zhan et al 2010). *C. robusta* and *C. intestinalis* coexist in sympatry in the English Channel and in some localities of the French Atlantic coasts (i.e. in Plymouth, UK, and in Brest, France; Sato et al 2012).

Adults of *P. mammillata* (Fig. 5C) are usually 20 cm long. The tunic is translucent, bluish-white colour and it is covered with irregular rounded lobes or mounds. It is distributed in north-eastern Atlantic Ocean, the North Sea, the English Channel and the Mediterranean Sea and can be found on rocky, sandy and muddy bottom up to the depth of 200 m. Some ascidians, including *P. mammillata*, accumulate the element vanadium in their blood cells (Ueki et al 2002), and it reaches a concentration ten million times higher than in the surrounding seawater.

C. robusta and *C. intestinalis* eggs are surrounded by a complex envelope formed by a layer of follicle cells, the vitelline coat or chorion, a perivitelline space and a layer of test cells on the surface of the egg (Satoh 1994). The eggs of *P. mammillata* are enclosed in a rigid acellular envelope, the vitelline coat (VC). Vacuolated follicle cells are attached to the outer, test cells to the inner surface of the VC (Honegger 1986).

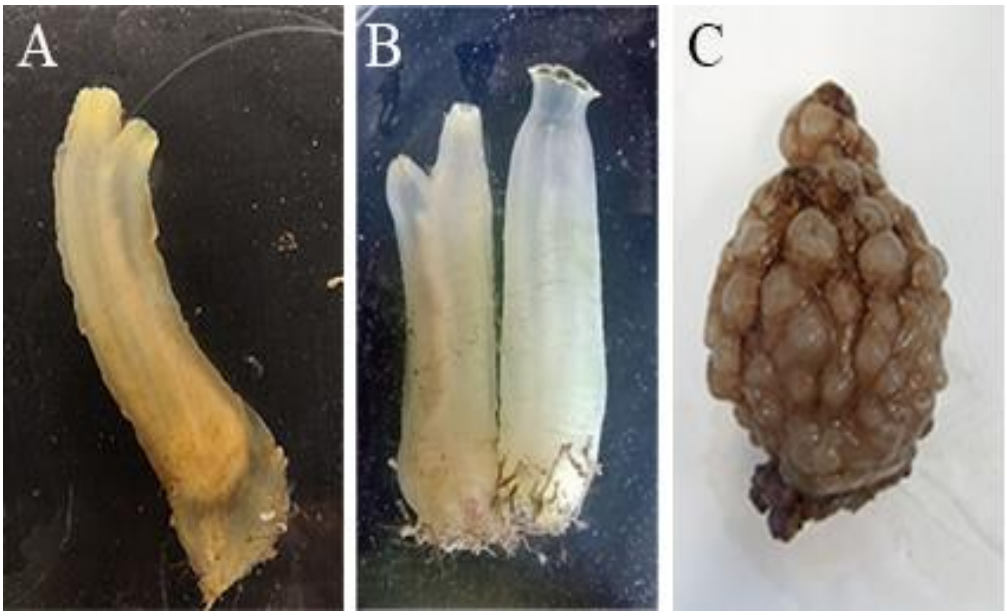

Figure 5: A) Adult of *Ciona robusta*. B) Adults of *Ciona intestinalis*. C) Adult of *Phallusia mammillata*.

1.2.2 Sea urchins

Sea urchins are members of one of the five traditional classes of the Phylum Echinodermata. This Phylum is the largest one that does not possess freshwater or terrestrial representatives and contains about 7000 living species and at least 13'000 extinct ones (Brusca & Brusca 2003). Echinoderms are found in all the oceans from shallow waters of intertidal zone to the deep abyssal plain, and with a few exceptions, they are all benthic. Echinoderms, along with Chordates and Emichordates, form the clade of Deuterostomia. The five traditional classes of echinoderms are: Crinoidea (sea lilies and feather stars), Asteroidea (starfish or sea stars), Ophiuroidea (brittle stars or snake stars), Echinoidea (sea urchins and sand dollars), and Holothuroidea.

Recently they have been proposed as marine target macroinvertebrates and useful test species in ecotoxicology (Sugni et al 2007). They constitute more than 90% of the benthic biomass, being a key-component of marine ecosystems and often performing a fundamental role in ecology, for example with algal community control (Brusca & Brusca 1990, Lawrence 1975). The extremely expanded external epithelia of adults allows a relevant uptake of dissolved substances making them easy targets of environmental contaminants, with particular reference to micropollutants stored in marine sediments (Candia Carnevali 2005). In addition, many echinoderm species are predators, and so are susceptible to biomagnifications processes. At last, their developmental biology is well-characterized and embryos and larvae are potentially useful for genetic manipulation.

The developmental biology of sea urchin embryos is well know thanks to the following characteristics:

- The wide distribution of these animals along the shores, particularly close to the marine biology laboratories of Stazione Zoologica of Naples and Marine Biological Laboratory of Woods Hole, Massachusetts, where the embryos of *Paracentrotus lividus* and *Arbacia punctulata* have been studied;
- The transparency of the eggs that allows to observe the morphology during development under a microscope;
- The easy manipulation of eggs;
- The relative synchronous development;
- The rapid development of embryos that reach the larval stage in about 2 days;
- The possibility to obtain a high number of eggs from each adults (Giudice 2012).

1.2.2.1. Paracentrotus lividus

Paracentrotus lividus, commonly known as purple sea urchin, is a regular echinoid, belonging to the family Parechinidae, characterized by a hemispherical body, densely clothed in long and sharply pointed spines, usually purple but that can have also colours including dark brown, light brown and olive green. *P. lividus* can be found all over the Mediterranean Sea and in the eastern Atlantic Ocean from western Scotland and Ireland to the Azores, Canary Islands and Morocco. In the Mediterranean coasts it is one of the most common sea urchins, widespread in the lower rocky shore, in the shallow sublittoral areas and in beds of seagrass of *Zostera marina* and *Posidonia oceanica* (Riedl 1991). Algae, seagrass, small animals and sponges compose its diet. This sea urchin possesses a circular theca, and the main organ systems are hosted in the body cavity that it is filled by a spacious coelom. *P. lividus* presents a water vascular system with a madreporite on the aboral side and the water canals along five main rows giving rise to the external tube feet (Hyman 1955). The oral side, that face the substrate, is provided by a conspicuous masticatory apparatus, the Aristotele's lantern. This calcareous structure is composed by several skeletal elements (e.g. pyramids), joint together through muscles and ligaments and it is mainly used to feed. The mouth leads to a short esophagus, followed by the stomach and the intestine which opens on the aboral side through the anus (Hyman 1955). The coelomic cavity hosts also the gonads. The nervous system is mainly composed by an ectoneural and a hyponeural system organized in five radial nerve cords with corresponding sinuses, and the sub-epidermal nerve plexus.

Between echinoderms, it was wide used for ecotoxicological experimentation in Europe thanks to its abundant distribution and its good adaptability to laboratory conditions (Menchaca et al 2011). Usually it presents a single annual gametogenic cycle (Pearse & Cameron 1991), with one or two periods of gametes release during the spring and the beginning of summer (Byrne 1990, Lozano et al 1995). However some populations, for example the Tyrrhenian one, display a first main reproductive event at the end of the spring, and a further minor and facultative spawning period in early autumn (Fenaux 1968).

P. lividus is a species with separate sexes even if male and female present the same external morphology. Secondary sex differences are related to the shape of the genital papillae and gonopores and they can be easily recognized only under a stereomicroscope when specimen are closing to the spawning events (Mercurio 2013). Each individual possesses five gonads positioned in the aboral hemisphere of the body (Pearse & Cameron 1991). The shape of gonads is elongated and they are composed by hundreds of acini that open into a central gonoduct. The

gonoduct reaches the gonopore, the opening from which the gametes are released in the seawater, which is located in one of the genital plates surrounding the anus. The unfertilized eggs of *P. lividus* (Fig. 6b) have an orange-yellow colour given by the carotenoid contents, measure about 90-100 µm and are surrounded by a jelly coat. When fertilization occurs, a "fertilization membrane" is lifted from the surface of the egg and within two minutes it becomes hard and prevents polyspermy (Fig. 6c; Giudice 2012). After 60 minutes, the embryo reaches the two cell stages (Fig. 6d) and the early blastula stage is reached 6 hour post fertilization (Hpf; Fig. 6j). Ten hpf, at the swimming stage, the fertilization membrane breaks up and the embryo hatches (Fig. 6m). After the gastrulation, the embryo goes through the prism stage (Fig. 6u) and then reaches the pluteus stage 48 hpf (Fig. 6w-x). Plutei are able to feed and possess a complete digestive system. After about 18-20 days, the larvae reach the competence to undergo metamorphosis (Gosselin & Jangoux 1996). They settle in response to macroalgae (Dworjanyn & Pirozzi 2008, Gosselin & Jangoux 1996, Juinio-Méñez & Bangi 2010), coralline algae (Grosjean et al 1998), marine and microbial biofilms (Dworjanyn & Pirozzi 2008, Rahim et al 2004). These substrates serve as primary food for juvenile sea urchins (Takahashi et al 2002).

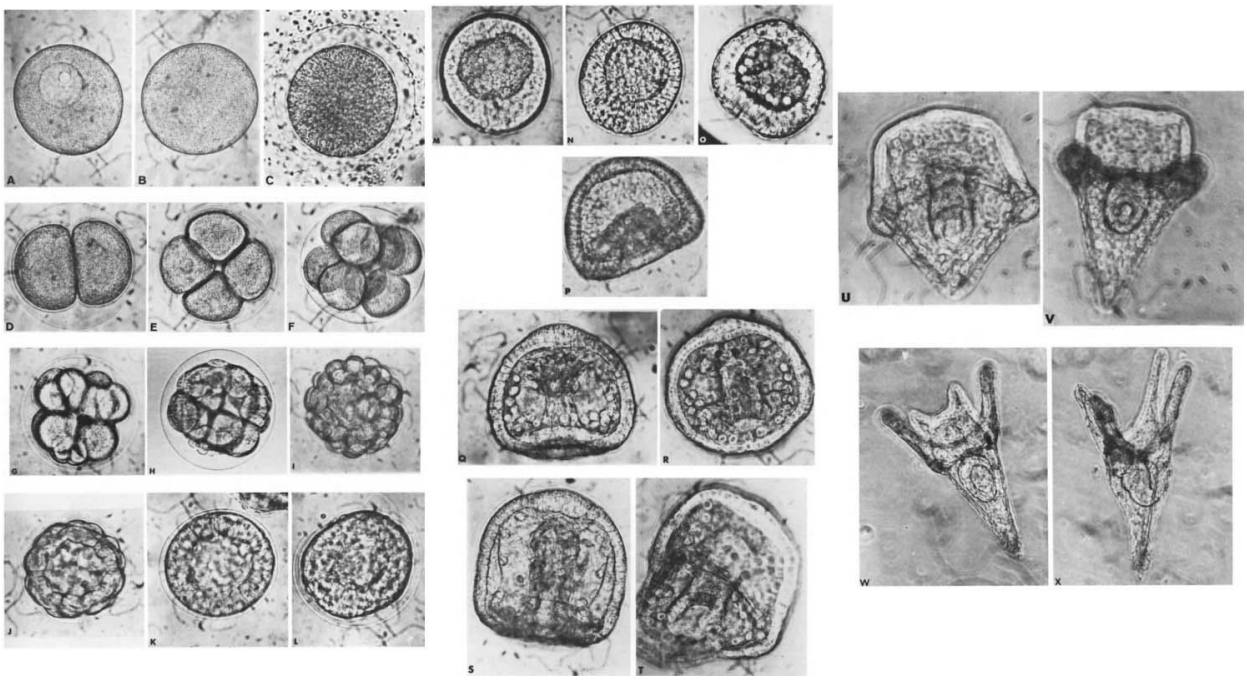

Figure 6: from Giudice, 2012. The early development of the sea urchin *Paracentrotus lividus* (x 400). (a) fully grown oocyte (slightly flattened with the coverslip); (b) unfertilized egg (slightly flattened with the coverslip); (c) fertilized egg; (d) 2-cell stage; (e) 4-cell stage, seen from the animal pole; (f) 8-cell stage; (g) 16-cell stage (the micromeres are toward the bottom); (h) 32-cell stage; (i) morula; (j) early blastula (the external edge has not yet become uniformly smooth); (k) blastula; (1) hatching blastula (10 hours after fertilization). (m) Swimming blastula; (n) early mesenchyme blastula; (o) late mesenchyme blastula; (p) early gastrula; (q) late gastrula; (r) early prism, viewed from the ventral and vegetal side; (s) prism viewed from the ventral side; (t) same as in (s), further rotated toward the vegetal side; (u) a developmental stage between the prism and the pluteus [same orientation as in (t)]; (v) pluteus [same orientation as in (t) and (u)]; (w) late pluteus (48 hours after fertilization) viewed from the vegetal side; (x) same as in (w), viewed from its right side. (Magnification of w, x: x 180.)

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Chapter 2 – Bisphenol A affects neural development of the ascidian *Ciona robusta*

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2.1. Abstract

Bisphenol A (BPA) is an organic pollutant derived from plastic degradation that has numerous and variable adverse effects on human health and wildlife. In particular, it has been reported that BPA can alter reproductive processes and nervous system development in vertebrates. Considering BPA presence in marine environment and the scant data available on its interaction with nervous system development, we analyzed the effect of BPA exposure on sperm viability, fertilization, embryogenesis and neural differentiation of the ascidian *Ciona robusta*. Ascidians are members of the Phylum Tunicata, the sister group of Vertebrata, sharing with them fundamental developmental processes. Our results showed that first cell division was altered starting from 5 µM concentration. LC_{50} was estimated to be 5.2 µM. Larvae developed from treated embryos showed specific malformations to the pigmented sensory organs even at 0.1 µM, corresponding to the highest environmental concentration reported so far. Moreover, GABAergic and dopaminergic neurons proved to be target organs of BPA teratogenic action, in accordance with similar results reported in vertebrate animal models. Overall, our results suggest that BPA can exert its effects on nervous system acting on different pathways and underline that *C. robusta* is a valuable invertebrate animal model for preliminary screenings of effects of pollutants on vertebrates.

2.2. Introduction

Bisphenol A (BPA; 2,2-bis-(4-hydroxyphenyl)-propane; CAS Registry No. 80-05-07) is an organic compound used in the manufacturing of polycarbonate plastics and epoxy resins, the former employed in the realization of food and beverage containers, the latter in dental materials, for lining food and beverage containers and water supply pipes (NTP 2008). In 2013, the global consumption of BPA exceeded 5.0 million tons (Lin et al 2017). In 2015, BPA was one of the highest volume chemicals produced worldwide (more than 3.0 million tons/year; Gao et al 2015). The European Food Safety Authority (EFSA) has evaluated BPA toxicity several times proposing a temporary Tolerable Daily Intake (t-TDI) of 4 µg/kg bw per day, but this value will be reconsidered in 2018 (EFSA 2017).

BPA can be released into the environment from degradation of BPA-based products, from sewage treatment effluent and landfill leachates (Crain et al 2007). It is also discharged from wastewater treatments in fresh and marine waters, where it can negatively interfere with animal physiology and development. BPA effects on living organisms are particularly numerous and variable, as it can act both as an endocrine disruptor by binding to different nuclear receptors

(Rubin 2011) and as teratogenic molecule (Iwamuro et al 2003, Kiyomoto et al 2006, Rubin 2011, Sone et al 2004, Yang & Chan 2015). Most of the previous research focused on BPA reproductive toxicity in aquatic vertebrates such as fish and amphibians in which it mainly caused reduction of sperm quality, alteration of sex steroid levels, embryonic deformities and intersex condition (reviewed in Flint et al (2012)). Studies on invertebrates showed the absence of reproductive effects at BPA environmental concentrations in cnidarians, nematodes and crustaceans (Hoshi et al 2003, Marcial et al 2003, Pascoe et al 2002, Tominaga et al 2003). However, chronic exposure altered or suppressed crustaceans development and growth (Andersen et al 1999, Mariager 2001), altered nematodes feeding behavior (Kohra et al., 2002) and caused sponges morphological deformities (Hill et al 2002). Moreover, recent studies demonstrated that BPA can have adverse effects on nervous system development. Although data are still very scarce, dopaminergic and GABAergic neurons seemed to be the principal targets of BPA neural action. In mice, BPA altered the postsynaptic regulation of dopaminergic neurotransmission (Suzuki et al 2003), as also demonstrated by the immunoreactivity increase of TH-positive cells in *nucleus accumbens* (Miyagawa et al 2007). Though, in rhesus monkeys, BPA caused a decrease in dopamine neurons in the midbrain (Elsworth et al 2013). GABAergic pathway is similarly affected by the exposure to BPA. In rats, BPA high and low doses resulted in opposing changes of mRNA expression of genes involved in GABA synthesis (GAD), reuptake (GAT), and signaling, with particularly complex modulatory effects on $GABA_A$ receptors (Choi et al 2007, Franssen et al 2016).

Considering BPA presence in marine environment and the scant data available on its interaction with nervous system development, in this study, we evaluated the effects of BPA exposure on the ascidian *Ciona robusta*. Ascidians were showed to be a reliable model organism for ecotoxicology bioassays due to their phylogenetic position, being sister group of vertebrates (Delsuc et al 2006, Putnam et al 2008), their wide distribution in marine ecosystems and their ecology (Gallo & Tosti 2015, Zega et al 2009). *Ciona* sessile adults develop through a swimming larva that presents all the main features of chordate body plan, comprising a hollow neural tube lying dorsal to a rod-like notochord (Passamaneck & Di Gregorio 2005). Larval central nervous system (CNS) arises by the enrolment of the neural plate and it is subdivided morphologically into the sensory vesicle, bearing two pigmented sensory organs (the otolith and the ocellus), the neck region, the visceral ganglion with five pairs of motoneurons, and the caudal nerve cord comprising primarily ependymal cells (Nicol & Meinertzhagen 1991). It consists of ~ 330 cells, of which ~100 are predicted to be neurons (Passamaneck & Di Gregorio 2005); even so small, CNS comprises different neural subpopulations that have been identified based on their

neurotransmitter content (Horie et al 2009). Moreover, expression patterns of transcription factors that regulate neural anterior–posterior patterning are conserved between ascidians and vertebrates (reviewed in Manni & Pennati (2016)), allowing to highlight conserved functional regions and neural circuits. In ascidians, fertilization occurs in seawater and so gametes and embryos may be subjected to the effects of many environmental pollutants (Villa et al 2003), making larvae suitable model organisms for testing neurotoxicity of environmental contaminants such as BPA. Previous studies have already analyzed BPA effects on ascidian embryonic development, reporting a general impartment of the process (Cangialosi et al 2013, Matsushima et al 2013). Fertilized eggs exposed to BPA concentrations higher than 1 µM did not reach the larval stage (Cangialosi et al 2013), while exposure from 2-cell stage decreased the larval hatching rate in a dose-dependent manner at concentrations higher than 3 µM (Matsushima et al 2013). Starting from these data, we further investigated adverse effects induced by BPA exposure on *Ciona robusta*, analyzing sperm viability, fertilization and embryogenesis. Particularly, we focused our attention on the effects on nervous system development to verify the existence of specific neural populations sensitive to BPA action.

2.3. Materials and methods

2.3.1. Animals and chemicals

Adults of *C. robusta* were collected from natural populations in Chioggia (Italy) and reared in 50 L aquaria equipped with mechanical, chemical and biological filters. Animals were maintained at 16±1°C and in constant light condition to avoid gametes release and stimulate their production (Lambert & Brandt 1967). Three animals were sacrificed each time, eggs and sperm were obtained by dissection of gonoducts and cross-fertilization was performed. All experimental procedures were carried out at 18±1°C. Treatments were performed in Petri glass dishes (Ø 4 cm). Bisphenol A (BPA, MW=228.29) was purchased from Sigma, Milan, Italy. Stock solution of 100 mM BPA was made in dimethyl sulfoxide (DMSO, Sigma, Milan, Italy) and then diluted in filtered artificial sea water with 1 M Hepes pH 8.0 (ASWH) to reach the final test concentrations (0.1, 0.5, 1, 5, 10, and 20 µM). Fresh solutions were prepared every time. As solvent control, a solution of 0.02% DMSO in ASWH was used.

2.3.2. Effects on sperm viability

To study the effects of BPA on sperm viability, 300 µl of sperm dilution (20 µl of dry sperm in 2.4 ml of ASWH) were added to vials containing 5 ml of testing solutions: ASWH as control; 0.1, 0.5, 1, 5, 10, and 20 µM BPA in ASWH; 0.02% DMSO in ASWH as solvent control. Sperm

was treated for 30 min before being used for fertilization. Then, 200 µl of these sperm solutions were added to Petri dishes containing 5 ml of ASWH and 60 eggs. When the controls reached the two-cell stage (approximately 1 hour post fertilization (hpf)), developing embryos were fixed in 4% paraformaldehyde in PBS (PFA) for 1 h at room temperature and then analyzed at a stereomicroscope. Fertilization rate (FR), determined as percentage of fertilized eggs which achieved the first division, was calculated. The experiment was repeated three times with different animal batches and results were considered reliable only when control FR was ≥ 80%.

2.3.3. Effects on fertilization

To test BPA effects on fertilization, 60 unfertilized eggs and 15 µl of sperm dilution (20 µl of dry sperm in 2.4 ml of ASWH) were added to Petri dishes containing 5 ml of: only ASWH for controls, different BPA concentrations of (0.1, 0.5, 1, 5, 10, and 20 µM) and DMSO control solution. When control embryos reached the two-cell stage (approximately 1 hpf), samples were fixed in 4% PFA in PBS and embryos completed the first cellular division were counted at a stereomicroscope. FR was then calculated as (number of 2-cell stage embryos / total number of embryos) x 100. The experiment was performed three times with different animal batches and results were considered reliable only when control FR was ≥ 80%.

2.3.4. Effects on embryogenesis

To analyze the effects of BPA on embryogenesis, three adult specimens were sacrificed for gametes collection. Cross-fertilization was performed in ASWH. When embryos reached the two-cell stage (~1 hpf), approximately 100 embryos were transferred in Petri dishes filled with 10 ml of 0.1, 0.5, 1, 5, 10, 20 µM BPA in ASWH. Control embryos were maintained in ASWH and in ASWH plus 0.02% DMSO. Each experiment was replicated at least three times and considered reliable only if 80% of control embryos hatched. When control embryos reached the swimming larva stage (~19 hpf), all specimens were fixed in 4% PFA in PBS and examined under a dissection microscope. For each treatment, the number of normal larvae, larvae with malformations and dead samples was counted and the corresponding percentage was calculated. Fifty larvae for each treatment were randomly chosen and observed under optical microscope: the pigmented organs were scored as normal (one otolith and one ocellus completely formed; Fig. 3B), mildly affected (one otolith and one ocellus with abnormal deposition of pigment; Fig. 3F) or severely affected (absence of one or both pigmented organs or presence of extra pigmented organs; Fig. 3G-H). Then, specimens were dehydrated to 70% ethanol and stored at -20°C for further analyses.

2.3.5. Immunohistochemistry

Immunolocalization experiments were performed as described in Pennati et al (2003). Briefly, after rehydration, specimens were rinsed in PBT (0.1% Tween-20 in PBS), permeabilized for 30 minutes in PBTT (PBS; 0.1% Tween-20; 0.25% Triton X-100) and incubated for 2 hours in 50% PBT / 50% normal goat serum, previously deactivated at 55°C for 30 min. Then, samples were incubated overnight at 4°C with anti-β tubulin monoclonal antibody (clone 2-28-33, Sigma, Italy), diluted 1:500 in PBT. After several washes in PBT, samples were blocked in 1% bovine serum albumine (BSA) in PBT for 2 h at room temperature and then incubated at 4°C overnight in Alexa Fluor 488 Anti-mouse IgG, diluted 1:800 in PBT. Specimens were washed multiple times in PBT and mounted in 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma, Italy) on microscope slides.

Samples were examined using a confocal laser scanning microscope Leica SP2 (Leica Microsystems, Heidelberg, Germany), equipped with an argon/krypton laser.

2.3.6. *In situ* hybridization

In order to evaluate the BPA effects on embryo neural development, expression pattern of neural marker genes was assessed by *in situ* hybridization using riboprobes for *Ci-ETR*, a pan-neural marker (Satou et al 2001), *Ci-GAD*, specific for GABAergic neurons (Zega et al 2008), *Ci-TH* for dopaminergic neurons (Moret et al 2005), synthetized using Dig-RNA labelling Kit (Roche, Germany) according to the manufactory instructions. In larvae with malformations on pigmented organs, *in situ* hybridization using *Ci-opsin1* riboprobe (Kusakabe et al 2001) was performed to discriminate between otolith and ocellus.

Whole mount *in situ* hybridization was performed as described in Boorman & Shimeld (2002) with some modifications. After rehydration, samples were permeabilized with 4 μg/ml proteinase K for 10 minutes at 37°C. After post-fixation in 4% PFA in PBT, embryos were washed twice in hybridization solution (50% formamide, 5× SSC, 100 μg/ml yeast RNA, 50 μg/ml heparin, 0.1% Tween 20) at room temperature and, then, pre-hybridization was performed for 2 h at 50°C. Hybridization was carried out overnight at 50°C. Embryos were washed 6 times for 20 minutes in 50% formamide, 5× SSC, 0.1% Tween 20 and after PBT washes they were transferred in blocking solution (sheep serum : PBT, 1:4) for 2 h. Embryos were incubated overnight at 4°C in blocking solution with anti DIG antibody (1:2000). After several washes in PBT, embryos were incubated in APT buffer (100 mm NaCl, 100 mm Tris HCl, pH 9.5, 50 mm MgCl2, 1% Tween 20) and then staining solution (APT buffer + 2.3 μl/ml NBT and 3.5 μl/ml

BCIP) was added. Staining reaction was performed in dark conditions at room temperature. When satisfactory signals were obtained, embryos were fixed with 4% PFA for 1 hour, mounted with 80% glycerol in PBT and photographed under a microscope equipped with Leica DFC-320 Camera.

ImageJ software (Schindelin et al 2015) was employed to further analyze and compare *Ci-TH* signal between controls and BPA treated samples. Stained area of each samples was measured and the size of the area was determined.

2.3.7. Statistical analysis

The analysis of variance (ANOVA) followed by HSD Tukey's post hoc test, was performed with R software (R-Core-Team 2013) and 'agricolae' package (de Mendiburu 2015) to evaluate the differences in fertilization rate and in larval development of samples treated with different BPA concentration. A Cochran test was performed to test the homogeneity and normality of the variances and percentage data were transformed when they did not meet the assumptions of the analysis (normality and homoscedasticity). The differences in pigmented organs development were statistically analyzed with $\chi 2$ test. Probit analysis was performed following the simple least squares regression method to calculate median lethal concentration (LC_{50}) and median teratogenic concentration (TC_{50}) using StatPlus software (AnalystSoft Inc. Vancouver, BC, Canada).

2.4. Results

2.4.1. Effects on sperm viability and fertilization

Three replicates of exactly 60 eggs were fertilized with sperm exposed to each of the BPA concentrations tested. As reported in Table 1, 30-minutes pre-exposure of sperm to different BPA concentrations, from 0.1 µM to 20 µM, had no effect on the sperm fertilization capability in *C. robusta* (ANOVA: F=0.7929; p=0.604). The mean fertilization rate was always higher than 91% and no significant difference between controls and treated groups was observed (Fig. 1A).

