



Teratogenic effects of environmental concentration of plastic particles on freshwater organisms

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ABSTRACT

Given the widespread presence of plastics, especially in micro- and nanoscale sizes, in freshwater systems, it is crucial to identify a suitable model organism for assessing the potential toxic and teratogenic effects of exposure to plastic particles. Until now, the early life stage of freshwater organisms and the regeneration capacity in relation to plastic particles exposure is a still poorly investigated topic. In this study, we examine the teratogenic effect on diatom *Cocconeis placentula* and cnidarian *Hydra vulgaris* under controlled exposure conditions of poly (styrene-co-methyl methacrylate) (P(S-co-MMA)) particles. Significant effects were observed at the lowest concentrations (0.1 µg/L). A significant increase in the teratological frequency in *C. placentula* and a significant decrease in the regeneration rate in *H. vulgaris* were found at the lowest concentration. The delay in hydra regeneration impaired the feeding capacity and tentacles reactivity at 96 h of exposure. No effects on diatom growth were observed upon exposure to P(S-co-MMA) particles (0.1, 1, 100, 10,000 µg/L) for 28 days and these findings agree with other studies investigating algal growth. The application of the Teratogenic Risk Index, modified for diatoms, highlighted a moderate risk for the lowest concentration evaluating *C. placentula* and low risk at the lowest and the highest concentrations considering *H. vulgaris*. This study suggests the importance of testing organisms belonging to different trophic levels as diverse teratogenic effects can be found and the need to evaluate environmentally relevant concentrations of plastic particles.

1. Introduction

Over the last few decades, plastic litter has become an increasing environmental concern as it accumulates in every environment on earth (Windsor et al., 2019; Cera et al., 2020a). World plastic production enhanced by over 300 million tonnes in 2020 and its production is expected to four-fold increase by 2050 (PlasticsEurope, 2020). The main properties that have made this material highly required worldwide are its high resistance and durability (Li et al., 2020a). However, due to low recycling rates and inadequate disposal practices, plastic waste enters and accumulates in aquatic ecosystems through various pathways (González-Fernández et al., 2021; Cesarini et al., 2023b).

One of the major threats is represented by the smallest particles, known as nanoplastics (NPs) and microplastics (MPs), which given their

sizes, can enter organisms and transfer along the food web (Koelmans, 2019). NPs include all plastic particles with a size between 1 and 1000 nm and MPs between 1000 nm – 5 mm (Gigault et al., 2018; Gallitelli et al., 2021). The primary sources of NPs and MPs are represented by commercial objects for daily use such as personal care products (cosmetics, lens cleaner, cleaning products, sunscreens), medicine items (drug delivery), biomedical and technical applications (electronics, biosensors) (Vance et al., 2015; Cera et al., 2020a; Gonçalves and Bebianno, 2021). The secondary sources of NPs and MPs are biodegradation, photodegradation, mechanical abrasion, weathering, and thermal oxidation degradation of larger-sized particles (Andrady, 2011; Wang et al., 2021). Both primary and secondary sources of NPs/MPs are leading to a rapidly increasing release and accumulation of countless plastic particles into aquatic ecosystems through sewage systems and

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inadequate disposal (Alimi et al., 2018). Given their sizes, among all plastics NPs are the less detectable in field and consequently the hardest to be removed from the environment (Zhang et al., 2021). The nano-micro size dimensions facilitate their uptake and translocation among organs and tissues in biota, from lower trophic level organisms to higher level organisms through the biomagnification process (Chae et al., 2018; Kukkola et al., 2021; Uddin et al., 2021).

This specifically raises concerns since aquatic organisms are exposed to the adverse effects produced by the direct (NPs/MPs ingestion and uptake) or indirect exposure (through predation and biomagnification) of NPs/MPs and their leaching additives (Casabianca et al., 2021; Town and van Leeuwen, 2020; Yin et al., 2021). Furthermore, due to the largest surface/volume ratio compared to larger size, NPs and MPs have the ability to adsorb on their surface a higher quantity of hazardous pollutants (persistent organic pollutants, heavy metals, endocrine disrupting chemicals) from the surrounding environment (Wan et al., 2018; Shen et al., 2019; Yu et al., 2019), causing biochemical harms still poorly investigated and known (Auta et al., 2017). This characteristic increases the reactivity of these plastic sizes in aquatic systems with different organic compounds and emphasizes the importance of NPs and MPs surface chemistry in interacting with biological systems (Tallec et al., 2019; Gigault et al., 2021; Liu et al., 2021).

Among all ranges of plastic polymers, polystyrene (PS), along with polypropylene (PP), polyethylene (PE), and polyethylene terephthalate (PET), is among the most commonly observed polymer types in aquatic environments (Cera et al., 2022; PlasticsEurope, 2020). It is frequently used for packaging and disposable utensils, and PS fragments have been abundantly documented to be found in fresh, marine water, and sediments (Wan et al., 2018). PS is frequently used in combination with other polymers to give higher mechanical and resistance properties forming a copolymer (Xu et al., 2003). A common copolymer of PS, is poly(styrene-co-methyl methacrylate) (P(S-co-MMA) nanocomposite, constituted by two components, styrene and methyl methacrylate, used for the realization of sophisticated optical, biomedical and technological products (e.g., contact lenses, dental and bone cements, and displays) (Otto et al., 2008; Schutzmann et al., 2008; Thomas et al., 2022). Considering the significant applications of this nanocomposite, its direct discharge into the environment, or indirect discharge through biomedical, industrial or urban wastewater of items containing nanosized plastics, can result in the release of P(S-co-MMA) into aquatic ecosystems (Das et al., 2021; Thomas et al., 2022).

Often the shape of NPs and MPs investigated is spherical given the facility of production and the concentrations used in laboratory studies are extremely high to obtain a result, thus losing ecological significance and link with the environmental situation (Stapleton, 2019; Zhang et al., 2021). The shapes of plastics mainly found in the environment are elongated, broken edges, and irregular (fibres, ellipses, granules) than perfect spheres (Baldwin et al., 2020; Boyle and Örmeci, 2020; Li et al., 2020b; Corami et al., 2022; Cesarini et al., 2022). Kukkola et al. (2021) suggest testing lower exposure concentrations of NPs/MPs and extending the time of exposure to better mimic environmentally relevant exposure. Some authors (Liu et al., 2020; Zhang et al., 2021; Seena et al., 2022; Xiong et al., 2023) suggested that environmental concentrations of NPs are in the range of 1–25 µg/L and MPs between 0.1 and 1 mg/L.

