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Trophic transfer of microplastics from producer (*Lemna minuta*) to primary consumer (*Cataclysta lemnata*) in a freshwater food chain

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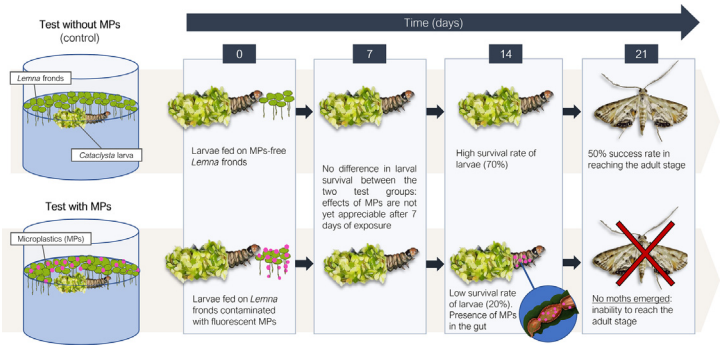
HIGHLIGHTS

- Aquatic larvae were fed with *Lemna* plants contaminated with microplastics (MPs).
- Larvae showed high mortality and inability to complete life cycle.
- MPs were found in gut and excrements of the larvae.
- MPs were transferred along the food chain from producer to primary consumer.
- Microplastic pollution proved a real threat to freshwater communities conservation.

GRAPHICAL ABSTRACT



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ABSTRACT

Contamination by microplastics (0.1 μm–5 mm plastic fragments) is currently one of the major threats to the conservation of aquatic and terrestrial ecosystems worldwide. Growing awareness on this issue has led to an increase in studies on the effects of microplastics on freshwater organisms, although there are still few investigations on possible transfer of this contaminant along water trophic chains from producers to primary consumers. In this study, aquatic herbivorous larvae of the moth *Cataclysta lemnata* were fed on microplastic-free (control) and contaminated (MPs treatment) *Lemna minuta* fronds. For treatments, *Lemna* fronds were grown in mineral water enriched with fluorescent microparticles of poly(styrene-co-methyl methacrylate) (MPs, 100 mg/L) and then fed to the larvae as a food source. Microplastics effects on larvae were tested at 0, 7, 14 and 21 days of exposure, corresponding to sensitive phases of the insect life cycle. Contaminant impact was assessed based on some parameters such as viability, larva body size/weight, feeding alterations and regularity of the insect life cycle. Using scanning electron and fluorescence microscopy, the presence of microplastics in the larvae was verified. The finding of fluorescent microplastics in both the intestinal lumen and excrement samples showed that larvae ingested contaminated *Lemna* fronds. In addition, larvae fed contaminated fronds were strongly affected by the presence of microplastic contaminant over time, showing high mortality (90 %) and total inability to complete the life cycle after 21 days by failing to reach the winged adult phase. In control tests, survival rates were higher than in treatments, and 50 % of the larvae managed to pupate and emerge as moths, reaching the adult phase. The results show that there was a trophic transfer of microplastics from producer to primary consumer along a freshwater food chain, generating negative effects on the life cycle of this aquatic herbivore.

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1. Introduction

Since the 1940s, the production and consumption of plastic items have grown exponentially (Pereao et al., 2020; Chen et al., 2021), with global plastic production exceeding 367 million tons in 2020, of which 55 million tons were produced in Europe, and 1.9 in Italy (Plastics Europe, 2021). Despite the undoubted benefits of using plastic products, the release of large amounts of this material into the environment has become a global pressing environmental problem to be addressed (Horton et al., 2017; Shen et al., 2020; Azeem et al., 2021).

Unauthorized dumping and inadequate waste management are among the main causes of the release of plastic material into the environment, which takes many years to degrade, accumulating in soil and water (Browne et al., 2011; Zbyszewski et al., 2014). In addition, plastic debris exposed to weathering (wind, precipitations, heat and cold) fragments into very small parts causing the formation of microplastics (MPs) (Barnes et al., 2009; Ryan et al., 2009), which are plastic particles between 1 and 1000 μm in size (Thompson et al., 2004; Hartmann et al., 2019).

Most studies on the impact of microplastics on freshwater communities have focused mainly on the effects of this contaminant on different plant and animal aquatic organisms (Kalčíková et al., 2017; Horton et al., 2018; Silva et al., 2019; Triebskorn et al., 2019; Wang et al., 2019; Windsor et al., 2019; Ceschin et al., 2021; Silva et al., 2022; Ceschin et al., 2023). To date, however, very little has been investigated on the transfer of microplastics along freshwater trophic chains, which closely link producers (plants) with primary consumers (Sabo et al., 2009; Mateos-Cárdenas et al., 2019; Kalčíková, 2020; Ceschin et al., 2023). In most cases, contaminants (including microplastics) first come into contact with plants that represent the first obligatory level in trophic chains. Thus, plants can adsorb and accumulate contaminants and adversely affect herbivores, and subsequently carnivores, through processes of trophic transfer, bioaccumulation and biomagnification along the food chain (Herrera et al., 2022; Rodrigues et al., 2022; Bhatt and Chauhan, 2023).

