



Comparative effects of polyvinyl chloride microplastics on the brittle star *Ophiactis virens* and the amphibian *Xenopus laevis*

Renato Bacchetta^a, Arianna Pica^a, Nadia Santo^b, Paolo Tremolada^{a,*}, Michela Sugni^a

^a Department of Environmental Science and Policy, Università degli Studi di Milano, Via Celoria, 26, 20133, Milano, Italy

^b Unitech NOLIMITS, Imaging Facility, Università degli Studi di Milano, Via Golgi, 19, 20133 Milano, Italy

ARTICLE INFO

Keywords:

PVC
Toxicity
Ecotoxicology
Echinoderm
Histology
Behaviour

ABSTRACT

In this study, we investigated the effects of PVC microplastics (PVC-MPs) using two different animal models: the brittle star *Ophiactis virens*, and the African clawed frog *Xenopus laevis*. This is the first study using an environmental relevant sample of PVC-MPs obtained through mechanical fragmentation of a common PVC plumbing pipe. Exposure experiments on brittle star were performed on the adult stage for a duration of 14 days, while those on African clawed frog were performed on the embryogenic developmental stage according to the standardized FETAX protocol (Frog Embryo Teratogenesis Assay-Xenopus). For both models, different endpoints were analysed: mortality, developmental parameters, behavioural assays and histological analyses on target organs by optical and electronic microscopy. Results showed that the concentration of $0.1 \mu\text{g mL}^{-1}$ PVC do not cause any adverse effects in both models (common NOEC concentration), while exposure to $1 \mu\text{g mL}^{-1}$ PVC adversely affected at least one species (common LOEC concentration). In particular arm regeneration efficiency was the most affected parameters in *O. virens* leading to a significantly lower differentiation pattern at $1 \mu\text{g mL}^{-1}$ PVC. On the contrary, in *X. laevis* larvae histopathological analyses and behavioural tests were the most susceptible endpoints, exhibiting several abnormal figures and different swimming speed at $10 \mu\text{g mL}^{-1}$ PVC. Histopathological analyses revealed a higher abundance of degenerating cells, pyknotic nuclei and cellular debris in the gut of exposed larvae in respect to control. The comparative analyses performed in this work allowed to characterize the specificity of action of the PVC-MPs on the two species, underlining the importance of exploring a large spectrum of endpoints to offer adequate protection in the emerging fields of microplastic research.

1. Introduction

Global production of plastics has been steadily increasing since 1950 and is projected to reach 34 billion tons by 2050, up from 9.2 billion tons in 2017 (Geyer, 2020). Among the various types of plastics, polyvinyl chloride (PVC) ranks as one of the most widely used worldwide (Benvenuto and Plaumann, 2021), third only to polyethylene and polypropylene in terms of production volume, 35 million metric tons in 2020 (Lu et al., 2023). This “success” can be attributed to the high versatility of PVC, which is considered as one of the most versatile polymers on the market. Due to the use of various additives, such as stabilisers, plasticisers, lubricants, fillers, and pigments, PVC can be tailored with the desired properties of flexibility and durability (Hahladakis et al., 2018; Markarian, 2007). Indeed, PVC’s mechanical properties are significantly influenced by the type and quantity of the additives used, with plasticised PVC applications incorporating up to 40 wt% of the final product

(Windels et al., 2022). PVC has a wide range of applications, both in its flexible and rigid forms. In its flexible form, when plasticisers are added, PVC is utilised in wire and cable insulation, flexible sheets and films, flooring, roofing, and toys, while in its rigid form, PVC is primarily used for pipes, fittings, and profiles for building applications (Zhang et al., 2015). Despite the large use of PVC, there is relatively little documentation of the occurrence, distribution and characterisation of PVC in the environment compared with other plastics (Turner and Filella, 2021).

The market success of PVC was soon accompanied by a significant environmental concern for several reasons: i) the toxicity of its vinyl chloride monomers (Li et al., 2016), ii) the poor biodegradability (Peng et al., 2020), and iii) the release of chemicals (leachates) throughout its entire life cycle (Thornton, 2002). For these reasons, the European Union (EU) proposed restrictions on the use of some PVC additives due to their potential human and environmental hazards, the import of PVC from extra-EU countries without such restrictions is expected to

* Corresponding author.

E-mail address: paolo.tremolada@unimi.it (P. Tremolada).

<https://doi.org/10.1016/j.aquatox.2024.106975>

Received 29 December 2023; Received in revised form 26 April 2024; Accepted 25 May 2024

Available online 27 May 2024

0166-445X/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

continue (European Chemicals Agency, 2016). Additionally, the fragmentation of PVC into microscale particles, characterised by high surface-to-volume ratios, facilitates the desorption of chemical additives, thus increasing the rate of release and altering exposure pathways, particularly in aquatic animals (Boyle et al., 2020). The ecological impact of PVC depends on several factors: to the leaching of additives in aqueous solutions (Boyle et al., 2020; Lithner et al., 2012; Oliviero et al., 2019; Shaikh et al., 2019; Turner and Filella, 2021; Ye et al., 2020; Yap et al., 2020; Zimmermann et al., 2020; Barkhau et al., 2022), to organic pollutants absorbed to PVC particles (Browne et al., 2013; Rist et al., 2016; Gomiero et al., 2018), and to adverse effects subsequent to microplastic ingestion (Wright et al., 2013; Pedà et al., 2016; Espinosa et al., 2017; Lei et al., 2018; Romano et al., 2018; Suckling, 2021; Liu et al., 2023), with increased toxicity observed as microplastic size decreases (Lei et al., 2018).

PVC-MPs were extensively analysed in fish (Pedà et al., 2016; Espinosa et al., 2017; Gokce et al., 2018; Lei et al., 2018; Romano et al., 2018; Boyle et al., 2020; Iheanacho and Odo, 2020; Xia et al., 2020; Vijayaraghavan et al., 2022, 2022; Wang et al., 2022; Liu et al., 2023), while, to our knowledge, only one study exists on amphibians (*Xenopus laevis*) recently done by Pekmezekmek et al. (2021). This study found no abnormalities in the lungs and intestines of adults, but reported malformations and decreased viability in embryos, along with significantly decreased expression levels of the Hsp70 and Pax6 genes in all PVC-exposed groups. Although amphibians are one of the most threatened groups of vertebrates, globally declining (Converse and Grant, 2019), they are currently poorly investigated regarding the impact of MPs. Recently, Balestrieri et al. (2022) highlighted that MPs could be a possible underrated threat to amphibian conservation, even if contrasting results are present in literature. Even on the same species, stage and concentrations, but different MPs, there were no effects at all (De Felice et al., 2018) or relevant ones (Bacchetta et al., 2021). Tadpoles, having opportunistic feeding habits (Altig et al., 2007), are largely exposed to MPs ingestion, as demonstrated by Balestrieri et al. (2022) with a mixture of MPs: expanded polystyrene (PS), high-density polyethylene (HDPE), polyester fibers (PES) and PVC. Similarly to tadpoles, benthonic invertebrates share the same habitat, and they showed a MPs contamination even higher than fish (Walkinshaw et al., 2020). Moreover, the marine environment is the final sink of plastic pollution and Mediterranean Sea is a hot-spot contamination area (Suaria et al., 2020). Therefore, to evaluate MPs impact to marine benthonic species, the Mediterranean invertebrate *Ophiactis virens* can be considered as a model species for MPs toxicological studies. The two species tested in this work (the brittle star *Ophiactis virens*, and the African clawed frog *Xenopus laevis*) can be considered both as a possible specific target of MPs toxicity, and, sharing similar feeding habits, their toxicological comparison could furnish interesting insight in studying MPs impact.

A common criticism of MP toxicity studies relates to the very high concentrations used in the tests (up to 150 mg L⁻¹ as in Yap et al., 2020), as well as the type of plastic used, which is often represented by standard MPs purchased on the market (Du et al., 2021; Jahnke et al., 2017). For these reasons, we exposed our test species to PVC concentrations lower than those used in most of the previous studies, and we used an environmental-relevant PVC-MPs sample, obtained through the mechanical fragmentation of a common PVC plumbing pipe. Given that commercially available PVC can vary widely in composition and different types of PVC can produce different levels of contamination, a chemical characterization of every PVC product is essential. PVC is known for having many additives including inorganic and organic compounds (Hahladakis et al., 2018). Among them, attention has been given to inorganic additives because, especially in the past, the addition of heavy metals, in particular lead (Pb), was the common solution to improve durability and resistance of PVC products. For example, Boyle et al. (2020) reported that PVC-MPs caused adverse effects in zebrafish mediated through desorption of Pb into the exposure water. Despite the restrictions in the European Union on the use of Pb in PVC formulation,

several Pb-contaminated PVC were present on the European market derived from the import from other countries, where no restrictions are presents (European Chemicals Agency, 2016). Therefore, we performed chemical and ecotoxicological analyses able to test the metal presence and the additional toxicity of our PVC-MPs sample due to leachates (both inorganic and organic).

PVC-MPs toxicity was tested on two different animal models (Fig. 1) with similar feeding habits, but living in two different aquatic environments i.e. marine and freshwater: the brittle star *Ophiactis virens* (Echinodermata: Ophiuroidea), and the freshwater African clawed frog *Xenopus laevis* (Amphibia: Anura), a well-known model organism widely used in toxicological tests (Bacchetta et al., 2021; Bantle et al., 1990), as well as in ecological risk assessment studies (Hoke and Ankley, 2005). By the comparison of the PVC-MPs toxicity on the two model species, we aimed to test the following hypotheses: i) the toxic effects toward the two species is expected to be different due to the different taxonomic groups and life stages of the two species; ii) the large spectrum of the considered toxicological endpoints and the considered behavioural parameters are expected to overcome the species specificity in the final toxicological evaluation; iii) the ingestion of PVC-MPs is expected to be the main input pathway for both species and thus iii) the digestive system is expected to be the main target of the PVC-MPs exposure.

2. Materials and methods

2.1. Preparation of PVC-MPs and testing solutions

PVC-MPs were obtained from a new commercial PVC building pipe by cryo-milling at 10,000 rpm for 30 s using a Pulverisette 11 mill (Fritsch, Idar-Oberstein, Germany). The obtained PVC-MPs were sorted through a 250 µm mesh filter to isolate the finest fractions for use in the exposures.

2.2. PVC-MPs characterisation

Ultrastructural and chemical analyses were performed to better characterise the PVC-MPs. A subsample of the cryomilled PVC-MPs was placed on standard aluminium stubs, platinum-sputtered with a Leica ACE600 sputter coater (Leica Microsystems, Germany), and photographed under a scanning electron microscope FE-SEM Sigma (ZEISS, Germany) at 2000x magnification. The perimeter and area of thousands of PVC-MPs ($n = 4099$) were measured from SEM images using the free image processing program ImageJ (Schneider et al., 2012) to characterise their size and external morphology. From the measured area, the diameter of the equivalent spherical particles (d_e , hereafter referred to as equivalent diameter of each particle) was geometrically calculated to uniformly characterise their mono-dimensional size. Instead, the PVC-MPs external morphology was expressed in terms of roughness index (R_i):

$$R_i = p/p_e$$

where p was the actual perimeter of each fragment measured by the software, while p_e was the perimeter of a spherical particle having the same area. For R_i close to 1, the fragment was considered similar to a spherical particle, whereas for $R_i \gg 1$ the particle had an irregular shape, with a perimeter twice ($R_i = 2$) or three times ($R_i = 3$) that of a spherical particle having the same area.

PVC-MPs were also chemically characterised to assess the presence of inorganic additives (e.g. heavy metals) and potential leachates in the exposure medium. The analysis was performed by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Three aliquots of about 10 mg each of PVC-MPs were mineralized in 50 mL of a mixture of ultrapure acids (HNO₃ and HCl with 4:2 ratio) and analysed using the Optima™ 8000 ICP-OES instrument (Perkin Elmer, Waltham, USA). A blank sample (MilliQ water and HNO₃ 2 %) and a calibration curve

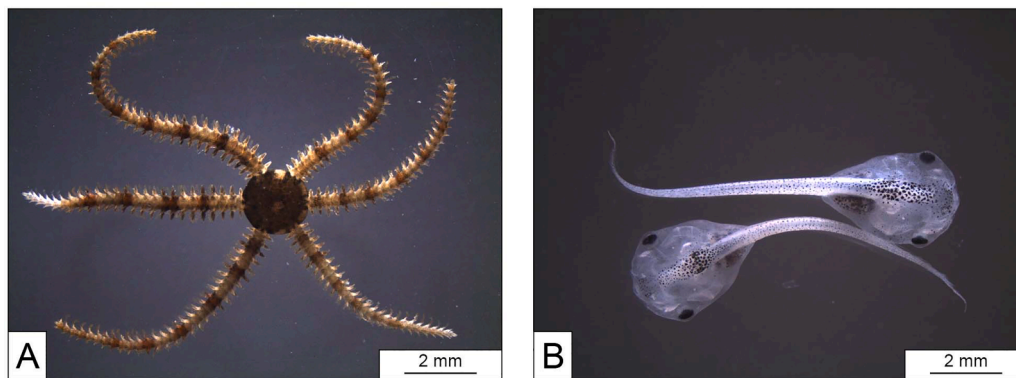


Fig. 1. Images of the two utilised animal models. (A) Adult of the brittle star *Ophiactis virens*; (B) Larvae of the African clawed frog *Xenopus laevis*.

between 0.005 and 1 mg kg⁻¹ were used for the quantification of the following elements: Chromium (Cr), Iron (Fe), Nickel (Ni), Aluminium (Al), Arsenic (As), Calcium (Ca), Cadmium (Cd), Copper (Cu), Manganese (Mn), and Lead (Pb). Relative Standard Deviation (RSD) of each quantification was obtained by 65 s of detection over 2 min of sample aspiration.

An estimate of the number of PVC-MPs per mL (MPs mL⁻¹) in the three tested concentrations (µg mL⁻¹) was also attempted. The number of microparticles was estimated from the ratio between the nominal exposure concentrations and the weight of PVC particles. Particle weight was obtained from the volume of the PVC-MPs and the PVC density (1.4 mg mm⁻³). The volume of PVC-MPs was calculated considering the relative abundance of each dimensional class of PVC-MPs and their mean volume (mm³). For each dimensional class a mean particle volume was geometrically derived considering the mean equivalent diameter (d_e) of each class.

2.3. *O. virens* test

2.3.1. Animal collection, maintenance and experimental design

Specimens of *O. virens* were collected from Le Grazie Gulf (La Spezia, Liguria, Italy) in January 2022. Animals were put in cooled boxes and immediately transported to the laboratory, where they were acclimated in an aerated 50 L aquarium filled with FASW (Filtered Artificial Seawater) for a three-week period. The temperature was gradually increased once a week up to 17 °C, which is the mean annual temperature of the Mediterranean Sea. Temperature, salinity and pH were measured daily, while levels of nitrite (NO₂-), nitrate (NO₃-), chlorine (Cl₂) and water hardness (KH, GH) were tested weekly and adjusted if necessary. The photoperiod was set at a 12 h light/dark cycle, and animals were fed grinded spirulina algae daily. After acclimatisation, brittle star juveniles (disc diameter 1–2 mm) were selected, avoiding specimens with regenerating arms and those that had recently undergone fission to reduce the risk of previous stress conditions. Animals were anaesthetised in a 3.5 % MgCl₂ solution and amputated of two adjacent arms at the junction between the third and fourth segments from the central disc, using a sterile scalpel under a Leica MZ75 stereomicroscope. Amputated brittle stars were then transferred to a glass container filled with FASW to recover from anaesthesia, and subsequently randomly distributed in the exposure groups: FASW for control and three concentrations of PVC-MPs (0.1, 1 and 10 µg mL⁻¹). For each experimental condition, 10 animals were placed in a 300 mL glass container, and groups were triplicates, resulting in a total of 120 brittle stars (10 brittle stars X 3 replicates X 4 experimental conditions). Each experimental group was provided with an oxygenator stone and mussel shells as shelters for brittle stars. A semi-static assay, with complete exposure medium renewal every 48 h, was performed for two weeks. Brittle stars were fed three times a week with spirulina algae, and mortality was checked daily. At the end of the exposure period, different

parameters were analysed and the samples processed as subsequently described.