Until now, the attention to NPs and MPs was mainly focused to investigate ingestion and toxicity effects, while other aspects are little investigated, such as the assessment of NP/MP exposure to early life stages, in particular on freshwater organisms. Teratogens are defined as physical, chemical, and biological agents that cause malformations in embryos or fetuses reducing individual fitness within a population (Wilson, 1973). Many laboratory studies investigate only one trophic level and secondary consumers, such as fish fauna that are the most studied, so there is a need to explore other trophic levels, like primary producers and different organisms (Kukkola et al., 2021). Here, we are interested in assessing the teratological effects of plastic particles on two species belonging to different trophic levels, primary producers and

secondary consumers. Diatoms and hydras are, indeed, both organisms sensitive to teratogens. Exposure to various teratogenic substances can cause modifications to the frustule (siliceous cell wall) of diatoms and to hydras in different ways, resulting in teratological forms and aberrations (Trottier et al., 1997; Falasco et al., 2009a; Pandey et al., 2017; European Commission, 2018; Marcheggiani and Puccinelli, 2019; Puccinelli et al., 2019a).

Microalgae (including diatoms) and invertebrates are benthic organisms that commonly colonize plastic in the environment (Zettler et al., 2013; Wilson et al., 2021; Nava et al., 2022; Taurozzi et al., 2022; Gallitelli et al., 2023). Diatoms are a key component in all types of aquatic ecosystems, at the base of food webs, considered responsible for 25 % of oxygen production and 50 % of global organic carbon fixation (Stevenson, 2014). Many invertebrates feed on diatoms and in this way, they are integrated into aquatic food webs (Taylor et al., 2007). Diatoms are used with other biological elements to assess the ecological status according to Water Framework Directive 2000/60/EC for their sensitivity to the variation of nutrients and organic pollution (CEC - Council of European Communities, 2000; Almeida et al., 2014; Puccinelli et al., 2019b). Hydras were used to evaluate teratogenic risk of chemicals contaminating freshwaters, such as heavy metals, for their high sensitivity (Trottier et al., 1997; Traversetti et al., 2017; Cera et al., 2020b). Thus, this study aimed to evaluate the teratological effects of P(S-co-MMA) plastic particles exposure to freshwater diatom, *Cocconeis placentula*, and cnidarian, *Hydra vulgaris*, under expected environmental concentrations. Specifically, for diatoms were evaluated growth rate and teratological frequency, while for hydras were evaluated regeneration rate, aberration frequency, feeding capacity and tentacles reactivity after regeneration. The concentrations chosen to conduct the teratogenicity assays were selected to start from a predicted environmental range of concentrations (Liu et al., 2020; Zhang et al., 2021; Seena et al., 2022).

2. Materials and methods

2.1. Polymeric particles preparation

Poly(styrene-co-methyl methacrylate) (462896-250G Lot#08119EJ; average Mw 100,000-150,000, pellets, styrene ~40 mol%; Aldrich) was used to prepare polymeric particles suspension for teratogenicity assays. The preparation process of plastic particles was carried out in Materials Chemistry Laboratory (LaChIM), following the OBM method (Chronopoulou et al., 2009). Briefly, 200 mg of polymer was dissolved in 10 mL dimethylformamide (DMF, C₃H₇NO, Merck) on a stirring plate for 24 h, then the solution (4.5 mL) was transferred into dialysis cellulose membranes (width 10 mm, D9277-100FT dialysis tubing cellulose membrane flat, Sigma Aldrich) for 96 h and further immersed into distilled water (200 mL). The procedure was carried out at constant temperature ($T = 24\text{ }^{\circ}\text{C}$), the precipitated polymer was recovered and used without size separation.

2.2. Polymeric particles characterizations

A drop of the suspension was deposited onto a corn glass substrate and then metallized for scanning electron microscopy (SEM) investigation. A Gemini 300 field emission SEM (Carl Zeiss AG, Jena, Germany) was used, at the electron microscopy laboratory of Roma Tre University (LIME, Rome, Italy), to characterize the particles suspension in terms of morphology and size (Fig. 1). Images were studied and edited on Fiji software. The images were adjusted by colour threshold, hue, saturation, and brightness, increasing the contrast and highlighting the item edges to be measured. Particles showed spheroidal shape, elliptical shape, and elongated shape (Fig. 1a). Particles had a size range from 100 nm and 2800 nm distributed in 89 % of NPs (<1000 nm) and 11 % of MPs (>1000 nm) (Fig. 1b). The weighted average size was 425.70 ± 175.02 nm, obtained by counting 16,463 particles. At the end of the procedure,