Once plastic microparticles are introduced into the aquatic environment, they occupy different portions of the water column depending on their density: some remain suspended, some float to the surface, and other with higher density settle to the bottom. Low-density microplastics (specific density < 1 g/cm), such as microspheres of polyethylene (PE), polystyrene (PS), and polypropylene (PP), localize in the upper layers of the water column (Eerkes-Medrano et al., 2015; Marchetto et al., 2017), where they can mainly come into contact with floating aquatic plants, including duckweeds. They are very small phanerogams that, free-floating on the water surface, expose most of the adsorption/absorption surface to water contact. Some duckweeds belonging to *Spirodela* and *Lemna* genera are very common in still or slow-flowing freshwaters, often playing a key role in these environments as trophic resource for several aquatic insects, fish and waterfowl (Draulans and Vanherck, 1987; Van Hoeck et al., 2015; Mariani et al., 2020a). Among these duckweeds, the American *Lemna minuta* Kunth is an invasive species that is rapidly spreading in wetlands throughout Europe (Ceschin et al., 2018), where it often produces dense free-floating stands on the water surface (Ceschin et al., 2016). Laboratory investigations on individuals of *L. minuta*, which were exposed to water contaminated with microplastics, showed both a high adsorption of the contaminant by the plant and negative effects on growth and chlorophyll content, appreciable especially over prolonged exposure times (28 days) (Ceschin et al., 2023). This plant is consumed in large quantities by the aquatic larvae of the moth *Cataclysta lemnata* Linnaeus 1758, which is the only aquatic arthropod known in the literature to feed on *L. minuta* (Mariani et al., 2020a). In this context, the present study aimed to verify the possible trophic transfer of fluorescent microplastics from a producer (*L. minuta*) to a primary consumer (aquatic larvae of *C. lemnata*) along a freshwater food chain. The emerging results from this study may help to better understand the type of impact that microplastics in water may have on the lower trophic levels (producer-primary consumer) of food chains occurring in aquatic ecosystems.

2. Materials and methods

2.1. Fluorescent microplastics production

Microplastic particles (MPs) were obtained from pellets of poly (styrene-co-methyl methacrylate) [P(S-co-MMA)] (Aldrich 462,896, pellets average Mw 100,000–150,000 pellets, styrene 40 %) following the osmosis base method (OBM) reported in a previous work (Chronopoulou et al., 2009). Subsequently, the produced MPs were made fluorescent to be tracked easily and clearly. Like other cytotoxicity and animal toxicity studies, rhodamine B-isothiocyanate (Sigma Aldrich) was used as dye to follow the biological effects caused by the plastics and not the fluorescent dye, which only acts as a tracker (Ruedas-Rama et al., 2012; Trubitsyn et al., 2019; Fournier et al., 2020; Skjolding et al., 2021). In fact, when rhodamine B-isothiocyanate is used, the dye is trapped within the polymer, drastically reducing its dispersion in the aqueous medium (Ruedas-Rama et al., 2012; Trubitsyn et al., 2019; Fournier et al., 2020). For the fluorescence of MPs, 300 mg of P(S-co-MMA) and 3 mg of the fluorescent dye (Rhodamine B-isothiocyanate, Sigma Aldrich) were dissolved in 100 mL of acetone (C_3H_6O, technical grade, Merck) and stirred for 24 h. Then an aliquot of 7 mL was transferred into a cellulose dialysis membrane (10 mm width, Sigma Aldrich D9277-100FT) and further immersed in 200 mL of distilled water for 5 days at constant temperature (T = 24 °C). MPs were observed under a scanning electron microscope (SEM) (Gemini 300, Zeiss, Jena, Germany), and their mean diameter was calculated on SEM images with ImageJ software vers. 1.53 t (National Institutes of Health, Bethesda, MD, USA) (Fig. 1). The MPs obtained had a mean diameter of 2.07 μm with a range of 0.21–11.71 μm.

A 5-L stock suspension was prepared with a concentration of 100 mg/L of these fluorescent MPs. This concentration, which is higher than that recorded in nature, was chosen both because it has been used in similar studies on the effects of microplastic (e.g., Kalčíková et al., 2017; Chae et al., 2018; Zimmermann et al., 2020; Ceschin et al., 2023), and thus useful for comparative purposes, and to stress the experimental system in order to obtain more obvious biological responses. The aqueous medium used to make the MP-stock suspension was bottled mineral water of a known composition with chemical and physical characteristics similar to that in which the producer (*L. minuta*) and the herbivore (*C. lemnata*) used in this study grow spontaneously in nature (Mariani et al., 2020b). The MP-stock suspension produced was used to set up a culture of the producer.

