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Toxicological effects of Micro- and Nano-plastics in different marine model organisms

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Riassunto

Ad oggi l'inquinamento da plastiche in mare viene universalmente riconosciuto quale minaccia globale, in grado di indurre danni alla salute, generare perdite economiche, depauperare il paesaggio naturale, oltre che impattare sull'ambiente. Se agli albori dello studio di tale materia, la ricerca si focalizzava principalmente sull'indagine della frazione macroscopica del *marine litter*, negli ultimi anni, l'attenzione degli scienziati si è concentrata, viceversa, sulla frazione più piccola, cioè le micro- (MP, particelle < 5 mm) e nano-plastiche (NP, < 1µm). Le ragioni di tale interesse sono varie: i) organismi marini appartenenti ad ogni livello trofico, dai produttori primari ai più alti ordini di consumatori, risultano capaci di ingerire e/o interagire con le plastiche e subire impatti a diversi livelli dell'organizzazione biologica; ii) tale frazione rappresenta una forma di inquinamento di lunga durata poiché la frammentazione delle macroplastiche già presenti in mare continuerebbe a produrre microplastiche secondarie anche qualora cessasse l'afflusso di nuovi rifiuti; iii) in riferimento alle nanoplastiche, la scarsità delle attuali conoscenze scientifiche impedisce una reale analisi del problema e di conseguenza una valutazione del rischio ambientale.

Questo progetto di dottorato si sviluppa su due linee di ricerca, ciascuna delle quali focalizzata su una particolare frazione del *marine litter:* le MICROplastiche e le NANOplastiche.

Gli obiettivi della prima linea di ricerca sono volti alla descrizione della variabilità della tossicità di eluati e sospensioni di microplastiche di polietilenetereftalato (PET), eletto come polimero di riferimento, al variare di alcuni fattori abiotici e biotici sospettati, in letteratura, di influenzarne il rischio chimico e fisico. In particolare, nel primo lavoro pubblicato e interamente riportato nella tesi, sono state investigate le possibili interazioni fra gli eluati e le sospensioni di MP di diversa dimensione (5-3,000 µm) con il pH dell'acqua di mare (Acido = 7.5 *vs*. Standard = 8.0) e la disponibilità di cibo (presenza/assenza); il tutto tramite l'allestimento di un test multispecie composto da *Vibrio fischeri* (Beijerinck, 1889)*, Phaeodactylum tricornutum* (Bohlin, 1898), e *Paracentrotus lividus* (Lamarck, 1816). Le analisi sono state poi ulteriormente estese a teleostei marini: giovanili di pesce pagliaccio *Amphiprion ocellaris* (Cuvier, 1830), sono stati esposti per 3 giorni a sospensioni di MP in presenza di cibo. La risposta biologica si è basata sulla misura dell'attività enzimatica di singoli *biomarker* legati allo stress

ossidativo (catalasi; glutatione-s-transferasi) e sulla capacità totale di scavenger ossidoradicale per mezzo del saggio TOSCA.

Dai risultati ottenuti nella prima linea di ricerca, e alla luce delle concentrazioni testate, risulta una bassa tossicità legata alle microplastiche di PET. In particolare: *i*) le risposte sono state specie-specifiche; *ii*) gli eluati si sono dimostrati generalmente più tossici delle sospensioni; *iii*) la taglia più piccola delle MP non sempre ha causato risposte biologiche più evidenti; *iv*) il pH e le variazioni di cibo sono in grado di modulare le risposte ecotossicologiche degli echinodermi secondo meccanismi non dimostrabili allo stato attuale delle conoscenze; *v*) non è stata evidenziata una risposta concentrazione-dipendente nei giovanili di pesce pagliaccio, infatti la concentrazione più bassa di MP ha indotto effetti 1.87 volte superiori rispetto alla concentrazione più alta.

Nei primi mesi del dottorato, la letteratura sull'inquinamento da nanoplastiche era ancora ridotta (solo 32 lavori pubblicati) ma si percepiva un graduale aumento di interesse della comunità scientifica verso le plastiche sub-micrometriche. Tale interesse si è presto concretizzato in un sensibile aumento della produzione scientifica sul tema, da cui è nato il primo obiettivo di questa seconda linea di ricerca: l'analisi bibliografica. Nella *review* pubblicata e interamente riportata nella tesi, viene indagata la conoscenza scientifica dell'inquinamento da nanoplastiche: dalla degradazione dei polimeri alla loro presenza in campioni di acqua marina, passando attraverso le evidenze in laboratorio della formazione e rilascio di NP. Si è provveduto alla classificazione dei lavori presi in esame sulla base degli aspetti trattati, dedicando particolare attenzione a quelli riguardanti gli effetti ecotossicologi e inoltre sono stati forniti suggerimenti per studi futuri. Il secondo obiettivo, viceversa, ha previsto lo sviluppo di competenze tecnico-sperimentali tramite allestimento di test tossicologici presso laboratori di ricerca nazionali e stranieri. I frutti di tale attività vengono descritti nel secondo lavoro riportato nella tesi, in preparazione per essere sottomesso, in cui giovanili di pesce pagliaccio sono stati esposti a nanoplastiche di polistirene (PS-COOH) per 7 giorni. Le possibili implicazioni biologiche vengono indagate per mezzo di un approccio multidisciplanare basato sulla risposta biochimica connessa allo stress ossidativo in aggiunta a quella molecolare basata su tecniche di Next Generation Sequencing (NGS) quali il sequenziamento del trascrittoma codificante (RNA-seq).

Da quanto appreso durante la preparazione della *review*, le nanoplastiche sembrerebbero essere un contaminante dagli effetti allarmanti, tuttavia, molte domande

(tra tutte, la quantificazione del fenomeno) rimangono irrisolte e dunque risulta prematuro prendere una posizione sul rischio reale che le NP eserciterebbero sulla biodiversità e sul funzionamento degli ecosistemi naturali. Il test realizzato su giovanili di pesce pagliaccio ha voluto colmare parzialmente la scarsità di conoscenze sugli effetti delle NP nei pesci marini (un target molto sottostudiato) ed ha evidenziato come: *i*) la catalasi, il glutatione-s-trasferasi, la glutatione reduttasi e il saggio TOSC idrossilico e perossilico, non siano stati significativamente alterati; *ii*) l'analisi integrata delle risposte biologiche (indice IBRv2) riporti una riduzione globale dello stato di salute dei pesci esposti alle NP dimostrando come tale indice possa rappresentare un approccio rapido, semplice ed informativo specialmente nel discriminare le differenze tra trattamenti che viceversa non verrebbero identificate dall'analisi statistica sui singoli *biomarker*; *iii*) la concentrazione più bassa sia in grado di suscitare risposte tossicogenomiche maggiori rispetto alle concentrazioni medie ed elevate, probabilmente a causa delle aggregazioni di NP a maggior concentrazione che si verificano nell'acqua di mare; *iv*) i geni con un cambiamento più elevato nei livelli trascrizionali siano stati quelli coinvolti nell'infezione virale e nella risposta infiammatoria (e in misura minore nella ciliogenesi, nel metabolismo energetico, nelle funzioni olfattive e riproduttive). In conclusione, questo studio ha rilevato una risposta minore allo stress ossidativo e ha contribuito a comprendere la cascata di eventi molecolari che vengono attivati dall'esposizione a PS-NP nei giovani del pesce pagliaccio.

Abstract

Nowadays, plastic pollution is recognised as a worldwide concern because it causes harm to the environment and generates adverse economic, health and aesthetic impacts. While at the beginning of the study of this subject, the research was focused mainly on the investigation of the macroscopic fraction of the marine litter, in recent years, the attention of scientists has extended also toward the smallest fraction, that is, the micro- (MP, particles < 5 mm) and nano- plastics (NP, < 1μm). The reasons for such emerging interest are diverse: *i*) marine organisms belonging to every trophic level, from primary producers to higher-orders of consumers have been reported to ingest and/or interact with plastic and suffer impacts at different levels of biological organization; *ii*) this fraction is a long-term form of pollution since the fragmentation of macroplastics already present at sea would continue to produce secondary microplastics even if the influx of new waste will cease; *iii*) with regard to nanoplastics, the scarcity of current scientific knowledge prevents a real analysis of the problem and consequently an environmental risk assessment.

Hence, this PhD project was developed on two parallel lines of research, each of which concentrated on a specific fraction of the marine litter: the MICROplastic and the NANOplastic.

The objectives of the first line of research are aimed at describing the variability of the toxicity of leachates and suspensions of polyethyleneterephthalate (PET) microplastics, elected as the reference polymer, as some abiotic and biotic factors suspected, in the literature, of influencing their chemical and physical risk vary. In particular, in the first full paper reported in this thesis were investigated the possible interactions between leachates and suspensions of different size (5-3,000 μm) of PET microplastic, the water pH (Ac=7.5 and St=8.0) and food variations (presence/absence). All through the setting up of a multispecies test composed by *Vibrio fischeri* (Beijerinck, 1889)*, Phaeodactylum tricornutum* (Bohlin, 1898) and *Paracentrotus lividus* (Lamarck, 1816). The analyses were subsequently extended also to marine teleosts: juveniles of *Amphiprion ocellaris* (Cuvier, 1830) were investigated in a 3-days water-exposure to suspensions of PET MPs under feeding conditions. Both single enzymes related to oxidative stress response

(catalase; glutathione-s-transferase) and total oxyradical scavenging capacity (TOSCA assay) were tested.

According to the final results obtained, considering the MP concentrations analyzed in these studies, a low toxicity is attributable to PET microplastics and, in particular: *i*) responses were species-specific; *ii*) leachates demonstrated to be more toxic than suspensions of MPs; *iii*) smaller sizes of plastics not always corresponded to stronger biological responses; *iv*) pH and food variations modulated the ecotoxicological responses of echinoderms according to mechanisms not demonstrable in the current state of knowledge; *v*) a concentration-dependent response was not evident in clownfish juveniles in which the lower concentration of MPs induced effects 1.87 times greater than the higher concentration.

Whereas the body of literature on nanoplastic pollution was very limited (only 32 published works) in the early months of the Doctorate, the scientific community's interest in plastic pollution gradually began to focus on sub-micrometric particles. This attention soon resulted in a significant increase in scientific production on the subject from which the first objective of this second line of research was born: bibliographical analysis. The first full paper reported in this thesis explores the scientific knowledge on the topic, from polymer degradation to occurrence in marine water, passing across laboratory evidence of nanoplastic formation. The works examined were classified on the basis of the aspects covered, with particular attention being paid to those relating to ecotoxicological effects, and suggestions were also made for future studies. The second objective, on the other hand, focused on the development of technical and experimental skills through the preparation of toxicological tests at national and foreign research laboratories. The fruits of this activity are described in the second work reported in the thesis, in preparation to be submitted, in which juveniles of clownfish were exposed to 100 nm polystyrene nanoplastics (PS-COOH) for 7 days. The possible biological implications were investigated by means of a multidisciplanar approach spanning from biochemical responses related to oxidative stress, to gene expression analysis by the Next Generation Sequencing (NGS) of the whole transcriptome (RNA-seq technique).

As we learn from the review, the framework on nanoplastics toxicity seems to be alarming. However, many knowledge gaps (quantification of the phenomenon first) still exist and therefore it is difficult to take a position about the risk they pose to the biodiversity and functioning of natural ecosystems. The experiment performed on

clownfish juveniles detected a minor response in oxidative stress and contributed in understanding the cascade of molecular events that are activated by the exposure to PS-NPs in the clownfish juveniles. Specifically: *i*) sensitive biomarkers such as catalase, glutathione S-transferase, glutathione reductase and TOSC Assay (hydroxylic and peroxylic) were not significantly altered; *ii*) the integrated analysis of biomarker responses reported a reduction in overall health status of fishes demonstrating how the IBRv2 index may represent a quick, useful and informative approach especially in discriminating against any differences between treatments that cannot be identified by statistical analysis on individual biomarkers; *iii*) RNA-seq analysis highlighted significant dysregulation of immune genes especially in the low condition which elicited higher toxicogenomic response than medium and high concentrations (likely due to NP aggregations occurring in sea water); *iv*) remarkably, the genes showing higher change in transcriptional levels were predominantly factors involved in viral infection and inflammatory response, and to less extent in ciliogenesis, energy metabolism, olfactory and reproductive functions.

Introduction

Plastic pollution, a recognized concern

Marine litter is defined as "any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment" (MSFD Technical Subgroup on Marine Litter 2013). Initially described in the marine environment in the 1960s, marine litter is nowadays commonly observed across all oceans (Ryan 2015). Plastic items represent the major part of litter found in different coastal and marine compartments and form sometimes up to 95 % of the waste that accumulates on the shorelines, the sea surface and the seafloor (Bergmann et al., 2015; Munari et al., 2016; Kamman et al., 2018). Between 1950 and 2015, the cumulative waste generation of primary and secondary (recycled) plastic waste accounted to 6,300 million tons (Geyer et al., 2017). Mainly due to leaks from production processes or to the use of inadequate waste disposal, in 2010 up to 12.7 million tons of plastics items entered the oceans (Jambeck et al., 2015).

Nowadays, plastic pollution is recognised as a worldwide concern by EU and global initiatives such as the United Nations Environment Programme (UNEP; see Sustainable Development Goal 14), the G7 and the G20 because it causes harm to the environment and generates adverse economic, health and aesthetic impacts. In this regard, the Marine Strategy Framework Directive (MSFD, European Union 2008), which represents the EU legal framework for the protection of the European Seas, and then the revised European Commission Decision COM/2017/848 (European Commission 2017), have included and deepened respectively, the marine litter as one of the Descriptors for the achievement of the Good Environmental Status (GES). Moreover, reducing plastic leakage into the environment is also one of the main goals of the EU Plastics Strategy (European Commission, 2018) which aims to address the challenges caused by plastic throughout its value chain, by taking into account its entire life cycle in order to progress toward a European Circular Economy.

Current knowledge about the plastic pollution

Sound scientific data are required in order to identify best measures and ascertain progress. Over the years, the scientific community has contributed to the comprehension

of an undoubtedly complex phenomenon collecting a substantial amount of data. In fact, in the past 10 years about 8,500 scientific contributions have been published on the topic "plastic pollution" (Fig 1, PubMed, November 2020). Consequently, the knowledge on such form of pollution greatly increased over the years.

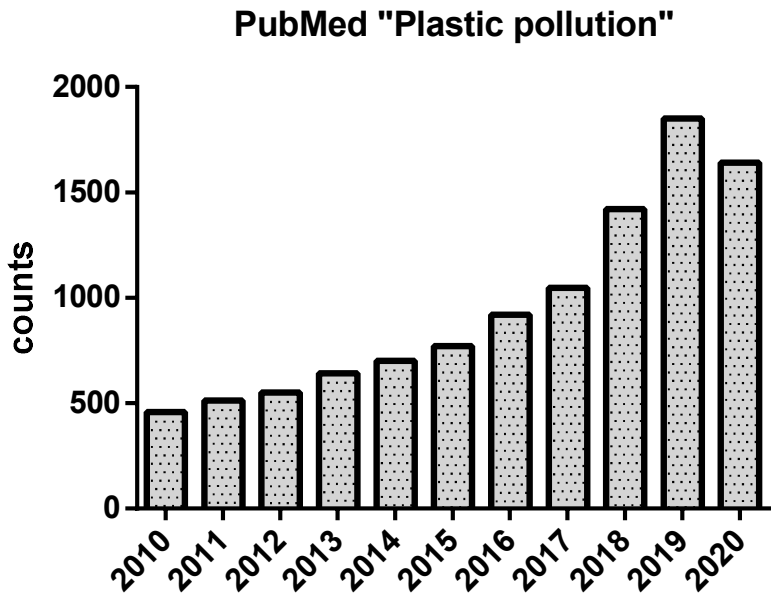

Fig 1. Number of papers published from 2010 to November 2020 interrogating PubMed for the keyword "plastic pollution".

Following the description given by Li et al. (2020), typical characteristics of plastic pollution can be summarized in: heterogeneity, ubiquity (global issues), long-term pollution (persistence), combined pollution and potential threats to organisms and human health.

The **heterogeneity** of plastic pollution is a consequence of the wide variety of plastic polymers formulated to date. In 2018, the most produced resins in the market were: PE (polyethylene, 29.7 %), PP (polypropylene, 19.3 %), PVC (polyvinylchloride, 10 %), PUR (polyurethane, 7.9 %); PET (polyethylene terephthalate, 7.7 %) and PS (polystyrene, 6.4 %) (Plastic Europe, 2019). In addition, plastic litter is also diverse in colour, sizes, and shapes, so much to have to talk about a "plastic soup" (Suaria et al., 2016).

Plastic is a global issue and is considered a **ubiquitous** contaminant in aquatic (Alimi et al., 2018; Pico et al., 2019), atmospheric (Prata 2018; Chen et al., 2020), and terrestrial systems (Horton et al., 2017; Ng et al., 2018), capable of polluting even remote areas such as Antarctic region (Isobe et al., 2015; Isobe et al., 2017; Lacerda et al., 2019) or tropical

islands (Imhof et al., 2017; Tan et al., 2020). The same features that make plastic so widespread, that is, durability and stability, are the reasons why plastic waste remains in the environment for a long time and consequently occupies all corners of the planet. Their distribution is firstly driven by the slight density: most synthetic polymers are, in fact, buoyant in seawater (e.g., the specific density of PE and PP ranges between 0.9-0.965 g/ml). This allows them to be transported by wind and currents for long distances and eventually washed ashore (Barnes et al., 2009; Thompson et al., 2009; Browne et al., 2011). On the contrary, the polymers that are denser than seawater (e.g., PVC and PET, specific density of 1.4 g/ml and 1.55 g/ml, respectively) tend to settle near the point where they entered the environment. However, other processes occur to the plastics once in the environment, influencing their fate and behaviour. Among others, the formation of microbial films could affect the fate of microplastics as well as their physico-chemical properties (i.e., surface hydrophobicity and buoyancy) (Lobelle and Cunliffe, 2011; Tu et al., 2020). Finally, some studies have shown the accumulation of plastic litter in seabed (Strafella et al., 2019; Krüger et al., 2020) that are nowadays considered the ultimate repository for microplastic debris, including those that were initially buoyant (Woodall et al., 2016; Barnes et al., 2009).

Plastic pollution is also a **long-term pollution.** Persistence in the environment is undoubtedly a typical feature of plastic contamination. There is virtually no data on kinetics of mineralisation of plastics in the marine environment (Andrady 2011; Law and Thompson, 2014). Despite this, it is well known that plastic is not immune to degradation processes. Degradation is generally classified according to the causing agent; environmentally-linked degradation processes can be classified as biodegradation (i.e., the action of living organisms, usually microbes), photo-degradation (i.e., the action of light, the sunlight outdoor), thermo-oxidative degradation (i.e., slow oxidative breakdown at moderate temperature), thermal degradation (i.e., due to high temperatures) and hydrolysis (i.e., reaction with water) (Andrady 2011). Light-induced oxidation is considered the most common and efficient mechanism in the marine environment (Bergmann et al., 2015). Degradation drastically reduces the average molecular weight of the polymer, and oxygen-rich functional groups are generated. Extensively degraded plastics become brittle enough to break down into powdery fragments forming the micro- and nano-plastics, a new subtle form of pollution.

Plastic Pollution is a **combined pollution.** Plastics are made by polymerizing monomers and other substances that enhance performance, including plasticizers that

make the plastic soft or flexible, antioxidants and stabilizers that prevent degradation of the plastic, in addition to flame retardants and pigments (Lithner et al., 2011; Engler 2012). Any toxicity associated with plastics in general can be attributed to one or more of the following factors:

(a) Residual monomers or toxic additives used in compounding of plastic. Such chemicals are not chemically but physically bound to the plastic, so they may leach from the ingested plastic. Specific compounds are phthalates from PVC, nonylphenol compounds from polyolefins, brominated flame retardants (BFRs) from ABS or urethane foam, and bisphenol A (BPA) from polycarbonate (Hermabessiere et al., 2017);

(b) Toxicity of some intermediates from partial degradation of plastics (for example, burning polystyrene can yield styrene and other aromatics);

(c) Toxicity of chemicals or microorganisms present in seawater, slowly adsorbed and concentrated in the plastic surfaces. In this case plastics, and in particular microplastics, may act as a "Trojan horse": once ingested, associated chemicals can become bioavailable to the organisms and exert their toxicity (González-Soto et al., 2019; Paul-Pont et al., 2016; Pittura et al., 2018; Guilhermino et al., 2018). Some environmental pollutants are for example, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), tetracycline, dichlorodiphenyltrichloroethane (DDT) and heavy metals (Cd, Pb, Cu, Zn, etc.). Finally, microorganisms and pathogens can colonize plastics (Carson et al., 2013; Virsek et al., 2017; Yang et al. 2020) and potentially affect organisms or simply accelerate their diffusion in the environment.

Threats to organism and human health. Plastic pollution may cause physical damage, chemical harm, and biological threats to organisms (Eerkes-Medrano et al., 2015). Plastic debris can be physically harmful to an animal *via* entanglement or ingestion, causing suffocation, lacerations and starvation (Gall and Thompson 2015). Entanglement mainly occurs in larger organisms, but nearly all the organisms in the different trophic levels are able to ingest microplastic debris, such as zooplankton (Sun et al., 2017), invertebrates (Digka et al., 2018; Renzi et al., 2018), fishes (Foekema et al., 2013; Romeo et al., 2015b; Garnier et al., 2019), turtles (Duncan et al. 2018), seabird (Reynolds and Ryan 2018) and whales (Jacobsen et al., 2010). Chemical and biological impacts are probably chronic effects which are not easily observed (Li et al., 2020). In laboratory studies microplastics have been shown to induce a variety of biological effects, posing risk to organisms across the full spectrum of biological organization from cellular to population level (Galloway et al., 2017). Moreover, under debate is the potential risk

for humans following exposure to microplastics which have been recently found in atmospheric fallout (Prata 2018), in different food and beverage products intended for human consumption (Lee et al., 2019; Diaz-Besantes al., 2020) and in seafood (Forrest and Hindell 2018).

Facing the plastic pollution and its possible implications at organismal levels requires a first distinction according to size, which is considered a crucial parameter in determining the impact. In fact, materials that are safe in their bulk form become active on biota if reduced in size to their nanoforms (Jeevanandam et al., 2018). This is due to the increase of the surface/volume ratio following the size reduction that, consequently, increases the surface that particles are able to expose to water.

Micro- and nano-plastics: a priority field of investigation

Internationally, plastic litter is divided in nano- (< 1 μm or < 100 nm), micro- (1 μm - 5 mm), meso- (5 mm - 2.5 cm) and macro-plastics (> 2.5 cm) (Hartmann et al. 2019). At the beginning, plastic litter research focused mainly on the investigation of the macroscopic fraction in the marine environments. Then, in recent years, the attention of scientists has shifted towards the smallest fraction, i.e. the micro- (MP, particles < 5 mm) and nano- plastics (NP, < 1μm). Smaller fractions are considered of particular interest for different reasons. For example, they interact with a huge variety of marine organisms, including those at the base of the trophic chain such as zoo- and phytoplankton, potentially accumulating in different tissues, translocating to inner organs, and causing effects at different levels of biological organization (Du et al. 2020; Peng et al. 2020). MP and NP quantities in the environment are recognized to increase as a consequence of further direct introductions of primary microplastic and fragmentation of larger items, representing a long-term form of pollution (Jambeck et al. 2015; Li et al. 2020). Actually, the greatest knowledge gaps concern these fractions: especially for nanoplastics, no quantitative data on environmental occurrence are available and eco-toxicological experiments are in their infancy (Gigault et al. 2016). Various open questions on micro- and nano-plastic still exist nowadays and a consensus has not been reached about their toxicity.

For the reasons given so far, this PhD project was organized in two parallel lines of research, each focussing on a particular fraction of the marine litter: the MICROplastic

and the NANOplastic. The objectives that have characterized each line of research will be presented in the following chapters.

MICROPLASTICS

Although the terminology is ambiguous and conflicting, for instance regarding size classes, the most frequently used definition is the one provided by NOOA (National Oceanic and Atmospheric Administration) stating that microplastics are all plastic particles < 5 mm in any dimension with an indeterminate lower limit (Hartmann et al., 2019). MPs are commonly divided into primary, which are originally produced in that size range (e.g. abrasive in cosmetics and cleaning products), and secondary, formed by the breakdown of larger plastic debris (Hartmann et al., 2019). Jambeck et al. (2015) reported that over 10 million metric tons of plastic waste enters the oceans every year, of which > 92% are MPs. If the situation remains unchanged, 250 million tons of marine plastic waste are estimated by 2025 and by 2050, plastic in the oceans will outweigh fish (World Economic Forum 2016; Jambeck et al., 2015). The Mediterranean basin can be considered a hot spot of plastic pollution, with an accumulation of floating plastic between 1,000 and 3,000 tons (Suaria and Aliani 2014; Cózar et al., 2015; Suaria et al., 2016). Moreover, MPs were also detected in remote areas of the world, from tropical islands (Garnier et al., 2019) to polar regions (Le Guen et al., 2020; Obbard et al., 2014) and mountain lakes (Imhof et al., 2013).

In the marine environment, organisms at every trophic level, from primary producers to higher-order consumers such as fish, turtles, sea birds and mammals, have been reported to ingest and/or interact with plastic (Wang et al. 2019). MPs exposes animals not only to the physical particles, but also to a “cocktail” of chemicals (Rochman et al. 2015). In laboratory studies predominantly in crustaceans and molluscs, MPs have been shown to cause a variety of biological effects, (Bucci et al., 2020). The main effects are inflammatory responses (Wright et al., 2013, Hu & Palić., 2020), disruption of feeding behaviour (Cole et al., 2015), decreases in growth (Sussarellu et al., 2016; Xiaohua et al., 2020) and in reproductive success (Xie et al., 2020), as well as larval abnormal development (Gandara e Silva et al., 2016). However, there are also studies that reported no effect (Batel et al. 2016; Espinosa et al., 2018; Hämer et al., 2014; Weber et al., 2018) and nowadays a consensus has not yet been reached with regard to their toxicity. In fact, for MPs the evidence surrounding effects is variable and seems to be context dependent (Bucci et al., 2020). The discrepancy between studies may be due to differences in

polymer type, shape, size, and chemicals associated, but also to other factors including species sensitivity and the dose and time of exposure (Fig 1). Another source of variability can be represented by variations of ocean water pH; pH variations can potentially modify the chemical equilibrium of MPs increasing or decreasing the leaching rate of the adsorbed chemicals (Seidensticker et al., 2018), and the combined stress (MP + water acidification) can influence the eco-toxicological responses for example by inhibiting, in mussels, the digestive enzymes (Wang et al. 2019) or altering lipid peroxidation (Provenza et al. 2020). In addition to pH variations, also the abundance of food was proven to affect the cycle and fate of MPs in marine environment and influence the ingestion/egestion rate of mussels (Chae et al., 2020).

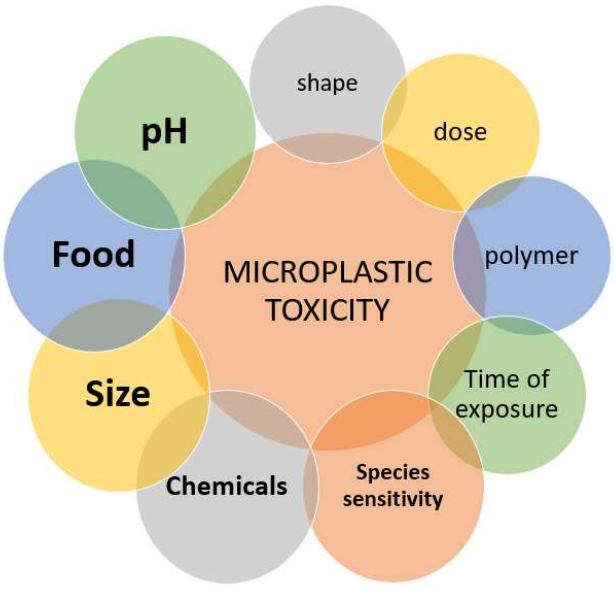

Fig 1. Some of the factors capable of influencing the MP toxicity; in bold factors studied in the first full paper reported in this thesis.

A part of this three PhD years is based on a cycle of experiments focused on the understanding of the eco-toxicological responses of diverse marine organisms following the exposure to PET (polyethylene terephthalate) MP. PET was selected as reference material for different reasons: 7.7 % of total European plastic production is represented by PET (Plastic Europe 2019), PET litter was found in diverse marine and coastal compartments from seabed to surface water and along the coasts (Munari et al., 2016; Mu et al., 2019; Suaria et al., 2016). Moreover, the handful of studies which used PET MPs report contrasting biological effects in freshwater organisms (Weber et al., 2018). The

selected particles were heterogeneous in shape and size, obtained by double trituration through industrial mills and characterized by irregular edges and surface irregularity, features that well simulate the secondary MPs present in the oceans (Lambert et al., 2017).

The first full paper reported in this thesis investigates the possible interactions between PET MPs and some other factors, namely the water pH (Ac=7.5 and St=8.0) and food variations (presence/absence), by testing the toxicity of leachates and suspensions of different size (5-3,000 μm) of MPs in a multispecies test composed by *Vibrio fischeri* (Beijerinck, 1889)*, Phaeodactylum tricornutum* Bohlin, 1898, and *Paracentrotus lividus* (Lamarck, 1816).

In order to complete the project and extend the analysis to marine teleosts, juveniles of *Amphiprion ocellaris* (Cuvier, 1830) were tested in a 3-days water exposure to suspensions of PET MPs under feeding conditions. Both single enzymes related to oxidative stress response (CAT, catalase; GST, glutathione-s-transferase) and total oxyradical scavenging capacity (TOSCA assay) were measured. The exposure to juveniles of clownfish, which will be further exposed with nanoplastics, was born in the context of the ASSEMBLE PLUS project (European Union's Horizon 2020 research and innovation program, personal Grant Agreement No. 730984) and resulted in a period abroad spent in the *Observatoire Océanologique* in *Banyuls sur mer* (Sorbonne University, France). ASSEMBLE Plus brings together 24 partners covering over 30 marine installations from 14 European and two associated countries, under the leadership of the European Marine Biological Resource Centre (EMBRC). The goal of the project is to stimulate fundamental and applied research excellence in Europe in the fields of marine biology and ecology, thereby improving the knowledge- and technology-base for the European bio-economy, policy shaping and education (http://www.assembleplus.eu/about).

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PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations

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Graphical abstract

Keywords: Polyethylene terephthalate; microplastic; Global changes; *Vibrio fischeri*; *Phaeodactylum tricornutum*; *Paracentrotus lividus*

Abstract

This study aims to evaluate effects induced by the exposure of key marine species to leachates and suspensions of different particle-size of PET microparticles, a plastic polymer that is actually considered safe for the environment. Leachates and suspensions of small (5–60 μm); medium (61–499 μm) and large (500–3000 μm) PET were tested on bacteria (*V. fischeri*; UNI EN ISO 11348-3:2009), algae (*P. tricornutum*; UNI EN ISO 10253:2016E), and echinoderms (*P. lividus*; EPA 600/R-95-136/Section 15) species both under standard (8.0) and acidified (7.5) pH conditions. Results obtained show that: i) conversely to larval stage of *P. lividus*, bacterial and algal tested species are not sensitive to PET pollution under all tested conditions; ii) different tested particle sizes of PET produce effects that are not always related to their particle-size; iii) differences comparing acidified and standard pH conditions were recorded; iv) concerning echinoderms, food availability produce significant differences compared to fasting conditions; v) special attention on the possible interactions between MPs and other stressors (e.g., food and pH) is needed in order to give a better picture of natural occurring dynamics on marine ecosystems especially in the future frame of global changes.

1. Introduction

An increase in plastic production resulted in an intensification in waste abandoned in the environment defacing the landscape, causing economic losses and threatening life. Legal or illegal dumps on coastal areas release waste polluting marine ecosystems (AEA, 2006). As consequence of the inadequate waste management performed, 6.2 items/linear meter of beach are recorded in Italy and plastic accounts for the 80% of the total amount collected (Carpentieri et al., 2018). Over than 40% of plastic production worldwide is used for packaging and largely for food packaging (UNEP, 2015) contributing to the marine litter (https://odims.ospar.org/maps/526/view). Marine environments are the ultimate stop for many pollutants including plastic litter. Without waste management infrastructure improvements, the cumulative amount of plastic waste available to enter the ocean is predicted to reach 250 million of metric tons by 2025 corresponding to an increase of an order of magnitude respect the level of 2015 (Jambeck et al., 2015). Plastic pollution represents a severe risk for marine ecosystems impacting marine species (Avio et al., 2015; Dehaut et al., 2016; Collard et al., 2017;) by direct ingestion (Cole et al., 2011), by trapping and by translocation of smallest MPs fractions inside cells (Lusher et

al., 2017) that could affect feeding habits and animals' reproductive success (Cole et al., 2014). Every year >12 million tons of plastic enter the oceans, of which >92% are particles generally smaller than 5 mm (Jambeck et al., 2015). These tiny particles, more commonly known as microplastics (MPs), may either result by the fragmentation of large items, or they can directly enter the environment as pellets, beads, and fibres (Li, 2018). MPs are ingested by a wide range of marine species (Dehaut et al., 2016), such as pelagic fish species (Renzi et al., 2018a), benthic species (Neves et al., 2015; Van Cauwenberghe et al., 2015; Courtene-Jones et al., 2017; Karlsson et al., 2017; Pellini et al., 2018; Renzi et al., 2018b; Renzi et al., 2018c), and marine mammals (Fossi et al., 2016). MPs are defined by GESAMP (2016) as "plastic pieces less than 5 mm in size" but any lower limits are defined. A more recent paper (Frias and Nash, 2019) defines the size-range interval 1–5000 μm as the most suitable to refer to microplastics. Lusher et al. (2017), in an extensive review, reported that particles larger than 150 μm are able to have low biological interactions while, particles smaller can migrate from the digestive apparatus of mammals towards their portal vein, their organs, and cells, also resulting able to overcome the brain barrier. Scientific interest towards microplastic in marine environment is quickly increased in the last five years; published researches on MPs related fields grew exponentially from 23 (2013) to 774 in 2019 (PubMed, searching "microplastic", publishing per year). Nevertheless, the largest number of papers is focused on toxicology and environmental chemistry and biological or ecological complex effects at the ecosystem level induced by the exposure to microplastic in marine environment represent yet a scarcely explored research field (Pauna et al., 2019).

In 2018, the global plastic production on Earth was estimated to be close to 359 million of ton. In the same year, according to Plastic Europe (2019), PET (PolyEthylene Terephthalate) represented one of the most produced polymer in Europe (7.7% of the total amount produced). Since PET is light in weight, cheap and with very low production costs, it is proved to be one of the best candidates for single use products for mass consumption. PET is considered a safe polymer for human health and it is largely utilized for the production of sheets, films, and fibres, for the packaging of foods and beverages but, also, to produce parts of electronics, automotive parts, sports goods, and textiles (Sinha et al., 2010; Webb et al., 2013; Singh et al., 2018). Although PET is extremely resistant to weathering, it is not immune to fragmentation processes. The abiotic weathering of PET in marine environments is likely to occur principally by photo-oxidation and by hydrolysis (Gewert et al., 2015). Different marine and coastal

compartments are contaminated by litter originated from PET and drinking bottles represent one of the items most frequently found in beach litter (Munari et al., 2016). Due to density driven mechanisms, PET can reach the seafloor (Mu et al., 2018). Nevertheless, in contrast to what expected on the polymer distribution based only on density features, PET-made microplastics are recorded in samples floating plastics collected in the Mediterranean Sea highlighting their contribution to the formation of the "soup" of polymers which affects the oceans worldwide (Suaria et al., 2016). Renzi et al. (in press) reported percentages of PET-made microplastics in sediments and holothurians respectively within 6.2-12.5% and 25.0-29.5%. Even if PET-made microplastics potentially affects both abiotic matrices and wild species, such records in natural water, sediments, and pelagic or benthic biota are scarce. Concerning water samples, the chemical composition of the fraction lower than 300 μm, which corresponds to the pore size of the manta net, is completely unknown. Concerning solid matrices, the European Technical Subgroup on Marine Litter (MSFD Technical Subgroup on Marine Litter, 2013) highlighted that this underestimation could be due to the low effective methods of extraction applied for microplastics. In fact, the density separation step, widely performed by literature using a hypersaline solution (NaCl, density of 1.2 g cm−3), although cheap, is not suitable for denser polymers such as PET (density of 1.37–1.45 g cm−3).