2.3.2. Behavioural responses

To analyse the potential effects of PVC-MPs exposure on *O. virens* behaviour, animal righting ability (righting time) and locomotion (speed) were evaluated. The righting time was the amount of time taken by one specimen to right itself from an inverted (oral surface up) to a fully correct (oral surface down) orientation. Using tweezers, each animal was flipped over, and the time for each specimen to completely return with all the five arms placed down was measured. Animal speed was evaluated by video tracking as follows: brittle stars were grouped in the centre of the experimental glass container and covered with a glass jar wrapped with a tin foil for 20 s. Then, the jar was removed and after 10 s, a 3-minute video was recorded. The 12 videos, from all the experimental conditions/replicates, were taken with a 1080p HD 30fps digital camera. Videos were then analysed by the image processing software ImageJ (Schneider et al., 2012) and the Animal Tracker plugin (Gulyás et al., 2016), considering for each specimen the distance moved (mm) and its relative speed (mm/sec).

2.3.3. Arm regeneration efficiency

After 14-days of exposure, brittle stars were anaesthetised in MgCl₂ and the regenerating arms of each specimen were photographed under a Leica MZ75 stereomicroscope equipped with a Leica EC3 camera. For each regenerating arm, the regenerate length (L) and the number of regenerated segments (s) were considered (Fig. 2). According to Candia

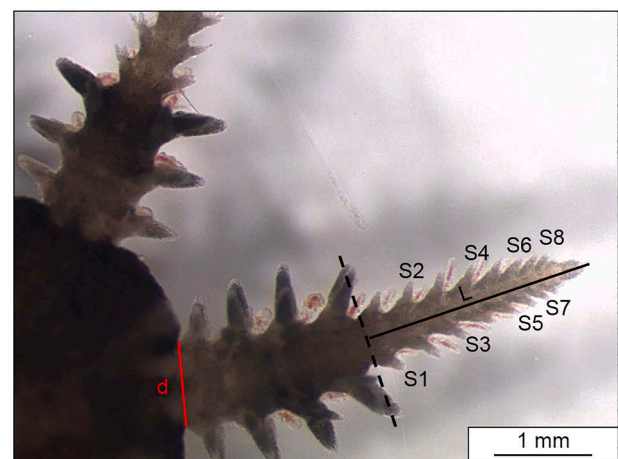


Fig. 2. Measured parameters used to calculate the arm differentiation index (DI) in *O. virens*. Red line = arm diameter (d); dashed line = amputation plane; black line = regenerate length (L); S1-S8 = regenerated segments.

Carnevali et al., 2024, the arm regeneration efficiency was expressed by the arm differentiation index (Di):

$$Di = s/Ln$$

where Ln is the normalised regenerate length calculated using the stump arm diameter (d), to eliminate age/size effect, as follows:

$$Ln = L/d$$

Afterwards, all the arms were amputated from the central discs, except for the regenerating arms, and samples were processed according to the specific protocols for light and electron microscopy analyses to assess the presence of PVC-MPs and the potential effects of PVC-MP exposure in terms of external or internal tissue anomalies (histopathology).

2.4. *X. laevis* test

2.4.1. Animals and experimental design

Adult *X. laevis* were maintained at the University of Milan in a TecnoPlus automatic breeding system (TecniPlast, Varese, Italy) under controlled conditions: $T = 20 \pm 2^\circ\text{C}$; $\text{pH} = 7.5 \pm 0.5$; conductivity = $1000 \pm 100 \mu\text{S}$; 12 h light/dark cycle and fed a semi-synthetic diet twice a week (XE40; Mucedola S.r.l., Settimo Milanese, Italy). The experiment ran according to the Frog Embryo Teratogenesis Assay-Xenopus, FETAX, protocol (ASTM, 1998), lightly modified. In particular, to investigate both the developmental toxicity of PVC particles and their possible effects due to ingestion, two different exposures were planned: i) a regular exposure, EXP1, from the classic blastula stage (stage 8–9, according to Nieuwkoop and Faber (1994), NF), and ii) a late exposure, EXP2, in which embryos were exposed prior to mouth opening, which occurs at NF stage 40.

Embryos for the two experiments were obtained from overnight natural mating; after breeding adults were removed and embryos collected in glass Petri dishes. Fertilised eggs were dejelled in a 2.25 % l-cysteine solution with an arranged pH of 8.0, and rinsed several times in FETAX solution, whose composition in $\mu\text{g mL}^{-1}$ was: 625 NaCl, 96 NaHCO_3 , 30 KCl, 15 CaCl_2 , 60 $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, and 70 MgSO_4 . Normally cleaved embryos at the blastula stage were selected for the regular exposure, while embryos at NF stage 36–37 were used for the late exposure. Ten embryos were seeded in 60 mm glass Petri dishes containing 10 mL of the control (FETAX solution) or test suspensions of 0.1, 1, and $10 \mu\text{g mL}^{-1}$ PVC. These were prepared in FETAX as well by serial dilution of the stock solution (1 L of $10 \mu\text{g mL}^{-1}$ PVC in FETAX previously prepared and maintained on a shaker under continuous agitation). All groups, controls included, were quadruplicated and allowed to develop in a thermostatic chamber at $23^\circ\text{C} \pm 0.5$ until NF stage 46, the free-swimming larva, end of the tests. For EXP1, two additional Petri were seeded for each group, thus a total of 240 embryos (10 embryos X 6 replicates X 4 experimental groups) were used, while for EXP2, 160 embryos (10 embryos X 4 replicates X 4 experimental groups) were selected. Each day, all solutions were renewed, and embryos checked for viability. At the end of the tests (NF stage 46), the FETAX endpoints, i.e., malformation, mortality and growth inhibition, were considered.

2.4.2. Behavioural responses

To evaluate the possible effects of PVC particles on the swimming activity of the larvae at the end of the two exposures, a swimming behavioural test was performed by video tracking analysis, according to Bacchetta et al. (2021). Briefly, 24 larvae per group, and in particular 4 larvae from the 6 groups of EXP1 and 6 from those of the corresponding 4 groups of EXP2, were randomly taken and individually transferred to a Petri dish filled with 10 mL of FETAX solution to be video tracked. Due to the high larval motility, each sample was placed into a 27 mm plastic cylinder, positioned inside a 60 mm Petri dish, and allowed 1 min to acclimate before being recorded for 30 s. Videos were taken from above

using a 1080p HD 30 fps digital camera and subsequently analysed using the Animal Tracker plugin (Gulyás et al., 2016), and the free image processing program ImageJ (Schneider et al., 2012). Total immobility time (sec), distance moved (mm), and mean swimming speed (mm sec^{-1}) were considered as swimming activity endpoints. At the end of the analysis, all larvae were anaesthetised with MS222 at a final concentration of $100 \mu\text{g mL}^{-1}$, evaluated for single malformations under a Leica EZ4 D stereomicroscope, and photographed for the growth retardation measurements, according to the standard FETAX protocol (ASTM, 1998). At the very end, all samples were fixed for the subsequent microscopical analyses (see below).

2.5. Light microscopy analyses

In *O. virens*, to evaluate the potential effects of PVC-MPs on the external anatomy of regenerating arms, exposed samples were compared to controls, mainly focusing on the presence and morphology of spines, tube feet, and oral/aboral shields. Furthermore, six central discs (housing the digestive system) from each experimental group were randomly selected and processed for histopathological analyses, according to Ben Khadra et al. (2015). Briefly, samples were fixed in Bouin's fluid for 3 weeks at 4°C , with a fixative renewal after 10 days, to allow complete decalcification. In *X. laevis*, 8–10 larvae from all the experimental groups, controls included, were randomly selected, anaesthetized, and fixed overnight in Bouin's fluid at room temperature.

After fixation, both *O. virens* (16 samples, 4 for each experimental condition) and *X. laevis* samples were rinsed in tap water, dehydrated in an ascending ethanol series, and embedded in Bio-plast tissue embedding medium (Bio-Optica Srl, Milano, Italy) and in paraffin (Paraplast Plus, Sigma-Aldrich) for *O. virens* and *X. laevis*, respectively, following the standard histological procedure. Seven-micron transverse serial sections of the samples were obtained using a Reichert rotative microtome (Reichert, Austria) and stained according to Milligan's trichrome staining (Milligan, 1946) and with Hematoxylin-Eosin (HE) for *O. virens* and *X. laevis*, respectively. Finally, all slides were mounted with Eukitt medium. The slides of *O. virens* specimens were observed under an Aus JENA light microscope equipped with a Leica Flexacam C3 camera, while those of *X. laevis* were examined under a Leica DMRA2 light microscope, and images were collected with a Leica DC300F digital camera. In *X. laevis*, a total of 73 larvae (37 from EXP1 and 36 from EXP2) were microscopically analysed covering all the larval organs and tissues.

2.6. Electron microscopy analyses

In *O. virens*, central discs and regenerating arms were fixed for 2 h in a mixture of FASW 85 % + glutaraldehyde 2 % at 4°C . Samples were then washed in FASW overnight at 4°C and post-fixed in a solution of 2 % OsO_4 in FASW 37 % and glucose 940 mOsm for 2 h, in the dark and at room temperature. After several washes, samples were dehydrated in an increasing ethanol series (25 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % and 100 %), and then dried using a critical point dryer CPD 020 (Balzers Union, Liechtenstein). In addition, eight *O. virens* samples (2 for each experimental condition) coming from light microscopy fixation, were half-sectioned, and the remaining disc halves were immersed in pure xylene until complete paraffin removal, left drying overnight at room temperature. At the very end, all specimens were mounted on standard aluminium stubs.

In *X. laevis* experiments, 20 larvae from each treatment group were randomly selected and fixed in a mixture of 4 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffered solution at pH 7.4. After several washes in the same buffer, larvae were post-fixed in 1 % OsO_4 for 2 h, at 4°C , dehydrated with an ascending ethanol series, and critical-point dried in a K850 apparatus (Quorum Technologies, Laughton, UK). The digestive tract of each larva was carefully dissected under a stereomicroscope, mounted onto standard aluminium SEM stubs. A total of 160 larvae (80 from the regular and 80 from the late

exposure) were processed, and half of them microscopically analysed, mainly at the small and large intestine level (Chalmers and Slack, 1998).

Both *O. virens* and *X. laevis* samples were platinum-sputtered with a Leica ACE600 sputter coater (Leica Microsystems, Germany), and observed under a FE-SEM Sigma (Zeiss, Jena, Germany) at 7 kV, WD 20–10 cm.

2.7. Statistical analyses

In *O. virens*, assumptions for normality were preliminary assessed using Shapiro-Wilk test, and no significant deviation from normality was observed for all the tested variables ($P > 0.05$). A chi-squared test with Yates' correction was used to analyse mortality during PVC-MPs exposure in *O. virens*, considering both concentration and time effects. Yates' correction was introduced because the total number of cases was >40 but <200 , while the condition for chi-squared test's validity (>5 expected cases for every experimental group) was respected. Logistic models were applied to evaluate the effects of concentrations and replicates on the mortality rate contemporarily. Analysis of variance (ANOVA) was used to test the effects of PVC-MPs on behavioural responses (righting time and speed) and arm regeneration efficiency (arm differentiation index). Significant ANOVA results were followed by a Tukey post-hoc test for pairwise comparisons.

In *X. laevis* exposure experiments, pair comparison of mortality and malformation frequencies between exposed and control batches at the same exposure interval (9–46 or 36–46) and between different exposure intervals (9–46 vs. 36–46) at the same exposure concentration were evaluated by means of Fisher exact test, because of the very low number of cases of mortality and malformation observed. The effects of "PVC concentration" and "exposure time interval" on the dependent variable "specimen length" was evaluated by two-way ANOVA, testing data distribution for normality by Kolmogorov-Smirnov test ($P > 0.05$). Three quantitative behavioural parameters: i) time spent in the inner or in the outer part of the arena; ii) distance travelled in the inner or in the outer part of the arena; and iii) movement speed in the inner or in the outer part of the arena were tested by the non-parametric Kruskal-Wallis and Mann-Whitney tests for multiple and pair comparison, respectively. R software, through Rstudio (version 4.2.0), was used for Shapiro-Wilk, Yates' χ^2 , ANOVA and Tukey post-hoc tests. Logistic models, Kruskal-Wallis and Mann-Whitney tests were performed with SPSS (version 15.0) software. All statistical analyses were carried out at the significance threshold of 0.05.

3. Results

3.1. Physico-chemical characterisation of PVC-MPs

Fig. 3 shows the appearance and primary characteristics of the material used in this study. A commercial PVC building pipe was first broken into large fragments (Fig. 3A), then cryo-milled and filtered to obtain PVC-MPs to be used in the exposure tests (Fig. 3B). Approximately 4100 PVC fragments were morphological characterised by SEM (Fig. 3C, as an example), and the size distribution frequencies of their equivalent diameters (d_e) were calculated as equivalent spherical particles (Fig. 3D). The d_e ranged from 0.1 to about 400 μm , with a calculated median diameter of 9.7 μm . Noteworthy the most prevalent size classes, accounting for approximately 50 % of the measured PVC fragments, included particles with $d_e < 10 \mu\text{m}$ (Fig. 3D), within which a small percentage of nanoplastics (slightly over 1 %) was detected. The external morphology of the PVC-MPs, assessed using the R_i parameter, indicated clear heterogeneity, with roughly 50 % being spherical particles ($R_i < 1.2$), about 45 % being non-uniform fragments ($1.2 < R_i < 3$), and approximately 5 % being highly irregular pieces ($R_i > 10$) (Fig. 3E).

As detailed in the Material and Methods section, an attempt was made to estimate the number of PVC particles for the three suspensions

of 0.1, 1, and 10 $\mu\text{g mL}^{-1}$ PVC-MPs. The indicative number of particles per exposure concentration was 161, 1610, and 16,100 particles mL^{-1} , respectively.

SEM EDX analysis showed the peaks of the different elements present within the PVC particles (Fig. 3F). The presence of Chlorine and Calcium peaks in the spectrum were evident, and confirmed the use of Calcium to improve the mechanical properties of PVC (Hounsham and Titow, 1984). Additionally, to assess the presence of possible inorganic additives embedded in PVC, which can eventually be released as leachates in the exposure mediums, ICP-OES analysis was performed. This showed that heavy metals concentration varied between 5 mg kg^{-1} for Cadmium and nearly 650 mg kg^{-1} for Copper (Fig. 3G).

