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

S. MESSINETTI[1], S. MERCURIO[1]*, G. SCARÌ[2], A. PENNATI[1], & R. PENNATI[1]

[1]*Department of Environmental Science and Policy, University of Milan, Milan, Italy, and* [2]*Department of Biosciences, University of Milan, Milan, Italy*

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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 hemocoelic cavity in just 8 days. As a defense mechanism, plastic spheres can also be phagocytized from specific circulating cells with phagocytic activity. Microplastics confirmed their potential as a threat to marine wildlife, interfering with food uptake and growth.

Keywords: *Polystyrene, translocation, tunicate, microplastics, development*

Introduction

Microscopic plastics, 1 μm–1 mm diameter (microplastics, MPs), are 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 (O'Brine & Thompson 2010; Browne et al. 2011; Davidson 2012; Napper et al. 2015).

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 zooplanktonic organisms (Cole et al. 2013).

Different species display disparate aptitudes in ingestion and retention of MPs, as demonstrated by the differences in stomach content reported in various 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-

*Correspondence: S. Mercurio, Department of Environmental Science and Policy, University of Milan, Via Celoria 2, Milan 20133, Italy. Tel: +39 0250314765. Email: sil.mercurio@gmail.com

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 the digestive system (Laist 1997; Derraik 2002; 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 performances 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 (>10 μm) 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 (<10 μm) 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 the 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.

Material and methods

Microplastics

Polystyrene spherical microparticles with a dark blue color 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 color 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 seawater (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 min 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.

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).

Development and survival rate

To test the effects of MPs presence on embryonic development, 30 embryos at two-cells stage (Hotta et al. 2007) were exposed to various 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 h 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.

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. One hundred microliters 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. Metamorphosis in *C. intestinalis* proceeds 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. Metamorphosis is completed by day 14 (stage 8) when the two atrial siphons fuse together, and the organs and tissue are almost the same as those of the adult (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 adhered to the dish and died soon after. Each experiment was performed in triplicate.

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% paraformaldehyde in phosphate buffer 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). Five micrometer 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.

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 2018) 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).

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 (Figure 1A; ANOVA: F = 0.9721, p = 0.4644). Under laboratory conditions, 4 days after attachment, more than 90% of control juveniles reached 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 increasing bead concentrations and was significantly different between samples exposed to 25 µg/ml MPs and control juveniles (Figure 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 (Figure 1B; ANOVA: F = 7.247, p = 0.026; Tukey's post hoc: CO vs 25 µg/ml p = 0.0328).

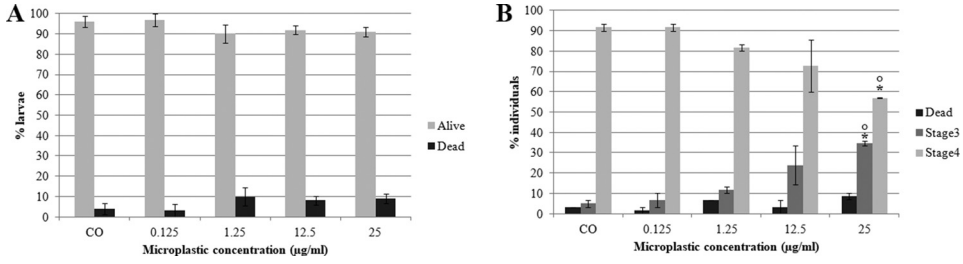

Figure 1. (A) Larval survival rate after exposure to microplastics. (B) Percentages of juveniles at different developmental stages observed after 4-day exposure to different concentrations of MPs. Data are means of 3 replicates ± standard error (SE). * = signicantly different from control; ° = significantly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

The transparency of ascidian juveniles allowed to clearly observe the gut content in whole mount specimens (Figure 2A). Juveniles were able to efficiently ingest small microplastics, that appeared clearly visible in their gut cavity, and to egest them in the fecal pellets (Figure 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 (Figure 2C).

Histological analyses showed that ascidian juveniles exposed to microparticles had accumulated polystyrene microspheres in their gut cavity (Figure 3). The microparticles were easily recognizable for their shape, color, and dimensions. They were found in the pharynx, the esophagus, the stomach and the intestine (Figure 3A–D). Some beads were present in the cytoplasm of fusiform cells localized in the wide hemocoelic cavity, characteristic of ascidian juveniles (Figure 3C). These cells presented dark granules in their cytoplasm, of ~1 μm in diameter (Figure 3C'', C''') and,