Concerning biological effects coming from the exposure to PET pollution, some recent studies demonstrated the bioavailability and toxicity of PET microplastics in different freshwater organisms such as amphipods, copepods, and fish (Heindler et al., 2017; Rochman et al., 2017; Weber et al., 2018). The main toxic effects recorded are the reduction in copepod egg production, the reduction of relative population size, and differences in feeding behavior of sturgeon when exposed to prey fed with microplastics. Several other endpoints such as gene expression, development of the molting, metabolism, condition factor, and mortality seem to be unaffected. In addition, PET eco-toxicity on marine organisms remains a poorly explored field of investigation to date.

Even if pH of marine ecosystems is strictly buffered to 8.0, coastal areas could be affected by pH fluctuations (Hofmann et al., 2011). In addition, surface ocean acidity increased by approximately 30% since preindustrial levels (Doney et al., 2009). Furthermore, a recent scenario models that, in 2100, as a result of global changes, ocean acidification will lead to a level of 7.5 pH units with loss of seawater buffer capacity (Raven, 2005). Recent papers suggested that changes of pH (Knutzen, 1981; Roleda et al., 2015) are able to determine, in marine species, unpredictable changes in eco-

toxicological responses. Variation of pH of ocean water can potentially modifies the chemical equilibrium of microplastics increasing or decreasing the leaching rate of chemical substances that are on their surface as a result of the production process or adsorption from the water. Therefore, the PET that is currently considered relatively safe for the environment could potentially become dangerous in the near future with different environmental conditions. Another possible factor of confusion on the evaluations of the eco-toxicological effects is represented by the availability of nutrition under natural conditions. Laboratory tests are generally performed in fasting conditions while species in nature are exposed to stressors and have access to trophic resources at the same time (Renzi et al., 2019a). Furthermore, the relationship between the toxicity of microplastics and their average size is little explored by the literature.

This study aims to fill some of the exposed knowledge gaps testing the effects induced on bacteria (*Vibrio fischeri*), algae (*Phaeodactylum tricornutum*), and on larval stage of echinoderms (*Paracentrotus lividus*) following exposure to PET microplastics of three different particle-sizes. Recently Foley et al. (2018) reported that MPs seem to exert a direct and indirect effects (i.e. chemical) threat to marine organisms. In this study, bacteria and algae species were exposed to leachates of PET MPs to evaluate chemical toxicity. Nevertheless, echinoderms were exposed both to leachates and suspensions of PET to evaluate also effects induced by the direct contact with MPs (i.e. ingestion for the smallest tested size; mechanical damages for the largest ones). In addition, all cases were tested in a standard (pH 8.0) or acidified scenario (pH 7.5), to simulate the phenomenon of sea acidification as a consequence of global warming. Finally, effects were measured in presence and absence of trophic resources.

The tested species were selected due to their representativeness of different trophic levels in marine ecosystems (ISPRA, 2011). Furthermore, laboratory experiments are largely standardized on these species and experimental variability can be controlled. *Vibrio fischeri* bioluminescence inhibition bioassay is widely used for toxicity evaluations in water, soil, and sediment samples and provides multiple advantages encompassing shorter test duration, sensitivity, cost-effective and ease of operation (Abbas et al., 2018). The genus *Vibrio* was previously used in other studies performed on polymers (Romeo et al., 2015; Gambardella et al., 2018). The algal growth inhibition test was used by previous literature to assess the MPs impact on primary producers (Zhang et al., 2017; Mao et al., 2018; Yi et al., 2019); nevertheless, PET has been never tested before. *P. lividus* has already been used in other similar studies. For instance, Oliviero et

al. (2019) reported a drastic reduction of larval length in plutei exposed to PVC leachates probably due to the presence of phthalates. Messinetti et al. (2018) recorded significant differences in body and arm length of *P. lividus* plutei reared at 25 mg/L of 10 μm polystyrene beads. Hence, the embryo toxicity test with *P. lividus* aims to investigate the possible implications on the pelagic phase of a commercially relevant and very sensitive species.

2. Material and methods

2.1. Microplastics production and pre-treatment characterization

PET micrometric flakes were obtained by double trituration of 1 mm pellets using industrial mills. The resulting gross-sized powder was sifted in order to obtain the three desired particle-sizes: small (5–60 μm; S-PET), medium (61–499 μm; M-PET), and large (500–3000 μm; L-PET). S-PET was selected as it resulted widely lower than 150 μm, the upper limit referred to cut off for migration from the digestive system within mammal's portal vein (Lusher et al., 2017). M-PET (61–499 μm) represents the cut off size between the fraction able to penetrate organs and the upper limit for visual identification of microplastic. L-PET (500–3000 μm) is the largely documented fraction in environmental matrices and animal tissues by literature (Suaria et al., 2016); furthermore, it is considered to be relatively safer than other fractions as it cannot penetrate biological barriers. Particle-sizes obtained were collected separately in glass bottles, then quickly washed in ethyl alcohol 96%, drain off and dried in oven at 35 °C up to complete evaporation of residual traces of alcohol. This pre-treatment was effective to remove trace of external pollution on MPs surfaces without changing structural MP chemical composition (Renzi et al., 2019a; Supplementary materials). Furthermore, ethyl alcohol is highly volatile (5.95 kPa, vapour pressure at standard atmospheric condition) and according to OECD (2014) it is not toxic to aquatic organisms at residual levels (low or absence of toxicity at concentration b 100 mg/L). The generic shape of MP s tested in this study can be approximated to very heterogeneous flakes, with jagged edges and surface irregularity (Fig. 1S – Supplementary materials). These shape features well simulate MPs particles present in environmental matrices (Lambert et al., 2017).

2.2. Preparation of solutions for the leaching test

Natural Standard Water (NSW) pre-filtered to 0.45 μm (38.0 ± 0.8 PSU; 7.0 ± 0.2 mg/L DO2; 20.0 ± 0.4 °C), stored in glass bottles was use as starting medium. Water pH was checked and correct to the desired level by the addition of opportune doses of HCl (0.1 M) to obtain both standard (St, pH = 8.0) and acidified (Ac, pH = 7.5) water starting medium stored separately (pH ± 0.1). MPs leachates of tested S-PET, MPET, and L-PET particle-sizes were prepared by the addition of 0.1 mg/mL of single tested MPs particle-size to 500 mL of both standard and acidified starting medium in a glass beaker and put in the incubator at 18 °C 12:12 light/dark cycle for 72 h. The MP dose selected in this study was superior to that expected to be present in the ocean but is suitable for detecting response/no response effects. After 72 h, the experimental leachates were obtained by filtering the suspensions on 0.45 μm nitrate cellulose fibre filters in order to remove plastic particles. A parallel filtration was carried on with Anodisc® filters (Whatman, lot n. A21184266; aluminum oxide membrane; 0.2 μm porosity) for μFT-IR analysis of the particle-size (see paragraph 3.1). Finally, filtered leachates solutions were aliquot to run eco-toxicological tests on *V. fischeri*, *P. tricornutum*, and *P. lividus*. Tests were performed immediately and residual aliquots were stored at −20 °C.

2.3. μFT-IR analysis and post-treatment characterization

A methodology based on microscopy associated to Fourier Transform Infrared Spectroscopy technique (μFT-IR; Nicolet, iN10 MX; Thermo Fisher Scientific) was run on Anodisc® filters for the identification of plastic features. The μFT-IR was equipped with liquid nitrogen cooled MCT-A operating within the spectral range 7800–650 cm−1 and with OMNIC Picta (Thermo Scientific, Waltham, MA, USA) users' interface. Filters were analysed by the Wizard-operating in transmission mode to determine particles mean and median sizes according to the 72 h of exposure to tested pH. PET particles thicker than 35 μm were analysed by ATR (Attenuated Total Reflection) using a germanium crystal (spectral range 3000–1300 cm−1). Chemical spectra of both 8.0 and 7.5 pH exposed MPs were collected to determine the occurrence of possible spectral differences. The threshold for IR spectra back-recognition was fixed over 65% of match. Limit of detection of chemical composition of targeted particles was 10 μm. The carbonyl index (CI) was used by literature to evaluate plastic degradation by acid exposure (Prata et al.,

2019). CI was calculated by dividing the intensity of the carbonyl peak (1715–1735 cm−1) by the intensity of the reference peak. Reference peak for PET was read at 1504 cm−1 (Pires et al., 2015).

MPs were also analysed by the Field Emission Scanning Electron Microscopy technique (FESEM, mod. Merlin II, Zeiss®) coupled with Wavelength Dispersive and Energy Dispersive spectrometer combined micro analyser (WD/ED mod. X-Max 50, Oxford Instruments®). FESEM was applied to check on targeted particles the occurrence of nano-changes on surface. This technique provides topographical and elemental information at magnifications within 10× - 300,000×, with virtually unlimited depth of field.

2.4. Eco-toxicity tests

2.4.1. Test on bacteria

Biological responses on bacteria were checked on the species *Vibrio fischeri* according to UNI EN ISO 11348-3:2009 using Microtox® (Ecotox) photometer and lyophilized bacteria purchased by Microbiotests Inc. The inhibition percentage of natural bioluminescence was measured after 5, 15, and 30 min of exposure to 90% of the concentration of the leachate, using two experimental replicates. Tests were performed both under standard and acidified pH conditions. Filtered NSW was used as control of the test. An experimental control was run also on acidified NSW.

2.4.2. Test on algae

Phaeodactylum tricornutum was selected as representative of effects on algal species. Growth inhibition percentage (I%) after 72 h of exposure was run on leachates under both standard and acidified conditions. An algal lot purchased by Ecotox® was tested after pre-enrichment in an ASPM culture medium. Illumination, temperature, salinity, and dark/light photo-cycles were set as reported in ISO 10253:2016 (E). Cell density measures were performed by spectrophotometer (Onda, mod. UV-30 scan; optical length 10 cm) and calculated by light absorbance at the wavelength 670 nm. The spectrophotometer response was calibrated using a cell density versus absorbance curve developed on tested algal stock by Burker's chamber counts at each of the 10 points scalar dilution of 106 cell/mL stock. Effects compared to controls were measured after 24 h, 48

h, and 72 h of exposure on three experimental replicates and calculated as reported by literature (Renzi et al., 2014).

2.4.3. Test on echinoderms

Paracentrotus lividus was selected as representative of effects on embryos. Tests were performed following EPA 600/R-95-136/Section 15; adapted according to ISPRA (2017) method. Percentage of abnormal larvae was measured after 72 h of exposure to both leachates and suspensions. The effects were assessed using sea urchin embryo-toxicity test and assessing both regular development and biometric impairment. The direct exposure test was performed using the single concentration of MPs (0.1 mg/mL) used to produce leachates. The exposure did not foresee the preparation of a stock solution to avoid both the influence in terms of toxicity of surfactants required to stabilize the suspension and possible variations in particle bioavailability. Thus, the desired mass of MPs was directly added to testing plates without the use of any surfactants. In this case the experimental design was developed both under standard and acidified conditions and, also, under fasting and feeding conditions to evaluate effects due to either ocean acidification and presence of feeding resources on toxicity of MPs. In details, fertilized eggs were exposed to four MP-enriched solutions (Ac NSW + food, St NSW + food, Ac NSW – food, and St NSW – food) and associated negative controls. Three replicates and corresponding controls for each of the tested size (S-PET, M-PET, and L-PET) were carried out. Mature specimens of sea urchin were caught in a natural marine area (Tuscany) and maintained in captivity until the starting of the experiment. In vitro fertilization was performed according to ISPRA protocol (ISPRA, 2011). Fertilized eggs were allowed to develop in incubator at 18 °C under darkness conditions. Daily, the experimental group under feeding conditions were fed with few drops of algal (*P. tricornutum*) suspension at 3 × 105 cell/mL density. The same correction of dilution was performed for fasting population by the addition of NSW drops. At 72 h post fertilization (hpf), samples were fixed with two drops of filtered Lugol solution, and 100 embryos for each plate were classified under the stereomicroscope (Nikon, mod. SMZ-800 N equipped with a digital camera interfaced to Nikon ACT-1 software) to perform counts of abnormal larvae. Larvae were considered abnormal if, at the developmental stage of 72 hour-old, *plutei* showed developmental arrest, all arms missing or with different length, additional arms cross lateral rods, asymmetrical body width and other anomalies

listed by literature (ISPRA, 2017). Additionally, on 15 normal larvae per plate (including negative controls), length reductions of both anterior oral arms (AOAs) and post-oral arms (POAs) were measured to evaluate effects on body size. This further endpoint was added as it resulted more sensitive to highlight early stress on exposed larvae during pre-screening tests than percentage of anomalies. The measures were carried out on larvae in the same spatial position in order to avoid perspective offsets. Micrometric measurements were performed by stereomicroscopy (Nikon, mod. SMZ-800 N equipped with a digital camera interfaced to Nikon ACT-1 software) and statistically analysed to evaluate solid differences compared to controls.

2.5. Quality assurance & quality control

Tests were performed in a certified laboratory (UNI EN ISO 9001:2015; UNI EN ISO 17025:2005) to ensure the quality of produced data. QA/QC tests were performed as described by the reference methods previously cited. Positive controls were performed by the direct exposure of tested species to standard toxicants: *V. fischeri* was exposed to 3,5′-dichlorophenol (I% 30 min = 21.7 ± 0.5); *P. tricornutum* was exposed to K2Cr2O7 (EC50 = 37.2, range 24.6–56.3 mg/L); *P. lividus* was exposed to Cu(NO3)2*3H2O (EC50 = 25.9, range 21.6–30.3 µg/L) resulting within the acceptability criteria defined by standard methods. Negative controls were performed on natural standard water under experimental conditions. Recorded data were within the acceptability of tests under standard conditions (pH = 8.00).

2.6. Statistical analyses

Data were statistically analysed by GraphPad Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Routines related to column statistics (mean, standard deviation, min-max ranges), one-way repeated measure ANOVA were run on *V. fischeri* dataset, 1-way and 2-way ANOVA (significance of observed differences between exposure and controls and within treatments) were performed on *P. tricornutum* and *P. lividus* dataset. Differences were considered significant at p-level < 0.05. Tukey's multiple comparison test was used to highlight significant differences within treatments and Dunnet's test between treatments and controls. Sidak's multiple comparisons test for disentangles differences between each size level of leachate with the other of suspension

was performed. Multivariate analyses were performed by Primer v7.0 (Primer-E Ltd., Plymouth Marine Laboratory, UK) following methods reported by Clarke and Warwick (1998). Euclidean matrix of distance was calculated on normalized data of effect expressed as percentage. Tested factors on mean effects were: particle-size (four levels, fixed, 0-PET, SPET, M-PET, L-PET), pH condition (two levels, fixed, 8.0 and 7.5), and food availability (two levels, fixed, feeding and fasting conditions).

3. Results

3.1. Microplastic characterization

Spectra collected by μFT-IR analyses confirmed the PET nature of the tested particles. Collected PETs spectra matched over than 80% with the spectra for PET from the reference library (Thermo Scientific®). In particular, matches recorded were: 82.8% for S-PET, 87.8% for M-PET, and 87.9% for L-PET. MP polymer may change due to degradation process including the exposure to acids (Da Costa et al., 2018). To evaluate polymer degradation, spectra of MPs collected under St pH conditions were saved as reference library and matched against spectra collected on Ac treated PETs. In this case, a significant reduction of matches was recorded as follow: -12.2% (S-PET), −1.8% (M-PET), and − 5.0% (L-PET) as resulted by Fig. 2S (Supplementary materials). CI index calculated for each tested PET particle-size are reported in Table 1. Values highlights a general reduction of levels after Ac exposure with the following intensity M-PET ≫ L-PET. Concerning levels in S-PET particles under Ac conditions, CI index showed an increase. Concerning PET characteristic peaks (1715 cm−1, C=O; 1245 & 1100 cm−1C-O) a significant reduction of intensity following acidification was recorded. S-PET and L-PET also highlight significant reduction of not characteristic peaks (3427, 2963, 2365, 1958, 1408, 1337, 1246 cm−1).

Table 1. Results of μFT-IR particle-size analyses. Mean size, standard deviation (SD), median, minimum and maximum (expressed in μm) of the three tested typologies of PET (S-PET; MPET; L-PET) MPs after 72 h of exposure to natural standard water both under pH 8.0 and pH 7.5. Statistics do not highlight significant differences between acidified and standard conditions. CI = Carbonyl Index.

Sample	Size-class (declared)	Treatment	CI	Mean	SD	Median	Min	Max
S-PET	5–60	7.5 pH	2.72	18.0	14.3	12.1	4.9	61.5
		8.0 pH	1.92	14.8	11.8	10.9	4.9	61.3
M-PET	61–499	7.5 pH	3.12	167.5	119.3	116.5	58.1	626.5
		8.0 pH	4.02	151.2	126.4	95.0	58.4	586.5
L-PET	>500	7.5 pH	2.46	737.6	185.1	719.5	494.6	1192.4
		8.0 pH	2.68	927.7	449.9	747.8	494.5	2345.6

The μFT-IR analyses of particle-size of tested PETs confirmed ranges theorized at the beginning of the experiment (Table 1). After 72 h of incubation, S-PET showed a mean length (±standard dev.) of 18 ± 14 μm (Ac) and of 15 ± 12 μm (St). Mean lengths of M-PET were: 168 ± 120 μm (Ac) and 151 ± 126 μm (St). L-PET under St conditions showed a longer mean length (928 ± 450 μm) compared to Ac exposed ones (738 ± 185 μm).

FESEM analyses showed topographic details and alterations occurring on MPs superficies when exposed to Ac pH conditions compared to control (St) as reported in Fig. 1. Jagged edges, surface irregularities and heterogeneity in size, that represent the main features of PET MP s under standard pH conditions (1c), are progressively modified under acidified condition. In particular, surface irregularities (1b) are notably increased after the exposure under strong acid pH = 4.0 (1a).

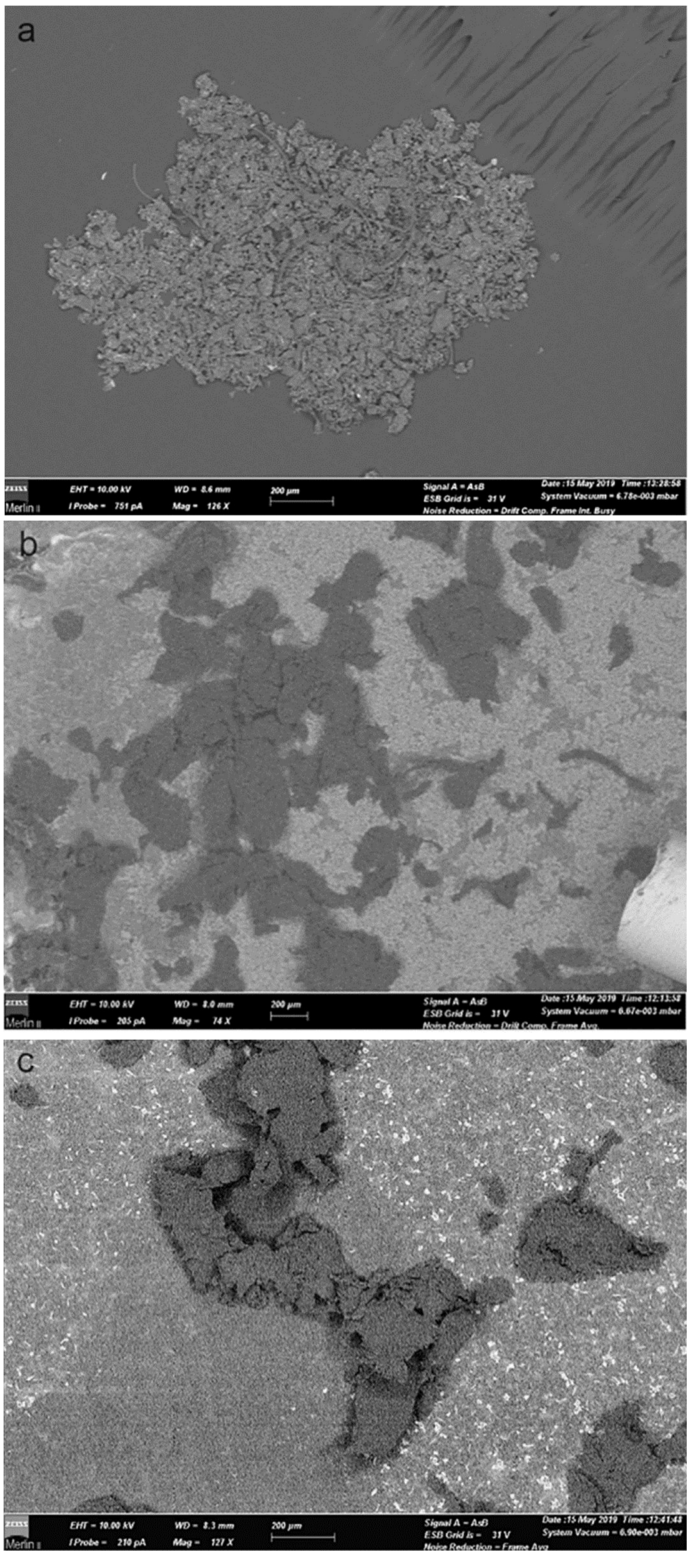

Fig. 1. FESEM topographic details and alterations occurring on MPs surfaces. Effects due to the exposure for 72 h to natural marine water at different pH conditions are reported: a) pH = 4.0; b) pH = 7.5; c) pH = 8.0. Images are referred to ethyl alcohol 96% quickly rinsed MPs particles.

3.2. Ecotoxicity of PET leaching

3.2.1. Tests on bacteria (*Vibrio fischeri*)

Results on inhibition percentages recorded after 5, 15, and 30 min of exposure of bacteria to leachates of S-PET, M-PET, and L-PET are represented in Fig. 2. Results obtained under St and Ac conditions are compared. Concerning St, mean values of effects ranged between 0% and -21.5% (biostimulation). Biostimulation was recorded in particular for the smallest particle-size tested (S-PET). The occurrence of biostimulation was confirmed under Ac conditions for S-PET (-19.8%) and M-PET (-8.1%). However, concerning L-PET, after 15 min a reversal of trend occurred causing a maximum inhibition of +18.2%. ANOVA (one-way) analysis revealed significant differences between M-PET and L-PET respect to control ($p < .01$) and between PET-size, under standard pH conditions.

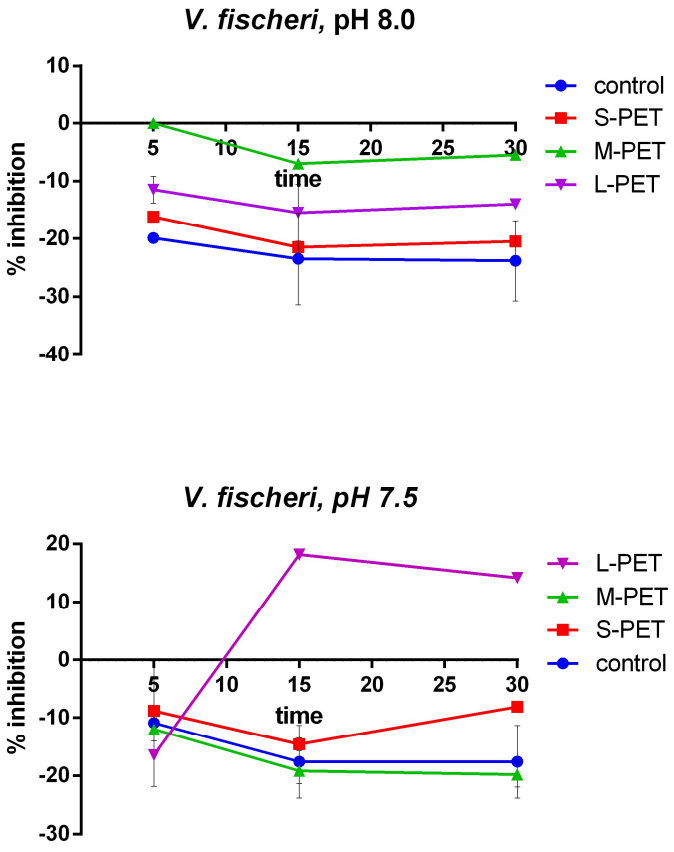

Fig. 2. Eco-toxicological effects on bacteria recorded after the exposure under different pH conditions to leachates of three particle-sizes of PET. Percentages of inhibitions of the natural biolominescence are reported after 5, 15, 30 min of exposure to leachates. Results are reported grouping data according to the pH of the leachate (pH = 8.0, standard condition; pH = 7.5,

acidified conditions). Each point represents mean value of two replicates (±SEM). Negative values mean biostimulation.

3.2.2. Tests on algal species (*Phaeodactylum tricornutum*)

There were no significant differences of growth rates between treatments and control, or within treatments after 72 h of exposure under both pH conditions. However, the ANOVA two-way calculated on growth rate inhibition (%) highlighted significant differences in all treatments respect to control at both pH conditions and differences between S-PET and both M-PET and L-PET at pH 7.5 (Fig. 3).

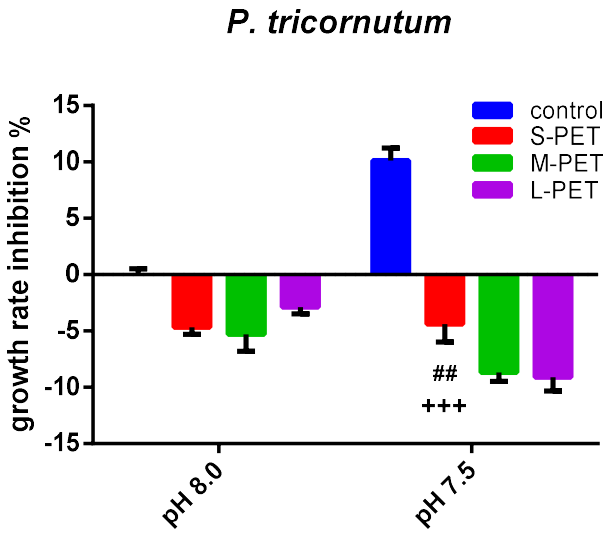

Fig. 3. Ecotoxicological effects on algae recorded after the exposure under different pH conditions to leachates of three particle-sizes of PET. Percentages of inhibitions of the natural growth rates recorded after 72 h of exposure to leachates are reported. Results are reported grouping data according to the pH of the leachate (pH = 8.0, standard condition; pH = 7.5, acidified conditions). Each bar represents mean value of three replicates (standard deviation). Negative values mean biostimulation. Although not graphically reported, all treatments differ from control (Dunnett test, p b .05). In addition, S-PET significantly differs from other treatments (Turkey test, p b .05). # means differences from M-PET; + means differences from L-PET.

3.2.3. Tests on echinoderms (*Paracentrotus lividus*)

A particle-size dependent response was recorded under St condition, where abnormal larvae (%) increased proportionally with the particle-size of MPs (Fig. 4). Under Ac conditions, except for L-PET, a similar trend was reported and significant differences

were found between controls, S-PET, and M-PET ($p < .01$). Under St conditions, L-PET ($p < .001$) and M-PET ($p < .05$) differ from controls. Tukey's multiple comparison tests highlighted significant differences between L-PET-SPET ($p < .05$) and L-PET-M-PET ($p < .01$) couples under Ac conditions. Under St, Tukey's multiple comparison tests evidenced significant differences between L-PET and S-PET ($p < .001$). Significant differences between controls and PET leachates were recorded for both AOAs and POAs of plutei under Ac and St conditions ($p < .0001$) (Fig. 5). As AOAs and POAs are strongly positive correlated ($p < .001$) only POAs correlations are reported. In general, treated *plutei* reported shorter POAs than control (up to -33.4%) and AOAs (up to -32.3%) when exposed to both pH scenarios (Table 2). Finally, the Ac scenario induced higher length reduction compared to St conditions, especially for M-PET (+18% of reduction) as represented in Fig. 6.

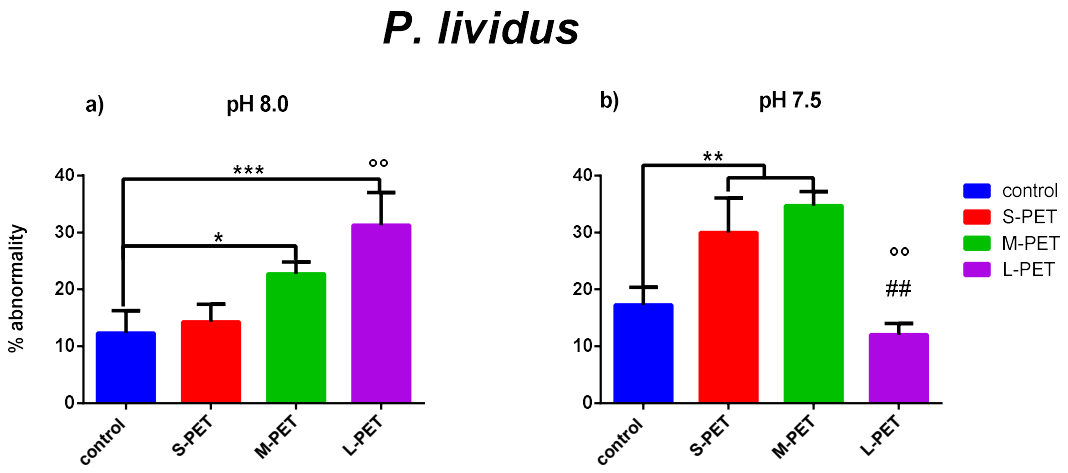

Fig. 4. Percentage of abnormalities (mean ± st. dev.; n = 3) of the sea urchin larvae after 72 h of incubation in different leaching solutions of PET MP s (S-PET; M-PET; LPET) at pH 8.0 (a) and 7.5 (b). Results were not normalized according to Abbott (1987), controls are represented. * = significant differences from control; ° = differences from S-PET; # = differences from M-PET. The repetition of * indicates the level of significance according to the following code: p b .05 *; p b .01 **; p b .001 ***; p b .0001 ****. Results from 1way-ANOVA (Tukey test for disentangle differences between treatments; Dunnett test for differences with control).

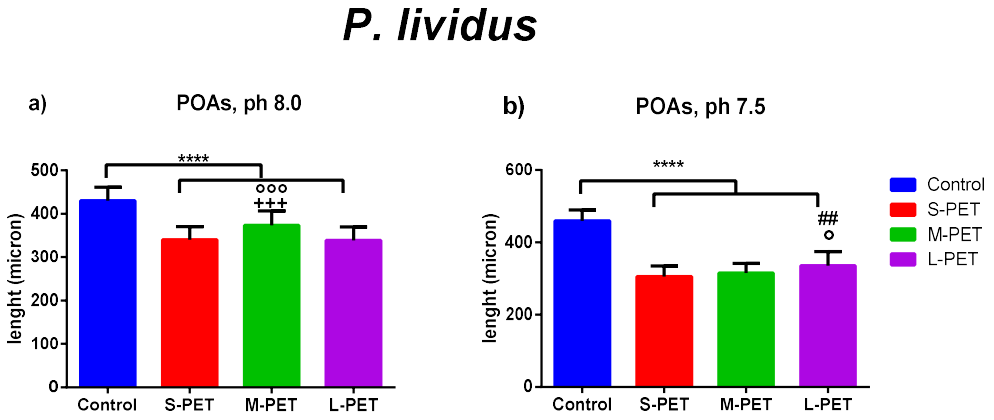

Fig. 5. Post oral arms (POAs) analysis of *plutei* exposed to different leaching solutions of PET MPs (S-PET; M-PET; L-PET) at pH 8.0 (a) and 7.5 (b). Results were not normalized according to Abbott (1987), controls are represented. * = significant differences from control; ° = differences from S-PET; # = differences from M-PET; + = differences from L-PET. The repetition of * indicates the level of significance according to the following code: p b .05 *; p b .01 **; p b .001 ***; p b .0001 ****. Results from 1way-ANOVA (Tukey test for disentangle differences between treatments; Dunnett test for differences with control).

Table 2. Length reduction (%; standard deviation) of anterior and posterior oral arms of *plutei* exposed for 72 h to different leaching solutions (S-PET; M.PET; L-PET). Differences between acidified and standard values highlight the pH-effect.

		S-PET		M-PET		L-PET	
		pH 7.5	pH 8.0	pH 7.5	pH 8.0	pH 7.5	pH 8.0
Post Oral Arms POA	Length reduction (%)	33.4 (3.5)	20.9 (4.3)	31.4 (7.9)	13.3 (5.4)	26.9 (3.2)	21.3 (2.4)
	Difference (Ac-St) %	12.5		18.1		5.6	
Anterior Oral Arms AOA	Length reduction (%)	32.3 (4.2)	19.6 (2.8)	31.4 (5.2)	15.1 (6.3)	25.0 (3.3)	23.6 (2.9)
	Difference (Ac-St) %	12.7		16.2		1.4	

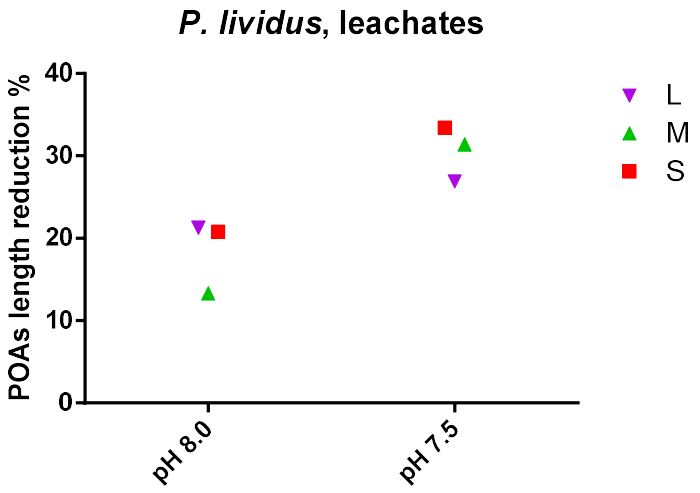

Fig. 6. Percentage of length reduction of POAs in different pH scenarios and PET article size. The acidified scenario seems to exert a stronger effect.

3.3. Ecotoxicity of suspensions on echinoderms

3.3.1. Fasting conditions

The direct exposure to the different particle-size of PET under St conditions seems not to affect the larval growth. However, under Ac conditions, L-PET treated larvae significantly differ both from control ($p < .05$) and S-PET and M-PET ($p < .01$) as represented in Fig. 7.