2.2. Producer: Lemna minuta

The American duckweed *L. minuta* (Alismatales: Lemnaceae) is a freshwater plant characterized by a reduced vegetative body (frond) and a high vegetative growth rate (Landolt, 1986; Ceschin et al., 2016). This duckweed was found to be particularly palatable to the aquatic larvae of the insect *C. lemnata* (Mariani et al., 2020a) (Fig. 2a), and highly adsorbing plastic microparticles floating on the water surface (Ceschin et al., 2023). For these reasons, this duckweed species was selected as ideal producer organism (first trophic level) for the laboratory reconstruction of a simple freshwater food chain along which to test the possible transfer of microplastic contaminants from producer to primary consumer.

Samples of *L. minuta* were collected from a natural pond within the Appia Antica Regional Park (Rome, Italy) and then transported to the laboratory. Here, they were placed in a glass tank (20 × 30 cm) filled with bottled mineral water for an acclimatization time of 7 days. The *Lemna* samples used in the experiment were neither pretreated nor grown under sterile conditions to keep them during the experiment under conditions as close to natural conditions as possible. Anyway, any algal growth in the tests was monitored during the experiment through observations under microscope (Standard 25, Zeiss) of *Lemna*

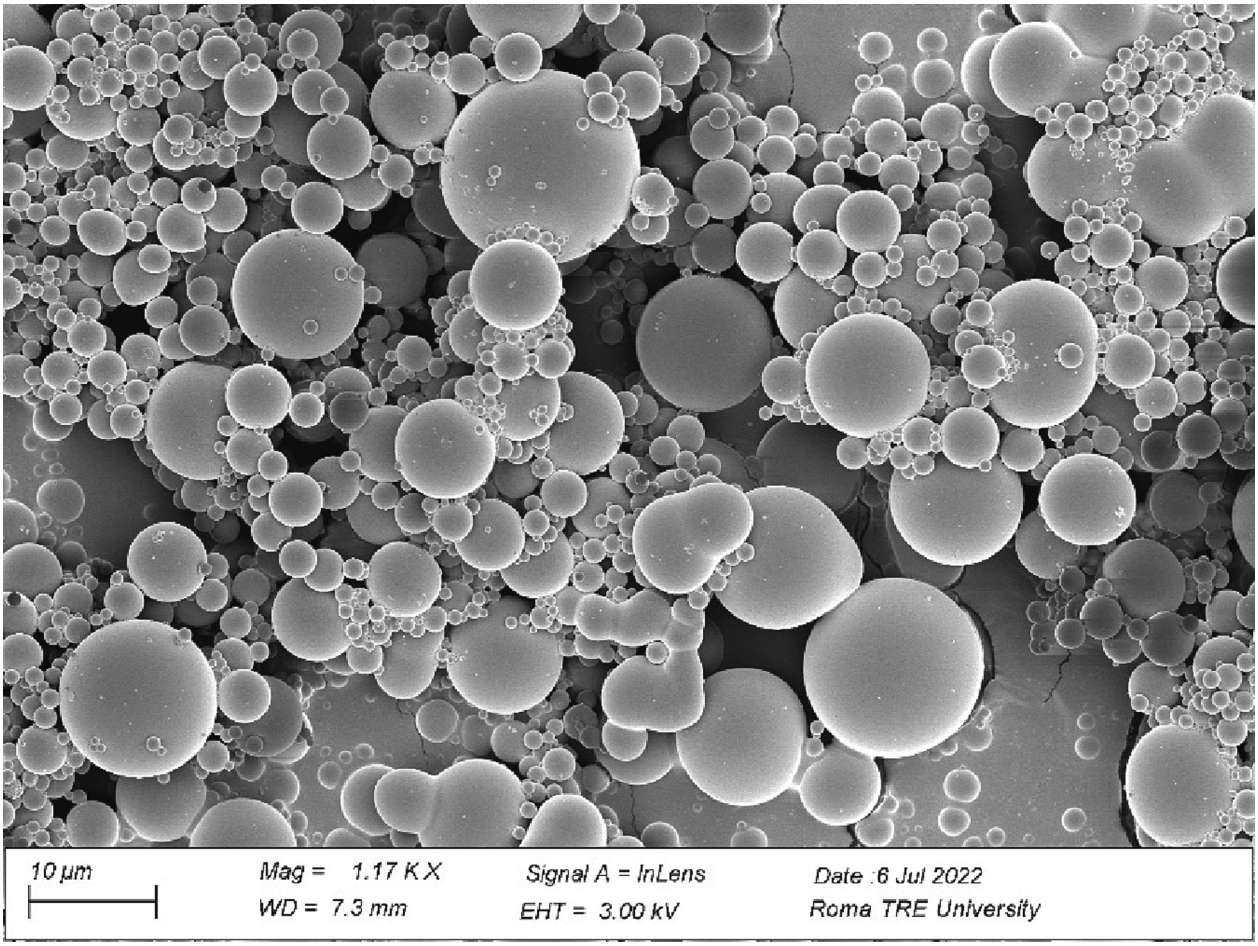

Fig. 1. SEM image of fluorescent MP-stock.

individuals and drops of the liquid medium to verify if it could become an affecting factor the experiment.