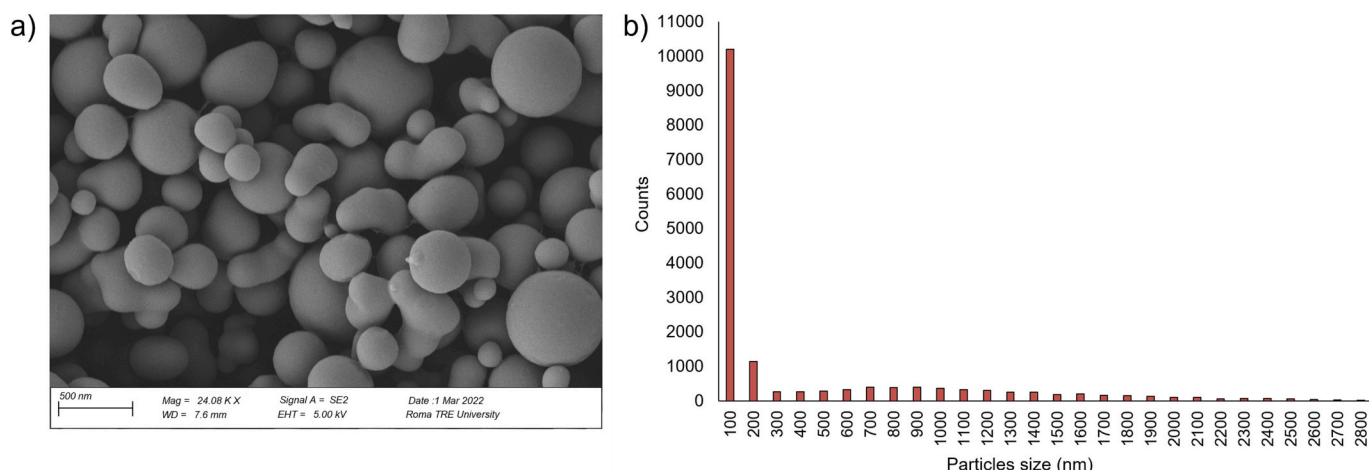


Fig. 1. (a) SEM image of poly(styrene-co-methyl methacrylate) particles. (b) Histogram showing the frequency distribution of the particles size.

the particles were washed and dried, then redispersed in water.

Moreover, Zetasizer Nano S® from Malvern Instruments equipped with a backscatter detection system at 173° was used for Dynamic Light Scattering (DLS) and Zeta potential (ζ pot) experiments, using quartz cuvettes at 25 °C. Samples at different concentrations in distilled water or culture media were tested. Hydrodynamic properties and characteristics were studied by DLS and ζ pot experiments, to assess the P(S-co-MMA) particles behaviour in solution. The diffusion coefficient was used to calculate the hydrodynamic mean diameter $\langle 2RH \rangle$ and Polydispersity Index (PDI) of the water and buffer media dispersed samples at different concentrations, before and after sonication. After sonication, the hydrodynamic diameter and PDI decreased and comparing different concentrations (0.1–100 and 10,000 $\mu\text{g/L}$), it was observed that by increasing the concentration, aggregation phenomena became more evident; broad and multimodal distributions in the size range 200 nm up to microns were observed with PDI values higher than 0.65 in all samples. High PDI values suggest a broad and non-monomodal distribution of P(S-co-MMA) particles size in solution in the investigated samples (Stetefeld et al., 2016). Aggregation phenomena were observed in more concentrated samples confirming a poor dispersion with the presence of big particles. Moreover, ζ pot measurements evidenced values at around -10 mV for the investigated samples with poor colloidal stability (Bhattacharjee, 2016). In Supplementary Information (Fig. S1) the DLS and Zeta potential measurements on distilled water suspensions of P(S-co-MMA) particles are reported.

DLS measurements of the samples dispersed in cell culture media, reported in Fig. S2, evidenced adsorption effects of the medium components and high fluctuations of $\langle 2RH \rangle$ values were observed, with PDI values higher than 0.65, even in the lower concentration. ζ pot measurements did not meet quality criteria probably due to the high polydispersity of the samples together with the presence of ions related to the culture media formulation.

2.3. Exposure conditions

A total of 4 concentrations of plastic particles were selected: 0.1, 1, 100, 10,000 $\mu\text{g/L}$ plus a control consisting only of diatom or hydra medium depending on the organism used. Stock solutions (i.e., 10,000 $\mu\text{g/L}$) were diluted in the test medium to reach the final concentrations. The stock solution was obtained by placing 5 mg of particles in a 500 mL flask containing culture media. The different concentrations were obtained by collecting an aliquot, previously stirred, of the stock solution calculated according to the formula for dilutions $C_1V_1 = C_2V_2$ (C = concentration, V = volume, 1 = start solution, 2 = final solution). Before the exposure, particle solutions were bath sonicated (Ultrasonic Cleaner 375H) for 2 min to ensure homogenization.

2.3.1. Diatom culture and teratogenicity assay

The freshwater diatom *Cocconeis placentula* var *lineata* Ehrenberg 1885 was selected for its important characteristics: i) widespread in freshwater ecosystems, pioneer species (Almeida et al., 2014; Marcheggiani et al., 2019) found as the most abundant species with a high percentage of teratological forms in a previous study (Cavallo et al., 2021); ii) teratological forms easy to identify given its size (Falasco et al., 2009a; Esquius et al., 2012; Lavoie et al., 2017; Falasco et al., 2021).

The *C. placentula* inoculum was purchased from the University of Texas at Austin (UTEX). The diatoms were cultured in Ag diatom medium (micronutrients, NO_3 , biotin, thiamine, vitamin B12; see Table S1) and maintained at 18 ± 1 °C and a 12:12 h light-dark cycle photoperiod under cool-white fluorescent bulbs (3000 lx).

All exposure experiments were performed in 50 mL glass bottles containing *C. placentula* at the initial growth phase with a starting concentration of 1.0×10^4 cells/mL in AG diatom medium using an automated cell counter (TC20, Bio-Rad). Diatoms were exposed to P(S-co-MMA) particles at 20 mL of the 4 concentrations plus control, each tested in triplicate, for 28 days to evaluate teratogenic forms. This time was selected because the teratological forms can be observed only following the life cycle of diatoms (Falasco et al., 2009a). Diatoms were manually aerated by rotating the glass bottles every day for the duration of the experiment. During the teratogenicity assay, the growth inhibition was also tested and 1 mL of diatom from each experimental condition was collected once a week. Samples were stored with Lugol's iodine solution at 4 °C. Cell concentration was determined by using a Sedgewick Rafter counting chamber (Marienfeld Superior) under a microscope at 40 \times magnification (MOTIC BA410E). Both growth rate (μ) and inhibition of growth rate (μ_i) were determined. Specifically, growth rate was calculated using the equation: $\mu = \ln(N_1/N_0)/(t_1 - t_0)$, where N_1 and N_0 are the biomass at time 1 and time 0, respectively (Wood et al., 2005). Inhibition of growth rate (μ_i) was then determined by calculating the equation mean value of $(\mu_c - \mu_i / \mu_c) \times 100$, where μ_c is referred to control and μ_i to the other treatment conditions.