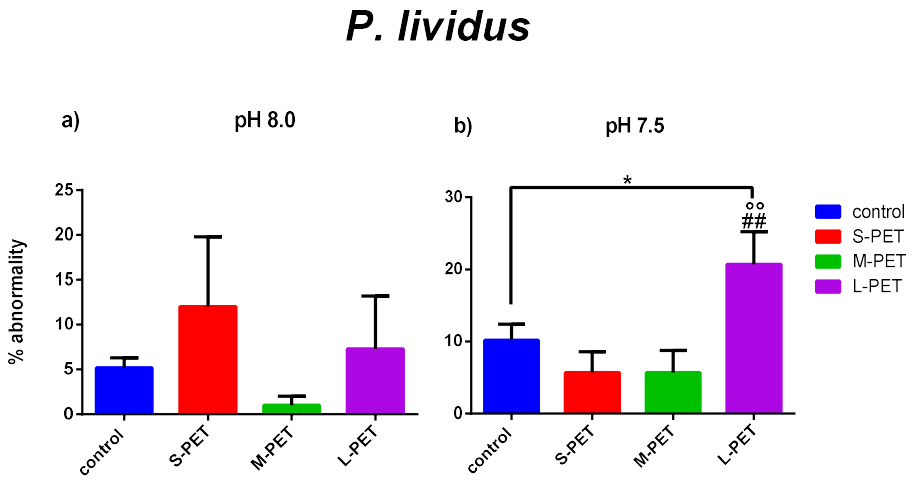

Fig. 7. Percentage of abnormalities (mean ± st. dev.; n = 3) of the sea urchin larvae after 72 h of incubation in different PET microplastics suspensions (S-PET; M-PET; L-PET) at pH 8.0 (a) and 7.5 (b). Results were not normalized according to Abbott (1987), controls are represented. * =

significant differences from control; ° = differences from S-PET; # = differences from M-PET. The repetition of * indicates the level of significance according to the following code: p b .05 *; p b .01 **; p b .001 ***; p b .0001 ****. Results from 1way-ANOVA (Tukey test for disentangle differences between treatments; Dunnett test for differences with control).

3.3.2. Feeding conditions

The direct exposure to PETs suspensions under Ac was not effective on larval growth if feeding conditions occurred ($p < .05$; Fig. 8). On the contrary, under St, M-PET significantly differs from control ($p < .01$). Concerning leachates, greater consequences have been recorded. Under Ac, S-PET ($p < .05$) and M-PET ($p < .001$) treated larvae significantly differed from controls. Also under St, *plutei* exposed to leachates differed from controls (M-PET, $p < .01$; L-PET, $p < .0001$). In the case of leachates, S-PET differed from others MPs under both pH conditions. Under Ac + feeding conditions, ANOVA (two-way) revealed significant differences respect to control both to suspension and leachate concerning POAs ($p < .0001$; Fig. 9). Under St + feeding conditions, strong significant differences were detected for all tested particle-size among each other and compared to controls.

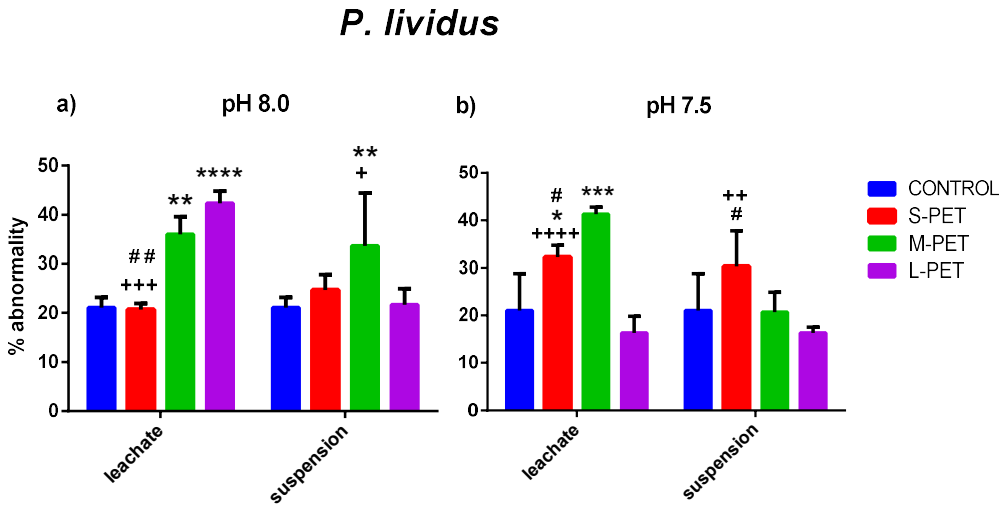

Fig. 8. Percentage of abnormalities (mean ± st. dev.; n=3) of the sea urchin larvae after 72h of incubation in different leachates and suspensions of PET MPs (S-PET; M-PET; L-PET) under feeding conditions at pH 8.0 (a) and 7.5 (b). Results were not normalized according to Abbott (1987), controls are represented. * = significant differences from control; # = differences from M-PET; + = differences from L-PET. The repetition of * indicates the level of significance according

to the following code: $p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***; $p < 0.0001$ ****. Results from 2way-ANOVA (Tukey test for disentangle differences between treatments; Dunnett test for differences with control). Results from 2way-ANOVA (Sidak's multiple comparisons test for disentangle differences between each size levels of leachate with the other of suspension) highlighted significant differences among leachate and suspension tested for L-PET ($p < 0.001$; St pH condition; data not represented) and for M-PET ($p < 0.001$; St pH condition; data not represented).

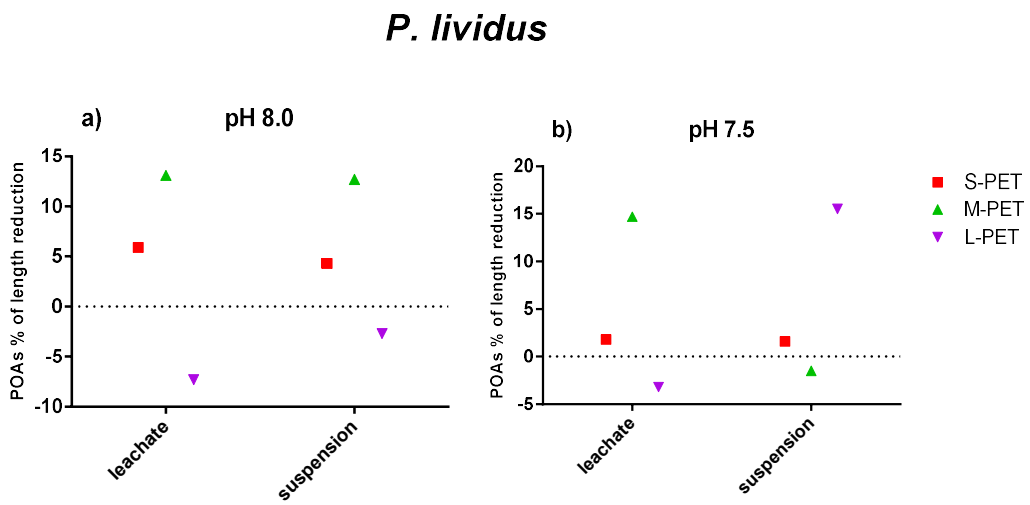

Fig. 9. Percentage of length reduction of posterior oral arms (POAs) of plutei exposed under feeding conditions to leachates and suspensions of PET MPs of different particle-size (S-PET; MPET; L-PET). One graph for each pH scenario: a) pH 8.0; b) pH 7.5. Negative values mean increase in length. A general reduction of POAs length is detectable at both pH conditions, except for L-PET at pH standard which seems to increase the length (up to +7.3%) and L-PET and M-PET in acidified conditions (+3.2%, +1.5%, respectively).

3.4. Overview by multivariate analyses

Non-metric multidimensional scaling (nm-MDS) performed on the whole database is represented in Fig. 10. The ANOSIM test performed on the factor "food availability" highlight significant segregations (Global R = 0.255, p b .01; Table 3) In this case, factors "particle-size", "treatments", and "pH conditions" should be tested separately respectively on feeding and fasting conditions datasets. Nevertheless, the amount of records did not allow testing factors of interest on two separate feeding resources

databases. A global overview on significant relation recorded in this study is summarized in Table 1S (Supplementary materials).

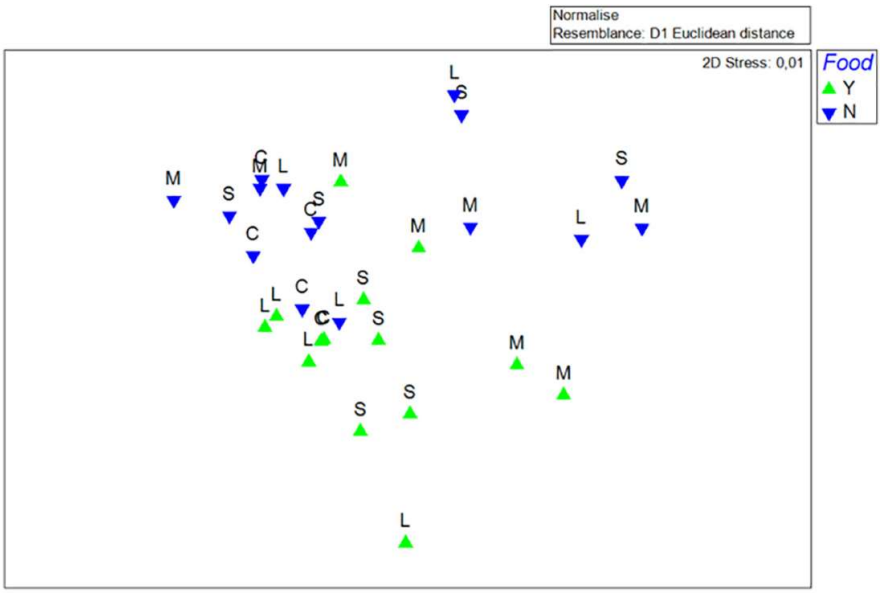

Fig. 10. Non-metric multidimensional scaling (nm-MDS) performed on normalized data. POA and % of anomalies data collected on echinoderms are represented.

Table 3. ANOSIM test one-way performed on factors affecting ecotoxicological responses on echinoderms. Notes: Pairwise test performed between couples of considered levels of tested factors is reported (Signif. Couples). Only significant relationships are reported. In bold are highlight significant relations, ** = high significant; * = slight significant.

Part a	Factors	Levels	Pairwise Couples	Global R	Signif. level (%)	Signif. couples
Global database	Size	4	S-M; S-L; M-L; S-C; M-C; L-C	0.057	11.6	M-C; S-C*
	Feeding	2	Y-N	**0.225**	**0.01****	–
	pH	2	Ac-St	−0.032	81.0	–

4. Discussions

4.1. Microplastic changes due to pH variation

The reduction of particle-size is usually associated to increasing toxicity as documented by studies performed on nanoparticles (Jeong et al., 2016; Lu et al., 2016; Cui et al., 2017; Karami et al., 2017). Materials that are safe in their bulk form become active on biota if reduced in size to their nanoforms (Jeevanandam et al., 2018). This is due to the increase of the surface/volume ratio following the size reduction that, consequently, increases the surface that particles are able to expose to water. MP is an "umbrella" term including any plastic-made particle with a dimensional range included within 1–5000 μm (Frias and Nash, 2019). Bioactivity within this wide size range could

be very different as focused by this study on tested species exposed to three different particle sizes. The chemical equilibrium of MPs could be affected by variations of ocean pH, as effect of global changes or human activities, which could exacerbate the release of chemicals modifying the toxicity of the polymers. Results obtained by analyses performed in this study support an ultrastructural change of PET MPs exposed to acidified conditions at the nanoscale-level. These results enhance the need for specific evaluation of eco-toxicological and ecological effects to better model risks for marine environments associated to packaging pollution under a global changing scenario. Performed analyses highlighted significant effects on PET material following exposure to Ac solution (size-trend M-PET ≫ L-PET). The reverse trend to increase CI index, which was recorded for S-PET particles, could be explaining as technical artefact due to the interference of size effect on spectra signal for smallest particles. In fact, the same occurrence was reported on virgin MPs by Raman spectroscopy and attributed to the impact point of the laser on the surface of the polymer (Dehaut et al., 2016). Following acidification, a significant reduction of intensity of PET characteristic peaks (1715 cm−1, C=O; 1245 & 1100 cm−1C-O) was recorded and explained by C=O and C-O degradation (Ioakeimidis et al., 2016). The reduction of intensity that resulted associated to the non-characteristic PET zone of spectra for all of the exposed particle-size, could be due to the release of chemical additives and structural modifiers. Information on such chemical additives requires additional chemical analysis, such as GC–MS (not performed in this context). Finally, the pre-treatment washing of PET-MPs with ethyl alcohol 96% is a widely used step in ecotoxicological study with MP (e.g. Jemec et al. 2016), showing effectiveness in reducing microparticles impurities (i.e. talc) absorbed on MP surfaces during their production without significantly alterations of μFT-IR spectra collected on both washed and unwashed MP (Renzi et al., 2019a; Supplementary materials). In spite of that, the extraction of some plastic additives during the preliminary rinsing performed, could not be a priori excluded and for this reason obtained results could underweight toxicity due to additive contribution. This specific aspect will be subject of further research aimed at achieving this goal.

4.2. Effects induced by leachates

4.2.1. Standard conditions (St, pH = 8.0)

Leaching of plastic packaging and MPs represents an indirect source of impact for marine ecosystems, as leaching could determine significant releases of chemicals in water (Rochman et al., 2013; Pedà et al., 2016). Our results reported that, under St condition, the exposure of marine species to leachates of the three different particle-sizes of PET tested are not sufficient to highlight significant acute effects on bacteria and algae species that pointed out, on the contrary, biostimulation. Results obtained in this study on bacteria are in agreement with literature. A handful of studies deployed the marine bacterium for eco-toxicity test of MPs (Booth et al., 2015; Gambardella et al., 2018) and *V. fischeri* often reported no effect or a scarce sensitivity. This study supported the idea that this species is not a sensitive model for plastic toxicity. Due to a major chemical affinity, MPs are able to absorb more organic compounds, but *V. fischeri*, is more sensitive to trace elements rather than to organic-due pollution and this could partially explain the weak sensitivity reported in this study. Control algae exposed to the acidified scenario highlight a slight increase (although not biologically significant) in growth rate inhibition, suggesting a sensitivity of such species to pH water variations. However, this early stress is totally buffered by the leachates of PET-MPs, which instead trigger biostimulation responses. Sjollema et al. (2016) tested effects following the exposure to nanoplastics (tested range within 0.05, 0.5 and 6 μm) and, reported adverse effects, were demonstrated to increase with decreasing particle size. Effects observed in this study concerning biostimulation on algal species (*P. tricornutum*), could represents an early sign of chronic stress (Renzi et al., 2014). A similar phenomenon was recorded by Mao et al. (2018) who tested the toxicity of polystyrene MPs on the growth inhibition of *Clorella pyrenoidosa* at the same concentration (100 mg/L). This study evidenced how the effects of plastic can impair or enhance the algal growth depending on the developmental stage of the algae. The algal growth is characterized by four stages: lag, logarithmic, stationary, and death (Tsai et al., 2017). When the algae reach the logarithmic phase, it generally shows the strongest ability to maintain its activities under biotic and abiotic stress. For this reason, the algal growth was stimulated from the end of the logarithmic stage to the stationary phase. In our study, the stimulation of algal growth occurred during logarithmic phase, suggesting *P. tricornutum* is a “plastic-resistant” species.

Tests performed on larval stage of *Paracentrotus lividus* highlight significant effect both on the two tested endpoints: % of anomalies after 72 h of exposure and body-size reduction in normal larvae. In particular, particle-size is directly related to the percentage of abnormal larvae recorded (toxicity of L-PET ≫ S-PET; M-PET). In our study, all leachates from different PET particle-size tested, evidenced significant reduction of POAs in normal larvae suggesting that all tested leachates are effective to determine a significant body-size reduction in exposed embryos also in normal phenotypes. These results are in agreement with literature. Oliviero et al. (2019) reported a drastic reduction of larval length (33%) in *plutei* exposed to PVC leachates probably due to the presence of phthalates. Our results support studies indicating *P. lividus* as a good bioindicator of marine pollution (Cunha et al., 2005; Alvarez et al., 2010; Sanchez-Marın et al., 2010). To evaluate more complex endpoint in eco-toxicological responses, embryo toxicity on sea urchin is reported to be more sensitive than acute tests on other species (Losso et al., 2004) and, also, than fertilization tests on the same species (Lera and Pellegrini, 2006). The exposure to low doses of chemicals acting as endocrine disruptors (i.e. nonyl- and octyl-phenols, bisphenol-A) resulted in this species able to affect the embryological development (Roepke et al., 2005; Arslan and Parlak, 2007; Ozlem and Hatice, 2007). These chemicals are common additives in plastic packaging because they increase the plastic performance. In conclusion, sea urchin larvae resulted to be the most sensitive organism and POAs length resulted to be a sensitive endpoint to detect early sub-lethal effects.

4.2.2. Acidified conditions (Ac, pH = 7.5)

Results obtained in this study on FESEM and infrared spectral microanalyses performed on PET MPs exposed to acidified marine water highlighted, at the nano-scale, the occurrence of disaggregation, structural changes (i.e. reduction of acute angles), and dissolution of plastics with losses of infrared peaks that are in the PET characteristics region of the infrared spectra. Results from dimensional analyses performed, supported the occurrence of a general fragmentation supported by the recorded reduction of mean dimension that was more significant in Ac L-PET that other particle-size tested. Literature reported microplastic degradation occurring during extraction process of microplastic by environmental matrices with acid solutions (i.e. 22.5 M of nitric acid; Avio et al., 2015) supporting the effectiveness of the exposure to acid solutions even for short times (20

min) in producing structural damages. Our results suggested that also the exposure to weak acids (Ac) for longer exposure times (72 h) could be effective to produce significant structural changes on MPs. Bacteria and algae exposed to MPs leachates under Ac conditions compared to their Ac controls showed any acute effects as also observed under St conditions. Concerning the algal species, in this case, a greater mean stimulation (+7.4%) was recorded than under St conditions (+4.5%). Results recorded by literature (Besseling et al., 2014) at exposure doses similar to ours, highlighted a maximum growth inhibition of 2.5% in the algal species *Scenedesmus obliquus* following to the exposure to nano-polystyrene. This suggests that eco-toxicological responses are strictly species dependent, particle-size dependent, and that generalization on the basis of models are scarcely realistic. Also, under Ac conditions, the larval stage of echinoderms represents a sensitive stage able to detect the tested stress. Concerning particle-size, Ac conditions produced a significant effect in S-PET and M-PET while LPET resulted not effective compared to Ac controls. This result is strictly linked to release in leachates of plastic and associated chemicals as reported by infrared spectra results. Normal larvae exposed to leachates resulted in a significantly lower POAs mean compared to Ac controls in all tested particle-size supporting results obtained under St conditions on this endpoint. Literature evidenced that early developmental stages of echinoderms are relatively robust to predicted ocean acidification if pH < 7.7 (Martin et al., 2011). Nevertheless, our study suggests that particle-size represents a key aspect to be considered concerning ecological and eco-toxicological effects under acidified scenarios. On the contrary, endpoints based on body-size (i.e. AOAs and POAs) resulted both very sensitive and effective under acidified conditions. Under changing scenarios concerning water acidification, POAs reductions in normal larvae could mean an increase of energy consumption during 72 h of development induced by the pH stress and consequently enhance the eco-toxicological response. This could be explained by the synergic effect of an increased energy consumption and gene transcription associated to chemical damages. Literature reported a temporal delay of the natural development of sea urchin exposed under acidified conditions that is due to 20-fold increase in gene up regulation and consequently an increase of energy consumption occurring at pH within 7.5–7.0 (Martin et al., 2011).

4.3. Effects induced by suspensions on echinoderms

It is known that exotrophy in sea urchin larvae starts around at 48 hpf (Giudice, 1986). The mouth of *plutei* that opens at this developmental stage is around 20 μm (Messinetti

et al., 2018). As reported by Beiras and Tato (2019) and Messinetti et al. (2018) *plutei* of sea urchin are able to ingest microplastic smaller than 10 μm, size compatible with the largest mass fraction of the smallest class (S-PET) used in this study. In this study, starved larvae reported an increased percentage of abnormalities when exposed to larger MPs (L-PET, N500 μm) and under feeding condition, M-PET exerts toxicity. M-PET and L-PET are not compatible with the buccal apparatus of the animals, so the statistically difference reported is more likely due to the greater concentration of compounds leached during the 72 h of exposure. This result is in agreement with leachate test results discussed above. Even in this test, POAs length reduction says the same information of % of abnormalities and is in agreement with results reported by Messinetti et al. (2018) who recorded significant differences in body and arm length of *P. lividus plutei* reared at 25 mg/L of 10 μm polystyrene beads. In conclusion, the adverse effects were not due to the polymer but, more probably, to the cocktail of contaminants presents in the PET flakes. Ingestion of the smallest PET particle-size did not determine significant effects compared to those recorded by the exposure to "not ingestible" PET fraction. This may also be due to the fact that the fraction that can be ingested (S-PET) is numerically lower than mean size of the S-PET fraction as determined in this study by microscopic
analyses. Further studies will need to better clarify these aspects.

5. Conclusions and future work

PET is a material actually considered safer than others plastic polymers and consequently widely used in single-to-use products. Its occurrence in different marine compartments has been proven (Munari et al., 2016; Suaria et al., 2016; Ter Halle et al., 2017; Mu et al., 2019). Nevertheless, especially for particles smaller than 150 μm (which are considered the more bioactive), its environmentally concentration is highly probable underestimated for the reasons presented in the introduction of this paper. In addition, PET eco-toxicological studies on marine species are poorly investigated by literature. For these reasons, in order to compare our results with those performed on other polymers at similar concentrations (Della Torre et al., 2014; Mao et al., 2018) and to highlight biological responses in the frame of global changes characterized by an increase of plastic pollution (Jambeck et al., 2015) and water acidification, we selected the concentration of 100 mg/L. However, it is important to point out that the concentration levels tested in this study are undoubtedly orders of magnitude higher than actual recorded levels in marine

water (1.25 ± 1.62 particles/m2; Suaria et al., 2016; SAPEA, 2019). Consequently, the authors suggest caution in the interpretation of the results here presented, inviting a reading in view of the future scenario of global changes.

Using this key to reading, from an ecological point of view, even if bacteria (detritivore) and algal species (primary producers) resulted not impacted by acute effects, larval stages of primary consumers (echinoderms) show increase of embryo toxicity under tested conditions. Consequently, consumers-linked trophic levels of marine trophic web could be significantly affected by PET MPs, directly and indirectly, by impacting the species fitness due to a significant reduction of larvae recruitment by the reproductive pool of the natural population. Furthermore, the significant reduction of body-size recorded on normal larvae species exposed to PET MPs, could represent a signal of subtle chronic stress that could affect significantly the survival rates on a longer temporal scale. Total energy transferred from zooplankton towards upper trophic levels could be also significantly affected. Concerning biostimulation recorded on exposed algal species, effects on primary producers on a longer temporal scale should also be better explored by future researches focusing on ecological possible consequences.

This study investigated if other stressors (i.e., food and pH variations) can influence the microplastic toxicity. Multivariate statistics performed highlighted a significant effect related to the factor food availability on eco-toxicological responses (% of anomalies and body-size length) of echinoderms. Food supply induces energy additional input compared to fasting treated species and could reduce larval stress induced by PET impacts. Furthermore, algal addition could actively affect chemical bioavailability by absorption with a significant lower impact. Otherwise, algal absorption on microplastic surfaces could affect palatability, buoyancy of MPs increasing or decreasing ingestion rates. For example, the availability of food influenced the ingestion of PE microplastic in *Tripneustes gratilla* larvae reducing the occurrence of MPs by a factor of 4–10 (Kaposi et al., 2014). Finally, algal absorption on MPs surfaces could reduce MPs/ water chemical exchanges reducing chemical releases from MPs towards water.

Plastic pollution could represent a significant threat for the conservation of marine ecosystems and for the associated losses of ecosystems service value following marine impacts by plastic packaging. Furthermore, such effects are not possible to be modelled on a theoretical basis without specific experimental data due to their specie-specificity response behaviour. Tested species resulted not all sensitive to PET leachates and data obtained in this study confirm the complexity of the microplastic toxicity mechanism,

able to be influenced by different stressors suggesting the needing to take them into account when an environmental risk assessment is carried out.

To sum up, results obtained in our study show that, in the context of a future scenario characterized by ocean acidification and increase in plastic pollution: *i) V. fischeri* and *P. tricornutum* are not responsive to PET induced stress under standard and acidified condition; *ii) P. lividus* larval stage are able to highlight effects following the exposure to leachate and suspension of PET MPs; *iii)* AOAs and POAs lengths on normal larvae resulted a suitable and more sensitive than anomalies endpoint to evaluate effects under both standard and acidified conditions; *iv)* different particle-size of PET produce different effects not always related to the particle size itself, suggesting the occurrence of more complex interactions than theorized ones; *v)* effects under acidified conditions are significantly different compared to standard pH suggesting changing ecotoxicity of plastic materials under global changing scenarios; *vi)* food availability produce significant different responses compared to fasting conditions; *vii)* ecological consequences following PET exposure could produce significant and not attended impacts on marine trophic webs; *viii)* special attention on the possible interactions between MPs and other stressors (e.g., food and pH) is needed in order to give a better picture of natural occurring dynamics.

Acknowledgements

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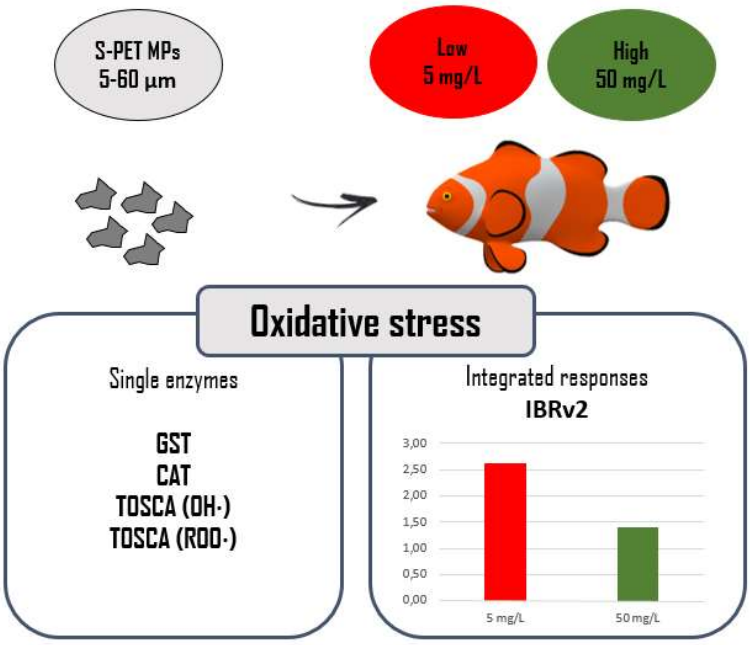
Oxidative stress responses in juveniles of *Amphiprion ocellaris* (Cuvier, 1830) after exposure to PET microplastics



1. Material and methods

1.1 Animals, experimental design and sampling

Juveniles of *Amphiprion ocellaris* (8.50 ± 0.43 mm standard length, 25.65 ± 6.01 mg weight) were bred in the facility of the *Observatoire Oceanologique in Banyuls sur Mer*. The test was performed in 1 L glass jars. Photoperiod was maintained at 13h/11h light/dark and NO_2^- levels were measured randomly once a day (<0.5 mg/L). Fishes were subjected to 24h of acclimatization. The exposure consisted in three experimental groups: 0 mg/L (control), 5 mg/L (low), 50 mg/L (high); each group composed by 4 jars with 3 animals per jar (N=36) and 0.5 L of artificial sea water (ASW) whose temperature was controlled (26.3 ± 0.1 °C) and a gentle aeration supplied. Animals were exposed to S-PET (5-60 µm), size compatible with the buccal apparatus of the juveniles, for 3 days (100% of daily water renewal). Fishes were fed twice a day *ab libitum*. At the end of the exposure 27 juveniles (9 for treatments) were randomly sampled and euthanized by immersion in a tricaine methanesulfonate (MS-222) bath (200 mg/L) for the analyses of

enzymatic antioxidants (Catalase, Glutathione S-transferases) and Total Oxyradical Scavenging Capacity (TOSC) assay. The remaining 3 fishes for treatment (N=9) were euthanized in ice bath, stored in Trizol at -80°C and destined for gene expression analysis. RNA was extracted following the same procedure adopted for nanoplastic experiment (for details, refer to Nanoplastic section pp 77, 78). At the moment, however, the procedure has not had any further developments.

1.2 CAT, GST, TOSCA and IBRv2 (Integrated Biological Response version2) index

The whole juvenile was homogenized (1:10 w:v ratio) in 100 mM K-phosphate buffer (pH 7.5), 0.008 TIU/ml aprotinin, 1 mg/ml leupeptin, NaCl 1.8%, and centrifuged at 110,000 G for 1 h at 4 °C. Measurements of enzymatic activities were made with spectrophotometer at a constant temperature of 20 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of 12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0. Glutathione S-transferases (GST) were determined at 340 nm using 1.5 mM 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). The TOSC assay measures the capability of cellular antioxidants to inhibit the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas in the presence of different forms of oxy-radicals, like peroxyl- radicals (ROO•) and hydroxyl- radicals (HO•) which are artificially generated at constant rate (Winston et al. 1998). Peroxyl- radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl- radicals (•OH) were produced by the Fenton reaction of iron-EDTA (1.8 µM Fe3+, 3.6 µM EDTA) plus ascorbate (180 µM) in 100 mM K-phosphate buffer. Under these conditions the different oxy-radicals produced quantitatively similar yields of ethylene in control reactions, thus allowing to compare the relative efficiency of cellular antioxidants toward a quantitatively similar radical flux (Regoli and Winston 1999). Ethylene formation in control and sample reactions was analyzed at 10-12 min time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 – (∫SA/∫CA x 100), where ∫SA and ∫CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a

specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

To integrate results from the different biomarkers, the Integrated Biological Response version 2 index (IBRv2) was calculated according to Sanchez, Burgeot, and Porcher (2013). Lower or higher index core values can be translated into the impact of PET MPs on organisms: higher index core values are indicative of a poorer health status (stressed organisms). For the IBRv2 calculation, individual biomarker data (Xi) were compared to the mean control data (X0), and a log transformation was applied to reduce the variance:

$$Yi = \log(Xi/X0)$$

In a second step, the general mean (μ) and standard deviation (s) of Yi were computed as previously described by Beliaeff and Burgeot (2002), and Yi standardized:

$$Zi = (Yi-\mu)/\text{st. dev.}$$

To create a basal line centered on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Zi) and the mean of control biomarker data (Z0) were used to define a biomarker deviation index (A):

$$A = Zi-Z0$$

To obtain an integrated multi-biomarker response named IBRv2, the absolute value of "A" parameters calculated for each biomarker in each tested concentrations were summed:

$$\text{IBRv2} = \sum |A|$$

For a single concentration, "A" parameters were reported in a star plot to represent the reference deviation of each investigated biomarker. The area up to 0 reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition.

1.3 Data analysis

Biochemical responses were statistically analysed by GraphPad Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com) by nonparametric Kruskal-Wallis

test. Differences were considered significant at p-level < 0.05. Dunn's multiple comparison test was used to highlight significant differences within treatments and between treatments and controls. The EXCEL software (Microsoft, Redmond, WA, USA) was used for the calculation of IBRv2 index and the creation of star plot.

2. Results

Kruskal-Wallis test between controls and treatments revealed no statistical relevance for CAT, GST and TOSCA OH· and ROO· (p-value = 0.56, 0.26, 0.79 and 0.65, respectively). Also the test performed within treatments confirmed the absence of oxidative stress responses in relation to the different concentrations tested (data shown in Fig. 1).

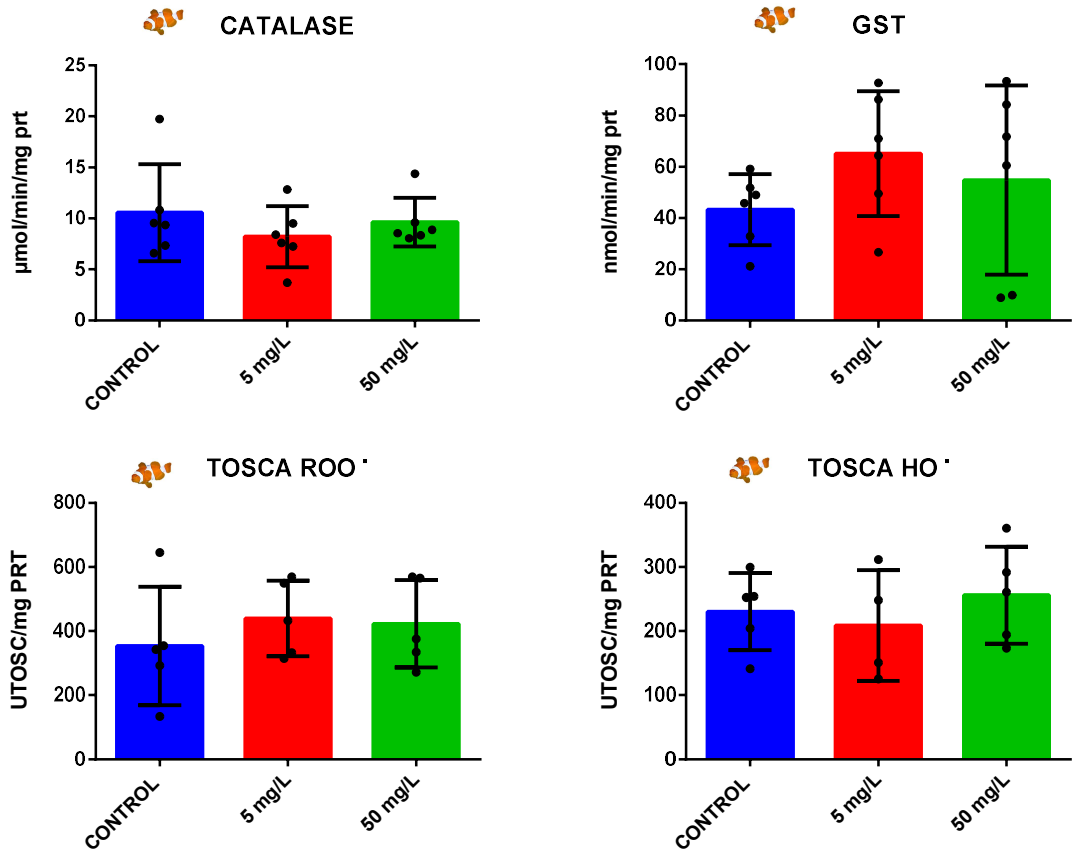


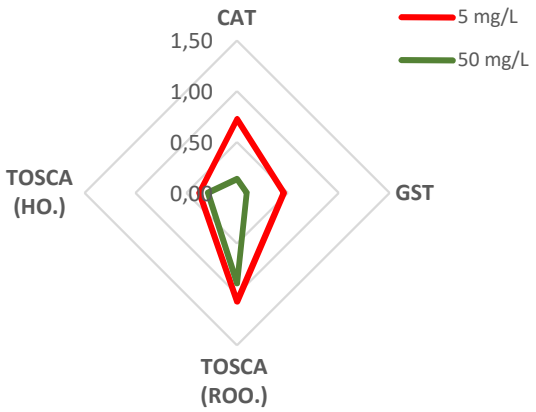
Fig. 1. Antioxidant defenses in clownfish juveniles exposed to 5 and 50 mg/L of S-PET MPs for 3 days. CATALASE, GST (glutathione S-transferase), n = 6; TOSCA (Total Oxydoradical Scavenging Capacity Assay, ROO· = peroxylic; OH· = hydroxylic), n = 5. Dot points represent individual values (mean ± st. deviation).