2.3. Primary consumer: Cataclysta lemnata

Cataclysta lemnata (Crambidae: Acentropinae) is a lepidopteran distributed throughout most of Europe, including Italy. Its biological cycle comprises a larval phase followed by a winged adult phase (Fig. 2b–e). The diet of the larvae is mainly based on the consumption of some duckweeds, such as *Lemna minor* L. and *Spirodela polyrrhiza* (L.) Schleid., but it has been shown that they are also voracious consumers of the American duckweed *L. minuta*, which also use it as plant material for the construction of protective cases (Mariani et al., 2020a) (Fig. 2a, b).

Individuals of *C. lemnata* were collected from a natural pond within the Appia Antica Regional Park (Rome, Italy) and transported to the laboratory where they were reared in a glass tank (28 × 19.5 × 13.2 cm) filled with 3.5 L of bottled mineral water and *L. minuta* fronds as a trophic source. The tank was covered with a fine-mesh net to prevent the winged adults from escaping. Once the adults emerged from the pupae, they were combined to form pairs. The larvae hatched from the eggs of these pairs were used for experiments when they reached the third larval stage (Mariani et al., 2021).

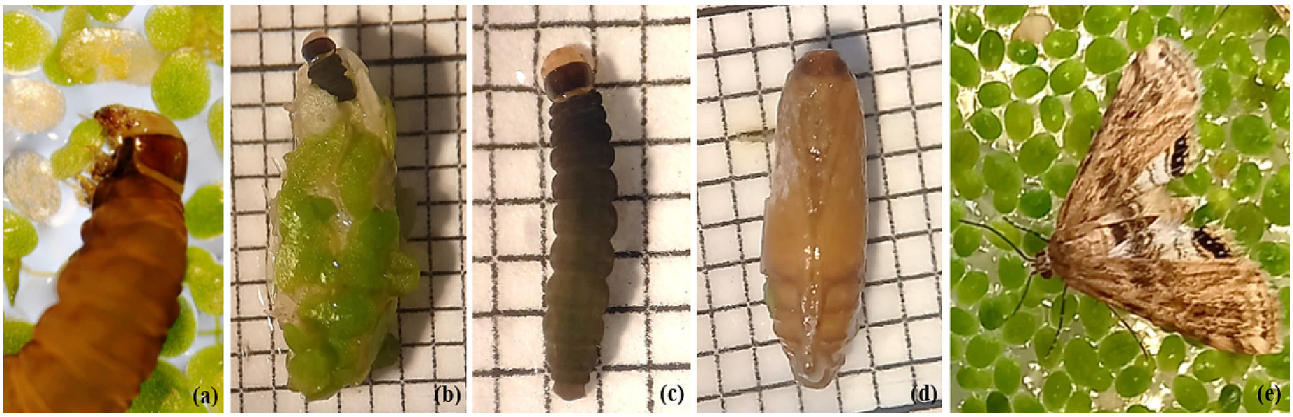

Fig. 2. Individual of *C. lemnata* eating *L. minuta* fronds (a). Larva of *C. lemnata* in case (b), larva (c), pupa (d) and adult (moth) (e).

2.4. Experimental design

At the beginning of the experiment, 15 cylindrical glass containers (7 × 7 mm, 240 mL capacity) were filled with 100 mL of bottled mineral water along with 3.0 g of *L. minuta* grown for 7 days in the stock suspension (fluorescent MPs, 100 mg/L) to ensure the adsorption of MPs on the surface of the fronds (Ceschin et al., 2023). Then, two larvae at the third larval stage were immersed in each container. Simultaneously to treatments with MPs, a control set was set up with 15 containers filled with 100 mL of mineral water, two larvae and MPs-free *Lemna*. The toxic effects of MPs on *Cataclysta* larvae were tested at 7, 14 and 21 days of exposure. These times were chosen because they correspond to some significant stages in the life cycle of *Cataclysta* based on the developmental timelines reported by Mariani et al. (2021). In addition, since MPs appear to cause primarily a chronic toxic effect on exposed organisms rather than acute one (Sharma and Chatterjee, 2017; Ceschin et al., 2023), longer exposure times than those generally used in toxicological analyses (Kalčíková et al., 2017; Mateos-Cárdenas et al., 2019; Wang et al., 2019) were chosen in this study. For each experimental time, five replicates (n = 5) were set up for a total of 15 controls and 15 treatments with contaminant; as biological material, 90 g of *Lemna* and 60 *Cataclysta* larvae of the same size and larval stage were used.