Similarly, three replicates of exactly 60 eggs were fertilized in presence of different concentrations of BPA and the co-exposure of eggs and sperm did not affect fertilization rate (Table 2; ANOVA: F=0.2434, p=0.9672; Fig 1B). However, concentrations higher than 5 µM caused incomplete egg division resulting in heart shaped embryos that did not develop further. As shown in Fig. 1C, the percentage of embryos that successfully completed the first division drastically fell at 5, 10 and 20 µM BPA and differences were statistically significant (Table 2;

ANOVA: F=101.92; p<0.0001; Tukey's post hoc test control *versus* 5 µM p<0.0002; control *versus* 10 µM p<0.0001; control *versus* 20 µM p<0.0001).

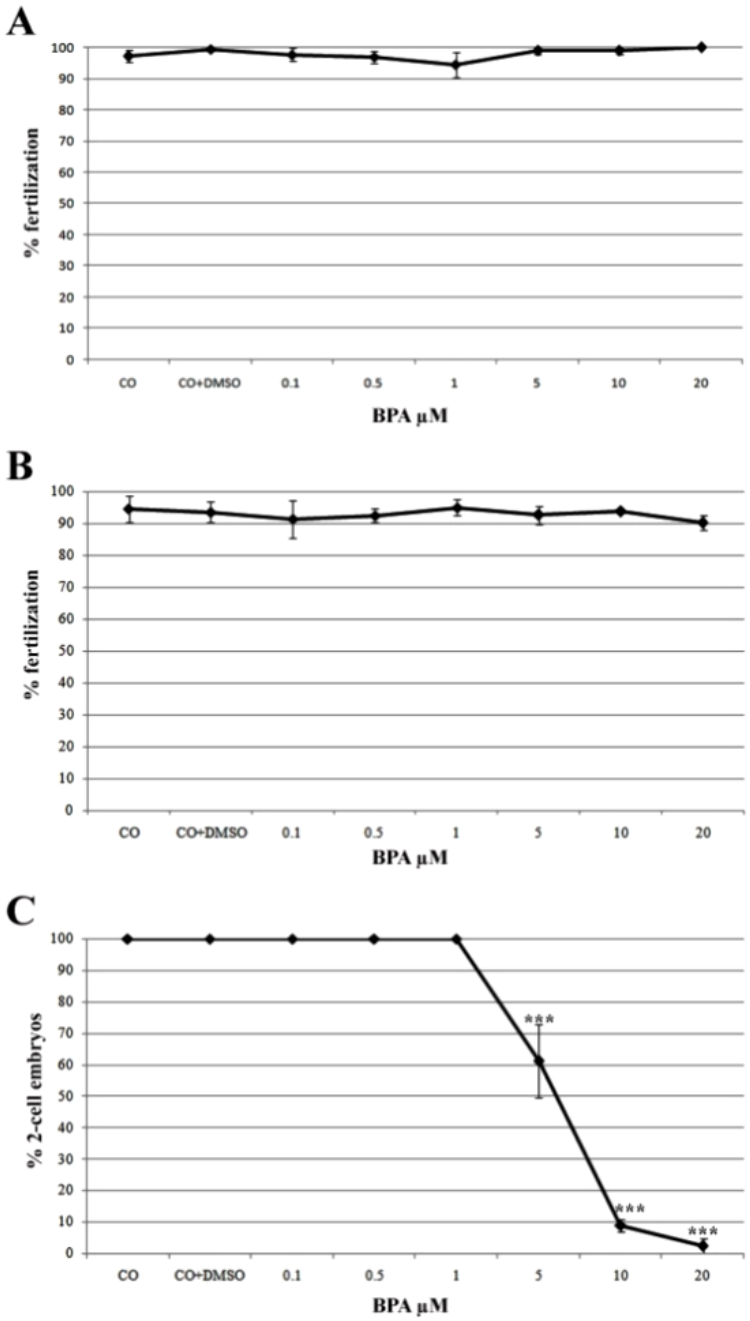

Figure 1: Effects of different concentrations of BPA on A) sperm viability, B) fertilization rate and C) first cell division in *Ciona robusta.* Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from control. The repetition of each symbol indicates the level of significance according to R significance codes: p<0.001 ***; p<0.01 **; p<0.05 *.

	Replicate 1		Replicate 2		Replicate 3	
	2-cell stage %	**eggs %**	**2-cell stage %**	**eggs %**	**2-cell stage %**	**eggs %**
Control	93.33	6.67	100.00	0.00	98.28	1.72
DMSO	100.00	0.00	100.00	0.00	98.31	1.69
0.1 µM BPA	93.33	6.67	100.00	0.00	100.00	0.00
0.5 µM BPA	93.33	6.67	100.00	0.00	97.78	2.22
1 µM BPA	86.67	13.33	96.67	3.33	100.00	0.00
5 µM BPA	96.67	3.33	100.00	0.00	100.00	0.00
10 µM BPA	100.00	0.00	100.00	0.00	96.72	3.28
20 µM BPA	100.00	0.00	100.00	0.00	100.00	0.00

Table 1: Percentage of fertilized (2-cell stage) and not fertilized eggs after sperm exposure to different BPA concentrations in *Ciona robusta*. Data for each replicate are reported.

	Replicate 1			Replicate 2			Replicate 3		
	2 cell stage (%)	**incomplete division (%)**	**unfertilized egg (%)**	**2 cell stage (%)**	**incomplete division (%)**	**unfertilized egg (%)**	**2 cell stage (%)**	**incomplete division (%)**	**unfertilized egg (%)**
Control	86.67	0.00	13.33	96.88	0.00	3.13	100.00	0.00	0.00
DMSO	91.89	0.00	8.11	88.89	0.00	11.11	100.00	0.00	0.00
0.1 µM BPA	80.00	0.00	20.00	93.75	0.00	6.25	100.00	0.00	0.00
0.5 µM BPA	90.70	0.00	9.30	96.88	0.00	3.13	90.00	0.00	10.00
1 µM BPA	97.50	0.00	2.50	97.37	0.00	2.63	90.00	0.00	10.00
5 µM BPA	72.92	20.83	6.25	58.06	29.03	12.90	37.50	59.38	3.13
10 µM BPA	6.25	87.50	6.25	6.67	86.67	6.67	12.12	81.82	6.06
20 µM BPA	6.25	84.38	9.38	0.00	93.75	6.25	0.00	86.11	13.89

Table 2: Percentage of embryos at 2 cell-stage. embryos with incomplete cell division and unfertilized eggs after 1 hour from fertilization in presence of different BPA concentrations in *Ciona robusta*. Data for each replicate are reported.

2.4.2. Effects on embryogenesis

Three replicates of about 100 embryos were exposed to different BPA concentrations during the development. BPA median lethal concentration (LC_{50}) for *Ciona robusta* resulted 5.2 µM (Fig. 2A). All embryos died when exposed to 20 µM BPA (Fig. 3E).

A control larva is showed in Fig. 3A and B. It developed normally, with an elongated trunk, a sensory vesicle containing two well-differentiated pigmented organs (otolith and ocellus); the tail appeared straight and elongated. BPA exposure strongly affected larvae morphology and two

main kinds of malformations were observed. The first type comprised short and kinked tail, malformed roundish trunk (Fig. 3C and D). The percentage of larvae displaying this phenotype increased with BPA concentration (Table 3; Fig. 2B) and was significantly higher at 10 µM BPA (ANOVA: F =15.806; p=0.00428, Tukey's post hoc test control *versus* 10 µM p<0.0001).

The second kind of malformation consisted of specific alterations of pigmented organs, namely absence of one or both pigmented organs or presence of supernumerary ones (Fig. 3F-I). 50 larvae for each treatment were observed under a microscope to classify the kind of malformations. Incidence of pigmented organs defects is showed in Fig. 2D and reported in Table 4. Mild alterations, characterized by abnormal deposition of pigments in otolith and/or ocellus (Fig. 3F) increased with BPA concentration until 5 µM and differences were statistically significant (p<0.001). At 10 µM BPA the percentage of severe malformations, including absence of one or both pigmented organs or extra ones, increased significantly (Controls *versus* 10 µM BPA: p<0.0001). TC_{50} for this malformation resulted 0.16 µM (Fig. 2C). The teratogenic index ($TI=LC_{50}/TC_{50}$) resulted 32.5.

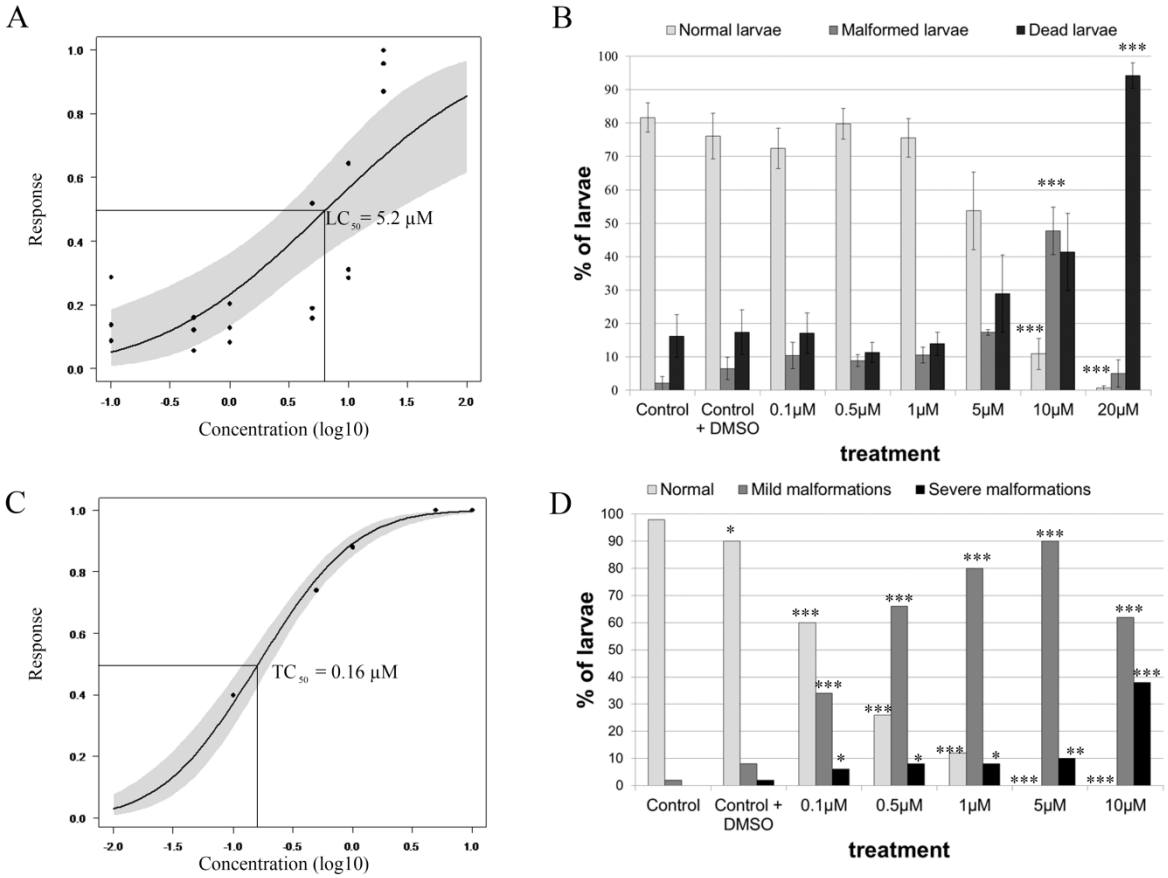

Figure 2: Effects of BPA on embryogenesis in *C. robusta*. A) LC_{50} predicted value calculated with probit analysis. B) Percentage of normal, malformed and dead larvae exposed to different concentrations of BPA. Mean values of three replicates and standard errors are indicated. C) TC_{50} predicted value for pigmented organs malformations, calculated with probit analysis. D) Incidence of pigmented organs malformations. Legend of symbols: *= differences from control. The repetition of each symbol indicate the level of significance according to R significance codes: p<0.001 ***; p<0.01 **; p<0.05 *.

	Replicate 1			**Replicate 2**			**Replicate 3**		
	Normal larvae (%)	**Malformed larvae (%)**	**Dead larvae (%)**	**Normal larvae (%)**	**Malformed larvae (%)**	**Dead larvae (%)**	**Normal larvae (%)**	**Malformed larvae (%)**	**Dead larvae (%)**
Control	81.25	0.00	18.75	89.58	6.25	4.17	74.19	0.00	25.81
DMSO	76.92	0.00	23.08	87.50	8.33	4.17	63.89	11.11	25.00
0.1 µM BPA	73.53	17.65	8.82	82.35	3.92	13.73	61.54	9.62	28.85
0.5 µM BPA	80.70	7.02	12.28	87.14	7.14	5.71	71.43	12.50	16.07
1 µM BPA	75.93	11.11	12.96	85.42	6.25	8.33	65.31	14.29	20.41
5 µM BPA	68.18	15.91	15.91	62.26	18.87	18.87	30.77	17.31	51.92
10 µM BPA	14.29	57.14	28.57	16.67	52.08	31.25	1.69	33.90	64.41
20 µM BPA	2.08	2.08	95.83	0.00	12.96	87.04	0.00	0.00	100.00

Table 3: Percentage of larvae with normal or malformed phenotype and of dead larvae after development in different BPA concentrations in *Ciona robusta*. Data for each replicate are reported.

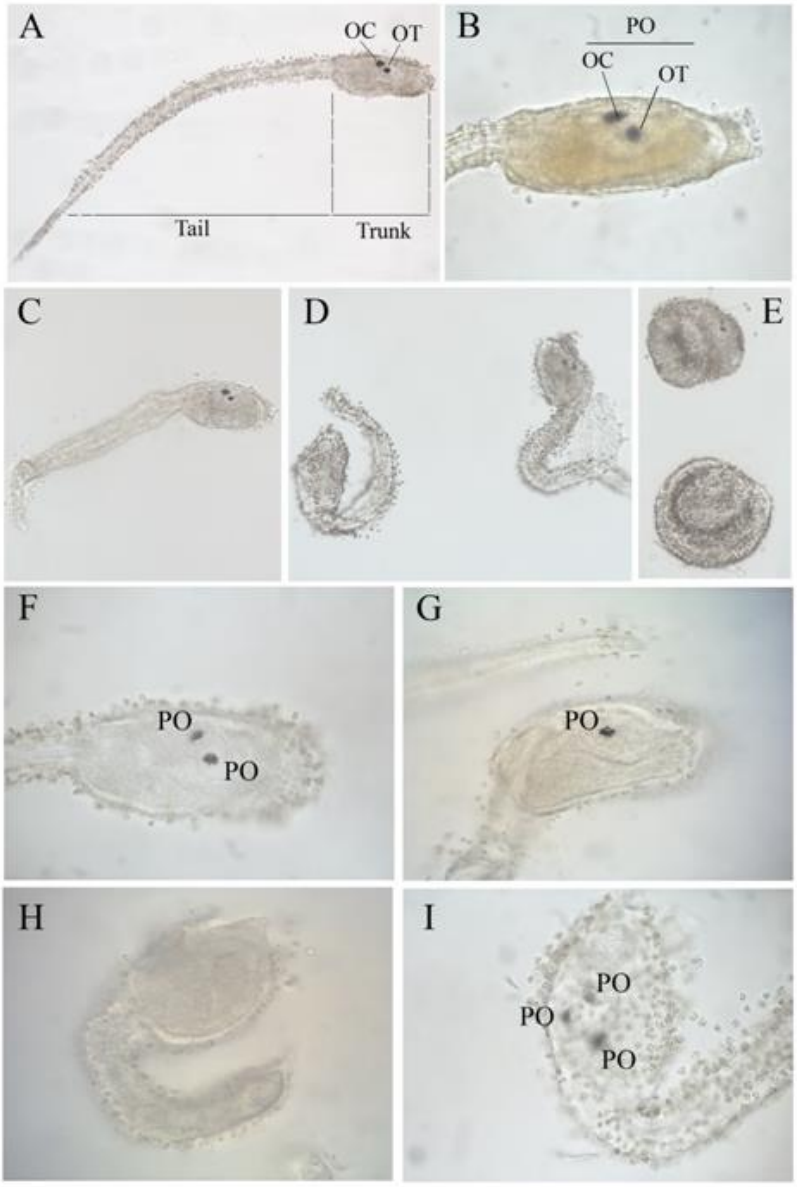

Figure 3: Phenotypes of BPA exposed larvae. A,B) Control larva. C, D), Malformed larvae exposed to 10µM BPA. E) Dead larvae. F) Mild malformation of pigmented organs with abnormal deposition of pigment. G-I) Severe malformations to pigmented organs. larvae display rispectively one, no or three pigmented organs.

	Normal phenotype	**Mild malformations**	**Absence of P.O**	**Supernumerary P.O.**	**One P.O.**
Control	96%	2%	0%	0%	2%
DMSO	90%	8%	2%	0%	0%
0.1 µM BPA	60%	34%	0%	0%	6%
0.5 µM BPA	26%	66%	0%	2%	6%
1 µM BPA	12%	82%	2%	2%	2%
5 µM BPA	0%	90%	0%	0%	10%
10 µM BPA	0%	62%	14%	2%	22%

Table 4: Incidence (%) of pigmented organ malformations in larvae exposed to different BPA concentrations. P.O. = pigmented organs.

2.4.3. Immunohistochemistry

The nervous fibers pattern of control and treated larvae was stained with anti-β tubulin antibody. This antibody labels the primary structure of nervous system composed by neurons of the palps, papillary nerves and the nervous fibers around the sensory vesicle and in the posterior dorsal neural tube (Fig. 4A and C). Larvae of *C. robusta* developed in 10 µM BPA showed a disruption of fibers in the tail and a disorganization of neurons in the sensory vesicle (Fig. 4B and D).

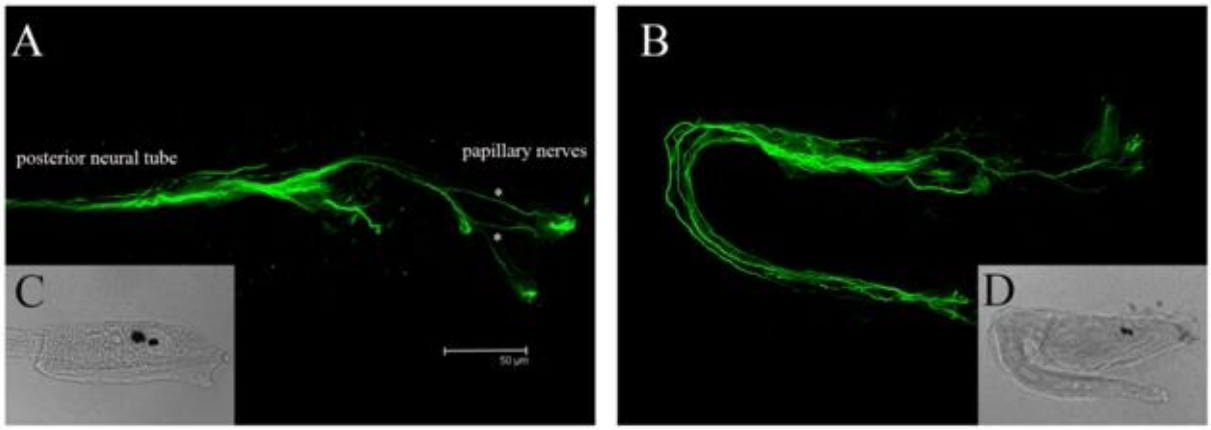

Figure 4: Immunostaining of the nervous system with anti β-tubulin antibody in *C. robusta*. Bright field images and confocal microscope. A,C) Control larvae; B,D) Larvae developed from embryos treated with 10 µM BPA. OC= Ocellus; OT=Otolith.

2.4.4. *In situ* hybridization

In situ hybridization analyses were performed only in samples displaying larval typical morphology to allow an accurate interpretation of the results.

In control larvae, normal expression of the pan-neural marker, *Ci-ETR,* was detectable in all the CNS regions (Fig. 5A). This expression pattern was not altered in larvae exposed to BPA at all the tested concentrations (Fig. 5B-D), suggesting that neurons differentiation occurred properly. When specific neural populations were investigated, differences in marker genes expression were observed. In control larvae, *Ci-GAD,* the synthesis enzyme of GABA, labelled GABAergic neurons located in a wide portion of the posterior sensory vesicle and in the visceral ganglion (Table 5; Fig. 5E). This expression was conserved in larvae exposed to 1 µM BPA (Table 5; Fig. 5F), whereas in larvae exposed to 5 and 10 µM BPA, this expression was disorganized or in few cases strongly reduced (Table 5; Fig. 5G and H). *Ci-TH* is the rate-limiting enzyme of dopamine synthesis and in control larvae it was expressed in few cells in the ventral region of the sensory vesicle (Table 6; Fig. 5I). The area of expression of *Ci-TH* was expanded in larvae treated with

1, 5 and 10 µM BPA (Table 6; Fig. 5L-N), probably due to a BPA-induced increase of *Ci-TH* positive cells (Fig. 6A and B). Extent of area occupied by *Ci-TH* positive neurons further suggested that dopaminergic neurons were altered in BPA treated embryos (Fig. 6C): the differences in the area occupied by *Ci-TH* positive neurons between control and all the other treatments were statistically significant (ANOVA: F=10.93; p =0.00038, Tukey's post hoc test controls *versus* 1 µM p=0.0199; control *versus* 5 µM p=0.0005; controls *versus* 20 µM p=0.0011).

As previously described in Horie et al., (2008), *Ci-Opsin1* expression in control larvae is observed in the photoreceptor cells surrounding the pigmented ocellus and in a group of cells belonging to the non-pigmented ocellus (Fig. 7A). Using this marker, the nature of pigmented cells of larvae exposed to 10 µM BPA was distinguished: 64% of samples showed a normal phenotype (Table 7), while 11% showed a single ocellus (Fig. 7B; Table 7), 15% showed a single otolith (Fig. 7C; Table 7). In a minority of samples, the two pigmented organs turned out to be two ocelli (2%; Fig 7D; Table 7) or two otoliths (4%; Fig. 7E; Table 7). At last individuals without pigmented organs showed a signal in the photoreceptor cells of the non pigmented ocellus (3%; Fig. 7F; Table 7). Due to the very low occurrence of supernumerary pigmented cells (Table 7), no information about *Ci-opsin1* expression could be reported.

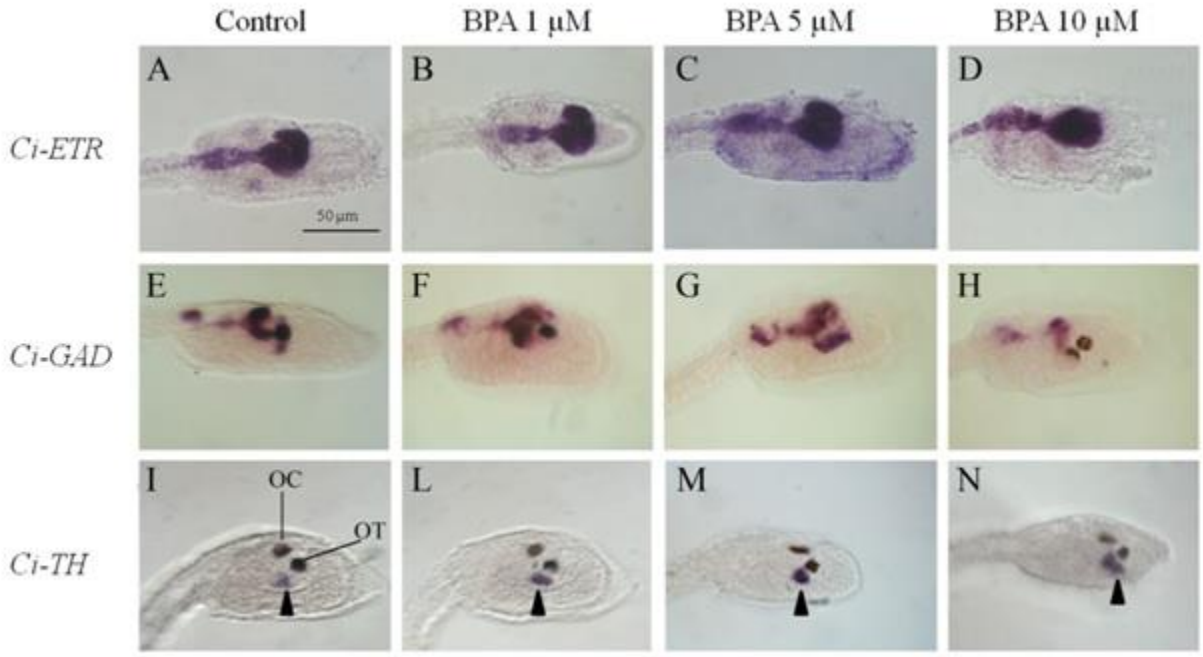

Figure 5: *Ci-ETR*, *Ci-GAD* and *Ci-TH* expression in *C. robusta* larvae exposed to different concentrations of BPA; OC= Ocellus; OT=Otolith.

	Normal signal	Disorganized signal	Reduced signal
Control	100%	0%	0%
1 µM BPA	91.6	8.4%	0%
5 µM BPA	13.3%	86.7%	0%
10 µM BPA	4.76%	76.19%	19.5%

Table 5: Number of individuals with normal. disorganized or reduced signal detected with *in situ* hybridization and *Ci-GAD* probe at different BPA concentrations.

	Normal signal	Increased signal
Control	100%	0%
1 µM BPA	0%	100%
5 µM BPA	0%	100%
10 µM BPA	0%	100%

Table 6: Number of individuals with normal or increased signal detected with *in situ* hybridization and *Ci-TH* probe at different BPA concentrations.

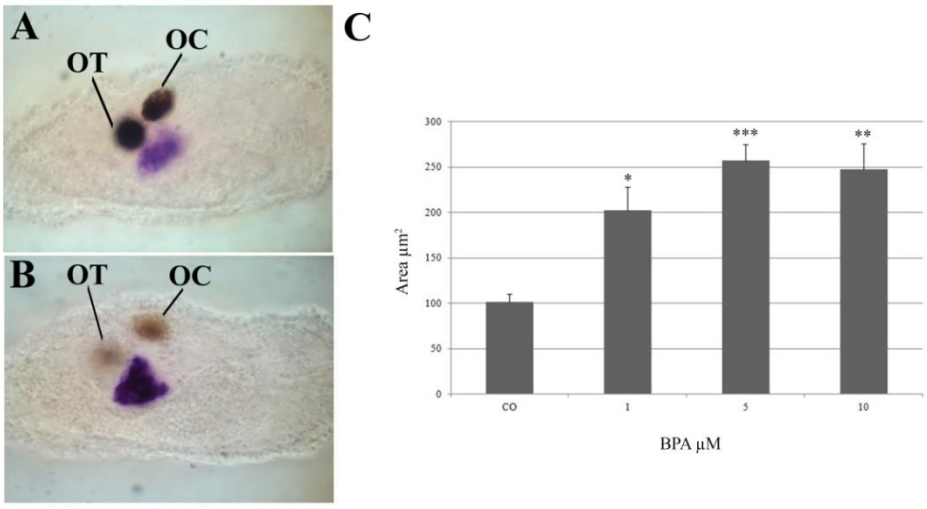

Figure 6: Details of *Ci-TH* area of expression in control larva (A) and 10 µM BPA exposed larva (B). C) Graph showing the extent of *Ci-TH* expressing area in control and BPA exposed larvae. Mean values and standard error are reported. Legend of symbols: *= differences from control. The repetition of each symbol indicates the level of significance according to R significance codes: p<0.001 ***; p<0.01 **; p<0.05 *.

