



Investigating on the toxicity and bio-magnification potential of synthetic glitters on *Artemia salina*

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ABSTRACT

Our research aims to assess the toxic impacts of polyethylene terephthalate (PET) glitters on *Artemia salina* as a model zooplankton. The mortality rate was assessed using a Kaplan Maier plot as a function of various microplastic dosages. The ingestion of microplastics was confirmed by their presence in digestive tract and faecal matter. Gut wall damage was confirmed by dissolution of basal lamina walls and an increase in the secretory cells. A significant decrease in the activities of cholinesterase (ChE) and glutathione-S-transferase (GST) were noted. A decrease in catalase activity could be correlated to an increase in the generation of reactive oxygen species (ROS). Cysts incubated in presence of microplastics exhibited delay in their hatching into 'umbrella' and 'instar' stages. The data presented in the study would be useful for scientists working on discovering new sources of microplastics, related scientific evidences, image data and model of study.

1. Introduction

Plastics find their application in various industries such as food, engineering manufacturing, automotive, fashion and others. Reports suggest that <10 % of plastics find their way towards being recycled (Jimenez et al., 2019). A total production of up to 367 million tons of plastics was estimated in the year 2021. This has been further estimated to rise up to 33 billion tons by the year 2050 (Suman et al., 2020). Issues such as persistence and low degradability could justify the categorization of plastics as an emerging pollutant (Schmaltz, 2020). Commonly plastics manufactured and utilized in bulk include synthetic polymers such as polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP), polyethylene (PE) and polyethylene terephthalate (PET), have been frequently traced in marine environments (Hsieh et al., 2021). Further degradation could be initiated by solar radiation and ocean waves and results in fragments, particles or fibers (Shim and Thomposon, 2015). A direct correlation between marine eco-system and synthetic glitters could justify the negative consequences of plastic wastes (which account for 60–80 %) on the marine ecosystem (Wang et al., 2020; Zhang et al., 2020a). Microplastics, resulting from the fragmentation of plastic wastes could pose a potential harm to the marine wildlife. Entanglement, blockage in digestive systems and gut wall damage could be reasons for

toxicity and have lethal effects on marine life (Hohn et al., 2020; Schmaltz, 2020). Their persistence in the aquatic ecosystem could be attributed to their typical sizes, shapes and structures. An accumulation of 4.85 trillion microparticles in the marine ecosystem has been estimated (Eriksen et al., 2013). Another aspect of being considered is their accumulation in the digestive tract, dead matter and faecal matter, which could lead to bio-magnification. In this regard, studies have reported the ingestion of microplastics at various trophic levels, such as zooplanktons, tuna and mussels (Botterell et al., 2019; da Costa Araújo et al., 2020). Additionally, microplastics could increase levels of heavy metals and persistent organic pollutants (POPS) owing to its hydrophobicity (Ivleva et al., 2016).

Synthetic glitter (SGPs) could be categorized as microplastics and has been widely used in the fashion industry (Perosa et al., 2021; Tagg and Ivar do Sul, 2019; Yurtsever, 2019). Synthetic glitter is comprised of three layers, namely (a) core layer (b) a reflective layer and (c) a transparent polymer-coated layer (Green et al., 2021). The size ranges between 50 and 5000 µm with a melting point of 260 °C, density of 1.38 g/cm³ and nil solubility in water (Perosa et al., 2021). Recent studies suggested the localization of glitters in sewage sludge samples in riverbed sediments of North England (Hurley et al., 2018; Harley-Nyang et al., 2022). As compared to spherical microbeads, research on

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exploring the toxic impacts of glitters is not much explored.

Research on synthetic glitters' presence, persistence and harmful impacts has been initiated (Yurtsever, 2019). Zooplankton, a major source of food for the secondary producers (such as fishes) in the aquatic ecosystem could be considered a model to assess the toxicity. These hold a high commercial value and could be fed on by other zooplankton species (Botterell et al., 2019). Brine shrimps such *Artemia salina* and *Artemia franciscana*, are being considered as model organisms for pursuing studies on microplastic toxicity (Jeyavani et al., 2022). A non-selective filter feeding activity and powerful osmotic pressure regulation could be possible reasons behind this choice (Álvarez-Alarcón et al., 2021). Changes in behavioral attributes of *Artemia salina* could provide insights on the toxic impacts of microplastics (Eom et al., 2020; Jeyavani et al., 2022; Suman et al., 2020).

We thus assessed the toxic impacts of synthetic glitter on adult brine shrimps and cysts. The specific objectives have been framed as a function of SGP dosage and exposure duration. These are: (i) assessment of survival, (ii) changes in swimming competencies as a measure of behavioral assessment, (iii) assessment of biochemical markers, (iv) ingestion and egestion and (v) hatching efficiencies in terms of development of pre-incubated cysts into 'umbrella stage' and 'instar stages' respectively. Based on the results from our study, deeper insights could be gained on the overall toxic impact of PET glitters on the marine ecosystem as well as their ability to bio-magnify and enter the food chain via marine zooplanktons.

2. Materials and methods

2.1. Characterization of synthetic glitter particles (SGPs)

Polyethylene terephthalate (PET) glitter particles were purchased from local craft stores and considered for the study. Dry SGP glitters were characterized by confirming their polymeric composition using an FT-IR in the ATR mode (Agilent Cary 630). Sizes were estimated by imaging them using a fluorescent microscope (EVOS Auto FL2; DAPI filter) and further analyzed using Image J software (Fig. S1).

2.2. In vivo studies

2.2.1. Acclimatization

Adult brine shrimps (*Artemia salina*) were purchased from Blades Biologicals (Kent, UK). Post random selection, their lengths were determined, and swimming competencies were estimated under bright field conditions (EVOS Auto FL 2). For this assessment, 4 % para-formaldehyde solution (for microscopy) was used as a tissue fixative. Artificial seawater (ASW) was prepared by dissolving 26.67 g of marine salt ('Sea-Life', Blades Biological, UK) in 1 L of tap water. The conductivity value was noted to be 3.76 ± 1.75 mS. The salinity was measured to be 24.9 ppt using a portable multimeter. Aliquots of 100 mL were prepared using artificial seawater to maintain the shrimps and perform the further experimentation on the exposure to microplastics. Five shrimps were distributed into each jar (total capacity of 250 mL). Experiments were carried out in a Fitotron plant growth chamber (SGC097). This included maintenance, acclimatization and exposure to pre-determined dosage of glitters. Parameters such as pH, total conductivity (mS) and dissolved oxygen (mg/L) were estimated using a multi-meter set-up (DTK 2017SD Multimeter, EDT037 for pH, DTK2017P/D for dissolved oxygen, DTK2017P/C for conductivity). Turbidity was also measured using turbidity meter (TR210 IR). Ammonia levels were checked at regular intervals using a kit (API brand). The chamber was programmed to maintain a temperature of $20.3 \text{ }^\circ\text{C} \pm 1.52$, relative humidity of 47 ± 2 % and a 12-h day-night cycle. Simultaneous aeration was maintained using 12 air-stones and an adequate lighting was maintained using fluorescent lamps. *Dunaliella salina*, a marine alga was chosen as the feed. For maintaining the shrimps, the microalgae was fed twice a day. The dissolved oxygen and

pH of ASW were noted to be 7.3 ± 0.4 mg/L and 8.4 ± 0.1 respectively. Residual baits and faeces were removed to prevent toxic build-up in the tank.