Every 7 days, to restore the level of partly evaporated water to the initial 100 mL, each container was refilled with the same aqueous medium in which the *Lemna* samples were grown.

2.5. Animal parameter analysis

Analysis of viability, growth, life cycle and feeding parameters of *Cataclysta* larvae were performed at each time (0, 7, 14, 21 days).

2.5.1. Viability and life stage assessment

To determine the viability status (alive/dead) of *Cataclysta* larvae, evidence of respiration and motility was noted for each individual through direct observations. Specifically, to assess whether the larvae were viable, and therefore breathing, the formation from the plastron (thin layer of air surrounding the larvae's body for oxygen exchange) of an air bubble near the cephalic capsule, was checked. In particular, a slight pressure was applied to the mid-terminal part of the larva's body, consequently causing the plastron to swell and possible form an air bubble. The reactivity of the larvae as a result of this slight pressure was considered evidence of motility.

To define the life stage at which individuals of *Cataclysta* were in, each individual was stripped of its protective case and its developmental stage at the time of observation was recorded (L — larva; P — pupa; A — adult). Considering that the larval phase includes 5 larval stages (Mariani et al., 2021), in order to determine the exact stage of each larva, the cephalic capsule was measured by taking stereomicroscope images of the head (Stemi 305, Zeiss) and measuring its dimensions with ImageJ software vers. 1.53 t (National Institutes of Health, USA).

2.5.2. Body size measurements

To assess the growth of *Cataclysta* individuals, weight and length of the larvae and length of their protective cases were measured. Larvae and cases were dried for 10 s on blotting paper and then weighed with a precision balance (Mark BEL Engineering Electronic Balance). To measure the length of the cases, the specimens were placed on graph paper, photographed, and measured using ImageJ software vers. 1.53t (National Institutes of Health, USA).

2.5.3. Feeding alterations assessment

To assess the ability of the larvae to feed on contaminated *L. minuta*, consumption of fronds by larvae was recorded by direct observation. An indirect estimate of the quantity of fronds ingested by the larvae was made by considering the production of excrements in the unit of time according to Gross et al. (2001). Specifically, each larva was transferred to a well (4 × 4 cm) containing clean, microplastic-free mineral water, where it was kept for 20 min. At the end of the time, the number of excrements in each well was recorded.

2.6. Observations of fluorescent microplastics in larvae and faecal samples of Cataclysta

At the end of each time period, once the biological parameters were analyzed, the *Cataclysta* larvae used in the treatments were first preserved in 95° alcohol, and then bleached in 30-volume hydrogen peroxide to be observed under a macroscope (Axiozoom v16, Zeiss) equipped with a HXP 200C metal halide lamp, PlanNeoFluor Z 1× objective and a color camera (Axiocam 503, Zeiss). A HE DsRed 43 filter (excitation: 550/25 nm, emission: 605/70 nm) was used for the fluorescence analysis of microplastics.

The possible presence of fluorescent microplastics was searched both on the external tegument of entire larval individuals and inside their dissected bodies. In particular, the dissection was carried out as follows: larvae were gently washed in distilled water, and it was checked by fluorescence analysis that no plastic remained adhering to the outside. Thereafter, using spring micro-scissors, two lateral incisions were made by superficially cutting the cuticle above and below the spiracles, from the prothoracic segment down to the last abdominal segment in order to obtain a sagittal view of the internal organs of the larva.

In addition, excrement samples of larvae exposed to microplastics were collected and placed in 90 % ethyl alcohol inside Eppendorf tubes (1.5 mL) for observations. In particular, the specimens were mounted on stubs (using self-adhesive carbon discs) and first observed under the aforementioned fluorescence macroscope. Fluorescent rhodamine dye allowed to evaluate the presence of microplastics and carry out a correlative analysis by observing the same samples under a SEM (Zeiss Gemini 300), after making them conductive by gold coating with a sputter coater (Emitech k550), allowing further morphological confirmation of the presence of MPs.

2.7. Data analysis

Data collected on the weight and length of the protective cases, and the width of the cephalic capsule of *Cataclysta* larvae, were statistically analyzed, and a linear mixed model was constructed for each parameter using the lmer function of the lme4 package (Bates et al., 2015). The best model for each parameter was selected comparing Akaike Information Criterion (AIC). Assumptions of normality and homoscedasticity were checked on the model residuals. A χ^2 test was then performed to identify possible alterations in *Cataclysta* life cycle. Statistical analyses were performed with R software (R Core Team, 2022).