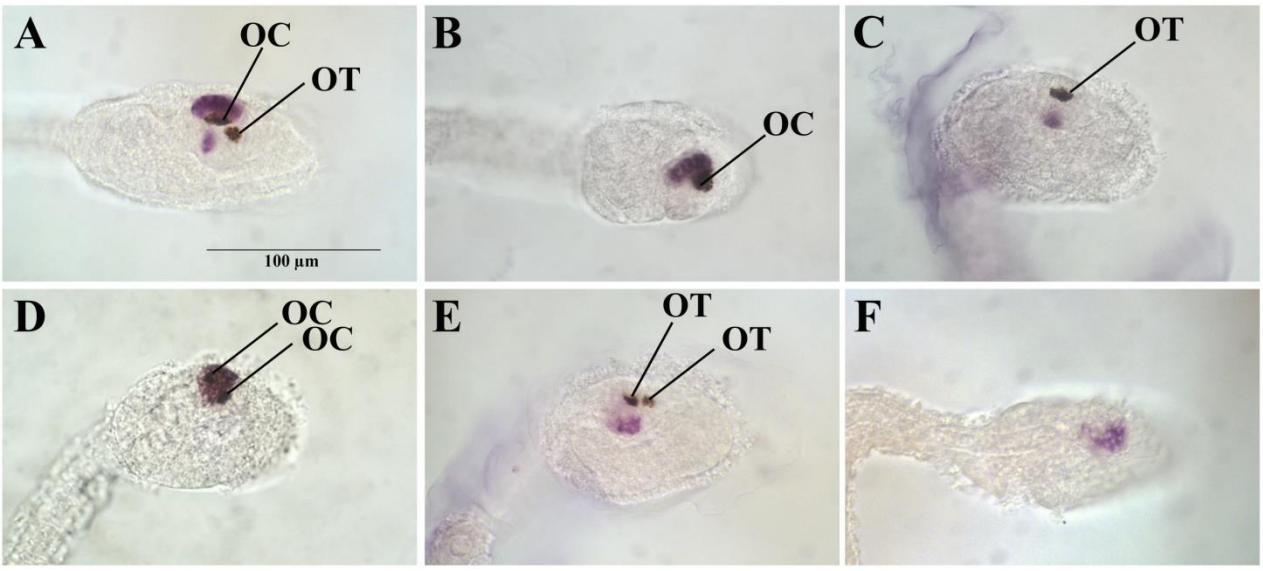

Figure 7: *Ci-opsin1* expression in *Ciona robusta*. A) Normal expression, B) larva with a single ocellus, C) larva with a single otolith, D) larva with 2 ocelli, E) larva with 2 otoliths, F) larva without pigmented organs.

	Total number	Percentage
one otolith and one ocellus	63	64.29
one ocellus	11	11.22
one otolith	15	15.31
two ocelli	2	2.04
two otoliths	4	4.08
only non pigmented ocellus	3	3.06

Table 7: Number and percentage of individuals developed in 10 µM BPA showing signals after *in situ* hybridization with *Ci-opsin1*.

2.5. Discussion

The exposure to the environmental pollutant BPA is known to cause adverse effects on both human health and wildlife (Crain et al 2007, Rochester 2013, Rubin 2011). Particularly, BPA affects different developmental processes (Flint et al 2012, Rubin 2011) but a comprehensive analysis of its effects is still lacking. In this study, we investigated BPA effects on fertilization and embryonic development of the ascidian species *C. robusta*, adding new important data about this pollutant. Due to their phylogenetic position (Delsuc et al 2006) and their ecology, ascidians represent a valuable model in ecotoxicology and particularly provide precious information about pollutant impact on chordate nervous system development (Gallo & Tosti 2015, Zega et al 2009).

First, BPA effects on ascidian fertilization was investigated. Although single gametes exposure did not seem to affect fertilization, the process appeared to be altered when fertilization occurred

in presence of this pollutant: the first cell division was, indeed, damaged at BPA concentrations higher than 5 µM. BPA deleterious effects on ascidians have been already described by Cangialosi et al (2013) which reported that in presence of 1 µM BPA *Ciona* embryos did not reach the gastrula stage, and more detrimental effects were observed at 10 µM BPA, as even morula stage was not achieved. Indeed, if exposure occurs immediately after fertilization, a concentration of 1 µM BPA is enough to prevent embryogenesis completion (Cangialosi et al 2013), probably because the first cell division is very sensitive to BPA presence, as demonstrated by our results. Even if we did not observe any impairments of first cell division at 1 µM, in these experiments we did not extend BPA exposure to later stages and thus our results could not be completely compared to those reported in Cangialosi et al. (2013). Taking into account these data, we exposed embryos after the first cell division to investigate BPA effects on larval development.

If the exposure started at 2 cell-stage, *C. robusta* embryos managed to reach the larval stage; even if the higher tested concentration of 20 µM BPA was lethal. Moreover, at 10 µM BPA most of the swimming larvae displayed different morphological alterations, such as rounded trunk and short and kinked tail, suggesting that BPA started to be toxic at this concentration. At lower concentrations, BPA seemed to affect specifically the development of pigmented sensory organs, otolith and ocellus. This effect was observed starting from 0.1 µM, corresponding to the highest environmental concentration reported so far (Belfroid et al 2002). Indeed, the high teratogenic index (TI = 32.5) indicates that this pollutant should be considered a teratogenic substance (Dawson & Bantlet 1987). Impairment of pigmented organs development was also reported in the ascidian *Phallusia mammillata* after exposure to 10 µM BPA (Dumollard et al 2017) suggesting that BPA interferes with the pathway of pigment cells differentiation.

In situ hybridization with *Ci-opsin1* probe revealed that the alterations of pigmented sensory organs are very variable: in individuals with two pigmented organs, they can be one otolith and one ocellus or two otoliths or two ocelli. In individuals with a single pigmented organ, it can be an otolith or an ocellus. Pigmented organs in ascidians originate from an equivalence group, so the two bilaterally positioned cells that will become the pigment cells are equipotent at least until mid gastrula stage (Nishida & Satoh 1989). In *Ciona* the inductive signals necessary for pigment cell precursor specification come from A-line cells (Nishida 1991, Nishida & Satoh 1989) and both FGF and Wnt signaling pathways have been demonstrated to be involved in the process. FGF signaling acts as a sequential inducer during pigment cell development, defining both their fate specification and later differentiation. Indeed, in absence of FGF successive inputs, pigment cell precursors turn their fate into anterior CNS cells (Racioppi et al 2014). FGF, via MAPK

cascade, regulates several aspects of early pigment cell formation (Haupaix et al 2014, Racioppi et al 2014), and crosstalk with other fundamental pathways is necessary to proper pigment cell development. ERK1/2 activation is restricted in pigment cell lineage by Eph/ephrin signals and their inhibition leads to supernumerary pigmented organs (Haupaix et al 2014). FGF/MAPK/Ets controls the transcription of *Ci-Tcf*, a Wnt downstream effector, making precursors pigment cells competent to Wnt signal. In particular, FGF signal is transmitted in the cells through the MAPK cascade which activates the transcription factor Ci-Ets1/2. This latter regulates *Ci-Tcf* expression, making pigment cell precursors ready to respond to Wnt canonical pathway (Squarzoni et al 2011). Furthermore, Wnt signaling was proved to play a key role in determining the differentiation in ocellus and otolith. Misexpression of Wnt7 resulted in the development of two ocelli while both melanocytes differentiate into otolith when Tcf was perturbed (Abitua et al 2012). The alterations we observed on embryos exposed to the lowest BPA concentrations were comparable to those described after perturbation of FGF and Wnt pathways, mainly concerning terminal differentiation, thus suggesting that BPA can interfere with these pathways. Further analyses are required to test at which level BPA acts in *Ciona robusta*.

BPA-induced alterations in brain and in sensory organs was also reported in zebrafish (Gibert et al 2011, Tohmé et al 2014), in which malformations were demonstrated to be due to the BPA interaction with Estrogen Related Receptor (ERR; (Tohmé et al 2014)). In *Ciona* genome, a single ERR is present (Aniseed database: https://www.aniseed.cnrs.fr/aniseed), supporting the hypothesis that also in ascidians part of BPA effects can be mediated by its binding with this receptor.

In the last years, BPA effects on CNS development have started to be explored. Although, immunostaining with anti-β tubulin showed alterations of fibers pattern in *C. robusta*, *Ci-ETR* expression showed that BPA did not alter the global organization of nervous system, even at 10 µM. However, hybridization with markers for specific neuronal populations revealed important modifications. BPA is known to act in a complex way on different elements of GABAergic pathways: at low doses it potentiated the response of $GABA_A$ receptors in *Xenopus*, but at high doses BPA inhibited this response (Aoshima et al 2001). Rat exposure to high and low doses resulted in opposing changes of mRNA expression of genes involved in GABA synthesis (*GAD*), reuptake (*GAT*), and signaling (*$GABA_A$* receptor subunits; Franssen et al., 2016); complex modulatory effects of BPA on $GABA_A$ receptors have been reported also by Choi et al (2007). In *Ciona*, the expression pattern of *Ci-GAD*, a marker for GABAergic cells, is strongly modified by BPA that induced an evident ectopic expression. BPA also acted on dopaminergic pathways, increasing D_1 receptor mRNA in mice brains (Suzuki et al 2003). It has been reported that it

decreased dopamine neurons in non-human primates (Elsworth et al 2013) and potentiated central dopaminergic neurotransmission, altering dopamine responsiveness in neurons and astrocytes (Miyatake et al 2006). Moreover, BPA increased TH and dopamine transporter like immunoreactivity in mouse (Miyagawa et al 2007). These data are in agreement with what we observed in ascidians. In these lower chordates, BPA showed to affect dopaminergic neural population: in larvae developed in 1, 5 or 10 µM BPA, *Ci-TH* signal appeared stronger and extended to a wider area. In particular, we noticed that BPA treated samples showed a group of Ci-TH positive cells just above the ones described in controls, suggesting that this pollutant induces an increase of the number of dopaminergic cells. While the effects on *Ci-TH* were also observable at concentrations lower than the LC_{50}, the effects on *Ci-GAD* expression were present only at higher concentrations and could be possibly imputed to the toxic effects of BPA. However, the absence of alterations in *Ci-ETR* expression at the higher concentrations suggest the hypothesis that the reported effects were BPA specific and not induced by the close-to-lethal concentration of exposure.

Taken together, these results showed that BPA specifically affects nervous system development being GABAergic, dopaminergic neurons and pigmented sensory organs its main targets. Although BPA molecular mechanism of action is still scarcely understood, our data suggest that it can interfere with different signaling pathways leading to neuron terminal differentiation and/or ERR regulation.

In conclusion, BPA was shown to influence different developmental processes, causing malformations, death and nervous system impairment in ascidians. Our data highlight the importance of deeply investigating the effects of this environmental pollutant on different biological targets. The similarity of effects reported in ascidians and in vertebrates underlines that *Ciona* is a valuable invertebrate model for testing pollutants and investigates their way of action.

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Chapter 3 – Effects of Bisphenol A on the development of pigmented organs in the ascidian *Phallusia mammillata*

Data published in Messinetti S., Mercurio S., Pennati R. (2018). Effects of Bisphenol A on pigmented organ development in the ascidian *Phallusia mammillata*. Invertebrate biology 137:329–338. https://doi.org/10.1111/ivb.12231

3.1. Abstract

Bisphenol A (BPA) is an organic compound that is used in the manufacture of polycarbonate plastic and epoxy resins and is increasingly being released into the environment. BPA can act as a teratogenic substance and an endocrine disruptor, raising concerns about its impact on humans and wildlife. Thus, in the present work, we evaluated the effects of different concentrations of BPA on ontogenetic processes in the ascidian *Phallusia mammillata*. The phylogenetic position of ascidians, their cosmopolitan distribution in marine ecosystems, and multiple technical advantages associated with their biology make ascidians reliable model organisms for ecotoxicology bioassays. Our investigations showed that BPA did not affect the capability of ascidian sperm to fertilize eggs, but it impaired embryonic development and caused a phenotype that was characterized by a short and kinked tail. Larvae developed from BPA-exposed embryos also presented malformations of pigmented organs such as altered pigment deposition, absence of one or both pigmented organs, and supernumerary organs. The co-exposure with 4-hydroxytamoxifen, an estrogen-related receptor (ERR) inverse agonist, rescued the normal phenotype of pigmented organs, thus supporting the hypothesis that, in ascidians, BPA exerts its teratogenic effects mainly by binding to ERR, as in vertebrate models.

3.2. Introduction

Bisphenol A (2,2-bis-(4-hydroxyphenyl)-propane; CAS Registry No. 80-05-7) is an organic compound originally developed as a synthetic hormone and then employed in the production of polycarbonate plastics and epoxy resins (Flint et al 2012). The production of BPA (Lin et al 2017) and its release into the environment have both increased recently (Flint et al 2012). This is a particular matter of concern, as BPA is a well-known teratogen and endocrine disruptor, exerting its action through different nuclear and cytoplasmic receptors (Rubin 2011, Tohmé et al 2014). Initially, BPA was considered to act by binding to estrogen and androgen receptors, even though its affinity to these receptors is extremely weak (Kuiper et al 1998, Rubin 2011). Subsequently, a competitive receptor binding assay revealed an extremely high binding affinity of BPA to the orphan nuclear estrogen-related receptor (ERR) γ in human cell lines (Okada et al 2008, Takayanagi et al 2006).

Estrogen-related receptors get their name from the sequence homology with estrogen receptors, but they do not bind to estrogens or other steroid hormones (Xie et al 1999). In humans, there are three types of ERRs: ERRα, β, and γ. The homologs of these receptors are also present in all other vertebrates, such as zebrafish, which have a genome with five ERR genes: ERRα, ERRβ,

ERRγ-A, ERRγ-B and ERRδ (lost in mammals). This diverse ERR gene family is the result of several gene duplications and losses. The genes are segmentally expressed in the hindbrain (Bardet et al 2005). A single ERR gene has been identified in basal chordates, and its expression appears to be conserved; in amphioxus, ERR is expressed in a segmented manner in a region considered to be homolog to the vertebrate hindbrain (Bardet et al 2005). ERRs have a very high level of constitutive transcriptional activity. The binding of BPA to ERRγ does not affect the receptor activity in humans (Takayanagi et al 2006), while in zebrafish it induces a dose dependent increase of ERRγ (but not of ERRβ) transcriptional activity (Okada et al 2008, Tohmé et al 2014). In zebrafish, both ERRγ genes are expressed in the otic vesicle (Bardet et al 2005, Tohmé et al 2014) and it was demonstrated that the binding of BPA to ERRγ induced alterations in the otic vesicle and in the otolith formation during the first stages of embryonic development. Moreover, these alterations were recovered by the co-exposure of zebrafish embryos to BPA and 4-hydroxytamoxifen (4-OHT; Tohmé et al 2014). 4-OHT is an inverse agonist of human ERRγ that is known to decrease the constitutive activity of the receptor (Coward et al 2001). Alterations of sensory organs induced by BPA were also observed in the ascidians *Ciona robusta* (Messinetti et al 2018) and *Phallusia mammillata* (Dumollard et al 2017).

Ascidians are marine invertebrates, characterized by filter feeding sessile adults and swimming lecitotrophic larvae, which display the typical chordate body plan. They have been confirmed as reliable model organisms for ecotoxicological bioassays because of their manipulable and rapid embryonic development, and high production of gametes (Gallo & Tosti 2015, Zega et al 2009). Moreover, they offer valuable advantages for exploring the molecular mechanism behind the action of pollutants because of their phylogenetic position and their small genome (Passamaneck & Di Gregorio 2005).

Thus, in this study, we studied the effects of the exposure to BPA on different ontogenetic processes in the solitary ascidian *Phallusia mammillata* Cuvier 1815, and evaluated whether different ascidian species display different sensitivities to this molecule. Then, we co-exposed embryos of *P. mammillata* to BPA and 4-OHT in order to gain insight into the molecular mechanism involved in the teratogenic effects of BPA and we tested whether the binding of this pollutant to ERR is responsible for the developmental impairment induced by BPA in these animals.

3.3. Materials and methods

3.3.1. Animals and chemicals

Adults of *P. mammillata* were collected from natural populations in Lerici (La Spezia, Italy). They were maintained in aquariums filled with artificial sea water (ASW, Instant Ocean®, Aquarium System) and equipped with mechanical, chemical and biological filters. Salinity was set at 36 ‰ and temperature fixed at 16±1 °C. Constant light promoted the production of gametes and prevented their premature release (Lambert & Brandt 1967). Eggs and sperm were collected by dissecting the gonoducts of three hermaphrodite adults in each experiment. Since self-fertilization decreases the development rate, we always performed cross fertilization. All procedures were carried out at 18±1°C in a thermostatic chamber. All exposure experiments were conducted in glass Petri dishes (diameter 4 cm) filled with 10 ml of the tested solutions. BPA (MW=228.29) and 4-hydroxytamoxifen (4-OHT, MW=387.51) were purchased from Sigma (Italy). Stock solution of 100 mM BPA was obtained by dissolving 11.4 mg in 500 µl of dimethyl sulfoxide (DMSO, Sigma), which was then diluted in filtered ASW with 0.005 M Hepes pH 8.0 (ASWH) to reach the final test concentrations (0.1, 0.5, 1, 5, 10, and 20 µM). We made 4-OHT stock solution (50 mM) by dissolving 10 mg in 500 µl of DMSO, which was then diluted in ASWH to obtain the test solutions (1 and 2.5 µM). A seawater control (CO; ASWH) and a solvent control (CO+DMSO; 0.02% DMSO in ASWH) were used in each experiment. Fresh solutions were prepared each time.

3.3.2. Effects on sperm viability

In order to analyze the effects of BPA on sperm viability, 20 µl of dry sperm was diluted in 2.4 ml of ASWH, and 300 µl of this solution was exposed to 5 ml of BPA at different concentrations for 30 minutes. For each of the BPA-sperm concentration, 200 was used to fertilize 60 eggs in 10 ml of ASWH in glass Petri dishes. One hour post fertilization (hpf), embryos were fixed in 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline solution, and the fertilization rate (FR) was calculated as follows: (number of embryos starting the first division / total number of initial eggs) x 100. The experiment was performed in triplicate and results were considered reliable only when FR of seawater control samples was ≥ 80%.

3.3.3. Effects on fertilization

In order to test the effects of BPA on fertilization, 60 unfertilized eggs and 15 µl of sperm dilution (20 µl of dry sperm in 2.4 ml of ASWH) were added to Petri dishes containing 10 ml of

a solution of BPA at different concentrations (0.1, 0.5, 1, 5, 10, and 20 µM) in ASWH. One hour later, embryos were fixed in 4% PFA and FR was calculated. The experiment was performed in triplicate and results were considered reliable only when FR of seawater control samples was ≥ 80%.

3.3.4. Effects on embryogenesis

In order to analyze the effects of BPA and 4-OHT and their co-exposure on embryogenesis, cross-fertilization was performed in Petri glass dishes containing ASWH and maintained at 18±1 °C for 1 hour, when the first division occurred. Subsequently, about 100 embryos at two-cell stage were exposed to different concentrations of BPA (0.1, 0.5, 1, 5, 10, 20 µM), two concentrations of 4-OHT (1 and 2.5 µM) and, based on observed incidence of malformations, also combinations of the two molecules (5, 10 or 20 µM BPA plus 1 or 2.5µM 4-OHT). Each experiment was performed in triplicate, and 100 embryos were used for each treatment (n: ~300). When controls reached the larva stage (~19 hpf), all specimens were fixed in 4% PFA and examined under a dissection microscope. For each treatment, the percentages of normal larvae, larvae with malformations and dead embryos were calculated.

3.3.5. Effects on pigmented organs

Fifty larvae exposed to BPA or to the mixtures of BPA and 4-OHT were observed under a dissection microscope to analyze malformations of pigmented organs. These organs were scored as normal (one otolith and one ocellus completely formed), mildly affected (one otolith and one ocellus with abnormal deposition of pigments) or severely affected (absence of one or both pigmented organs or supernumerary pigmented organs).

3.3.6. Immunohistochemistry

We also performed immunostaining of the nervous system, as described by Pennati et al (2003), on fixed larvae treated with different concentrations of BPA. Monoclonal anti β-tubulin (clone 2-28-33, Sigma) antibody developed in mouse was used to label nervous system fibers, while polyclonal anti GABA antibody developed in rabbit (Sigma) was specific for GABAergic neurons. In short, specimens were rinsed in 0.1% Tween-20 in phosphate buffered saline (PBT), permeabilized with three washes for 10 min in a solution of 0.25% Triton X-100 in PBT and incubated for 2 hours in 50% PBT : 50% normal goat serum (NGS), previously deactivated at 55°C for 30 min. Then, samples were incubated overnight at 4°C with primary antibody diluted 1:500 in 10% NGS in PBT. After several washes in PBT, the samples were incubated in 1%

bovine serum albumin in PBT for 2 hr at room temperature and then incubated at 4°C overnight in PBT, in which Alexa Fluor 488 anti mouse IgG or anti rabbit IgG antibody, diluted 1:800, was added. The specimens were then washed multiple times in PBT and mounted in 1,4-diazabicyclo [2,2,2] octane (DABCO, Sigma) on microscope slides. Samples were examined using an argon confocal laser scanning microscope Leica SP2 (Leica Microsystems, Heidelberg, Germany), equipped with argon/krypton laser.

3.3.7. Statistical analysis

We used analysis of variance, followed by HSD Tukey's post hoc test, performed with R software (R-Core-Team 2013) and the agricolae package (de Mendiburu 2015) to evaluate the statistical significance of the differences in FR and in larval development of samples treated with different concentrations of BPA and 4-OHT and controls. A Cochran test was performed to test the homogeneity and normality of the variances, and percentage data were transformed when they did not meet the assumptions of the analysis (normality and homoscedasticity). The differences in the development of pigmented organs were statistically analyzed with χ^2 test.

3.4. Results

3.4.1. Effects on sperm viability and fertilization

The capability of sperm to fertilize was not affected by the 30-min pre-exposure to BPA (from 0.1 µM to 20 µM); the differences in FR were not statistically significant ($F=0.7665$; $p=0.623$; Fig. 1A).

Fertilization rate was not altered even when fertilization occurred in the presence of BPA (Fig. 1B). However, when eggs were fertilized at concentrations >5 µM, some of them did not complete the first cell division; fertilization occurred, but the embryos appeared arrested mid-way through the division process and were therefore unable to complete the first cellular division and develop a heart shape (Fig. 1C). The number of these aberrant embryos increased with increasing BPA concentration and, in particular, the percentage of these malformed two-cell embryos was significantly different from that of control embryos, at concentrations as low as 5 µM BPA ($F=262.77$; $p<0.0001$; Tukey's post hoc test control vs. 5 µM $p<0.0002$; control vs. 10µM $p<0.0001$; control vs. 20 µM $p<0.0001$).

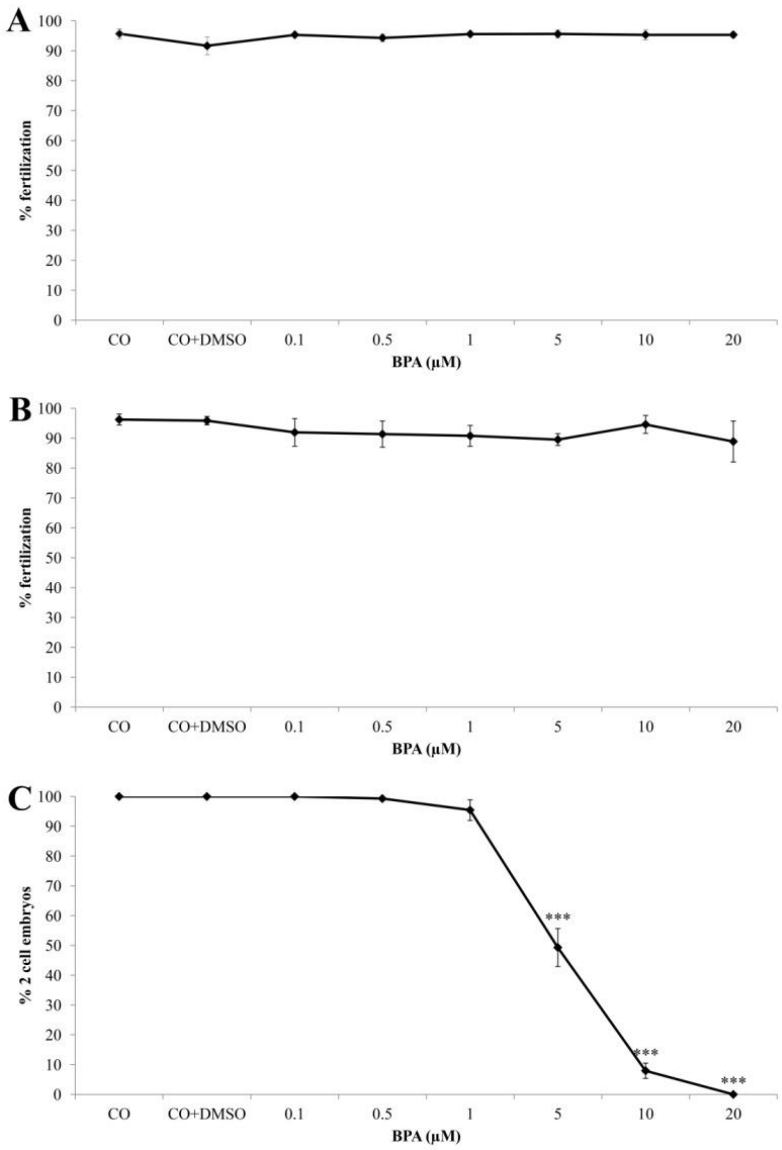

Figure 1: Effects on sperm viability (A), on fertilization rate (B) and proportion of fertilized eggs that reach correctly the two cell stage (C) at different concentrations of BPA in *P. mammillata*. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: p<0.001 '***' ; p<0.01 '**'; p<0.05 '*'.

3.4.2. Effects on embryogenesis

Exposure to BPA at concentrations <10 µM did not significantly affect the general anatomy of embryos of *P. mammillata*. The body shape of the larvae appeared under light microscopy to be comparable to that of control larvae (Fig. 2A). At 10 µM BPA, the percentage of body malformation reached 17.7%, even if this value was not statistically different from the 4% rate of malformations in controls. By contrast, exposure to 20 µM BPA resulted in a high percentage (94.3%) of severely malformed embryos. Such incidence was significantly higher than in

controls (F =98.338 p<0.0001, Fig. 2A). The anatomical malformations consisted of a short and kinked tail, associated with a malformed trunk and a reduction of papillae.

Immunolabeling of the nervous fibers by the anti β-tubulin antibody highlighted the impairment of the nervous system induced by the highest concentration of BPA. In control larvae, the antibody labeled the neurons in several structures including the papillae, the papillary nerves that connect the sensory cells of the papillae to the sensory vesicle, the complex of nervous fibers around the sensory vesicle, and in the posterior dorsal neural tube (Fig. 3A,D). Larvae exposed to 20 µM BPA showed an altered nervous system, with disorganized fibers around the sensory vesicle and reduced or absent papillary sensory neurons (Fig. 3B,C,E,F).

