Science of the Total Environment 806 (2022) 150965

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A comparative investigation of toxicity of three polymer nanoparticles on acorn barnacle (*Amphibalanus amphitrite*)

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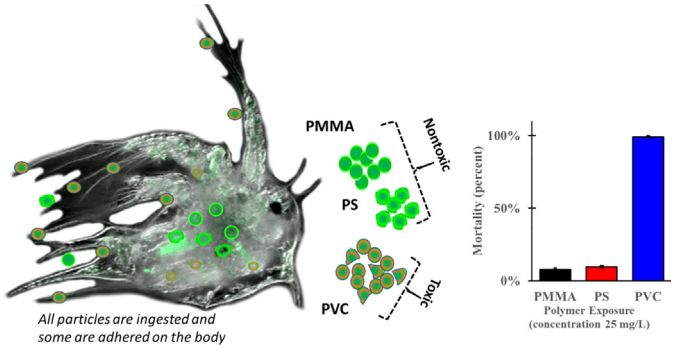
HIGHLIGHTS

- Fluorescent nanoparticles of PMMA, PS and PVC were prepared and characterised.
- Comparative toxicity investigations of three polymer nanoparticles were done using barnacle nauplii.
- PVC nanoparticles showed 99% mortality among exposed nauplii at 25 mg/L.
- Chemical toxicity of oligomers and adherence of nanoparticles to naupliar carapace contribute to the toxicity.
- Toxicity investigation of multiple polymer nanoparticles on single animal model shows interesting results.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 August 2021
Received in revised form 2 October 2021
Accepted 9 October 2021
Available online 15 October 2021

Editor: Jay Gan

Keywords:
Nanoplastics
Marine pollution
Contaminants of emerging concern
Barnacles

ABSTRACT

Pollution from plastic waste is increasingly prevalent in the environment and beginning to generate significant adverse impact on the health of living organisms. In this study, we investigate the toxicity of polymer nanoparticles exposed to Acorn Barnacle (*Amphibalanus amphitrite*) nauplii, as an animal model. Highly stable aqueous dispersion of luminescent nanoparticles from three common polymers: polymethylmethacrylate (PMMA), polystyrene (PS), and polyvinylchloride (PVC), were prepared via nanoprecipitation and fully characterised. Exposure studies of these polymer particles to freshly spawned barnacle nauplii were performed within a concentration range from 1 to 25 mg/L under laboratory-controlled conditions. The exposure to PMMA and PS nanoparticles did not show detrimental toxicity and did not cause sufficient mortality to compute a LC_{50} value. However, PVC nanoparticles were significantly toxic with a mortality rate of up to 99% at 25 mg/L, and the calculated LC_{50} value for PVC nanoparticles was 7.66 ± 0.03 mg/L, 95% CI. Interestingly, PVC nanoparticle aggregates were observed to adhere to the naupliar carapace and appendages at higher concentrations and could not be easily removed by washings. To explore the possibility of chemical toxicity of polymer nanoparticles, analysis of the polymer powders which was used to prepare the nanoparticles was conducted. The presence of low molecular weight oligomers such as dimers, trimers and tetramers were observed in all polymer samples. The chemical nature and concentration of such compounds are likely responsible for the observed toxicity to the barnacle nauplii. Overall, our study shows that care should be exercised in generalising the findings of exposure studies performed using one type of plastic particles, as the use of different plastic particles may elicit different responses inside a living organism.

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https://doi.org/10.1016/j.scitotenv.2021.150965
0048-9697/

1. Introduction

Pollution from plastic waste has been increasing in the aquatic environments, which is causing significant adverse ecological and health impacts to numerous living organisms (Jambeck et al., 2015). Plastics waste in the environment has been known to degrade to form smaller particles in the micron and nanometre range (Barnes et al., 2009). The exposure and subsequent uptake of particles of common plastics by various animals have largely found detrimental effects on their physiology and physical health (Pitt et al., 2018; Varó et al., 2019; Wu et al., 2019).

However, the extent of acute toxicity due to nanoplastics exposure appears to vary significantly based on surface chemistry (Bergami et al., 2017; Della Torre et al., 2014), and size (Jeong et al., 2016).