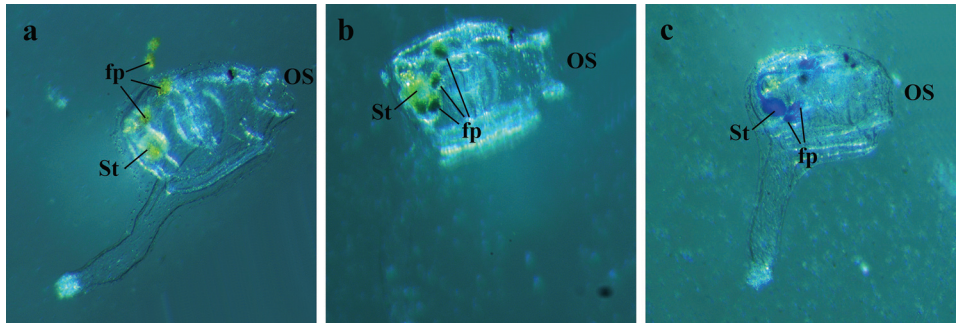

Figure 2. (A) Control juvenile that ingested microalgae. (B) Juvenile exposed to 25 μg/ml MPs. (C) Juvenile exposed to 1.25 μg/ml MPs. St = stomach; OS = Oral Siphon; fp = fecal pellets.

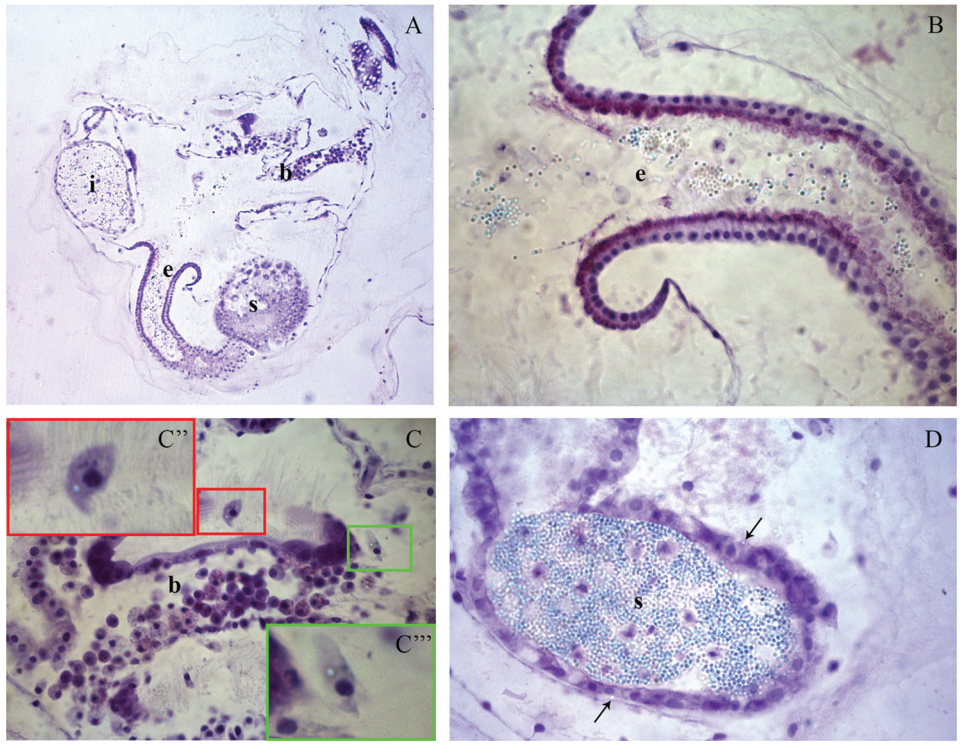

Figure 3. (A) Section of an ascidian juvenile. (B) Details of esophagus containing MPs. (C) MPs particles phagocytized by amoebocytes. (C'', C''') Magnification of amoebocytes containing MPs. (D) Stomach of juvenile containing MPs particles. Arrows indicate MPs that translocated through the stomach wall into the hemocoelic cavity. i = intestine; b = hemolymph lacuna; e = esophagus; s = stomach.

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 (Figure 3D). Most interestingly, some microparticles were found outside the gut cavity, in the extracellular fluid, next to the stomach wall (Figure 3D).

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 their feeding activity, as their stomach was 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 smaller ones can be more easily expelled. Altogether 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 only 8 days. Particles translocation can occur through two alternative pathways: the paracellular pathway, implying the passage 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). In histological sections, we never observed MPs inside the enterocytes but always in strict contact with the gut basal membrane so that we cannot exclude either of the alternative mechanisms. Further works are required 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 in recognizing MPs as nonself particles and in clearing them. In fact, we observed MPs inside the cytoplasm of fusiform circulating 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 cells can ingest 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 phagocytize 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 the 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 the additives used in their production (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.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Ballarin L, Cima F, Sabbadin A. 1994. Phagocytosis in the colonial ascidian *Botryllus schlosseri*. Developmental and Comparative Immunology 18:467–481.