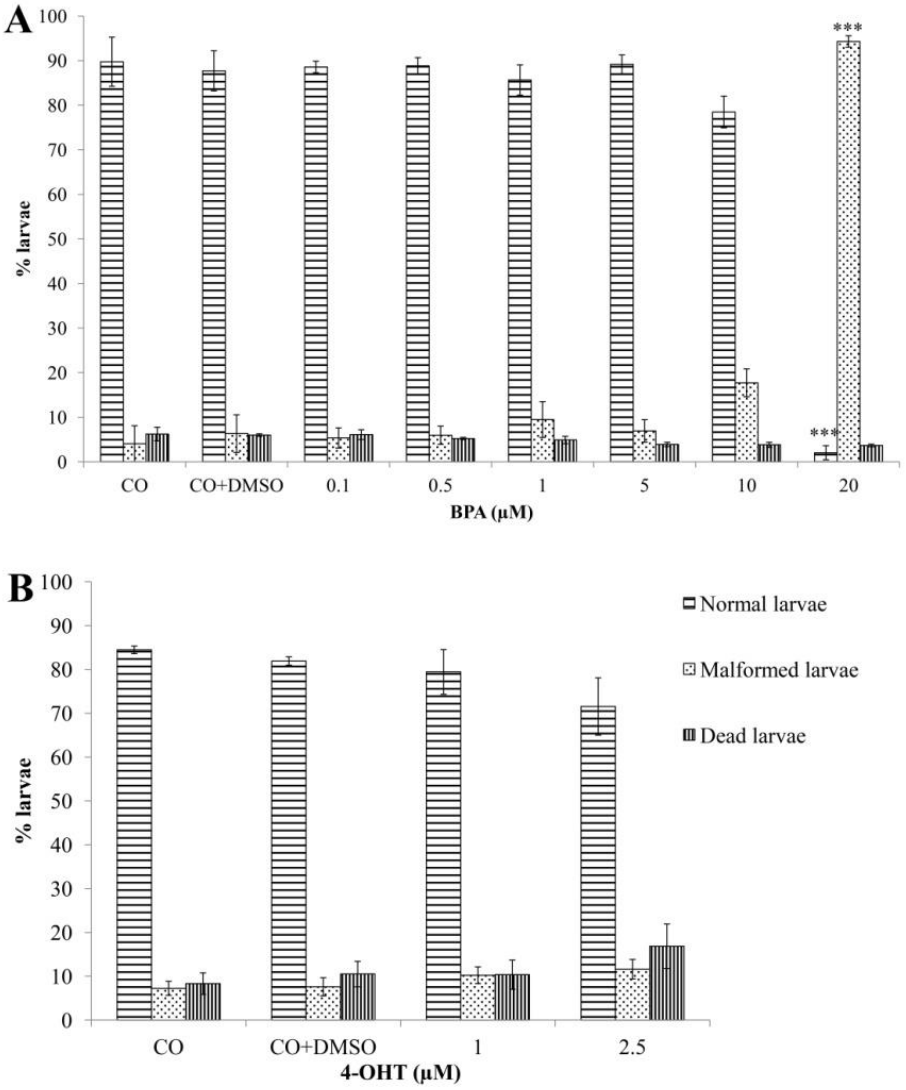

Figure 2: Effects of BPA (A) and 4-OHT (B) on embryogenesis in *P. mammillata*. Mean values of three replicates and standard errors are indicated. Legend of symbols: *= differences from Control. The number of each symbol indicate the level of significance according to R significance codes: p<0.001 '***' ; p<0.01 '**'; p<0.05 '*'.

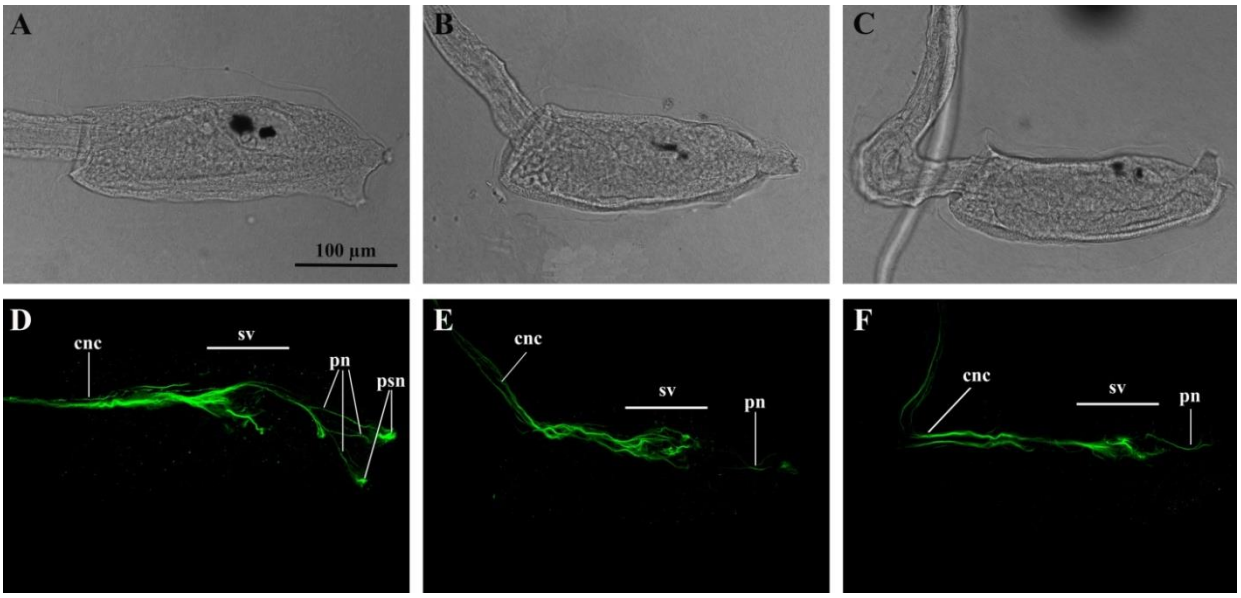

Figure 3: Immunostaining of the nervous system with anti β-tubulin antibody in *P. mammillata*. Bright field images and confocal microscope. (A,D) Control larvae, (B,C,E,F) Larvae developed from embryos treated with 20 µM BPA. psn=Papillary sensory neurons; pn= papillary nerves; sv= sensory vesicle; cnc= caudal nerve cord.

3.4.3. Effects on pigmented organs

In addition to the previously described body malformation, abnormal development of pigmented organs was observed in larvae exposed to 5, 10 and 20 µM BPA. A statistically significant increase in the incidence of affected pigmented organs was recorded in BPA-treated larvae compared to controls ($p<0.001$; Fig. 4A-C). We identified mild malformations consisting of abnormal deposition of pigment, and severe malformations consisting of the presence of supernumerary pigmented organs or absence of one or both organs (Fig. 5). Larvae with normal pigmented organs were almost absent in samples exposed to the three highest concentrations, and the percentage of larvae with severe malformations increased with the increase in BPA concentrations (Fig. 4A-C).

In order to characterize these malformations more clearly, we performed an immunolocalization of GABA. GABA immunoreactivity in larvae not exposed to BPA was associated with pigmented organs. In particular, GABA was localized in a dorsal fan of positive cells on the ocellus and in few cells of the stalk of the otolith, forming a cup-shaped structure (Fig. 6A,D). In larvae exposed to low concentrations of BPA, the localization of GABA was similar to that of control larvae, and this localization was always recognizable a signal associated with the ocellus and a signal associated with the otolith (Fig. 6B,C,E,F). In larvae exposed to 20 µM BPA, a concentration at which a higher percentage of severe malformations was recorded, we observed different results: (a) GABA was localized in a dorsal fan of cells that corresponded to the single

pigmented organ, which was identified as an ocellus (Fig. 6G,J); (b) the peculiar cup-shaped signal of GABA allowed us to identify the presence of supernumerary otoliths (Fig. 6H,K); (c) it was not possible to discern which pigmented organ was differentiated because the signal was distorted by the development of pigment organs outside the sensory vesicle (Fig. 6I,L).

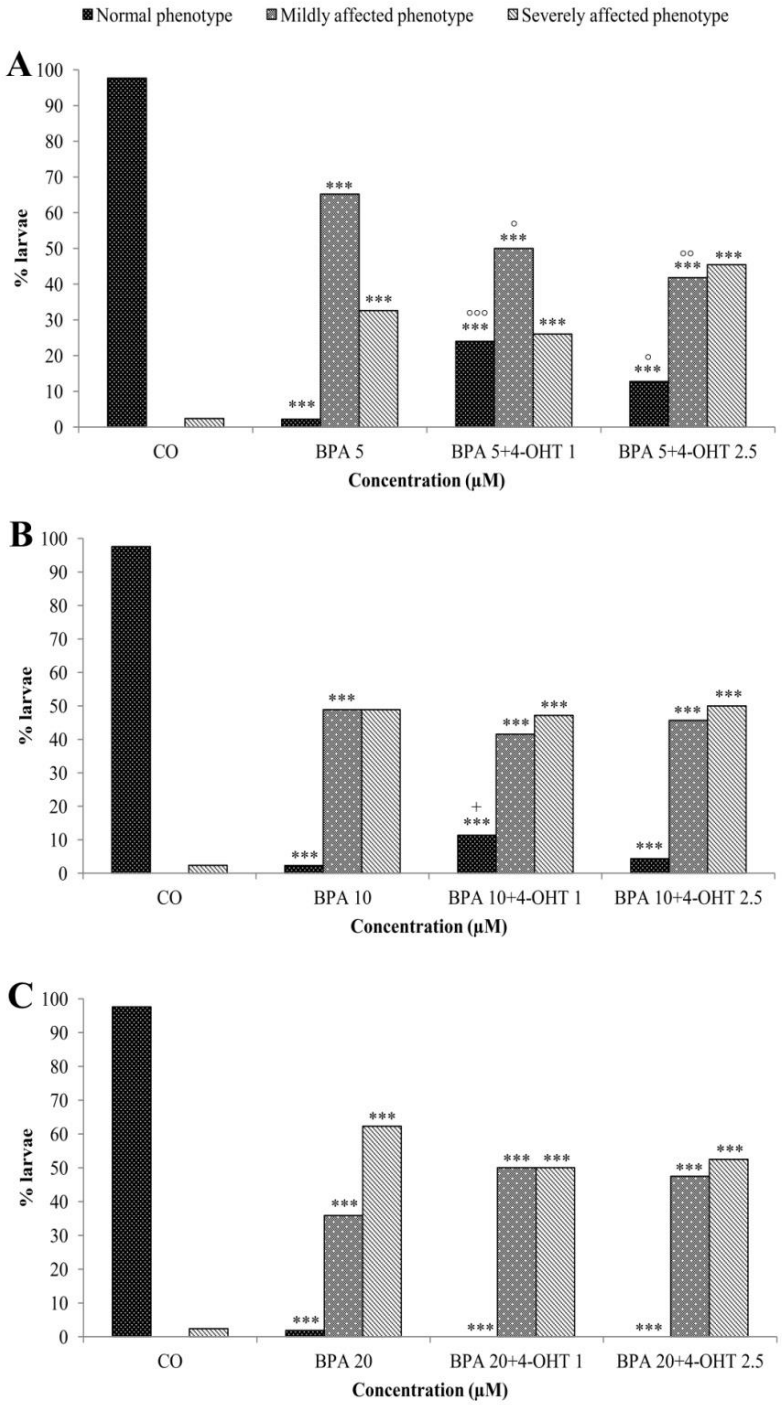

Figure 4: Effects of co-exposure to BPA and 4-OHT on pigmented organs at 5 µM BPA (A), 10 µM BPA (B) and 20 µM BPA (C). Legend of symbols: *= differences from Control; °=differences from 5 µM BPA; +=differences from 10 µM BPA. The number of each symbol indicate the level of significance according to R significance codes: p<0.001 '***' ; p<0.01 '**'; p<0.05 '*'.

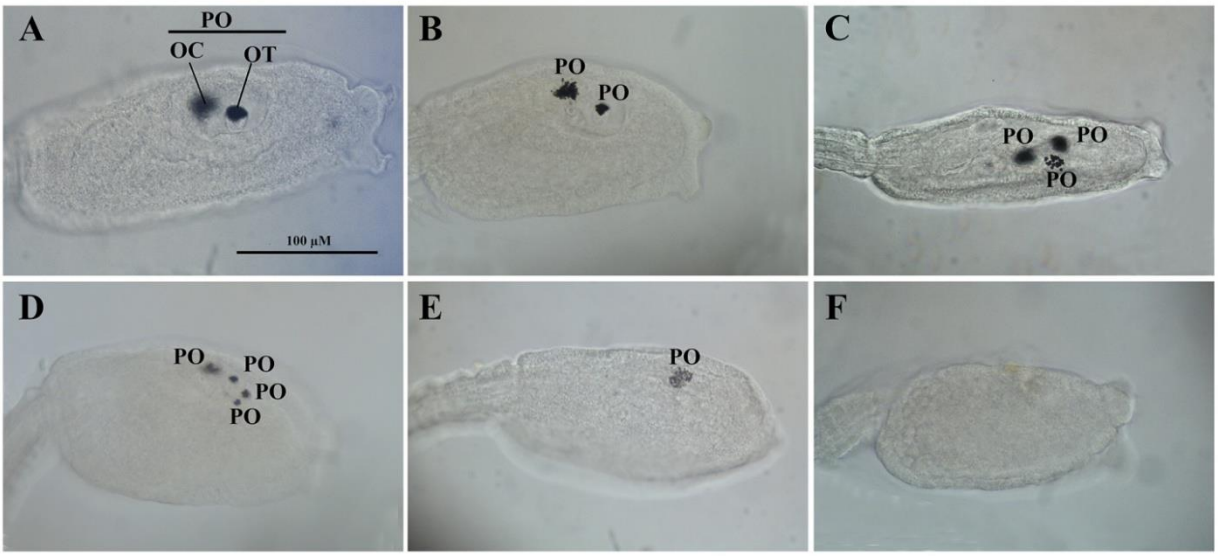

Figure 5: Malformation of pigmented organs. (A) normal phenotype, (B) mildly affected phenotype, (C-F) severlely affected phenotype, particularly (C-D) extranumerary pigmented organs, (E) one pigmented organ, (F) absence of pigmented organs.

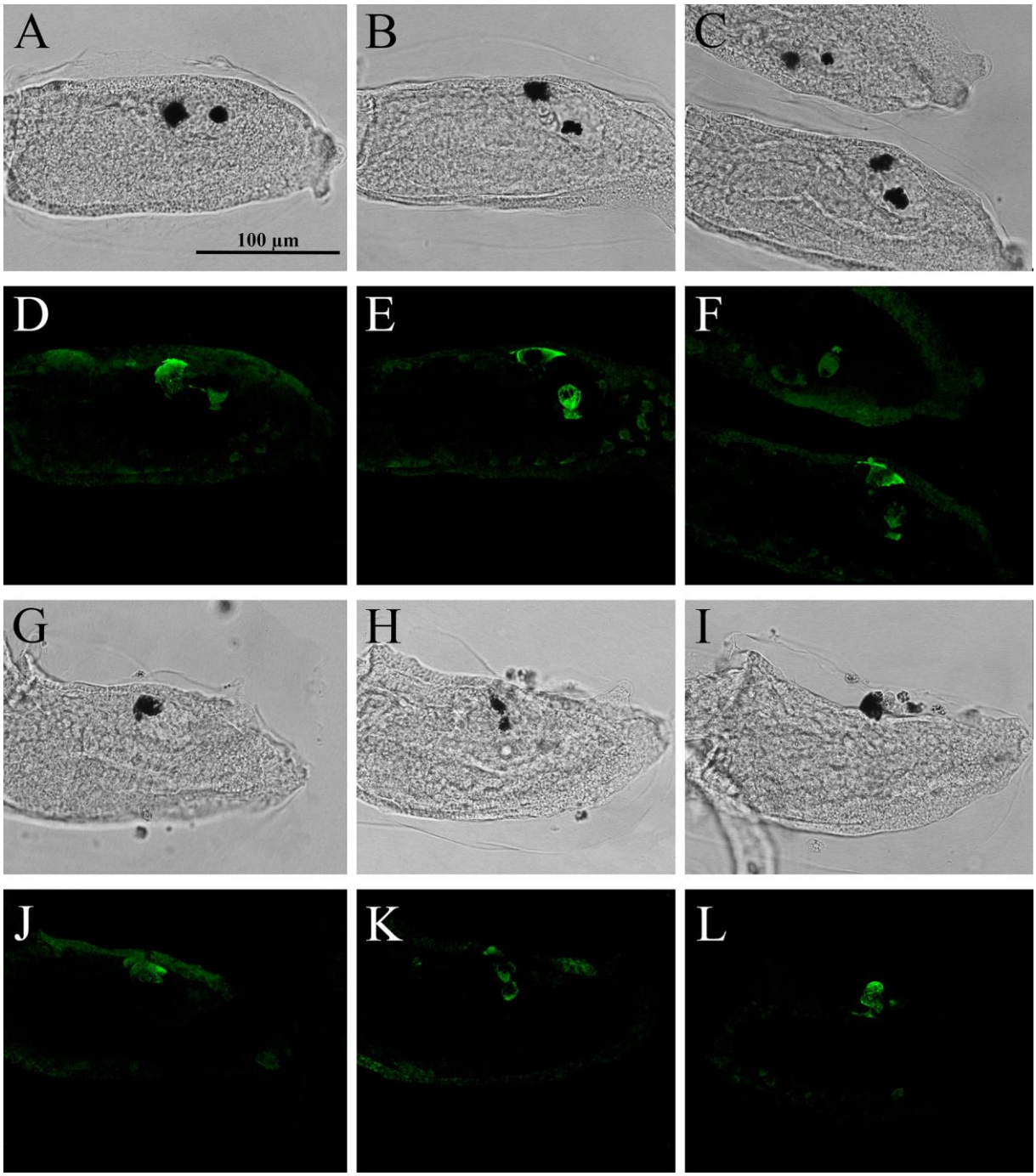

Figure 6: Immunostaining of the nervous system with anti GABA antibody in *P. mammillata*. Bright field images and confocal microscope. (A,D) Control larvae, (B,E,C,F) Larvae with mild malformation of pigmented organs, (G-L) Larvae with severe malformation of pigmented organs.

3.4.4. Effects of 4-OHT co-exposure on pigmented organs

The incidence of malformations to sensory organs in *P. mammillata* were similar to the incidence of otolith abnormalities observed in zebrafish treated with BPA. Thus, we considered that these alterations were due to a specific action of the molecule, possibly the action of binding to ERRs. To test this hypothesis, we co-exposed embryos to both BPA and 4-OHT, an inverse agonist of ERR. Before testing the effects of the co-exposure to BPA and 4-OHT, we verified that the chosen 4-OHT concentrations did not affect the development of *P. mammillata*: the exposure to 1 and 2.5 µM 4-OHT did not alter the percentage of normally developed larvae compared to controls ($F=1.776$, $p=0.2293$; Fig. 2B). Because malformations to the anatomy of larvae and to their pigmented organs were absend at the lowest BPA doses, co-exposure experiments were made combining 5, 10 or 20 µM of BPA with the two 4-OHT concentrations. The co-exposure to 5 µM BPA and 4-OHT produced a partial rescue of the normal phenotype at the pigmented organ level. In fact, the percentage of normal phenotypes increased from an average of 2.17% in larvae exposed to 5 µM of BPA to 24% in larvae co-exposed to 1 µM of 4-OHT and 12.73% in larvae co-exposed to 2.5 µM of 4-OHT. The differences in the incidence of normal phenotypes between co-exposed larvae and larvae treated only with BPA were statistically significant ($p<0.001$ and $p=0.0126$ respectively) (Fig. 4A). The potential of 4-OHT to rescue the phenotypes decreased with increasing BPA concentrations. After co-exposure to 10 µM of BPA and 1 µM of 4-OHT, 11.32% of larvae showed a normal phenotype, a percentage which was significantly different from that observed after exposure to 10 µM of BPA alone ($p=0.0218$; Fig. 4B). Although there was a decrease in the number of severe malformations and an increase in mild malformations after co-exposure to 20 µM of BPA and 4-OHT, the differences between co-exposure and BPa alone were not statistically significant ($p>0.1150$; Fig. 4C).

3.5. Discussion

There is high concern about the effects induced by the organic pollutant BPA on human and wildlife health (Crain et al., 2007; Rochester, 2013; Rubin, 2011). In order to understand the possible consequences of an increase of this pollutant in aquatic environments, ecotoxicological bioassays have been performed on numerous species and report different levels of sensitivity to BPA (Flint et al 2012). For example, doses as low as 0.1 µg/L and 0.08 µg/L caused developmental inhibition and delayed larval emergence in the crustacean *Tigriopus japonicus* and in chironomids respectively (Marcial et al 2003, Watts et al 2003), while concentrations

ranging 0.83-100 µg/L administrated for 90 days, did not produce any effects in the amphibian *Xenopus laevis* (Pickford et al 2003). Such different effects at similar concentrations could be simply an artifact of phylogenetic distance. However, variable results have also been observed between more closely related species: a dose of 250 mg/kg/day produced variable responses among mammalian species, including an increase of testosterone levels in the shrew *Microtus agrestis* (Nieminen et al 2002b), but no effects at all in polecat *Mustela putorius* (Nieminen et al 2002a), highlighting the fact that BPA may be tolerated differently among members of the same phylogenetic group. On the other hand, the effects of BPA may vary even within a single species; in *X. laevis*, the observed effects of BPA varied among studies, depending on concentration, duration of exposure, and exposure during different development stages (Iwamuro et al 2003, Kloas et al 1999, Levy et al 2004, Oka et al 2003, Sone et al 2004).

In ascidians, previous studies conducted on *Ciona intestinalis* and *C. robusta* (Cangialosi et al 2013, Matsushima et al 2013, Messinetti et al 2018), reported various effects of BPA on developmental processes. In the present work, we explored the ontogenetic impairments induced by BPA in another ascidian species, *P. mammillata*. Comparably to what has been observed in the studies on *C. robusta*, the exposure of sperm to BPA did not alter the FR in cultures of *P. mammillata,* but the exposure of both sperm and eggs to BPA compromised the first cell division at concentrations of BPA >5 µM. When the exposure occurred after the first cell division, embryos of *P. mammillata* could reach the larval stage in the presence of BPA, even at the highest tested concentration of 20 µM BPA, although all larvae were malformed. By contrast, in *C. robusta,* 20 µM BPA was lethal (Messinetti et al 2018), suggesting that differences in the tolerance to BPA action are also common in these marine invertebrates. Immunostaining with anti-β tubulin showed an alteration of neural fiber pattern in *P. mammillata*, comparable to that observed in *C. robusta*. These malformations can be ascribed to a pleiotropic toxic action of the molecule (Wetherill et al 2007). Moreover, we observed that in *P. mammillata* the sensory organs, otolith and ocellus, showed a wide range of alterations at concentrations of BPA that did not produce external morphological malformations. These alterations were mild, such as the abnormal deposition of pigmentation, or severe, such as the presence of supernumerary pigmented organs, the presence of only a single organ, or the absence of pigmented organs. The highly reproducible malformations we observed may be the result of a specific action of BPA. Alterations of the sensory organs were also reported in zebrafish after their exposure to BPA (Gibert et al 2011, Tohmé et al 2014). It was demonstrated in these fish that induced malformations were due to the interaction between BPA and an ERRγ (Tohmé et al 2014). The genome of *C. intestinalis/robusta* contains 17 nuclear receptors, including a single ERR

(GenBank Accession Number NM_001078232.1). Also, in the ascidians *Halocynthia roretzi* and *Herdmania curvata.* an ERR gene was reported and the expression profile was assessed through *in situ* hybridization (Devine et al 2002, Park et al 2009). The phylogenetic reconstruction revealed that *Hr-ERR* could be an ancestral form of vertebrate ERRs. This ERR was able to bind to estrogen responsive elements and estrogen related receptor responsive elements and showed a constitutive activity (Park et al 2009). Moreover, 4-OHT and DES were able to suppress the receptor activity in a dose-dependent manner (Park et al 2009). An ERR sequence is also present in the transcriptome of *P. mammillata* and it is available within the databank ANISEED (https://www.aniseed.cnrs.fr). The partial rescue of the phenotype that we observed after co-exposure of embryos to BPA and 4-OHT suggests that, like in fish, the teratogenic mechanism of BPA in ascidians may be triggered by binding to this nuclear receptor. The rescue was observed in larvae treated with 5 µM of BPA and 1 µM of 4-OHT. Higher concentrations of BPA were toxic and were not rescued by co-exposure to 4-OHT. It was interesting to observe the presence of supernumerary otoliths and ocelli and the absence of one or both pigmented organs, as revealed also by GABA immunolabelling. Since ascidians develop with a fixed cell lineage, it has been possible to trace the embryological origin of the pigmented organs. In *C. intestinalis*, the pigmented organs are derived from a symmetrical pair of cell from the a-line, particularly from the a8.25 pair (Nicol & Meinertzhagen 1988a, Nicol & Meinertzhagen 1988b, Nishida 1987). The fate of the two pigmented cells is induced by A-line nerve cord precursors (Nishida 1991) through a crosstalk between FGF and Wnt signaling pathways (Squarzoni et al 2011), but the left-right position is not correlated to the final structure (otolith or ocellus; Nishida 1987). After neural tube closure, the two a10.97 cells line up along the dorsal midline and the anterior one becomes the otolith and the posterior one the ocellus (Darras & Nishida 2001). Perturbations to different elements of the aforementioned pathways, such as FGF-receptor, ERK1/2, Ci-Ets1/2, Ci-Tcf or Wnt7 pathways, produced different alterations in pigment cells development (Abitua et al 2012, Haupaix et al 2014, Racioppi et al 2014, Squarzoni et al 2011). More specifically, blocking FGF signals changed the developmental fate of pigment cells that become CNS cells (Racioppi et al 2014). Misexpression of Wnt7 and Tcf led to the development of larvae with two ocelli or two otoliths, respectively (Abitua et al 2012). Furthermore, overexpression of ERK1/2 resulted in larvae with supernumerary pigment cells (Haupaix et al 2014). It has yet to be established which step of the pathway that leads to the correct differentiation of the two organs is altered by the action of BPA. The mechanism is finely tuned by extracellular and intracellular environment, and it is possible to suppose that, depending on which step is altered by the action

of the BPA, changes to the pathway can produce either an extra otolith or an extra ocellus or neither.

The findings of this work suggest that the effects of BPA on sperm viability and fertilization in *P. mammillata* are qualitatively similar to the results obtained in *C. robusta*, even though *P. mammillata* tolerated higher concentrations of BPA during embryogenesis. Indeed, in a previous study, we tested BPA effects on ontogenetic processes of *Ciona robusta*, and, similar to this work, we also observed alteration of pigmented organs in that species, suggesting that this malformation was specifically caused by BPA teratogenic potential. In *P. mammillata* we explored the mode of action of BPA through co-exposure with an inverse agonist of ERR, and obtained a partially rescued phenotype. Thus, our results suggest that, in ascidians, BPA interferes with ERR, as already demonstrated in tests on zebrafish. As far as we know, our is the first work describing BPA mode of action in ascidians. Furthermore, the different sensitivity to BPA observed across ascidian species is of particular importance for future ecotoxicological studies in that it highlights the great variability in sensitivity to pollutants even among closely related species. The reasons of such different sensitivity are still unknown, but it may be the result of different detoxification capabilities or different permeability of egg coats that could partially block the diffusion of the pollutants through the chorionic membrane. Finally, this study supports the emerging role of ascidians as model organisms to predict the effects and define the mode of action of pollutants on humans and other vertebrates.