2.2.2. Exposure to SGPs

As a measure of exposure of shrimps to various SGP dosages, the studies carried were categorized as (i) survival assay, (ii) feeding behaviour, (iii) hatching efficiencies, and (iv) changes in swimming competencies, as described next.

- (i) *Survival assay*. Post acclimatization, shrimps were fasted for a period of 24 h and randomly assigned to glass jars with a total volume of 250 mL. A working volume of 100 mL of ASW was considered for the study. The feeding dosage range for synthetic glitter particles were chosen to be 10 mg/L–100 mg/L based on the previous reports available on their discharge limits (0.022 – $33,500$ $\mu\text{g/L}$ (Law et al., 2010; Eriksen et al., 2013; Susarellu et al., 2016)). These were by added replacement of equivalent volume of ASW. Adult and fully grown brine shrimps (*Artemia salina*) was considered for the study and experiments were conducted under controlled conditions in a Fitotron growth chamber (Humidity: 45–50 %; Temperature: 20 $^\circ\text{C}$; 12 h-Day night cycle). The total conductivity and pH values were noted to be 49.7 ± 1.75 mS and 8.4 respectively. A sample number 5 was maintained such that each jar had one shrimp and experimentation was carried out in triplicates. For each set of experiments, a control set was maintained in which shrimps were maintained under similar conditions as mentioned above, however, without any trace of microplastics. The LD₅₀ values were determined by varying the SGP dosage values between range of 10 mg/L–100 mg/L. Ten different dosages were considered and experiments were performed, for 5 days of exposure to glitters. Results were assessed using a GraphPad prism software tool.
- (ii) *Feeding behaviour*. Changes in feeding behaviour were estimated in terms of average cell counts/mL of the microalgal suspension fed to the shrimps. In the present study, *Dunaliella salina* was provided to the adult shrimps as a food source. The microalgae were cultured under similar conditions as specified in previously reported literature (Albano et al., 2021). Experimentation was conducted in 250 mL glass jars containing 200 mL of freshly prepared ASW. Each jar consisted of 3 shrimps and were arranged in triplicates. Treatment groups could be divided as control (microalgae with shrimps and nil SGPs), group A (microalgae with shrimps and 10 mg/L SGPs), group B (microalgae with shrimps and 50 mg/L SGPs) and group C (microalgae with shrimps and 100 mg/L SGPs). The total conductivity value were noted to be 46.2 ± 1.51 mS and a pH of 8.1 was recorded. The microalgae concentration in terms of average cells per mL was counted in triplicate for all solutions before the administration as verification during experimental phases of feeding rate, using a Bürker Türk counting chamber, under a bright field microscope (Evos Auto FL2) at a magnification of 20 \times .
- (iii) *Hatching efficiency*. The assessment of hatching efficiencies was performed in a 12-well microplate (Giuliana et al., 2021). Each jar contained 10 mg of dry cysts which were suspended in 50 mL of freshly prepared and pre-characterized ASW. Experiments were performed at different dosage values of SGPs (namely 0 mg/L, 10 mg/L, 50 mg/L and 100 mg/L). The jars were incubated for 18 h at 28 $^\circ\text{C}$ for acclimatization, under continuous illumination and intermittent shaking. Incubation was conducted in a Fitotron chamber with 50 % relative humidity. Post incubation, cysts were manually selected using a micropipette and placed in a glass bottom 8-well plate. The well plates were placed and secured in the chamber of a Widefield Deconvolution microscope (Olympus IX83) and assessed for their hatchability over a period of 60 min. The hatching efficiencies were calculated as per the equation:

$$h(\%) = \frac{n}{n+c} \times 100\% \quad (1)$$

where n is the number of Instar I developed and c is the number of cysts in umbrella stage. The stages are explained in Supplementary Fig. S1. Release of aluminium ions from SGPs was performed by dispersing the required dosage of SGPs in 100 mL ASW. The set-up was placed in a Fitotron chamber under similar conditions mentioned for pre-incubation of *A. salina* cysts. Experiments on release of aluminium was carried out for a period of 48 h and in triplicates. For estimation of aluminium ions, ASW was filtered using a Whatman No.2 filter paper, followed by calibration and estimation using standard aluminium tablets in a portable Lovibond MD-640 tintometer.

(iv) *Swimming competencies*. Estimation of swimming competencies of adult *A. salina* were carried out as a function of SGP dosages and exposure periods of 24 and 48 h, respectively. Parameters such as conductivity, pH and turbidity were determined for freshly prepared ASW solutions using a portable multimeter (DTK 2017SD). Traces of residual salt were thoroughly checked. Treatment groups were labelled as control (exposed to nil SGPs), A (exposed to 10 mg/L SGPs), B (exposed to 50 mg/L SGPs) and C (exposed to 100 mg/L SGPs). At the 0th hour, shrimps were carefully placed in a 12-well plate containing a volume of 0.75 mL of ASW to prevent drying out of shrimps and abnormal swimming behaviour. Bright field microscopy (Evos Auto FL2) was employed to assess the rhythmic flapping of their fore and hind legs. The flaps were recorded for a total duration of 60 s. Conditions such as poor flapping and nil mobility were recorded as 0 swimming competency.

2.2.3. Microplastic ingestion and localization studies

Shrimps were exposed to various dosages of SGPs to assess their non-selective filter feeding nature. With a set-up similar to that maintained while assessment of swimming competencies, the shrimps were fed with a combination of both microplastics and microalgae. Exposure period was maintained at 24 and 48 h, post which, the shrimps were imaged under both fluorescence and bright-field conditions. Localization of microparticles in the digestive tract and faecal matter was traced using fluorescence microscopy (Evos Auto FL2, 2× and 4× magnifications, DAPI filter) and the sizes were estimated using an imageJ software tool. Internal damages were assessed in terms of changes in the gut wall structure. Imaging was done at a 10× magnification using an Evos Auto FL2 under bright field conditions.

2.3. Biochemical markers

2.3.1. Sample preparation

Adult shrimps from control and treatment groups were thoroughly rinsed with phosphate buffered saline (PBS) for the removal of adhered microplastics. Further, homogenization was performed in PBS buffer by intermittent vortexing. Protein supernatant samples were collected by performing centrifugation for 10 min at 13,000 rpm (Thiagarajan et al., 2020). The supernatant samples were used for studying changes in biochemical markers. The supernatant samples were stored at -20°C refrigerator for further analyses.