After 28 days of exposure, 5 mL taken from each experimental condition were treated as indicated by standard protocols (ISPRA, 2014), oxidized using H_2O_2 and HCl then fixed on a glass slide, using a mountain resin with a high reflective index (Naphrax) and morphologically analyzed under a microscope at 100 \times magnification to investigate teratological forms. The teratogenic parameters considered were the Teratological Frequency (TF) and the type of aberrations. TF was calculated as the ratio between the relative frequency of diatom valves with morphological aberrations and the total number of diatom valves. The classification of abnormal forms followed the 7 types recorded by Falasco et al. (2009a, 2009b). Moreover, a Teratogenic Risk Index (TRI)

has been developed for diatoms, modifying the already existing one for hydras. Therefore, the value of TF (vertical entry) and GR (horizontal entry) were inserted in a double-entry matrix and their match provided the score of the TRI, divided into 5 risk classes from no risk to very high risk (Table S2). The GR range has been set considering the highest growth value observed in our experiments.

2.3.2. Hydra culture and teratogenicity assay

The freshwater cnidarian *Hydra vulgaris* Pallas 1766 was selected due to its: (i) high sensibility to a variety of contaminants (organic and inorganic); (ii) morphological aberrations easy to identify and regenerative abilities (Trottier et al., 1997; Wilby, 1988; Quinn et al., 2012; Traversetti et al., 2017).

The *H. vulgaris* organisms used in this study belong to an established laboratory culture maintained at the Department of Sciences, University Roma Tre. The culture was reared in hydra medium (H_2O , CaCl_2 , NaHCO_3 ; see Table S3) at $18 \pm 1^\circ\text{C}$ and 18:6 h light-dark photoperiod within $30 \times 30 \times 30$ cm glass tanks. The hydra cultures were fed ad libitum with *Artemia salina* nauplii, obtained from commercially available cysts. After feeding, hydra cultures were gently washed to remove undigested food and transferred into tanks with clean medium.

The teratogenicity assay was performed in glass Petri dishes exposed for 96 h to the 4 different concentrations of plastic particles and under control. Before the start of teratogenicity assay, hydras without buds were cut by a bistoury under a stereomicroscope (Nikon C-LEDS) in two parts: *hypostoma* (mouthpart, head, and tentacles) and *columna* (remaining body portion). Each treatment (including control) was conducted in 3 replicates and for each replicate, 5 *columnae* were placed in 10 mL of tested solution. During the entire exposure, there was no media turnover or food supplied. Organisms were checked, under a stereomicroscope, to assess the regeneration every 24 h until the end of assay, 96 h, the time to regenerate a whole specimen (Wilby, 1988). The teratogenic parameters morphologically considered were the regeneration rate (RR) and aberration frequency (AF), which were scored and classified according to the method outlined by Traversetti et al. (2017). AF was computed as the ratio between the relative frequency of individuals with morphological aberrations and the total number of individuals. The AF value is the vertical entry and RR value the horizontal entry of a double-entry matrix and their match provided the TRI score (see Traversetti et al., 2017; Table S4). At each TRI score corresponds a class of teratogenic risk, the same used for diatoms, associated with different particle concentrations.

In addition to morphological biomarkers, behavioural endpoints were also considered. Post-exposure feeding and reactivity of tentacles were assessed to obtain information about the complete regeneration of the nervous system. Each hydra, after 96 h, was transferred alone in a well with 2 mL of exposure medium. The feeding rates were evaluated by providing 10 *Artemia* nauplii to each hydra. After 30 min, the remaining brine shrimp were counted, and feeding was assessed evaluating the difference between the initial 10 nauplii and the remaining (Venancio et al., 2021). The reactivity was evaluated by pricking the tentacle with a thin pin and evaluating if it was bent and brought towards the mouth.

2.4. Data analysis

The normality of each data set was checked by Shapiro-Wilk test. If data was parametric, a One-Way ANOVA followed by Dunnett's test was applied to assess significant differences between control and P(S-co-MMA) particle concentrations. If data was non-parametric, Kruskal-Wallis test was applied, followed by Dunn's test. Correlation analyses were performed between values of Teratogenic Risk Index, teratological/aberration frequency and growth/regeneration rate to evaluate what parameter was correlated between the Index. The statistical analysis was conducted with GraphPad Prism version 8.0.1 and the significance was set at $p < 0.05$. All statistical analyses and relative

results are shown in Table S5.

3. Results

3.1. Teratogenic effects of nanoplastics on freshwater diatom *Cocconeis placentula*

There was a significant increase (K-W = 9.667; $p < 0.05$) in the teratological frequency (51 %) of *C. placentula* in the lowest concentration (0.1 $\mu\text{g/L}$) compared to the control (Fig. 2). In the highest concentration, the teratological frequency was 32 %, although with no statistical difference (Dunn's; $p = \text{ns}$). At intermediate concentrations (1–100 $\mu\text{g/L}$), it was possible to see the lowest teratological frequencies of particles exposure (30 % and 24 %, respectively). Teratological forms have also been observed in the CTL but with a very low frequency $< 10\%$ negligible, due to artificial growth conditions.

In Fig. 2 are presented the types of teratological forms found in *C. placentula* after the P(S-co-MMA) particles exposure of 28 days. Overall, the most common teratological form was the deformed valve outline (Type 1) with the highest percentage (46 %), followed by the changes in the shape and size of the longitudinal and central area (Type 3) with 35 % and by mixed type in which one valve shows more than one kind of teratology (Type 7) with 16 %. Sporadic teratological form characterized by modifications of the raphe (Type 4) with only 3 % was observed. The other types of teratological forms were not found.

Concerning growth rates and inhibition, no effects on diatom growth were observed after the exposure to P(S-co-MMA) particles for 28 days (One-way ANOVA = 1.433; $p = \text{ns}$ and K-W = 2.684; $p = \text{ns}$, respectively). Average growth rates were in the range of 0.52–0.68 between exposed diatoms and control (Fig. 3).

3.2. Teratogenic effects of nanoplastics on freshwater cnidarian *Hydra vulgaris*

The results of the 96 h regeneration assay with *H. vulgaris* are shown in Fig. 4. The AF between treatments and control has been found not to be significant (K-W = 4.948; $p = \text{ns}$).