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Chapter 4 – Bisphenol A alters anterior nervous system patterning and pigment cell development in the ascidian *Ciona intestinalis*

Paper in preparation Messinetti S., Mercurio S., Pennati A., Pennati R. Bisphenol A alters anterior nervous system patterning and pigment cell development in the ascidian *Ciona intestinalis*

4.1. Abstract

Bisphenol A is a chemical compound that can be released in the environment after the degradation of plastic products. Other than general negative impacts on reproduction and nervous system differentiation both in vertebrates and invertebrates, some specific effects on sensory organs development have been reported in zebrafish, *Xenopus laevis* and in the ascidian species *Ciona intestinalis*, *Ciona robusta* and *Phallusia mammillata*. To investigate the molecular mechanisms underlining BPA teratogenic activity, we analyzed the effects on expression of genes involved in pigment cells specification and differentiation in the ascidian *Ciona intestinalis*. After BPA exposure, an ectopic expression of *Ci-Six3/6* and *Ci-Pax6*, and a reduction of cells expressing the melanin synthesizing enzyme *Ci-tyr* were reported. Similar effects were reported after the perturbation of FGF signaling pathway, leading to suppose that BPA acts at some point of the cascade activated by FGF signaling.

4.2. Introduction

Bisphenol A (BPA) is a commercial compound used for the production of polycarbonate plastics and epoxy resins that spreads into different environmental compartments, mainly via wastewater effluents. In 2015, Bisphenol A was one of the highest volume chemicals produced worldwide. Several studies demonstrated BPA reproductive toxicity in aquatic vertebrates, such as fish and amphibians, in which it mainly causes reduction of sperm quality, alteration of sex steroid levels, embryonic deformities and intersex condition (reviewed in Flint et al (2012)). Studies on invertebrates showed that chronic exposure altered or suppressed crustacean development and growth (Andersen et al 1999, Mariager 2001), altered nematode feeding behavior (Kohra et al 2002) and caused morphological deformities in sponges (Hill et al 2002). It has been proposed that BPA interferes with animal physiology and development by acting both as an endocrine disruptor (Rubin 2011) and as a teratogenic molecule (Iwamuro et al 2003, Kiyomoto et al 2006, Rubin 2011, Sone et al 2004, Yang & Chan 2015).

To properly monitor the effects of a teratogenic molecule in the environment and to formulate appropriate surveillance protocols, is opportune to know the molecular mechanisms affected by the pollutant. We previously reported that ascidian embryos exposed to low doses of BPA developed into larvae with altered pigmented organs (Messinetti et al 2018a). Impairment of pigment cell development was also reported in the ascidian *Phallusia mammillata* (Dumollard et al 2017, Messinetti et al 2018b). In this paper, we exploited the ascidian model to investigate the molecular mechanisms underlining BPA teratogenic activity.

Tadpole larva of ascidians has been proposed as a valuable miniaturized model to study basic mechanisms of development in chordates (Passamaneck & Di Gregorio 2005).

Ascidians are marine invertebrates with a key phylogenetic position, being members of the sister group of vertebrates (Delsuc et al 2006). Adults are sessile and develop through a swimming larva that displays the typical chordate body plan, comprising a dorsal hollow neural tube, a notochord and a locomotory tail. The larvae of most ascidian species present two sensory pigmented organs in the brain vesicle, namely the otolith, for gravity reception and the ocellus, for light reception (Barnes 1971, Dilly 1964, Dilly 1962, Dilly 1961, Eakin & Kuda 1970).

In *Ciona intestinalis*, one of the most studied ascidians, otolith is composed by an unique cell and contains a single large melanin granule with a diameter of approximately 15 µm (Dilly 1962), while ocellus is composed by 3 lens cells, around 30 photoreceptor cells and a cup-shaped pigmented cell that holds several hundreds of small melanin granules with a diameter of 1–2 µm (Dilly 1964, Horie et al 2005).

At a low concentrations BPA specifically affected the development of pigment cells of sensory organs of the cogeneric species *Ciona robusta* (Messinetti et al 2018a). Indeed, the high teratogenic index (TI = 32.5) indicates that this pollutant should be considered a teratogenic substance thus acting on a specific developmental mechanism (Dawson & Bantlet 1987).

Ascidian embryos develop with an invariable cell fate that allowed to trace the lineages of each embryonic precursor cell up to 10^{th} round of cell division. In *C. intestinalis*, the pigment cells of the otolith and ocellus derive from the paired a8.25 blastomeres (Nishida 1987). During gastrulation, these blastomeres divide and form a9.49 and a9.50 cell pairs. The two a9.50s (left and right) are located in the row IV of the neural plate and are progenitor cells of the anterior brain, while the two a9.49 are located in the row III and are the pigment cell precursors (PCPs; Squarzoni et al 2011). During successive cleavages, a9.49s undergo two further divisions giving origin to two cell pairs (a10.98 and a10.97) at mid gastrula stage and to eight cells (four on each side) at tailbud stage (a11.196; a11.195, a11.194, a11.193; Racioppi et al 2014). These cells are characterized by the expression of the melanogenic enzyme-coding genes tyrosinase (*tyr*) and *tyr*-related proteins in a fashion similar to the vertebrate pigment cells (Esposito et al 2012). Nevertheless, among these cells, only the two most posterior become ocellus and otolith pigment cells.

During development, FGF signaling, through MAPK/ERK cascade, is required for the correct differentiation of the pigment cells and antero-posterior patterning of the *Ciona* central nervous system (CNS). In absence of FGF signaling, PCPs change their fate into anterior CNS cells as demonstrated by the ectopic expression of genes involved in anterior specification. The

transcription factor *Ci-Six3/6* is normally expressed in cells of the row IV of the neural plate including the daughters of the a9.50 cells, sisters of the a9.49 PCPs located in the row III. In embryos transfected with a dominant negative form of the FGF receptor, FGF inhibition caused ectopic *Ci-Six3/6* expression in a posterior domain that includes *Ci-tyrp1/2a* positive PCPs (Racioppi et al 2014). Similarly, the anterior CNS markers *Ci-Pax3/7*, *Ci-Pax6* and *Ci-COE* are ectopically expressed in PCPs domain when FGF signaling is inhibited (Racioppi et al 2014). Moreover, for pigment cell differentiation, Wnt signaling is also required and these two pathways crosstalk, as *Ci-Tcf*, a downstream effector of Wnt, is regulated by FGF cascade-dependent transcriptional factor *Ci-Ets1/2* (Squarzoni et al 2011). The alteration of *Wnt7* or *Tcf* produced larvae with two ocelli or two otolith respectively (Abitua et al 2012). At last, supernumerary pigmented cells can be produced by alteration of ERK1/2 (Haupaix et al 2014).

To test the hypothesis that BPA teratogenic action in ascidians may be due to its interference with FGF signaling, we analyzed the expression pattern of genes involved in the specification of pigment cells and anterior nervous system in embryos and larvae exposed to different concentrations of the molecule.

4.3. Materials and methods

4.3.1. Animals and chemicals

Adults of *C. intestinalis* were collected from natural populations in Roscoff (France), and reared in 50 L aquaria equipped with mechanical, chemical and biological filters. Animals were maintained at 16±1°C and in constant light conditions to avoid gamete release and stimulate their production (Lambert & Brandt 1967). Three animals were sacrificed for each experiment, eggs and sperm were obtained by dissection of gonoducts and cross-fertilization was performed. All experimental procedures were carried out at 18±1°C. Treatments were performed in Petri glass dishes (diameter of 4 cm). Bisphenol A (BPA, MW=228.29) was purchased from Sigma, Italy. Stock solution of 100 mM BPA was made in dimethyl sulfoxide (DMSO, Sigma) and then diluted in filtered artificial sea water with 1 M Hepes pH 8.0 (ASWH) to reach the final test concentrations (0.1, 0.5, 1, 5 and 10 µM). Fresh solutions were prepared every time. As solvent control, a solution of 0.02% DMSO in ASWH was used.

4.3.2. BPA treatments

Embryos at two-cell stage (~1 hpf) were exposed to 0.1, 0.5, 1, 5 and 10 µM BPA in ASWH. Control embryos were maintained in ASWH and in ASWH plus 0.02% DMSO. Samples at the desired developmental stage were fixed in MOPS fix (4% PFA, 0.5 M NaCl, 0.1 M MOPS pH 7.5) in PBS for 1 h at room temperature. Before fixation, embryos at mid tailbud stages (12hr post fertilization (hpf)) were dechorionated by gentle rocking in a solution of 1% sodium thioglycolate and 0.05% pronase in ASWH previously activated with 2.5 M NaOH.

4.3.3. RNA extraction and cDNA synthesis

RNA was extracted from samples at different developmental stages using ReliaPrepTM RNA Tissue Miniprep System (Promega, Italy), following manufacturer's instructions. The quality and integrity of RNA was checked on 1% agarose gel, to assure it can be used for subsequent analysis. cDNA was synthesized according to instruction of InProm-IITM Reverse Transcription System (Promega, Italy).

4.3.4. Primer design and probe synthesis

Sequences of the genes were downloaded from GenBank with the following Accession numbers: *Ci-opsin1* (AB058682.1), *Ci-tyr* (XM_002123004.4), *Ci-gsx* (AF305500.1), *Ci-six3/6* (AB210683.1), *Ci-Pax6* (AB079883.1). Primers were designed using Primer-BLAST (Ye et al

2012) and are listed in Table 1. Ci-opsin1 Fw1 and Ci-opsin1 Rev1 were designed according to Kusakabe et al (2001). T7, T3 or SP6 promoter sequences were added to 5' ends for the transcription protocol.

Primer name	Sequence 5'--> 3'	Position
Ci-opsin1 Fw 1	AATTAACCCTCACTAAAGAAACGCAGCATTATGAATCAGTG	1
Ci-opsin1 Rev 1	ATTTAGGTGACACTATAGGTCAAGACTTTTTATTTTCAGAATATATAG	1547
Ci-opsin1 Fw 2	AATTAACCCTCACTAAAGTGCTAACGGTGCGGGATATT	414
Ci-opsin1 Rev 2	ATTTAGGTGACACTATAGCCAAGCGCGTAATCGATCTG	981
Ci-tyr Fw 1	TAATACGACTCACTATAGGGACGACAGAGCATTCTGGC	238
Ci-tyr Rev 1	ATTTAGGTGACACTATAGGACGTCATCGACCTCCTGTG	1078
Ci-tyr Fw 2	TAATACGACTCACTATAGGACGAGTCAAAACGCGTCAC	434
Ci-tyr Rev 2	ATTTAGGTGACACTATAGGACTACGTCACGCAGCTCTT	790
Ci-gsx Fw1	AATTAACCCTCACTAAAGAGACGTCATCACCACGTCAC	213
Ci-gsx Rev1	ATTTAGGTGACACTATAGGGGATCTGGTGTTACCCGAC	895
Ci-gsx Fw2	AATTAACCCTCACTAAAGAGAACCAGCTCTCCGCAAAA	326
Ci-gsx Rev2	ATTTAGGTGACACTATAGGGGCGTGTGAGTATGAGCTT	743
Ci-six3/6 Fw 1	AATTAACCCTCACTAAAGTCGAACCCTAGACCGGACAT	135
Ci-six3/6 Rev 1	ATTTAGGTGACACTATAGACGATTGTTCTTTGCGGCAG	813
Ci-six3/6 Fw 2	AATTAACCCTCACTAAAGTCACCCAACCCTTTGCTACC	223
Ci-six3/6 Rev 2	ATTTAGGTGACACTATAGGGCTTCGGGTTCGTTCCTTA	661
Ci-pax6 Fw 1	TAATACGACTCACTATAGGAATTTGCACATAACGGTGCG	221
Ci-pax6 Rev 1	ATTTAGGTGACACTATAGGCGCTGGCTACGTATTTTCTC	953
Ci-pax6 Fw 2	TAATACGACTCACTATAGAATCGCTTCCTACAAGCGGG	389
Ci-pax6 Rev 2	ATTTAGGTGACACTATAGAGGTTCGGTTTCGTTGGAGT	774

Table 1: List of primers used for the PCR, with relative position on the corresponding sequence available on GenBank. T7, T3 and SP6 sequences added at the 5' ends are underlined.

For the first PCR with Fw1 and Rev1 primers pair, the following conditions were used: an initial DNA denaturation at 94°C for 4 minutes followed by: 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 or 2 minutes, followed by a final extension of 5 min at 72°C. A nested PCR with the Fw2 and Rev2 primers pair was performed for each gene with the same PCR protocol, but with 30 seconds of extension. 5 µl of PCR products were used as a template for the probe synthesis using the DIG RNA labeling Kit (Roche, Germany) with T7 or T3 promoter to produce the sense probes and SP6 promoter to produce the antisense probes according to the manufacture protocol. 78 µl of water, 10 µl of 3 M sodium acetate and 300 µl of absolute ethanol were added to the 22 µl of the final product of transcription, and the probes were left to precipitate overnight at -20°C. The following day, the probes were resuspended and checked on 1% agarose gel.

4.3.5. *In situ* hybridization

Whole mount *in situ* hybridization was performed as described in Boorman & Shimeld (2002) with some modifications. After rehydration, samples were permeabilized with 4 µg/ml proteinase K for 10 minutes at 37°C and post-fixed in 4% PFA in PBT for 1h. Then, samples were washed twice in hybridization solution (50% formamide, 5× SSC, 100 µg/ml yeast RNA, 50 µg/ml heparin, 0.1% Tween 20) at room temperature. Pre-hybridization was performed for 2 h at 50°C, hybridization was carried out overnight at 50°C in prehybridization solution plus 1µl/ml of DIG-labelled probe. Samples were washed 6 times for 20 minutes in 50% formamide, 5× SSC, 0.1% Tween 20 and then transferred in blocking solution (Normal sheep serum: PBT, 1:4) for 2h. Samples were incubated overnight at 4°C in blocking solution with anti DIG antibody (1:2000). After several washes in PBT, samples were incubated in APT buffer (100 mm NaCl, 100 mm Tris HCl, pH 9.5, 50 mm MgCl2, 1% Tween 20) and then staining solution (APT buffer + 2.3 µl/ml NBT and 3.5 µl/ml BCIP) was added. Staining reaction was performed in dark conditions at room temperature. When satisfactory signals were obtained, samples were fixed with 4% PFA for 1 hour, mounted with 80% glycerol in PBT and photographed under a Leica DMRB microscope equipped with Leica DFC-320 Camera.

4.4. Results

4.4.1. Tailbud stage

C. intestinalis embryos exposed to tested concentrations of BPA from 2-cell stage showed an overall normal phenotype at gross morphological analysis. In control tailbud embryos (12 hpf) the expression of *Ci-tyr* is restricted to 4 aligned cells, the pigment cell precursors (PCPs). The stain is more intense in the two posterior cells and slighter in the two anterior ones (Fig. 1A, D). The same expression could be observed in embryos treated with low concentrations of BPA (0.1, 0.5 and 1 µM) (Fig. 1B, E). Embryos treated with higher BPA doses, 5 and 10 µM, showed only three *tyr* positive cells in 33% and 90% of treated embryos respectively, in which the intensity of the signal was comparable (Fig. 1C, F).

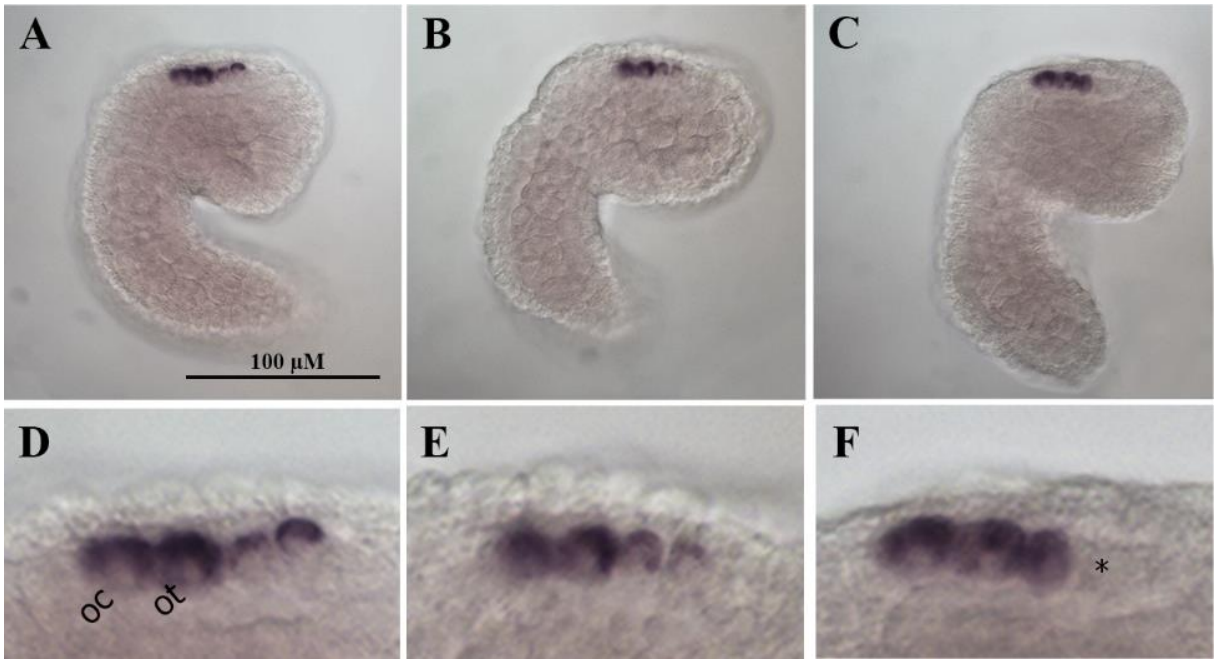

Figure 1: *Ci-tyr* expression in *C. intestinalis* tailbud (12 hpf) exposed to different concentrations of BPA. (A, D) Control tailbud, (B, E) tailbud developed in 1 µM BPA, (C, F) tailbud developed in 5 µM BPA. Asterisk highlight the cell without expression of *Ci-tyr*.

In tailbuds (12 hpf), *Ci-Six3/6* is expressed in the anterior presumptive sensory vesicle including derivates of the a9.50 blastomeres, adjacent but not overlapping with a9.49 *tyr* positive PCPs (Fig. 2A, D). In all embryos treated with low concentrations of BPA (starting from 0.1 µM), *Ci-Six3/6* expression was ectopically expressed in a single cell, posteriorly to the main stained area, corresponding to the anterior most PCPs (Fig. 2B-C, E-F).

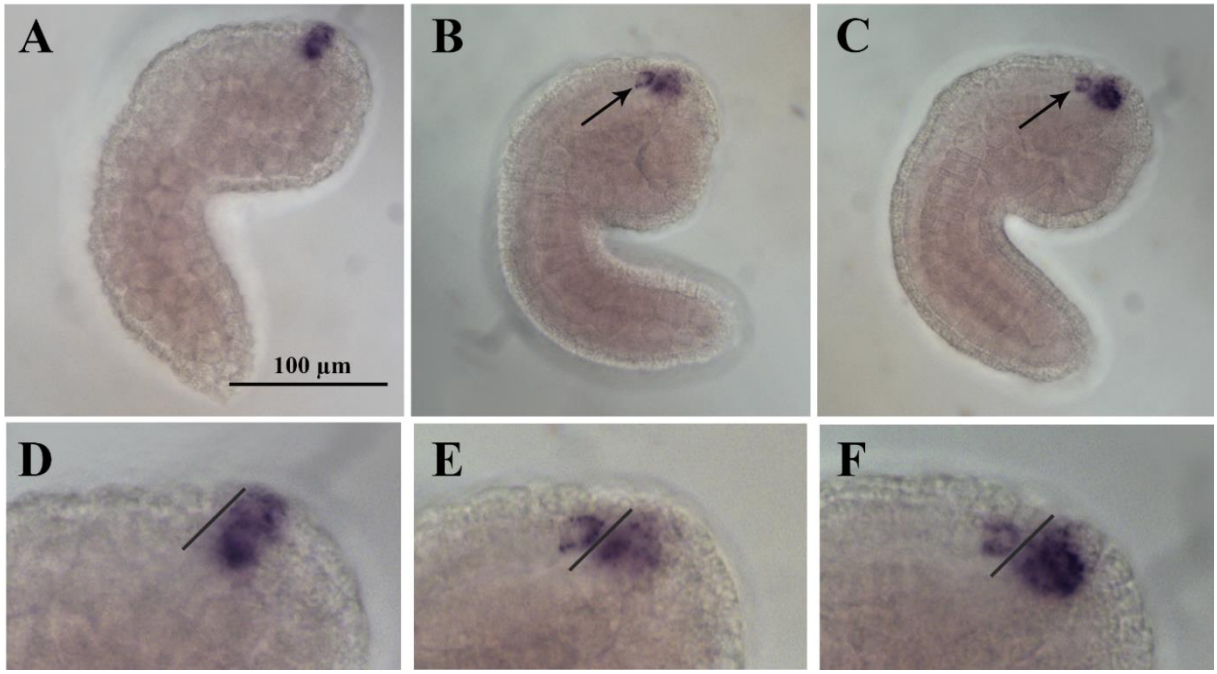

Figure 2: *Ci-Six3/6* expression in *C. intestinalis* tailbud (12 hpf) exposed to different concentrations of BPA. (A,D) Control tailbud, (B,E) tailbud developed in 1 µM BPA, (C,F) tailbud developed in 5 µM BPA. Arrows indicate the ectopic expression of the gene.

In the anterior presumptive sensory vesicle of tailbuds (12 hpf), the expression of *Ci-Pax6* is localized into two distinct patches separated by a small region where the gene is not expressed (Fig. 3A, D). Embryos exposed to low and mid concentrations of BPA (0.1, 0.5 and 1 µM) showed *Ci-Pax6* expression comparable to that of controls with the two positive regions separated by a *Ci-Pax6* negative region (Fig. 3B, E). Exposure to a higher concentration of BPA (5 µM) caused an ectopic expression of the gene in the region between the two patches so that an uninterrupted region of expression was detected in more than 50% of treated embryos (Fig. 3C, F).

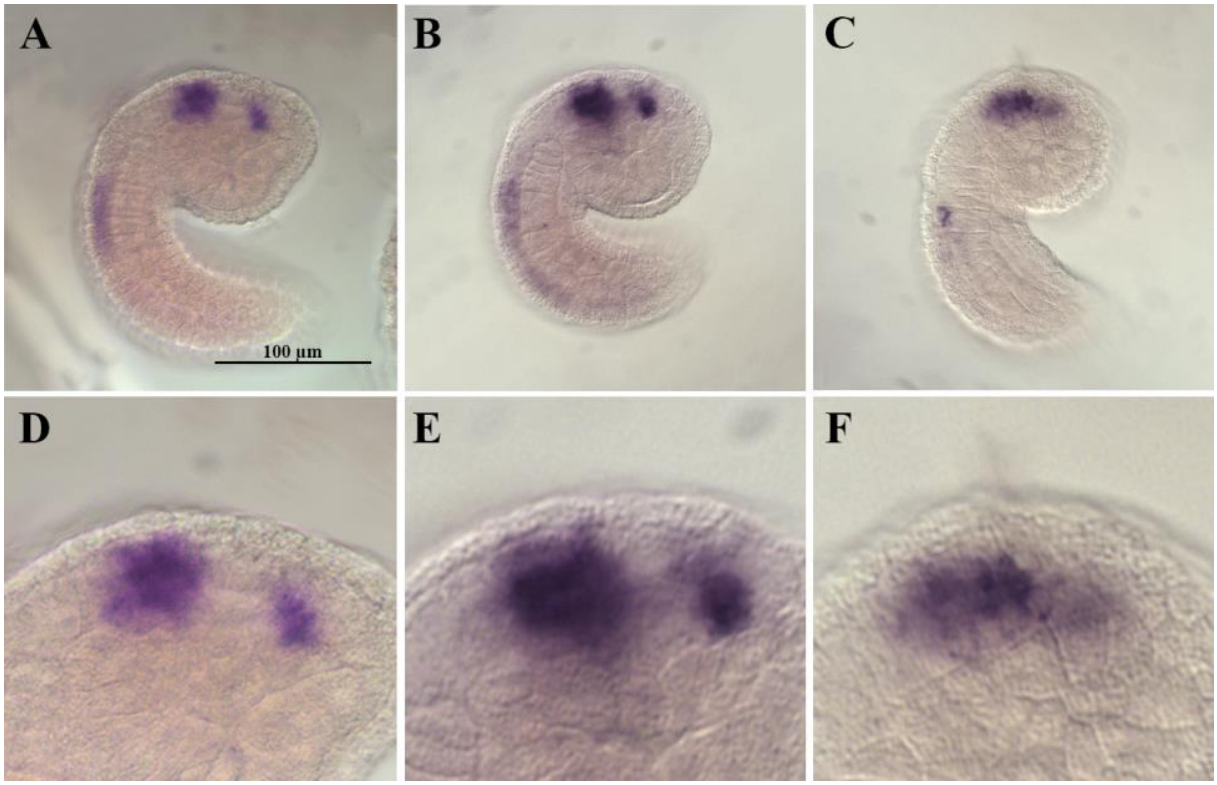

Figure 3: *Ci-Pax6* expression in *C. intestinalis* tailbud (12 hpf) exposed to different concentrations of BPA. (A,D) Control tailbud, (B,E) tailbud developed in 1 µM BPA, (C,F) tailbud developed in 5 µM BPA.

To verify that BPA action affects specifically pigment cells, we analyzed the expression of genes involved in the differentiation of the other components of the sensory organs, such as the photoreceptor cells of the ocellus.

Ci-gsx belongs to ParaHox homeobox family of transcription factors; in *C. intestinalis* it is expressed at mid-gastrula stage in the a9.33 pairs that will become the photoreceptor cell precursors (Hudson & Lemaire 2001). *Ci-gsx* expression territories at tailbud stage include two distinct symmetric domains along the anterior posterior axis bordering the midline (Fig. 4A). Exposure to BPA at all the tested concentrations did not altered the expression of this gene (Fig. 4B-C).

Ci-opsin1 is a gene coding for the visual pigment used by ascidians as a photoreceptor molecule. During tailbud stages, *Ci-opsin1* is expressed in a group of cells belonging to the developing sensory vesicle that will differentiate into the photoreceptor cells of the larva (Fig. 4D). The stained area and the intensity of the signal were not affected by the exposure to any tested concentrations of BPA (Fig. 4E-F).