2.3.2. Biochemical markers

Biochemical markers considered for the study include cholinesterase (ChE), glutathione S-transferase (GST) and catalase (CAT). Experiments were performed for control and various treatment groups. Estimation of cholinesterase was conducted in a reaction mixture containing protein supernatant (2.5 mL), 100 mM potassium phosphate buffer (10 mL; pH 7.4) containing 5,5 dithiobis-2-nitrobenzoic acid and acetylthiocholine chloride at concentration values of 0.05 mM and 1 mM respectively. Absorbance values were recorded using a spectrophotometer at

absorbance of 405 nm. Incubation was performed at a temperature of 25°C for a duration of 15 min (Jeyavani et al., 2022). Assay for catalase (CAT) involved a reaction mixture containing 2.5 mL of protein supernatant and 12.5 mL of 10.8 mM of hydrogen peroxide (prepared in 50 mM potassium phosphate buffer (pH 7.0)). The activities were expressed in nmole/min.mg of protein. A multi-plate reader was employed to record absorbance values at 240 nm. Estimation of glutathione-S-transferase involved reaction between 1 mM glutathione reductase (prepared in 4 mM ethanol) and 1 mM of 1 chloro-2,4 dinitrobenzene (CDNB). A wavelength of 340 nm was chosen to note the absorbance values and results were expressed in nmole/min.mg protein. Quantification of reactive oxygen species as a measure of oxidative stress was performed as per pre-established protocols (Thiagarajan et al., 2020). A chemical named 2,7-dichlorodihydrofluorescein (DCDHF) was used to fluorescently label the shrimps and image them under GFP filter using a fluorescence microscope. Fluorescence intensities of homogenized samples were measured using a benchtop fluorimeter (Fluoromax).

2.3.3. Statistical analyses

Appropriate comparisons were drawn using one-way ANOVA multiple comparisons using Dunnett's test. GraphPad Prism (version 9) was used for the statistical data analysis. The level of significance was represented as *, **, ***, or **** for $p \leq 0.01$, $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.0001$, respectively.

3. Results and discussion

3.1. Characterization of synthetic glitter particles (SGPs)

The glitter particles considered in the study were primarily composed of polyethylene terephthalate (PET) as suggested by the position of $-\text{C}-\text{H}$ bond, methylene group and ester groups as per the FT-IR spectra (Chen et al., 2012) (Supplementary Fig. S2). The particles could thus be identified as synthetic glitters comprising a plastic core with a coating of PET and aluminium. The total area was calculated to be $0.771 \pm 0.07 \text{ mm}^2$. A size range of 277–1035 μm was noted for secondary particles. Peaks were noted at 2929 cm^{-1} , 3071 cm^{-1} , 2849 cm^{-1} , 1452 cm^{-1} and 1376 cm^{-1} all assigned to $-\text{C}-\text{H}$ bonds, thus confirming the presence of aldehyde, ethyl and methylene groups, and phenyl ring. Spherical microparticles have been reported for their toxic impacts (Jeyavani et al., 2022; Suman et al., 2020; Albano et al., 2021; Eom et al., 2020). Microplastics predominantly exist as debris-based fragments in aqueous environments (Hamm and Lenz, 2021). Reports are scanty on investigating the toxic impacts of irregular-shaped microplastics (Choi et al., 2018; Hamm and Lenz, 2021; Xia et al., 2022). This justified our choice of materials to fill the lacunae in research on microplastics.

3.2. Survival rate

A Kaplan Maier plot as shown in Fig. 1 was used to explain the impacts of SGPs on survival of the adult shrimps. The number of live and dead shrimps were noted for each day (from day 0 to day 5). In the case of the control group, all the shrimps survived, thereby suggesting no lethal effect and thus, the survival rate was seen to be maintained at 100 %. Survival rates were noted to decrease for other treatment groups. In case of group A, a decrease in survival rate was noted on day 3 (80 %) and 5 (60 %) (Table S1). For group B, the survival was noted to be 20 % on day 5. The highest mortality was noted for shrimps in group C. To determine the lethal dosage₅₀ (LD₅₀) values the survival percentages were calculated over a period of 5 days for a concentration range of 10–100 mg/L. The LD₅₀ values were noted to be 75.65, 55.20, 58.58, 41.84 and 14.78 mg/L for day 1, day 2, day 3, day 4 and day 5, respectively (Supplementary Fig. S3). Contrastingly, the team of Choi et al. reported nil effects of polystyrene microplastics on *Tigriopus japonicus* when they were exposed to 20 mg/L of microplastics for a

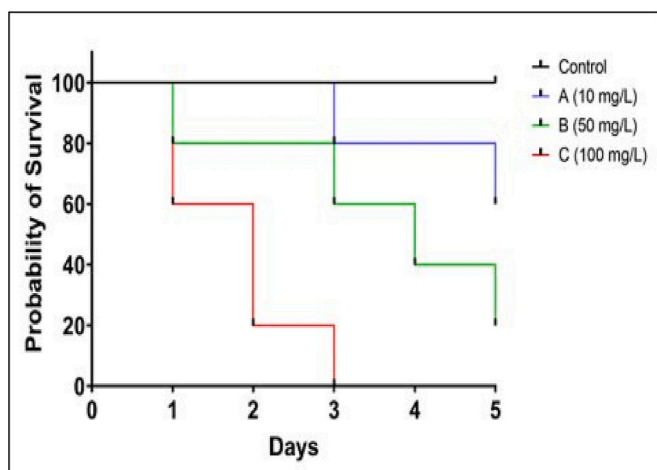


Fig. 1. Kaplan-Meier survival plot.

*Control indicates the condition where the shrimps were exposed to 0 mg/L of SGPs; treatment groups A, B and C indicated treatment conditions where the shrimps were exposed to 10 mg/L, 50 mg/L and 100 mg/L dosage of SGPs respectively.

duration of 48 h (Choi et al., 2020). Similar to the present study, carboxylated polystyrene were reported to increase the mortality rate of *Neomysis japonica* when exposed to the microplastics at a dosage range of 10–6250 $\mu\text{g/L}$ (Wang et al., 2020). In our study, an SGP dosage of 100 mg/L had a lethal effect on the survival rate. Microplastic dosage and survival rate were reported to be inversely related, based on experiments performed on *Artemia franciscana* in the presence of polyethylene terephthalate (PET) fibers and polypropylene (PP), for exposure period of 48 h, at 5 varying dosages (Kim et al., 2021). Based on the data obtained from the survival rate, it was relatively simpler to understand the changes in the microalgal content explained in the following section.

3.3. Behavioural assessment

Swimming competencies of adult *Artemia salina* have been estimated in the presence of SGPs. It could be considered an important behavioral trait, vital to estimate the SGP toxicity on shrimps. Results have been reported showing both graphical and tabulated representations of the data acquired (Fig. 2a; Table S2). In the present study, an appreciable

change in the swimming competencies were not noted. However, at SGP values of 50 and 100 mg/L, abnormal swimming behaviors (such as wriggling motions and curling up at abnormal swimming speed) could be related to cholinesterase levels. However, the size of microplastics plays a vital role in affecting the overall swimming behaviour of shrimps. The inhibition in swimming competencies was higher in the case of shrimps exposed to microplastics of lower size ranges.