Data on feeding capacity and tentacles reactivity are reported in Fig. 4. The tentacles' reactivity was impacted only in the lowest concentration (K-W = 34.53; $p < 0.0001$), characterized by 27 % of reactivity. Regarding the feeding assay carried out after the regeneration assay showed significant differences (K-W = 38.79; $p < 0.0001$). Specifically, hydras exposed to the lowest concentration were no able to ingest *Artemia* nauplii (Dunn's; $p < 0.0001$), at 1 $\mu\text{g/L}$ ingested with an average of 1.13 nauplii, at 100 $\mu\text{g/L}$ ingested as the control group with an average of two nauplii, and at the highest concentration ingested an average of 1.6 nauplii.

Regarding RR, a significant difference was found in the case of the lowest concentration (RR = 3.33) compared to the control (K-W = 40.36; $p < 0.0001$; Fig. 5). At intermediate and highest concentrations, it was possible to see a similar regeneration trend with respect to the control group (RR = 3.93 for 1 $\mu\text{g/L}$ and RR = 4 for 100 and 10,000 $\mu\text{g/L}$; Fig. 5).

3.3. Application of teratogenic risk index on nanoplastic exposure

The application of the modified TRI for diatoms highlighted a moderate risk for the lowest concentration 0.1 $\mu\text{g/L}$, while the other concentrations resulted in low risk (Fig. 6a). Investigations of correlations between diatom TRI scores and both Teratological Frequency (TF) and Growth Rate (GR) revealed a significant relationship only between TRI and TF (Pearson $r = -0.96$, $p < 0.01$).

The application of the TRI for hydras highlighted a low risk for the lowest (0.1 $\mu\text{g/L}$) and highest concentration (10,000 $\mu\text{g/L}$), while no risk resulted in the other concentrations (Fig. 6b). Investigations of correlations between hydra TRI scores and both AF and RR revealed a

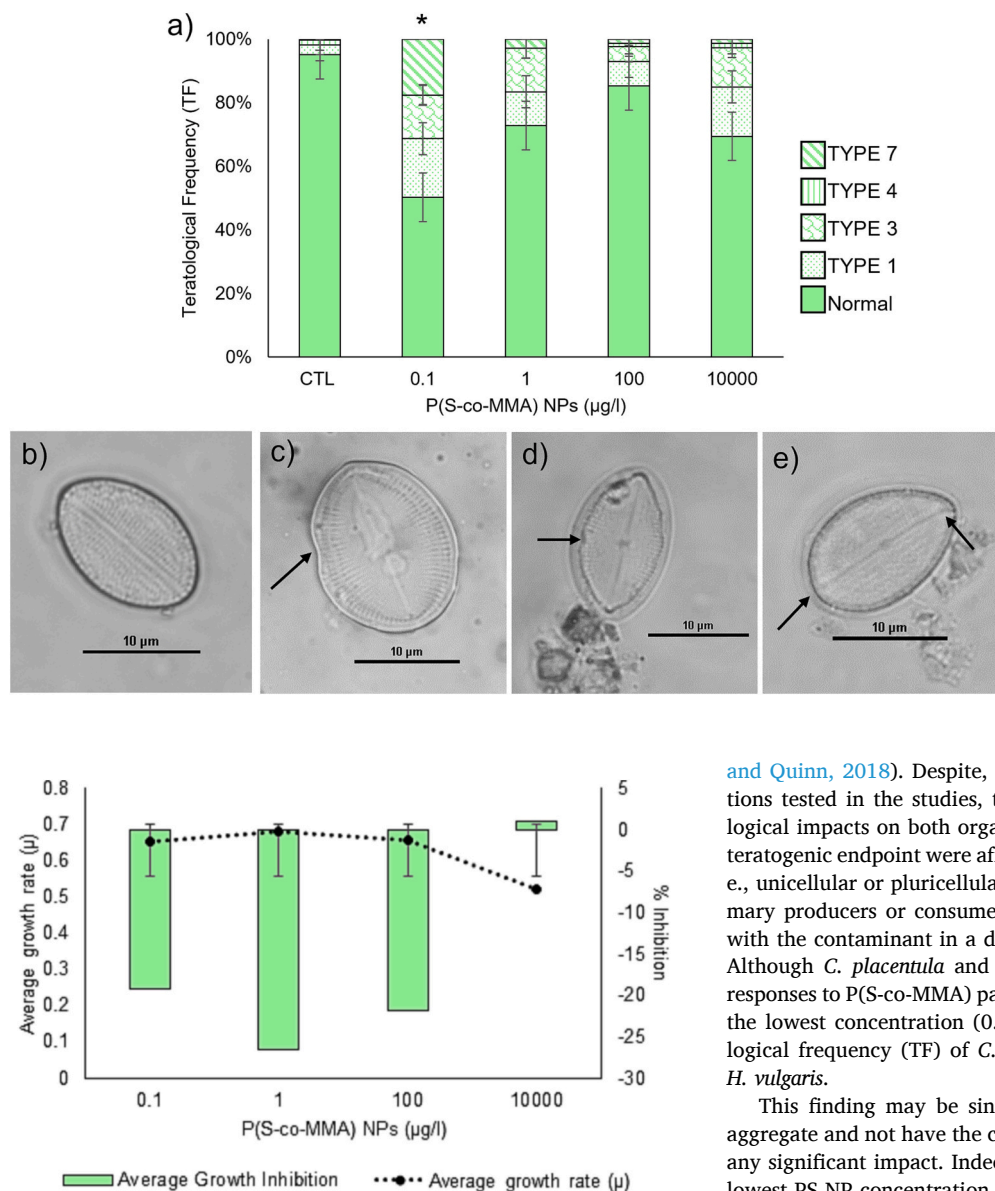


Fig. 3. Average growth rate (circles) and percentage of growth inhibition compared to control (bars) after 28 day exposure of *Cocconeis placentula* exposed to different poly(styrene-co-methyl methacrylate) nanoplastic concentrations. Data shown as mean \pm standard deviation.

significant relationship only between TRI and AF (Spearman $r = -0.92$, $p < 0.05$).