3. Results and discussion

3.1. Effects of microplastics on Cataclysta larvae

During the experiment, the survival rate of *Cataclysta* larvae showed differences between control and treatment tests depending on the exposure time of the larvae to food (*Lemna* fronds) contaminated with microplastics. Specifically, at 7 days, no significant difference in larval survival was found between the two test groups (Fig. 3), suggesting that effects of MPs are not yet appreciable after 7 days of exposure of larvae to contaminated food. At 14 days, i.e. after longer exposure, control and treatment tests differed significantly with survival rates of 70 % and 20 %, respectively, a difference that remained high even at 21 days (Fig. 3). These results clearly demonstrate that the exposure of *Cataclysta* larvae to contaminated *Lemna* fronds causes an increase in insect mortality. On the contrary, in a similar study on the trophic transfer of polyethylene microplastics from the duckweed *L. minor* to the amphipod *Gammarus duebeni* Lilljeborg 1852, no obvious impact on the survival of the crustacean was observed despite having ingested contaminated *Lemna* (Mateos-Cárdenas et al., 2019). However, in this latter case, the absence of effect on the animal is probably due to the short time of exposure to the contaminant, which was 24–48 h, much shorter than that considered in the present study.

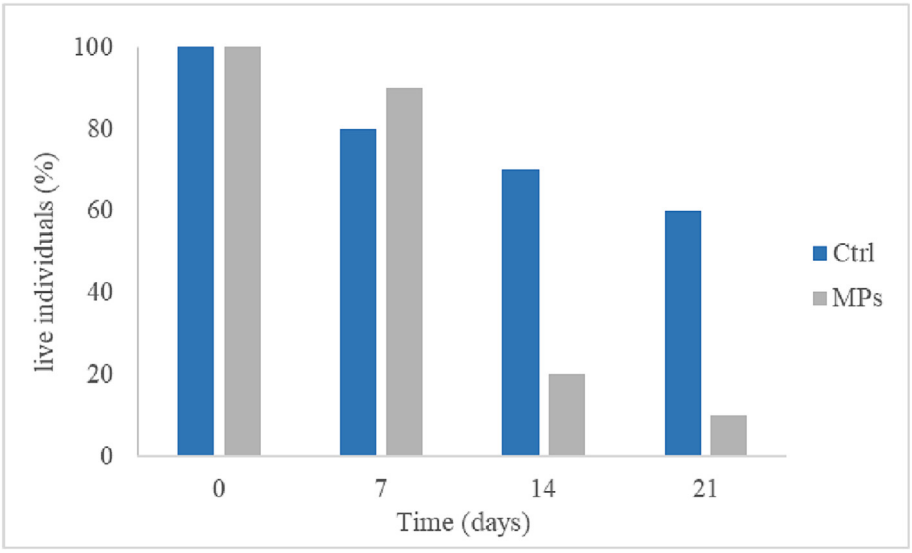

Fig. 3. Percentage of live individuals of *Cataclysta* in control (Ctrl) and treatment (MPs) tests at different times (0, 7, 14, 21 days).

Regarding life cycle, after 7 days, in the control tests, all the larvae were at the larval stage predicted by the timeline described by Mariani et al. (2021), while in the treatment tests 20 % of the larvae had been pupated early. Furthermore, at 21 days, while in the controls 50 % of pupae had managed to complete the life cycle by reaching the adult stage, in the treatment tests no individuals had emerged as moths. Statistical analysis revealed that these differences between control and treatment were significant (χ^2 with $p < 0.05$).

These results suggest that exposure of larvae to MPs may alter the regular development of the insect, with the pupal stage occurring earlier and an impediment to completing the life cycle, as individuals die prematurely and do not reach the adult stage (Fig. 4). Therefore, it is possible that the ingestion of the contaminant constitutes a disturbance factor for the insect, to the point that the larval stage, which is also the only one in which the insect feeds, is shortened, earlier transitioning to the pupal stage in which the insect does not eat. Anyway, ingestion of microplastics by larvae, albeit for a short time, seems afterwards to completely impair the insect's ability to emerge as an adult.

There was no difference in the number of excrements produced by larvae in control and treatment tests, suggesting that the larvae consumed both contaminated and uncontaminated *Lemna* fronds, showing no ability to select between the two different *Lemna* types. There were also no significant differences between larvae fed with contaminated and non-contaminated *Lemna* fronds in terms of weight and length of the cases and size of cephalic capsule of larvae. This is probably due to the high mortality of individuals in tests with MPs at 14 and 21 days, and thus the lack of sufficient numbers of individuals to perform adequate statistical analyses.