3.2. Effects of PVC-MPs on *O. virens*

3.2.1. Mortality and arm regeneration efficiency

No significant differences in mortality were observed among control and PVC-MP-treated groups due to either treatment (χ^2 test with Yates' correction; $\chi^2 = 1.02$, d.f. = 3, $P = 0.80$) or time effect (χ^2 test with Yates' correction; $\chi^2 = 1.98$, d.f. = 3, $P = 0.58$). Logistic regression analysis of the number of deaths indicated that neither the treatment nor the replicate effects were statistically significant ($P = 0.47$ and $P = 0.31$, respectively). The highest percentage of mortality registered at the end of the exposure period was observed in ophiuroids exposed to 1 $\mu\text{g mL}^{-1}$ PVC-MPs (26.7 %), while the lowest in samples exposed to 0.1 $\mu\text{g mL}^{-1}$ PVC-MPs (13.3 %).

The PVC-MPs treatment adversely affected the arm regeneration efficiency of *O. virens* resulting in a lower differentiation index in the exposed groups compared to the controls ($F_{3,91} = 9.838$, $P < 0.001$; Fig. 4). Significant differences were observed between the control group and samples from the 1 and 10 $\mu\text{g mL}^{-1}$ PVC-MPs groups ($P = 0.048$ and $P < 0.001$, respectively).

3.2.2. Morphology and histopathological analyses

Morphological analyses, performed on all brittle stars that survived to the 14 days-exposure, did not reveal marked differences when compared to the controls. The external anatomy and the regenerated arm tips of samples from the PVC-exposed groups closely resemble those of the untreated samples in terms of general morphology (e.g., presence, shape, size of spines, podia, segments) and length of the regenerated arms (Fig. 5). Histopathological examination of randomly selected samples from the treatment groups also did not show significant differences compared to the controls. The internal disc anatomy was similar and no tissue was apparently affected by PVC-MPs exposure. One sample exposed to 10 $\mu\text{g mL}^{-1}$ PVC showed a small PVC particle in the preoral cavity (Fig. 6E) while few samples from both the 1 and 10 $\mu\text{g mL}^{-1}$ PVC groups displayed fragments or little clusters of PVC inside their digestive sac. These particles/clumps were observed either free in the stomach lumen (Fig. 6F) or lying within the folds of the digestive epithelium (Fig. 6G-H). However, no evident damages to the apical portion of the cells were detected (Fig. 6F-H), and the overall structure of the stomach wall was maintained: the epithelium, much taller aborally than orally, was provided with a regular brush border and laid on a thin layer of connective tissue. More externally, a few muscle fibres were present followed by the cuboidal coelomic lining (Hyman, 1955). Some samples from all the experimental groups were subjected to a more detailed analysis by SEM to look for possible ultrastructural damages due to PVC-MPs. Also in this case, no morphological abnormalities were observed. The digestive cells of samples from the three PVC-exposed groups were as normal as in controls, and no damage was found in the stomach or other organs/tissues. As by the histological analysis, a PVC fragment was detected in the preoral cavity of one sample exposed to 10 $\mu\text{g mL}^{-1}$ PVC-MPs (Fig. 7), but no microplastics were observed in samples treated with lower PVC-MP concentrations.

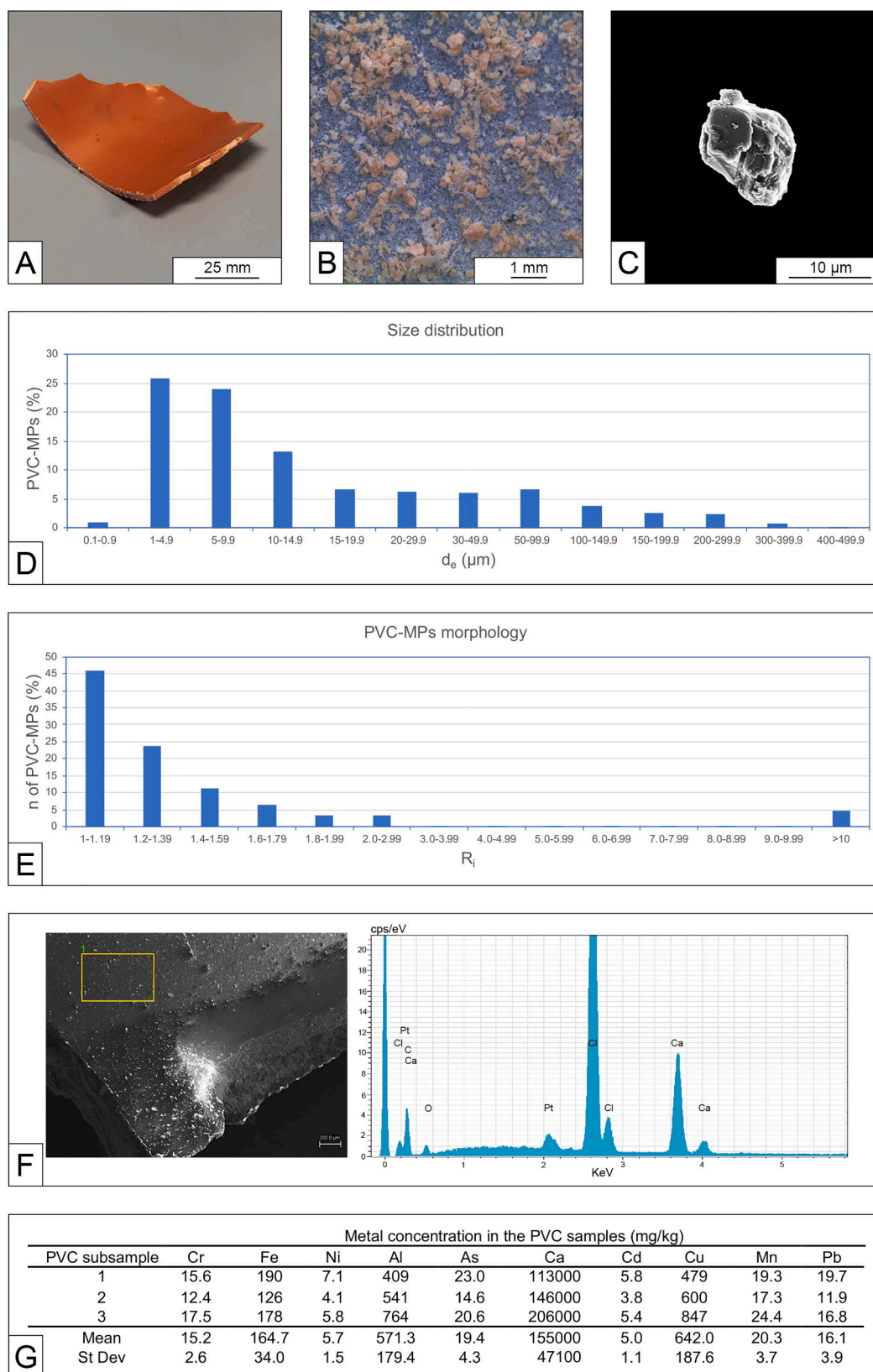


Fig. 3. Characterisation of PVC-MPs. (A) Piece of a PVC building pipe; (B) PVC fragments obtained through cryo-milling by the stereomicroscope; (C) Image of a PVC microparticle by SEM; (D) Size distribution histogram of PVC-MPs by using the equivalent diameter (d_e); (E) Histograms showing external PVC-MP morphology expressed as roughness index (R_i); (F) EDX spectrum from a PVC fragment; (G) Metal concentration in PVC-MPs as measured by ICP-OES.

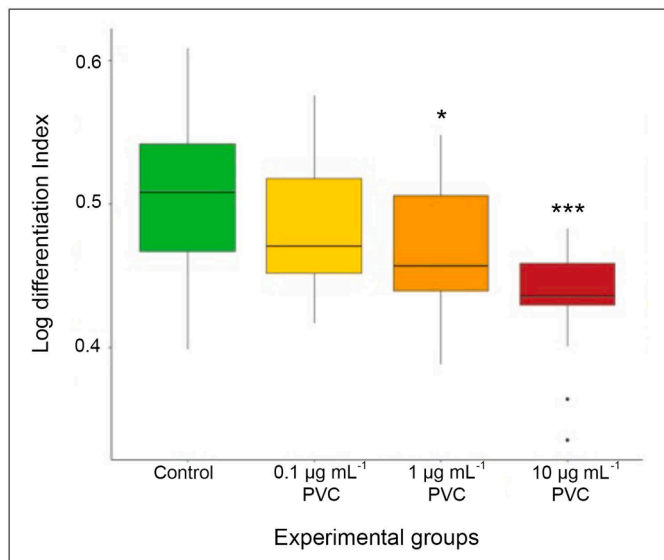


Fig. 4. Boxplot showing the arm regeneration efficiency measured in controls and in the groups exposed to PVC-MPs.

3.2.3. Behavioural effects of PVC-MPs

After the 14-days exposure, all surviving brittle stars underwent two different behavioural tests to assess possible impairments in their locomotion abilities and righting time. Analysis of the calculated speed data from samples in the different experimental groups indicated an increase in median values from controls to the 0.1 and 1 µg mL⁻¹ PVC groups. In

contrast, the locomotion abilities of samples exposed to 10 µg mL⁻¹ PVC were very similar to those recorded in the control group (Fig. S1). Statistical analyses did not reveal any significant effect of PVC-MPs on the locomotion capability of treated samples and no differences were observed between these samples and the control group ($F_{3,89} = 0.744$, $P = 0.528$). Focussing on the median times taken by brittle stars to right themselves and achieve a fully correct orientation, a decreasing trend was apparent: controls were slower (i.e., higher righting times) than all other groups, while samples exposed to the highest PVC concentration were the fastest (i.e., exhibited the lowest righting time; Fig. S2). However, as with locomotion speed, no statistical differences were observed among groups, and no effects due to PVC-MPs on the righting behaviour of treated samples could be identified ($F_{3,91} = 1.797$, $P = 0.153$).

3.3. Effects of PVC-MPs on *X. laevis* embryos

3.3.1. Mortality, malformations, and growth inhibition: comparison between the two exposures

No differences were observed between the regular exposure (NF stage 9-46, EXP1) and the late exposure (NF stage 36/37-46, EXP2) regarding mortality, external malformations, and growth inhibition.

All larvae from both control and PVC-MP-treated groups survived until NF stage 46 (end of the test), one specimen from the 10 µg mL⁻¹ PVC group in EXP1 excluded. Fisher's exact test for pairwise comparison did not reveal any significant effects ($P > 0.5$), neither for the concentration (exposure vs control) nor for the exposure interval (EXP1 vs EXP2, at the same exposure concentration).

Regarding external malformations, no differences were observed in comparison to controls in both treatments, with almost all larvae

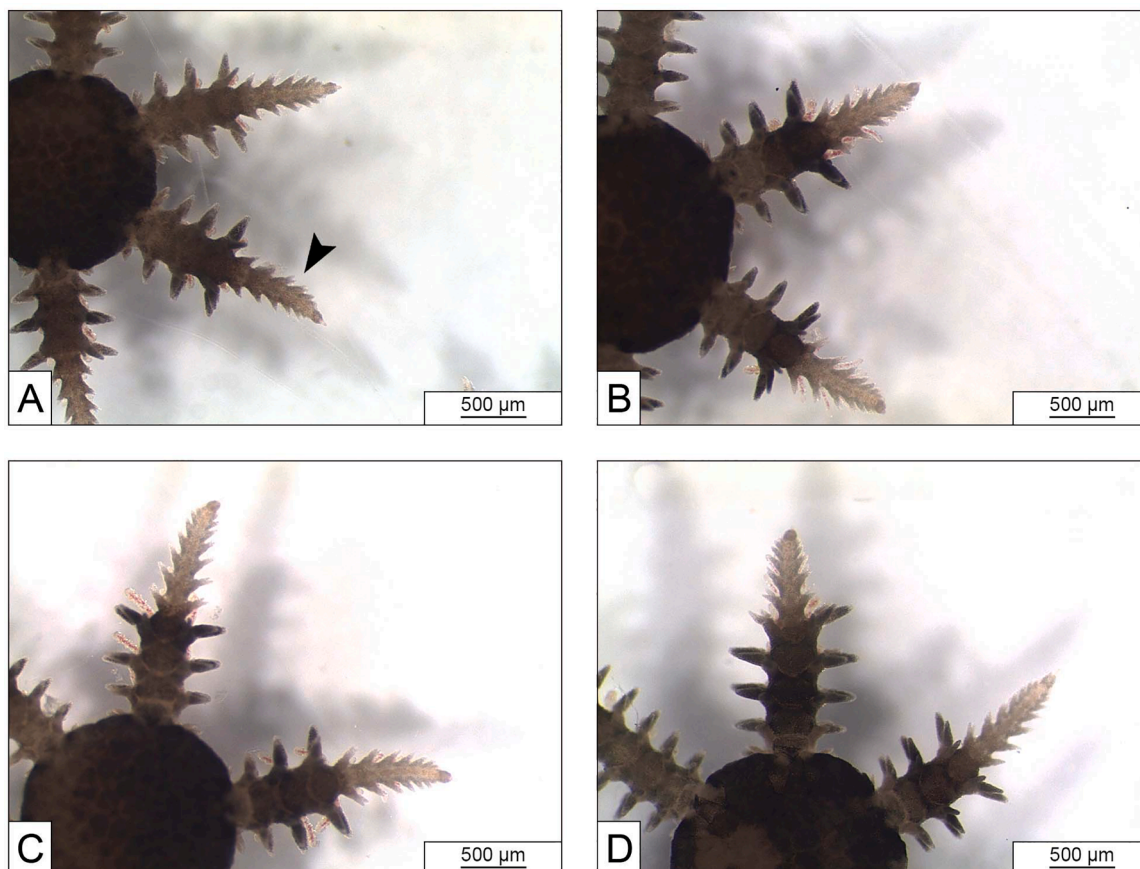


Fig. 5. External anatomy of the regenerated arm tips by the stereomicroscope. (A) Control sample; (B, C, and D) Samples exposed to 0.1, 1 and 10 µg mL⁻¹ PVC-MPs, respectively. Black arrowhead = regenerated arm tip.