4. Discussion

This is the first research on the potential teratogenic effects of plastic particles of the freshwater diatom *C. placentula* and cnidarian *H. vulgaris*. Tested particles have different negative effects on these organisms: in diatoms increased the teratological frequency and in hydras delayed the regeneration rate. The studies available on the teratogenicity of plastic particles on aquatic organisms mainly investigated zebrafish and tested high concentrations (e.g., 50–100 mg/L). Instead, the concentrations considered environmentally relevant are about 1–25 µg/L, selected as start concentrations in this study. Also, the size can result significant for the effects observed, in our case most particles were in the range > 100 and > 200 nm, while other studies tested particles with fewer sizes (e.g., ~ 40 nm, Venâncio et al., 2021) or higher sizes (e.g., ~ 400 µm, Murphy

Fig. 2. (a) Teratological frequency (%) observed in diatom *Cocconeis placentula* exposed, for 28 days, to different concentrations of poly(styrene-co-methyl methacrylate) nanoplastics plus control (CTL). * denotes statistical differences between poly(styrene-co-methyl methacrylate) nanoplastic treatments and control (Dunn's; $p < 0.05$). Some examples of the most frequent types of teratological forms found: (b) normal morphology; (c) deformed valve outlines (Type 1); (d) aberrated longitudinal area (Type 3); (e) mixed teratologies (Type 7). Data shown as mean \pm standard deviation.

and Quinn, 2018). Despite, the possible different experimental conditions tested in the studies, the P(S-co-MMA) particles showed teratological impacts on both organisms considered. Different aspects of the teratogenic endpoint were affected according to the organism studied (i.e., unicellular or pluricellular) and trophic level of belonging (i.e., primary producers or consumers). Indeed, the two organisms interacted with the contaminant in a different way resulting in a diverse impact. Although *C. placentula* and *H. vulgaris* showed significantly different responses to P(S-co-MMA) particles exposure, the effect was observed at the lowest concentration (0.1 µg/L) with significant effects in teratological frequency (TF) of *C. placentula* and regeneration rate (RR) of *H. vulgaris*.

This finding may be since plastic particles at high concentration aggregate and not have the capacity to cross biological barriers causing any significant impact. Indeed, Ripken et al. (2020), reported that the lowest PS NP concentration (10 µg/L) caused the highest inhibition of growth rate in dinoflagellate, while increased concentrations (100 and 10,000 µg/L) have a higher possibility of self-aggregation producing fewer effects and these concentrations are the same used in our study. A review highlighted that the combination of the properties of NPs and medium solution influences the aggregation of NPs and the subsequent deposition or suspension, causing different effects on aquatic organisms at different depths (Zhang et al., 2021). High concentrations of NPs or elements such as nitrogen and phosphorus increase self-aggregation and the possibility of sinking, so the higher risk is for benthic organisms (Li et al., 2016; Ripken et al., 2020). In our study, although both the organisms studied are benthic, no significant effects at increasing plastic particle concentrations were found probably due to the small size of the organisms and the higher size that the aggregation of particles can cause. In literature, another effect commonly found in studies of MPs (≤ 10 µm) and NPs (≤ 50 nm) exposure highlighted hormetic dose response in several organisms (e.g., algae, invertebrates, fish) testing different polymers (see Agathokleous et al., 2021). Therefore, the hormetic effect, i.e., low-dose stimulation and high-dose inhibition, can occur independently of the polymer employed.

Investigate possible interactions of diatoms with plastic polymers is fundamental given that diatoms represent the most common colonizers found on plastic substrates in all types of aquatic ecosystems (Zettler et al., 2013; Puccinelli et al., 2014; Di Pippo et al., 2020; Nava and Leoni,

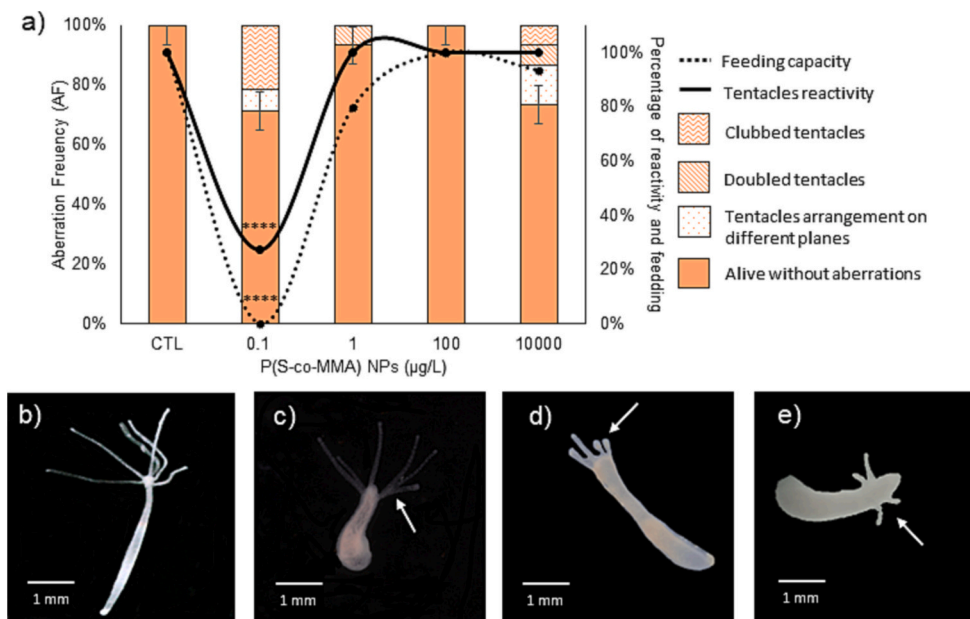


Fig. 4. (a) Aberration frequency (bars) and tentacles reactivity and feeding capacity (lines) observed in cnidarian *Hydra vulgaris* exposed, for 96 h, to different concentrations of poly(styrene-co-methyl methacrylate) plus control (CTL). * denotes statistical differences between poly(styrene-co-methyl methacrylate) nanoplastic treatments and control (Dunn's; $p < 0.05$). Some examples of types of teratological forms found: (b) normal morphology; (c) tentacles arrangement on different planes; (d) doubled tentacles; (e) clubbed tentacles. Data shown as mean \pm standard deviation.