Various studies have shown that the surface chemistry and charges of polymer nanoparticles could influence how they can effect toxicity on the organisms. Bergami et al. (2017) found that the exposure of *Dunaliella tertiolecta* to cationic (positively charged) 40 nm polystyrene (PS) nanoparticles caused growth inhibition, adsorption to appendages and antennules, while ingestion by *Artemia franciscana* resulted in increased *clap* and *cstb* gene expression, which relate to larval growth and moulting, indicating a reaction to an environmental stressor. On the other hand, exposure to anionic (negatively charged) 50 nm PS nanoparticles did not affect the growth of both *D. tertiolecta* and *A. franciscana,* thereby suggesting that cationic PS nanoparticle toxicity could be attributed to direct toxicity from simple exposure, or strong adsorption of the positively charged PS particles to the microalgal feed used, resulting in ingestion of the cationic PS nanoparticles (Bergami et al., 2017). The acute toxicity of nanoparticles reported in the aforementioned studies may have been influenced by surface functionalisation or surface charges on the particles. In general, cationic particles, surface-functionalised with animo groups, tend to elicit greater toxic response as seen in *D. tertiolecta* and *A. franciscana* (Bergami et al., 2017), and *D. magna* (Mattsson et al., 2017). While anionic particles, surface-functionalised with carboxylic groups or stabilised with the anionic surfactant sodium dodecylsulfate, showed comparatively lower toxicity in *D. tertiolecta* and *A. franciscana* (Bergami et al., 2017), delayed toxicity in *C. elegans* (Zhao et al., 2017), or no detectable toxicity in developing *A. amphitrite* larvae (Bhargava et al., 2018a). Ingestion of uncharged PVC, PS, and polyamide microspheres by *Sparus aurata* over 45 days did not show any adverse effect after depuration (Jovanović et al., 2018). Exposure of *Amphibalanus amphitrite* (*A. amphitrite*) barnacle nauplii to submicron anionic (−35.1 mV zeta potential) polymethylmethacrylate (PMMA) particles also did not show detrimental toxicity, albeit exhibiting extensive translocation and accumulation of the particles inside the tissues (Bhargava et al., 2018a). *Crassostrea gigas* larvae ingesting PS particles as small as 70 nm, and as large as 20 μm generally did not show any detrimental effects, even though cationic amino-functionalised PS particles were found to be more readily ingested and retained (Cole and Galloway, 2015).

However, another study exposing *A. amphitrite* and *Artemia franciscana* to 100 nm anionic (−53.1 mV zeta potential) polystyrene nanoparticles found that both model organisms showed oxidative stress and neurotoxicity in exposure concentrations as low as 0.001 mg/L (Gambardella et al., 2017). *Mytilus edulis* larvae exposed to 100 nm was found to have higher instances of abnormal larval development, even though the PS particle exposure did not affect larval growth, and PS particle egestion (Rist et al., 2019).

Although particle surface charges were not reported in some of the studies, the colloidal polymer nanoparticles used in nanoplastics investigations require a surface charge to maintain stable suspension in an aqueous medium (Selvamani, 2018), hence the nanoparticles used in most studies were likely charged. Environmental aging of plastics in the environment causes chemical changes to the plastic surface, and through oxidation, introduces carboxyl groups to plastic surfaces (Liu et al., 2019; Luo et al., 2020), hence environmental nanoplastics are also likely to be anionic.

Exposure of unfunctionalised PS nanoparticles to *Brachionus koreanus* has been reported to negatively affect growth rate, fecundity, lifespan, reproduction time, and body size, as compared to exposure to micron-sized particles implying that the size of polymer particles had a significant effect on organisms' physiology (Jeong et al., 2016). Microparticles of various polymers like PE and styrene-acrylonitrile copolymers within the size range of 124 μm and 438 μm have been detected inside the liver tissues of *Engraulis encrasicolus* via Raman microscopy (Collard et al., 2017), demonstrating that microscale particles can absorb into internal organs. The translocation of polymer particles from the digestive tract to other parts of an animal's body is also an important problem investigated in the literature, in particular, on the reproductive process (Cui et al., 2017; Zhao et al., 2017). Polystyrene (PS) nanoparticles (100 nm) with a measured zeta potential of −9.698 mV were found in the gonads of *Caenorhabditis elegans* after ingestion, which caused behavioural changes in third generation offspring (Zhao et al., 2017). Translocation of PS nanoparticles (52 nm) into the ovaries and brood chamber of *Daphnia galeata*, which resulted in lower hatching rates and higher lipid storage in embryos was also reported (Cui et al., 2017). Similarly, polymer nanoparticles exposed to different human cell lines also induced significant metabolic changes under in vitro conditions (Lim et al., 2019; Mahadevan and Valiyaveettil, 2021a, 2021b; Schirinzi et al., 2017).