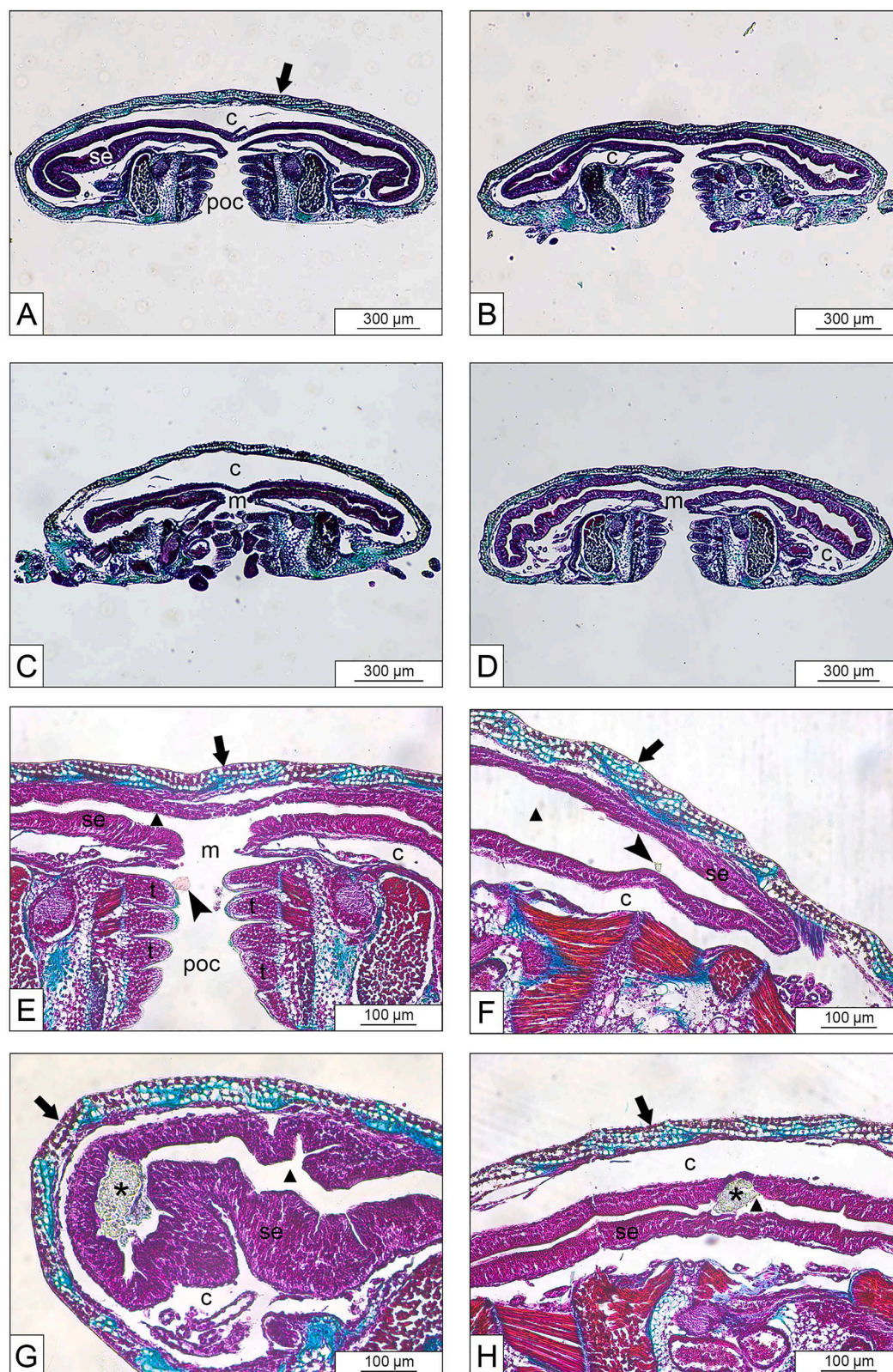


Fig. 6. Histological sections of controls and PVC-MP-exposed groups at low (A-D) and high magnification (E-H). Sections through the disc at mouth level show the internal anatomy of the brittle stars. Black arrow = epidermis; c = coelom; se = stomach epithelium; poc = pre-oral cavity; m = mouth; black arrowhead = PVC fragment; t = teeth; black triangle = stomach lumen; * = PVC clump.

A = Control; B = 0.1 µg mL⁻¹ PVC-MPs; C and G = 1 µg mL⁻¹ PVC-MPs; D, E, F and H = 10 µg mL⁻¹ PVC-MPs.

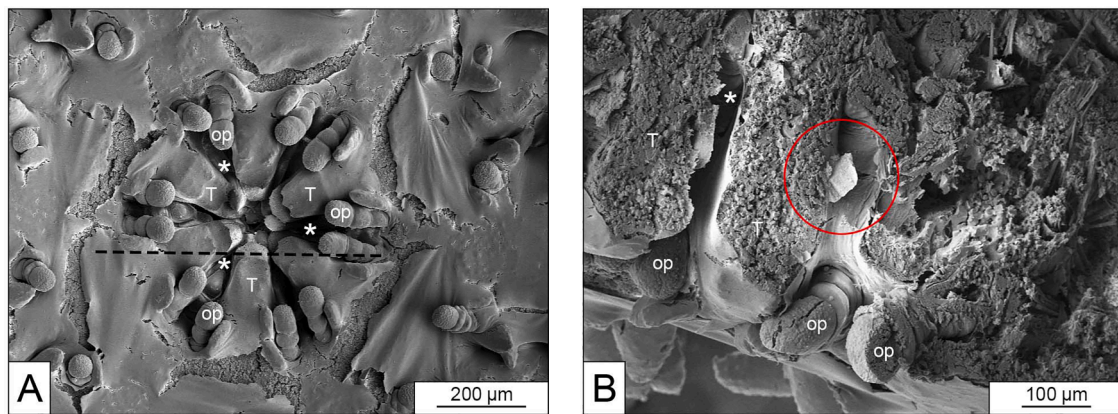


Fig. 7. SEM images of two *O. virens* specimens, exposed to $10 \mu\text{g mL}^{-1}$ PVC-MPs. The dashed line in (A) shows the section plane visible in (B). * = pre-oral cavity; op = oral podia; T = teeth; red circle = PVC-MP.

showing regular development until the end of the tests. Very few cases of external malformations were observed in some experimental groups from both EXP1 and EXP2 (Table S1), and Fisher's exact test did not indicate any significant effects, neither for the concentration nor for the exposure time interval ($P > 0.22$).

Data from larval measurements conducted at the end of the tests indicated that growth was not inhibited by PVC-MPs either. The effect of PVC-MP concentration and exposure time interval were evaluated by two-way ANOVA: marginal means of the length of the larvae among the different exposure concentrations and between the two exposure time intervals were very similar (Table S2), and none of the considered

factors (PVC-MP concentration and exposure time interval) nor their interaction showed a significant effect on larval size ($F_{3;305} = 0.51$ and $P = 0.67$, $F_{1;305} = 3.27$ and $P = 0.072$ and $F_{3;305} = 2.41$ and $P = 0.067$, respectively). Larvae from the two exposures had very similar mean lengths (10.81 ± 0.39 mm SD for EXP1 and 10.70 ± 0.62 mm SD for EXP2) with a total larval length of 10.76 ± 0.52 mm SD.

Morphological analyses initially performed under a stereomicroscope on all larvae that reached NF stage 46 did not reveal significant differences compared to controls. Very few alterations were observed among all the experimental groups, and they were recorded only sporadically, independently from the treatment (Table S1). Even though

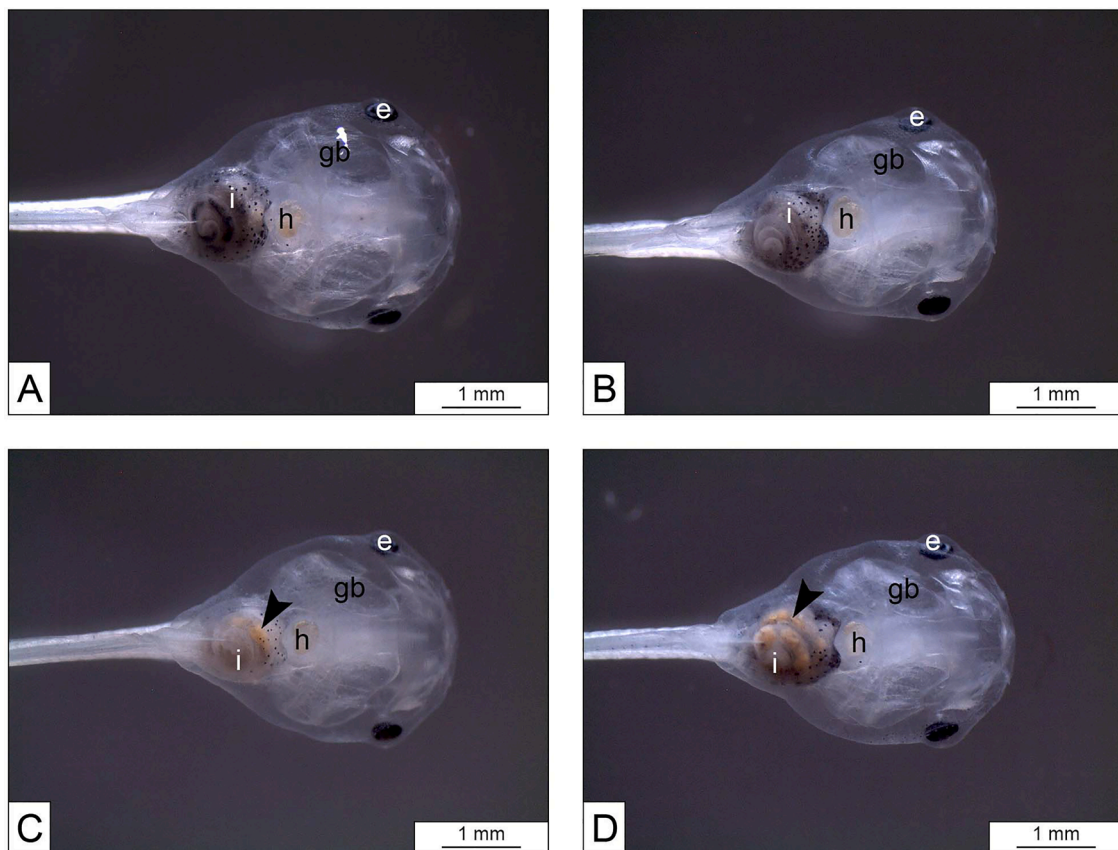


Fig. 8. *Xenopus laevis* larvae at NF stage 46. (A) Ventral view of a control larva; (B) ventral view of a larva exposed to $0.1 \mu\text{g mL}^{-1}$ PVC-MPs; (C-D) ventral view of larvae exposed to 1 and $10 \mu\text{g mL}^{-1}$ PVC-MPs, respectively, showing cluster of PVC-MPs in the gut (black arrowhead). i = intestine; h = heart; e = eye; gb = gill basket.

samples exposed to the two highest PVC-MP concentrations displayed a normal development as controls, PVC-MPs could be detected in their digestive systems. PVC-MPs were visible by transparency across the intestinal wall in samples exposed to $1 \mu\text{g mL}^{-1}$ PVC-MPs and was more evident in samples exposed to $10 \mu\text{g mL}^{-1}$ PVC-MPs. In larvae from these two groups, PVC-MPs appeared as orange material inside the gut. No PVC-MPs were visible by external stereomicroscopic observation in the intestine of $0.1 \mu\text{g mL}^{-1}$ PVC-MP-exposed larvae (Fig. 8).

A more detailed investigation was conducted by processing some samples (8–10 from each experimental group) to standard histopathological procedures and considering all organs/tissues of the larvae. This analysis revealed the presence of fragments and/or clusters of PVC-MPs inside the digestive systems of a significant number of larvae from all the exposed groups, including samples from $0.1 \mu\text{g mL}^{-1}$ PVC-MPs (Table 1, Fig. 9). In this group, only two samples showed clusters of PVC-MPs inside their gut (one from EXP1 and one from EXP2), with most of the remaining larvae showing only small particles or little PVC fragments. With increasing PVC-MPs concentration, the number of clusters significantly increased, reaching 100 % of samples in the groups exposed to the highest PVC-MPs concentrations for EXP1 and 88 % for EXP2 (Table 1). Some samples from the $10 \mu\text{g mL}^{-1}$ PVCMP groups showed a thinner epithelium compared to controls (Fig. 9A and D), and while this still displayed a regularly-defined brush border, sometimes it had a few adjacent cells characterised by pyknotic nuclei, whose presence was statistically different from controls only in EXP2 (Table 1). Interestingly, these fields were observed only at the large intestine level (Chalmers and Slack, 1998), and were never observed in other gut regions. In about half of the samples exposed to $10 \mu\text{g mL}^{-1}$ PVC-MPs, a significant number of larvae with cellular debris inside the intestinal lumen was recorded for both EXP1 and EXP2 (Fig. 9E). Some larvae exposed to $10 \mu\text{g mL}^{-1}$ PVC-MPs displayed a gut completely filled with PVC-MPs, either leaning on the apical portion of the intestinal cells or compressing the digestive epithelium so strongly that they deformed the regular architecture of the organ (Fig. F and G). In few cases, the intestinal loops had very thin walls with areas characterised by degenerating epithelia in which the microvillar layer was no longer recognizable; sometimes these fields coexisted in the same sample with areas in which the intestine displayed apparently normal tissue (Fig. H). No damage to other organs/tissues was observed, confirming the digestive system as the most affected apparatus, with the gut being the preferential site of PVC-MPs accumulation.

Ten additional samples per group, including controls, underwent a more detailed analysis by SEM to identify possible damages to the most affected tissues/organs, as indicated by the histological investigations. From 0.1 to $10 \mu\text{g mL}^{-1}$ PVC-MPs, despite the increasing presence of MPs inside their digestive tracts, the treated samples did not exhibit any ultrastructural alterations (Fig. 10). Interestingly, PVC-MPs were almost embedded in a mucous material, which was well visible already in samples (EXP1 and EXP2) exposed to the lowest PVC-MP concentration. However, no morphological abnormalities were observed: the digestive cells formed a regular columnar epithelium, higher in the small intestine

if compared to the large intestine, with a well-conserved and defined brush border. Even in regions where the PVC-MPs came into contact with the apical portions of the enterocytes, no damages were found and intact microvilli were always observed. The presence of PVC-MPs in the most posterior section of the gut in some samples exposed to the highest PVC-MP concentration, as well as in faeces partially expelled from the anus, not only demonstrates that PVC-MPs can accumulate in the intestine, but also that they can be eliminated. In order to confirm the chemical composition of the cluster observed by SEM, detailed analyses were performed by an EDX detector. Fig. S3 shows an example of the EDX spectrum mapped on the gut content from a *X. laevis* sample exposed to PVC-MPs.

3.3.2. Behavioural responses to PVC-MPs

Swimming activity was recorded in 192 larvae from the different treatment groups to examine possible behavioural alterations due to PVC-MPs in the two exposure experiments (EXP1 and EXP2). Analysed parameters were the time spent in the inner and outer portions of the experimental arena, immobility time, and the total distance eventually travelled in the arena by each larva, as shown in Table S3 and Table S4 for EXP1 and EXP2, respectively.

Control larvae spent almost all their time in the outer circle of the arena in both EXP1 and EXP2 (mean \pm SD of 26.4 ± 6.2 and 28.6 ± 2.0 s, for a recording time of 30 s, respectively). Differences between the two control groups were not statistically significant (Mann-Whitney test, $z = -1.08$; $P = 0.28$). Considering control and exposure groups at the three tested concentrations (0.1 , 1 and 10 mg L^{-1} PVC-MPs) for both exposure intervals, no significant differences were observed (Kruskal-Wallis test, $\chi^2 = 3.02$; d.f. = 3; $P = 0.39$ and $\chi^2 = 2.08$; d.f. = 3; $P = 0.56$, for EXP1 and EXP2, respectively), indicating that exposure to PVC-MPs did not affect the positioning of the larvae inside the arena.

During the time spent in the outer portion of the arena, larvae from control groups swam for almost half the time, with high variability among specimens (mean \pm SD of 9.7 ± 10.2 and 14.5 ± 11.5 s for EXP1 and EXP2, respectively). Some samples swam all the time, while others remained immobile for the entire recording period. The minimum-maximum intervals of swimming time for EXP1 and EXP2 groups were $0 - 28.6$ and $0 - 29.9$ s, respectively. Differences between the two control groups were not significant (Mann-Whitney test, $z = -1.06$; $P = 0.29$). No significant differences were observed between control and exposed groups at the three tested concentrations and for both exposure intervals (Kruskal-Wallis test, $\chi^2 = 4.6$; d.f. = 3; $P = 0.20$ and $\chi^2 = 2.7$; d.f. = 3; $P = 0.44$, for EXP1 and EXP2, respectively), indicating that exposure to PVC-MPs did not affect the swimming time of the larvae in the outer circle of the arena.