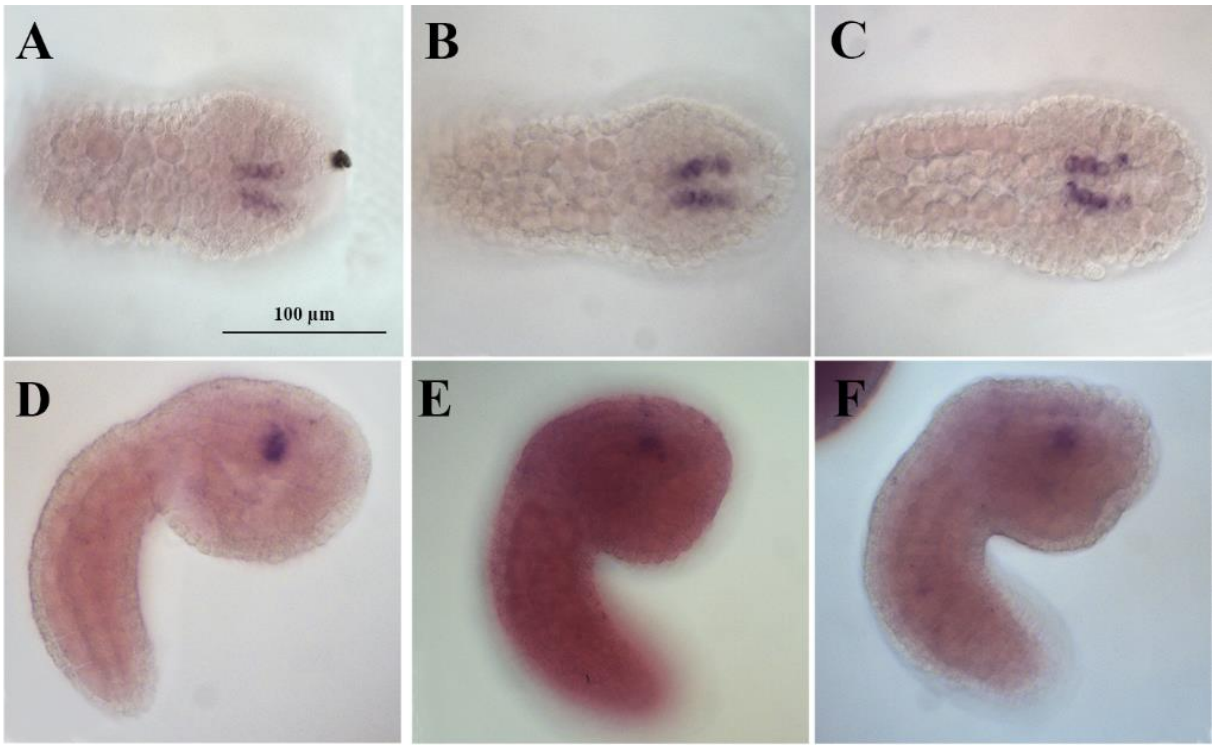

Figure 4: (A-C) *Ci-gsx* expression in *C. intestinalis* tailbud (12 hpf) exposed to different concentrations of BPA. (A) Control tailbud, (B) tailbud developed in 1 µM BPA, (C) tailbud developed in 5 µM BPA. (D-F) *Ci-opsin1* expression in *C. intestinalis* tailbud (12hpf) exposed to different concentrations of BPA. (D) Control tailbud, (E) tailbud developed in 1 µM BPA, (F) tailbud developed in 5 µM BPA.

4.4.2. Larvae

In swimming larvae (20 hpf), *Ci-six3/6* positive cells are located in the anterior part of sensory vesicle (Fig. 5A). Larvae exposed to low concentration of BPA (0.1 µM), showed a stained area that was comparable to controls. In larvae exposed to higher BPA concentrations (starting from 0.5 µM), *Ci-Six3/6* was ectopically expressed in a second group of cells in the posterior region of the sensory vesicle (Fig. 5B). This ectopic expression was observed also in larvae in which the pigmented cells were only mildly affected.

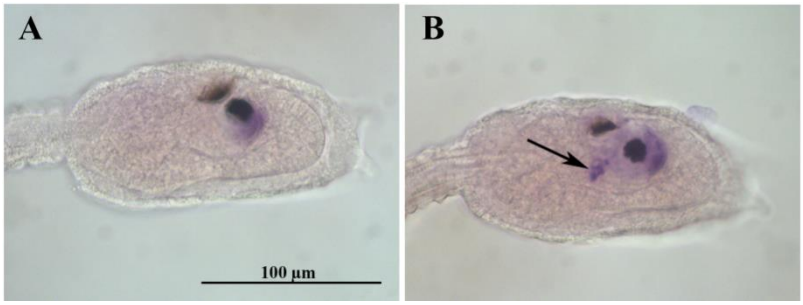

Figure 5: *Ci-Six3/6* **e**xpression in *C. intestinalis* larvae (20 hpf) exposed to different concentrations of BPA. (A) Control larva, (B) larva developed in 1 µM BPA.

During larval stage, *Ci-opsin1* expression is localized in the photoreceptor cells surrounding the upper part of the ocellus and in some cells in the posterior-ventral part of the sensory vesicle belonging to the non-pigmented ocellus (Fig. 6A). The expression was not altered by the exposure to all the tested doses of BPA, even when the deposition of pigment in pigment cells was severely affected (Fig. 6B-C).

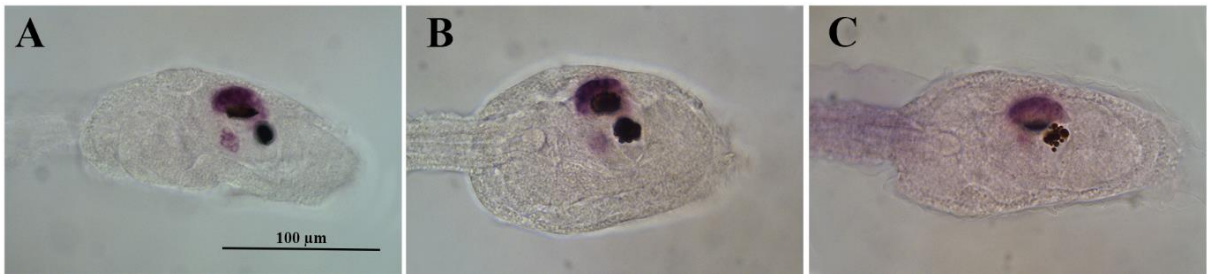

Figure 6: *Ci-opsin1* **e**xpression in *C. intestinalis* larvae (20 hpf) exposed to different concentrations of BPA. (A) Control larva, (B) larva developed in 1 µM BPA, (C) larva developed in 5 µM BPA.

4.5. Discussion

In this work, we analyzed the effects of BPA exposure on the expression of genes involved in pigment cells differentiation of the ascidian *Ciona intestinalis*, as *Ci-Six3/6* and *Ci-Pax6*, and known to be under the control of FGF pathway (Racioppi et al 2014), and on genes involved in

pigment synthesis as *Ci-tyr*, to test the hypotheses that BPA can interfere with this signaling mechanism.

Exposure to low concentrations of BPA was sufficient to induce an ectopic expression of *Ci-Six3/6* in tailbuds. The most anterior cell of the pigment cell progenitors (PCPs), which normally does not express this genes, became *Ci-Six*3/6 positive. Higher concentrations of BPA were necessary to lost *Ci-tyr* expression in anterior PCPs. Similarly, the anterior neural marker *Ci-Pax6* was expressed ectopically in posterior PCPs domain in BPA treated embryos. Ectopic expression of *Ci-Six3/6* was maintained also at larval stage. The correct expression of these genes requires FGF signaling molecules from adjacent *Ci-FGF8/17/18* and *Ci-FGF9/16/20* positive cells. Interestingly, other genes involved in photoreceptor differentiation were not affected by BPA, suggesting the existence of a specific teratogenic mechanism involved in the specification of pigment cells. Our results are similar to those obtained after FGF inhibition by transfection with a dominant negative form of FGF receptor (Racioppi et al 2014) and suggest that BPA exerts its teratogenic effects by acting at some point of the cascade activated by FGF signaling.

A possible candidate gene involved in this mechanism is *Ci-ERK1/2*, a downstream factor of FGF involved in pigment organ differentiation that is continuously and differentially activated in the pigment cell lineage following each successive cell division (Haupaix et al 2014). In fact, it has been demonstrated that BPA can modulate ERK activity in breast and endometrial cancer cells binding, among others, to the orphan nuclear estrogen related receptor (ERR) γ (Shafei et al 2018, Yaguchi 2018).

Indeed, in zebrafish, defects in otolith caused by exposure to BPA are mediated by binding to ERRγ (Tohmé et al 2014). ERRs (α, β and γ) are a group of the nuclear receptor (NR) superfamily of transcription factors closely related to ERs. Like other NRs, the ERRs regulate gene expression binding to a specific sequence in the regulatory regions of target genes. The transcriptional activity of the ERRs is constitutive and independent to exogenously added ligands. Although natural ERR agonists may not exist, the ER agonist diethylstilbestrol (DES) and the selective ER modulator (4-hydroxytamoxifen; 4-OHT) have been identified as ERR antagonists (Coward et al 2001, Tremblay et al 2001).

Ascidians as other invertebrates have a unique ERR gene (*Halocynthia* and *Ciona*). A study on the ascidian *Halocynthia roretzi* suggested that tunicate ERRs are the ancestral forms of vertebrate ERRs, since a phylogenic analysis based on amino acid sequences of Hr-ERR placed it before the duplication of vertebrate ERR into ERRα and β/γ (Park et al 2009). Gene reporter assays showed that *Hr-ERR* activates transcription through ERRE and its activity is antagonized

by 4-OHT (Park et al 2009). *Hr-ERR* transcripts as well as ERR of *Ciona intestinalis* (Imai et al 2004) are abundant during early embryogenesis and decreased during their late stages, from neural plate to larva (Devine et al 2002, Park et al 2009).

We previously demonstrated that co exposure with 4-OHT is able to rescue BPA effects on pigment cells in the ascidian *Phallusia mammillata* (Messinetti et al 2018b). This suggests that BPA exert its activity by binding to ERR also in ascidians, possibly activating ERK1/2 and ultimately altering FGF pathway downstream genes.

Even if direct evidences of ERK activation after BPA exposure are required, these results provide the first indication that BPA can interfere with basic mechanisms of cells differentiation, shared by a vast majority of animals, rising concern about the consequences of massive presence of this pollutant in the environment and urging the request of its use regulation.

4.6. References

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Chapter 5 – Microplastic effects on two marine invertebrates with different feeding strategies

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5.1. Abstract

Nowadays, microplastics represent one of the main threats to marine ecosystems, being able to affect organisms at different stages of their life cycle and at different levels of the food web. Although the presence of plastic debris has been reported in different habitats and the ability to ingest it has been confirmed for different taxa, few studies have been performed to elucidate the effects on survival and development of marine animals. Thus, we explored the effects of different environmental concentrations of polystyrene microbeads on the early stages of two invertebrate species widespread in the Mediterranean shallow waters: the pelagic planktotrophic pluteus larvae of the sea urchin *Paracentrotus lividus* and the filter-feeding sessile juveniles of the ascidian *Ciona robusta*. We evaluated the effects on larvae and juvenile development and determined the efficiency of bead ingestion. The feeding stages of both species proved to be extremely efficient in ingesting microplastics. In the presence of microbeads, the metamorphosis of ascidian juveniles was slowed down and development of plutei altered. These results prompted the necessity to monitor the populations of coastal invertebrates since microplastics affect sensitive stages of life cycle and may have consequences on generation recruitment.

5.2. Introduction

Microplastics (MPs) are small plastic particles (1 µm - 1 mm; Andrady 2015) some of which are specifically produced at the micro-level scale, such as sandblasting media, virgin pellet, cosmetics (Fendall & Sewell 2009), while others originate from the degradation of bigger plastic debris, such as polyester fibers, polyethylene plastic bags and polystyrene particles from buoys and floats (Browne et al 2011, Davidson 2012, O'Brine & Thompson 2010). Over the past 50 years, an incredible amount of plastic has reached the marine environment and its presence has been recorded in different aquatic habitats at all latitudes (Thompson et al 2004). The potential negative impact of this big debris on the ecosystems has been taken into consideration since the 1980s (Stefatos et al 1999) while the MPs have been neglected until recent time, when numerous studies flourished, highlighting the presence of these particles in different environmental compartments. In North American Great Lakes, 43,000 microplastic particles/km^2 have been registered (Eriksen et al 2013), whereas the sediments of Italian Garda Lake contain more than 1,000 particles/ m^2 (Imhof et al 2013). According to Eriksen et al (2014) there are over 5 trillion microplastics floating in the oceans. More in detail, their amount in the coastal waters has been estimated to vary from 3 to 100,000 item/m^3 (Carpenter et al 1972, Doyle et al 2011, Norén & Naustvoll 2010) and to exceed 67,000 particles/ km^2 in the open oceans (Colton et al 1974).

However, this measurement has been determined through the employment of plankton nets that have a mesh size between 80 and 330 μm, generating an important underestimation of the abundance and distribution of the smaller particles, which probably represent the highest threat (Andrady 2015). Moreover, the influence of tide, wind, wave action and oceans currents determines a high variability of spatial and temporal distribution of particles, making extremely difficult a real quantification of MPs abundance. Nowadays, the ecological impacts of MPs are of particular interest but our knowledge about their effects on marine organisms is still very limited. MPs ingestion has been demonstrated in different taxa including fish (Boerger et al 2010, Davison & Asch 2011, Lusher et al 2013), seabirds (van Franeker et al 2011), benthic polychaetes (Wright et al 2013), amphipods, lugworms, barnacles (Thompson et al 2004), mussels (Browne et al 2008), decapods crustaceans (Murray & Cowie 2011) and in different zooplanktonic organisms (Cole et al 2013). The negative effects of MPs oral uptake vary from damaging and blocking the feeding appendages and digestive system (Derraik 2002, Laist 1997, Murray & Cowie 2011), to limiting the food intake and transferring pollutants in living organisms (Mato et al 2001, Oehlmann et al 2009, Talsness et al 2009, Teuten et al 2009). In copepods, the copresence of MPs beads and algae reduces the ingestion rate of the latter (Cole et al 2013) and ultimately causes fertility reduction, probably due to insufficient nutrition (Lee et al 2013). However, other species, such as polychaete worms, are able to ingest and expel plastic microspheres without any apparent detrimental effects, underlying the variety of responses in the different taxa (Cole et al 2011). When MPs reach the marine environment, they can interact with a wide range of organisms (Barnes et al 2009). Animals inhabiting different compartments of the marine environment and displaying different feeding strategies can be differently affected by MPs. Moreover, different stages of the life cycle can have diverse sensitiveness, being the larvae usually the most vulnerable. Very scant data are available regarding the effects of MPs on the development of aquatic organisms, as most studies have focused on the chemical aspects of MPs. In fact, the toxic effects of leachate from particles have been analyzed in different organisms, such as mussels (e Silva et al 2016), fish (Lonnstedt & Eklov 2016) and sea urchins (Nobre et al 2015), whereas the physical effects are still scarcely explored (Kaposi et al 2013). To fill this important gap of knowledge, we evaluated the effects of environmental MPs concentrations on developmental stages of two different invertebrate species. *Ciona robusta* (Brunetti et al 2015, Pennati et al 2015) is a solitary ascidian, inhabiting shallow waters, in particular coastal areas rich of organic material (Satoh 1994), and proposed as model organism to test water pollution (Zega et al 2009). The adults develop through tadpole non-feeding larvae which swim for few hours before adhering to a substrate and metamorphosing into sessile juveniles (Chiba et al 2004,

Hotta et al 2007). Both ascidian adults and juveniles are filter feeding and exploit the pharyngeal gill slits in muco-ciliary plankton feeding (Burighel & Cloney 1997). *Paracentrotus lividus* is a common echinoid of the Mediterranean and North Atlantic coasts. This herbivorous sea urchin has an important role in coastal ecosystem maintenance as its foraging activity remarkably affects the compositions and the dynamics of algal and rocky littoral pools (Lawrence 1975). Mature gametes are released directly in seawaterand the larvae are pelagic until they become competent and undergo metamorphosis after about three weeks. Feeding activity starts slightly before the pluteus stage is reached, 48 h post fertilization (Giudice 1986). The larvae feed on plankton employing external ciliary bands (Strathmann 1971). The chosen species offered the unique opportunity to evaluate and compare the impact of MPs on the development of two different suspension feeders: the pelagic suspension feeding plutei and the sessile filter-feeding ascidian juveniles. These allowed us to investigate if MPs differently affect animals with different feeding strategies. This information is of key importance to evaluate the existence of a specific sensitive compartment in the marine environment and properly manage its natural resources.

5.3. Materials and methods

5.3.1. Microplastics

Polystyrene spherical microparticles with a dark red colour and a diameter of 10 mm were used in the experiments; chemical and physical properties of the MPs were provided by the supplier (Sigma, Italy). The red colour allowed us to follow the beads track inside the transparent tested animals. The size of microbeads was chosen to be compatible with the plutei mouth opening (about 20 μm) and ascidian juvenile oesophagus (20-30 μm). Polystyrene microbeads were preferred because, unlike other plastics, they have a negligible styrene release in suspension (Cohen et al 2002), ensuring that the observed effects can be ascribed to the physical presence of plastic particles and not to monomer contamination. The commercial standard was an aqueous suspension with a particle concentration of 50 mg/ml, that was diluted 1:1000 in artificial sea water buffered with 5 mM Hepes pH 8 (ASWH) to produce a stock suspension of 50 μg/ml of beads from which the final exposure suspensions were made. As published protocols suggested (Cole et al 2013, Kaposi et al 2013), all the suspensions were freshly prepared every time and sonicated for 10 min before use to ensure a homogenous distribution of the beads in the medium. Based on previous works (Lee et al 2013), four different microparticles concentrations were

tested: 0.125, 1.25, 12.5 and 25 µg/ml. For both the experimental models, all the experiments were performed in triplicate.

5.3.2. Ascidians

Adults of the ascidian *C. robusta* were collected from natural populations in Chioggia bay (Venice, Italy) and maintained in aquaria at 18 ± 1 C. Constant light condition was preferred to promote gamete production and avoid spawning (Lambert & Brandt 1967). For each experiment, at least three adults were sacrificed. Eggs and sperms were obtained by dissection of gonoducts and cross fertilization was performed in vitro. Embryos were cultured at 18 ± 1 C in ASWH until they reached the desired developmental stages (see below).

5.3.2.1. Development and larval survival rate

To test the effects of MPs presence on ascidian embryonic development, 30 embryos at 2-cells stage were exposed to the different bead concentrations in ASWH. Each exposure was performed in triplicate (n = 450). They were reared at 18 ± 1 °C until control larvae (CO), maintained in ASWH, reached the hatching larva stage (~ 18 h post fertilization (hpf); Hotta et al 2007). To keep MPs in suspension, embryos were maintained in gently rocking condition. After ~ 18 hpf, the survival rate was evaluated: each experimental group was carefully observed under a stereoscope and the percentage of alive larvae was calculated as: (number of alive larvae/total exposed embryos) x 100. Subsequently, larvae were fixed in 4% paraformaldehyde in standard Phosphate buffer saline (PBS) supplemented with 0.5 M NaCl, for 1.5 h at room temperature. After a few washes in PBT (PBS + 0.01% Tween 20), larvae were mounted on slides and observed under a dissection microscope to evaluate the presence of malformations.

5.3.2.2. Metamorphosis

Ascidian embryos develop into tadpole swimming larvae that, after few hours, metamorphose into sessile juveniles, in which adult tissues and organs differentiate. To determine the effects of MPs on metamorphosis, embryos were allowed to develop in ASWH until they reached the hatching larva stage. Then, 30 larvae for each treatment were transferred into 5.5 cm Petri dishes and allow to attach to the substrate. After adhesion, ASWH was replaced with the testing suspensions (0.125, 1.25, 12.5 and 25 µg/ml microbeads in ASWH). Control animals (CO) were maintained in fresh ASWH. 100 µl of a concentrated suspension of algae (*Tetraselmis* sp. 4-10 µm of diameter) were added to each treatment. The media were changed every day with freshly prepared ones. Animals were left to develop in the experimental conditions for four days. Then,

each individual was observed under a stereoscope to estimate the proceeding of juvenile development. Each treatment was performed in triplicate (n = 90). The metamorphosis in *C. robusta* is a complex series of events (Chiba et al 2004), mainly consisting in tail reabsorption, organs rotation and development of protostigmata or gill slits. By day 4, juveniles normally reach stage 4, characterized by completed organs rotation and the presence of two pairs of protostigmata (for a comprehensive description of *Ciona* metamorphosis process see Chiba et al 2004). To evaluate MPs effects on metamorphosis and juvenile development, we assigned a developmental stage, roughly corresponding to those described in Chiba et al (2004), to each individuals, mainly evaluating organs rotation and the dimension of the axial complex. Stage 4 juveniles had a small almost negligible axial complex; stage 3 juveniles had completed organ rotation but retained a big axial complex; stage 2 samples did not start the organs rotation. Moreover, we counted animals that had adhered to the dish and died soon after (Fig. 1A-D).

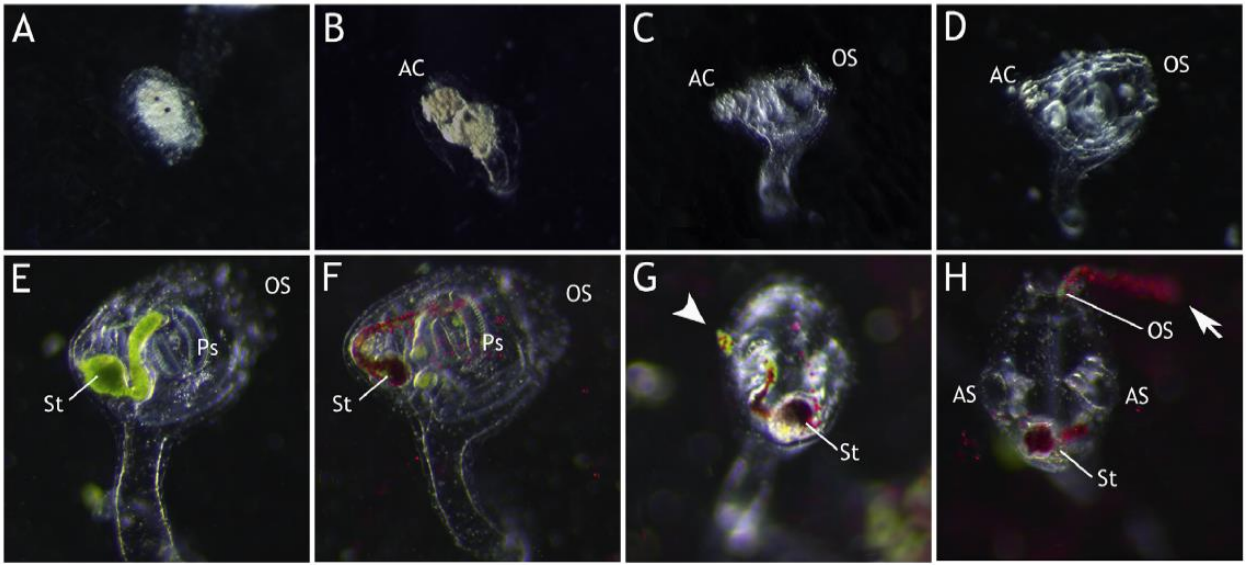

Figure 1: *C. robusta* developing juveniles. A-D: Different phenotypes observed after exposure to microplastics. A: Dead individual. B: Stage 2. C: Stage 3. D: Stage 4, see text for description. E-H: Feeding juveniles observed 24 h after exposure. E: Lateral view of a control juvenile with algae in the digestive tract. F: Lateral view of a juvenile exposed to 25 µg/ml MPs showing MPs and algae in the digestive tract. G: Dorsal view of a juvenile exposed to 12.5 µg/ml showing MPs and algae in the digestive tract and in a fecal pellet. H: Dorsal view of a juvenile while rejecting MPs from the oral siphon. AC, axial complex; AS, atrial siphon; OS, oral siphon; Ps, protostigmata; St, stomach. Arrowhead = faecal pellet; arrow = rejected MPs.

5.3.2.3. Juvenile survival

Effects on juvenile survival were evaluated exposing ~ 90 metamorphosed individuals (30 for each replica) at stage 3 to the different concentrations of MPs for 8 days. Stage 3 was chosen

since it was the stage just before the oral siphon started contracting (Chiba et al 2004). The suspensions were supplemented with 100 µl of concentrated algae and renewed every day with freshly prepared ones. A control group (CO) was reared in ASWH plus algae. Then, each individual was observed under a stereoscope and the percentage of alive juveniles was recorded.

5.3.2.4. Feeding behavior and ingestion rates

To measure the ingestion rate, 30 juveniles at stage 4 (Chiba et al 2004) were exposed to MPs. As soon as the exposure suspensions were added, the single individuals were observed under a stereoscope and the feeding activity was recorded for 1 min using a Leica DFC-450-C camera. The ingestion rate was calculated as the number of particles ingested in the first minute of exposure. The juveniles were maintained in the exposure media for 12 h then the suspensions were replaced with ASWH plus algae. After 24 h, the percentage of juveniles with MPs in their digestive tract and/or in fecal pellets was calculated to estimate the expelling efficiency.

5.3.3. Sea urchins

Adults of *Paracentrotus lividus* were collected in the Gulf of Lerici (La Spezia, Italy) and reared in aquaria as previously described (Mercurio et al 2014). Gametes were obtained by intraoral injection of 1e2 ml of 1 M KCl (Kaposi et al 2013) from adults of homogenous size (Ø ~ 4 cm). Fertilization and embryos culture were performed at 18 °C with 12 h dark/light cycle in 300 ml of gently aerated ASWH. As sea urchin larvae start eating between 36 and 48 h post fertilization (hpf), Phyto Reef (SHG, Italy), a nutritional supplement based on microalgae (2-8 µm) was added to the media just before feeding activity began.

5.3.3.1. Development and larval survival rate

Sea urchin embryos were allowed to develop in ASWH for 24 hpf. Then, 25 ml of ASWH with embryos were added to 25 ml of MPs suspensions in order to reach the final testing concentrations (0.125, 1.25, 12.5 and 25 µg/ml microbeads in ASWH + CO). When the larvae reached the 4-armed pluteus stage (72 hpf), 1 ml of each treatment suspension plus plutei was collected and the percentage of alive larvae was evaluated under a microscope as: (number of larvae with ciliary movements/total number of larvae) x 100. To assess the effects on development, 1 ml of each suspension was collected and larvae were fixed in 4% paraformaldehyde in PBS supplemented with 0.5 M NaCl. Ninety randomly chosen animals for each tested concentration were photographed under an optical microscope and body length, post-

oral arm length and body width (Fig. 6A) were measured to verify the presence of developmental delay or malformations as reported by (Kaposi et al 2013).

5.3.4. Comparison between ascidian and sea urchin feeding activity

To compare the ingestion efficiency of ascidian filter-feeding juveniles and sea urchin pelagic plutei, we exposed for an hour stage 4 juveniles and 72 hpf plutei to the different concentrations of MPs. Subsequently, juveniles and plutei were observed under a stereomicroscope and number of individuals displaying MPs in their digestive tract was recorded. Feeding efficiency was calculated as: (number of fed individuals/total number of individuals scored) x 100.

5.3.5. Statistical analysis

All data were analyzed using the software R (R-Core-Team 2013). A single way Analysis of Variance (ANOVA) was performed, after a Cochran Test to test the homogeneity of variance. If H0 of homoscedasticity was rejected, the data were square root transformed to meet the assumptions of the analysis. If the results of ANOVA were significant ($P < 0.05$), a Tukey's Post-hoc Test was performed to disentangle differences among groups.

5.4 Results

5.4.1. Effects on ascidians

5.4.1.1. Development and larval survival rate

Larval survival rate was not affected by MPs exposure at all the tested concentrations ($F_{4,10}$ = 2.0433; p = 0.1639; Fig. 2). Moreover, exposed larvae showed a normal phenotype, perfectly comparable to control samples (Fig. 3).