There is no consensus on the toxicity of micro- and nanoplastics in living systems due to the use of different organism models in studies, differences in chemical properties of polymers, purity standards of particles used, different assessment protocols and data reporting methods.

To date, no comparative studies using different polymer nanoparticles have been reported towards understanding their toxicity in marine organisms. In the present study, we hypothesise that different polymer nanoparticles should show various levels of toxicity based on their chemical structure, provided the initial particles have similar composition, size and properties. The acorn barnacle, *A. amphitrite* is a common fouling organism found along shorelines of marine environment in Singapore, where plastic pollution is known to be high. Based on our previous publication, we found the transparent *A. amphitrite* nauplii larvae to be a useful and effective model organism and therefore selected for our study (Bhargava et al., 2018a). Freshly spawned nauplii were exposed to nanoparticles of PMMA, PS, and polyvinylchloride (PVC) at a range of concentrations to compare the impact and toxicity of the three polymers, as well as describe the intake and translocation of nanoparticles by the transparent nauplii at 1, 5, 15, and 25 mg/L polymer nanoparticle concentration.

Luminescent PMMA, PS, and polyvinylchloride (PVC) nanoparticles were prepared in our lab using the nanoprecipitation method reported earlier (Bhargava et al., 2018b, 2018a). To visualise the nanoparticles inside the organism, fluorescence markers encapsulated inside the polymer nanoparticles were used. Fluorophore tagging of polymer nanoparticles has been scrutinized for its flaws such as leaching of fluorescent dye out of the particle and potentially staining cells and tissues which results in false identification of particles (Catarino et al., 2019; Schür et al., 2019), the perylenetetraester (PTE) dye used in the present study is hydrophobic in nature with zero or negligible solubility in water and homogenously dispersed inside the polymer matrix of the nanoparticles (Bhargava et al., 2018b). We also know that if PTE dye molecules leach from the polymer nanoparticle into an aqueous environment, they quickly aggregate and precipitate as solid particles, causing aggregation induced quenching of emission, mitigating interference in the imaging process (Bhargava et al., 2018b, 2018a).

The use of polymer nanoparticles in the mg/L range, as seen in many nanoplastics studies (Brandts et al., 2021; Tallec et al., 2020), is significantly higher compared to predicted environmentally relevant concentrations based on microplastics samples found in environmental surveys (Lenz et al., 2016). Some recent studies reported using environmentally relevant nanoplastics concentrations at 15 μg/L (Al-Sid-Cheikh et al., 2018), 1 μg/L (Liu et al., 2020; Qu et al., 2019), and 0.04 ng/L to 34 μg/L (Guimarães et al., 2021).

The use of relatively high polymer nanoparticle concentrations in the present study served two purposes. *Amphibalanus amphitrite* has been shown in a previous studies to be relatively tolerant to polymer particle exposure, and the coastal environment in which the barnacle is found would usually have higher plastics contamination (Bhargava et al., 2018a; Yu and Chan, 2020). Therefore, a higher contaminant dose was needed to elicit a measurable toxic response within a short timeframe. Confocal fluorescence imaging of the polymer nanoparticle exposed nauplii is also relatively insensitive for detecting fluorescent polymer nanoparticles due to interference from background noise, autofluorescence, and natural fluorescence in biological tissues (Al-Sid-Cheikh et al., 2018; Schür et al., 2019), despite the high fluorescence quantum yield of the fluorophore (Bhargava et al., 2018b).

Stabilised by a negative zeta potential, induced by the sodium dodecylsulfate incorporated during the nanoprecipitation process (Bhargava et al., 2018b), the anionic polymer nanoparticles are expected to form micron sized aggregates in the high ionic strength environment of seawater, as observed in another study (Tallec et al., 2020). Nanoplastics form heteroaggregates in the presence of dissolved ions and organic matter in seawater (Canesi and Corsi, 2015; Chen et al., 2018). Nonetheless, ingested microparticles and nanoparticle aggregates can undergo digestive fragmentation to give rise to submicron particles, as demonstrated by a study with Antarctic Krill (Dawson et al., 2018). It is hence possible that organisms ingesting micron sized plastic particles or nanoplastics aggregates, are ultimately exposed to nanoplastics, regardless of the particle aggregation conditions in the water column. However, after fragmentation and translocation from the digestive tract, Nanoplastics may also interact with proteins in physiological conditions (Kihara et al., 2019), which is expected to occur after ingestion and translocation by an organism. This complicates the determination of effective particle sizes within physiological conditions.