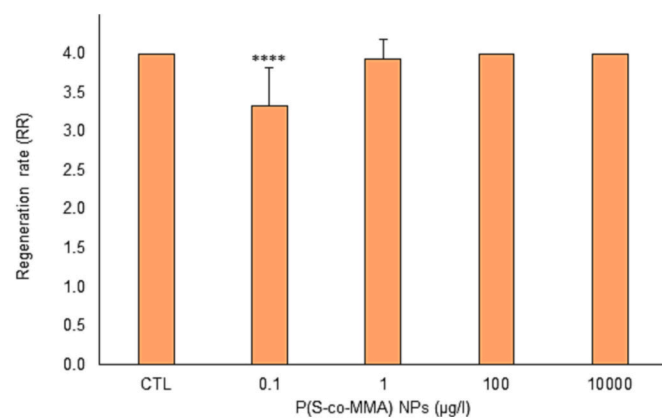


Fig. 5. Regenerative rate (mean \pm s.d) observed in *Hydra vulgaris* after 96 h of poly(styrene-co-methyl methacrylate) exposure and in the control (CTL). **** denotes statistical differences between poly(styrene-co-methyl methacrylate) nanoplastic treatments and control (Dunn's; $p < 0.0001$).

2021; Ryabushko et al., 2021). Diatoms are known to have abnormal forms caused by different environmental stress, among which drought conditions, light intensity, water temperature, attacks by grazers, and contaminants, such as heavy metals, cyanide, polycyclic aromatic

hydrocarbons, and pesticides (Debenest et al., 2008; Falasco et al., 2009a, 2009b; Morin et al., 2009; Renzi et al., 2014; Pandey et al., 2017; European Commission, 2018). The studies available about MPs and NPs have not explored possible teratogenic effects on microalgae. These studies have evaluated algal growth, algal photosynthesis, algae lipid, and fatty acid composition (e.g., Zhang et al., 2017; Bellingeri et al., 2019; Guschina et al., 2020; Prata et al., 2022). As regards the diatom growth, no effects were observed in this study as well as other research on marine diatoms testing polystyrene on *Skeletonema marinoi* and polyethylene on *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* (Baudrimont et al., 2020; Bellingeri et al., 2020; Guo et al., 2020). Instead, testing freshwater green algae, *Scenedemus subspicatus*, at concentrations of polyethylene similar to ours, growth inhibition was observed at all the concentrations tested (Baudrimont et al., 2020). A review highlighted that green algae seem more sensitive than diatoms to pollutant (e.g., herbicides) exposure due to the presence of carotenoids and xanthophylls in diatoms that have antioxidant properties (Debenest et al., 2008). Moreover, the sizes of MPs and NPs tested and the concentrations of exposure could be the dominant factors influencing the microalgae growth. In fact, the effects of MPs and NPs seem to increase with smaller sizes due to the possibility of particles of entering the microalgae cells. A study that tested NPs of 50 nm showed that microalgal (*Chorella vulgaris*) growth was negatively affected but only at high concentrations (250 mg/L) (Sjollema et al., 2016). Therefore, it is possible to state that by testing environmentally relevant

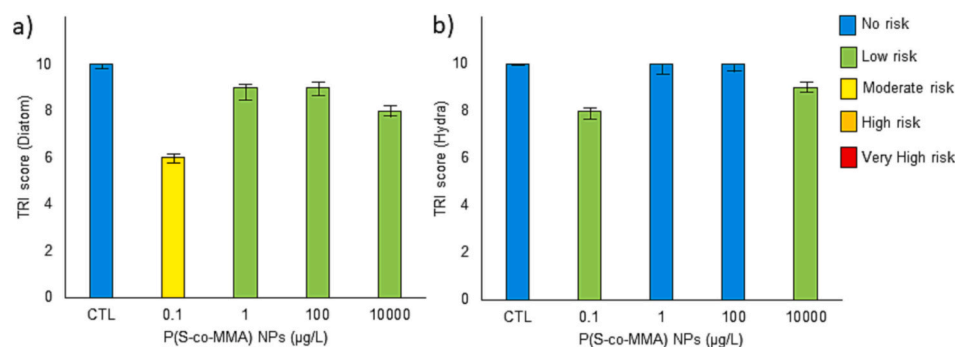


Fig. 6. Application of Teratogenic Risk Index to evaluate poly(styrene-co-methyl methacrylate) exposure on (a) diatoms (modified version of TRI) and (b) hydras. The colours refer to Teratogenic Risk Index (TRI) classes from no risk to very high risk. Data shown as mean \pm standard deviation.

concentrations, P(S-co-MMA) particles have no effect on the growth of diatoms.

Regarding the teratological forms found in *C. placentula* after 28 days of P(S-co-MMA) particles exposure, valve outlines deformation (Type 1) was the most common, as highlighted by several studies testing different stress conditions and contaminants compared to plastic particles (see Falasco et al., 2009a). Abnormal diatoms are potentially impacted from an ecological point of view. For instance, deformed valve outlines could prevent the correct connection of the spine (a pointed silica extension of the valve) during colony formation (Lavoie et al., 2017). Bellingeri et al. (2020) highlighted a reduction in chain length of marine diatom *S. marinoi* as a consequence of PS NPs adhesion that impacts on fultoportula processes involved in the cell connections. Similar effects could impact the primary production of aquatic ecosystems and the cycle of carbon (Miao et al., 2019; Bellingeri et al., 2020). Modifications of the raphe (Types 4 and 7) can limit the locomotion and diatom ability to be attached to a substrate, reducing the possibility to survive in unstable environmental conditions (Lavoie et al., 2017). Artificial conditions, mainly due to light stress, inadequate culture medium or osmotic stress, can also lead to the onset of teratogenic forms, as occurred in our study but with a frequency not relevant. Therefore, a contaminant such as plastic particles can affect the first stages of the development of organisms, causing malformations and reducing individual fitness within a population (Falasco et al., 2009a).