On the other hand, *Artemia Salina* did not exhibit a significant reduction in swimming competencies when exposed to microplastics of higher size ranges (<5 mm) (Lin et al., 2019; Wang et al., 2020; Jeyavani et al., 2022). The shape of microplastics also played a significant role in affecting swimming competencies. Spherical microparticles negatively affected the swimming competencies in *Neomysis japonica* larvae. Similarly, polyethylene (PE) spherical particles exhibited an inhibitory effect on *Cyprinodon variegatus*. As a novel approach, our study provides deeper insights into hexagonal microplastics. Feeding behaviour was assessed in terms of changes in the microalgal count (average cells/mL) (Fig. 2b). Comparisons could be made based on the day or type of treatment. In the case of control I, where microalgal cells were grown in ASW without SGPs and shrimps, their growth reduced from $3.2 \times 10^5 \pm 14,047$ cells on day 0 to $1.8 \times 10^5 \pm 18,330$ cells. The reduction was noted to be up to 42 %. However, in the presence of microplastics, the reduction was up to 29.9 %, which could be justified in terms of providing a support matrix for the growth of *D. salina*. However, a maximum reduction in microalgal content was noted in case of control III (microalgae with shrimps and nil SGPs). The reduction was calculated to be 99.7 % and justified by a decrease in microalgal cell count from $1.3 \times 10^5 \pm 15,096$ cells/mL to $4.0 \times 10^3 \pm 4000$ cells/mL. The reductions in average microalgal cell count were noted to decrease from 67.9 % (for treatment group A) to 24.6 % (for treatment group B) and 15.1 % (for treatment group C), respectively. In case of the last two reduction values, it is evident that shrimps did not hold a significant effect on reducing microalgal cell count, unlike that of control group III and treatment group A. A negative influence of microplastics on feeding activity has been reported earlier (Albano et al., 2021); however, the present study provides further detailed and illustrative evidence.

3.4. Hatching efficiencies

The hatching efficiencies were assessed in terms of the number of *A. salina* cysts that developed into ‘umbrella’ and ‘instar I’ stages, respectively (Supplementary Fig. S4). The presence of toxic chemicals or compounds could be lethal to marine life, and assessing hatching

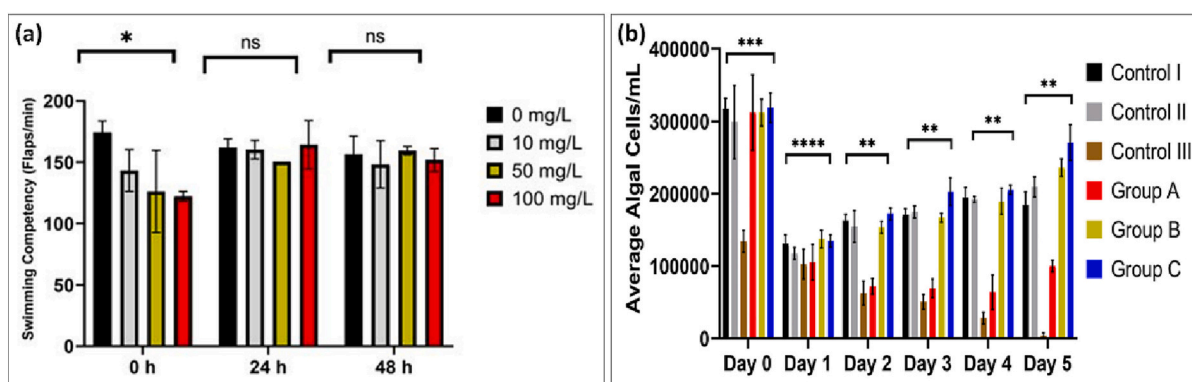


Fig. 2. Behavioral properties assessed in terms of changes in (a) swimming competencies^a (b) feeding behaviour^{a,b}.

^aSwimming competencies at 0 h, 24 h and 48 h of shrimps exposed to varied dosages of synthetic glitter particles (Overall significance analysis: ns — $P > 0.05$; * — $P \leq 0.05$).

^bControl I: Microalgae without shrimps and SGPs; Control II: Microalgae with 100 mg/L of SGPs; Control III: Microalgae with adult *Artemia salina* and nil SGPs (0 mg/L); Group A: microalgae with adult *Artemia salina* and 10 mg/L dosage of SGPs; Group B: microalgae with adult *Artemia salina* and 50 mg/L dosage of SGPs; Group C: microalgae with adult *Artemia salina* and 100 mg/L dosage of SGPs.

*One sample *t*-test was performed for the algal cells on the respective days. The *p*-values can be represented as follows—Day 0: 0.0002; Day 1: <0.0001; Day 2: 0.0013; Day 3: 0.0029; Day 4: 0.0060; Day 5: 0.0089.

efficiency is one of the techniques to assess toxicity. The presence and bioavailability of heavy metals have been reported to affect the hatching efficiencies of *A. salina* cysts (Liu and Chen, 1987; Wang et al., 2018). The team of Liu and Chen reported the toxic effects of cadmium (90–100 $\mu\text{mol/L}$) on the hatching efficiencies of *A. salina* cysts, which could be attributed to the absorption of the heavy metal on the wall of the cyst. Similarly, inorganic complexes of zinc could also affect the hatching process of *A. salina*. The order of toxicity reported by Long and Angino could be $\text{Zn}^{2+} < \text{Zn}(\text{OH})^+ < \text{ZnCl}^+$ (Long and Angino, 1977), thus enhancing the absorption of zinc and altering enzymatic systems involved in hatching (Alayse-Danet et al., 1979). The present study reports the effect of synthetic glitter particles on the hatching efficiencies of *A. salina* cysts. We studied the hatching efficiencies in the development of cysts in ‘hydrated’ stage to ‘umbrella’ and ‘instar I’ stages respectively. Fig. 3(a)–(l) shows a detailed routes of development as a function of SGP dosages. Live images of developing cysts were recorded using a widefield de-convolution microscope. A total of 7–12 cysts were considered to calculate the hatching efficiencies. While the control (0 mg/L SGP) showed prominent development of instars after 60 min, more cysts were noted to be in hydrated and umbrella stages when pre-incubated with varying dosages of SGPs. The data on the hatching efficiencies have been calculated in terms of development into umbrella (hatching_{US} (%)) and instar stages (hatching_{IS} (%)). A graphical representation is shown in Fig. 3(m)–(n). Results have been tabulated in Supplementary Table S3. A parallel comparison between the figures showed a linear decrease in the umbrella stage and an increase in the instar stage from a period of 0–60 min for cysts pre-incubated without SGPs. A significant difference in the hatching efficiencies were noted at 60th minute for cysts pre-incubated with 10 mg/L and 50 mg/L of SGPs ($75.4 \pm 21.8\%$ vs. $36.7 \pm 7.6\%$). In the case of cysts incubated with 100 mg/L of SGPs, the values for hatching efficiencies were noted to be the least ($30.0 \pm 5.0\%$). The results suggested a negative impact of SGP dosage on the hatching efficiencies. It has already been documented that the presence of heavy metals negatively affected these. A careful study of the leaching of aluminium from SGP particles justified the impact of

SGPs on the *A. salina* cysts (Supplementary Fig. S5). Studies were conducted on the time-based release of aluminium ions from SGPs. The release of aluminium ions was initiated from 6th hour onwards for all three dosage values of SGPs. Post 12 h, a significant rise in aluminium ions was noted to be released in the case of SGP dosages of 50 mg/L and 100 mg/L. The maximum release of aluminium ions was recorded until 48 h and reached a concentration of 0.3 ppm. As per the EPA, a secondary maximum permissible limit has been accepted to be the range of 0.05–0.2 ppm. The values of aluminium released from the glitter particles were slightly above the permissible range and could possibly have been absorbed by the cysts in their pre-incubation period (12 h), which could account for their slow hatching efficiency.