Lastly, we considered the distance moved, and the speed of each larva calculated as the ratio between the distance travelled in the arena and the swimming time, considering both portions separately and combined. During the time they swam, controls from EXP1 and EXP2 displayed very similar swimming speeds (mean \pm SD of 7.35 ± 2.0 and

Table 1
Results from the histopathological analysis on *X. laevis* larvae.

| Experimental group | Exposure time | Specimens n | Picnosis n | Debris n | PVC | |
|------------------------------|---------------|-------------|------------|----------|------------|-----------|
| | | | | | Fragment n | Cluster n |
| CTRL | EXP1 | 8 | 0 | 0 | 0 | 0 |
| | EXP2 | 10 | 0 | 0 | 0 | 0 |
| $0.1 \mu\text{g/mL}$ PVC-MPs | EXP1 | 10 | 0 | 0 | 5* | 1 |
| | EXP2 | 8 | 0 | 0 | 3 | 1 |
| $1 \mu\text{g/mL}$ PVC-MPs | EXP1 | 10 | 0 | 1 | 4 | 3 |
| | EXP2 | 10 | 2 | 3 | 1 | 7** |
| $10 \mu\text{g/mL}$ PVC-MPs | EXP1 | 9 | 2 | 5* | 0 | 9*** |
| | EXP2 | 8 | 4* | 4* | 1 | 7*** |

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; Fisher's Exact Test.

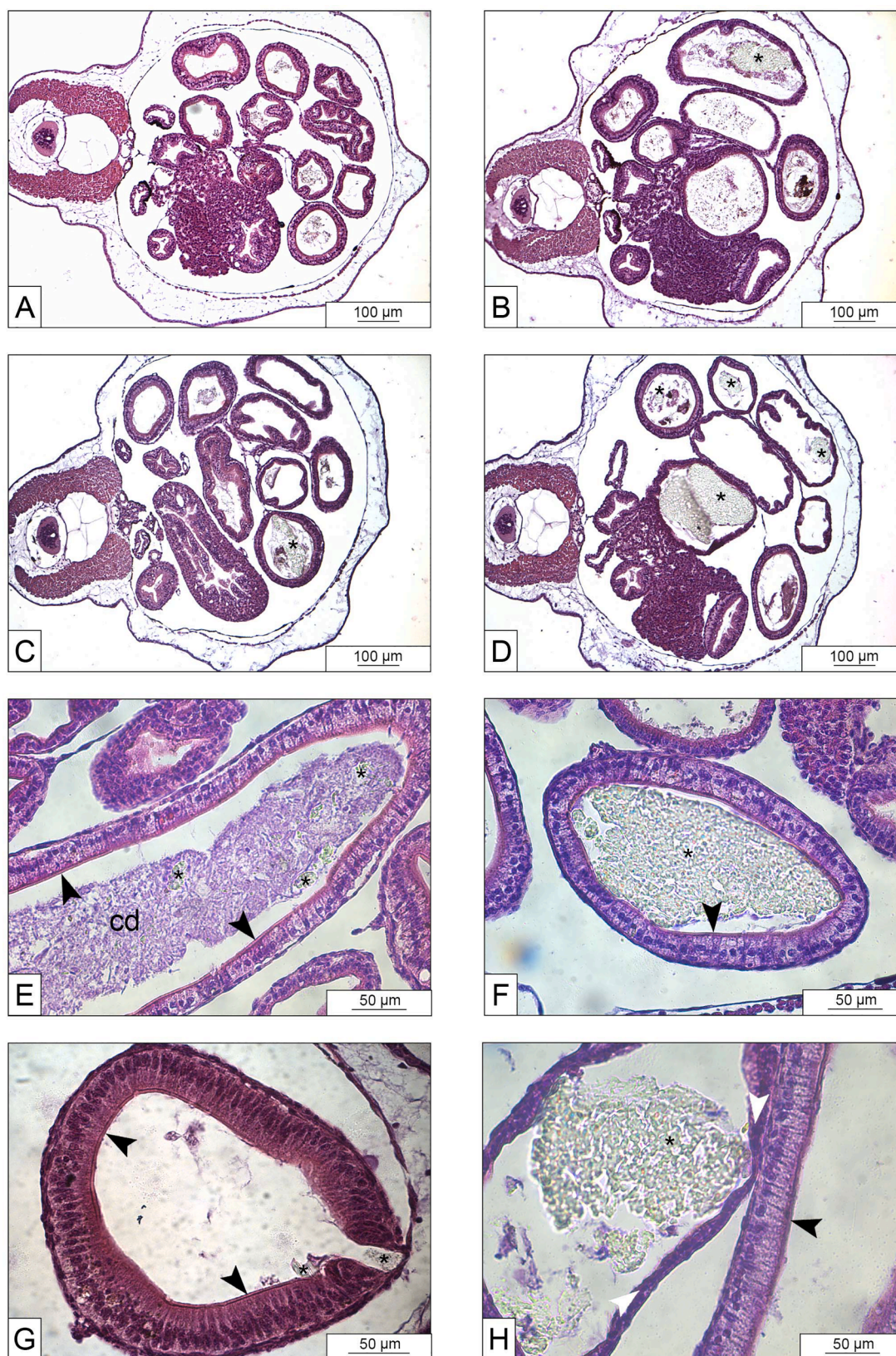


Fig. 9. Histological sections of *X. laevis* larvae at NF stage 46 at low (A-D) and high magnification (E-H). (A) Transversal section of a control larva; (B, C, and D) Transversal sections of larvae exposed to 0.1, 1, and 10 $\mu\text{g mL}^{-1}$ PVC-MPs, respectively. * = cluster of PVC-MPs inside the intestinal loop; black arrowhead = brush border; cd = cellular debris; white arrowhead = degenerating epithelium.

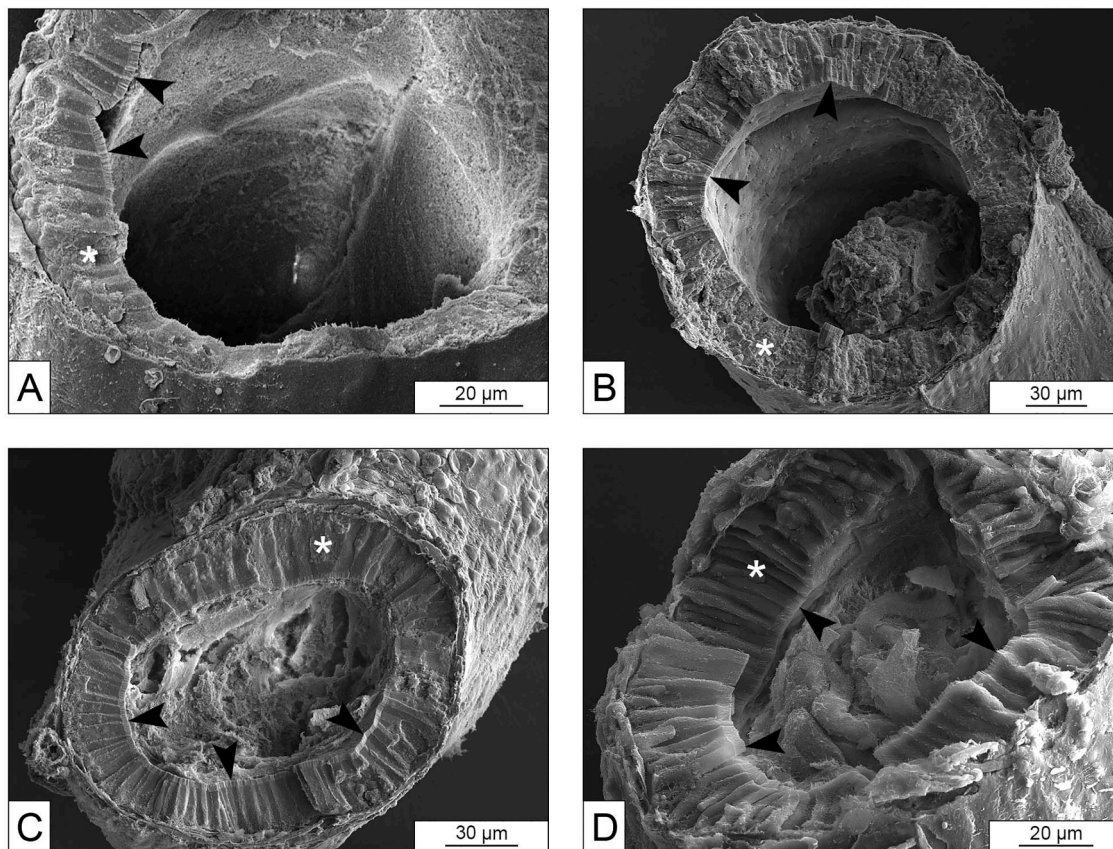


Fig. 10. SEM images of the intestine from *X. laevis* larvae at NF stage 46. (A) Control larva; (B, C, and D) Detail of the intestine from larvae exposed to 0.1, 1, and 10 $\mu\text{g mL}^{-1}$ PVC-MPs, respectively. Notice the heavy presence of PVC-MP clusters within the intestinal lumen already at the lowest PVC-MP concentration. * = intestinal wall; black arrowhead = brush border.

$8.91 \pm 3.1 \text{ mm sec}^{-1}$, respectively); no statistical differences were detected between them (Mann-Whitney test, $z = -1.77$; $P = 0.076$). In contrast, considering control and exposure groups at the three tested concentrations for both exposure intervals, significant differences among experimental groups were observed (Kruskal-Wallis test, $\chi^2 = 19.5$; d.f. = 3; $P < 0.001^{***}$ and $\chi^2 = 15.1$; d.f. = 3; $P = 0.002^{**}$, for EXP1 and EXP2, respectively). Swimming speed in controls was similar between the two exposure times, but it was lower than that observed in the exposed groups.

To confirm these results, the behaviour in the inner part of the arena was also considered, but not alone due to the limited time spent there by the samples from control groups (mean \pm SD of 3.6 ± 6.2 and 1.3 ± 2.0 s for EXP1 and EXP2, respectively) and from all the experimental groups (mean \pm SD of 1.6 ± 2.6 and 1.8 ± 2.8 s for EXP1 and EXP2, respectively). Therefore, observations taken here could not be considered representative of the behaviour of the larvae and were added to those taken in the outer portion, providing an overall behaviour assessment in the 30-sec recording time. Thus, considering the entire arena, control samples from both EXP1 and EXP2 swam for almost half of the time, with high variability among specimens (mean \pm SD of 11.2 ± 10.4 and 15.7 ± 12.3 s, respectively). Some larvae swam all the time, while others remained immobile for the entire recording period. The minimum-maximum intervals of swimming time for EXP1 and EXP2 were 0 - 29.5 and 0 - 29.9 s, respectively. Differences between the two control groups were not significant (Mann-Whitney test, $z = -1.23$; $P = 0.22$). No significant differences were observed considering control and exposure groups at the three tested concentrations for both exposures, (Kruskal-Wallis test, $\chi^2 = 4.3$; d.f. = 3; $P = 0.23$ and $\chi^2 = 2.6$; d.f. = 3; $P = 0.46$, for EXP1 and EXP2, respectively), confirming that exposure to PVC-MPs did not cause any effect on the swimming time in *X. laevis*.

Regarding swimming speed, control samples in both EXP1 and EXP2 displayed very similar behaviour, with a mean \pm SD of 7.6 ± 2.4 and $9.0 \pm 3.1 \text{ mm sec}^{-1}$, respectively. Differences between the two control groups were not significant (Mann-Whitney test, $z = -1.52$; $P = 0.13$); on the contrary, considering control and exposed groups at the three tested concentrations for both exposure intervals, significant differences were observed (Kruskal-Wallis test, $\chi^2 = 15.8$; d.f. = 3; $P = 0.001^{**}$ and $\chi^2 = 16.6$; d.f. = 3; $P = 0.001^{**}$, for EXP1 and EXP2, respectively). Analysis of swimming speed in the whole arena confirmed the results found in the outer circle of the arena.

4. Discussion

The novelty of this work was to evaluate the potential effects caused by PVC-MPs at low concentrations until $0.1 \mu\text{g mL}^{-1}$, using an environmental-relevant PVC-MPs sample, “mimicking” the environmental mechanical degradation of a commonly used plumbing pipe. These elements are considered essential for testing MPs toxicity as recommended by Du et al. (2021), although a full comparability between laboratory testing and environmental occurrence is very difficult to be achieved. Indeed, laboratory testing needs to have a relatively homogeneous MPs sample of known dimensional and chemical composition and use relevant-toxicological concentrations, while in the environment, we always found a heterogeneous mixture of MPs, very often at very low concentrations, mainly expressed as number and not as weight of MPs. In freshwater environment, for example, Winkler et al. (2022) found in water and sediment an average concentration of 33 and 11 MPs m^{-3} or kg^{-1} , respectively; detected polymers were EVA, PP, PE, PET and PVC, with a majority of fragments (83.5 %), followed by fibres (12.7 %) and spheres (3.8 %); PVC particle dimension ranged between 0,11 to 1.2

mm. In UK marine sediments, Bakir et al. (2022) reported a concentration range from 133 to 6933 MPs kg⁻¹; detected polymers were PS, PE, PA, PVC, Acrylic, EMAA and PET, with a majority of fragments (73 %), followed by fibres (19 %) and spheres (8 %). Plastic fragments ranged from 46 to 3276 µm. A global perspective review on MPs pollution reported that the extreme heterogeneity of MPs environmental concentrations and size may depend *in primis* by sampling methodologies (Hale et al., 2020). Generally, the larger size found in environmental monitoring in relation to our PVC-MPs sample depends on sampling and analytical methodologies that have, as lower detection limit, a threshold of tens or even hundreds of micrometres, while most of our PVC-MPs were below 100 µm in size. In this scenario the comparison of our PVC-MPs sample with environmental concentrations and size is challenging. In addition, we performed exposure experiments with two different species representatives of different aquatic compartments (marine and freshwater) to compare and integrate PVC-MPs effects. Moreover, to investigate the possible effects of release of additives in the test medium in respect to the PVC particle ingestion, two exposure experiments were performed with *X. laevis*. In fact, EXP1 started at the early developmental stage of *X. laevis* embryos, while EXP2 started later at the stage of the mouth opening (details are reported in Material and Method section). By the difference between EXP1 and EXP2 we would be able to detect the eventual effects of the additives released during the early developmental stage (additional toxicity in EXP1) in respect to those occurred later after the MPs ingestion (toxicity in EXP2). The two test species, the two exposure experiments with *X. laevis* larvae and the variety of endpoint tested make quite complex the discussion of the results, but they can furnish a quite complete picture of the toxicity of this PVC-MPs sample.