IBRv2 values were 2.63 and 1.41 for 5 mg/L and 50 mg/L treatment groups, respectively (Fig 2a). Animals exposed to the lower concentration reported IBRv2 value 1.87 times higher than 50 mg/L (Fig 2c), indicative of more stressed conditions as clearly represented in the star plot (Fig 2b). Albeit very slight variations, all the biomarkers analyzed show stimulation and not inhibition. Regarding the sensitivity of biomarkers to detect any effects, TOSCA ROO· demonstrated to be more sensitive in both treatment groups, followed by CAT, GST and TOSCA HO· in 5 mg/L group, while in 50 mg/L group the second more sensitive biomarker was TOSCA HO· followed by CAT and GST (Fig. 2a and b).

a)

	CAT	GST	TOSCA-ROO.	TOSCA-HO.	IBRv2
5 mg/L	0.73	0.46	1.07	0.37	2.63
50 mg/L	0.14	0.09	0.89	0.29	1.41

b)



c)

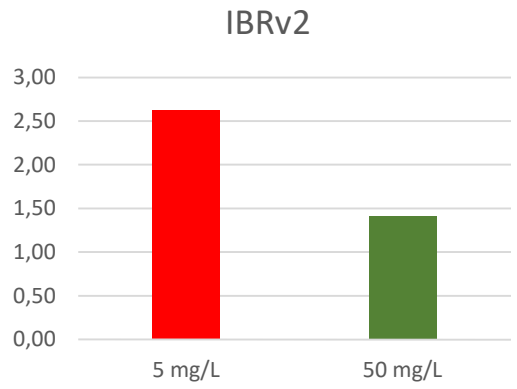


Fig. 2. a) Table with the reference deviation of each investigated biomarker calculated to obtain the IBRv2 index in different treatment groups (5 and 50 mg/L). b) Star plot based on the reference deviation of each investigated biomarker: CAT = catalase; GST = glutathione S-transferase; TOSCA (ROO·) = Total Oxydoradical Scavenging Capacity Assay (peroxylic); TOSCA (HO·) = Total Oxydoradical Scavenging Capacity Assay (hydroxylic). c) IBRv2 values for each concentration of PET MPs.

3. Discussions

PET microplastics ingestion by marine fishes collected from natural environments has been reported by several studies (Rummel et al. 2016; Alomar and Deudero 2017; Wang et al. 2021), some of which focused on tropical reef systems (Ding et al. 2019) where microplastics abundance in seawater samples reached values up to 45.2 items L^{-1}. According to Ding et al. (2019), in tropical fish samples, particles between 20-330 μm represented the largest fraction, mainly characterized by fibers or fragments and PET corresponded to the second most abundant polymer (16.5%). Despite this, a handful of studies tested the toxicity of PET MPs in fishes: Boyle et al. (2020) studied the role of chemical additives in the aqueous toxicity of PET MPs (500 mg/L) in early-life stage zebrafish with no effects in transcriptional levels of *metallothionein 2* (*mt2*), *cytochrome P450* 1a (*cyp1a*) and *vitellogenin 1* (*vtg1*); Jakubowska et al. (2020) reported no effects of 3000 μm PET MPs (0.055 pellets L^{-1}) on development of the sea trout *Salmo trutta* early life stages and only genotoxic responses (nuclear buds, micronuclei and blebbed nuclei cells). To our knowledge the present study represents the first record about the effect of PET MPs fragments in tropical fishes.

Considering the lack of knowledge on PET toxicity at the moment of the analysis, and based on the assumption that PET is considered among the safest polymers so much that is used in food packaging, we decided to set up an acute test of 3 days with a not environmentally realistic high concentrations of MPs (5 and 50 mg/L). In order to detect any effects, we focused the attention on oxidative stress responses by measuring the activities of specialized enzymes, namely catalase and GST, but also quantifying the total oxyradical scavenger capacity of the organism by TOSC assay. Oxidative stress represents an endpoint widely used in MP toxicity experiments (roughly 33.3 %, of research publications on MPs exposure in zebrafish used oxidative stress responses). Some reports show the ability of MPs to interfere with ROS metabolism and to cause oxidative stress by altering the antioxidant levels of CAT and SOD (Lu 2016; Qiao et al. 2019) or inducing higher lipid peroxidation (Barboza et al. 2019).

Analysing biomarker by biomarker, our findings did not evidence statistically relevant differences between control and treated animals or between concentrations. Discrepancies between our results and other studies could be due to different experimental conditions such as polymer type, size, shape, target species, dose and time of exposure. On the contrary, the integrated analysis of biomarker responses has proved to be a quick, useful

and informative approach, especially in discriminating against any differences between treatments that cannot be identified by statistical analysis on individual biomarkers. A simple method to summarize biomarker responses and simplify their interpretation was adopted: the "Integrated Biological Responses version 2" index (IBRv2). The IBRv2 index is based on the reference deviation concept and it was selected because it solves the two major weak points related to the previous index version (IBR) of Beliaeff and Burgeot (2002): (1) the strongly dependency on the arrangement of the biomarkers on the star plot, and (2) the only up- or downregulation of biomarkers. In our experiment, IBRv2 index allowed to detect a very slight reduction in overall health status of fishes tested with 50 mg/L, but interesting was the severe effect induced by the lower concentration of MPs (1.87 times greater). The absence of a concentration-dependent response is not rare in microplastic exposure (Brandts et al. 2018; Brandts et al. 2021) and could be indicative of a mechanism of toxicity not strictly dependent on contaminant concentration. Higher values of TOSCA (ROO·) and catalase may suggest a beginning of stress mainly due to peroxyradicals and H_2O_2, and eventually detectable only on longer temporal scale (chronic exposure). The high sensitivity of CAT reported in this study is in accordance with results of Huang et al. (2020) who reported for the catalase activity the highest index in response to MPs (polystyrene; 32−40 μm diameter; 100 and 1000 μg/L) exposure in guppy (*Poecilia reticulata*) for 28 days. In conclusion, no acute (3 days) effect due to exposure of S-PET (5-60 μm) microplastics was detected in juveniles of clownfish, even if calculation of IBRv2 revealed a slight oxidative stress response especially for the lower concentration (5 mg/L) and toward peroxyradicals and hydrogen peroxyde.

Finally, a special attention should be put on MP bioavailability and the consequent probability to be ingested by juveniles under laboratory conditions. In this regard, several precautions have been taken: particles were selected in a size range compatible with the buccal apparatus of the juveniles and provided jointly with the food during the exposure; in addition, a continuous resuspension of the particles was insured by a bottom-up gentle aeration in order to avoid the sedimentation of the PET due to its higher density (1.37–1.45 g cm $^{-3}$) respect to ASW. Despite this, no data have been acquired to confirm the microplastic uptake and consequently, two different hypothesis can be formulated: *i*) despite the precautions, the ingestion of particles did not occur or the egestion rate was > than the ingestion rate without obvious oxidative stress responses; *ii*) ingestion occurred and did not cause statistically significant oxidative stress. Further investigations are

necessary to first demonstrate the PET MPs accumulation in juveniles of clownfish and consequently assess the eventually toxicity on longer temporal scale.

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NANOPLASTICS

Three years ago, the body of literature on nanoplastic pollution was very limited (only 32 papers). The hypothesis that degradation of plastic waste could continue and lead to the formation of particles in the nano-range had recently been formulated and laboratory evidence of nanoplastic release from bigger plastic items started to appear. The collection of nano-fraction of marine litter in seawater samples was very recently reported and few eco-toxicological tests were available. The scientific community's interest in plastic pollution was slowly shifting its focus to sub-micrometric particles. In this context, our research group decided to focus its attention also on this new understudied form of pollution and therefore started a new line of research.

For these reasons, the research activity of this PhD is also based on state-of-the-art analysis of nanoplastic pollution and, the understanding of the possible eco-toxicological effects of polystyrene nanoplastics in the tropical reef fish *Amphiprion ocellaris* (Cuvier, 1830) previously tested with PET microplastics.

The first full paper reported in this thesis is a review revised and completed during the quarantine period due to the Sar-Covid-2 emergency. The contribution explores the scientific knowledge on the topic, from polymer degradation to occurrence in marine water, passing across laboratory evidence of nanoplastics formation. Main fields of research effort, eco-toxicological aspects and suggestions for future studies are further discussed.

In the second full paper reported in this thesis, a 7-days exposure to 100 nm PS-COOH nanoplastics in juveniles of clownfish explores their possible biological implications both from a biochemical and molecular point of view. The paper is being prepared to be submitted.

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Review

Nanoplastics in the oceans: theory, experimental evidence and real world

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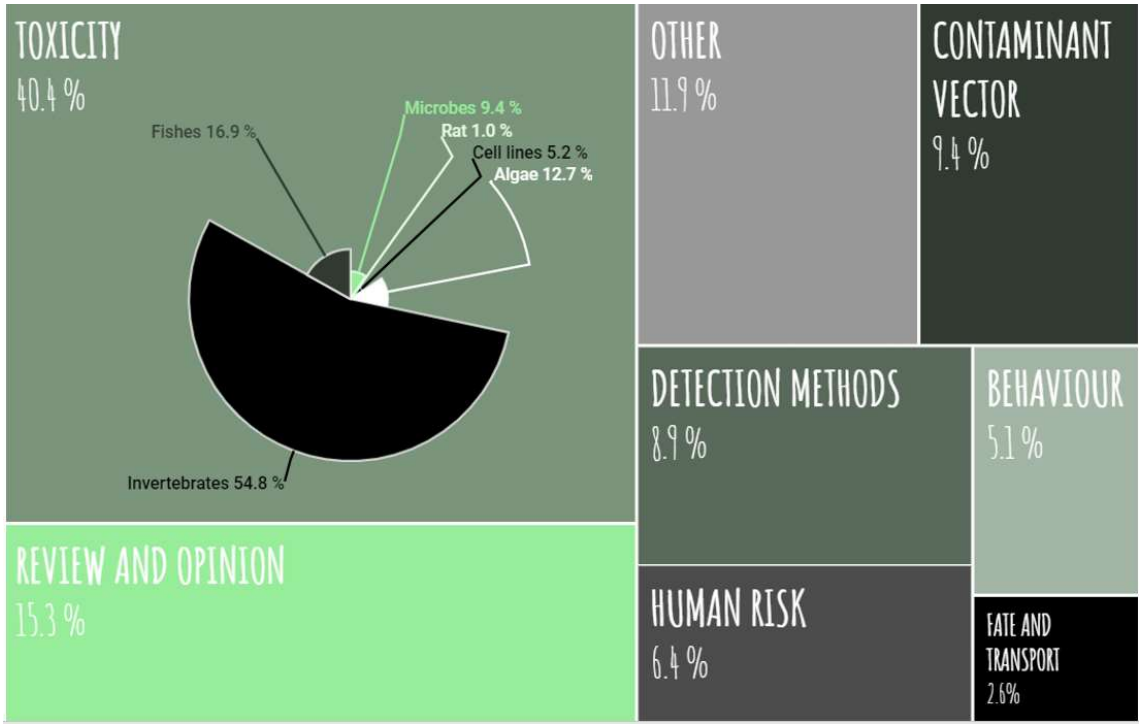
Graphical abstract



Keywords: Nanoplastics; Polystyrene; Reference materials; Knowledge gaps; Laboratory experiments; Dynamic phenomenon

Abstract

This review critically analyses more than 200 papers collected by searching on Pubmed the word “nanoplastics”, a group of emerging contaminants which are receiving growing attention. The present review intends to provide an overview of current knowledge on nanoplastic pollution starting with the theory of polymer degradation, passing to laboratory confirmation of nanoplastic formation and ending with the possible occurrence in sea water samples. Most of the observations proposed focus the attention on polystyrene (PS) because the majority of research knowledge is based on this polymer. Moreover, we thoroughly describe what effects have been observed on different organisms tested in controlled conditions. Nanoplastics formation, fate and toxicity seems to be a very dynamic phenomenon. In light of this, we identify some aspects retained crucial when an eco-toxicological study with nanoplastics is performed and which elements of nanoplastics toxicity could be deeper covered.

1. Introduction

Searching on PubMed the word “nanoplastics” (NPs) a list of 224 papers appears, among which, the oldest dates back to 2012 (research conducted on February 5th, 2020). Respect to microplastics, nano-fraction of the marine litter, represent a new infant field of investigation as suggested by the number of papers published in 2019 which doubled the studies of the previous year and that the first two weeks of 2020 have recorded almost the entire 2018 scientific production on this topic (Figure 1).

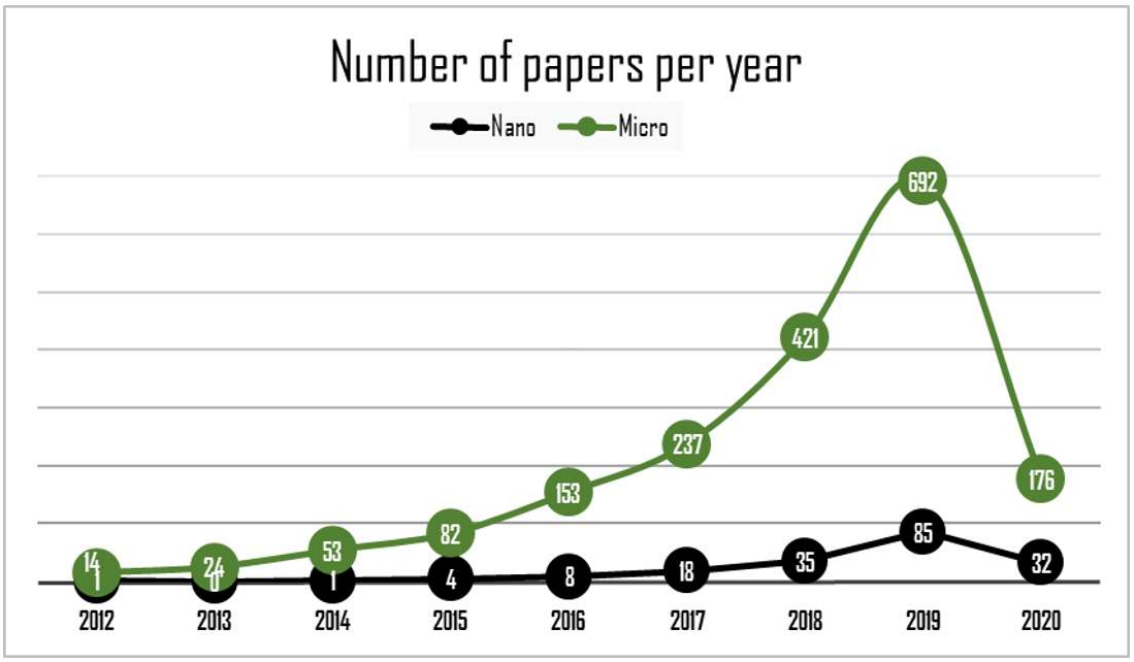


Fig. 1. Number of papers published, from 2012 to 2020, searching the term "nanoplastics" and "microplastics" on PubMed (February 5th, 2020).

Some authors adopted the definition taken from the nanomaterial including particles smaller than 100 nm, others prefer to raise the upper size limit to 1 μm, typical border of colloids (Cole and Galloway 2015; Pinto da Costa et al. 2016; Alimi et al. 2018; Gigault et al. 2018). In addition, most researchers include in the definition, either primary (manufactured) and secondary (originated from degradation) NPs. Personal care products, industrial abrasives, paints, and particles used in drug delivery can be considered primary NPs (Guterres, Alves, and Pohlmann 2007; Alimi et al. 2018; Hernandez, Yousefi, and Tufenkji 2017). Also recent technologies, as 3D printing, have proved to be sources of ultrafine particles (Azimi et al. 2016). Nevertheless, although nanomaterials production is ever-expanding (Inshakova and Inshakov 2017), NPs represents only a thin slide of the market (Vance et al. 2015).

Mostly because of technological limitations and dilution effect, nowadays, it is laborious to extract and quantify NPs in marine environment. Despite the impetus in the research which took place in recent years, there are still many lights and shadows on the subject which, this review aims to highlight. The majority of the observations proposed in this review focus the attention on polystyrene (PS) because most of research knowledge (about 97%) is based on this polymer. It will be discussed what are the knowledges on

polymer degradation, initially reviewing laboratories experiments and consequently reporting evidence of the occurrence in marine samples. Laboratory evidence of biological effects on aquatic organisms will be also briefly reported. Finally, some aspects retained crucial for eco-toxicological studies with NPs will be pointed out and elements of research on NPs which could be deeper investigated will be suggested.

2. Polymer degradation theory, lab and field evidence

2.1. The theory of polymer degradation

A full-bodied bibliography on polymer degradation is available on Pubmed. The degradation of plastic polymers is induced by either abiotic or biotic paths (Gewert et al., 2015). Generally abiotic degradation precedes biodegradation following the photo-oxidative degradation, the thermal and ozone-induced degradation, the mechano-chemical degradation and the catalytic degradation (Singh and Sharma, 2008). Such mechanisms lead to changes in polymer properties resulting in bond scissions and consequent chemical transformations.

First, the modification involves the surface of the item: the visual effects are micro-cracking, discoloration, erosion and crazing. Subsequently, the inside polymer, can be subjected to further degradation such as microbial attach. Two different pathways exist, depending on whether we consider carbon-carbon backbone plastic (polyethylene, PE; poly- propylene, PP; polystyrene, PS; polyvinylchloride, PVC) or plastics with heteroatoms (polyethylene terephthalate, PET; polyurethane, PUR). Henceforth, the attention will be put on PS.

The European demand of polystyrene in 2018 was 6.4%, mainly destined for the production of food packaging (e.g. egg tray), coffee cup, lid, eyeglasses frames and building insulation (Ho et al., 2018; Plastic Europe, 2019). Once released into the environment, the polystyrene is subjected to degradation. Specifically, when irradiated with UV-light, the phenyl ring got excited and the excitation energy is transferred to the nearest C-H bond (Yousif and Haddad, 2013). The PS is thus deeply modified: the cleavage of the hydrogen, the formation of a polymer radical cross-linking and chain scission lead to the formation of ketones, olefins and styrene monomers as the main volatile product (Singh and Sharma, 2008; Yousif and Haddad, 2013). Although PS is susceptible to outdoor weathering, it is considered a polymer very resistant to

biodegradation (Mor and Sivan, 2008). Furthermore, additives and UV-stabilizers decrease its degradability (Ho et al., 2018).

An interesting suggestion on additional polymer degradation pathway is given by the physical size alteration of virgin polyethylene microplastics (31.5 μm) occurred after ingestion by Antarctic krill (Dawson et al., 2018). Following a 4-day static assay, the mean particle size isolated from the krill was 78% smaller than the original plastic particles (7.1 μm ± 6.2 SD). The paper suggests a new pathway of degradation that it would be interesting to deeper investigate even with other polymers.

The theory of abiotic polymer degradation is well known, but less is the knowledge about laboratory confirmation of such phenomenon in the nanoscale. Few studies reproduce realistic conditions for the marine environment.

2.2. Laboratory confirmation of nanoplastics formation

Laboratory evidence shows that the formation of nanoscale particles from bigger plastic items is very plausible. Shim et al. (2014) firstly reported fragmentation of expanded polystyrene (EPS) beads to micro- and nano-sized particles in laboratory. The study mimed conditions at beached or riverbanks, by submitting infield expanded polystyrene (EPS) beads to a monthly accelerated mechanical abrasion with glass beads and sand. Subsequently, they combined effects of UV exposure and mechanical abrasion on different polymer types (polyethylene PE, polypropylene PP and expanded polystyrene EPS) demonstrating that, in laboratory conditions, a large proportion of the particles had fragmented into undetectable submicron particles, within 12 months (Song et al., 2017). In particular, after 2 months the surface of the EPS pellets became yellow, brittle and showed cracks. In addition, powder-like white fine particles were produced on the surface of EPS. After 12 months, micrometer-sized fine particles were detected inside the cracks. Finally, aggregations of small particles (lower than 1 μm) were confirmed on the inner surface of cracks under high magnification. The study by Lambert and Wagner (2016) is a further laboratory example reporting how the degradation of plastic materials can lead to the release of nano-sized plastic particles to the environment. They used a Nanoparticle tracking analysis to characterize the formation of NPs during the degradation of a polystyrene (PS) disposable coffee cup lid. In detail, the PS sample was cut into 1 cm squares, placed in glass vials, immersed in 20 mL demineralized water, and placed in a weathering chamber. After 56 days' exposure the concentration of NPs was

1.26 × 108 particles/mL (average particles size 224 nm) compared to 0.41 × 108 particles/mL in the control. Nevertheless, the exposure conditions were kept static with the temperature set to 30 °C, 24 h exposure to light in both the visible and ultra-violet (UV; 320 and 400 nm) range; conditions quite distant from those found in the environment. In addition to photo- and mechanical degradation, the biodegradation pathway of PS particles has been also investigated. PS is considered a polymer very resistant to biodegradation. Tian et al. (2017) calculated the mineralization of 14C-PS polymers in liquid medium (pH 7.5) with the fungi *Penicillium variabile* for 16 weeks after ozonation pre-treatment. The study reports only a very slow mineralization ability expressed by the terrestrial fungi. Most of other studies have been conducted burring in soil the PS (Atiq et al., 2010; Oliveira et al., 2010). Marine-like experiments are still missing. Whilst there is evidence on PS degradation into nano-size particles, less is known about other polymers. Recently Enfrin et al. (2020) demonstrated how polyethylene microplastics extracted from a commercial facial scrub exposed to a wide range of shear forces may fragmented into NPs. Specifically, the study reports that a daily use of 4 g of scrub could release up to 1011 particles of 400 nm in size per litre of wastewater. The mechanism at the base of the fragmentation into NPs smaller than 10 nm is supposed to be a combination of turbulences created by mixing or pumping followed by a crack propagation and failure mechanism. Finally, only strong, and far-removed from reality methods have been applied in order to produce PET NPs (Magrì et al., 2018). The details will be provided in the Section 4.2.

Some studies report the fragmentation and degradation of plastic to NPs under laboratory conditions but evidence of their occurrence in environmental samples is still poor. In addition, the relative importance of this process still has to be validated in the field.

2.3. Nanoplastics occurrence in marine samples

Ter Halle et al. (2017) first reported the occurrence of the nano-fraction (1–999 nm) of the marine litter in environmental samples. Sea water samples collected in the North Atlantic Subtropical Gyre, were ultra-filtrated and analysed under a dynamic light scattering (DLS). After a concentration of 200 times, the DLS showed a relaxation of the light intensity over time, indicating the presence of colloidal materials. They didn't obtain an accurate size distribution, due to sample dilution, but the analysis reports the presence

of particles between 1 and 1000 nm. In order to obtain a chemical fingerprint of colloidal samples and confirm their plastic nature, they performed a pyrolysis coupled to gas chromatography–mass spectrometry. PE, PS, PVC and PET have been detected in the sample. There are no other studies demonstrating the occurrence of NPs in marine samples, by so far.

3. Biological effects in aquatic organisms

Given the lack of protocols of extraction and detection of nano-plastics in wild organisms, the only possible effects of NP interaction with biological systems are confined to laboratory contexts. Due to their small size < 1 μm, lower than the average dimension of vegetal and animal's cellular mean diameter (10–30 μm), they are potentially able to cross the contact surfaces (gills, gastrointestinal tract, cellular wall) translocate to inner organs and directly interact at a cellular level (Rossi et al., 2014; Forte et al., 2016). Similar to microplastics, PS nanoparticles can exert an intrinsic toxicity or act as vectors for other pollutants (Ma et al., 2016; Chen et al., 2017a, 2017b; Chen et al., 2017a; Cui et al., 2017; Barría et al., 2019; Shen et al., 2019a; Shen et al., 2019b). Further, they can interact with organisms at the base of trophic chain and be transferred to top consumers (Cedervall et al., 2012; Chae et al., 2018). More than half of the experiments (54.8%) deployed invertebrates followed by fish (16.9%), algae (12.7%), microbes (9.4%), cell lines (5.2%) and rat (only one paper) (Fig. 2). Within of the huge, and biologically speaking extremely different category of invertebrate, crustaceans have been tested in almost half of the cases (43.1%) followed by worms (23.5%), molluscs (21.6%), rotifers (7.8%), and sea urchin (3.9%).

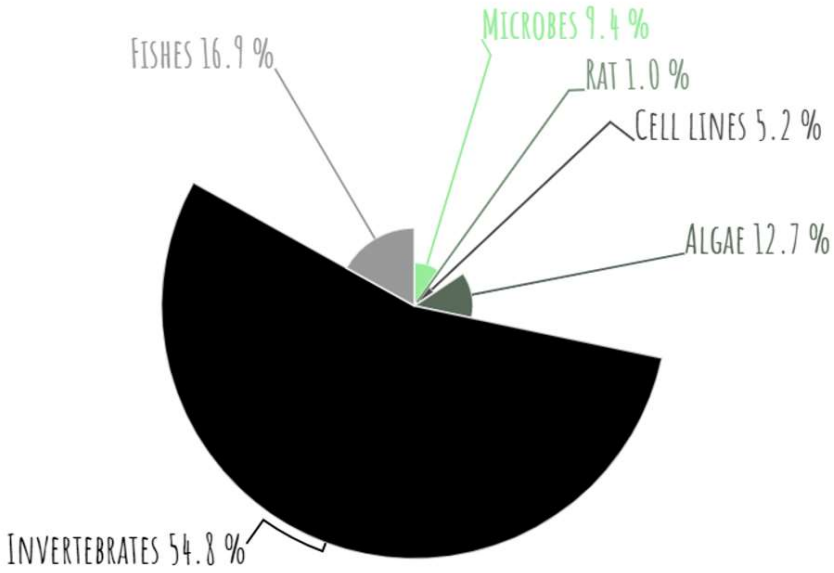


Fig. 2. Percentage of biological categories used in eco-toxicological studies with nanoplastics. Invertebrates (divided in crustaceans, molluscs, worms, rotifers and sea urchins) are deployed in more than half of the cases.

3.1. Toxicity to microbes

Some studies highlighted negative effects (especially on gut microbiome biodiversity) but others did not exhibit any observable toxicity. Among the first, particles from 50 to 100 nm induced significant impacts on gut microbiome of soil oligochaete *Enchytraeus crypticus* after a 7-day exposure of PS nanoplastics leading to a reduction in the relative abundance of *Rhizobiaceae, Xanthobacteraceae* and *Isosphaeraceae* families (Zhu et al., 2018). However, the majority of the effects were found in the highest exposure rate of 10% nano-polystyrene, which is roughly 250–10,000 times bigger than the estimated environmental concentration (as suggested by the authors themselves). The disrupted microbiome in *E. crypticus* is also reported by Ma et al. (2020) who, in addition, investigated the combined effect of NPs and tetracycline on antibiotics resistance genes (ARGs). ARGs increased after 14 days of exposure and their diversity and abundance was not completely restored after a period of recovery. Changes in the gut microbiota and contents of amino acids and fatty acids in the shrimp *Litopenaeus vannamei* were reported by Chae et al. (2019) suggesting possible consequences on nutritional values of the shrimp. The single and combined effects of amino polystyrene NPs (70 nm; 0.2 mg/L) and perfluorooctane sulfonate (PFOS: 0.1, 1.0, and 5.0 mg/L) on metabolism of thermophilic bacteria has been investigated by Chen et al. (2020). NPs alone resulted in

a 53.9% reduction in hydrogen production, however, the combined exposure revealed an antagonistic effect (31.6%). One of a kind, PS-NH2 at 5.0 mg/L had no effect on the growth of *Microcystis aeruginosa* but combined with glyphosate showed antagonistic effects significantly alleviating the inhibitory effect of the herbicide. Sun et al. (2018) studied the toxic effects of 50-nm PS-NPs on the marine bacterium *Halomonas alkaliphila*. The results showed growth inhibition and differences on chemical composition and ammonia conversion efficiencies at concentration of 80 mg/L. Moreover, oxidative stress and increased extracellular polymeric substances were detected. Finally, *Vibrio fischeri* did not exhibit any observable toxicity to 26 or 100 nm dialysed-PS (< 100 mg/L) or poly-methyl-methacrylate (PMMA) nanoparticles (86–125 nm; ≤1000 mg/L) (Booth et al., 2016; Heinlaan et al., 2020).

3.2. Toxicity to algae

How NPs can interact and affect algae has been the object of some studies. Generally, toxicity seems to be influenced by a variety of factors such as the surface charge of the particles, the medium hardness and the particle concentration (Bergami et al., 2017; Nolte et al., 2017), the presence of natural organic polymer (Liu et al., 2019a), the size of the particles (Sjollema et al., 2016) and the algal life stages (González-Fernández et al., 2019). Chae et al. (2018) studied the trophic transfer and individual impact of 51 nm PS NPs in a four-species freshwater food chain included the alga *Chlamydomonas reinhardtii*. After an exposure to a maximum 100 mg/L, they reported little or no mortality although the confocal laser microscopy showed the attachment to the surface of zoospores and the penetration of the outer layer during cell division. Other experiments reported the adsorption of NPs on microalgae (Bergami et al., 2017; Nolte et al., 2017). In some case algal growth was reduced (Besseling et al., 2014; Sjollema et al., 2016; Bergami et al., 2017; González- Fernández et al., 2019; Sendra et al., 2019b), in others it was not affected (Venâncio et al., 2019; Baudrimont et al., 2020; Heinlaan et al., 2020). The decrease of photosynthesis activity of *Chlorella* and *Scenedesmus*, probably caused by the light shading effect of the adsorbed PS particles (20 nm) and/or by obstructed CO2 gas flow and nutrient uptake pathways has been reported by Bhattacharya et al. (2010). ROS production, morphological alterations, decreases in chlorophyll content and esterase activity, DNA damage and depolarization of mitochondrial and cell membrane are some

of the impacts reported by freshwater and marine algae (Bellingeri et al., 2019; González-Fernández et al., 2019; Sendra et al., 2019a, 2019b).

3.3. Toxicity to invertebrates

Rotifers are major components of zooplankton in freshwater and coastal marine ecosystems throughout the world and could be useful indicator species. A handful of studies have been conducted on rotifers. For example, Jeong et al. (2016) evaluated the accumulation and adverse effects of PS micro- and nanoplastic (6 μm, 500 nm, 50 nm) in the rotifer *Brachionus koreanus*. Using different concentration (0.1-1-10- 20 mg/L), all sizes led to significant size- dependent effects, including reduced growth rate, reduced fecundity, decreased lifespan and longer reproduction time. They reported also significant differences in anti-oxidant enzymes such as SOD, GR, GPX and GST. Manfra et al. (2017) reported an increase of mortality rate of *Brachionus plicatilis* after 24 h and 48 h of exposure to cationic (−NH2) PS. Significant differences were detected at concentrations upper than 5 mg/L. Also PMMA-NPs were able to induce mortality in rotifers at concentrations higher than 4.69 mg/L with an estimated 48 h median lethal concentration of 13.27 mg/L (Venâncio et al., 2019).

Della Torre et al. (2014) investigated the disposition and toxicity of two surface modified polystyrene nanoparticles (-COOH and -NH2, 40 and 50 nm respectively) in early development of sea urchin embryos (*Paracentrotus lividus*). PS-COOH accumulated inside embryo's digestive tract but no embryotoxicity was observed up to 50 mg/L. PS-NH2 were more dispersed and caused severe developmental defects in addition to induce *cas8* gene at 24 h post fertilization (at the concentration of 3 mg/L), suggesting an apoptotic pathway. A significant concentration and time-dependent decrease in lysosomal membrane stability and apoptotic-like nuclear alterations were observed in phagocytes of sea urchin upon exposure to 50 nm PS-NH2 at concentration of 10 and 25 mg/L, although the multi-xenobiotic resistance phenotypes were not altered (Marques-Santos et al., 2018).

Copepods are widely used as model species in eco-toxicity and nano-toxicity test too (Baun et al., 2008; Jarvis et al., 2013; Bergami et al., 2016; Luo et al., 2018). A size-dependent effect of micro- and nano-polystyrene particles in the marine copepod *Tigriopus japonicas* has been investigated by Lee et al. (2013). The study reported some effects on survival and development of first (F0) and second (F1) generation, after

administration of 50 nm PS particles at concentration higher than 1 mg/L. Further, the 500 nm PS treated individuals, reported a decrease in fecundity, which was not recorded for the smaller 50 nm particles. Several studies demonstrated the accumulation of NPs in different organs and developmental stages of *Daphnia spp*. (Brun et al., 2017; Cui et al., 2017; Rist et al., 2017; Liu et al., 2018; Liu et al., 2019b; Saavedra et al., 2019; Liu et al., 2020;). A wide range of effects have been recorded: the decrease in survival, reproduction and body size (Besseling et al., 2014; Cui et al., 2017), abnormal development, low hatching rate and differences in levels of genes encoding key stress defence enzymes (Liu et al., 2018; Lin et al., 2019a; Liu et al., 2020) as well as induction of the heat shock proteins HSP70 and HSP90 (75 nm; ≤1 mg/L) (Liu et al., 2019a, 2019b, 2019c). Accumulation but no mortality and toxicity effects on *Daphnia magna* have been reported in a trophic transfer experiment (60 nm) (Chae et al., 2018) as well as in experiment performed with PMMA nanoparticles (86–125 nm; ≤1000 mg/L) (Booth et al., 2016) or with dialyzed PS-NPs (26 and 100 nm; ≤100 mg/L) (Heinlaan et al., 2020). Finally, during a 14-days experiment, the presence of 50 nm NPs significantly enhanced the bioaccumulation of phenanthrene-derived residues in daphnia body (NPs: 5 mg/L; Phe: 0.1 mg/L) (Ma et al., 2016). *Artemia spp.* has been also deployed in eco-toxicological studies highlighting the ability to bioaccumulate NPs and several sub-lethal effects (Bergami et al., 2016; Bergami et al., 2017; Mishra et al. 2019; Sendra et al., 2020).

Bivalve molluscs are abundant from freshwater to marine ecosystems, where they are extensively used in biomonitoring programs but also in studies of nanoparticles toxicity (Canesi et al., 2012; Rocha et al., 2015). Among the first studies focused on possible effects of NPs on feeding behaviour, Wegner et al. (2012) recorded an increase in the total weight of the *faeces* and *pseudo-faeces* in specimens of *Mytilus edulis* exposed to 30 nm PS NPs and a reduction in their filtering activity quantified as valve opening. However, it is necessary to note that the exposure concentrations were very high: 0.1–0.2-0.3 g/L. More recently, the increase in faecal production after exposure to polyethylene nanoparticles on specimens of *Corbicula fluminea* has been demonstrated by Baudrimont et al., 2020 using concentrations more environmentally relevant, namely 1000 µg/L. A handful of studies highlight the variability of ingestion and egestion rates of micro and nanoplastic particles describing a mechanism that is dependent on multiple variables such as developmental stage, particle size and surface properties of the particles (Cole and Galloway, 2015; Al-Sid-Cheikh et al., 2018; Rist et al., 2019). Decrease in fertilization success and in embryo-larval development (50, 500 nm, 2 µm; 0.1, 1, 10 and

25 mg/ mL) (Tallec et al., 2018), significant alterations in the expression of genes associated with biotransformation, DNA repair, cell stress-response and innate immunity (110 nm; ≤50 mg/L) (Brandts et al., 2018a), consequences on haemocytes in terms of motility, apoptosis, ROS and phagocytic capacity (Canesi et al., 2015; Canesi et al., 2016; Sendra et al., 2019a) are some of the impacts induced by NPs with different surface functionalization (NH2, COOH). The presence of amino- or carboxylic functional groups on the surface of the particles greatly affects their behaviour, stability and potentially their toxicity. In fact, different responses were recorded by González-Fernández et al. (2018) who tested the toxicity of 100 nm PS-COOH and PS-NH2 on oyster *Crassostrea gigas* gametes. A significant increase of ROS production was observed in sperm cells after 1 h of exposure at concentration of 1, 10 and 100 mg/L of PS-COOH. On the contrary, spermatozoa exposed to PS-NH2 were not significantly affected and no significant differences were observed in the percentage of motile spermatozoa or movement linearity after exposure.