3.5. Microplastic ingestion and localization

The presence of microplastics in live shrimps and dead matter could provide valid inferences on the bio-magnification potential as zooplanktons are consumed by higher marine organisms. In this regard, the genus *Artemia* could serve as a suitable model organism, as also considered in some of the published researches (Kim et al., 2021; Varó et al., 2019; Jeyavani et al., 2022; Suman et al., 2020; Albano et al., 2021; Eom et al., 2020; Albano et al., 2021). Nano- and microplastics of size range 50 nm–300 μm , respectively, could easily be ingested by the zooplankton of genus *Artemia*. Thus it was further important to understand the consequences, like the size of the ingested microparticles in the digestive tract and egested particles in faecal matter, as an outcome of the filter-feeding mechanism. Figures 4 confirmed the ability of adult *A. salina* to ingest and retain SGPs at all respective dosage values. Interestingly, the size of the retained SGP particles varied with the dosage. A prominent presence of accumulated SGPs could be seen in Fig. 4 (images, o, p, a4, a5, a6). Due to the non-selective filter feeding, an increase in size of accumulated particles was noted with an increase in dosage of SGPs ($25.01 \pm 13.04\ \mu\text{m}$ at 10 mg/L to $95.22 \pm 55.55\ \mu\text{m}$ at 100 mg/L at 48 h). Similarly, faecal matter collected after 24 h and 48 h showed presence of egested glitter particles (Fig. S6). Fig. 4 (panels f, g,

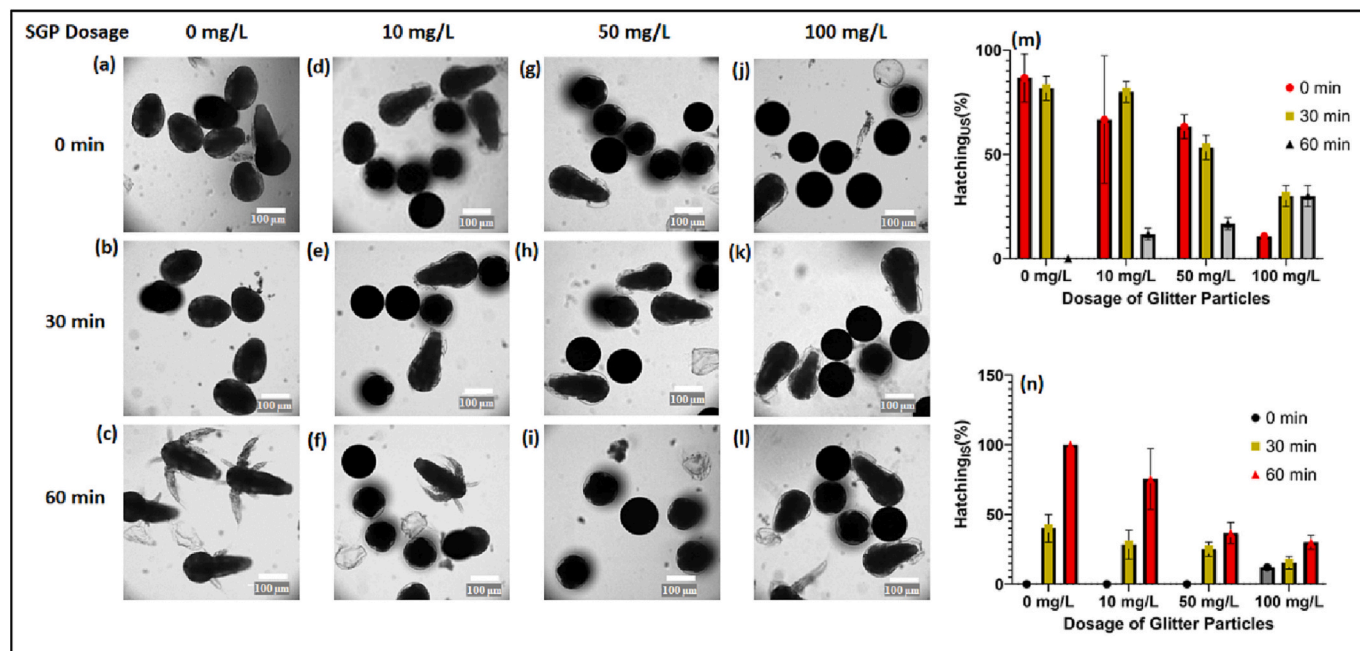


Fig. 3. Hatching efficiencies assessed in terms of development of hydrated cyst into ‘umbrella’ and ‘instar I’ stages. (a)–(l) represent 3D images obtained from live imaging of cysts post incubation with SGPs; (m)–(n) graphical representation of data. *Hatching (%)_{US} represents number of cysts that developed into umbrella stage from hydrated stage and Hatching (%)_{IS} represents number of cysts that developed into instar I stage from hydrated stage.

*Statistical analyses were performed using a one-sample *t*-test. The *p*-values are mentioned as follows: H_{US}: *p*-value (0th min)—0.0709; *p*-value (60th min)—0.0022; *p*-value (90th min)—0.1450; H_{IS}: *p*-value (0th min)—0.2138; *p*-value (60th min)—0.0352; *p*-value (90th min)—0.0372.