The use of an actual environmental contaminant (PVC building pipe) prompted us to put considerable efforts in the characterization of the PVC-MPs under investigation, because different PVC products are characterised by a distinct and specific composition of a wide number of different chemicals, such as plasticisers, stabilisers, colourants, and more generally "additives", which can be potentially released into the environment at any stage of the material's life (Foss Hansen et al., 2007). This PVC heterogeneity necessarily results in a potentially diversified environmental impact and in a wide range of effects. As expected for an environmental PVC-MPs sample, the analysis of the external morphology of our particles revealed clear heterogeneity in shape, with almost half of the particles being non-uniform or even highly irregular (Fig. 3E). De Sá et al. (2018) recommended the use of MPs with sizes falling within the most common size ranges found in biological samples (200–1600 µm). However, in their review they noted a lack of data related to MPs smaller than 50 µm, attributed to challenges in sampling procedures and detection methodologies, which may lead to their underestimation. The size range of particles we have used (0.13–449 µm) and their shape meet their recommendations and further supports their environmental relevance. Considering the chemical composition of the PVC-MPs we used, as obtained by SEM EDX analysis (Fig. 3F), the presence of Calcium is evident and confirms the use of Calcium-based stabilisers in many PVC products, which have recently replaced the historically employed lead-based ones. Since 2016, the use of lead stabiliser for PVC formulations has been banned in most EU countries in favour of cheaper and less hazardous materials (European Commission, 2022). Considering the results of metal analyses performed on our sample (Fig. 3), and an extreme scenario of complete release of heavy metals from the highest tested PVC-MPs dose (10 µg mL⁻¹), the resulting heavy metal concentration would range between 0.05 and 0.2 µg L⁻¹ (this range refers to the concentrations of Cd and As, respectively, while those of Ni, Cr, and Pb fall within this range). The potential toxicity of these maximum theoretical concentrations of PVC leachates was compared to the water quality levels defined by the European Water Framework Directive 2000/60 (EEA EWFD, 2000) and by the Italian Decree 2010/260 (GU MATTM, 2011), which established Annual Average-Environmental Quality Standards (AA-EQSS) for surface

waters, including coastal marine waters. The cited legislation indicates the maximum concentrations of chemical substances that should not be exceeded for a healthy biological and ecological water status. The estimated maximum concentration of heavy metals potentially released from the highest tested PVC-MP dose is far less below the established AA-EQSSs (i.e., 0.2 µg L⁻¹ for Cd and 5 µg L⁻¹ for As), indicating that PVC-MPs leachates should not pose a risk in our experimental conditions. Our behavioural results with *X. laevis* confirmed the absence of an additional toxicity due to additive leaching (comparison of EXP1 and EXP2) and agree with data from Zimmermann et al. (2020), who reported no chemical toxicity in the aqueous medium of *Daphnia magna* over a 21-day exposure period to PVC-MPs. According to these data, the effects observed in both *Ophiactis virens* and *Xenopus laevis*, should be consequently attributed mainly to a physical effect of the PVC-MPs after their ingestion.

The main effects observed in the model species used in the present study are summarized in Table 2; as shown, mortality and external malformations were not impacted by PVC-MPs exposures, neither in *O. virens* sub-adults, nor in larvae of *X. laevis*. According to our findings, Yap and co-workers (Yap et al., 2020) reported no differences in the survival rates of the Mediterranean mussel *Mytilus galloprovincialis* exposed for 35 days to 1.5, 15, and 150 mg L⁻¹ PVC particles, but a slightly lowered Body Condition Index, BCI, resulting in a reduction of their general condition. More recently, Barkhau et al. (2022) observed no mortality in the mussel *Semimytilus algosus* exposed up to 150 mg L⁻¹ PVC-MPs, but they reported a significant reduction of BCI in samples exposed to the same concentration comparing to those exposed to 15 mg L⁻¹ PVC. These authors hypothesised that the energy balance of samples with lower BCI was impaired, this possibly being related to a reduction in energy uptake or to an increased energy consumption. The effects observed in *O. virens* samples on arm regeneration efficiency ("Growth inhibition" section in Table 2), could be similarly explained. Regeneration is, indeed, a post-embryonic developmental process that shares common cellular events, such as proliferation, differentiation, migration, and more, with embryogenesis and body growth. In our samples, the exposure to PVC-MPs caused a decreasing trend in the arm regeneration index at increasing PVC-MPs doses, with significantly lower values in 1 and 10 µg mL⁻¹ PVC-MP groups when compared to controls. Similar results have been reported by Oliviero et al. (2019), who observed a decrease of larval length in plutei exposed to low concentrations of micronized PVC plastics and a complete block of larval development in embryos exposed to the highest doses. In echinoderms, regeneration is known to be sensitive to various environmental stress, such as: i) variation in oxygen concentration (Nilsson and Sköld, 1996), pH (Schram et al., 2011), ii) salinity (Talbot and Lawrence, 2002), iii) turbulence (McAlister and Stanczyk, 2003), and exposure to harmful substances, such as endocrine disruptors (Candia Carnevali et al., 2001; Sugni et al., 2008). Barbaglio et al. (2006) reported alterations in the modality and timing of arm regeneration in the crinoid *Antedon mediterranea* after Triphenyltin chloride and Fenarimol exposure, while D'Andrea et al. (1996) observed a slowed regeneration after exposure to Cadmium in the brittlestar *Microphiopholis gracillima*. In the present study, the treated samples showed greater arm elongation in respect to its differentiation. This regeneration impairment can be considered a signal of interference of PVC-MPs with the development process, according to what proposed by Dupont and Thorndyke (2006) to explain the regeneration variability in the brittle star *Amphiura filiformis*.

X. laevis larvae exposed to PVC-MPs did not show any differences in developmental stage and in the head-tail length when compared to controls, suggesting no effects on amphibian development by PVC-MPs, at least at our experimental conditions. The diversity of results obtained in our two experimental models highlights that comparable developmental processes involving cellular processes such as proliferation, differentiation, and apoptosis can be differently affected by xenobiotics. Considering this endpoint, regeneration process in *O. virens* appeared to be more sensitive than the development of *X. laevis* larvae.

Table 2
Comparative effects of PVC-MP exposure in the two model organisms.

| | <i>Ophiactis virens</i> | <i>Xenopus laevis</i> |
|--------------------------|---|---|
| |  |  |
| Mortality | - | - |
| External Malformations | - | - |
| Growth Inhibition | + | - |
| Histological Alterations | - + | + |
| Ultrastructural Damage | - | - + |
| Behavioural Impairment | - | + |

Under the “Histological Alterations” line in Table 2 we have considered the damages to organs and tissues. As regards *O. virens* no difference with control samples has been detected, nevertheless, a few specimens from the two highest PVC-MP-exposed groups showed fragments or little PVC-MP clusters inside their digestive systems. As shown in Fig. 6G and H, the PVC-MPs lie on the digestive epithelium between its normal folds, but it is also evident that some portions of those clusters infiltrate the tissue, pressing against the stomach wall, thus causing a thinning of the epithelium (more visible in Fig. 6H). Thus, even though these fields have been observed in few samples, our scoring highlights the capability of PVC-MPs to induce histological alterations in the digestive tissue of *O. virens*. When considering the larvae of *X. laevis*, the effects of the tested materials on their gut are more pronounced. Larvae from all treated groups exhibited the presence of PVC-MPs inside their digestive systems, and while at the lowest PVC-MP concentration larvae mainly showed only fragments of PVC in the lumen, and rarely they presented clusters, at the highest concentrations almost all larvae had their intestinal loops engulfed with PVC-MPs. The number of samples with such features did not vary in larvae from EXP1 and EXP2, indicating that PVC-MP can cause these effects after only 40 h of exposure. In fact, according to the NF developmental stages, stage 46 (end of the exposure test) occurs 106 h post fertilisation, while stage 40 (at which larvae open their mouth) occurs 66 h post fertilisation (Nieuwkoop and Faber, 1994). The presence of: *i*) pyknotic nuclei in some areas of the digestive epithelium, *ii*) cellular debris inside the intestinal lumen, and *iii*) areas of the intestinal loops characterised by thin walls and degenerating epithelia (Fig. 9E-H) are clear consequences of the physical interaction of PVC-MPs with the digestive epithelia of the larvae. Similar effects have been previously reported following PVC-MPs exposure in other animal models: Lei et al. (2018) in *Danio rerio* observed diffuse damage to the intestine in 80 % of samples exposed for 10 days to 70 µm sized PVC particles. These authors observed ruptures of villi and splitting of the digestive cells, suggesting intestinal damage as a key effect of MP exposure. Gokce et al. (2018) in the same species observed increased apoptotic figures in different portions of the body of zebrafish embryos exposed for 96 h to increasing PVC-MP concentration. Liu et al. (2023) reported that *Cyprinus carpio* larvae exposed for 60 days to a diet rich in PVC-MPs showed vacuolation, swelling, partial rupture and even destruction of the intestinal villi. Pedà et al. (2016) demonstrated histopathological alterations to the regular structure of the entire intestinal wall in *Dicentrarchus labrax* fed with a PVC-MP-based diet for 30, 60, and 90 days. Although severe effects on the gut were observable after 60 days, these authors reported the most adverse conditions in samples exposed to polluted PVC-MP pellets for 90 days, some of which displayed completely compromised intestinal functions. Interestingly, the same authors observed the worst conditions in the distal intestine of fish fed with polluted PVC-MP pellets. This well agrees with our observations

in *X. laevis* larvae exposed to PVC-MPs, where the primary damage was observed in the large (i.e., distal) intestine and not in other portions of the gut. This could be explained by the reduced height of the epithelial cells normally occurring in the large intestine compared to the small intestine, which could make the large intestine more sensitive to the abrasive action of the PVC fragments. This well agrees with data from Xia et al. (2022) who attributed the toxicity of PVC particles in the fish *Oryzias melastigma* to their irregular shape, and also with data from Romano et al. (2018), who exposed *Barbodes gonionotus* fry to smooth PVC particle for 96 h but did not observe any kind of tissue alteration. Indeed, shape has been recognized to play a key role in the nano-bio interactions (Bacchetta et al., 2018; Elder et al., 2009; Nel et al., 2009), and the importance of surface geometry in determining toxicity seems to be valid also for microplastic particles (Qiao et al., 2019; Tursi et al., 2022). More studies are certainly needed to understand the role of material shape on toxicity, considering that the effects caused by differently shaped particles are reported to be species-specific and composition-dependent (Suckling, 2021; Zimmermann et al., 2020).

Considering the high number of *X. laevis* larvae with histological damage at the large intestine, some ultrastructural damages at the digestive cells of the small intestine were expected as well. Instead, SEM analyses did not reveal any particular alterations in the proximal intestinal portion, and the digestive cells consistently displayed their regular brush border (Fig. 10). The only thing worth noting was the presence of mucous material surrounding the PVC-MPs which can be interpreted as a response of the *X. laevis* larvae digestive cells to xenobiotic overload. This initial defence mechanism (Pelaseyed et al., 2014) has already been reported for this species (Bacchetta et al., 2021, 2012; Bour et al., 2015), as well as in other species, such as zebrafish when exposed to MPs (Qiao et al., 2019). These authors reported that the accumulation of MPs in the fish intestine led to multiple toxic effects, including mucosal damage, increased permeability, inflammation, and metabolism disruption. Pedà et al. (2016) also found similar results for PVC-MPs in the sea bass *Dicentrarchus labrax*. After 30 days of exposure to a PVC-MP-based diet, these authors observed an increased number of goblet cells along with an overproduction of mucus in the intestine of the exposed fish. Concerning *O. virens* samples, we were not surprised that they exhibited no ultrastructural alterations, as the presence of fragments or small PVC-MP clusters was only sporadic in specimens of this species. In fact, digestive cells in the samples from the three groups exposed to PVC-MPs were just as healthy as those in the controls, and no evident damage was detected in the stomach or other organs/tissues.

Under the “Behavioural Impairment” line in Table 2 we have considered the righting time and the locomotion ability of *O. virens*, while the swimming activity, immobility time and distance moved by *X. laevis* larvae. The righting behaviour, which is the ability of echinoderms to return to their correct orientation if flipped over accidentally or

following a predator attack, enables them to survive and offers valuable insights into the overall health of these organisms. In fact, this movement requires a remarkable neuromuscular coordination and for this reason it has been already used as a reliable and easily measurable behavioural response to stress conditions (Hyman, 1955; Meretta et al., 2021; Ubaldo et al., 2008). The righting response can be influenced by several factors, including temperature (Buccheri et al., 2019; Ubaldo et al., 2008), pH (Clements and Comeau, 2019), light intensity (Sun et al., 2019), diet (Vega Fernández et al., 2019), and sediment contamination (Lane and Riddle, 2004). During the righting behaviour, the time required to complete the flipping can either increase or decrease in response to the type of stressor, or organisms may be unable to perform it. For example, the ophiuroid species *Ophiocoma scolopendrina* and *Macrophiothrix longipeda* in Ubaldo et al. (2008) righted themselves faster at higher temperatures, while fuel contamination in the sediment caused a loss of righting ability in *Ophiura crassa* (Lane and Riddle, 2004). Interestingly, the righting response of our brittle stars was not significantly affected by PVC exposure in any of the tested treatments, this being in line with results from Suckling (2021) who reported no influence of PVC-MPs exposure on the righting time of sea urchins *Psammechinus miliaris* and *Paracentrotus lividus*.

Another commonly investigated behavioural endpoint in exposure trials is locomotion capability, measured as speed of the specimens. This has been assessed in various species including the brittle star *Amphiura filiformis* and the purple urchin *Strongylocentrotus purpuratus* (Chan et al., 2016), as well as in the sea urchin *P. lividus* (Morgana et al., 2016), exposed to ocean acidification and chemical pollution, respectively. As observed for the righting behaviour, the impact of stressors on organism speed can lead to various outcomes, including negative effects (i.e., decreased speed), positive effects (i.e., increased speed), or no effect at all. In our study, the speed of the tested *O. virens* samples did not exhibit any significant differences when compared to controls. On the contrary, control and PVC_MP-exposed *X. laevis* larvae showed significant differences of swimming time and speed when compared to control larvae. This was in accordance with data from Vijayaraghavan et al. (2022) who reported increased behavioural alterations in the fish *Etroplus suratensis* with increasing PVC-MP concentrations. These authors reported fin flickering, burst swimming and jerking movements in fish exposed for 42 days to 3.32, 5.98, and 10.78 mg L⁻¹ PVC-MPs, confirming data from previously published papers which suggest that MPs can impair the normal activity of fish (Chen et al., 2020, 2017; Yin et al., 2018). To our knowledge, two other previously published papers have reported altered swimming activity in *X. laevis* larvae following exposure to MPs (Bacchetta et al., 2021; De Felice et al., 2018), even though their results were inconsistent. De Felice and colleagues did not observe any changes in larval behaviour as a result of polystyrene-MP exposure, whereas Bacchetta and co-workers reported a significant reduction in motility in samples exposed to polyester microfibres when compared to controls. The differences among our results and those from the cited papers could be due to the difference in shape of the MPs used in the exposure. In fact, as previously underlined, the impact of shape on MPs toxicity is a widely recognized factor, as it can affect retention times, accumulation rates, and physical damage (Jemec et al., 2016). Qiao and colleagues in zebrafish reported a shape-dependent accumulation in the gut with the order: fibers > fragments > beads (Qiao et al., 2019). Additionally, it must be considered that the longer MPs remain inside the intestine, the greater the chance they have of causing damage to the epithelium throughout the digestive system. In any case, whatever the mechanism of toxicity may be, the changes observed in the normal behaviour of fish and amphibian larvae are highly significant and impactful, as they can alter predator-prey relationships and potentially disrupt the balance within aquatic ecosystems.