Bigelow LK, Lasenby DC. 1991. Particle size selection in cadmium uptake by the opossum shrimp, *Mysis relicta*. Bulletin of Environmental Contamination and Toxicology 47:790–796.

Boerger CM, Lattin GL, Moore SL, Moore CJ. 2010. Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. Marine Pollution Bulletin 60:2275–2278. doi:10.1016/j.marpolbul.2010.08.007.

Bonello G, Varrella P, Pane L. 2018. First evaluation of microplastic content in benthic filter-feeders of the gulf of La Spezia (Ligurian Sea). Journal of Aquatic Food Product Technology 27:284–291. doi:10.1080/10498850.2018.1427820.

Browne MA, Crump P, Niven SJ, Teuten E, Tonkin A, Galloway T, Thompson R. 2011. Accumulation of microplastic on shorelines woldwide: Sources and sinks. Environmental Science & Technology 45:9175–9179. doi:10.1021/es201811s.

Browne MA, Dissanayake A, Galloway TS, Lowe DM, Thompson RC. 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environmental Science & Technology 42:5026–5031. doi:10.1021/es800249a.

Cajaraville MP, Pal SG. 1995. Morphofunctional study of haemocytes of the bivalve mollusc *Mytilus galloprovincialis* with emphasis on the endo lysosomal compartment. Cell Structure and Function 20:355–367.

Carr KE, Smyth SH, McCullough MT, Morris JF, Moyes SM. 2012. Morphological aspects of interactions between microparticles and mammalian cells: Intestinal uptake and onward movement. Progress in Histochemistry and Cytochemistry 46:185–252. doi:10.1016/j.proghi.2011.11.001.

Chiba S, Sasaki A, Nakayama A, Takamura K, Satoh N. 2004. Development of *Ciona intestinalis* juveniles (through 2nd ascidian stage). Zoological Science 21:285–298. doi:10.2108/zsj.21.285.

Cohen JT, Carlson G, Charnley G, Coggon D, Delzell E, Graham JD, Greim H, Krewski D, Medinsky M, Monson R, Paustenbach D, Petersen B, Rappaport S, Rhomberg L, Ryan PB, Thompson K. 2002. A comprehensive evaluation of the potential health risks associated with occupational and environmental exposure to styrene. Journal of Toxicology and Environmental Health, Part B 5:1–263. doi:10.1080/10937400252972162.

Cole M, Lindeque P, Fileman E, Halsband C, Goodhead R, Moger J, Galloway TS. 2013. Microplastics ingestion by zooplankton. Environmental Science & Technology 47:6646–6655. doi:10.1021/es400663f.

Cole M, Lindeque P, Halsband C, Galloway TS. 2011. Microplastics as contaminants in the marine environment: A review. Marine Pollution Bulletin 62:2588–2597. doi:10.1016/j.marpolbul.2011.09.025.

Davidson TM. 2012. Boring crustaceans damage polystyrene floats under docks polluting marine waters with microplastic. Marine Pollution Bulletin 64:1821–1828. doi:10.1016/j.marpolbul.2012.06.005.

Davison P, Asch RG. 2011. Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. Marine Ecology Progress Series 432:173–180. doi:10.3354/meps09142.

de Mendiburu F. 2015. Agricolae: Statistical procedures for agricultural research. R Package Version 1.2-3. http://CRAN.R-project.org/package=agricolae

Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439:965–968. doi:10.1038/nature04336.

Derraik JGB. 2002. The pollution of the marine environment by plastic debris: A review. Marine Pollution Bulletin 44:842–852.

Fernández F. 1979. Particle selection in the nauplius of *Calanus pacificus*. Journal of Plankton Research 1:313–328. doi:10.1093/plankt/1.4.313.

Gallo A, Tosti E. 2015. The Ascidian *Ciona intestinalis* as model organism for ecotoxicological bioassays. Journal of Marine Science: Research & Development 5:e138.

Hotta K, Mitsuhara K, Takahashi H, Inaba K, Oka K, Gojobori T, Ikeo K. 2007. A web-based interactive developmental table for the ascidian *Ciona intestinalis*, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. Developmental Dynamics 236:1790–1805. doi:10.1002/dvdy.21188.

Jang M, Shim WJ, Han GM, Rani M, Song YK, Hong SH. 2016. Styrofoam debris as a source of hazardous additives for marine organisms. Environmental Science & Technology 50:4951–4960. doi:10.1021/acs.est.5b05485.