In the last two years, several research groups put the attention on the possible interaction and toxicity of NPs on soil worms (Dong et al., 2018; Qu et al., 2018; Zhu et al., 2018; Kim et al., 2019; Qu et al., 2019; Shao et al., 2019; Kim et al., 2020; Qiu et al., 2020; Yang et al., 2020), however, how marine worms could be affected by this new form of contamination remain poorly clarified. Bioaccumulation study of NPs and polycyclic aromatic hydrocarbons (PAHs) in the clamworm *Perinereis aibuhitensis* has been conducted by Jiang et al. (2019), who indicated a NP-adsorbed pyrene of < 1% of the total pyrene accumulation in the clamworm body when the concentration of NPs in seawater was as low as 0.4 mg/L. Cholinesterase activity and burrowing are endpoints considerable impacted on Polychaeta *Hediste diversicolor* which, on the contrary, did not exhibit differences in most of the parameters associated with oxidative stress (100 nm; 0, 0.005, 0.05, 0.5, 5, 50 mg/L) (Silva et al., 2020).

3.4. Toxicity to fish

Laboratory experiments demonstrate the trophic transfer of polystyrene NPs from primary producers to fishes which, in turn, highlighted effects. In particular, Cedervall et al. (2012) reported weight loss, differences in triglycerides/cholesterol ratio in blood serum and differences in the distribution of cholesterol between muscle and liver of *Carassius carassius*. Moreover, the time it took the fish to consume 95% of food was

more than doubled, indicating behavioural disorders in fishes exposed to 24 nm polystyrene nanoparticles. *Oryzias sinensis* and *Zacco temminckii* after a 7-days trophic transfer exposure to 51 nm PS, reported several abnormalities with differences in liver histopathology, slight increase in the total amount of cholesterol in the blood serum and locomotor deficits (Chae et al., 2018). Kashiwada (2006) reported the accumulation of 39 nm PS particles in gills, brain, testis, liver, blood, and intestine of *Oryzias latipes* when exposed at a concentration up to 10 mg/L. In vitro experiment on fathead minnow (*Pimephales promelas*), demonstrate significant degranulation increase and induction of neutrophil extracellular trap release at a dose of 0.1 g/ LPS (Greven et al., 2016). Evidence of immune system of fish compromised by exposure to 45 nm PMMA NPs (≤ 20 mg/L) are reported by Brandts et al., 2018b who also evidenced the increase of m-RNA transcripts related to lipid metabolism, *pparα* and *pparγ*. Behavioural changes have been detected in *Sebastes schlegelii* specimens exposed to 0.5 and 15 μm PS particles (190 μg/L), which reported cluster, reduction of swimming speed, but also increasing in oxygen consumption and ammonia excretion and lower protein and lipid contents (Yin et al., 2019).

Danio rerio, better known as zebrafish, proved to be a good model organism in nanoparticle toxicity studies (Chakraborty et al., 2016). Nano PS distribution and accumulation has been studied at different developmental stages (Chen et al., 2017a, 2017b; Van Pomeren et al., 2017; Pitt et al., 2018a, 2018b) highlighting the ability to localize in different tissues (e.g. head, yolk sac, gastrointestinal tract, gall bladder, liver, pericardium, pancreas, etc.). Some effects reported are altered larval behaviour, significant inhibition of acetylcholinesterase (AChE) and genotoxic effects (50 nm; 1 mg/L) (Chen et al., 2017a, 2017b), but also decreased glucose level (25 nm; 0.2, 2, 20 mg/L) (Brun et al., 2019). Chen et al. (2017a, 2017b) reported an upregulation of *gfap* and *α1-tubulin*, nervous system related genes, after 5-days exposure at 1 mg/L 50 nm PS. Pitt et al. (2018a, 2018b) demonstrated the maternal transfer of 42 nm PS particles to offspring in zebrafish through dietary exposure (at a final NPs concentration of 10% of the food by mass). The co-parental exposure did not significantly affect the reproductive success but reduced glutathione reductase activity. They concluded that PS NPs could bioaccumulate and passed on to the offspring but does not cause major physiological disturbances. Finally, the combined effects of gold ions, PAH and PS NPs was investigated by Lee et al. (2018) and Trevisan et al. (2019). In the first case, PS NPs alone (50, 200, and 500 nm; 0.1 mg/ml) induced only marginal effects on survival, hatching

rate, developmental abnormalities and cell death of zebrafish embryos, but exerted a synergistic effect on gold toxicity. The second experiment reports opposite results: NPs adsorbed contaminants but potentially decrease their uptake due to particle agglomeration (PS:44 nm; 0.1, 1, 10 ppm; PAHs: 5.07 to 253.65 ng/ml).

4. What's been done and future studies

Fig. 3 summarizes the main field of interest on nanoplastic issue, suggesting which ones have been more done and what remain to be investigated. Of 224 papers, 40.4% of research efforts regarded eco-toxicological studies as reviewed above, followed by reviews often including considerations on microplastics (15.3%) and papers on methods of detection (8.9%). The possible role of NPs as vector of contaminants has been examined by 9.4% of cases. Studies on NPs behaviour, degradation, fate and transport represents only a small slice (5.1%, 2.6% and 2.6%, respectively). Human risk related publications represent 6.4% of the total (Table 3, Suppl. Materials). Undoubtedly, the possible implications of nanoplastic contamination in terrestrial and freshwater ecosystems are understudied (5.5%), however, this percentage does not take into account the toxicological studies performed on zebrafish, a freshwater species used in 11 out of 18 cases when fishes are tested (Table 1, Suppl. materials), Daphnia spp. (17 papers), soil worms (10 papers) and freshwater microalgae/cyanobacterium (7 papers). Terrestrial/freshwater related studies investigated, for example, the transport of polystyrene NPs in natural soils, their formation in agricultural ecosystems, the inhibition of activated sludge by PS NPs, but also review and toxicological effects in freshwater dipteran or plants (Table 4, Suppl., materials). Maybe due to practical difficulties, trophic transfer of NPs has been examined only by Chae et al. (2018) in a four-species freshwater food chain and by Cedervall et al. (2012) reproducing a food chain transport *Scenedesmus sp.- Daphnia magna - Danio rerio*. Taking into account the results obtained in laboratory experiments on different organisms, the framework on NPs toxicity seems to be alarming. However, it is proper to highlight how the laboratory context represents only an oversimplification of a phenomenon, in many ways, unknown and complex. In the following paragraphs we aim to pointing out some aspects which are retained crucial when an eco-toxicological study with NPs is performed and which elements of NPs toxicity could be deeper covered.

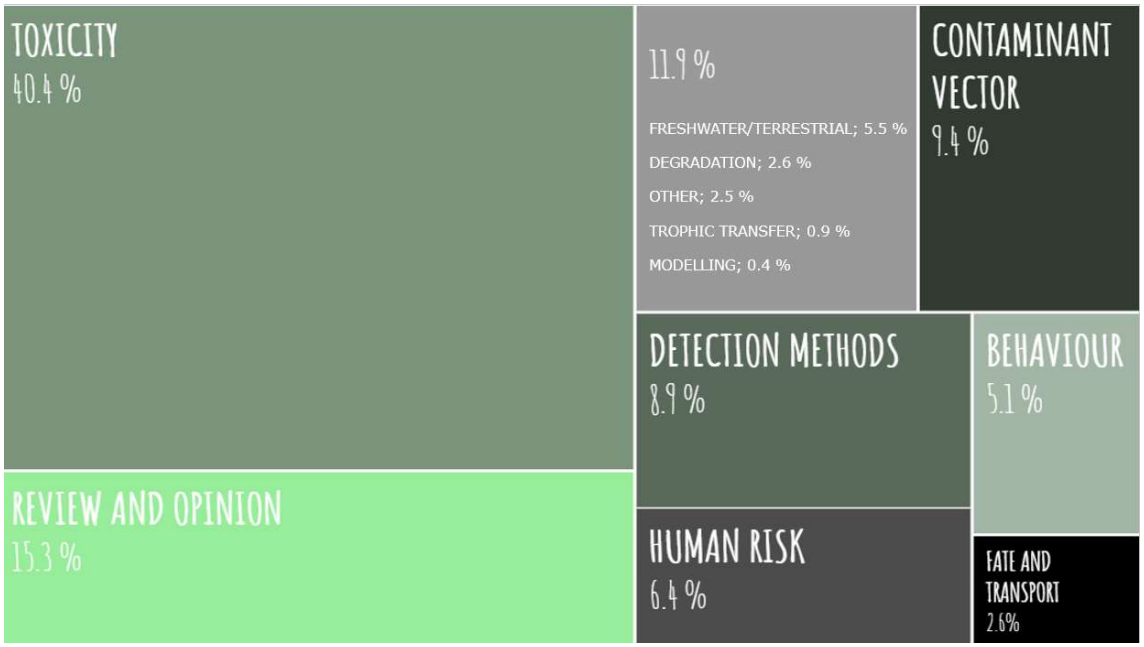


Fig. 3. Paper published on "nanoplastics" divided for main categories. "Other" includes studies on nanomaterials, protein interaction and leaching of fluorescent dye.

4.1. Concentration

Since the first record of Ter Halle et al. (2017) which reported the occurrence of NPs in marine samples from the North Atlantic Sea, the detection and quantification of the nano-fraction remains a challenge. To date, twenty papers proposed different methods for the analysis of sub-micrometric particles in the environment (Table 2, Suppl. materials). However, its concentration and distribution are still unknown and, accordingly, it is difficult to size the phenomenon spotting the time and space scales. For these reasons, the concentrations used in laboratory studies are based on evidence on microplastics concentrations. It remains to clarify whether they are overlapping and if the relative polymers abundance is the same. Further, as reported above, many studies used rather high concentrations, sometimes up to g/L. However, the use of such high concentrations in dose-response experiments could be justified because it provides important insights on toxicity thresholds (Paul-Pont et al., 2018). Always in the context of laboratories studies, some possibilities have been recently proposed for the tracking of very low concentrations (< 15 μg/L) of nanoparticles in order to assess their bioaccumulation in aquatic organisms and/or to investigate their fate and behaviour in complex matrices. In the first case Al-Sid-Cheikh et al. (2018) used 14C-radiolabelled nano-polystyrene for studying the

uptake, distribution and depuration of 24 nm and 250 nm NPs in *Pecten maximus*. In the second case, a metal tracer made of palladium was added to a polyacrylonitrile (PAN) core ad spiked into sludge (Mitrano et al., 2019).

Further studies are needed to better understand these aspects.

4.2. Reference materials

Most studies used polystyrene as reference materials, mainly because it is quite cheap and the only available in the market. Only 7 papers (out of 224) are based on polymers different by polystyrene. In detail: regarding polyethylene nanoplastic Paço et al. (2017) focused the attention on the ability of marine fungus *Zalerion maritimum* to biodegrade PE microplastics; Baudrimont et al., 2020 explored the eco-toxicity of PE nanoplastic on different marine organisms; Panizon et al. (2015) developed coarse-integrated models of polyethylene and polypropylene aimed at the study of the interaction with lipid membrane. Regarding on PMMA nanoparticles, Booth et al. (2016); Brandts et al. (2018a, 2018b) and Venâncio et al. (2019) tested the toxicity on different organism (algae, rotifer and fish). Finally, Magrì et al. (2018) explored the possible toxic effects of 100 nm PET NPs by in vitro studies on human Caco-2 intestinal epithelial cells.

If polystyrene can be considered the dominant polymer, the same applies to the bead shape. Gigault et al. (2018) state that secondary NPs completely differ from the manufactured polystyrene nanomaterials used as reference material in eco-toxicological researches. Nanomaterials are characterized by a homogeneity of size (< 100 nm), type of polymer, physical/chemical surface properties and often are sold in dispersion spiked with surfactants (e.g. Sodium dodecyl sulfate, SDS) and antimicrobials that affect their water behaviour. On the opposite, secondary NPs are supposed to be very motley as the result of the degradation process (Potthoff et al., 2017). NPs, similar to microplastics, in natural soils and water are supposed to undergo various transformation as result of interaction with inorganic and organic factors present in natural water (e.g. differences in density, surface properties, charge, particles size distribution, particle shape distribution) (Potthoff et al., 2017). This heterogeneity plays a crucial role within nanoparticles behaviour and can affect the fate once in the environment.

Attempts on PS- alternative nanoparticles production only recently begin to appear. Magrì et al. (2018) applied a top-down approach which allows obtaining PET nano-sized particles starting from bulk scale materials. In detail, laser ablation of commercial PET

film was performed and allowed to mimic real environmental NPs (e.g. size and shape heterogeneity, weak acid group). Nanoparticles of polymethylmethacrylate (PMMA) prepared by microemulsion polymerization of styrene with sodium dodecyl sulfonate have been also tested on *Mytilus galloprovincialis* and *Dicentrarchus labrax* (Brandts et al., 2018a; Brandts et al., 2018b). Baudrimont et al., 2020 produced PE nanoparticles through the ultra-sonication of a solution consisting of PE reference powder and Milli-Q water for 1 h with pulses of 5 μs in an ice bath. After sonication, the solution was filtered at 0.45 μM and then evaluated with total organic carbon measurements and trace amounts of titanium by ICP-MS.

The development of new techniques for the nanoplastic production is needed to expand the knowledge on other polymers (e.g. PE, PP) and shapes (e.g. fragments) in order to make experiments more realistic. Meanwhile, the use of PS beads can help to investigate some aspects believed to be possible drivers in nanoparticle behaviour in water matrices. Anyway, a primary (focused on the particle itself) and secondary (on its interactions once in the medium) characterization of NP, before the eco-toxicological experiment is strongly required (Fabrega et al., 2011; Bergami et al., 2016; Lambert et al., 2017). Electron microscopy, Dynamic Light Scattering (DLS) or Nanoparticle Tracking Analysis (NTA) are essential tools for achieving these objectives (Ter Halle et al., 2017).

4.3. Interactions with surrounding medium, ageing effect and vector of contaminants

Due to their small size, nanoparticles have a larger surface area relative to mass, and this confers them an enhanced reactivity (Baun et al., 2008). In aquatic environment NPs can form hetero-aggregates (e.g. with suspended particulate matter) or homo-aggregates with particles of the same type (Alimi et al., 2017). Aggregation, for example driven by surface charge, can convert the nano-sized particles to micro-sized (Manfra et al., 2017). NPs may also interact with dissolved organic and inorganic colloids to form stable and unstable aggregates (Cai et al., 2018; Gigault et al., 2018). The marine environment has a wide variety of colloids and natural organic matter (Klaine and Alvarez, 2008). Summers et al. (2018), after an incubation study, revealed the importance of marine bacterial glycoprotein (EPS) on the formation and abundance of plastic agglomerates. Aggregation determines the fate, persistence, mobility and bioavailability of NPs (Alimi et al., 2017). How the bioavailability can change has been investigated by Ward and Kach

(2009) who studied the ingestion and egestion dynamics in two bivalve molluscs (namely, *Mytilus edulis* and *Crassostrea virginica*) after an exposition to 100-nm PS NPs. The plastics were delivered either dispersed or embedded within aggregates generated in the laboratory. Results show that aggregates significantly enhanced the uptake of nanoparticles. In this case, incorporation of NPs into aggregated material increased the bioavailability to suspended-feeding molluscs.

Little interest has been placed on the ageing effect to the binding of NPs with contaminants. For example, Liu et al., 2019c demonstrated how the surface oxidation of PS NPs induced by UV/ozone ageing drastically affected the mobility and contaminant-mobilizing ability. Further, NPs demonstrated the ability to bind different types of contaminants from naphthalene, fullerene, PCBs, PAHs, metals, POPs, glyphosate, perfluorooctane sulfonate to silver (Lin et al., 2011; Velzeboer et al., 2014; Liu et al., 2016; Jeong et al., 2018; Town et al., 2018; Zhang et al., 2018; Lin et al., 2019b; Wan et al., 2019; Chen et al., 2020; Hu et al., 2020; Ma et al., 2020). Finally, one of a kind, the possible superimposed effects of NPs and an antibiotic (i.e. tetracycline) on the microbiome of *Enchytraeus crypticus* have been investigated by Ma et al. (2020). In particular, the exposure significantly perturbed the abundance of some bacterial families and the microbiome was reversibly impacted.

In the light of above, it is recommended to recreate a more realistic exposure medium in order to not oversimplify the possible interactions and conduct always a secondary characterization (e.g. by DLS and/or electronic microscope).

5. Conclusions

The greater attention on nanoplastic pollution is a physiological convergence between the growing awareness on microplastic pollution and the open debate on nanomaterials toxicity. To anyone considering entering this field to embrace a blended approach between chemistry, engineering, ecotoxicology, and physics is required. This review aimed to critically analyse the body of literature in order to point out lights and shades of the phenomenon. The first reason of reflection regards the definition of NPs. Gigault et al. (2018) prefer to consider NPs as: “particles within a size ranging from 1 to 1000 nm resulting from the degradation of industrial plastic objects and can exhibit a colloidal behaviour”. Others are more stringent to the definition taken from the nanomaterials. The latter definition leaves a link with the nanomaterials and allows the use of knowledges of

decades and decades of studies in these field, but the former is maybe more consistent with the natural phenomenon. The second huge gap is represented by the quantification of the phenomenon. The occurrence of particles lower than 1 μm in marine samples suggests how 60–70 years from the boom in plastic production were sufficient to transform bigger items in colloidal (< 1 μm) particles. It still remains unclear how many NPs are in the ocean and what are the rates of degradation. Koelmans et al. (2015) calculate the time scales required to reach the 100 nm scale as a function of initial plastic size. They estimated ca. 320 years needed to bring 1 mm microplastics to the 100 nm nanoscale, by joint photo-oxidation and biodegradation. In the ocean this estimation could be greater. The dilution effect, the technological limitations and the complexity of biological samples make the extraction from marine samples difficult. First attempts have been done and many others are needed.

Taking into account the results obtained in laboratory experiments on different organisms, the framework on NPs toxicity seems to be alarming. Reduction in the relative abundance of gut microbiome, reduced growth and fecundity, longer reproduction time and differences in antioxidant enzymes in rotifers, inducing of *cas8* gene in *Paracentrotus lividus*, effects on survival and development in copepods, algal growth inhibition; behavioural disorders, abnormalities in liver histopathology, degranulation increase, inhibition of acetylcholinesterase and genotoxic effects in fishes are all aspects already highlighted. However, how benthic or endo-benthic organisms (such as marine worms) could be affected by this new form of contamination remain poorly investigated. Results obtained are all related to PS or PMMA NPs an aspect which pose a debate regarding how they are a good reference material and how many important are the system conditions on bioavailability of NPs and their consequent toxicity.

NPs formation, fate and toxicity seem to be a very dynamic phenomenon. It is not possible to reproduce and control all the variables in a laboratory context but even an oversimplification is counterproductive. It is therefore advisable to recreate a more realistic exposure medium in order to not oversimplify the possible interactions and conduct always a secondary characterization.

Our paper proposes a recent update in a rapidly evolving area of scientific knowledge. Linking laboratory results to real effects, estimating natural levels is essential. To date the problem of the impact of NPs in the marine environment has been assessed on the basis of laboratory exposure studies. The question relating levels of NPs in the natural aquatic environment must be explored with techniques that allow reliable and

reproducible real levels to avoid to under- or overestimate the problem. The currently available methodological approaches need to be improved to allow for accurate and reliable determinations of nanoparticles in abiotic and biotic matrices. Nanoplastic pollution hides lights and shadows and it is difficult to take a position at the moment about the risk they pose to the biodiversity and functioning of natural ecosystems. A precautionary principle is appropriate, but this must be accompanied by solid and clear scientific evidence. At this moment they are missing, and it is not even clear how they can be obtained in a short time.

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In preparation to be submitted

Viral-like toxicity of polystyrene nanoplastics on coral reef fish juveniles

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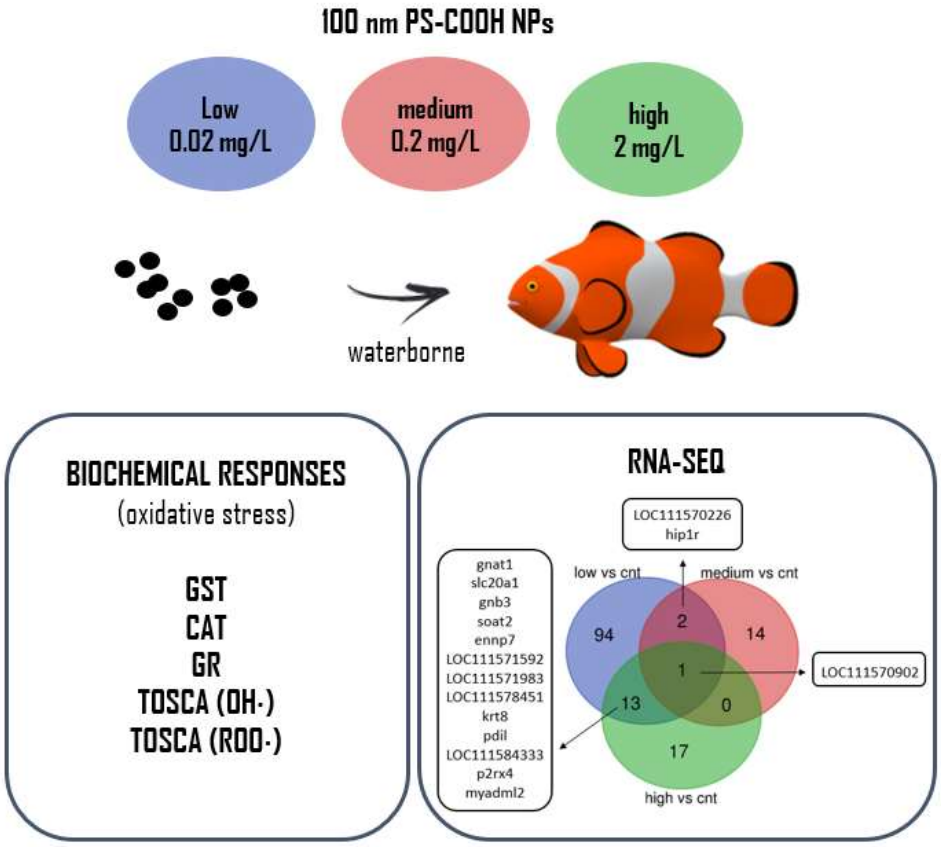
Graphical abstract



Keywords: nanoplastics; clownfish; virus-like toxicity; IBRv2 index; transcriptomics

Abstract

This study aims to extend the knowledge about the NP toxicity in marine fish performing the first eco-toxicological assessment of 100 nm PS-COOH NPs in juveniles of *Amphiprion ocellaris.* Clownfish were tested in a 7-days waterborne exposure at 3 concentrations (low = 0.02, medium = 0.2; high = 2 mg/L of daily exposure), adopting a multidisciplinary approach including biochemical responses and gene expression analyses. Whereas the activity of single adaptive responses to oxidative stress were not significantly altered, the integrated analysis of such biomarkers (IBRv2 index) reported a reduction in overall health status of fishes exposed to NPs. Importantly, low concentration elicited higher toxicogenomic responses (110 DEGs; 27 enriched GOs) than medium and high concentrations, likely due to NP aggregations occurring in sea water. The genes showing higher change in transcriptional levels were factors involved in viral infection and inflammatory response, and to less extent in ciliogenesis, energy metabolism, olfactory and reproductive functions. In conclusion, this study detected a minor response in oxidative stress and contributed in understanding the cascade of molecular events that are activated by the exposure to PS-NPs in the clownfish *A. ocellaris* juveniles.

1. Introduction

The plastics industry is an ever-expanding sector with a global average production of > 300 million tons (Mt) per year (Plastic Europe 2019). Mainly due to inadequate waste management, up to 12 Mt of plastic enter the oceans every year (Jambeck et al. 2015) exposing worldwide ecosystems to risks whose nature and magnitude is far from being fully understood. Plastic pollution is now recognized as a global concern with dramatic socio-economic and ecological implications (Bergmann, Lars, and Klages 2015). If the situation remains unchanged in terms of high production and poor waste management, the cumulative amount of plastic waste available to enter the ocean is predicted to reach 250 Mt by 2025 (roughly 12,000 Mt of plastic litter will accumulate in landfills or in the natural environment by 2050; Geyer, Jambeck, and Law 2017).

Despite its resistance in the environment, plastic is subjected to chemical-physical and biological degradation (Gewert, Plassmann, and Macleod 2015; Potthoff et al. 2017) in a process which gradually reduces the size down to the formation of so called microplastics (MPs, < 5 mm) and nanoplastics (NPs, < 1 μm or < 100 nm). Respect to microplastics, the nano-fraction of the marine plastic litter is at the center of a new field of investigation.

While there is still disagreement about the size range (Cole and Galloway 2015; Pinto da Costa et al. 2016; Alimi et al. 2017; Gigault et al. 2018), NPs are considered those particles less than one micron in diameter. Similar to MPs, NPs can be divided into primary (particles manufactured in such dimensions) and secondary (originated from degradation). Personal care products, industrial abrasives, 3D printing, paints and particles used in drug delivery can be considered primary NPs (Guterres, Alves, and Pohlmann 2007; Alimi et al. 2017; Azimi et al. 2016; Hernandez, Yousefi, and Tufenkji 2017). However, NP concentration and distribution in the environment are still strongly elusive. After the first report on the occurrence of polystyrene enriched NPs (1-999 nm) in environmental samples collected in the North Atlantic Subtropical Gyre (Ter Halle et al. 2017), NP presence has been only very recently reported in sand water extracts (Davranche et al. 2020) and soil (polyethylene, polystyrene and polyvinyl chloride; Wahl et al. 2021). Polystyrene (PS) is one of the most produced polymers worldwide, being the 6th most demanded plastic in Europe in 2018 and having a similar position on the global demand of plastic in 2015 (Morten W. Ryberg, Alexis Laurent 2018; Plastic Europe 2019). The global production capacity of PS is expected to remain stable in a five-year projection, from 15.5 million metric tons in 2018, to a slight increase by 2023, reaching 15.6 million metric tons (Statista 2019). PS is mainly destined for the production of food packaging (e.g. egg tray), coffee cup, lid, eyeglass frames, building insulation etc. (PlasticsEurope 2017; Ho, Roberts, and Lucas 2018). Laboratory evidence shows that the formation of PS nanoparticles from bigger plastic items is plausible (Shim et al. 2014; Gigault et al. 2016; Lambert and Wagner 2016; Song et al. 2017). In fact, once released into the environment, PS is subjected to degradation processes such as ultraviolet radiation that may cause photo-oxidative degradation of the polymer with abundant surface O-functional groups, varying in epoxy/hydroxyl, carbonyl, and carboxyl groups (Liu et al. 2019). In the context of this study, carboxyl-modified NPs were used to address ageing due to weathering.

Currently, the only possible effects of NP interaction with biological systems are confined in a laboratory context, using concentrations known for MPs and, in most of cases employing PS beads. Although it is unclear whether PS beads represent a good reference model, they are the preferred choice on the public market. For this reason, over the years, they have been widely used in eco-toxicological studies on different marine organisms. Consequently, a consistent body of literature allows to make comparisons and to acquire knowledge that is potentially extendable to more environmentally realistic

particles (Lenz et al. 2016; Piccardo, Renzi, and Terlizzi 2020). PS nanoparticles (PS-NPs) can exert an intrinsic toxicity or act as vectors for other contaminants (Ma et al. 2016; Chen et al. 2017; Chen, Gundlach, et al. 2017; Cui, Kim, and An 2017). Currently, knowledge on PS-NP bioaccumulation and biological impact is most concerned with freshwater fish, in which a host of PS-NP effects have been reported. Laboratory experiments demonstrated the trophic transfer of PS-NPs from primary producers to fishes (Cedervall et al. 2012; Chae et al. 2018) and their ability to localize in different tissues during embryo development (Van Pomeren et al. 2017; Chen, Yin, et al. 2017; Pitt, Kozal, et al. 2018). Exposure to NPs causes weight loss, changes in triglycerides/cholesterol ratio and levels in muscle and liver, behavioral disorders in C*arassius carassius*, (Cedervall et al 2012); liver histopathology, cholesterol increase in the blood serum, locomotor deficits in *Oryzias sinensis* and *Zacco temminckii* (Chae et al. 2018); behavioral alterations, inhibition of acetylcholinesterase, genotoxic effects, upregulation of nervous system related genes *(gfap* and *α1-tubulin*) in zebrafish larvae (Chen, Gundlach, et al. 2017; Q. Chen, Yin, et al. 2017) .

Zebrafish is undoubtedly the most studied fish in NP exposure research, with 18 published papers (Bhagat et al. 2020). As far as the marine world is concerned, NP studies have been conducted on *Dicentrarchus labrax* (Brandts et al. 2018), *Sparus aurata* (Brandts et al. 2021) and *Sebastes schlegelii* (Yin et al. 2019). In our work, we extended the knowledge on the impact of NPs on marine fishes by focusing on *Amphiprion ocellaris* juveniles, who have been recently proved to be able to ingest MPs in laboratory exposure (Nanninga, Scott, and Manica 2020). *A. ocellaris* belongs to clownfishes (or anemonefishes; subfamily *Amphiprioninae*, genera *Amphiprion* and *Premnas*), an iconic group of coral reef fishes that include 28 species (Ollerton et al. 2007). Their distribution spans the whole tropical belt of the Indo-West Pacific Ocean, but their highest species richness is situated in the Coral Triangle region (Elliott and Mariscal 2001). We tested specimens in the juvenile phase since early life stages of fishes are known to be exceptionally vulnerable to contaminants due to their small size and less developed immune system (Weis and Weis 1989). The juvenile period in clownfish corresponds to the shift from partially pelagic to epibenthic life phases, from 15 to 17 day post hatching (dph) until settlement in the sea anemones on 17^{th} to 20^{th} dph (Madhu, Madhu, and Retheesh 2012). Plastic litter has been found in all marine and coastal compartments from pole to tropic (Bergmann, Lars, and Klages 2015; Lacerda et al. 2019; Reed et al. 2018; Garnier et al. 2019). Tropical reef systems, already subjected to different natural and

human activities, are not immune to plastic pollution as reported by Ding et al. (2019) who found MPs smaller than 330 µm in diameter in tropical fishes. NP contamination in tropical fishes is thus plausible although additional research effort and methodological improvements are necessary to confirm this hypothesis.

For all the above reasons, this study aims to perform the first eco-toxicological assessment of 100 nm PS-COOH NP effects in juveniles of *Amphiprion ocellaris* in a 7-days waterborne exposure at concentrations ranging from 0.02 to 2 mg/L (of daily exposure). We adopted a multidisciplinary approach including biochemical and gene expression analyses. Biochemical responses related to antioxidant defenses were evaluated by measuring the activities of specially adapted enzymes (catalase, CAT; glutathione S-transferase, GST; glutathione reductase, GR) and TOSCA (Total Oxyradical Scavenging Capacity Assay) for quantifying ROS scavenging capacity. A simple method to summarize biomarker responses and simplify their interpretation was further adopted: the "Integrated Biological Responses version 2" index (IBRv2) (Sanchez, Burgeot, and Porcher 2013). Lower or higher index core values can be translated into the impact of PS NPs on organisms: higher index core values are indicative of a poorer health status (stressed organisms). We also used RNA sequencing (RNA-seq), the principal Next-generation sequencing (NGS) platform, for a transcriptome-wide analysis of differential gene expression (Chen, Shi, and Shi 2017; Hrdlickova, Toloue, and Tian 2017; Stark et al 2019) in order to link transcriptional changes to physiological conditions in response to NP exposure.

2. Materials and methods

2.1 Nanoplastics

Carboxyl-modified polystyrene nanoparticles beads (PS-COOH NPs) with 100 nm in size and internally fluorescent labelled (ρ = 1.05 g/ml; green: 441 nm excitation, 486 nm emission) were purchased from Polysciences Inc. (Eppelheim, Germany). Seller declare the presence of residual surfactant (SDS) in the storage buffer to ensure ease of handling. PS NPs were selected because of their wide use in nano-toxicological studies performed on marine organisms (Manfra et al. 2017; Nolte et al. 2017; Tallec et al. 2018); the carboxyl functional groups on the surface of the particles should simulate the age effect due to weathering making the particles more realistic (Lambert et al. 2017). Exposure consisted by vortexing the stock solution for 5 minutes and directly adding the proper

volume into each beaker to obtain the experimental concentrations of 0.02 mg/L (3.64 10^7 particles/ml), 0.2 mg/L (3.64 10^8 particles/ml) and 2 mg/L (3.64 10^9 particles/ml), following the procedure adopted by Auguste et al. (2020). Particle characterization was performed in MilliQ water and in testing medium (artificial sea water, ASW) by assessing Z-average and Polydispersity Index (PdI) by dynamic light scattering (DLS, Zetasizer Nano-ZS) at hour 0, 1 h and 24 h (time of water renewal during the exposure).

2.2 Animals and experimental design

Juveniles of *Amphiprion ocellaris* (8.83 ± 0.57 mm standard length, 27.65 ± 6.04 mg weight) were bred in the facility of the *Observatoire Oceanologique de Banyuls sur Mer*. The test was performed in 1 L glass jars. During the treatments, jars were held in a water bath for temperature control and a gentle aeration were supplied for ensuring both the proper oxygenation necessary for the fish welfare and to maintain NP mixing. Photoperiod was maintained at 13/11 light/dark cycle, and temperature and nitrate (NO^-_2) levels were measured randomly once a day. Temperature was always kept at 26.3 ± 0.1 °C and NO^{-2} within acceptable ranges (< 0.5 mg/L). 24 h before the onset of the exposure, fishes were randomly distributed in the jar system for the acclimatization period. Because the environmentally relevant levels of NPs in the environment are unknown, the exposure concentrations were based on reported environmental concentrations of MPs (Koelmans, Besseling, and Shim 2015; Greven et al. 2016) and selected according to similar studies performed on aquatic organisms (Lenz, Enders, & Gissel, 2016; Pitt, Kozal, et al., 2018; Brun et al., 2019). The daily exposure consisted in four experimental groups: 0 mg/L (control), 0.02 mg/L (low), 0.2 mg/L (medium), 2 mg/L (high). Each experimental group consisted of 6 jars with 6 animals per jar, containing 0.5 L of ASW. Juveniles were exposed to PS-COOH NPs for 7 days and the test medium was renewed every 24 h (80%). Fishes were fed with dry pellet (in the morning) and *Artemia salina* nauplii (in the evening), *ab libitum*. Mortality was checked every day and dead animals removed. At the end of the experiment, termination count of number alive and dead was recorded. All the experimental procedures involving fish have been carried out in compliance with the national law on the protection of animals for scientific purposes.