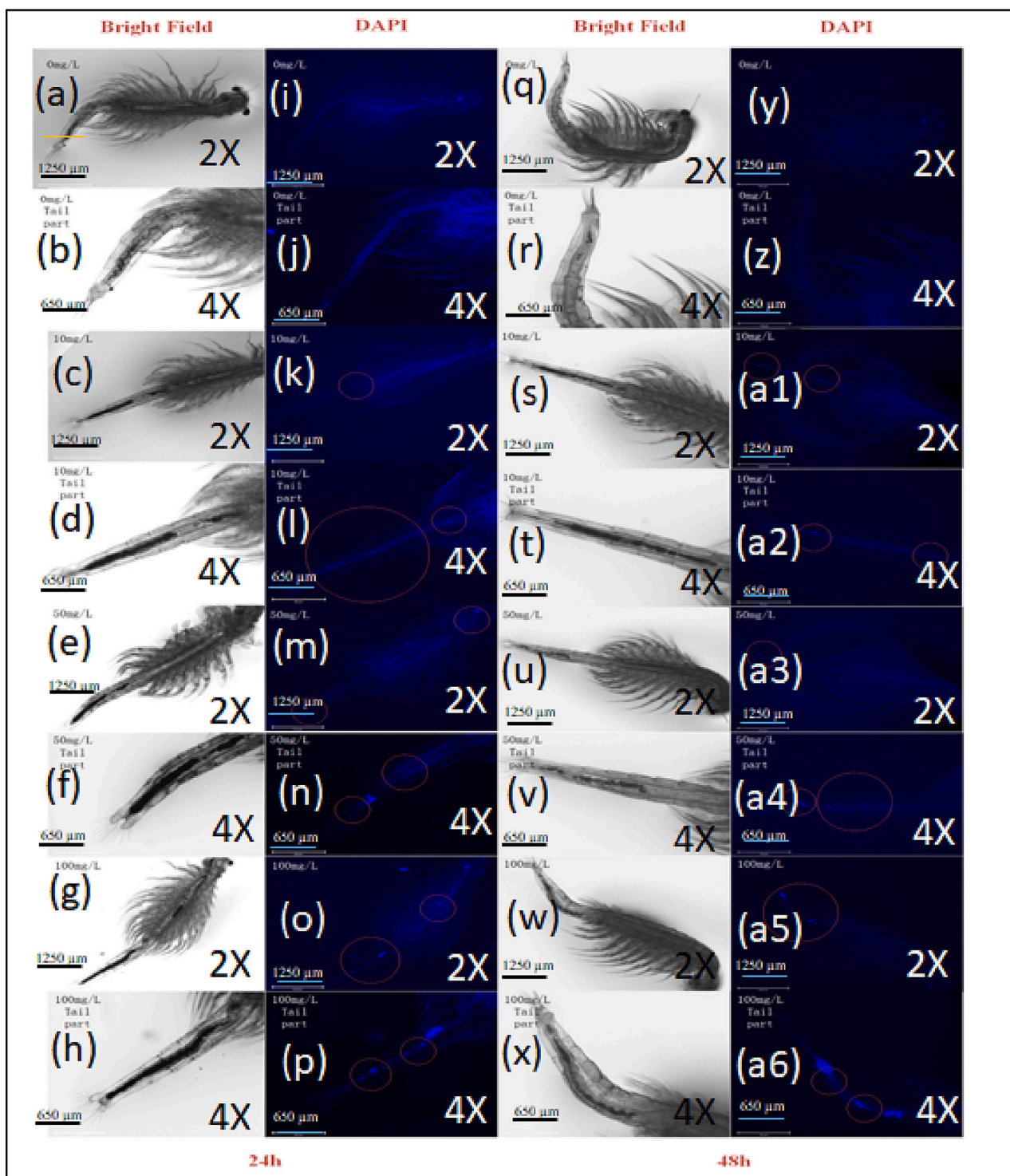


Fig. 4. Bright-field and fluorescence imaging of SGPs accumulated in the digestive tract of *Artemia salina* exposed to 10, 50 and 100 mg/L dosage of SGPs at 24th h and 48th h respectively.

*The shrimps were fixed in 4 % para-formaldehyde solution. The SGPs exhibited an auto-fluorescence under DAPI filter. Images were obtained using an EVOS Auto FL2 at magnifications 2 \times and 4 \times respectively.

h, n, o, p) confirmed the localization of the microparticles. The results highlighted the biomagnification potential of the organism towards synthetic glitters. Similar reports were noted in the amphipod *Gammarus fossarum* when exposed to the microfibers of polyamide (500 \times 20 μ m) for 16 h (Blarer and Burkhardt-Holm, 2016). The increase in sizes of bioaccumulated microparticles could be attributed to the dosage and exposure time. Changes in the microalgal count was noted to be minimal

when shrimps were exposed to higher SGP dosage, which suggested an increase in selectivity towards microparticles irrespective of the presence of microalgae. The dosage of microplastic residues in faecal matter and the dosage of fed microplastics have been correlated in previous reports (Blarer and Burkhardt-Holm, 2016; Wang et al., 2020). A dosage and time-dependent bioaccumulation of polystyrene (PS) variants in *Neomysis japonica* has been reported (Wang et al., 2020). Egested SGPs in

the faecal matter showed a drastic decrease in size after 48 h which could be attributed to the filter-feeding activity (Table S4). The size of ingested SGPs was calculated to be $95.23 \pm 55.55 \mu\text{m}$ (when exposed to SGP dosage of 100 mg/L for 48 h). Exposure to lower dosage values (like 10 mg/L) resulted in size ranges up to 150 times less than the size of particles fed ($6.20 \pm 0.36 \mu\text{m}$). The results revealed chances of bio-magnification of *Artemia salina* when exposed to glitters. However, statistical analyses revealed no significant difference in microparticle sizes in gut and faecal matter when compared.

3.6. Assessment of gut damage

Internal changes in the gut wall structure could provide meaningful inferences on the toxic effects of microplastics (Zhang et al., 2020b). Changes in the inner gut wall in response to *Artemia salina* exposures to synthetic glitters have been reported in the present study and represented in Fig. 5. As dosage value of 100 mg/L was found exhibit a toxic effect, this dosage was chosen to visualize the internal damages in contrast to the control (exposed to nil SGPs). Significant damages in the basal lamina (BL) were noted which could be justified by an increase in secretory cells (SE). Secretory cells are tall columnar cells which contain secretory granules (composed of lysosomes and acid) in their cytoplasm. Their components are responsible for the reduction in lipid droplets and destruction of epithelial and basal lamina (BL) layers (Wang et al., 2019a; Wang et al., 2019b). This could negatively affect metabolic functions (Jeyavani et al., 2022; Suman et al., 2020). As a result of being exposed to 100 mg/L of SGPs, significant changes in gut wall structure were noted, which were visualized under $20\times$ magnifications and reported in terms of abnormalities in the distribution of secretory cells (SE) and dissolution of the basal lamina (BL) layers (Schmaltz, 2020). A drastic increase in the secretory cells was noted as a comparison between treated and control groups (Fig. 5(a)). This was followed by a

blurred appearance of the basal laminal layer (Fig. 5(b)). As per the present study, irregular shaped microparticles could have a significantly high toxic effect in terms of internal gut wall damages.