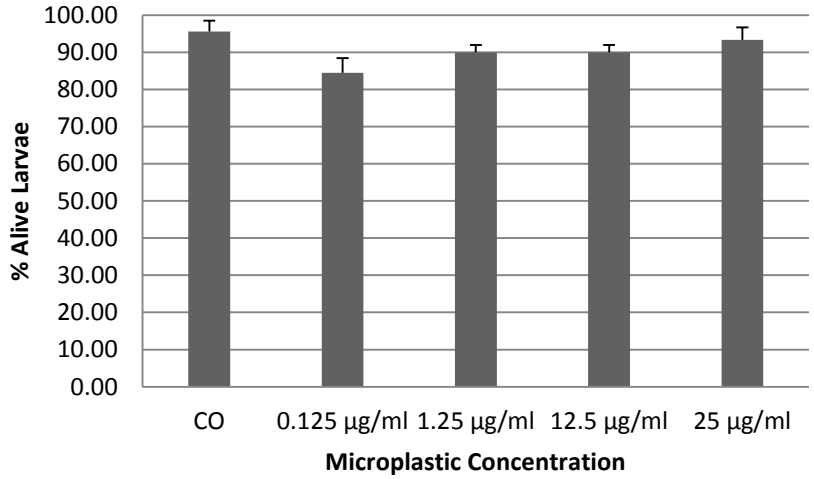

Figure 2: Effects of microplastic presence on ascidian larval survival rate. Data are means of 3 replicates with ±SE. No differences were observed.

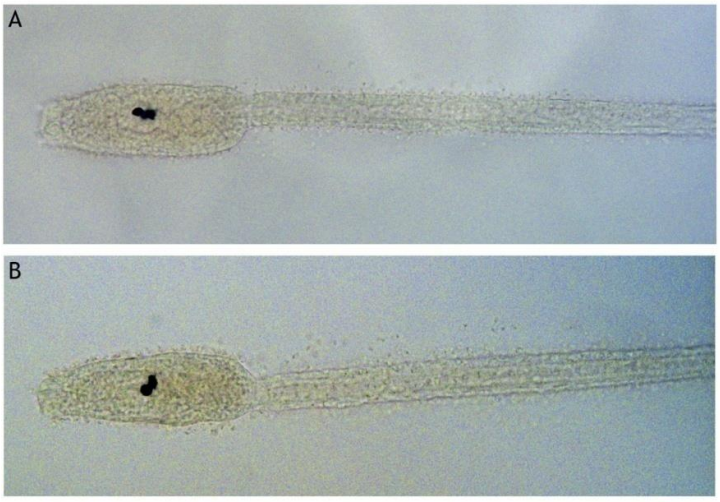

Figure 3: A) Control larva; B) Larva developed in 25 µg/ml of microplastics. No differences in the phenotypes were observed.

5.4.1.2. Metamorphosis

We recorded in all the experimental groups the presence of dead individuals and juveniles at different developmental stages (Fig. 1A-D). Dead animals were observed in all the experimental groups and no statistically significant difference was reported. The percentage of individuals that after 4 day reached stage 4 (Fig. 1D) was significantly lower in all the exposed groups compared to control group ($F_{4,10}$ = 11.388, p = 0.0009; Fig. 4A). Moreover, the percentage of juveniles scored as stage 3 (Fig. 1C) was significantly higher at 12.5 and 25 µg/ml concentrations than in control group ($F_{4,10}$ = 4.6175, p = 0.0226; Fig. 4A). Metamorphosis was slowed down but manifest malformations were not observed at all the tested concentrations.

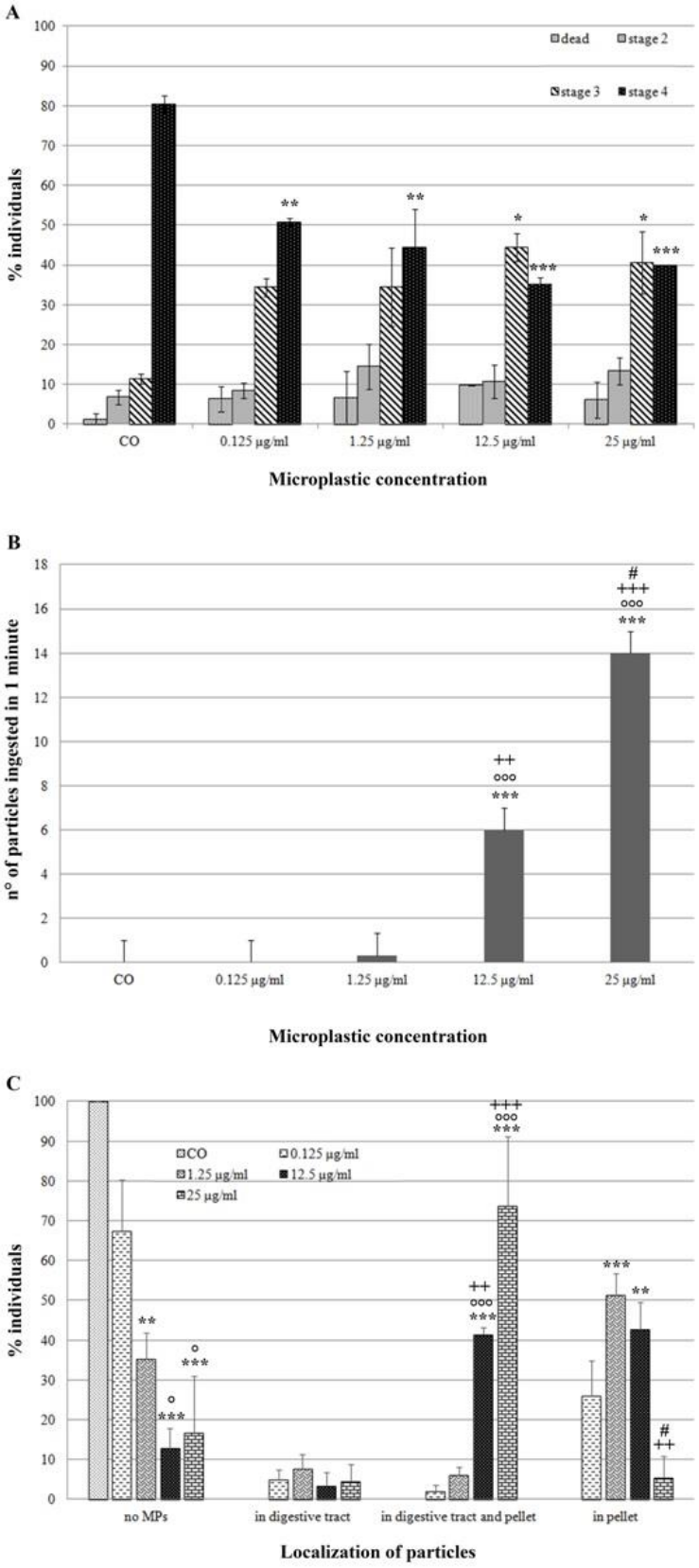

Figure 4: Effects of polystyrene microplastics on C. *robusta*. Data are means of 3 replicates ± standard error (SE). A: Percentages of juveniles at different developmental stages observed after 4-day exposure to different concentrations of MPs. B: Number of MPs ingested after 1 min of exposure at different concentrations. C: Localization of MPs after 24 h from exposure. Legend of symbols: * = differences from control; ° = differences from 0.125 µg/ml; + = differences from 1.25 µg/ml; # = differences from 12.5 µg/ml. The repetition of each symbol indicate the level of significance according to R significance codes: $p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *.

5.4.1.3. Juvenile survival

No statistically significant difference in juvenile survival was observed between control and treated samples: survival rate ranged from 83.7%, observed in control group and 67.5% registered at the highest MPs concentration ($F_{4,10}$ = 1.147; p = 0.3894; Fig. 5).

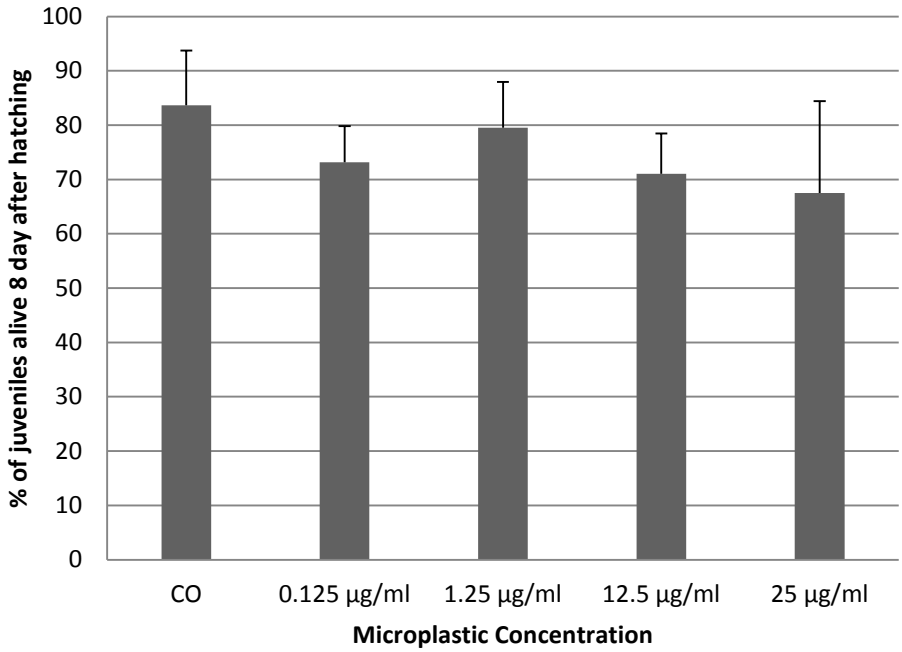

Figure 5: Effects of microplastics presence on juveniles survival rate 8 dph. Data are means of 3 replicates with ± SE. No differences were observed.

5.4.1.4. Feeding behavior and ingestion rates

After 1 min of exposure to the three highest concentrations, juveniles had already MPs in their digestive tract (Fig. 4B). The number of styrene MPs ingested increased with particle concentration ($F_{4,10}$ = 40.804, p = $3.66*10^{-6}$). In particular, the number of MPs ingested in juveniles exposed to 12.5 and 25 µg/ml was significantly higher than that ingested by juveniles exposed to 1.25 µg/ml (Fig. 4B). After 24 h (Fig. 1E-G, 4C), very few individuals had MPs only in the digestive tract and the differences were not significant for all the concentrations ($F_{4,10}$ = 0.7388, p = 0.5865). Most of the juveniles exposed to 0.125 and 1.25 µg/ml had no MPs or had expelled them in the fecal pellets. At the two highest concentrations (12.5 and 25 µg/ml), a significant higher percentage of individuals displayed MPs both in the pellets and in the digestive tract (12.5 and 25 µg/ml vs the other three treatments: $F_{4,10}$ = 32.217, p = $1.092*10^{-5}$). Some juveniles were observed while rejecting MPs and algae from oral siphon (Fig. 1H).

5.4.2. Effects on sea urchins

5.4.2.1. Development and larval survival rate

Plutei can efficiently ingest MPs that can be observed in their digestive tract (Fig. 6B). After 72 h exposure, no difference in the survival rate was observed between control larvae and those developed in presence of MPs beads at all tested concentrations ($F_{4,10}$ = 0.6298; p = 0.6524; Fig. 7). Significant differences were found in body length and arm length of plutei reared at different MP concentrations (Fig. 8A-C). Plutei exposed to 12.5 µg/ml were significantly longer than control ones ($F_{4,445}$ = 20.24, p < 0.0001; Tukey's post hoc test p = 0.0001), while plutei exposed to 25 µg/ml were shorter ($F_{4,445}$ = 20.24, p < 0.0001; Tukey's post hoc test p = 0.0002). Differences in body width were found between control group and 0.125 µg/ml, between 0.125 µg/ml and all the other treatments and between 12.5 µg/ml and 25 µg/ml ($F_{4,445}$ = 18.29, p < 0.005). At last, treated plutei had longer arms than control group when developed at 0.125 and 12.5 µg/ml ($F_{4,445}$ = 39.67, p < 0.0001; Tukey's post hoc test p < 0.0001). Plutei developed at 1.25 µg/ml had shorter arms compared to 0.125 µg/ml (Tukey's post hoc test p = 0.0336) and those developed at 25 µg/ml had shorter arms compared to all the other treatments (Tukey's post hoc test p < 0.0001).

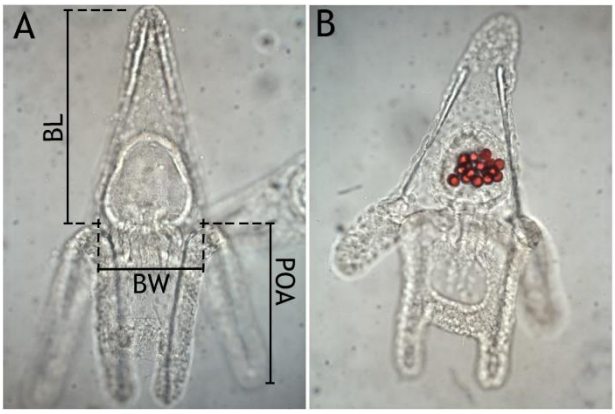

Figure 6: *Paracentrotus lividus* plutei. A) Control pluteus 72 h post fertilization. B) Pluteus developed from an embryo exposed to 25 µg/ml MPs. BL: body length; BWbody width POA: post oral arm.

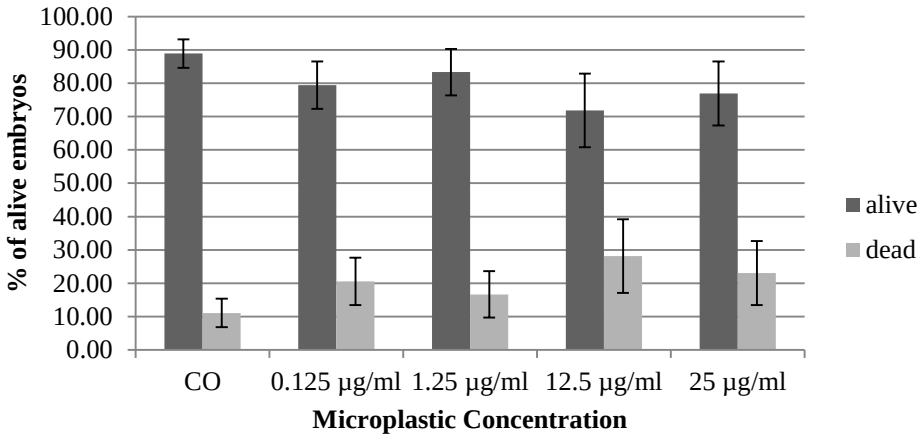

Figure 7: Effects of microplastic presence on sea urchin embryos' survival rate. Data are means of 3 replicates with ±SE. No differences were observed**.**

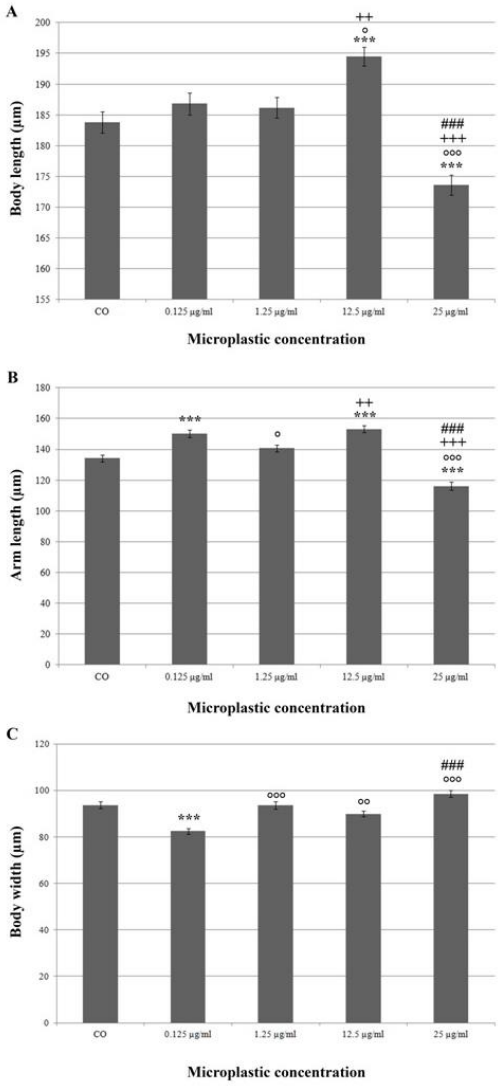

Figure 8: Morphometric analysis of plutei exposed to MPs. Legend of symbols: * = differences from control;° = differences from 0.125 µg/ml; + = differences from 1.25 µg/ml; # = differences from 12.5 µg/ml. The repetition of each symbol indicates the level of significance according to R significance codes: $p < 0.001$ ***; $p < 0.01$ **; $p < 0.05$ *.

5.4.3. Comparison between ascidian and sea urchin feeding efficiency

After 1 h exposure to MPs, both ascidian juveniles and plutei ingested beads at all tested concentrations but they displayed different efficiencies. In both species, the percentage of individuals with beads in their digestive tract increased with MP concentration. At 1.25, 12.5 and 25 µg/ml, more than 87% of ascidian juveniles had MPs in their digestive tract. These values were significantly higher than the percentage of juveniles that ingested MPs at 0.125 µg/ml concentration and in control group ($F_{4,10}$ = 106.1, p = $3.08*10^{-8}$; Fig. 9). Only 20% of plutei ingested MPs at 0.125 µg/ml, while 100% of plutei at 25 µg/ml showed them in the digestive tract ($F_{4,10}$ = 283.55, p < 0.0001). At highest concentrations, there was not a significant difference in the efficiency of ingestion between the two species, as almost all individuals fed on MPs (ANOVA: 12.5 µg/ml: $F_{1,4}$ = 0.4895, p = 0.5227; 25 µg/ml $F_{1,4}$ =1, p = 0.3739). On the other hand, at the lowest concentrations, the efficiency of ingestion of planktonic plutei resulted significantly lower than that of filter-feeding benthic juveniles of *C. robusta* (ANOVA: 0.125 µg/ ml: $F_{1,4}$ = 7.9014 p = 0.04827; 1.25 µg/ml: $F_{1,4}$ = 27.888 p = 0.006166; Fig. 9).

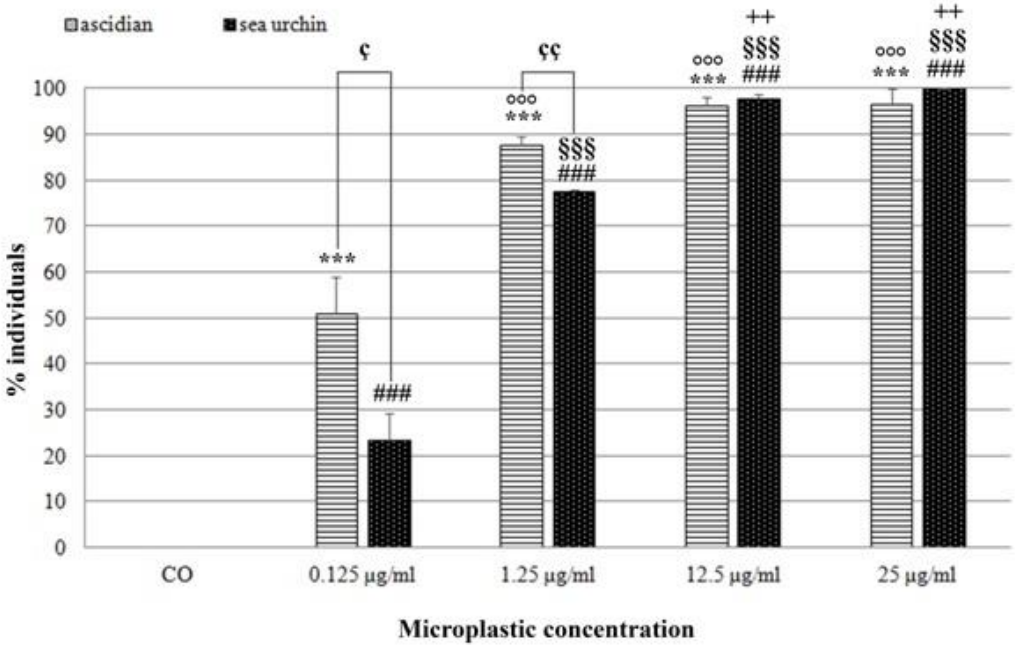

Figure 9: Percentage of *C. robusta* juveniles and *P. lividus* plutei with MPs in their digestive tracts, after 1 h of exposure to particles. Data are means of 3 replicates with ±SE. Legend of symbols: * = differences of *C. robusta* from control;° = differences of *C. robusta* from 0.125 µg/ml; # = differences of *P. lividus* from control; §= differences of *P. lividus* from 0.125 µg/ml; + = differences of *P. lividus* from 1.25 µg/ml; ç = differences between the two species at the same concentration. The repetition of each symbol indicates the level of significance according to R significance codes: p < 0.001 ***; p < 0.01 **; p < 0.05 *.

5.5. Discussion

Plastic pollution represents one of the main threats to aquatic ecosystems: the high production level and the slow degrading time of plastic have led to an increasing amount of plastic-derived debris in oceans and seas. After degradation, these remains are fragmented into micro sized particles that can be ingested by marine organisms and enter the food web (Andrady 2015). In this study, we evaluated the effects of polystyrene MPs on the development of two ecologically relevant marine invertebrates: the ascidian *C. robusta* and the sea urchin *P. lividus*. Our results add new important information about the effects of these emerging pollutants on early life stages of invertebrates with different feeding strategies. As already reported for many other taxa, the feeding stages of both the analyzed species are able to efficiently ingest MPs. In particular, ascidian juveniles and plutei were fed with a suspension of MPs and algae and they appeared to be unable to discriminate between food and inorganic particles, as other invertebrates proved to do (Fernández 1979, Paffenhofer & Vansant 1985). Indeed, when ascidian juveniles are exposed to high particles concentrations, the MPs ingestion occurred as soon as beads were added to ASWH, suggesting that juveniles, filtering the water, did not make any kind of selection. However, some individuals were observed rejecting microbeads and algae from the oral siphon, with a series of whole body contractions. This is probably a reflex triggered by sensorial organs upon siphon tentacles (Mackie et al 2006, Rigon et al 2013) and due to the excessive intake of particles. In contrast, nauplii of the copepod *Calanus pacificus* strongly selected and consumed almost exclusively algal cells, when fed with mixtures of planktonic algae and plastic beads of different or similar sizes (Fernández 1979). The ability of *C. pacificus* to select organic particles could be due to the restrictive diet of naupilii that feed only upon plant material (Fernández 1979), while ascidian juveniles filter water uptaking any kind of particles. Thus, the different sensitiveness to MPs observed in these two animals could be related to their specific feeding behavior. Furthermore, the appearance of the rejection reflex in *Ciona* juveniles at high MPs concentration was probably caused by an adaptive behavior of the sensorial organs. Indeed, different sensory structures have been described in ascidian siphons. In the atrial cavity, mechanoreceptors, the cupular organs, send their axons to the central nervous system. At the oral siphon base, the coronal organ, consisting of secondary neurons, borders the velum and tentacles. In addition, scattered sensory cells are located in siphons inner and outer wall tentacles (Mackie et al 2006, Rigon et al 2013). Previous works reported that numerous marine organisms are able to ingest and expel plastic microspheres without any apparent negative effects (Thompson et al 2004). On the contrary, our results suggested that MPs effects depend on animal feeding strategy

and life stages. After an hour of exposure, a significant percentage of ascidian juveniles and plutei showed plastic beads in their stomach at all tested concentrations. However, comparing MPs intake in the two animal models, the higher ingestion ability of the filter-feeding juveniles appeared evident. Although at the two highest concentrations almost all individuals ingested MPs, regardless of the species, at the lowest concentrations a significant higher percentage of ascidian juveniles ate microbeads compared to sea urchin plutei. This result suggest that the presence of MPs in the environment, even at low concentrations, can impact sessile filtering organisms more than pelagic suspension feeders. This consideration is strengthened by the fact that juvenile and adult ascidians employ the pharyngeal basket to filter a huge amount of water per day, estimated around 46.4 ml/min for adults with a total dry weight of 0.84 g (Randløv & Riisgård 1979). After 24 h, almost all ascidian juveniles that ingested MPs at the concentrations of 0.125 µg/ml and 1.25 µg/ml, managed to expel them with fecal pellets. However, with the increase of particle concentration and, therefore of ingestion, it became more difficult for animals to expel them as demonstrated by the higher percentage of individuals that even after pellet expulsion retained particles in their stomach. Moreover, at the highest concentrations, some ascidian juveniles tried to expel their stomach content by rejecting it from the oral siphon, with a series of contractions of the whole body. Also this behaviour was not completely efficient to eliminate MPs from the digestive tract, suggesting that a higher particles intake can determine a higher risk of damages and digestive system block. Studies on MPs effects on animal development are particularly scarce, even if different stages of life cycle can have diverse sensitiveness, being the larvae usually the most vulnerable one. Thus, we explored the impact of MPs presence on the early life stages of ascidians and sea urchin. Although MPs exposure did not affect the survival rate of *C. robusta* juveniles and *P. lividus* embryos, plastic beads appeared to be detrimental during some developmental phases. We analyzed three different standard parameters to characterized MPs effects on plutei body growth and we observed values that significantly differed from control group. A clear trend was not observable: the absence of a dose-dependent effect between bead concentrations and measured parameters is not unusual in aquatic organisms, being already reported in other species of sea urchin (Kaposi et al 2013), copepods (Cole et al 2013) and marine worms (Wright et al 2013). In our case, the reduction of body and arm length and the increase of body width reported in plutei exposed to 25 µg/ml highlighted that the co-ingestion of beads and algae compromised the correct development of the body shape. More controversial results were the increase of body length at 12.5 µg/ml, the increase of arm length at 0.125 and 12.5 µg/ml and the reduction of body width at 0.125 µg/ml. The high variability in particles intake and release occurring at the lower tested concentrations

may be responsible for the variability of the measured responses. In contrast, at the highest tested concentration, MPs distribution was probably more homogenous making the exposure more uniform and the effects more consistent. Moreover, the same kind of results have been found for the echinoderm species *Tripneustes gratilla* in which a reduction of body width was observed at the lowest and highest concentration tested but not at the intermediate ones (Kaposi et al 2013). In *Ciona*, the metamorphosis proved to be the most sensitive process to MPs presence. Exposed juveniles showed a significant slowdown of metamorphosis, displaying a high percentage of individual at an earlier developmental stage than controls. Since MPs did not affect the embryonic development of the non-feeding ascidian larvae, we supposed that the effects observed on the other stages were mainly due to the physical presence of the beads rather than to chemical released by plastic degradation. It was proposed that the physical presence of microbeads can alter the development of a planktotrophic larva, probably reducing the food intake: in copepods, MPs presence appeared to decrease food ingestion (Cole et al 2013), eventually leading to fertility reduction (Lee et al 2013). Moreover, it was suggested that the selective feeding behavior of *C. pacificus* nauplii could be an energy input optimization, so the animals could achieve a larger input of energy per unit time than would be if no selection occurred (Fernández 1979). Thus, the non-selection behavior displayed by ascidian juveniles and plutei suggested that MPs ingestion can cause a reduction of nutritional uptake, responsible for the developmental and growth alterations observed in ascidians and plutei. These considerations suggested that sensitiveness to MPs differ among species, indicating the necessity to extend the studies to a wider range of species in order to elucidate MPs impact on marine ecosystem. In conclusion, according to our results, the developing stages of both ascidian and sea urchin are able to ingest MPs. Particularly, comparing the intake efficiency of the two species, the sessile filtering organisms appeared more vulnerable to MPs effects than pelagic suspension feeders. Even if MPs exposure do not seem to influence specimens survival, their development was affected as plastic beads ingestion slowed down metamorphosis of *C. robusta* and altered postembryonic development or/and growth of *P. lividus*. These results appear particularly worrying when we consider that the ability to clear the gut of plastic decreases with the increase of MPs concentration. In long-term exposure, this could cause MPs accumulation in the digestive tract, definitively compromise the nutritional uptake and ultimately lead to the animal death. Thus, our results are particularly relevant as they revealed that developmental stages of different species are highly sensitive to MPs presence, prompting the necessity to monitor coastal invertebrate populations since MPs can alter generation recruitment.