2.3 Biochemical analysis

2.3.1 CAT, GST, GR and TOSCA

At the end of the exposure 24 juveniles (6 for treatments) were randomly sampled and euthanized by immersion in a tricaine methanesulfonate (MS-222) bath (200 mg/L). The whole juvenile was homogenized (1:10 w:v ratio) in 100 mM K-phosphate buffer (pH 7.5), 0.008 TIU/ml aprotinin, 1 mg/ml leupeptin, 1.8% NaCl, and centrifuged at 110,000 G for 1 h at 4 °C. Measurements of enzymatic activities were made with spectrophotometer at a constant temperature of 20 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of 12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0. Glutathione S-transferases (GST) were determined at 340 nm using 1.5 mM 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). Glutathione reductase (GR) was determined at 340 nm, from NADPH oxidation during the reduction of 1 mM GSSG (λ = 340 nm, $\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 µM NADPH. The TOSC assay measures the capability of cellular antioxidants to inhibit the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas in the presence of different forms of oxy-radicals, like peroxyl-radicals (ROO•) and hydroxyl- radicals (HO•) which are artificially generated at constant rate (Winston et al. 1998). Peroxyl-radicals (ROO•) were generated by the thermal homolysis of 20 mM 2-2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl- radicals (•OH) were produced by the Fenton reaction of iron-EDTA (1.8 µM Fe3+, 3.6 µM EDTA) plus ascorbate (180 µM) in 100 mM K-phosphate buffer. Under these conditions the different oxy-radicals produced quantitatively similar yields of ethylene in control reactions, thus allowing to compare the relative efficiency of cellular antioxidants toward a quantitatively similar radical flux (Regoli and Winston 1999). Ethylene formation in control and sample reactions was analysed at 10-12 min time intervals by gas-chromatographic analyses and the TOSC values are quantified from the equation: TOSC = 100 – ($\int$ SA/$\int$ CA x 100), where $\int$ SA and $\int$ CA are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay

and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

2.3.2 Integrated Biomarker Response version 2 index (IBRv2)

To integrate results from the different biomarkers, the Integrated Biomarker Response version 2 (IBRv2) index was calculated according to Sanchez, Burgeot, and Porcher (2013). The IBRv2 index is based on the reference deviation concept and it was selected because solves the two major weak points related to the previous index version (IBR) of Beliaeff and Burgeot (2002): (1) the strongly dependency on the arrangement of the biomarkers on the star plot, and (2) the only up- or downregulation of biomarkers. For the IBRv2 calculation, individual biomarker data (Xi) were compared to the mean control data (X0), and a log transformation was applied to reduce the variance:

$$Yi = \log(Xi/X0)$$

In a second step, the general mean (μ) and standard deviation (st. dev.) of Yi were computed as previously described by Beliaeff and Burgeot (2002), and Yi standardized:

$$Zi = (Yi-\mu)/\text{st. dev.}$$

To create a basal line centered on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Zi) and the mean of control biomarker data (Z0) were used to define the biomarker deviation index (A):

$$A = Zi-Z0$$

To obtain an integrated multi-biomarker response named IBRv2, the absolute value of A parameters calculated for each biomarker in each tested concentration were summed:

$$\text{IBRv2} = \sum |A|$$

For a single concentration, “A” parameters are reported in a star plot to represent the reference deviation of each investigated biomarker. The area up to 0 reflects biomarker induction, and the area down to 0 indicates biomarker inhibition. Lower or higher index core values can be translated into the impact of PS NPs on organisms: higher index core values are indicative of a poorer health status (stressed organisms).

2.4 Molecular analysis

2.4.1 RNA extraction

At the end of the exposure 24 juveniles (6 for treatment) were randomly sampled and euthanized with thermal shock, thus stored in TRIzol (Thermo Fisher Scientific, Waltham, MA) and put at -80 °C until the moment of the analyses. A phenol-chloroform based protocol was specifically drawn for the RNA extraction. Briefly, each juvenile was thawed, crushed in 1 ml TRIzol with Ultraturrax (Janke & Kunkel), centrifuged 15 min at 4 °C and 10,000 rpm. The supernatant was transferred in a new tube and left 5 min at room temperature (RT). Then, 200 µl/1 ml TRIzol of chloroform were added to homogenates, vortexed few seconds and left 3 min at RT. A subsequent centrifugation at 12,000 G for 20 min at 4 °C was run. The supernatant was transferred to a new tube and 500 µl/1ml TRIzol of isopropanol added. The sample was fast vortexed and left at RT for 10 min, then centrifuged at 12,000 G for 10 min at 4 °C. The supernatant was removed and the transparent pellet washed with 1 ml of ethanol 75%, vortexed and finally centrifuged at 7,500 G for 5 min at 4 °C. A second washing with 200 µl of ethanol 75% was performed in order to remove reagent and salts. The ethanol was carefully removed and sample left to dry for 20/30 min at RT under the lamp until complete alcohol evaporation. Then, the RNA samples were solubilized adding 30 µl RNAse-free H_2O, vortexed, spin and RNA quantity and quality checked by NanoDrop 2000 (Thermo Fischer Scientific, Waltham, MA) and by electrophoresis. The RNA quality was further checked by Agilent 2100 Bioanalyzer System.

2.4.2 RNA-seq and bioinformatics analysis

Four samples per treatment were destined for RNA-seq analysis. Next Generation Sequencing experiments were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). RNA concentration in each sample was assayed with a ND-1000 spectrophotometer (NanoDrop) and its quality assessed with the TapeStation 4200 (Agilent Technologies). Indexed libraries were prepared from 800 ng/ea purified RNA with TruSeq Stranded mRNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 (Agilent Technologies) and Qubit fluorometer (Invitrogen Co.), then pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled

samples of 2 nM. The pooled samples were subject to cluster generation and sequencing using an Illumina NextSeq 550 System (Illumina) in a 2x75 paired-end format at 1.8 pmol final concentration. The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Downstream analyses were performed using the CLC Genomics Workbench 20.0 software (CLC bio, Aarhus, Denmark). After the quality control of reads and the trimming procedure, reads were aligned to *A. ocellaris* genome (GCF_002776465.1, Mabuchi et al. 2007). Details on reading process, trimming procedure and mapping statistics are available as supplementary materials. Differential gene expression between the control and exposed fishes was evaluated allowing for no more than 2 mismatches per read (fold change > |1.5|, FDR *p*-value ≤ 0.05). Annotations were downloaded from NCBI. Gene ontology (GO) were downloaded from ebi.ac.uk/GOA/index. Genes with significant changes in expression were further tested for the GO enrichment test to determine enrichment of biological pathways (DEGs compared to coding genes and sorted by FDR *p*-value ≤ 0.05 and observed gene count > 3).

2.5 Data analysis

Biochemical responses were statistically analysed by GraphPad Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com) by nonparametric Kruskal-Wallis test. Differences were considered significant at p-level < 0.05. Dunn's multiple comparison test was used to highlight significant differences within treatments and between treatments and controls. The EXCEL software (Microsoft, Redmond, WA, USA) was used for the calculation of IBRv2 index and the creation of star plot. CLC Genomics Workbench 20.0 software (CLC bio, Aarhus, Denmark) was used for the quality control of reads, the trimming procedure, the analysis of differential gene expression (fold change > |1.5|, FDR *p*-value ≤ 0.05) and the GO enrichment test (FDR *p*-value ≤ 0.05 and observed gene count >3). Venn diagram was generated using the Bioinformatics and Evolutionary Genomics custom Venn Diagram generator (http://bioinformatics.psb.ugent.be/webtools/Venn/). Volcano plots of significant genes for each concentration were generated using the GraphPad Prism software.

3. Results

3.1. Nanoplastics dispersion

The characterization of PS-COOH NPs through DLS analysis showed an optimal dispersion in MilliQ, with Z-average values of 125.1 ± 3.5 nm (mean ± st. dev.) and PdI of 0.2 ± 0.1 (mean ± st. dev.) with meaningless differences over the time and between concentrations. On the contrary, PS-COOH NPs in ASW showed extremely higher Z-average (1457.4 ± 573.9 nm, mean ± st. dev.) and PdI (0.6 ± 0.1, mean ± st. dev.) indicative of a particular aggregation pattern (Fig 1). The size of such aggregates seems to change over 24 h but it is important to notice that samples in ASW were characterized by low quality DLS data due to their high polydispersion or to the presence of large or sedimenting particles (as suggested by the quality report of the instrument).

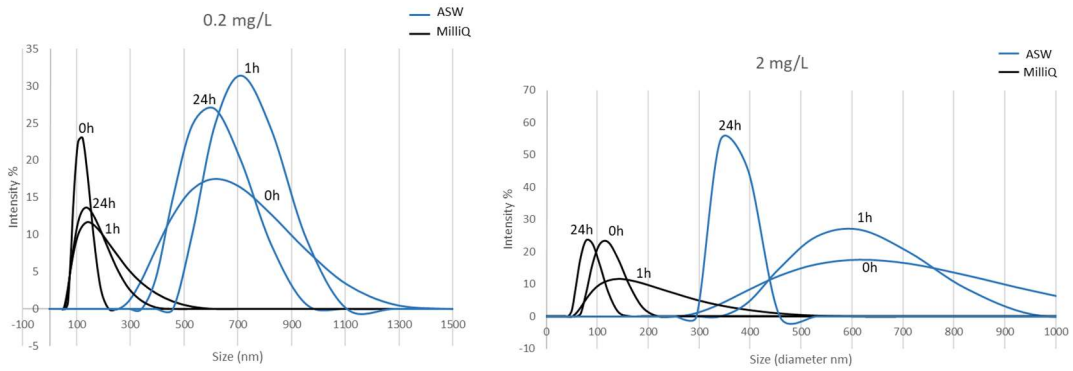


Fig 1. DLS analysis, on medium (0.2 mg/L) and high (2 mg/L) concentrations**,** shows an optimal dispersion in MilliQ with meaningless differences over the time. On the contrary, PS-COOH NPs in ASW showed higher Z-average and PdI indicative of a particular aggregation pattern. Data on lower concentration (0.02 mg/L) are not available because of the technological limits of the instrument.

3.2 Biomarkers of oxidative stress

Kruskal-Wallis test between controls and treatments reveals no statistically relevant difference for CAT, GST, GR and TOSCA OH· and ROO· (*p*-value = 0.45, 0.08, 0.93, 0.32 and 0.25 respectively) as shown in Fig 2. The limited variations of oxidative stress responses in relation to the different concentrations was confirmed by the test performed within treatments.

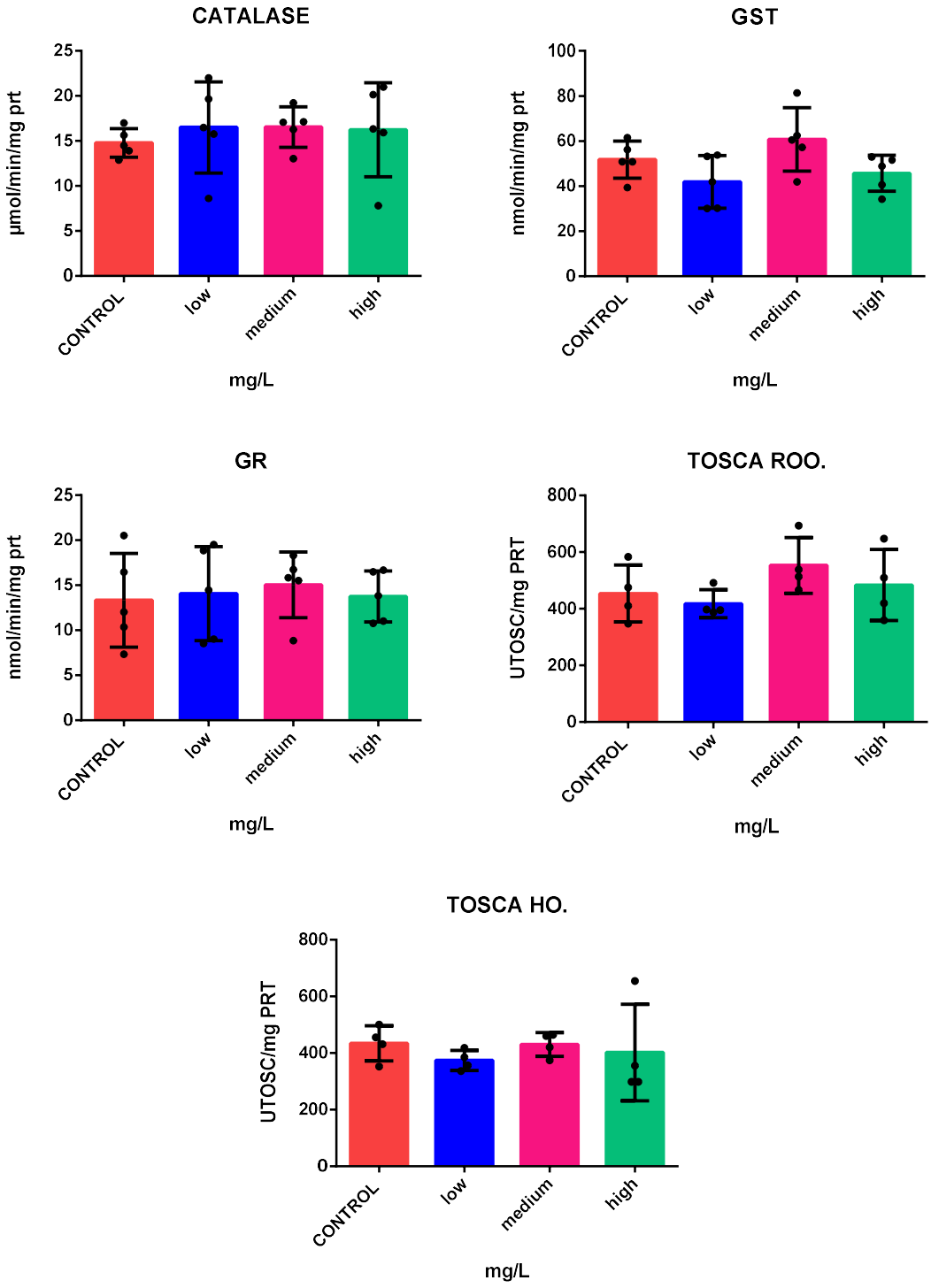


Fig 2. Biochemical responses in juveniles of clownfish exposed for 7 days to a daily concentration of 0.02-0.2-2 mg/L (low-medium-high) of 100 nm PS-COOH. Catalase, GST (glutathione S-transferase), GR (glutathione reductase) n = 5; TOSCA (Total Oxydoradical Scavenging Capacity Assay, ROO· = peroxylic; OH· = hydroxylic) n = 4. Dot points represent each individual's value (mean ± st. dev.).

Clownfish exposed to NPs show a global deterioration in health status respect to controls, as highlighted by IBRv2 values that were 2.43, 2.53 and 1.83 for low, medium and high groups, respectively (Fig 3a, c). Lower and medium concentration of NPs seemed to represent more stressful conditions than higher treatment (Fig 3c). Whereas all biomarkers analyzed show stimulation and not inhibition, a different order of biomarker sensitivity was recorded for each concentration, and specifically:

Low-treatment = GST > TOSCA (HO·) > TOSCA (ROO·) > CAT > GR

Medium-treatment = TOSCA (ROO·) > GR > CAT > GST > TOSCA (HO·)

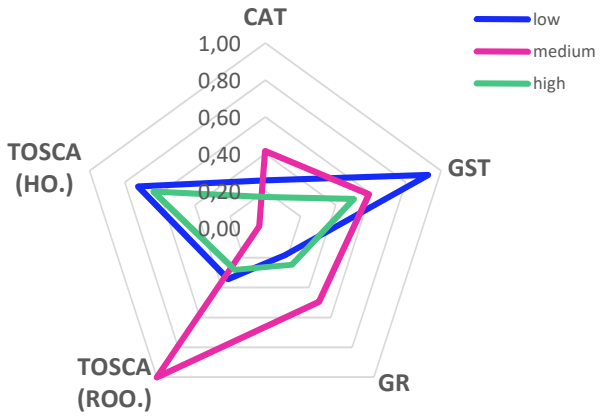
High-treatment = TOSCA (HO·) > GST > TOSCA (ROO·) > GR > CAT

Low and high concentration treatments seem to modulate the activation of biomarkers in a more similar way with respect to the medium group (Fig 3b) which appears to be characterized by a different biochemical response.

a)

	CAT	GST	GR	TOSCA (ROO·)	TOSCA (HO·)	IBRv2
low	0.26	0.93	0.18	0.34	0.72	2.43
medium	0.42	0.59	0.49	1.00	0.03	2.53
high	0.17	0.50	0.25	0.28	0.64	1.83

b)



c)

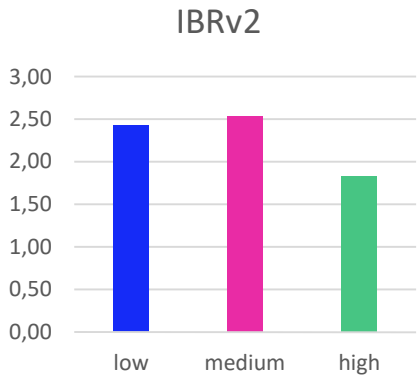


Fig 3. a) Table with the reference deviation of each investigated biomarker calculated to obtain the IBRv2 index in different treatment groups (low=0.02 mg/L; medium= 0.2 mg/L; high=2

mg/L). b) Star plot based on the reference deviation of each investigated biomarker: CAT = catalase; GST = glutathione S-transferase; GR = glutathione reductase; TOSCA (ROO·) = Total Oxydoradical Scavenging Capacity Assay (peroxylic); TOSCA (HO·) = Total Oxydoradical Scavenging Capacity Assay (hydroxylic). c) IBRv2 values for each concentration of PS NPs.

3.3 Transcriptomics analysis

Exposure to lower concentration of NPs (0.02 mg/L) resulted in higher amount of differentially expressed genes (DEGs, fold change > |1.5|, FDR *p*-value ≤ 0.05): 110 (49% upregulated), while 0.2 mg/L exposures resulted in 17 DEGs (59% upregulated) and 2 mg/L in 31 DEGs (58% upregulated) as represented by volcano plots (Fig 4) (details on Table S1, S2, S3).

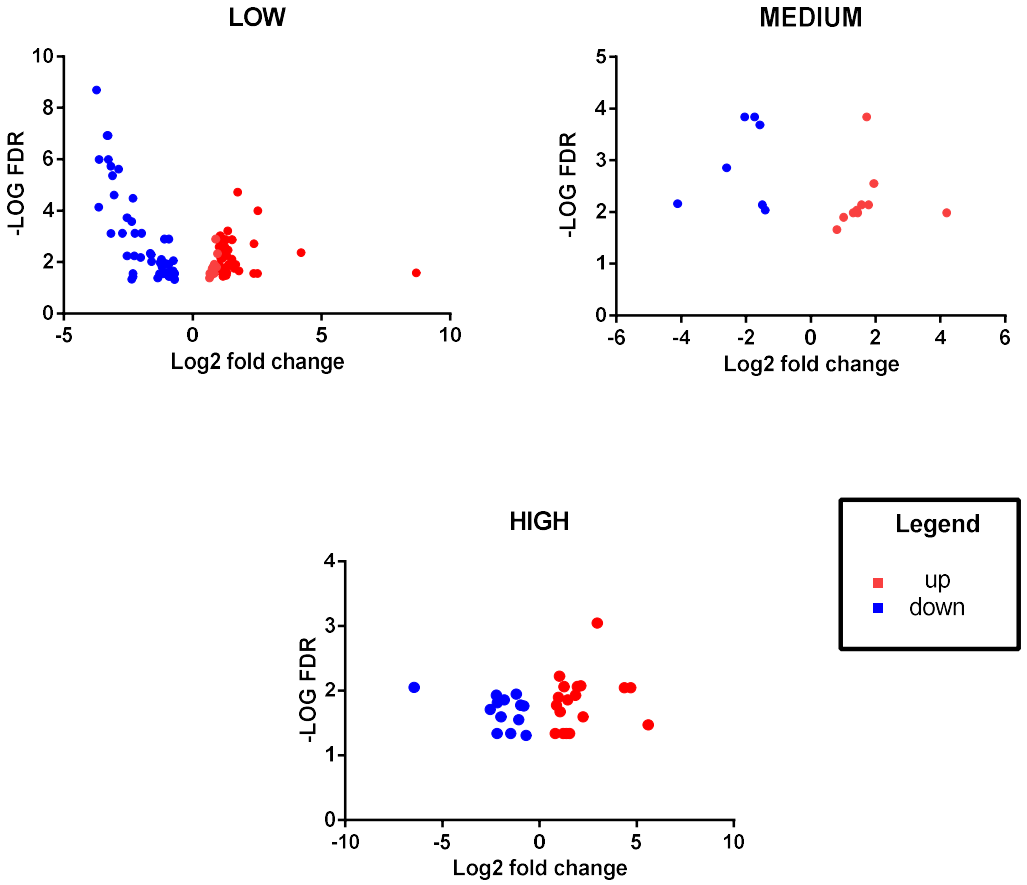


Fig 4. Volcano plots of significant genes (FDR <0.05) for each concentration (low = 0.02 mg/L; medium = 0.2 mg/L; high = 2 mg/L).

In heat maps displaying segregation by gene expression, treated and control samples are distinguished clearly from one another while grouping highly within their own sample types (Fig 3).

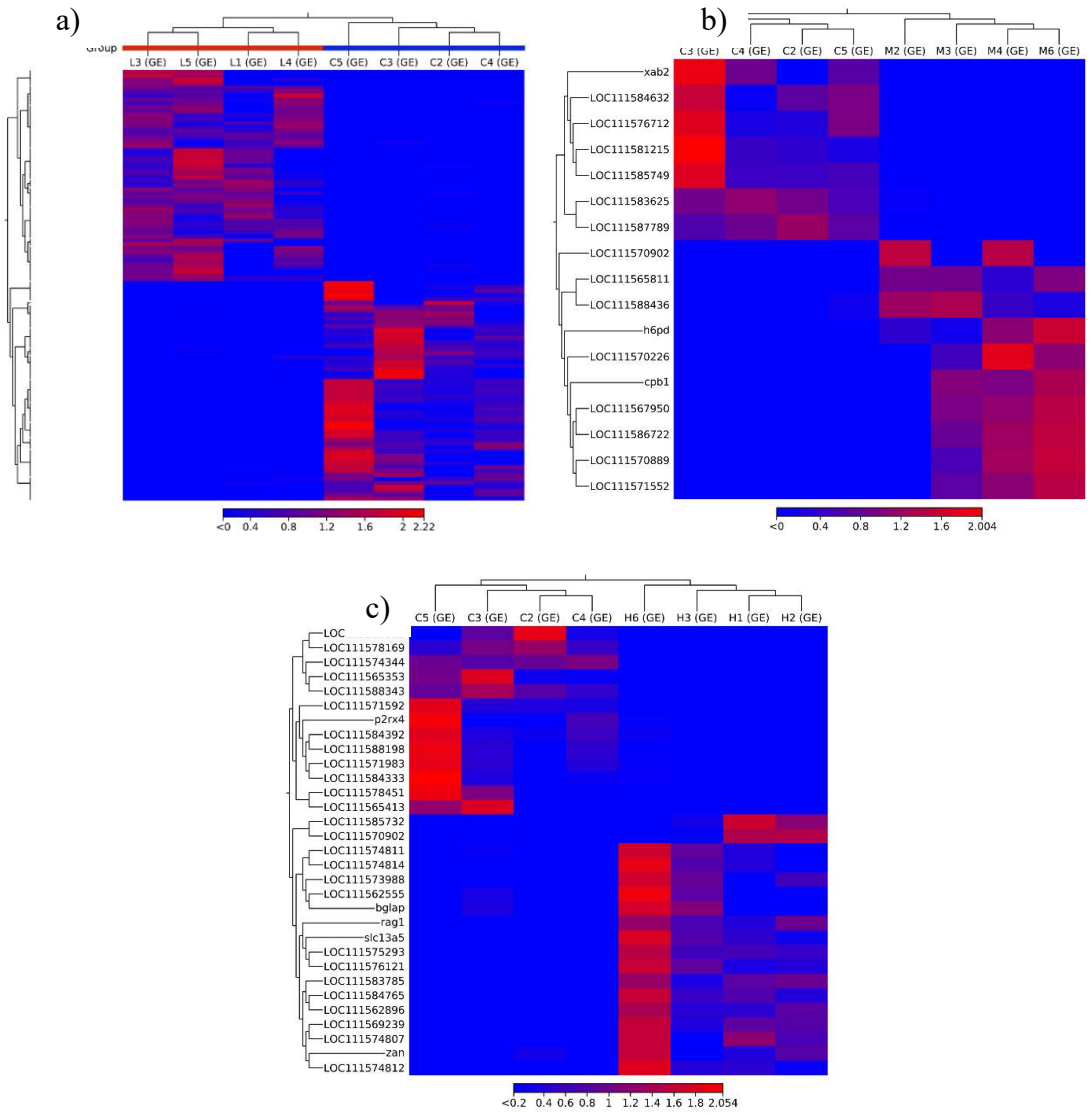


Fig 3. Heat maps show the segregation of samples by gene expression: a) low *vs* control; b) medium *vs* control; c) high *vs* control. Colours mean different intensity in gene regulation changes.

We further investigated if these three concentrations could affect the same set of genes, as shown in Venn diagrams (Fig 4). Not considerable overlap between all treatments was evident, with only 1 DEG (LOC111570902, uncharacterized gene) up-regulated in all three experimental fish groups. Besides, 13 DEGs were in common between low and

high, and 2 DEGs between low and medium (details on Table 1).

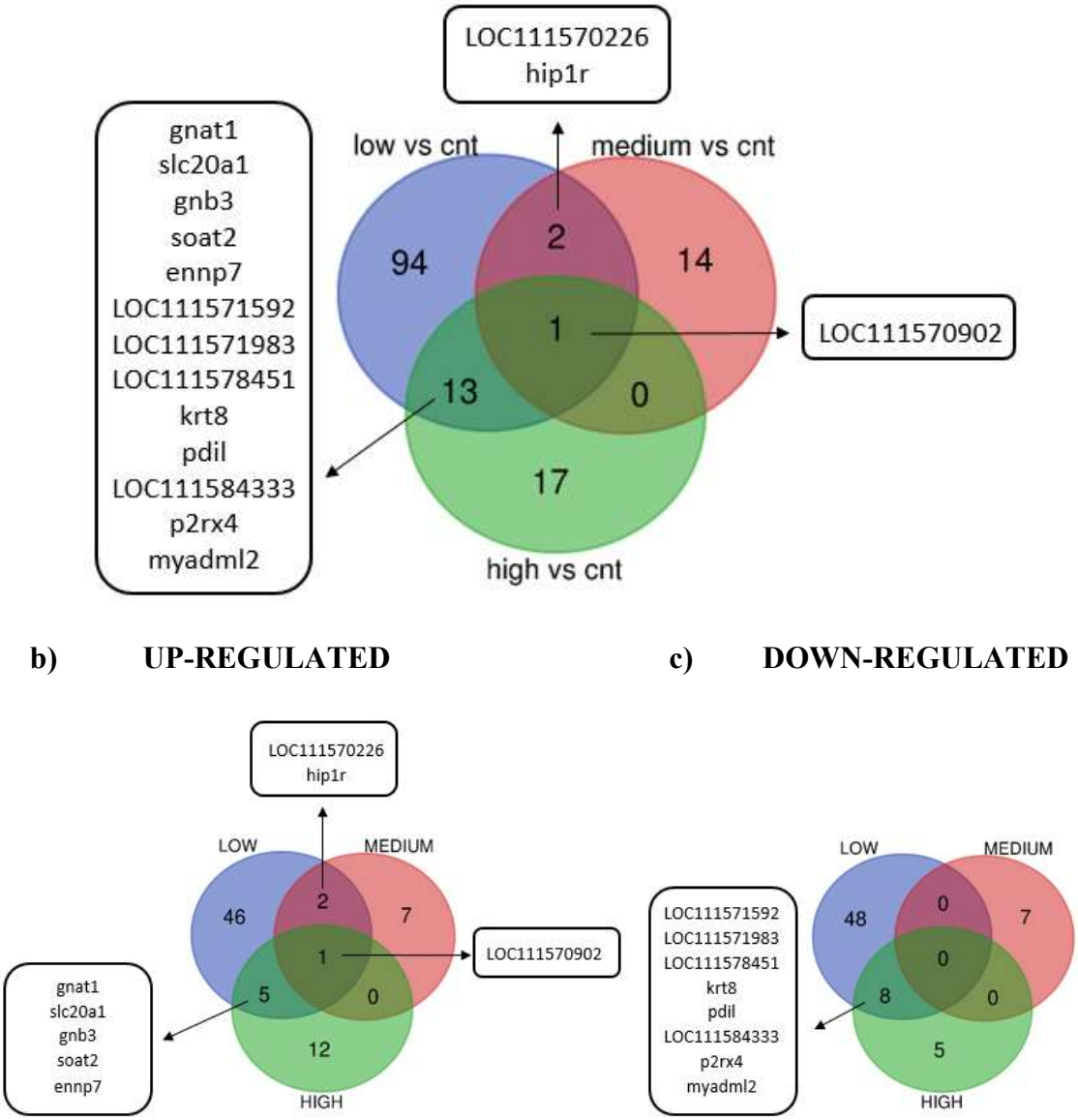


Fig 4. Venn diagrams indicating overlap among a) all differentially expressed genes between treatments, b) between up-regulated DEGS, c) between down-regulated DEGs (generated using the Bioinformatics and Evolutionary Genomics custom Venn Diagram generator). Gene names in common are specified.

Table 1. Differentially expressed genes shared between different treatment groups (low = 0.002 mg/L; medium = 0.2 mg/L; high = 2 mg/L) associated with relative products and level of expression.

groups	total genes in common	gene	fold change			product	regulation
			high	low	med		
high_ low_ medium	1	LOC111570902	20.5	18.6	18.3	uncharacterized	up
low_medium	2	LOC111570226		2.5	3.0	pancreatic alpha-amylase-like	
		hip1r		3.2	3.9	huntingtin-interacting protein 1-related protein-like	
high_low	13	gnat1	1.8	1.8		guanine nucleotide-binding protein G(t) subunit alpha-1	
		slc20a1	2.3	3.4		sodium-dependent phosphate transporter 1-A-like	
		gnb3	1.8	1.7		guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3-like	
		soat2	2.6	2.5		sterol O-acyltransferase 2-like	
		enpp7	2.4	2.2		ectonucleotide pyrophosphatase/phosphodiesterase family member 7-like	
		LOC111571592	-4.5	-8.7		endonuclease domain-containing 1 protein-like	down
		LOC111571983	-4.7	-10.0		endonuclease domain-containing 1 protein-like	
		LOC111578451	-2.8	-3.1		lectin-like	
		krt8	-1.6	-1.7		keratin type II cytoskeletal 8-like	
		pdil	-3.5	-5.0		protein disulfide-isomerase-like	
		LOC111584333	-4.5	-12.4		endonuclease domain-containing 1 protein-like	
		p2rx4	-3.9	-5.8		purinergic receptor P2X 4	
		myadml2	-5.8	-12.5		myeloid-associated differentiation marker-like protein 2	

A greater similarity between "high" and "low" groups is also confirmed by PCA (Principal Component Analyses) performed on levels of expression, that highlights segregation between treatments along the PC2 axis, in particular between "high" and "low" treated animals, and between control and "medium" groups (Fig 5).

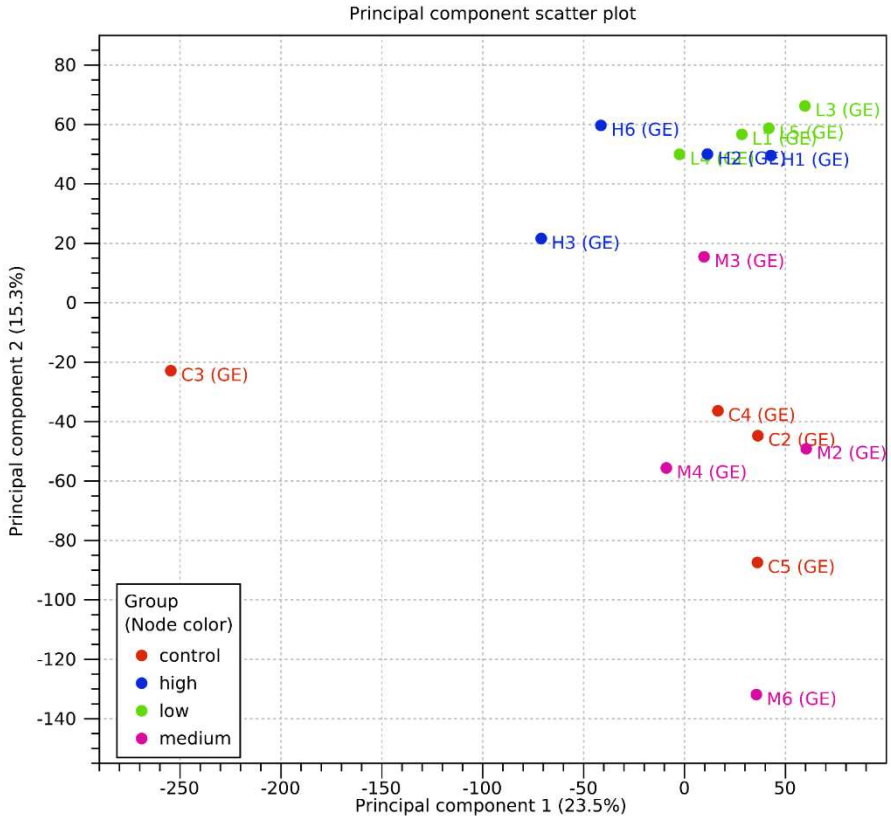


Fig 5. Principal Components Analysis performed on levels of gene expression.

In agreement with the differential analysis, GO enrichment test on low group revealed 27 enriched pathways, the majority of which (44%) related to biological processes such as platelet degranulation (10 DEGs, down-regulated), visual perception and post-translational protein modification (6 DEGs, both up-regulated), adaptive immune response (5 DEGs, down-regulated), response to calcium ion, bacterium, cold-induced thermogenesis (4 DEGs), endocytosis, gene expression, negative regulation of osteoblast differentiation and lipid metabolism (4 DEGs). Cellular component-related GO (33%) was dominated by intra- and extracellular region and molecular function (22%) with significant enrichment in calcium ion binding (10 DEGs) (Table 2).