5. Conclusion

Considering the hypotheses of this work, we confirmed that the

observed PVC-MPs toxicity was highly species-specific: while in *O. virens* the main effects were observed on developmental processes (i.e., regeneration), in *X. laevis* the most evident effect was a direct physical damage to the intestinal epithelium. According to the literature, the physical damage at cellular level in the intestine can be attributed to the irregular and pointed shape of particles and their concentration/dimensions. This intricate phenomenon in MPs toxicity is commonly referred to as the "shape effect". The reason of the absence of such effect in *O. virens* might be attributed to their ability to select materials based on MPs dimensions and the availability of alternative food sources. *X. laevis* larvae ingested a significant amount of PVC-MPs because they fell within the appropriate size range and no other food alternatives were present. In contrast, *O. virens* ingested fewer PVC particles, possibly due to a stricter food selection, and the presence of other food options. The observed effects are highly dependent on the model species as well as on the exposure conditions.

Concerning with the second hypothesis, the large spectrum of the considered toxicological endpoints was able to overcome the species specificity giving a final toxicological evaluation mainly coherent between the two model species. PVC-MPs exhibited adverse effects on both species at a concentration of 10 µg mL⁻¹ and no adverse effects were observed in both species at the concentration of 0.1 µg mL⁻¹ (NOEC value for this PVC-MPs sample). The significant effect observed on the arm regeneration process in *O. virens*, established the LOEC values for this PVC-MPs sample to the concentration of 1 µg mL⁻¹.

The ingestion of PVC-MPs was confirmed as the main input pathway for both species, but only for *X. laevis* the digestive system was the main target of the PVC-MPs exposure. The reason of this was already mentioned. The results and the comparative analysis presented in this work emphasise the importance of conducting careful comparisons among the findings of various studies and provide possible explanations for the highly divergent results in MPs toxicity reported in the literature.

The outcomes of our research underscore the importance of implementing the classical toxicity protocols with histological and behavioural analyses, in order to significantly increase our understanding of the mechanisms of action of these pollutants. This knowledge is invaluable for understanding and mitigating the environmental consequences of MPs pollution. We believe that our findings will contribute to the scientific community's understanding of the multifaceted impacts of MPs and, since the adverse effects of microplastics are becoming increasingly clear, we ask for a strict regulation of plastic pollution in order to substantial decrease their presence in the environment.

Appendix A. Supplementary data

Supplementary data to this article can be found in the online version of the paper.

CRedit authorship contribution statement

Renato Bacchetta: Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing. **Arianna Pica:** Investigation, Writing – original draft, Writing – review & editing. **Nadia Santo:** Methodology, Investigation, Resources, Writing – review & editing. **Paolo Tremolada:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing. **Michela Sugni:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing.

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.

Acknowledgements

The authors would like to express their gratitude to Dr. Stefania Marzorati for her assistance in sample preparation, Dr. Marco Schiavoni for his support in chemical characterization, Dr. Lorenzo Quattrini for his help during the experimental procedures, and Dr. Francesca Di Renzo and Prof. Elena Menegola for kindly providing *X. laevis* embryos for the tests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.106975.

References

- Altig, R., Whiles, M.R., Taylor, C.L., 2007. What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. *Freshw. Biol.* 52, 386–395.
- ASTM, American Society for Testing and Materials, 1998. Standard guide for conducting the frog embryo Teratogenesis Assay-Xenopus (Fetax).
- Bacchetta, R., Santo, N., Valenti, I., Maggioni, D., Longhi, M., Tremolada, P., 2018. Comparative toxicity of three differently shaped carbon nanomaterials on *Daphnia magna*: does a shape effect exist? *Nanotoxicology* 12, 201–223. <https://doi.org/10.1080/17435390.2018.1430258>.
- Bacchetta, R., Tremolada, P., Di Benedetto, C., Santo, N., Fascio, U., Chirico, G., Colombo, A., Camatini, M., Mantecchia, P., 2012. Does carbon nanopowder threaten amphibian development? *Carbon* 50, 4607–4618. <https://doi.org/10.1016/j.carbon.2012.05.047>.
- Bacchetta, R., Winkler, A., Santo, N., Tremolada, P., 2021. The toxicity of polyester fibers in *Xenopus laevis*. *Water* 13, 3446. <https://doi.org/10.3390/w13233446>.
- Balestrieri, A., Winkler, A., Scribano, G., Gazzola, A., Lastrico, G., Grioni, A., Pellitteri-Rosa, D., Tremolada, P., 2022. Differential effects of microplastic exposure on anuran tadpoles: a still underrated threat to amphibian conservation? *Environ. Pollut.* 303, 119137.
- Bakir, A., Doran, D., Silburn, B., Russell, J., Archer-Rand, S., Barry, J., Maes, T., Limpenny, C., Mason, C., Barber, J., Nicolaus, E.E.M., 2022. A spatial and temporal assessment of microplastics in sea floor sediments: a case study for the UK. *Front. Mar. Sci.* 9. <https://www.frontiersin.org/articles/10.3389/fmars.2022.1093815>.
- Bantle, J.A., Fort, D.J., Rayburn, J.R., DeYoung, D.J., Bush, S.J., 1990. Further validation of FETAX: evaluation of the developmental toxicity of five known mammalian teratogens and non-teratogens. *Drug Chem. Toxicol.* 13, 267–282. <https://doi.org/10.3109/01480549009032286>.
- Barbaglio, A., Mozzì, D., Sugni, M., Tremolada, P., Bonasoro, F., Lavado, R., Porte, C., Carnevali, M.D.C., 2006. Effects of exposure to ED contaminants (TPT-Cl and Fenarimol) on crinoid echinoderms: comparative analysis of regenerative development and correlated steroid levels. *Mar. Biol.* 149, 65–77. <https://doi.org/10.1007/s00227-005-0205-0>.
- Barkhau, J., Sanchez, A., Lenz, M., Thiel, M., 2022. Effects of microplastics (PVC, PMMA) on the mussel *Semimytilus algosus* differ only at high concentrations from those of natural microparticles (clay, celite). *Mar. Pollut. Bull.* 177, 113414. <https://doi.org/10.1016/j.marpolbul.2022.113414>.
- Ben Khadra, Y., Ferrario, C., Benedetto, C.D., Said, K., Bonasoro, F., Candia Carnevali, M. D., Sugni, M., 2015. Re-growth, morphogenesis, and differentiation during starfish arm regeneration: starfish arm regeneration. *Wound Rep. Reg.* 23, 623–634. <https://doi.org/10.1111/wrr.12336>.
- Benvenuto, M.A., Plaumann, H., 2021. Chapter 35 Polyvinyl chloride (PVC). Chapter 35 Polyvinyl Chloride (PVC), pp. 145–148. <https://doi.org/10.1515/9783110542868-035>. De Gruyter.
- Bour, A., Mouchet, F., Verneuil, L., Evariste, L., Silvestre, J., Pinelli, E., Gauthier, L., 2015. Toxicity of CeO₂ nanoparticles at different trophic levels – Effects on diatoms, chironomids and amphibians. *Chemosphere* 120, 230–236. <https://doi.org/10.1016/j.chemosphere.2014.07.012>.
- Boyle, D., Catarino, A.L., Clark, N.J., Henry, T.B., 2020. Polyvinyl chloride (PVC) plastic fragments release Pb additives that are bioavailable in zebrafish. *Environ. Pollut.* 263, 114422. <https://doi.org/10.1016/j.envpol.2020.114422>.
- Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Curr. Biol.* 23, 2388–2392. <https://doi.org/10.1016/j.cub.2013.10.012>.
- Buccheri, E., Foellmer, M., Christensen, B., Langis, P., Ritter, S., Wolf, E., Freeman, A., 2019. Variation in righting times of *Holothuria atra*, *Stichopus chloronotus*, and *Holothuria edulis* in response to increased seawater temperatures on heron reef in the Southern GBR. *J. Mar. Biol.* 2019, 1–6. <https://doi.org/10.1155/2019/6179705>.
- Candia Carnevali, M., Galassi, S., Bonasoro, F., Patrino, M., Thorndyke, M., 2001. Regenerative response and endocrine disruptors in crinoid echinoderms: arm regeneration in *Antedon mediterranea* after experimental exposure to polychlorinated biphenyls. *J. Exp. Biol.* 204, 835–842. <https://doi.org/10.1242/jeb.204.5.835>.
- Candia Carnevali, D., Sugni, M., Bonasoro, F., 2024. Regeneration Potential in Echinoderms: Revisiting the Regeneration concept. *Frontiers in Invertebrate Physiology: A Collection of Reviews*. Apple Academic Press eBook ISBN 9781003405184.
- Chalmers, A.D., Slack, J.M.W., 1998. Development of the gut in *Xenopus laevis*. *Dev. Dyn.* 212, 509–521. [https://doi.org/10.1002/\(SICI\)1097-0177\(199808\)212:4<509::AID-AJA4>3.0.CO;2-L](https://doi.org/10.1002/(SICI)1097-0177(199808)212:4<509::AID-AJA4>3.0.CO;2-L).
- Chan, K.Y.K., Grünbaum, D., Arnberg, M., Dupont, S., 2016. Impacts of ocean acidification on survival, growth, and swimming behaviours differ between larval urchins and brittlestars. *ICES J. Marine Sci.* 73, 951–961. <https://doi.org/10.1093/icesjms/fsv073>.
- Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Sci. Total Environ.* 584–585, 1022–1031. <https://doi.org/10.1016/j.scitotenv.2017.01.156>.
- Chen, Q., Lackmann, C., Wang, W., Seiler, T.B., Hollert, H., Shi, H., 2020. Microplastics lead to hyperactive swimming behaviour in adult zebrafish. *Aquat. Toxicol.* 224, 105521. <https://doi.org/10.1016/j.aquatox.2020.105521>.
- Clements, J.C., Comeau, L.A., 2019. Behavioral defenses of shellfish prey under ocean acidification. *shre* 38, 725–742. <https://doi.org/10.2983/035.038.0324>.
- Converse, S.J., Grant, E.H.C., 2019. A three-pipe problem: dealing with complexity to halt amphibian declines. *Biol. Conserv.* 236, 107–114.
- D'Andrea, A.F., Stancik, S.E., Chandler, G.T., 1996. Sublethal effects of cadmium on arm regeneration in the burrowing brittlestar, *Microphiopholis gracillima*. *Ecotoxicology* 5, 115–133. <https://doi.org/10.1007/BF00119050>.
- De Felice, B., Bacchetta, R., Santo, N., Tremolada, P., Parolini, M., 2018. Polystyrene microplastics did not affect body growth and swimming activity in *Xenopus laevis* tadpoles. *Environ. Sci. Pollut. Res. Int.* 25, 34644–34651. <https://doi.org/10.1007/s11356-018-3408-x>.
- De Sá, L.C., Oliveira, M., Ribeiro, F., Rocha, T.L., Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? *Sci. Total Environ.* 645, 1029–1039. <https://doi.org/10.1016/j.scitotenv.2018.07.207>.
- Du, J., Zhou, Q., Li, H., Xu, S., Wang, C., Fu, L., Tang, J., 2021. Environmental distribution, transport and ecotoxicity of microplastics: a review. *J. Appl. Toxicol.* 41, 52–64. <https://doi.org/10.1002/jat.4034>.
- Dupont, S., Thorndyke, M.C., 2006. Growth or differentiation? Adaptive regeneration in the brittlestar *Amphiura filiformis*. *J. Exp. Biol.* 209, 3873–3881. <https://doi.org/10.1242/jeb.02445>.
- Elder, A., Vidyasagar, S., DeLouise, L., 2009. Physicochemical factors that affect metal and metal oxide nanoparticle passage across epithelial barriers. *Wiley. Interdiscip. Rev. Nanomed. Nanobiotechnol.* 1, 434–450. <https://doi.org/10.1002/wnan.44>.
- Espinosa, C., Cuesta, A., Esteban, M.A., 2017. Effects of dietary polyvinylchloride microparticles on general health, immune status and expression of several genes related to stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 68, 251–259. <https://doi.org/10.1016/j.fsi.2017.07.006>.
- European Commission, 2022. Directorate-General for environment. The Use of PVC (poly vinyl chloride) in the Context of a Non-Toxic environment: Final Report. Publications Office, LU.
- European Chemicals Agency, 2016. Annex XV Restriction Report – Lead Stabilisers in PVC. Helsinki, Finland.
- European Environment Agency. European water framework directive, 2000. European Water Framework Directive 2000/60, 2000/60EC.
- Foss Hansen, S., Larsen, B.H., Olsen, S.I., Baun, A., 2007. Categorization framework to aid hazard identification of nanomaterials. *Nanotoxicology* 1, 243–250. <https://doi.org/10.1080/17435390701727509>.
- Gazzetta Ufficiale. Ministero dell'Ambiente e della Tutela del Territorio e del Mare, 2011. DECRETO 8 novembre 2010, n. 260.
- Geyer, R., 2020. Chapter 2 - Production, use, and fate of synthetic polymers. In: Letcher, T.M. (Ed.), *Plastic Waste and Recycling*. Academic Press, pp. 13–32. <https://doi.org/10.1016/B978-0-12-817880-5.00002-5>.
- Gokce, B., Onen, O., Kiran, M., Uuncu, S.I., 2018. The effects of polyvinyl chloride microparticles on the apoptotic processes in zebrafish (*Danio rerio*). *Embryos* 4.
- Gomiero, A., Straffella, P., Pellini, G., Salvalaggio, V., Fabi, G., 2018. Comparative effects of ingested PVC micro particles with and without adsorbed Benzo(a)pyrene vs. spiked sediments on the cellular and sub cellular processes of the benthic organism *Hediste diversicolor*. *Front. Mar. Sci.* 5, 99. <https://doi.org/10.3389/fmars.2018.00099>.
- Gulyás, M., Bencsik, N., Pusztai, S., Liliom, H., Schlett, K., 2016. AnimalTracker: an ImageJ-based tracking API to create a customized behaviour analyser program. *Neuroinformatics* 14, 479–481. <https://doi.org/10.1007/s12021-016-9303-z>.
- Hahladakis, J.N., Velis, C.A., Weber, R., Iacovidou, E., Purnell, P., 2018. An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling. *J. Hazard. Mater.* 344, 179–199. <https://doi.org/10.1016/j.jhazmat.2017.10.014>.
- Hale, R.C., Seeley, M.E., La Guardia, M.J., Mai, L., Zeng, E.Y., 2020. A global perspective on microplastics. *J. Geophys. Res. Oceans* 125. <https://doi.org/10.1029/2018JC014719> e2018JC014719.
- Hoke, R.A., Ankley, G.T., 2005. Application of frog embryo teratogenesis assay- *Xenopus* to ecological risk assessment. *Environ. Toxicol. Chem.* 24, 2677–2690. <https://doi.org/10.1897/04-506R.1>.