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Chapter 6 – Ingested microscopic plastics translocate from the gut cavity of juveniles of the ascidian *Ciona intestinalis*

Messinetti S., Mercurio S., Scarì G., Pennati A., Pennati R. (submitted). Ingested microscopic plastics translocate from the gut cavity of juveniles of the ascidian *Ciona intestinalis*

6.1. Abstract

Small plastic particles, named microplastics, are abundant in the marine environment and can be ingested by marine organisms. Species with different feeding strategies can be differently affected by the presence of microplastics. Moreover, the impact of these particles can depend on their size. In this study, we analyzed the effects of 1 µm polystyrene particles on larval and juvenile development in the ascidian *Ciona intestinalis*. As previously reported for 10 µm beads, smaller particles caused a delay in the growth of juveniles, even if this delay was registered only at the highest concentration tested. Instead, larval development was not affected by the presence of microplastics. Histological analysis of juveniles revealed that 1 µm particles, after ingestion, can translocate from the gut to the internal extracellular compartment in just 8 days. As a defense mechanism, plastic spheres can also be phagocytized from specific blood cells whit phagocyte activity. Microplastics confirmed their potential as a threat to marine wildlife, interfering with food uptake and growth.

6.2. Introduction

Microscopic plastics, 1 µm-1 mm diameter (microplastics, MPs), are an abundant and widespread marine pollutants of increasing environmental and economic concern. They derive from the fragmentation of larger plastic debris, such as plastic bottles and bags, and may also be directly produced by cosmetic industries (Browne et al 2011, Davidson 2012, Napper et al 2015, O'Brine & Thompson 2010).

Biomonitoring studies revealed the widespread ingestion of microplastic particles by marine organisms including fish (Boerger et al 2010, Davison & Asch 2011, Lusher et al 2013), benthic polychaetes (Wright et al 2013), amphipods, lugworms, barnacles (Thompson et al 2004), mussels (Browne et al 2008), decapod crustaceans (Murray & Cowie 2011) and different zooplantoktonic organisms (Cole et al 2013).

Different species display different aptitudes in ingestion and retention of MPs, as demonstrated by the differences in stomach content reported in different invertebrates sampled in the same locality. For example, *Ascidia* spp. specimens retained a number of MPs five-fold higher than bivalve species (*Crassostrea gigas*; *Mytilus galloprovincialis*; *Anomia ephippium;* Bonello et al 2018).

In fact, the ability to select particles varies among species and depends on their buccal specialization and feeding mechanisms. No effect of MPs on survival and growth has been reported when crustaceans or fish, which have selective feeding strategies, have been analyzed

(Fernández 1979, Paffenhofer & Vansant 1985). Similarly, polychaete worms are able to ingest and expel plastic microspheres without any apparent detrimental effects (Cole et al 2011). On the contrary, filter-feeders are more sensitive to microplastics pollution as filter-feeding is a less selective strategy than predation (Gallo & Tosti 2015, Rummel et al 2016).

The effects of MPs oral uptake vary among taxa ranging from damaging and blocking the feeding appendages and digestive system (Derraik 2002, Laist 1997, Murray & Cowie 2011), to limiting the food intake and ultimately causing fertility reduction (Mato et al 2001, Oehlmann et al 2009, Talsness et al 2009, Teuten et al 2009). The size of microplastics plays an important role in determining their effects on marine organisms. The crustacean *Mysis relicta* showed different performance in ingesting MPs of different sizes, the smallest (0.75 µm) and largest (40-50 µm) being less favored than intermediate sizes (Bigelow & Lasenby 1991). Moreover, it has been demonstrated that large microplastics (> 10µm) accumulate in the digestive tracts and are eventually discarded with the feces (Cole et al 2011), while only few studies have investigated whether small microplastics (<10µm) can translocate from the gut cavity to the circulatory system and body tissues. Mussels (*Mytilus edulis*) exposed to 3 µm and 9.6 µm polystyrene beads for 3 days presented MPs particles of both sizes in their circulatory system, where they persisted for over 48 days (Browne et al 2008). This long permanence in the animal body greatly enhanced the possibility of trophic transfer throughout the food web and hindered the efforts to elucidate MPs fate and impacts on marine ecosystem.

Ascidians are filter feeding organisms evolutionarily close to vertebrates (Delsuc et al 2006) and they are an important component of benthic assemblages worldwide (Zega et al 2009). Adults and juveniles of the ascidian *Ciona intestinalis* employ the pharyngeal basket to filter a huge amount of water per day, estimated around 46.4 ml/min for adults with a total dry weight of 0.84 g (Randløv & Riisgård 1979), and they appeared to be unable to discriminate between food and inorganic particles (Messinetti et al 2018). In a previous paper, we tested the effects of 10 µm diameter polystyrene beads in *C. robusta* and demonstrated that exposure to these MPs was not detrimental during larval and juvenile development. In laboratory conditions, juveniles could efficiently ingest them even when they are present at low concentrations (0.125 µg/ml). At high concentrations (12.5 and 25 µg/ml), 10 µm MPs persisted in the digestive system and affected the growth-rate during metamorphosis, probably by decreasing the energy intake (Messinetti et al 2018).

In this paper, we tested the effects of small polystyrene MPs (1 µm diameter) on larval and juvenile development of *C. intestinalis* in order to compare their effects with those obtained with larger MPs. Moreover, we tested whether small MPs can translocate in the circulating fluids.

6.3. Material and methods

6.3.1. Microplastics

Polystyrene spherical microparticles with a dark blue colour and a diameter of 1 μm were used in the experiments; chemical and physical properties of the MPs were provided by the supplier (Sigma, Milano, Italy). The blue colour allowed us to track the beads inside the transparent tested animals. Polystyrene microbeads were preferred as they determine a negligible amount of styrene release when in aqueous suspension (Cohen et al 2002). The commercial standard, an aqueous suspension of 50 mg particles/ml, was diluted 1:1000 in filtered sea water (FSW) to produce a stock suspension of 50 μg/ml of beads from which the final exposure suspensions were made. All the suspensions were freshly prepared each time and sonicated for 10 minutes before use to ensure a homogenous distribution of the beads in the medium. Based on previous work (Messinetti et al 2018), four different MPs concentrations were tested: 0.125, 1.25, 12.5 and 25 μg/ml.

6.3.2. Ascidians

Adults of *Ciona intestinalis* were collected in the water near Roscoff (France) and maintained in aquaria at 18 ± 1 °C. Constant light condition was preferred to promote gamete production and avoid spawning (Lambert & Brandt 1967). For each experiment, at least three adults were sacrificed. Eggs and sperms were obtained by dissection of gonoducts and cross fertilization was performed *in vitro*. Embryos were cultured at 18 ± 1°C in FSW until they reached the desired developmental stages (see below).

6.3.3. Development and survival rate

To test the effects of MPs presence on embryonic development, 30 embryos at 2-cells stage (Hotta et al 2007) were exposed to the different bead concentrations in FSW. Samples were reared at 18 ± 1 °C. Larval survival rate was evaluated when control embryos (CO), maintained in FSW reached the hatching larva stage (∼18 hours post fertilization (hpf); Hotta et al 2007). Each experimental group was carefully observed under a stereoscope and the percentage of alive samples was calculated as: (number of alive sample / total exposed samples) x 100. Each experiment was performed in triplicate.

6.3.4. Metamorphosis

To determine the effects of small MPs on metamorphosis, 30 larvae for each treatment were transferred into 5.5 cm Petri dishes and allowed to attach to the substrate. After adhesion, FSW was replaced with the testing suspensions (0.125, 1.25, 12.5 and 25 µg/ml microbeads in FSW). Control animals (CO) were maintained in fresh FSW. 100 µl of a concentrated suspension of algae were added to each treatment group. The media were changed every day with freshly prepared ones. Animals were left to develop in the experimental conditions for four days. Then, each individual was observed under a stereoscope to estimate the proceeding of juvenile development. The metamorphosis in *C. intestinalis* proceed following a series of events (Chiba et al 2004), mainly consisting in tail reabsorption, organs rotation and development of protostigmata or gill slits. By day 4, juveniles normally reach stage 4, characterized by completed organs rotation and the presence of two pairs of protostigmata (for a comprehensive description of *Ciona* metamorphosis process see Chiba et al. 2004). To evaluate MPs effects on metamorphosis, we assigned a developmental stage, roughly corresponding to those described in Chiba et al. (2004), to each individual, mainly evaluating organ rotation and the dimension of the axial complex (Messinetti et al 2018). We also counted animals that had adhered to the dish and died soon after. Each experiment was performed in triplicate.

6.3.5. Tracking the uptake of polystyrene microbeads

To determine whether polystyrene microspheres had accumulated in the gut, juveniles exposed for 8 days to 25 µg/ml microplastics were fixed in 4% PFA and sectioned for detailed localization of microbeads. Briefly, animals were dehydrated in ethanol series, stained in alcoholic eosine and embedded in Technovit resins (Heraeus Kulzer, Werheim, Germany). 5 µm sections were cut with a microtome, counterstained with ematossilin and mounted with entellan (Merck, Whitehouse Station, N.J.). Samples were observed under a light microscope and photographed using a Leica DFC-320-C camera.

6.3.6. Statistical analyses

To evaluate if the different MPs concentrations significantly affect animal survival and development, the analysis of variance (ANOVA), followed by HSD Tukey's post hoc test, was performed using R software (R-Core-Team 2013) and 'agricolae' package (de Mendiburu 2015). A Cochran test was performed to test the homogeneity and normality of the variances and

percentage data were transformed when they did not meet the assumptions of the analysis (normality and homoscedasticity).

6.4. Results

Exposure of ascidian embryos to different concentrations of 1 µm MPs from two cells to larval stage did not affect development and larval survival (Fig. 1A; ANOVA: F=0.9721, p=0.4644). In laboratory conditions, 4 days after attachment, more than 90% of control juveniles reached the stage 4 of development, characterized by completed organs rotation and the presence of two pairs of protostigmata. The percentage of juveniles that reached stage 4 after exposure to the MPs decreased with bead concentrations and was significantly different between samples exposed to 25 µg/ml MPs and control juveniles (Fig.1B; ANOVA: F= 6.1473, p=0.03612; Tukey's post hoc: CO vs 25 µg/ml p=0.0431). At the same time the percentage of juveniles at stage 3 significantly increased (Fig. 1B; ANOVA: F= 7.247, p=0.026; Tukey's post hoc: CO vs 25 µg/ml p=0.0328).

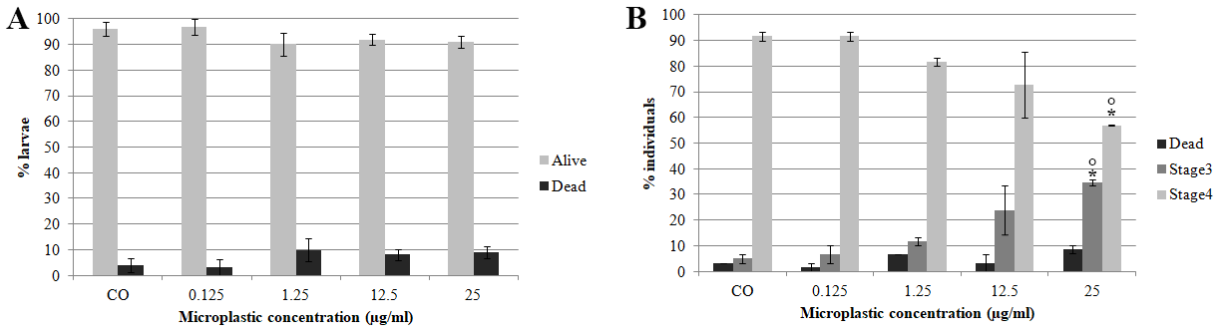

Figure 1: A) Larval survival rate after exposure to microplastics. B) Percentages of juveniles at different developmental stages observed after 4-day exposure to different concentrations of MPs. *= differences from control; °= differences from 0.125 µg/ml. Data are means of 3 replicates ± standard error (SE).

The transparency of ascidian juveniles allowed to clearly observe the gut content in whole mount specimens (Fig. 2A). Juveniles were able to efficiently ingest the small microplastics, that appeared clearly visible in their gut cavity, and to egest them in the fecal pellets (Fig. 2B). When both MPs and algae were present in the medium, juveniles were not able to discriminate between them. Algae and MPs were both present in the gut cavity and in the fecal pellets of exposed individuals (Fig. 2C).

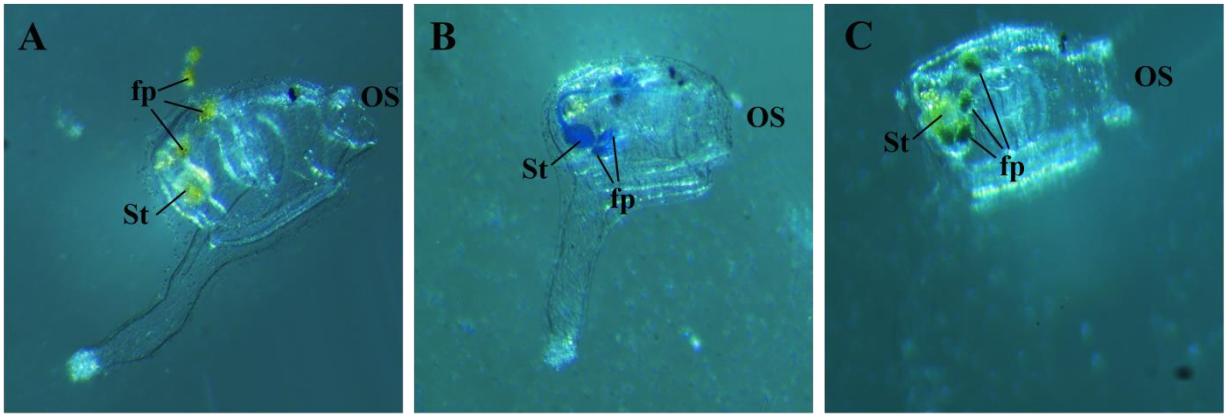

Figure 2: A) Control juvenile that ingested microalgae. B) Juvenile exposed to 25 µg/ml MPs. C) Juvenile exposed to 1.25 µg/ml MPs. St= stomach; OS= Oral Siphon; fp= fecal pellets.

Histological analyses showed that ascidian juveniles exposed to microparticles had accumulated polystyrene microspheres in their gut cavity (Fig. 3A). The microparticles were easily recognizable for their shape, colour and dimensions. They were found in the pharynges, the esophagus, the stomach and the last tract of the intestine (Fig. 3A-D). Some beads were present in the cytoplasm of fusiform cells localized in the wide extracellular space, characteristic of ascidian juvenile tissues (Fig. 3C). These cells presented dark granules in their cytoplasm, of ~1 µm in diameter (Fig. 3C”-C’’’) and, based on these characteristics, they were identified as

granular amoebocytes with phagocytic activity (Rowley, 1982). The microbeads were particularly abundant in the stomach where they appeared densely packed (Fig. 3D). Most interestingly, some microparticles were found outside the gut cavity, in the extracellular fluid, next to the stomach wall (Fig. 3D).

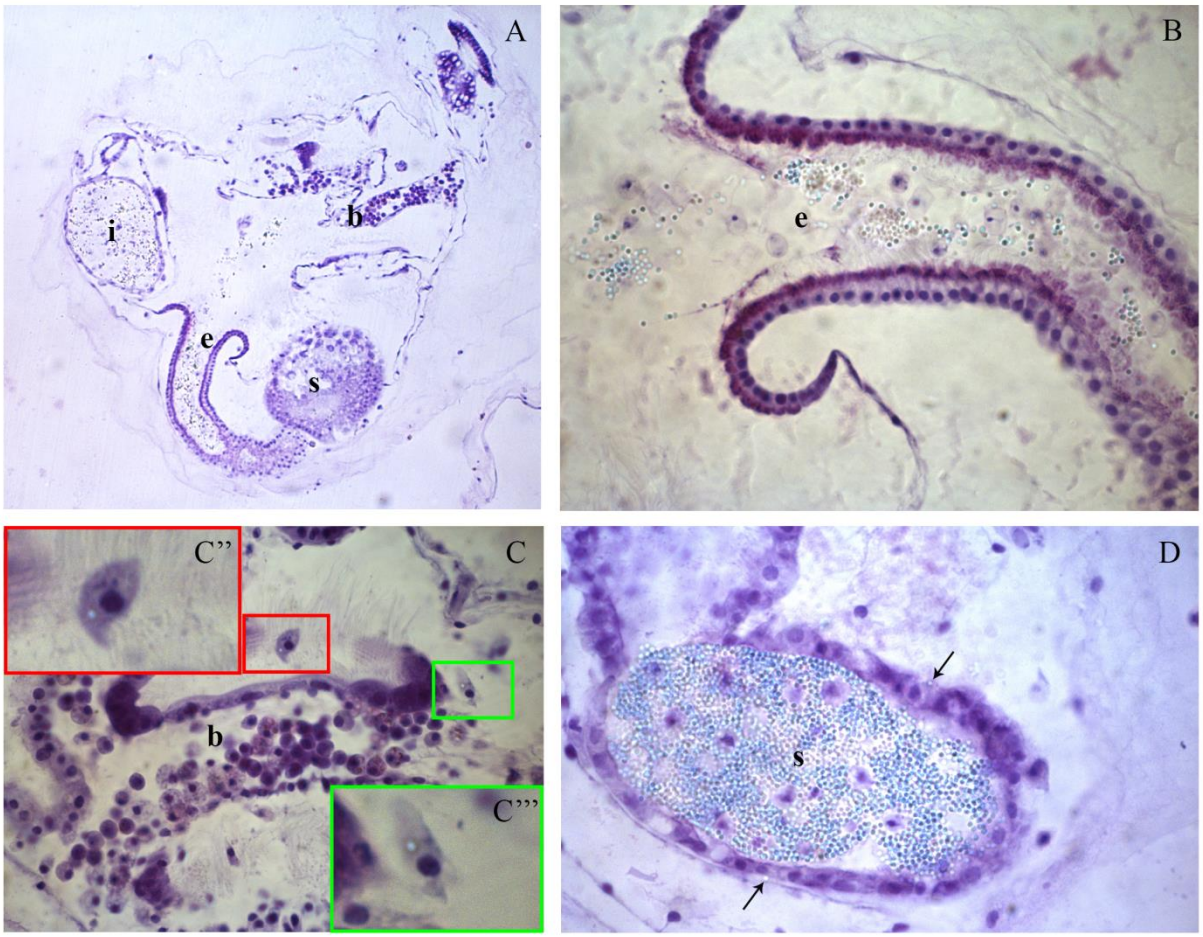

Figure 3: Histological section of juveniles fed with microplastics. A) Longitudinal section. B) Enlargement of the esophagus containing MPs. C) MPs particles phagocyted by amoebocytes. C'', C'') Magnification of amoebocytes containing MPs. D) Stomach of a juvenile containing MPs particles. Arrows indicate MPs outside the stomach wall. i= intestine; b= blood; e= esophagus; s= stomach.

6.5. Discussion

The present work demonstrates that juveniles of *Ciona intestinalis* can efficiently ingest 1 µm microplastics which accumulate in stomach and intestine.

The presence of MPs in the gut delayed juvenile development probably due to lower food intake and insufficient energy supply. Control individuals, fed with algae could obtain all the energy necessary for the development, while juveniles that co-ingested MPs and algae obtained a lower amount of energy from the feeding activity, as their stomachs were filled with MPs and the amount of ingested algae was drastically reduced. Our data are consistent with those previously

reported exposing ascidian juveniles to larger (10 µm) plastic particles (Messinetti et al 2018). However, the effects on juvenile development were more severe when animals were fed with large MPs, probably because smallest ones can be more easily expelled. All together these results confirm that underfeeding may be a detrimental consequence of microplastics presence in the marine environment for filter feeder invertebrates.

Though MPs have been shown to accumulate in the gut cavity of several marine animals, particles translocation from the gut to the circulatory system is still poorly investigated in invertebrates. In our study, particles of polystyrene translocated from the gut cavity to the internal extracellular compartment in just 8 days. Particles translocation can occur through two alternative pathways: the paracellular pathway, implying uptake between cells, through intercellular junctions and spaces, and the transcellular pathway, involving the absorption of particles by the enterocytes, which release them through the basolateral membrane (for a review see Carr et al (2012)). Since in histological sections we never observed MPs inside the enterocytes but always in strict contact with the gut basal membrane, we cannot exclude either of the alternative mechanisms. Further works are needed to elucidate the mechanism by which MPs cross the gut barrier and move to the internal compartment.

After translocation, phagocytosis may play an important role to defend the organism from MPs presence. In fact, we observed MPs inside the cytoplasm of fusiform blood cells, probably phagocytes. In ascidians, several blood cell types were identified, including macrophage-like cells with large vacuoles and devoid of amoeboid activity and amoebocytes of the hyaline/ microgranular type involved in phagocytosis (Ballarin et al 1994). Granular amoebocytes are readily recognizable as they are the only amoebocyte type that contains large granules (0.5-1.5 µm in diameter; Rowley 1981). Since the MPs containing cells have also dark cytoplasmic granules, they could be reasonably identified as granular amoebocytes.

In the colonial ascidian *Botryllus schlosseri*, hyaline amoebocytes could phagocyte latex granules of 1 and 3 µm in diameter, confirming the capability of ascidian phagocytes to internalize particles of that size. Granulocytic hemocytes of *Mytilus galloprovincialis* are able to phagocyte polystyrene particles up to 800 nm in *in vitro* trials (Cajaraville & Pal 1995) suggesting that the surface properties of polystyrene beads can be recognized by invertebrate phagocytes.

Considering our results, we cannot exclude the possibility that ingestion and/or translocation of plastic into animal body may induce toxicological effects. We treated the animals only for 8 days with one type of plastic particles; in the environment, animals are exposed to different kinds of particles characterized by different chemical properties. Plastics can potentially release various

types of contaminants, especially additives used in their production (e.g. Jang et al (2016)). Moreover, persistent organic pollutants may accumulate on plastic fragments and plastic pellets (Rios et al 2007), possibly leading to further adverse effects after particles ingestion.

Therefore, future studies should be addressed to survey the toxicological effects induced by long-term exposure to various plastic particles usually found in marine habitats.

6.6. References

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Chapter 7 – Conclusions and future perspectives

Since plastic pollution in the marine environment is a growing problem whit negative impact on marine organism, in the present work we aimed to analyze some of the chemical and physical effects caused by plastic pollution on marine invertebrates. Plastic debris release in the environment additives, plasticizer, flame retardants and other chemicals. Moreover, after fragmentation, they are reduced to small size particles termed microplastics. To understand the impact of plastics on marine wildlife, the effects of the organic compound Bisphenol A (BPA) and of microplastic particles (MPs) with 1 or 10 µm of diameter were studied on different organisms, namely the sea urchin *Paracentrotus lividus* and the ascidians species *Ciona robusta*, *Ciona intestinalis* and *Phallusia mammillata*.

BPA is an organic compound, released after plastic products degradations, that can be found in different environmental compartment and, in surface water, it reaches concentrations in the low µg/L range (Crain et al 2007, Flint et al 2012). The exposure during the very early development of ascidians embryos, namely the first cell division, compromised the further development starting from 5 µM BPA concentration. If the exposure occurred soon after the two cells stages, BPA revealed teratogenic effects on all tested ascidian species. The molecule clearly affects a specific molecular pathway involved in the development and specification of pigment cells of sensory organs and nervous system. After hatching, ascidians larvae initially swim upward toward more illuminated surface waters and then start to swim downward until they find a suitable substrate for the metamorphosis (Svane & Young 1989). The sensory organs otolith and ocellus are necessary for this behavior and their alteration after BPA exposure could alter and interfere with the recruitment of new generations. Moreover, we collected some hints suggesting that the mechanism of action of this molecule is activated by its binding to estrogen related receptor (ERR). The same mechanism was responsible for otolith alterations in zebrafish (Tohmé et al 2014), highlighting similarities between vertebrates and tunicates. Also the alterations reported on nervous system development, involving GABAergic and dopaminergic neurons, are comparable to the data collected in mice, rats and monkeys (Choi et al 2007, Elsworth et al 2013, Franssen et al 2016, Miyagawa et al 2007, Miyatake et al 2006, Suzuki et al 2003).
Results from this work revealed that BPA caused effects similar to those obtained by altering FGF signaling cascade, and that most probably its action is mediated by binding to ERR. Further analyses will be necessary to detect the exact step of the pathways in which BPA acts. Taken together these data underline that ascidians are a good model to assess the effects of pollutants also at molecular level. The wide range of alterations and the possible subsequent negative effects of BPA on recruitment make clear that a monitoring of its concentrations in natural

environment is required. Moreover, results from this work prompt the necessity of a reduction of its use for plastic production in order to decrease its input in the environment.

A further physical threat for marine life given by plastic is due to its increasing production and formation of small particles named microplastics. Our analyses revealed that both sessile filter-feeders and free swimming suspension-feeder organisms are able to ingest microbeads. Even if the survival rate was not affected, other negative impacts of MPs have been registered. First, MPs ingestion caused a delay of growth during ascidians metamorphosis, affecting the possibility to successfully achieve the adult stages. Then, the body shape of sea urchin larvae was altered with probably further negative effects on the later developmental stages. The retention time of beads increased with the particle concentration in the seawater. At last, the ability of small particles with 1 μm diameter to move from digestive system to other tissues and to be phagocytized opened the possibility to more severe impact on the animals that is necessary to further analyze. As for BPA, these data highlight the importance of new rules and policies on plastic wastes and on MPs production, to avoid an increasing of abundance and distribution of these new pollutants that could negatively affect marine wildlife at different levels.

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ANNEX I

Abstracts and publications

Messinetti S., Mercurio S., Parolini M., Pennati R. (2018). Effects of microplastics presence on different developmental stages in invertebrate species. Oral Communication at Micro2018 congress, Lanzarote 19-23 November 2018

Messinetti S., Mercurio S., Pennati A., Pennati R. (2018). Effects of Bisphenol a on pigmented organ development in ascidians. Poster at 79° congress Unione zoologica Italiana, Lecce 25-28 September 2018

Messinetti S., Mercurio S., Pennati R. (2018). Effects of Bisphenol A on pigmented organ development in the ascidian *Phallusia mammillata.* Invertebrate biology 137:329–338. https://doi.org/10.1111/ivb.12231

Messinetti S., Mercurio S., Pennati R. (2018). Bisphenol A affects neural development of the ascidian *Ciona robusta.* Journal of experimental zoology - Part A: 1-12 https://doi.org/10.1002/jez.2230

Messinetti S., Mercurio S., Parolini M., Sugni M., Pennati R. (2018). Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environmental pollution 237: 1080-1087 https://doi.org/10.1016/j.envpol.2017.11.03

Messinetti S., Mercurio S., Pennati R. (2017). Effects of exposure to microplastics on the development and metamorphosis of *Ciona robusta.* Poster at 9th Tunicate International meeting, New York 17-21 July 2017

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