Table 2. GO enrichment test from exposure to low concentration of NPs (0.02 mg/L); GO related to biological process, molecular function and cellular component (with each gene involved) are listed (observed counts > 3; FDR *p*-value ≤ 0.05).

	observed counts	GO_description	*p* -value	FDR	genes
ALL					
biological process					
GO:0002576	10	platelet degranulation	5.85E-09	2.27E-06	LOC111570736, LOC111586536, LOC111586982, LOC111583735, LOC111574540, LOC111571592, LOC111571578, LOC111574802, LOC111571983, LOC111584333
GO:0007601	6	visual perception	0.017149	0.034079	LOC111566243, LOC111585780, rbp3, pde6c, LOC111568803, LOC111565835
GO:0043687	6	post-translational protein modification	0.003213	0.01052	klhl30, kbtbd12, asb10, fgf19, LOC111579831, LOC111586982
GO:0002250	5	adaptive immune response	0.001631	0.006954	LOC111586536, LOC111575620, btk, LOC111571004, LOC111584005
GO:0006898	4	receptor-mediated endocytosis	0.008104	0.019775	LOC111565811, LOC111578844, LOC111583735, LOC111575608
GO:0071277	4	cellular response to calcium ion	0.001876	0.007785	LOC111585780, LOC111569776, LOC111571004, LOC111584005
GO:0010467	4	gene expression	0.000153	0.002171	umodl1, kbtbd12, LOC111568594, LOC111570736
GO:0006260	4	DNA replication	0.000765	0.004547	grwd1, gins1, pole2
GO:0009617	4	response to bacterium	0.029137	0.049857	LOC111570226, LOC111568594, atg9b, fgf19
GO:0045668	4	negative regulation of osteoblast differentiation	0.000268	0.002832	fgf19, LOC111570994, LOC111571004, LOC111584005
GO:0120162	4	positive regulation of cold-induced thermogenesis	0.001145	0.005678	LOC111568594, fgf19, LOC111571004, LOC111584005
GO:0006629	4	lipid metabolic process	0.005652	0.015749	LOC111568594, fgf19, LOC111571004, LOC111584005
molecular function					
GO:0005509	10	calcium ion binding	0.012897	0.027494	umodl1, LOC111570226, LOC111585780, LOC111568594, LOC111565835, LOC111569776, LOC111563066, LOC111571004, LOC111584005, LOC111583735
GO:0005516	6	calmodulin binding	0.005519	0.01546	LOC111572110, LOC111569776, LOC111571004, LOC111584005, LOC111582951, LOC111576402
GO:0003676	5	nucleic acid binding	0.002056	0.008039	LOC111574540, LOC111571592, LOC111571578, LOC111571983, LOC111584333
GO:0004519	5	endonuclease activity	1.79E-05	0.001114	LOC111574540, LOC111571592, LOC111571578, LOC111584333, LOC111571983
GO:0016787	4	hydrolase activity	0.002153	0.008395	LOC111562896, LOC111572110, LOC111571004, LOC111584005
GO:0042981	4	regulation of apoptotic process	0.022953	0.042057	umodl1, LOC111565811, asb10, LOC111583735
cellular component					
GO:0005576	21	extracellular region	0.001234	0.005752	umodl1, LOC111570226, LOC111568594, fgf19, LOC111562896, rbp3, LOC111570736, LOC111563066, LOC111564232, LOC111572448, LOC111586982, LOC111583735, LOC111575608, LOC111574540, LOC111582951, LOC111571592, LOC111571578, LOC111574802, LOC111576475, LOC111571983, LOC111584333,
GO:0031410	7	cytoplasmic vesicle	0.02001	0.038198	umodl1, LOC111583080, atg9b, LOC111581091, LOC111586536, LOC111578844, btk
GO:0043204	7	perikaryon	0.003621	0.011685	klhl30, LOC111576545, LOC111565835, LOC111566251, LOC111571004, LOC111584005, LOC111582951
GO:0005765	6	lysosomal membrane	0.007337	0.018729	LOC111570736, LOC111575620, LOC111578844, LOC111571004, LOC111584005, LOC111574802
GO:0030659	5	cytoplasmic vesicle membrane	0.00695	0.018037	LOC111583080, atg9b, LOC111563805, LOC111571004, LOC111584005
GO:0005764	5	lysosome	0.023364	0.04256	LOC111586536, LOC111570736, LOC111571004, LOC111584005, LOC111574802
GO:0031902	4	late endosome membrane	0.002182	0.008467	atg9b, LOC111575620, LOC111578844, LOC111574802
GO:0005770	4	late endosome	0.006901	0.01797	atg9b, LOC111586536, LOC111570736, LOC111574802
GO:0001750	4	photoreceptor outer segment	0.003048	0.010283	LOC111566243, LOC111585780, pde6c, LOC111565835

GO enrichment test selectively performed on down-regulated DEGs shows enrichment of 17 GOs mainly related to platelet degranulation (10 DEGs), endonuclease activity (5 DEGs), extracellular region (15 DEGs) and lysosome-related GO (11 DEGs). On the contrary, visual perception, signal transduction, calcium ion binding (6 DEGs) and

obsolete cell (6 DEGs) were enriched according to the GO enrichment test selectively performed on up-regulated DEGs (Table S4). Animals exposed to medium and high concentrations of NPs did not show enrichment in pathways related to biological processes, rather to cellular component such as extracellular region (up-regulated in medium; down-regulated in high), intracellular membrane-bounded organelle, Golgi apparatus and membrane (Table 3 and 4).

Table 3. GO enrichment test from exposure to medium concentration of NPs (0.2 mg/L); GO related to biological process, molecular function and cellular component (with each gene involved) are listed (observed counts >3; FDR *p*-value ≤ 0.05).

	observed counts	GO_description	*p* -value	FDR	genes
ALL					
cellular component					
GO:0005576	7	extracellular region	0.001	0.002	LOC111571552, LOC111586722, LOC111570226, LOC111570889, cpb1, LOC111585749, LOC111584632
GO:0005615	6	extracellular space	0.001	0.003	LOC111571552, LOC111586722, LOC111570226, cpb1, LOC111584632, LOC111570889
GO:0043231	4	intracellular membrane-bounded organelle	0.010	0.014	LOC111565811, h6pd, xab2, LOC111571552
GO:0005794	4	Golgi apparatus	0.025	0.031	LOC111565811, LOC111571552, LOC111588436, LOC111584632

Table 4. GO enrichment test from exposure to high concentration of NPs (2 mg/L); GO related to biological process, molecular function and cellular component (with each gene involved) are listed (observed counts > 3; FDR *p*-value ≤ 0.05).

	observed counts	GO_description	*p* -value	FDR	genes
ALL					
molecular function					
GO:0070062	8	extracellular exosome	5.05E-05	0.0002	LOC111562555, LOC111562896, LOC111576121, slc13a5b, LOC111565353, LOC111565413, LOC111571592, LOC111584333, LOC111571983
GO:0046872	7	metal ion binding	0.0002	0.0005	rag1, LOC111562896, LOC111566839, LOC111571592, LOC111571983, LOC111574344,
GO:0005576	6	extracellular region	0.0004	0.0011	LOC111562896, LOC111565353, LOC111565413, LOC111571592, LOC111584333, LOC111571983
GO:0004519	4	endonuclease activity	9.57E-09	5.57E-07	rag1, LOC111571592, LOC111584333, LOC111571983
GO:0005615	4	extracellular space	0.0043	0.0068	rag1, LOC111584333, LOC111571592, LOC111571983
GO:0005887	4	integral component of plasma membrane	0.0072	0.0102	LOC111573988, LOC111571592, LOC111584333, LOC111571983
GO:0005623	4	obsolete cell	0.0092	0.0124	LOC111573988, LOC111575293, LOC111565353, LOC111565413
cellular component					
GO:0016020	8	membrane	0.0005	0.0012	LOC111562555, LOC111562896, slc13a5b, LOC111565353, LOC111565413, LOC111566839, LOC111571592, LOC111584333, LOC111571983
GO:0005829	7	cytosol	0.0325	0.0382	LOC111562555, rag1, slc13a5b, LOC111566839, LOC111571592, LOC111584333, LOC111571983, LOC111574344

Finally, in order to test the enrichment of pathways due to the exposure to plastic treatment without distinction of concentration (low + medium + high= treatment), we performed a new differential analysis followed by the GO enrichment test. Results highlights an exclusive involvement of extracellular region/space (9 DEGs) and integral component of plasma membrane (4 DEGs), details on Table 5.

Table 5. GO enrichment test from exposure to plastic treatment (low + medium + high = treatment); GO related to biological process, molecular function and cellular component (with each gene involved) are listed (observed counts > 3; FDR *p*-value ≤ 0.05).

	observed counts	GO_description	*p* -value	FDR	genes
ALL					
cellular component					
GO:0005576	5	extracellular region	0.008108109	0.011722567	LOC111570226, LOC111571552, LOC111577098, LOC111586722, LOC111562896
GO:0005615	4	extracellular space	0.011873471	0.01606936	LOC111570226, LOC111571552, LOC111586722, LOC111577098
GO:0005887	4	integral component of plasma membrane	0.020907928	0.026317672	LOC111568026, LOC111571552, LOC111586385, LOC111562896

The precise functional characterization of DEGs is a key in the understanding the precise biological functions responsive to NP exposure. For further confirmation of GO biological processes, and with the purpose of gleaning additional insights, literature concerning most upregulated and downregulated DEGs within each specific experimental condition was scrutinized. The genes showing higher change in transcriptional levels (Suppl. Table S1-S3) were predominantly factors involved in viral infection and inflammatory response, and to less extent in ciliogenesis, energy metabolism, olfactory and reproductive functions. The most upregulated gene in the low condition is the interferon-stimulated gene coding for the coiled-coil domain containing 92 lipoproteins, *ccdc92* (f.c. +409.26). This DEG is highly conserved in the animal phylogeny and is expressed in several tissues and cell types (e.g. monocytes, T cells, blood cells, spinal cord, retina, heart, pancreas, ovary), where it has a role in lipid metabolism and ciliogenesis (Chasman et al 2009; Bernatik et al 2020). Recently, CCDC92 has been shown to interfere with virus entry and to inhibit viral transcription, lending support to the hypothesis that it can serve as a baseline cell susceptibility sentinel during infection (Kuroda et al 2020). The DCX family member dyslexia-associated gene coding for DCDC2 (*dcdc2*; f.c. +5.76) is known to play a role in ciliogenesis and, redundantly with other Dcx genes, in neurogenesis and neuronal migration (Wang et al 2011; Schueler et

al 2015). The gene *clcnka* (f.c. +5.76) is a member of the ClC family of voltage-gated chloride channels with functions in urinary concentration and excretion mechanisms in kidney physiology (Wingo and Stockhand, 2016; Tomilin et al 2018). The olfactory receptor 1 (*ora1*, f.c. +5.20) codes for a factor implicated in smell (Sammeta et al 2010). Of note, the Ora1 protein elicits olfactory-mediated oviposition in zebrafish (Ahuja and Korsching, 2014). The greatest downregulated gene in the low NP concentration was the one encoding the protein S100-A1-like (S100A1; f.c. -13.27), a Ca^{2+} signal transducer that generates antimicrobial peptides in viral and bacterial infections also in fishes (Kraemer et al 2008; Li et al 2016; Zi et al 2018; Zhang et al 2019). Additional inflammatory response genes that modulate immune signaling in response to the low treatment and that show transcriptional level decrease are *endod1* (f.c. -12.39), *cd63* (f.c. -9.67), *p2x4* (f.c. -5.83), *pla2r1* (f.c. -5.83) and *tsetseEP* (f.c. -5.19). In the medium condition, the greatest upregulated gene codes for huntingtin-interacting protein 1-related protein-like, a well-known modulator of presynaptic activity, that has been recently demonstrated to mediate viral infection across animal evolution (Jiang et al 2020). Of note, genes encoding digestive enzymes such as the bile salt activated lipase (*Cel*; f.c. 3.46), chymotrypsin A (f.c. 3.31) and pancreatic alpha-amylase-like (f.c. 2.98) shows consistent upregulation in medium tratment. Conversely, the transcriptional regulator encoding gene *RPB3* (DNA-directed RNA polymerase II subunit RPB3; f.c. -17.25) is the one showing higher downregulation in the intermediate concentration, followed by the innate immunity-related gene transcobalamin 1 (f.c. -6.07) (Nakamura et al., 2020). Finally, the effect of high NP concentration on clownfish juveniles highlights significant opposite change in the expression level of the genes encoding HBB (haemoglobin subunit beta; f.c. +48.22) and ADPRHL1 (ADP-ribosylarginine hydrolase 1; f.c. -87.37), two proteins implicated in oxygen transport and cardiac myofibrillogenesis, respectively (Pillai et al 2020; Smith et al 2020). Another immune gene whose expression is upregulated in response to high treatment is *Rag1* (f.c. 3.85). Vice versa, greatest downregulation is shown by ADPRHL1 (protein ADP-ribosylarginine hydrolase-like; f.c. -87.37), a gene involved in the developing myocardium (Smith et al., 2020).

4. Discussions

A biochemical and transcriptomics approach was used to identify the effects of polystyrene NP exposure on juveniles of a coral reef iconic fish, *Amphiprion ocellaris*.

Whereas the activity of sensitive adaptive biomarkers of oxidative stress were not particularly altered, RNA-seq highlighted significant dysregulation of immune genes especially in the low condition, consistent with the analysis of particle size distribution.

4.1 NP behavior and implications to bioavailability

DLS analysis reported in this study confirms the different behavior of NPs in fresh and seawater. Specifically, once in ASW, NPs tend to immediately form aggregates higher in size than in MilliQ water where, instead, they remain essentially monodisperse. Several studies support our results (Kashiwada S. 2006; Della Torre et al. 2014; Bergami et al. 2016; Manfra et al. 2017) and multiple implications can be deduced. For example, it is reasonable to speculate a different bioavailability between freshwater and seawater systems. In zebrafish, a well-known freshwater fish, nano PS distribution and accumulation has been studied at different developmental stages (Pitt, Kozal, et al. 2018; Van Pomeren et al. 2017; Q. Chen, Yin, et al. 2017), highlighting the ability to localize in different tissues (e.g. head, yolk sac, gastrointestinal tract, gall bladder, liver, pericardium, pancreas, etc). To our knowledge, bioaccumulation data on seawater fish are uniquely represented by the study of Kashiwada (2006) who reported the accumulation of 39 nm PS-NPs in gills, intestine, brain, testis, liver and blood ability of medaka *(Oryzia latipes)* adults at particle concentration of 10 mg/L. The limited number of NP studies available is integrated by the reliability of bioaccumulation studies performed using externally fluorescent-labelled NPs. In this regard, Catarino et al. (2019) stated that the use of externally fluorescent nanoPS is inadequate to provide absolute conclusive evidence of particle absorption. In support of this claim, Catarino and co-workers experimentally demonstrated that commercial fluorescent-labelled nanoPS can leach their fluorophores, and the fluorophore alone can accumulate within internal tissues of zebrafish larvae. In medaka, is not clear if NPs were internally or externally labelled (Kashiwada 2006). Since it is believed that bioaccumulation analysis represents an essential part in eco-toxicological studies, we used internally fluorescent labelled NPs to measure the ability of clownfish juveniles to ingest the particles. Unlikely, the protocol based on tissues digestion in KOH followed by the reading of fluorescence at spectrofluorimeter did not permit to discriminate the background from the particle signal. Confounding factors such as food provided to tested animals and the low exposure concentrations likely played a determinant role in this negative result. In conclusion,

working with environmentally relevant concentrations reduces the possibility to estimate the NP uptake. In this regard, the use of 14C-radiolabelled particles or a metal tracer may represent an alternative as suggested by Al-Sid-Cheikh et al. (2018) and Mitrano et al. (2019). Future experiments on bioaccumulation of nanoplastics in seawater fish are warmly requested.

4.2 Biochemical responses to oxidative stress

Environmental pollution may increase the cellular reactive oxygen species load, because many xenobiotics exert part of their toxicity *via* the formation of reactive oxygen species (Lackner 1998). The additional load of reactive oxygen species requires that the animals adapt to the specific situation and increase detoxification capacity including both detoxifying enzymes and low molecular scavengers. Fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as mammalian species do. The activity of sensitive biomarkers such as catalase (CAT), glutathione S-transferase (GST) and glutathione reductase (GR) was not significantly altered in juveniles exposed to NPs during our experiment. Our results are in accordance with those reported by Chen et al. (2017) and Pitt, Trevisan, et al. (2018). The first study tested the toxicity of NPs in zebrafish larvae (50 nm; 1 mg/L): CAT and GPx levels were similar to control animals. The second, used 42 nm PS-NPs (at the concentration of 10% of food by mass) in adult zebrafish (male and female) reporting no statistical differences in catalase activity. On the contrary, antioxidant-related genes (e.g. *gpx* 1, *sod 2*, *gr*) were up-regulated in seabream (*Sparus aurata*) specimens treated with polymethylmethacrylate (PMMA) NPs. Discrepancies could be due to different time of exposure (96 h *vs* 7 days in our study), polymer type (PMMA *vs* PS) and organ analyzed (liver *vs* whole body). In addition to this, we further evaluated the capability of the system to neutralize two potent cellular oxidants (peroxyl and hydroxyl radicals) by TOSC assay; no statistical differences were recorded.

Analyzing biomarker for biomarker, our findings did not identify statistically relevant differences. On the contrary, the integrated analysis of biomarker responses has proved to be a quick, useful and informative approach especially in discriminating against any differences between treatments that cannot be identified by statistical analysis on individual biomarkers. In our experiment, IBRv2 index allowed to detect a reduction in overall health status of fishes tested with NPs, and specifically, low and medium

concentrations seemed to represent more stressful conditions than the high treatment.

The absence of a typical dose-response pattern is not rare in plastic exposure. This is consistent with results described by Brandts et al. (2018, 2021) who tested the toxicity of PMMA nanoparticles in gilthead seabream and european seabass. This could be indicative 1) of a mechanism of toxicity not strictly dependent on contaminant concentration, 2) of different response capacity of biomarkers (increased or decreased activity) depending on the intensity of the stressor (Hook et al. 2014), 3) lower probability of particle collision at low concentrations that lead to a lower agglomeration/aggregation.

According to our findings, while all the biomarkers analyzed show stimulation and not inhibition, a different order of biomarker sensitivity was recorded for each concentration: low and high treatment seem to modulate the activation of biomarker in a more similar way than medium group, with a particular involvement of GST and TOSCA HO·. In conclusion, the minor response in oxidative stress detected in clownfish juveniles was confirmed by gene expression analysis lacking oxidative stress-related DEGs (see below).

4.3 Transcriptomics analysis reveals a viral-like response at low NP dose

NGS platforms such as transcriptomics represents a very sensitive technique for more rapid toxicological characterizations (Merrick 2019). RNA-seq has been already adopted for studying the toxicity of a variety of chemicals in fish larvae (Chen et al. 2016; Zheng et al. 2019; Wu et al. 2017; Pedersen et al. 2020). The sensitivity and discovery potential of RNA-seq has found applications in biomarker discovery and environmental monitoring that can be relevant for many stages of risk assessment. There are few bibliographic references about the transcriptomics effects of NPs in fish (reviewed in Barría et al. 2020; Piccardo et al. 2020). Even fewer are the studies that attempted to evaluate the toxicity of NPs in fish at molecular level (Brun et al. 2019; Chen et al. 2017a, 2017b; Brandts et al. 2021; Brandts et al. 2018). At our knowledge, Pedersen et al. (2020) reported the only transcriptomic analysis after the exposure to polystyrene NP (50 nm and 200 nm) in freshwater fish larvae (zebrafish, 6-120 hpf). In particular, these authors tested two different concentrations, i.e. 0.1 and 1 mg/L, comparable with medium and high treatments used in our study. Considering their transcriptomics study from a quantitative point of view, treatment with 200 nm particles resulted in 734 and 864 DEGs (0.1 and 1 mg/L, respectively), which corresponds to amounts of differential expressed genes greater

than those reported in our study (17 and 31 DEGs, 0.2 and 2 mg/L, respectively). This discrepancy could be explained by the more advanced annotation level of the zebrafish genome with respect to *A. ocellaris* genome. On the contrary, smaller NPs (50 nm) were able to differentially express only 2 zebrafish DEGs (*hsd11b2, slc3a2b*). From a qualitative point of view, Pedersen reported multiple enriched pathways, namely, organismal injury and abnormalities, endocrine system disorder, neurological disease, skeletal/muscular and nervous system development, dysregulated metabolic, cardiac and hepatic pathways, gastrointestinal functioning affected.

The findings of our transcriptomics analysis in a marine fish suggest novel mechanisms that had not been linked to NP exposure previously. The results of this study show a robust relationship with DEGs that are key components in the innate and adaptive immune response to bacterial and, namely, viral infections (i.e. *ccdc92*, *S100A1*, *hip1r*), and in anti- and pro-inflammatory mechanisms (e.g. *endod1*, *cd63*). Remarkably, we did not observe dysregulation of canonical immune and inflammatory genes such as chemokines, cytokines and toll-like receptors. Of course, validation of these results by qRT-PCR analyses will start in a short term.

We further investigated whether these three concentrations could affect the same set of genes, and no considerable overlap between all treatments was evident, with only 1 DEG LOC111570902 (uncharacterized gene) up-regulated in all three groups of fish. Low and high treatment shared the greater number of DEGs (13) as also supported by principal component analysis. Within the 13 DEGs, 5 were up-regulated and consist in membrane or intracellular proteins with important role in signal transduction, protein transport, nucleic acid, cellular and lipid metabolism. The remaining 8 down-regulated DEGs code for endonuclease (enzymes that cleave the phosphodiester bond within a polynucleotide chain), lectins (carbohydrate-binding proteins with a role in recognition on the cellular and molecular level and play numerous roles in biological recognition phenomena involving cells, carbohydrates, and proteins), keratin type II cytoskeletal 8 protein (structural proteins of the intermediate filament family mainly expressed in epithelial cells, Martorana et al. 2001), protein disulfide-isomerase-like (an enzyme that catalyzes disulfide formation and isomerization and a chaperone that inhibits aggregation, Wilkinson and Gilbert 2004), purinergic receptor P2X4 (one of the most sensitive purinergic receptors widely expressed in central and peripheral neurons, in microglia, and also found in various epithelial tissues and endothelial cells. It is preferentially localized in lysosomes, where it is protected from proteolysis by its glycosylation, Suurvali et al.

2017) and myeloid-associated differentiation marker-like protein 2. Intriguingly, one of the five DEGS upregulated both in high and low condition is *slc20a1*/*pit1*, a sodium-dependent phosphate transporter whose main function as a receptor is in viral binding and entry (Farrell et al 2002) as well as in bacterial-induced inflammation (Koumakis et al 2019). Likewise, *hip1r* is one of the two DEGs upregulated in low and medium treatments.

Similar to results obtained by Brandts et al. (2021), a concentration-dependent trend has not been highlighted in our study, following a level of severity of low >> high > medium. Low concentration corresponded to greater effect with 110 DEGs respect to control, high group resulted in 31 DEGs and medium to 17 DEGs. If, on the one hand, a clear concentration-dependent trend is not evident, on the other, gene expression data are coherent with the mortality rate recorded in our experiment. In fact, higher mortality (13.9 %) was recorded in the low group, followed by medium and high (8.3 %, both).

GO enrichment test on low-treated animals revealed 27 enriched pathway, in particular, NPs seems to exert a depressive effect on both hemostasis mechanisms (platelet degranulation down-regulated) and adaptive immune system (5 DEGs, down-regulated), an activation of visual perception and post-translational protein modification (6 DEGs, both up-regulated), response to calcium ion, bacterium, cold-induced thermogenesis (4 DEGs), endocytosis, gene expression, negative regulation of osteoblast differentiation and lipid metabolism (4 DEGs). Also, cellular component-related GO (33%) dominated by intra- and extra-cellular region and molecular function (22%) including calcium ion binding (10 DEGs) resulted significantly enriched.

5. Conclusions

In this study, we contribute in understanding the cascade of events that are activated by the exposure to PS-NPs in the clownfish *A. ocellaris* juveniles. Full comprehension of NP impact on the life cycle of marine fish could represent an important turning point to predict the long term effects of NP production and uncontrolled waste on marine biodiversity. These findings led us to propose that the NPs at low concentration elicit higher toxicogenomic response than medium and high concentrations, likely due to NP aggregations occurring in sea water. Remarkably, the viral infection-specific molecular response may reflect the degree of physical similarity between NPs, as those used in this

work (100 nm), and viruses (20-300 nm), thus indicating a broad commonality in the way NPs and viruses are perceived by the cells.

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General conclusions

Microplastics

Through a number of experiments, the interactions between different sizes of polyethylene terephthalate (PET) MPs and some other factors, namely the water pH (7.5; 8.0) and food variations (presence/absence) were investigated. Tests were performed on leachates and suspensions of MPs in a multispecies test composed by *Vibrio fischeri* (Beijerinck, 1889), *Phaeodactylum tricornutum* Bohlin, 1898, and *Paracentrotus lividus* (Lamarck, 1816) known to be target species representative of different trophic levels. The toxicity of suspensions of MPs was also explored in juveniles of *Amphiprion ocellaris* (Cuvier, 1830), an iconic coral reef fish better known as clownfish.

In conclusion, comparing the concentrations tested in these studies with the actual levels of MP contamination, a low toxicity is attributable to PET MPs and:

i) Responses were species-specific: bioluminescence of bacteria (detritivore) and growth of algal species (primary producers) resulted not impacted by acute effects; biochemical responses related to oxidative stress in clownfish juveniles (secondary consumers) were statistically not relevant even if exposure to MPs increased the level of stress as suggested by the integrative biomarker responses index; larval stages of opportunistic herbivores (echinoderms) showed increase of embryo toxicity at concentration of 100 mg/L;

ii) Leachates demonstrated to be more toxic than suspensions of MPs;

iii) Smaller size of plastics not always corresponded to a stronger biological response;

iv) pH and food variations modulated the eco-toxicological responses of echinoderms (normal larvae exposed to leachates at pH 7.5 resulted in a significantly shorter mean length of Posterior Oral Arms compared to controls, in all tested particle-size) according to mechanisms not demonstrable in the current state of knowledge;

v) A concentration-dependent response was not evident in clownfish juveniles (the lower concentration of MPs induced effects 1.87 times greater than the higher concentration).

The work of this thesis confirms the complexity of MP toxicity mechanism, able to be influenced by different stressors highlighting the difficulty to make *a priori* predictions and suggesting the need of taking them into account when an environmental risk assessment is carried out, especially in the future frame of global changes.

Nanoplastics

Respect to MPs, nano-fraction of the marine litter represents a new infant field of investigation. Despite the impetus research is having in recent years, there are still many open questions on the subject. The review fully reported in this thesis aimed to highlight some on these. The first reason of reflection has regarded the definition of NPs: are NPs plastic particles smaller than 100 nm or than 1 μm? The second huge gap was represented by the quantification of the phenomenon; it still remains unclear how many NPs are in the ocean and what are the rates of degradation. Taking into account the results obtained in laboratory experiments on different organisms, the framework on NPs toxicity seems to be alarming. However, how benthic or endo-benthic organisms could be affected by this new form of contamination remains poorly investigated and results obtained are all related to PS (about 97%) or PMMA NPs (an aspect which poses a debate regarding the availability of good reference materials). In conclusion, the current level of knowledge does not make it possible to take a position about the risk that NPs pose to the biodiversity and functioning of natural ecosystems.

The experiment performed on clownfish juveniles attempted to partially fill the knowledge gap about the effects of NPs in marine fishes (an overlooked target) and highlighted that:

i) working with environmentally relevant concentrations reduces the possibility to estimate the NP uptake, strongly limiting the assessment of eco-toxicological risk;

ii) sensitive biomarkers such as catalase, glutathione S-transferase, glutathione reductase and TOSC Assay (hydroxylic and peroxylic) were not significantly altered;

iii) the integrated analysis of biomarker responses reported a reduction in overall health status of fishes tested with NPs demonstrating how the IBRv2 index may represent

a quick, useful and informative approach especially in discriminating against any differences between treatments that cannot be identified by statistical analysis on individual biomarkers;

iv) molecular analysis revealed that a concentration-dependent trend was not evident following the level of severity of low >> high > medium group. This pattern was consistent with the mortality rate and supported by the analysis of particle size distribution;

v) low concentration elicited higher toxicogenomic responses (altering 110 DEGs and enriching 27 GO) likely due to NP aggregations occurring in sea water;

vi) The genes showing higher change in transcriptional levels were predominantly factors involved in viral infection and inflammatory response. To less extent, other affected gene functions are implicated in ciliogenesis, energy metabolism, olfactory and reproductive functions. The detected viral infection-specific molecular response may reflect the degree of physical similarity between NPs (100 nm) and viruses (20-300 nm), thus indicating a broad commonality in the way NPs and viruses are perceived by the cells.

In conclusion, this study detected a minor response in oxidative stress and contributed in understanding the cascade of molecular events that are activated by the exposure to PS-NPs in the clownfish *A. ocellaris* juveniles.

Appendix

Other papers published

Provenza, F., Piccardo, M., Terlizzi, A., & Renzi, M. (2020). Exposure to pet-made microplastics: Particle size and pH effects on biomolecular responses in mussels. *Marine Poll*, *156*, 111228. https://doi.org/10.1016/j.marpolbul.2020.111228

Pignatelli, S., Broccoli, A., Piccardo, M., Felline, S., Terlizzi, A., Renzi, M. (2021). Short-term physiological and biometrical responses of *Lepidium sativum* seedlings exposed to pet-made microplastics and acid rain. *Ecotoxicology and Environmental Safety, 208, 111718*. https://doi.org/10.1016/j.ecoenv.2020.111718

Conference contributions

Piccardo M., Renzi M., Pittura L., Gorbi S., Terlizzi A. The complexity of microplastic toxicity: a case study on PET toxicity in marine organisms. 50th SIBM Conference. Trieste, 8-12 June 2020. Contribution accepted for oral communication but deleted because of SARS-CoV-2 emergency.

Piccardo M., Pittura L., Gorbi S., Terlizzi A., Sordino P., Laudet V. PLASTICNEMO: First eco-toxicological assessment of nanoplastics in the clownfish *Amphiprion ocellaris* (Cuvier, 1830). 50th SIBM Conference. Trieste, 8-12 June 2020. Contribution accepted for poster communication but deleted because of SARS-CoV-2 emergency.

Supplementary materials of

PET microplastics toxicity on marine key species is influenced by pH, particle size and food variations

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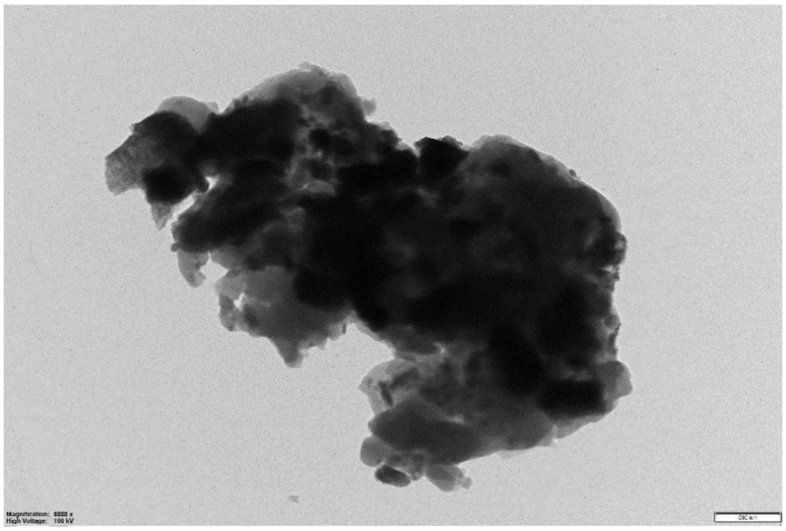


Fig. 1S. TEM image of the smallest fraction of PET microplastic used in the experiments. Jagged edges, surface irregularities and heterogeneity in size are the main features.

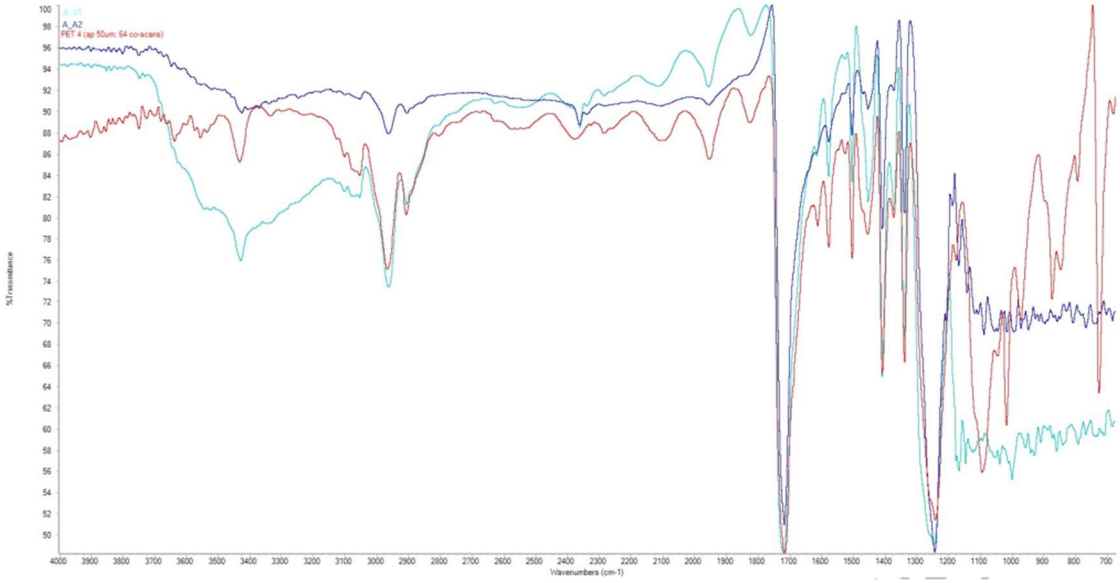


Fig. 2Sa. µFT-IR spectra acquired in transmission mode of PET small MPs (S-PET). Spectra are referred to 72h of exposure to natural standard water. Light blue line means S-PET at pH= 8.0; blue line means S-PET at pH= 7.5; red line means library standard PET spectra.

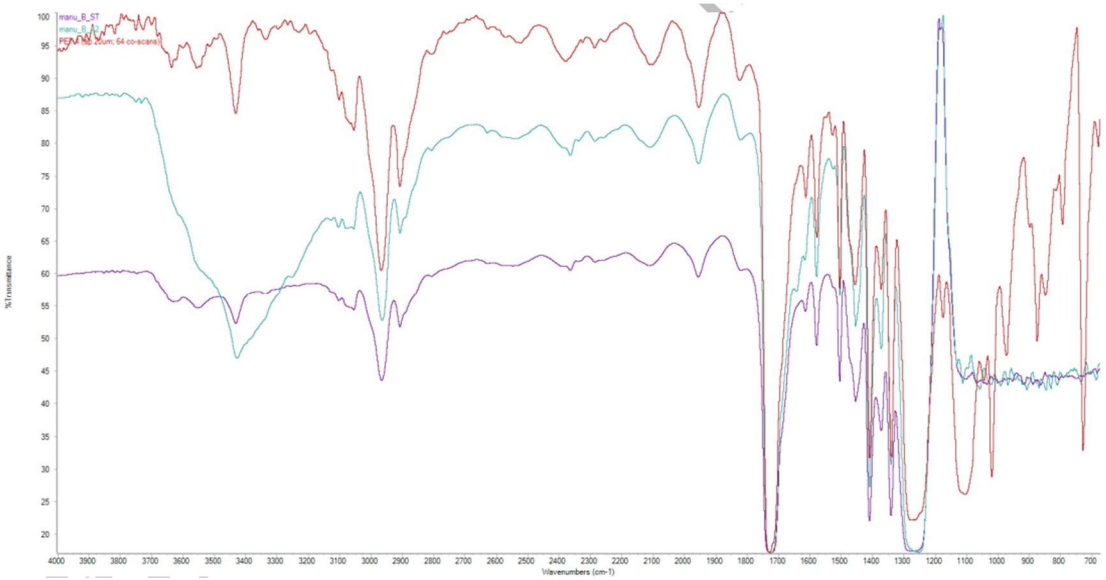


Fig. 2Sb. μFT-IR spectra acquired in transmission mode of PET medium MPs (M-PET). Spectra are referred to 72h of exposure to natural standard water. Purple line means M-PET at pH= 8.0; light blue line means M-PET at pH=7.5; red line means library standard PET spectra.