3.2. Microplastic transfer from producer (Lemna) to primary consumer (Cataclysta)

Larvae of *C. lemnata* exposed to contaminated fronds showed a negligible presence of MPs on the outer portions of the body, while it was consistent inside the gut lumen (Fig. 5). In addition, microplastics were observed within the excrement samples of the larvae fed with contaminated *Lemna* (Fig. 6), confirming that the ingestion of contaminated fronds by the larvae occurred. Although fluorescence analysis did not prove cellular internalisation of MPs nor their translocation to larval tissues, the presence of MPs both within the gut lumen and excrements is nevertheless an evidence for the transit of this contaminant through the digestive tract and

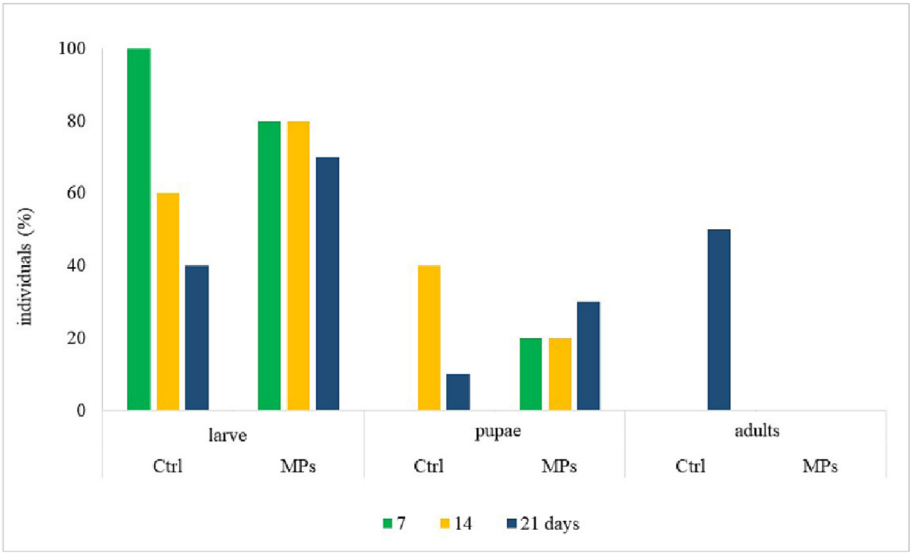

Fig. 4. Percentage of *Cataclysta* individuals at larval, pupa and adult stage in control (Ctrl) and treatment (MPs) tests at different exposure times (0, 7, 14, 21 days).

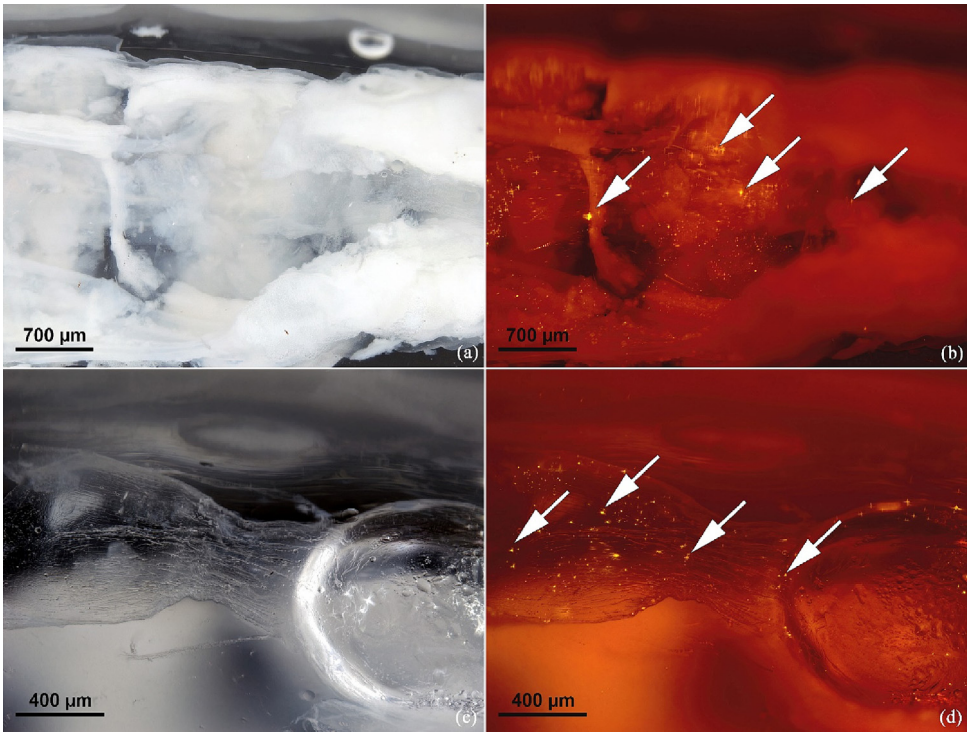

Fig. 5. Sagittal views depicting details of the digestive tract of a *C. lemnata* larva dissected by removal of the left pleural region and illustrating the transit of MPs along the gut. Light (a) and fluorescence (b) images of the mid-gut region; partial deterioration of the tissue led to exposure of the lumen and subsequent leakage of its contents, including fluorescent microplastics. Light (c) and fluorescence (d) images of the hind-gut; numerous MPs confined within the lumen can be observed. The white arrows indicate some MPs.

its subsequent release in the environment by excrements. In addition to the demonstrated negative impact on larval survival rate, the ingestion of MPs by *Cataclysta* larvae highlights that there has been a transfer of this contaminant from the producer to the primary consumer, suggesting the hypothesis that such a transfer may potentially also involve other aquatic consumers linked to the same trophic chain. Indeed, it is possible to hypothesise a transfer of MPs either through the direct predation of larvae with microplastics occurring in the gut lumen, or through use of their contaminated excrements as a source of food by detritivores or coprophagous organisms.