The P(S-co-MMA) particles exposure after the cut of *hypostoma* caused no significant effects in AF of *H. vulgaris*. Other studies found toxicity and mortality in diverse species of *Hydra* testing different polymers at higher concentrations. For instance, aberrations as clubbed or malformed tentacles were found in *Hydra viridissima* after the toxicity tests using polymethylmethacrylate NPs at 1, 5, and 80 mg/L (Venâncio et al., 2021). Murphy and Quinn (2018) showed that MPs exposure caused significant changes to the morphology of *Hydra attenuata* at polyethylene concentrations ranging from 0.02 to 0.08 mg/L. Murugadas et al. (2019) evaluated the regeneration of *H. vulgaris* after Bisphenol A, an additive of plastic material, exposure finding inhibition at 15 µM concentration. NPs also caused in *Hydra* several molecular effects, such as decreased biomass, lipid peroxidation, and increased polar lipid levels (Auclair et al., 2020).

The regeneration ability of *H. vulgaris* was significantly impacted at the lowest concentration (0.1 µg/L), causing a slower RR compared to the control, as explained above this finding could be due to the aggregation of particles at the highest concentrations. Moreover, feeding capacity and tentacles reactivity were inhibited at the same lowest concentration, maybe due to the delayed RR (3 score) that has not allowed to have fully regenerated tentacles able to catch the prey. Venâncio et al. (2021) reported dead individuals after 96 h regeneration assay in all polymethylmethacrylate NPs treatments, with about 60 % of mortality in the highest concentration (40 mg/L). During our regeneration assay, attachment on particles agglomerate to the basal disk of *H. vulgaris* was observed in particular at the highest concentration (10,000 µg/L). Although it is currently only an observation, the potential decrease in attachment to substrates due to the presence of plastic particles and its resulting ecological impacts highlight the importance of further exploring this aspect in future studies.

Teratogenic effects were also investigated in the early life stages of fish. Polyethylene MPs cause a lower larval survival rate after egg hatching and teratological aberrations (Malafaia et al., 2020). The co-exposure of PS NPs and phenanthrene caused more negative effects on teratogenicity and mortality of zebrafish embryos than the single contaminant (Xu et al., 2022). Irregular-shaped and aged polyvinyl chloride MPs caused higher teratogenic effects compared to virgin microplastics in *Oryzias melastigma* embryos (Xia et al., 2022). Studies on regeneration ability of planarians found that MPs of polyethylene and PS reduced growth rate and caused a significant delay in the regeneration of planarians (Gambino et al., 2020; Gao et al., 2022; Cesarini et al., 2023a).

Regarding the feeding, no effects were found in *H. viridissima* (Venâncio et al., 2021), while in *H. attenuata* was significantly reduced (Murphy and Quinn, 2018). In preliminary tests, *H. attenuata* did not ingest perfect microsphere, but only irregular flakes of MP as more similar to their prey *A. salina*. Therefore, the shape is a very important parameter to be considered to testing conditions like the real ones. MPs and NPs uptake is very common in several aquatic organisms such as filter bivalves, primary and secondary consumers, apex predators (Mateos-Cárdenas et al., 2019; D'Souza et al., 2020; Cesarini et al., 2022; Kim et al., 2022). The environmental presence of MPs was also recorded in marine Cnidaria and Ctenophora (Devereux et al., 2021). Therefore, the potential accumulation of MPs and NPs represents an imminent risk in the food web transfer contaminating different trophic levels (Carbery et al., 2018; Gambardella et al., 2018; Redondo-Hasselherm et al., 2018; Bellasi et al., 2020; Zhu et al., 2021).

NP/MP surface can absorb and become a vector of environmental contaminants, such as heavy metals, pesticides, and persistent organic pollutants, which act as teratogens on diatoms and hydras (Falasco et al., 2009a; Quinn et al., 2012). In this way, plastic particles increase their toxicity to the exposed organisms (Xu et al., 2020).

The application of TRI highlighted a different sensitivity of organisms considered to the particles exposure: the new TRI for diatoms showed a moderate teratogenic risk of P(S-co-MMA) particles at the lowest concentration, while the TRI applied considering hydras reported a low teratogenic risk at the lowest and the highest concentrations. Therefore, although the teratogenic risk from particles exposure has not been found to be high, this finding is of concern given the low concentrations analyzed and given that the environmental concentration of NPs and MPs is expected to increase in the future.

5. Conclusions

We evaluated the teratogenic effects of exposure to plastic particles on the freshwater diatom *C. placentula* and cnidarian *H. vulgaris*, focusing on a crucial yet often overlooked aspect in the literature: the impact on the most vulnerable life stage of these organisms. Our findings highlighted, significant impacts on teratological frequency of diatoms and regeneration rate of hydras at the lowest concentrations, while no significant impacts on the algal growth rate and aberration frequency of hydras were found. In addition, feeding capacity and tentacles reactivity in hydras were inhibited at the lowest concentrations. The different interactions found depending on the organism considered, highlighted the importance of testing organisms belonging to different levels. Moreover, this study raises the question of testing low concentrations, as they may be more impactful than previously expected. The found effects can have ecological implications as are caused by predicted environmental concentrations and therefore are possible to be observed in nature. Clearly, even if in the laboratory some simplifications are necessarily used, the researchers must try to investigate as much as possible relevant environmental conditions, for example testing low concentrations, polymers abundant in nature, common shapes such as ellipse, granules or fibres, or plastics aged with biofouling. Further studies are necessary to understand if there is a correlation between a specific type of NP/MP and teratological form, investigating the teratogenic effects in combination with other polymers, sizes, and shapes.

Moreover, the two TRI indices applied to environmental data could detect the potential teratological risk on freshwater ecosystems, which may arise not only from plastic pollution. These tools, in conjunction with assessments of environmental concentrations of plastic particles and other contaminants, can provide valuable insights into the effects of nano-microplastics on aquatic ecosystems.

CRedit authorship contribution statement

G.C.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing -

review & editing. **S.S.:** Data curation, Investigation, Writing - review & editing. **D.T.:** Data curation, Investigation, Writing - review & editing. **I.V.:** Methodology, Resources, Writing - review & editing. **C.B.:** Methodology, Resources, Writing - review & editing. **S.M.:** Supervision, Writing - review & editing. **L.M.:** Supervision, Writing - review & editing. **I.F.:** Formal analysis, Methodology, Writing - review & editing. **M.S.:** Funding acquisition, Conceptualization, Methodology, Resources, Supervision, Visualization, Writing - review & editing. **C.P.:** Conceptualization, Methodology, Resources, Supervision, Visualization, Writing - review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.165564>.

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