Laist DW. 1997. Impacts of marine debris: Entanglement of marine life in marine debris including a comprehensive list of species with entanglement and ingestion records. In: Coe JM, Rogers DB, editors. Marine debris. Springer Series on Environmental Management. New York, NY: Springer. pp. 99–139

Lambert CC, Brandt CL. 1967. The effect of light on the spawning of *Ciona intestinalis*. Biological Bulletin 132:222–228. doi:10.2307/1539890.

Lusher A, McHugh M, Thompson R. 2013. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. Marine Pollution Bulletin 67:94–99. doi:10.1016/j.marpolbul.2012.11.028.

Mato Y, Isobe T, Takada H, Kanehiro H, Ohtake C, Kaminuma T. 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. Environmental Science and Technology 35:318–324.

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. doi:10.1016/j.envpol.2017.11.030.

Murray F, Cowie PR. 2011. Plastic contamination in the decapod crustacean *Nephrops norvegicus* (Linnaeus, 1758). Marine Pollution Bulletin 62:1207–1217. doi:10.1016/j.marpolbul.2011.03.032.

Napper IE, Bakir A, Rowland SJ, Thompson RC. 2015. Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics. Marine Pollution Bulletin 99:178–185. doi:10.1016/j.marpolbul.2015.07.029.

O'Brine T, Thompson RC. 2010. Degradation of plastic carrier bags in the marine environment. Marine Pollution Bulletin 60:2279–2283. doi:10.1016/j.marpolbul.2010.08.005.

Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, Wollenberger L, Santos EM, Paull G. 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philosophical Transactions of the Royal Society B: Biological Sciences 364:2047–2062. doi:10.1098/rstb.2008.0242.

Paffenhofer GA, Vansant KB. 1985. The feeding response of a marine planktonic copepod to quantity and quality of particles. Marine Ecology: Progress Series 27:55–65. doi:10.3354/meps027055.

Randløv A, Riisgård HU. 1979. Efficiency of particle retention and filtration rate in four species of ascidians. Marine Ecology Progress Series 1:55–59. doi:10.3354/meps001055.

R-Core-Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rios LM, Moore CG, Jones PR. 2007. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Marine Pollution Bulletin 54:1230–1237. doi:10.1016/j.marpolbul.2007.03.022.

Rowley AF. 1981. The blood cells of the sea squirt, *Ciona intestinalis*: Morphology, differential counts, and in vitro phagocytic activity. Journal of Invertebrate Pathology 37:91–100. doi:10.1016/0022-2011(81)90060-4.

Rowley AF. 1982. The blood cells of *Ciona intestinalis*: An electron probe X-ray microanalytical study. Journal of the Marine Biological Association of the United Kingdom 62:607–620. doi:10.1017/S0025315400019780.

Rummel CD, Mgj L, Fricke NF, Lang T, Griebeler E-M, Janke M, Gerdts G. 2016. Plastic ingestion by pelagic and demersal fish from the North Sea and Baltic Sea. Marine Pollution Bulletin 102:134–141. doi:10.1016/j.marpolbul.2015.11.043.

Talsness CE, Andrade AJM, Kuriyama SN, Taylor J, Vom Saal FS. 2009. Components of plastic: Experimental studies in animals and relevance for human health. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 364:2079–2096. doi:10.1098/rstb.2008.0281.

Teuten EL, Saquing JM, Knappe DRU, Barlaz MA, Jonsson S, Bjrn A, Rowland SJ, Thompson RC, Galloway TS, Yamashita R, Ochi D, Watanuki Y, Moore C, Viet PH, Tana TS, Prudente M, Boonyatumanond R, Zakaria MP, Akkhavong K, Ogata Y, Hirai H, Iwasa S, Mizukawa K, Hagino Y, Imamura A, Saha M, Takada H. 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philosophical Transactions of the Royal Society B 364:2027–2045. doi:10.1098/rstb.2008.0284.

Thompson RC, Olsen Y, Mitchell RP, Davis A, Rowland SJ, John AWG, McGonigle D, Russell AE. 2004. Lost at sea: Where is all the plastic? Science 304:838. doi:10.1126/science.1094559.

Wright SL, Rowe D, Thompson RC, Galloway TS. 2013. Microplastic ingestion decreases energy reserves in marine worms. Current Biology 23:R1031–R33. doi:10.1016/j.cub.2013.10.068.

Zega G, Pennati R, Candiani S, Pestarino M, De Bernardi F. 2009. Solitary ascidians embryos (Chordata, Tunicata) as model organisms for testing coastal pollutant toxicity. Invertebrate Survival Journal 6:S29–S34.