4. Conclusions

This study showed evidence of trophic transfer of MPs from the producer to the primary consumer by finding the presence of MPs both in the gut lumen and excrements of herbivorous larvae fed MPs-contaminated *Lemna*. Although the microplastics ingested by the larvae were not shown to translocate from the gut lumen to other tissues, bioaccumulating in the larvae's body, the presence and passage of MPs in the gut lumen of the larvae was found to be significantly correlated with increased larval mortality and the inability of the insect to

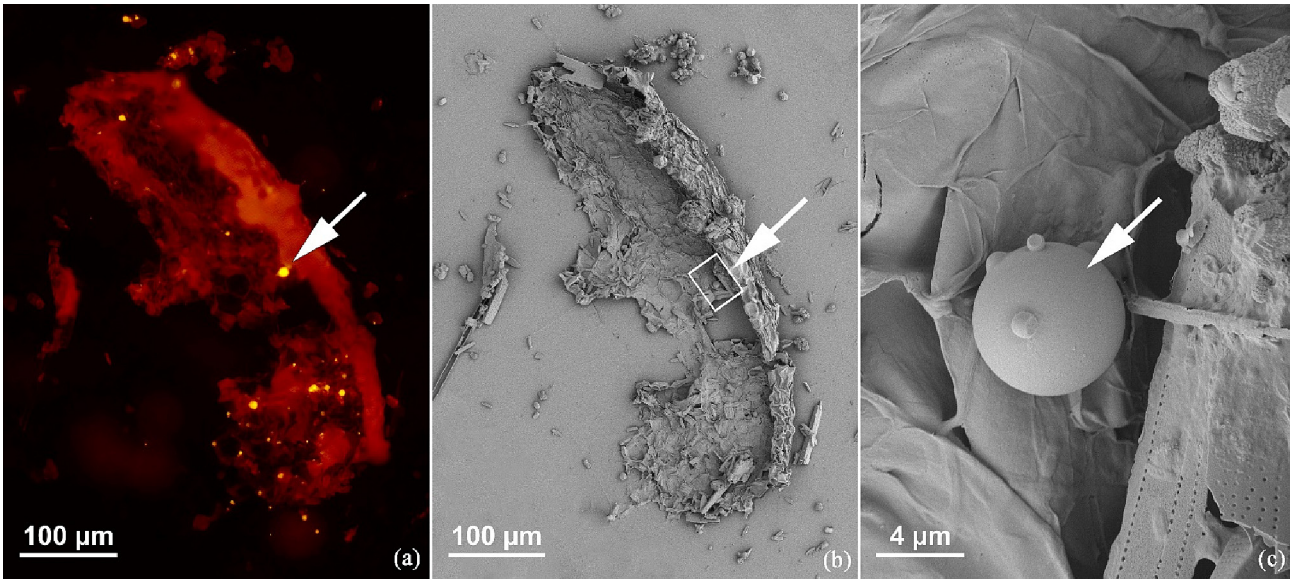

Fig. 6. Excrement samples of *C. lemnata* larvae fed by MPs-contaminated *Lemna* under fluorescence microscope (a) and SEM (b, c). Arrows indicate fluorescent MPs.

complete its life cycle by failing to transition from the pupa to the adult stage.

Certainly, one aspect to be investigated further, as already pointed out by Windsor et al. (2019), is whether ingested plastic material bioaccumulates over time within the bodies of these organisms without instead being totally released externally through excrements. Furthermore, it would be interesting to test whether larvae that have ingested food contaminated with microplastics can 'fragment' the microplastics passing through their gut into even smaller particles. Therefore, further fragmentation of the MPs could potentially lead to a higher possibility of bioaccumulation of this contaminant in larval tissues due to the smaller size, with possible further negative consequences.

Anyway, based on the evidence emerging from this study, it is clear that impact of MPs on aquatic herbivore insect selected cannot be considered negligible, and consequently the risk of MPs contamination along freshwater trophic chains should not be underestimated.

CRediT authorship contribution statement

Mariani Flaminia: Conceptualization, methodology, validation, investigation, data curation, writing—review and editing, visualization. Dario di Lernia: Conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft preparation, visualization. Iole Venditti: methodology, validation, writing—review and editing. Pelella Emanuele: formal analysis, writing—review and editing. Muzzi Maurizio: writing—review and editing, visualization. Di Giulio Andrea: writing—review and editing, supervision. Ceschin Simona: Conceptualization, methodology, validation, investigation, resources, data curation, writing—original draft, writing—review and editing preparation, visualization, supervision.

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Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare no conflict of interest.

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