3.7. Biochemical markers and oxidative stress

In the present study, the biochemical markers considered were cholinesterase (ChE), glutathione-S-transferase (GST) and catalase (CAT). A graphical representation showing changes in the enzymatic activities have been recorded and represented in fig. S7. Significant and linear reductions in cholinesterase activity as a function of SGP dosage values post-exposure periods of 24 and 48 h were noted (Fig. S6(a); Table S5). Similar reductions were noted in case of GST activities, when shrimps were exposed for duration of 24 and 48 h. (Fig. S6(b); Table S6). Catalase is an antioxidant enzyme, and changes in its activity could be correlated to changes in the reactive oxygen species level. No significant inhibition was noted for shrimps from treatment group A. However, for treatment group B (shrimps exposed to SGP dosage of 50 mg/L), significant ($p \leq 0.01$) inhibition in catalase activities was noted for an exposure period of 24 h, while treatment group C exhibited inhibitions at both exposure periods of both 24 and 48 h (Fig. S6(c), Table S7). Microplastics have been reported to be the cause of oxidative stress in zooplankton (Jeyavani et al., 2022; Kim et al., 2021; Lin et al., 2019). Thus, reactive oxygen species (ROS) could serve as an important biomarker for the assessment of oxidative stress. In the present study, an increase in ROS levels were noted in case of *Artemia salina* from all the three treatment groups, for a period of 24 h and 48 h. Cellular inflammation and apoptosis could be an outcome of elevated ROS levels (Jeyavani et al., 2022). Changes in fluorescence intensities could be correlated to the alterations in the levels of reactive oxygen species (ROS) (Fig. 6(a)–(b); Table S8). A recent study reported apoptosis *Artemia salina* exposed to polypropylene at dosage values ranging

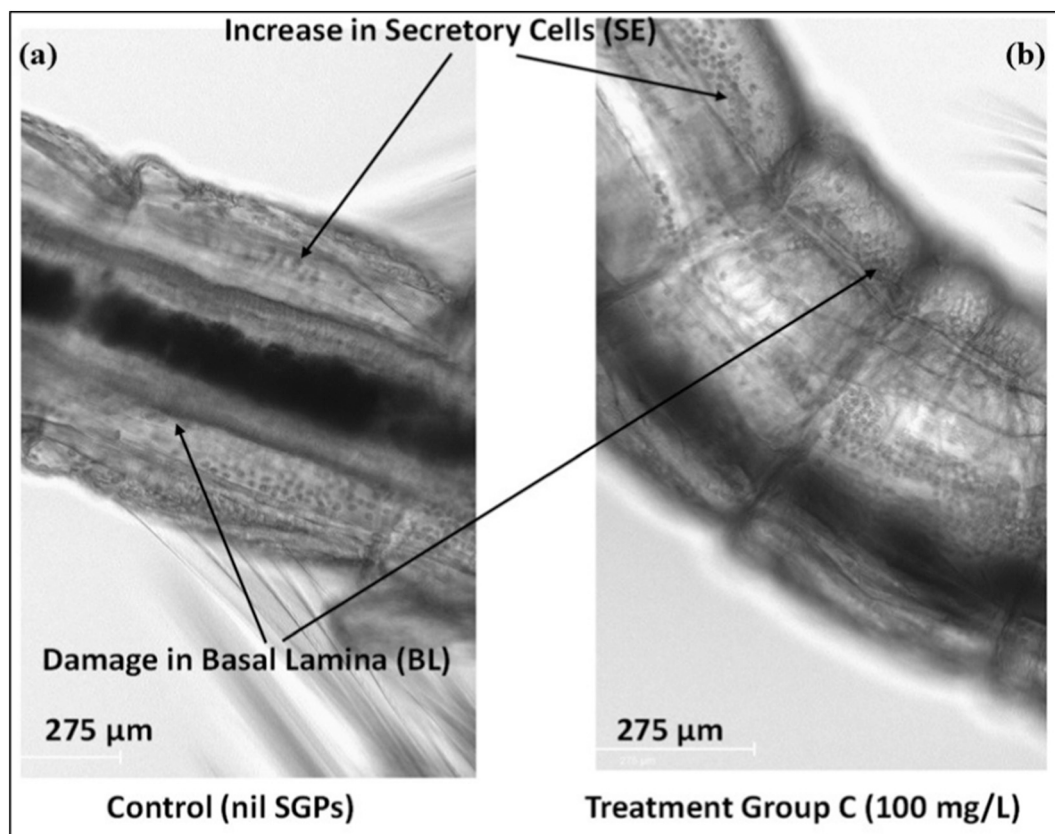


Fig. 5. Changes in the inner tract of gut in *Artemia salina* when exposed to (a) 0 (control) and (b) 100 mg/L dosage of SGPs.

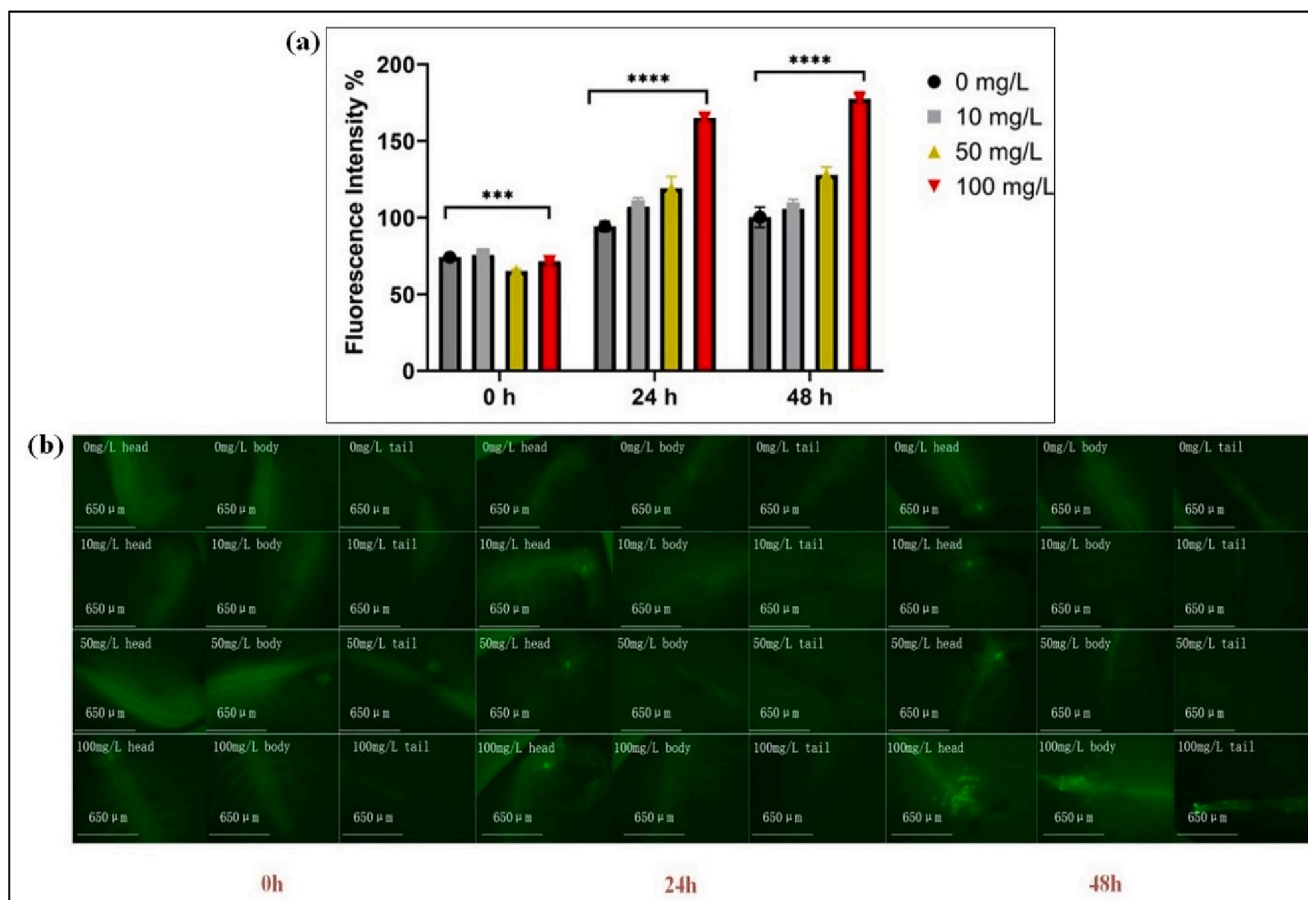


Fig. 6. (a) Graphical representation for changes in ROS levels of *Artemia salina* exposed to different dosage values of SGPs (b) bio-imaging (under GFP filter) of different body portion.