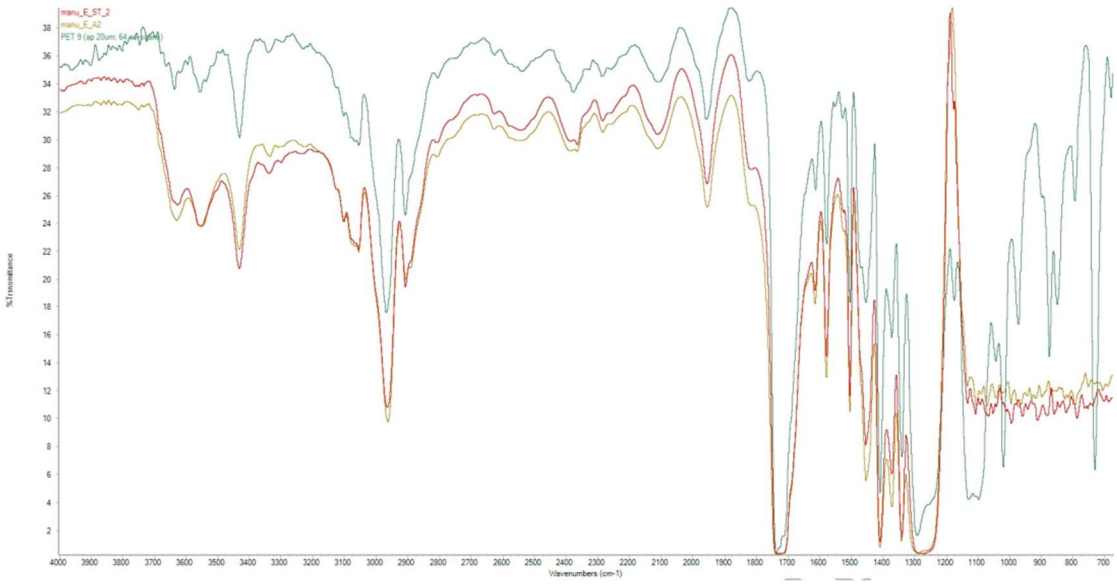


Fig. 2Sc. μFT-IR spectra acquired in transmission mode of PET large microplastics (L-PET). Spectra are referred to 72h of exposure to natural standard water. Red line means L-PET at pH= 8.0; yellow line means L-PET at pH=7.5; green line means library standard PET spectra.

Appendix

Supplementary materials of

Nanoplastics in the oceans: theory, experimental evidence and real world

Manuela Piccardo, Monia Renzi, Antonio Terlizzi

Below is the list of paper published on some aspects related to nanoplastic contamination analysed in this review. Data are updated to February 5th, 2020.

Table 1: eco-toxicological studies on fishes
Table 2: methods of detection
Table 3: human-risk related studies
Table 4: freshwater/terrestrial system studies

Table 1. Eco-toxicological studies with nanoplastics performed on **fishes** from 2006 to 2020.

YEAR OF PUBL.	TITLE	AUTHORS
2006	Distribution of nanoparticles in the see-through Medaka (*Oryzias latipes*)	Kashiwada et al.
2016	Polycarbonate and polystyrene nanoplastic particles act as stressors to the innate immune system of fathead minnow (*Pimephales promelas*).	Greven et al.
2017	Exploring uptake and biodistribution of polystyrene (nano)particles in zebrafish embryos at different developmental stages	Van Pomeren et al.
2017	Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity.	Chen et al.
2017	Enhanced uptake of BPA in the presence of nanoplastics can lead to neurotoxic effects in adult zebrafish.	Chen et al.
2018	Effects of polymethylmethacrylate nanoplastics on *Dicentrarchus labrax*.	Brandts et al.

2018	Maternal transfer of nanoplastics to offspring in zebrafish (*Danio rerio*): A case study with nanopolystyrene.	Pitt et al.
2018	Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (*Danio rerio*).	Pitt et al.
2019	Bioaccumulation of polystyrene nanoplastics and their effect on the toxicity of Au ions in zebrafish embryos.	Lee et al.
2019	Nanoplastics Decrease the Toxicity of a Complex PAH Mixture but Impair Mitochondrial Energy Production in Developing Zebrafish.	Trevisan et al.
2019	Microplastics and nanoplastics: would they affect global biodiversity change?	Hu et al.
2019	Polystyrene nanoplastics alter the cytotoxicity of human pharmaceuticals on marine fish cell lines.	Almeida et al.
2019	Polystyrene nanoplastics (20 nm) are able to bioaccumulate and cause oxidative DNA damages in the brain tissue of zebrafish embryo (*Danio rerio*).	Sökmen et al.
2019	Polystyrene nanoplastics disrupt glucose metabolism and cortisol levels with a possible link to behavioral changes in larval zebrafish.	Brun et al.
2019	Evaluation of the infiltration of polystyrene nanobeads in zebrafish embryo tissues after short-term exposure and the related biochemical and behavioral effects	Paracenti et al.
2019	Ecotoxicological effects on *Scenedesmus obliquus* and *Danio rerio* Co-exposed to polystyrene nano-plastic particles and natural acidic organic polymer.	Liu et al.
2019	Impacts of polystyrene microplastics on the behavior and metabolism in a marine demersal teleost, black rockfish (*Sebastes schlegelii*).	Yin et al.
2020	Establishment of a brain cell line (FuB-1) from mummichog (*Fundulus heteroclitus*) and its application to fish virology, immunity and nanoplastics toxicology.	Ruiz-Palacios et al.

Table 2. Publications on **methods of detections** of nanoplastics, from 2017 to 2020.

YEAR OF PUBL.	TITLE	AUTHORS
2017	Nanoplastic in the North Atlantic Subtropical Gyre.	Ter Halle et al.
2017	Asymmetrical flow field flow fractionation methods to characterize submicron particles: application to carbon-based aggregates and nanoplastics.	Gigault et al.
2018	Detection of nanoplastics in food by asymmetric flow field-flow fractionation coupled to multi-angle light scattering: possibilities, challenges and analytical limitations.	Correia et al.
2018	Laser Ablation as a Versatile Tool To Mimic Polyethylene Terephthalate Nanoplastic Pollutants: Characterization and Toxicology Assessment.	Magrì et al.
2018	Marine debris, plastics, microplastics and nano-plastics: What next?	Agamuthu P.
2019	Synthesis of metal-doped nanoplastics and their utility to investigate fate and behavior in complex environmental systems.	Mitrano et al.
2019	Outlook on optical identification of micro- and nanoplastics in aquatic environments.	Peiponen et al.
2019	Raman Tweezers for Small Microplastics and Nanoplastics Identification in Seawater.	Gillibert et al.
2019	Detection of polystyrene nanoplastics in biological samples based on the solvatochromic properties of Nile red: application in *Hydra attenuata* exposed to nanoplastics.	Gagné et al.
2019	Detection of polystyrene nanoplastics in biological tissues with a fluorescent molecular rotor probe.	Gagné
2019	Cloud-Point Extraction Combined with Thermal Degradation for Nanoplastic Analysis Using Pyrolysis Gas Chromatography-Mass Spectrometry.	Zhou et al.
2019	Challenge for the detection of microplastics in the environment.	Lv et al.

2019	Membrane Processes for Microplastic Removal.	Poerio et al.
2019	Assessing the environmental transformation of nanoplastic through 13C-labelled polymers.	Sander et al.
2019	Trace analysis of polystyrene microplastics in natural waters.	Schirinzi et al.
2019	The marine nano- and microplastics characterization by SEM-EDX: The potential of the method in comparison with various physical and chemical approaches.	Gniadek et al.
2019	Toxicological Evaluation and Quantification of Ingested Metal-Core Nanoplastic by Daphnia magna Through Fluorescence and Inductively Coupled Plasma-Mass Spectrometric Methods.	Vicentini et al.
2019	Separation and Analysis of Microplastics and Nanoplastics in Complex Environmental Samples.	Nguyen et al.
2019	Development of methods for extraction and analytical characterization of carbon-based nanomaterials (nanoplastics and carbon nanotubes) in biological and environmental matrices by asymmetrical flow field-flow fractionation.	Abdolahpur Monikh et al.
2020	Managing the analytical challenges related to micro- and nanoplastics in the environment and food: filling the knowledge gaps.	Alexy et al.

Table 3. **Human risk- related** nanoplastic studies published from 2015 to 2020.

YEAR OF PUBL.	TITLE	AUTHORS
2015	Potential Health Impact of Environmentally Released Micro- and Nanoplastics in the Human Food Production Chain: Experiences from Nanotoxicology.	Bouwmeester et al.
2017	Cytotoxic effects of commonly used nanomaterials and microplastics on cerebral and epithelial human cells.	Schirinzi et al.
2018	Airborne microplastics: Consequences to human health?	Prata et al.
2018	Laser Ablation as a Versatile Tool To Mimic Polyethylene Terephthalate Nanoplastic Pollutants: Characterization and Toxicology Assessment.	Magrì et al.
2018	Plastic contamination of the food chain: A threat to human health?	Waring et al.
2018	Occurrence of microplastics in raw and treated drinking water.	Pivokonsky et al.
2019	Potential adverse health effects of ingested micro- and nanoplastics on humans. Lessons learned from in vivo and in vitro mammalian models.	Rubio et al.
2019	Emergence of Nanoplastic in the Environment and Possible Impact on Human Health.	Lehner et al.
2019	Are Micro- or Nanoplastics Leached from Drinking Water Distribution Systems?	Xu et al.
2019	Internalization and toxicity: A preliminary study of effects of nanoplastic particles on human lung epithelial cell.	Xu et al.
2019	Plastic Teabags Release Billions of Microparticles and Nanoparticles into Tea.	Hernandez et al.
2019	Hazard assessment of small-size plastic particles: is the conceptual framework of particle toxicology useful?	Heddagaard & Møller
2019	In Vitro Genotoxicity of Polystyrene Nanoparticles on the Human Fibroblast Hs27 Cell Line.	Poma et al.

2019	Multi-endpoint toxicological assessment of polystyrene nano- and microparticles in different biological models in vitro.	Hesler et al.

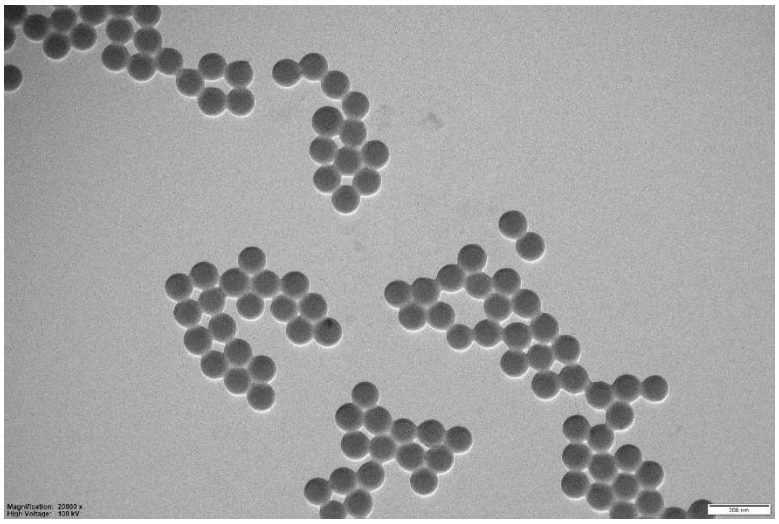
Table 4. Publications on **freshwater/terrestrial system**, from 2017 to 2020.

YEAR OF PUBL.	TITLE	AUTHORS
2017	Microplastic Incorporation into Soil in Agroecosystems.	Rillig et al.
2018	Role of extracellular polymeric substances in the acute inhibition of activated sludge by polystyrene nanoparticles.	Feng et al.
2018	Exposure to polystyrene nanoplastic leads to inhibition of anaerobic digestion system.	Fu et al.
2018	Micro- and Nanoplastic Analysis in Soils.	Bigalke et al.
2018	Effects of polystyrene nanoparticles on the microbiota and functional diversity of enzymes in soil.	Awet et al.
2019	New Perspective on the Nanoplastics Disrupting the Reproduction of an Endangered Fern in Artificial Freshwater.	Yuan et al.
2019	Microplastic effects on plants.	Rillig et al.
2019	Transport of polystyrene nanoplastics in natural soils: Effect of soil properties, ionic strength and cation type.	Wu et al.
2019	Mechanical formation of micro- and nano-plastic materials for environmental studies in agricultural ecosystems.	Astner et al.
2019	Toxicity of microplastics and natural particles in the freshwater dipteran *Chironomus riparius*: Same same but different?	Scherer et al.
2020	Behavior of microplastics and plastic film residues in the soil environment: A critical review.	Qi et al.
2020	Transport of Nano- and Microplastic through Unsaturated Porous Media from Sewage Sludge Application.	Keller et al.
2020	Impact of polystyrene nanoplastics (PSNPs) on seed germination and seedling growth of wheat *(Triticum aestivum L.*).	Lian et al.

Supplementary materials of

Viral-like toxicity of polystyrene nanoplastics on coral reef fish juveniles

M. Piccardo, L. Pittura, R. M. Sepe, F. Capanni, N. Roux, M. Renzi, A. Pallavicini, S. Gorbi, V. Laudet, A. Terlizzi, P. Sordino



Supplementary Fig 1. Image of the nanoplastics tested in the experiment, captured by the Transmission Electron Microscope (magnification 20,000 x, voltage 100 kV).

Transcriptomics analysis

Sequence read processing. On average, 33 ± 6 million reads of 76 bp were generated per sample. No evidence of low-quality reads based on Illumina's scoring was observed spanning the 76 bp of sequence (mean PHRED > 34). However, base sequence content across the reads indicated an abnormal pattern spanned the first 12 bases sequenced. Thus, we removed the first 12 nucleotides improving both the mean PHRED (= 36) and the G/C content.

RNA-seq mapping statistics. Of the total reads, an average of 69.6% (approximately 32 million reads per sample) mapped to annotated portions of the reference genome. Among these, 92.0 % of the reads mapped to genes and 8.0 % to intergenic region.

Supplementary Table S1: All differentially expressed genes from exposures to NPs at low daily concentration (i.e. 0.02 mg/L) with annotations.

Gene name	Log2 fold change	Fold change	*p*-value	FDR *p*-value	product
ccdc92	8.68	409.26	8.54E-05	0.026165	coiled-coil domain containing 92
LOC111570902	4.21	18.55	6.17E-06	4.23E-03	uncharacterized LOC111570902
LOC111566243	2.53	5.76	5.69E-08	1.02E-04	doublecortin domain-containing protein 2-like
clcnka	2.52	5.73	1.05E-04	0.027806	chloride voltage-gated channel Ka
LOC111588247	2.38	5.20	2.34E-06	1.94E-03	olfactory receptor 1-like
LOC111588262	2.37	5.19	1.00E-04	0.027413	olfactory receptor 1F1-like
umodl1	1.80	3.47	6.23E-05	0.02177	uromodulin like 1
LOC111586335	1.75	3.36	6.36E-05	0.02177	uncharacterized LOC111586335
LOC111585732	1.745	3.35	7.36E-09	1.90E-05	sodium-dependent phosphate transporter 1-A-like
LOC111565811	1.67	3.19	2.93E-05	0.012408	huntingtin-interacting protein 1-related protein-like
klhl30	1.61	3.05	4.61E-05	0.01718	kelch like family member 30
LOC111578993	1.53	2.89	1.46E-06	1.34E-03	transmembrane protein 41A-B-like
LOC111586852	1.52	2.87	1.49E-05	7.70E-03	xin actin-binding repeat-containing protein 1-like
LOC111576338	1.41	2.66	2.69E-05	0.012167	AMP deaminase 3-like
LOC111579968	1.38	2.60	3.21E-05	0.013353	actin-binding Rho-activating protein-like
cunh10orf71	1.38	2.60	4.48E-06	3.36E-03	chromosome unknown C10orf71 homolog
trpa1	1.37	2.58	4.19E-07	6.09E-04	transient receptor potential cation channel subfamily A member 1
lrrc2	1.33	2.52	1.02E-05	5.82E-03	leucine rich repeat containing 2
LOC111570226	1.32	2.50	8.95E-05	0.026381	pancreatic alpha-amylase-like
LOC111583785	1.31	2.48	1.38E-04	0.033078	sterol O-acyltransferase 2-like
kbtbd12	1.29	2.45	6.74E-05	0.021804	kelch repeat and BTB domain containing 12
LOC111583080	1.29	2.44	1.50E-06	1.34E-03	serine/threonine-protein kinase ULK1-like
LOC111580169	1.27	2.42	6.69E-05	0.021804	uncharacterized LOC111580169
asb10	1.25	2.39	3.35E-06	2.60E-03	ankyrin repeat and SOCS box containing 10
LOC111585780	1.25	2.38	6.65E-06	4.42E-03	guanylyl cyclase-activating protein 1-like
LOC111563754	1.20	2.30	6.68E-05	0.021804	RNA-binding protein 24
LOC111568594	1.19	2.27	8.83E-05	0.026362	uncharacterized LOC111568594
atg9b	1.18	2.27	1.22E-04	0.03024	autophagy related 9B
psme4	1.18	2.27	1.54E-04	0.035916	proteasome activator subunit 4
fgf19	1.17	2.25	9.20E-05	0.026772	fibroblast growth factor 19
LOC111572085	1.15	2.22	1.21E-04	0.030165	eukaryotic translation initiation factor 4E type 3-like
LOC111562896	1.15	2.22	1.38E-05	7.29E-03	Ectonucleotide pyrophosphatase/phosphodiesterase family member 7-like
LOC111564035	1.13	2.19	4.19E-05	0.015972	UV excision repair protein RAD23 homolog A-like

LOC111567416	1.10	2.14	1.30E-05	7.01E-03	arrestin-C-like
rbp3	1.07	2.10	8.41E-07	9.32E-04	retinol binding protein 3
pde6c	1.07	2.10	5.52E-06	3.90E-03	phosphodiesterase 6C
LOC111572110	1.07	2.10	5.25E-06	3.82E-03	calcium-independent phospholipase A2-gamma-like
LOC111576545	1.07	2.10	4.65E-05	0.01718	potassium voltage-gated channel subfamily V member 2-like
LOC111581091	1.04	2.06	1.73E-06	1.49E-03	ubiquitin-protein ligase E3A-like
LOC111568803	1.03	2.04	3.15E-06	2.53E-03	retinal cone rhodopsin-sensitive cGMP 3'5'-cyclic phosphodiesterase subunit gamma-like
rp1l1	1.01	2.02	2.87E-05	0.012383	RP1 like 1
LOC111566377	1.01	2.01	5.96E-05	0.021329	synaptotagmin-2-like
LOC111568026	0.96	1.95	7.50E-06	4.72E-03	solute carrier family 13 member 5-like
LOC111575224	0.93	1.90	3.99E-05	0.01567	uncharacterized LOC111575224
LOC111565835	0.90	1.86	1.28E-06	1.28E-03	recoverin-like
grk7	0.83	1.78	9.83E-05	0.027291	G protein-coupled receptor kinase 7
LOC111584765	0.83	1.78	2.76E-05	0.012167	guanine nucleotide-binding protein G(t) subunit alpha-1
LOC111586052	0.80	1.74	3.85E-05	0.015464	peripherin-2-like
LOC111569239	0.79	1.73	8.30E-05	0.02612	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3-like
cplx3	0.76	1.69	5.06E-05	0.018416	complexin 3
LOC111563805	0.72	1.65	1.34E-04	0.032527	aquaporin-11-like
gtpbp2	0.71	1.64	1.45E-04	0.034446	GTP binding protein 2
LOC111579831	0.66	1.58	9.85E-05	0.027291	protein tyrosine phosphatase type IVA 3-like
LOC111569776	0.65	1.57	1.92E-04	0.042044	synaptotagmin-2-like%2C transcript variant X1
LOC111579144	-0.71	-1.63	9.72E-05	0.027291	proteasome subunit beta type-7-like
LOC111580715	-0.71	-1.63	2.28E-04	0.048181	glutathione S-transferase omega-1-like
LOC111585886	-0.71	-1.64	1.08E-04	0.02826	transcription factor BTF3 homolog 4-like
LOC111586536	-0.72	-1.65	1.92E-04	0.042044	src-like-adapter
grwd1	-0.73	-1.66	1.90E-04	0.042044	glutamate rich WD repeat containing 1
LOC111588343	-0.75	-1.68	1.77E-05	8.75E-03	keratin type II cytoskeletal 8-like
LOC111564133	-0.76	-1.70	8.51E-05	0.026165	cysteine and glycine-rich protein 1-like
LOC111570736	-0.76	-1.70	6.32E-05	0.02177	prosaposin-like
LOC111563066	-0.77	-1.71	6.73E-05	0.021804	ependymin-1-like
polr2j	-0.79	-1.73	1.02E-04	0.027475	RNA polymerase II subunit J
snrpd1	-0.80	-1.74	8.83E-05	0.026362	small nuclear ribonucleoprotein D1 polypeptide
csrp2	-0.80	-1.74	1.47E-04	0.034476	cysteine and glycine rich protein 2
msmb	-0.85	-1.80	9.38E-05	0.02695	microseminoprotein beta
cdk1	-0.88	-1.84	1.23E-04	0.03024	cyclin dependent kinase 1
s100p	-0.89	-1.86	1.58E-04	0.036135	S100 calcium binding protein P
pfdn1	-0.92	-1.89	1.22E-06	1.28E-03	prefoldin subunit 1
LOC111586621	-0.93	-1.90	2.77E-05	0.012167	protein PBDC1-like
LOC111575620	-0.93	-1.90	7.50E-05	0.023927	H-2 class II histocompatibility antigen E-S beta chain-like
gins1	-0.93	-1.91	1.58E-04	0.036135	GINS complex subunit 1

LOC111564232	-1.02	-2.02	1.13E-04	0.028623	lysozyme g-like
LOC111586496	-1.05	-2.07	2.41E-05	0.011237	zymogen granule membrane protein 16-like
LOC111566251	-1.09	-2.13	1.32E-06	1.28E-03	high affinity choline transporter 1-like
LOC111578844	-1.14	-2.20	1.12E-04	0.028623	dispanin subfamily A member 2b-like
pole2	-1.18	-2.26	4.04E-05	0.01567	DNA polymerase epsilon 2%2C accessory subunit
l3hypdh	-1.19	-2.29	3.31E-05	0.013534	trans-L-3-hydroxyproline dehydratase
btk	-1.21	-2.31	1.56E-05	7.91E-03	Bruton tyrosine kinase
LOC111580431	-1.26	-2.39	2.23E-05	0.010583	putative protein TPRXL
LOC111572448	-1.29	-2.44	1.12E-04	0.028623	saxitoxin and tetrodotoxin-binding protein 2-like
LOC111583651	-1.36	-2.56	1.93E-04	0.042044	transcription factor E2F8-like
LOC111570994	-1.60	-3.03	1.97E-05	9.55E-03	DNA-binding protein inhibitor ID-2-like
kiaa1324	-1.61	-3.06	8.37E-06	5.12E-03	KIAA1324 ortholog
LOC111578451	-1.65	-3.14	7.04E-06	4.55E-03	lectin-like
LOC111586982	-1.98	-3.95	6.17E-07	7.56E-04	alpha-1-antitrypsin homolog
tmem44	-2.02	-4.06	1.20E-05	6.63E-03	transmembrane protein 44
LOC111571004	-2.24	-4.73	5.97E-07	7.56E-04	transient receptor potential cation channel subfamily M member 5-like
LOC111584005	-2.26	-4.80	1.02E-05	5.82E-03	sterol O-acyltransferase 2-like
LOC111571464	-2.31	-4.97	1.65E-04	0.037364	mitotic apparatus protein p62-like
LOC111568967	-2.32	-4.98	1.04E-04	0.027806	taste receptor type 1 member 2-like
LOC111584392	-2.32	-4.99	1.58E-08	3.34E-05	protein disulfide-isomerase-like
LOC111563047	-2.36	-5.13	1.70E-07	2.64E-04	SE-cephalotoxin-like
LOC111583735	-2.36	-5.14	2.17E-04	0.046759	macrophage mannose receptor 1-like
LOC111574167	-2.38	-5.19	2.25E-04	0.047997	protein TsetseEP-like
LOC111575608	-2.54	-5.83	1.01E-05	5.82E-03	secretory phospholipase A2 receptor-like
p2rx4	-2.54	-5.83	1.13E-07	1.88E-04	purinergic receptor P2X 4
LOC111582951	-2.73	-6.62	5.64E-07	7.56E-04	alpha-16-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A-like
LOC111574540	-2.87	-7.33	7.40E-10	2.46E-06	endonuclease domain-containing 1 protein-like
LOC111576402	-3.05	-8.30	1.06E-08	2.47E-05	myosin-11-like
LOC111571592	-3.11	-8.66	1.50E-09	4.36E-06	endonuclease domain-containing 1 protein-like
LOC111571578	-3.17	-9.02	6.61E-07	7.69E-04	endonuclease domain-containing 1 protein-like
LOC111563058	-3.18	-9.05	4.77E-10	1.85E-06	SE-cephalotoxin-like
LOC111574802	-3.27	-9.67	1.87E-10	1.01E-06	CD63 antigen-like
LOC111576475	-3.29	-9.79	1.14E-11	1.20E-07	kallikrein-14-like
LOC111571983	-3.32	-10.01	1.55E-11	1.20E-07	endonuclease domain-containing 1 protein-like
LOC111584333	-3.63	-12.39	2.16E-10	1.01E-06	endonuclease domain-containing 1 protein-like
LOC111588198	-3.65	-12.51	3.76E-08	7.29E-05	myeloid-associated differentiation marker-like protein 2
LOC111571656	-3.73	-13.27	8.70E-14	2.03E-09	protein S100-A1-like

Supplementary Table S2: All differentially expressed genes from exposures to NPs at medium daily concentration (i.e. 0.2 mg/L) with annotations.

Gene name	Log2 fold change	Fold change	*p*-value	FDR *p*-value	product
LOC111570902	4.20	18.34	6.69E-06	0.010384	uncharacterized LOC111570902
LOC111565811	1.95	3.86	7.22E-07	2.80E-03	huntingtin-interacting protein 1-related protein-like
LOC111571552	1.79	3.46	2.77E-06	7.23E-03	bile salt-activated lipase-like
LOC111586722	1.73	3.31	1.14E-08	1.45E-04	chymotrypsin A-like
LOC111570226	1.57	2.98	2.98E-06	7.23E-03	pancreatic alpha-amylase-like
LOC111567950	1.45	2.72	5.82E-06	0.010384	chymotrypsin B-like
LOC111570889	1.42	2.68	4.75E-06	9.21E-03	chymotrypsin-like elastase family member 2A
cpb1	1.31	2.48	6.51E-06	0.010384	carboxypeptidase B1
h6pd	1.02	2.03	8.69E-06	0.012643	hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase
LOC111588436	0.81	1.75	1.60E-05	0.021834	NADPH oxidase organizer 1-like
LOC111585749	-1.40	-2.63	4.48E-06	9.21E-03	cornifelin homolog A-like
xab2	-1.50	-2.83	3.11E-06	7.23E-03	XPA binding protein 2
LOC111587789	-1.57	-2.96	3.56E-08	2.07E-04	interferon-induced very large GTPase 1-like
LOC111584632	-1.73	-3.32	1.83E-08	1.45E-04	carbonic anhydrase 4-like
LOC111576712	-2.04	-4.10	1.87E-08	1.45E-04	phospholipase A2-like
LOC111581215	-2.60	-6.07	2.99E-07	1.39E-03	transcobalamin-1-like
LOC111583625	-4.11	-17.25	2.07E-06	6.88E-03	DNA-directed RNA polymerase II subunit RPB3

Supplementary Table S3: All differentially expressed genes from exposures to NPs at high daily concentration (i.e. 2 mg/L) with annotations.

Gene Name	Log2 fold change	Fold change	*p*-value	FDR *p*-value	product
LOC111574807	5.59	48.22	3.48E-05	0.033721	hemoglobin subunit beta-like
bglap	4.69	25.77	3.09E-06	8.98E-03	bone gamma-carboxyglutamate protein
LOC111570902	4.36	20.52	2.91E-06	8.98E-03	uncharacterized LOC111570902
LOC111574812	2.97	7.85	3.86E-08	8.99E-04	hemoglobin embryonic subunit alpha-like
LOC111574811	2.24	4.71	2.31E-05	0.025446	hemoglobin embryonic subunit alpha
LOC111562555	2.13	4.37	1.09E-06	8.44E-03	actin alpha cardiac
rag1	1.94	3.85	1.58E-06	8.69E-03	recombination activating 1
LOC111574814	1.85	3.60	5.16E-06	0.011801	hemoglobin subunit beta-like
zan	1.55	2.93	5.22E-05	0.04604	zonadhesin
LOC111573988	1.45	2.73	8.34E-06	0.013869	red-sensitive opsin
LOC111583785	1.38	2.60	5.20E-05	0.04604	sterol O-acyltransferase 2-like
LOC111562896	1.26	2.40	1.87E-06	8.69E-03	ectonucleotide pyrophosphatase/phosphodiesterase family member 7-like

LOC111585732	1.22	2.32	5.88E-05	0.04604	sodium-dependent phosphate transporter 1-A-like
LOC111576121	1.06	2.09	1.82E-05	0.021137	sodium-dependent neutral amino acid transporter B(0)AT3-like
slc13a5	1.03	2.04	5.13E-07	5.97E-03	solute carrier family 13 member 5
LOC111575293	0.97	1.96	6.54E-06	0.012691	green-sensitive opsin-like
LOC111584765	0.87	1.83	1.23E-05	0.016814	guanine nucleotide-binding protein G(t) subunit alpha-1
LOC111569239	0.81	1.75	5.93E-05	0.04604	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3-like
LOC111588343	-0.69	-1.62	6.54E-05	0.049137	keratin type II cytoskeletal 8-like
LOC111565353	-0.81	-1.76	1.34E-05	0.017274	leukocyte elastase inhibitor-like
LOC111565413	-0.97	-1.95	1.19E-05	0.016814	leukocyte elastase inhibitor-like
LOC111578169	-1.07	-2.10	2.81E-05	0.028397	fibroblast growth factor-binding protein 2-like
LOC111566839	-1.20	-2.30	4.39E-06	0.011349	N-chimaerin-like
LOC111578451	-1.48	-2.79	5.36E-05	0.04604	lectin-like
LOC111584392	-1.82	-3.52	8.34E-06	0.013869	protein disulfide-isomerase-like
p2rx4	-1.98	-3.94	2.41E-05	0.025446	purinergic receptor P2X 4
LOC111571592	-2.17	-4.49	9.91E-06	0.015376	endonuclease domain-containing 1 protein-like
LOC111584333	-2.18	-4.52	5.81E-05	0.04604	endonuclease domain-containing 1 protein-like
LOC111571983	-2.22	-4.65	5.58E-06	0.011801	endonuclease domain-containing 1 protein-like
LOC111588198	-2.54	-5.81	1.60E-05	0.01959	myeloid-associated differentiation marker-like protein 2
LOC111574344	-6.45	-87.37	2.32E-06	8.98E-03	protein ADP-ribosylarginine hydrolase-like

Supplementary Table S4. GO enrichment test from exposure to low concentration of NPs (0.02 mg/L) relative to up and down-regulated DEGs; GO related to biological process, molecular function and cellular component (with each gene involved) are listed (observed counts > 3; FDR *p*-value ≤ 0.05).

UP-regulated					
	observed counts	**GO_description**	***p* -value**	**FDR**	**genes**
biological process					
GO:0007601	6	visual perception	0.000155	0.001023	LOC111566243, LOC111585780, rbp3, pde6c, LOC111568803, LOC111565835
GO:0007165	6	signal transduction	0.030626	0.043743	LOC111588262, LOC111583080, LOC111585780, fgf19, pde6c, LOC111565835
GO:0043687	5	post-translational protein modification	0.000186	0.001173	klhl30, kbtbd12, asb10, psme4a, fgf19, LOC111579831
GO:0009617	4	response to bacterium	0.000865	0.003124	LOC111570226, LOC111568594, atg9b, fgf19
molecular function					
GO:0005509	6	calcium ion binding	0.005924	0.012109	umodl1, LOC111570226, LOC111585780, LOC111568594, LOC111565835, LOC111569776
cellular component					
GO:0005623	8	obsolete cell	0.029745	0.043112	umodl1, LOC111570226, LOC111585780, LOC111568594, LOC111565835, LOC111569776
GO:0031410	4	cytoplasmic vesicle	0.014749	0.024283	umodl1, LOC111583080, atg9b, LOC111581091
GO:0001750	4	photoreceptor outer segment	6.79E-05	0.000798	LOC111566243, LOC111585780, pde6c, LOC111565835

DOWN-regulated					
	observed counts	**GO_description**	***p* -value**	**FDR**	**genes**
biological process					
GO:0002576	10	platelet degranulation	1.80E-11	5.81E-09	LOC111586536, LOC111570736, LOC111586982, LOC111583735, LOC111574540, LOC111571592, LOC111571578, LOC111574802, LOC111571983, LOC111584333
GO:0002250	5	adaptive immune response	2.41E-05	0.000423	LOC111586536, LOC111575620, btk, LOC111571004, LOC111584005
GO:0043312	5	neutrophil degranulation	0.010649	0.019936	LOC111570736, LOC111586982, LOC111571004, LOC111584005, LOC111574802
GO:0006260	4	DNA replication	6.21E-05	0.000631	grwd1, gins1, pole2
GO:0014070	4	response to organic cyclic compound	0.00676	0.014447	LOC111586536, LOC111570994, LOC111583735
GO:0030335	4	positive regulation of cell migration	0.020644	0.032908	LOC111586536, LOC111582951, LOC111574802, LOC111588198
GO:0001701	4	in utero embryonic development	0.025438	0.038882	LOC111585886, LOC111566251, LOC111578844, LOC111576402
GO:0015031	4	protein transport	0.034377	0.049417	LOC111585886, LOC111566251, LOC111578844, LOC111576402
molecular function					
GO:0003676	5	nucleic acid binding	0.000102	0.000862	LOC111574540, LOC111571592, LOC111571578, LOC111571983, LOC111584333
GO:0004519	5	endonuclease activity	5.56E-07	5.37E-05	LOC111574540, LOC111571592, LOC111571578, LOC111571983, LOC111584333
GO:0005516	4	calmodulin binding	0.007595	0.015811	LOC111571004, LOC111584005, LOC111582951, LOC111576402
cellular component					
GO:0005576	15	extracellular region	0.000427	0.002032	LOC111570736, LOC111563066, LOC111564232, LOC111572448, LOC111586982, LOC111583735, LOC111575608, LOC111582951, LOC111574540, LOC111571592, LOC111571578, LOC111574802, LOC111584333, LOC111571983, LOC111576475
GO:0070062	13	extracellular exosome	0.018881	0.030552	LOC111580715, LOC111586536, LOC111570736, LOC111586982, LOC111582951, LOC111574540, LOC111576402, LOC111571592, LOC111571578, LOC111574802, LOC111571983, LOC111584333
GO:0005765	6	lysosomal membrane	0.000322	0.001778	LOC111570736, LOC111575620, LOC111578844, LOC111571004, LOC111584005, LOC111574802
GO:0005764	5	lysosome	0.001775	0.005515	LOC111586536, LOC111570736, LOC111571004, LOC111584005, LOC111574802
GO:0045121	5	membrane raft	0.009819	0.019084	LOC111586536, btk, LOC111571004, LOC111584005, LOC111588198
GO:0043204	4	perikaryon	0.014466	0.024954	LOC111566251, LOC111571004, LOC111584005, LOC111582951

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