- Hounsham, I.D., Titow, W.V., 1984. Fillers in PVC. In: Titow, W.V. (Ed.), PVC Technology. Springer, Netherlands, Dordrecht, pp. 215–254. https://doi.org/10.1007/978-94-009-5614-8_8.
- Hyman, 1955. The Invertebrates: Echinodermata. McGraw-Hill Book Company, New York.
- Iheanacho, S.C., Odo, G.E., 2020. Neurotoxicity, oxidative stress biomarkers and haematological responses in African catfish (*Clarias gariepinus*) exposed to polyvinyl chloride microparticles. *Compar. Biochem. Physiol. Part C: Toxicol. Pharmacol.* 232, 108741 <https://doi.org/10.1016/j.cbpc.2020.108741>.
- Jahnke, A., Arp, H.P.H., Escher, B.I., Gewert, B., Gorokhova, E., Kühnel, D., Ogonowski, M., Potthoff, A., Rummel, C., Schmitt-Jansen, M., Toorman, E., MacLeod, M., 2017. Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment. *Environ. Sci. Technol. Lett.* 4, 85–90. <https://doi.org/10.1021/acs.estlett.7b00008>.
- Jemec, A., Horvat, P., Kunj, U., Bele, M., Kržan, A., 2016. Uptake and effects of microplastic textile fibers on freshwater crustacean *Daphnia magna*. *Environ. Pollut.* 219, 201–209. <https://doi.org/10.1016/j.envpol.2016.10.037>.
- Lane, A., Riddle, M., 2004. Toxicity of dispersed and undispersed diesel in sediments to the Antarctic ophiuroid *Ophiura crassa*. *Australas J. Ecotoxicol.* 10, 115–121.
- Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. *Sci. Total Environ.* 619–620, 1–8. <https://doi.org/10.1016/j.scitotenv.2017.11.103>.
- Li, H.X., Getzinger, G.J., Ferguson, P.L., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. *Environ. Sci. Technol.* 50, 924–931. <https://doi.org/10.1021/acs.est.5b02781>.
- Lithner, D., Nordensvan, I., Dave, G., 2012. Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile-butadiene-styrene, and epoxy to *Daphnia magna*. *Environ. Sci. Pollut. Res.* 19, 1763–1772. <https://doi.org/10.1007/s11356-011-0663-5>.
- Liu, X., Liang, C., Zhou, M., Chang, Z., Li, L., 2023. Exposure of *Cyprinus carpio* var. larvae to PVC microplastics reveals significant immunological alterations and irreversible histological organ damage. *Ecotoxicol. Environ. Saf.* 249, 114377 <https://doi.org/10.1016/j.ecoenv.2022.114377>.
- Lu, L., Li, W., Cheng, Y., Liu, M., 2023. Chemical recycling technologies for PVC waste and PVC-containing plastic waste: a review. *Waste Manag.* 166, 245–258. <https://doi.org/10.1016/j.wasman.2023.05.012>.
- Markarian, J., 2007. PVC additives – What lies ahead? *Plastics Addit. Compound.* 9, 22–25. [https://doi.org/10.1016/S1464-391X\(07\)70153-8](https://doi.org/10.1016/S1464-391X(07)70153-8).
- McAlister, J.S., Stancik, S.E., 2003. Effects of variable water motion on regeneration of *Hemipholis elongata* (Echinodermata, Ophiuroidea). *Invertebrate Biol.* 122, 166–176. <https://doi.org/10.1111/j.1744-7410.2003.tb00082.x>.
- Meretta, P.E., Rezende-Ventura, C.R., Meretta, P.E., Rezende-Ventura, C.R., 2021. Locomotion and righting behavior of sea stars: a study case on the bat star *Asterina stellifera* (Asterinidae). *Rev. Biol. Tropical* 69, 501–513. <https://doi.org/10.15517/rbt.v69isuppl.1.46392>.
- Milligan, M., 1946. Trichrome Stain for Formalin-Fixed Tissue. *Am. J. Clin. Pathol.* 16, 184–185. <https://doi.org/10.1093/ajcp/16.11.ts.184>.
- Morgana, S., Gambardella, C., Falugi, C., Pronzato, R., Garaventa, F., Faimali, M., 2016. Swimming speed alteration in the early developmental stages of *Paracentrotus lividus* sea urchin as ecotoxicological endpoint. *Mar. Environ. Res.* 115, 11–19. <https://doi.org/10.1016/j.marenvres.2016.01.007>.
- Nel, A.E., Mädlar, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the nano-bio interface. *Nature Mater.* 8, 543–557. <https://doi.org/10.1038/nmat2442>.
- Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin): a Systematic and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis. Garland Pub, New York.
- Nilsson, H.C., Sköld, M., 1996. Arm regeneration and spawning in the brittle star *Amphiuira filiformis* (O.F. Müller) during hypoxia. *J. Exp. Mar. Biol. Ecol.* 199, 193–206. [https://doi.org/10.1016/0022-0981\(95\)00188-3](https://doi.org/10.1016/0022-0981(95)00188-3).
- Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., Beiras, R., 2019. Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin *Paracentrotus lividus*. *Environ. Pollut.* 247, 706–715. <https://doi.org/10.1016/j.envpol.2019.01.098>.
- Pedà, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T., Maricchiolo, G., 2016. Intestinal alterations in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) exposed to microplastics: preliminary results. *Environ. Pollut.* 212, 251–256. <https://doi.org/10.1016/j.envpol.2016.01.083>.
- Pekmezkekmek, A.B., Emre, M., Erdogan, S., Yilmaz, B., Tunc, E., Sertdemir, Y., Emre, Y., 2021. Effects of high-molecular-weight polyvinyl chloride on *Xenopus laevis* adults and embryos: the mRNA expression profiles of Myf5, Esr1, Bmp4, Pax6, and Hsp70 genes during early embryonic development. *Environ. Sci. Pollut. Res.* <https://doi.org/10.1007/s11356-021-16527-1>.
- Pelaseyed, T., Bergström, J.H., Gustafsson, J.K., Ermund, A., Birchenough, G.M.H., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñero, A.M., Nyström, E.E.L., Wising, C., Johansson, M.E.V., Hansson, G.C., 2014. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol. Rev.* 260, 8–20. <https://doi.org/10.1111/imr.12182>.
- Peng, B.Y., Chen, Z., Chen, J., Yu, H., Zhou, X., Criddle, C.S., Wu, W.M., Zhang, Y., 2020. Biodegradation of Polyvinyl Chloride (PVC) in *Tenebrio molitor* (Coleoptera: tenebrionidae) larvae. *Environ. Int.* 145, 106106 <https://doi.org/10.1016/j.envint.2020.106106>.
- Qiao, R., Deng, Y., Zhang, S., Wolosker, M.B., Zhu, Q., Ren, H., Zhang, Y., 2019. Accumulation of different shapes of microplastics initiates intestinal injury and gut microbiota dysbiosis in the gut of zebrafish. *Chemosphere* 236, 124334. <https://doi.org/10.1016/j.chemosphere.2019.07.065>.
- Rist, S.E., Assidqi, K., Zamani, N.P., Appel, D., Perschke, M., Huhn, M., Lenz, M., 2016. Suspended micro-sized PVC particles impair the performance and decrease survival in the Asian green mussel *Perna perna*. *Mar. Pollut. Bull.* 111, 213–220. <https://doi.org/10.1016/j.marpolbul.2016.07.006>.
- Romano, N., Ashikin, M., Teh, J.C., Syukri, F., Karami, A., 2018. Effects of pristine polyvinyl chloride fragments on whole body histology and protease activity in silver barb *Barbodes gonionotus* fry. *Environ. Pollut.* 237, 1106–1111. <https://doi.org/10.1016/j.envpol.2017.11.040>.
- Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675. <https://doi.org/10.1038/nmeth.2089>.
- Schram, J.B., McClintock, J.B., Angus, R.A., Lawrence, J.M., 2011. Regenerative capacity and biochemical composition of the sea star *Luidia clathrata* (Say) (Echinodermata: Asteroidea) under conditions of near-future ocean acidification. *J. Exp. Mar. Biol. Ecol.* 407, 266–274. <https://doi.org/10.1016/j.jembe.2011.06.024>.
- Shaikh, M.M., Hanafiah, M.M., Basheer, A.O., 2019. Leaching of organic toxic compounds from PVC water pipes in medina Al-Munawarah, Kingdom of Saudi Arabia. *Processes* 7, 641. <https://doi.org/10.3390/pr7100641>.
- Suaria, S., Achtypi, A., Perold, V., Lee, J.R., Pierucci, A., Bornman, T.G., Aliani, S., Ryan, P.G., 2020. Microfibers in oceanic surface waters: a global characterization. *Sci. Adv.* 6 <https://doi.org/10.1126/sciadv.aay8493> eay8493.
- Suckling, C.C., 2021. Responses to environmentally relevant microplastics are species-specific with dietary habit as a potential sensitivity indicator. *Sci. Total Environ.* 751, 142341 <https://doi.org/10.1016/j.scitotenv.2020.142341>.
- Sugni, M., Manno, V., Barbaglio, A., Mozzi, D., Bonasoro, F., Tremolada, P., Candia Carnevali, M.D., 2008. Echinoderm regenerative response as a sensitive ecotoxicological test for the exposure to endocrine disruptors: effects of p,p'DDE and CPA on crinoid arm regeneration. *Cell Biol. Toxicol.* 24, 573–586. <https://doi.org/10.1007/s10565-008-9057-y>.
- Sun, J., Chi, X., Yang, M., Ding, J., Shi, D., Yu, Y., Chang, Y., Zhao, C., 2019. Light intensity regulates phototaxis, foraging and righting behaviors of the sea urchin *Strongylocentrotus intermedius*. *PeerJ* 7, e8001. <https://doi.org/10.7717/peerj.8001>.
- Talbot, T.D., Lawrence, J.M., 2002. The effect of salinity on respiration, excretion, regeneration and production in *Ophiophragmus filigraneus* (Echinodermata: ophiuroidea). *J. Exp. Mar. Biol. Ecol.* 275, 1–14. [https://doi.org/10.1016/S0022-0981\(02\)00208-3](https://doi.org/10.1016/S0022-0981(02)00208-3).
- Thornton, J., 2002. Environmental Impacts of Polyvinyl Chloride Building Materials. Healthy Building Network, Washington, D.C.
- Turner, A., Filella, M., 2021. Polyvinyl chloride in consumer and environmental plastics, with a particular focus on metal-based additives. *Environ. Sci.: Process. Impacts* 23, 1376–1384. <https://doi.org/10.1039/D1EM000213A>.
- Tursi, A., Baratta, M., Easton, T., Chatzisyseon, E., Chidichimo, F., De Biase, M., De Filipo, G., 2022. Microplastics in aquatic systems, a comprehensive review: origination, accumulation, impact, and removal technologies. *RSC Adv.* 12, 28318–28340. <https://doi.org/10.1039/D2RA04713F>.
- Ubaldo, J., Uy, F., Dy, D., 2008. Temperature tolerance of some species of Philippine intertidal echinoderms. *Philippine Scientist* 44. <https://doi.org/10.3860/psci.v44i0.381>.
- Vega Fernández, T., Badalamenti, F., Bonaviri, C., Di Trapani, F., Gianguzza, P., Noè, S., Musco, L., 2019. Synergistic reduction of a native key herbivore performance by two non-indigenous invasive algae. *Mar. Pollut. Bull.* 141, 649–654. <https://doi.org/10.1016/j.marpolbul.2019.02.073>.
- Vijayaraghavan, G., Neethu, K.V., Aneesh, B.P., Suresh, A., Saranya, K.S., Bijoy Nandan, S., Sharma, K.V., 2022. Evaluation of toxicological impacts of Polyvinyl Chloride (PVC) microplastics on fish, *Etroplus suratensis* (Bloch, 1790), Cochin estuary, India. *Toxicol. Environ. Health Sci.* 14, 131–140. <https://doi.org/10.1007/s13530-021-00120-7>.
- Walkinshaw, C., Lindeque, P.K., Thompson, R., Tolhurst, T., Cole, M., 2020. Microplastics and seafood: lower trophic organisms at highest risk of contamination. *Ecotoxicol. Environ. Saf.* 190, 110066 <https://doi.org/10.1016/j.ecoenv.2019.110066>.
- Wang, H., Wang, Y., Wang, Q., Lv, M., Zhao, X., Ji, Y., Han, X., Wang, X., Chen, L., 2022. The combined toxic effects of polyvinyl chloride microplastics and di(2-ethylhexyl) phthalate on the juvenile zebrafish (*Danio rerio*). *J. Hazard. Mater.* 440, 129711 <https://doi.org/10.1016/j.jhazmat.2022.129711>.
- Windels, S., Diefenhardt, T., Jain, N., Marquez, C., Bals, S., Schlummer, M., De Vos, D.E., 2022. Catalytic upcycling of PVC waste-derived phthalate esters into safe, hydrogenated plasticizers. *Green. Chem.* 24, 754–766. <https://doi.org/10.1039/D1GC03864H>.
- Winkler, A., Antonioli, D., Masseroni, A., Chiarcos, R., Laus, M., Tremolada, P., 2022. Following the fate of microplastic in four abiotic and biotic matrices along the Ticino River (North Italy). *Sci. Total Environ.* 823, 153638 <https://doi.org/10.1016/j.scitotenv.2022.153638>.
- Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. *Curr. Biol.* 23, R1031–R1033. <https://doi.org/10.1016/j.cub.2013.10.068>.
- Xia, B., Sui, Q., Du, Y., Wang, L., Jing, J., Zhu, L., Zhao, X., Sun, X., Booth, A.M., Chen, B., Qu, K., Xing, B., 2022. Secondary PVC microplastics are more toxic than primary PVC microplastics to *Oryzias latipes* embryos. *J. Hazard. Mater.* 424, 127421 <https://doi.org/10.1016/j.jhazmat.2021.127421>.
- Xia, X., Sun, M., Zhou, M., Chang, Z., Li, L., 2020. Polyvinyl chloride microplastics induce growth inhibition and oxidative stress in *Cyprinus carpio* var. larvae. *Sci. Total Environ.* 716, 136479 <https://doi.org/10.1016/j.scitotenv.2019.136479>.

- Yap, V.H.S., Chase, Z., Wright, J.T., Hurd, C.L., Lavers, J.L., Lenz, M., 2020. A comparison with natural particles reveals a small specific effect of PVC microplastics on mussel performance. *Mar. Pollut. Bull.* 160, 111703 <https://doi.org/10.1016/j.marpolbul.2020.111703>.
- Ye, X., Wang, P., Wu, Y., Zhou, Y., Sheng, Y., Lao, K., 2020. Microplastic acts as a vector for contaminants: the release behavior of dibutyl phthalate from polyvinyl chloride pipe fragments in water phase. *Environ. Sci. Pollut. Res. Int.* 27, 42082–42091. <https://doi.org/10.1007/s11356-020-10136-0>.
- Yin, L., Chen, B., Xia, B., Shi, X., Qu, K., 2018. Polystyrene microplastics alter the behavior, energy reserve and nutritional composition of marine jacopecover (*Sebastes schlegelii*). *J. Hazard. Mater.* 360, 97–105. <https://doi.org/10.1016/j.jhazmat.2018.07.110>.
- Zhang, M., Buekens, A., Jiang, X., Li, X., 2015. Dioxins and polyvinylchloride in combustion and fires. *Waste Manage Res.* 33, 630–643. <https://doi.org/10.1177/0734242X15590651>.
- Zimmermann, L., Göttlich, S., Oehlmann, J., Wagner, M., Völker, C., 2020. What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to *Daphnia magna*. *Environ. Pollut.* 267, 115392 <https://doi.org/10.1016/j.envpol.2020.115392>.