*(Overall significance analysis: Ns — $P > 0.05$; *** $P \leq 0.001$; **** $P \leq 0.0001$).

between 25 and 100 mg/L (Suman et al., 2020). A graphical representation of data showed linearity in the increase in ROS levels as a function of increase in glitter dosage values. As expected, maximum generation of ROS was noted in case of treatment group C (shrimps exposed to 100 mg/L SGPs) for a duration of 48 h ($177.58 \pm 4.62\%$) (Fig. 6(a)). Fig. 6 (b) represents variations in fluorescence accumulation based on exposure duration, body part and SGP dosage values. Enhanced values of ROS could be positively correlated to an overall increase in the fluorescence accumulation head portion of the shrimps. Maximum accumulation was noted at the end of the exposure duration of 48 h which justified the enhanced oxidative stress in the zooplankton.

Catalase (CAT), an antioxidant enzyme, could hydrolyze hydrogen peroxide, thereby eliminating the accumulation of free radicals. At dosage values of 50 and 100 mg/L, an inhibition in CAT activity was noted after an exposure period of 48 h. The inhibition could be due to the aggregation of hydrogen peroxide, which led to an overall increase in ROS levels. Glutathione-S-transferase (GST), on the other hand, plays an important role towards protection against oxidative damage. Results showed a decrease in the activities of both enzymes, demonstrating an impairment in the oxidative barrier caused due to exposure to SGPs. Similar results have been reported in the case of spherical microparticles of varied polymeric natures (Varó et al., 2019; Clasen et al., 2018; Wang et al., 2019c). Cholinesterase (ChE) activity is a biomarker for the assessment of neurotoxicity. Under inhibition, it could result in affecting neurotransmission (Lin et al., 2019). In our study, similar to the results of catalase and glutathione-S-transferase, all three treatment groups exhibited a reduction in ChE activity (except for a dosage of 50 mg/L and exposure period of 24 h). This could be positively correlated to the

increase in the build-up of oxidative stress and abnormal swimming behaviors (Eom et al., 2020; Jeyavani et al., 2022; Lin et al., 2019; Peixoto et al., 2022; Varó et al., 2019). Enhanced generation of reactive oxygen species (ROS) could account for internal gut wall damage (Wang et al., 2019c). Per previous reports, certain biomarkers (like GST) do not show significant changes when model organisms are exposed for 48 h (Eom et al., 2020; Varó et al., 2019). In our study, significant changes in the biomarker activities was noted at all three dosage values of SGPs, which suggested their enhanced toxicity. This could be due to their irregular shape, polymeric composition and aluminium/heavy metal leaching property.

Statistical analyses of the data obtained from the studies on biochemical markers showed that variations in activities over 0, 24 and 48 h were significant for SGP dosages. However, variations were noted based on the type of biochemical marker. In case of cholinesterase and catalase, variations in activities were significant at dosage values of 0 mg/L, 10 mg/L and 100 mg/L respectively. The variations in reactive oxygen species generation were noted to be significant for SGP dosage values of 0 mg/L, 50 mg/L and 100 mg/L. On the contrary, variations in GST activities showed no significant difference over SGP dosages. The results confirmed the impact of SGP dosage on the metabolic activities of shrimps.

4. Conclusion

The toxicity of synthetic glitters on *Artemia salina* could be explained in terms of definite behavioral and biochemical responses and as a function of exposure duration as well as dosage. Attributes such as

irregular hexagonal shape, size, polymeric nature and heavy metal leaching property affected the behavioral and biochemical traits at a given exposure dosage and exposure period. At a maximum dosage value of 100 mg/L, a decrease in survival rate was noted. While only 20 % of shrimps survived on day 2, there were nil survivors on day 3. No significant effects on swimming competencies were noted, which could be attributed to the size of the particles (277–1035 μm). A significant reduction in feeding rate was noted. In absence of SGPs, a reduction in microalgal content was noted from $1.3 \times 10^5 \pm 15,096$ cells/mL to $4.0 \times 10^3 \pm 4000$ cells/mL (99.7 %). However, a reduced feeding rate was inversely related to the microalgal count. In the presence of SGPs, the reduction in algal count was noted from 67.9 % (for treatment group exposed to 10 mg/L SGPs) to 24.6 % (for treatment exposed to 50 mg/L SGPs) and 15.1 % (for treatment group exposed to 100 mg/L SGPs) respectively. Microplastics were traced in the digestive tract and faecal matter. The size of ingested particles in the digestive tract was directly proportional to dosage. The size of microparticles in the faecal matter was found to reduce with a decrease in SGP dosage and an increase in exposure time. Due to its filter-feeding nature, microparticles of size 6–17 μm were confirmed in the faecal matter when shrimps were exposed to the least dosage of 10 mg/L for a maximum exposure duration of 48 h. These sizes were approximately 90–150 times smaller than those fed to the shrimps, which justified their potential to bio-magnify them in the aquatic ecosystem. Hatching efficiencies were reduced for cysts pre-incubated in the presence of SGPs. This was attributed to the absorption of aluminium ions (released from SGPs) by the hydrated cysts. A maximum of 0.3 ppm of aluminium was released from a dosage of 100 mg/L of SGPs. Hatching efficiencies as assessed in the form of H_{US} (%) and H_{IS} (%), suggested that hydrated cysts remained in the same or umbrella stage when pre-incubated in the presence of SGPs, while cysts from the control group developed into instar stages rapidly. The results indicated significant levels of SGP-induced toxicity in the aquatic ecosystem. An enhancement in the generation of reactive oxygen species was confirmed by fluorescence accumulation in the head portion and alterations in other biochemical markers. Increased oxidative stress could be correlated to the internal damage caused in the gut wall structure, which was marked by an increase in the secretory cells and dissolution of the basal lamina wall. The results from the present study provided evidence of the toxic impacts of irregularly shaped glitter particles and insights into the routes of bio-magnification.

CRedit authorship contribution statement

Dr. Devlina Das Pramanik: Conceptualization, Plan of Work, Investigation, Formal analyses, Resources, Supervision, Manuscript- Writing, Review & Editing; Sihan Lei: Investigation, Formal analysis; Prof. Paul Kay: Supervision, Review & Editing; Prof. Francisco M. Goycoolea: Hosting and Supervision; Resources, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2023.114828>.

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