2. Methods and materials

2.1. Preparation of polymer nanoparticles

Polymeric nanoparticle dispersions were prepared using a nanoprecipitation method based on a published procedure (Bhargava et al., 2018a), using commercially available PMMA, PS and PVC powders. In a typical procedure, PMMA (400 mg, $M_w = 15$ kDa, Sigma Aldrich) was dissolved in acetone (100 mL). PVC (400 mg, $M_w = 120$ kDa, Scientific Polymer Products) and PS (400 mg, $M_w = 45$ kDa, Scientific Polymer Products) were dissolved in tetrahydrofuran (THF, 50 mL), before dilution with acetone to prepare a stock solution (100 mL). The stock solutions also contains the stabilizer, sodium dodecylsulfate (SDS, 20 mg, 5 wt% of the polymer), and the PTE dye (4 mg, 1 wt% of the polymer). For nanoprecipitation, an aliquot (5 mL) of the appropriate polymer stock solution was quickly added into ultrapure water (50 mL), stirred for 20 h for the slow evaporation of the organic solvent (i.e. acetone or THF) and filtered using a cotton plug to remove large precipitates, if any. The precipitation of polymer nanoparticles occurred immediately after addition of the polymer solution to the water (Zhao et al., 2020). Due to the hydrophobicity of PTE, all molecules are encapsulated inside the polymer matrix instead of remaining in the polar aqueous environment (Bhargava et al., 2018b; Markwalter et al., 2019).

The hydrodynamic size, and zeta potential of the polymer nanoparticles dispersed in ultrapure water were characterised using Dynamic Light Scattering (DLS) on a Malvern Zeta Sizer instrument (Fig. S1 & S2). The optical characterisation of the particle dispersions was performed on UV-1800 Shimadzu UV–Visible spectrophotometer, and an Agilent Cary Eclipse Fluorimeter. Morphological characterisation of all three polymer nanoparticles were performed using a JEOL JSM-6701F Field Emission Scanning Electron Microscope (Fig. 1). Briefly, the drop-cast films of the polymer nanoparticle dispersions on a glass slide were dried and sputter coated with platinum prior to imaging via electron microscopy. The theoretical polymer nanoparticle concentration in the suspension was calculated based on the mass of the polymer dissolved and precipitated.

2.2. Aggregation of polymer nanoparticles in seawater

To understand the aggregation characteristics of the polymer nanoparticles in seawater, the plastic nanoparticles were dispersed in filtered seawater adjusted to 27 practical salinity units (PSU) and allowed to stand for 24 h. The hydrodynamic size of the nanoparticle aggregates dispersed in the seawater was measured using DLS. Due to high ionic strength, the zeta potentials of the polymer nanoparticles were not measured in seawater. All values are reported as an average of the data from three consecutive measurements performed using the DLS instrument on the same polymer nanoparticle dispersion.

2.3. Collection of acorn barnacle nauplii

Adult barnacles were collected from mangroves along the northwest coast of Singapore and maintained at the indoor aquaria facility at St Johns National Marine Laboratory and fed daily with freshly hatched brine shrimp. To induce spawning of nauplii, adult barnacles were brushed clean, rinsed with fresh water and left to air dry overnight at ambient temperature. Stage II nauplii were released the following day upon immersion of adults in fresh seawater, concentrated in a corner of the tank by a point source of light and collected with a pipette (van Dam et al., 2016). The collected nauplii were dispersed in 1 μm aged seawater (27 PSU) and concentration of nauplii was adjusted to 80 individuals/mL.

2.4. Exposure of polymer nanoparticles to barnacle nauplii and statistical analysis of mortality

The toxicity or interaction of PMMA, PS, and PVC nanoparticles was determined by exposing the freshly spawned stage II nauplii to polymer nanoparticles over 24 h. All treatments were conducted in triplicates and average values are reported. Four concentrations, 10, 50, 150, and 250 mg/L of each polymer nanoparticles were first prepared by dilution of the main stock solution with ultrapure water. For each polymer and concentration level experiment, 40 nauplii were used in 2 mL glass vials. Each vial contain aged seawater (900 μL) and appropriate amounts (100 μL) of polymer nanoparticle dispersion in ultrapure water to yield final concentrations of 1, 5, 15 and 25 mg/L. A control experiment was set up by using the same number of nauplii and experimental conditions with no polymer nanoparticles added into the solution. All vials were incubated inside a growth chamber maintained at 28 °C, with a 12-hour light/12-hour dark cycle. After 24 h of exposure, the contents of each vial was transferred onto a Bogorov tray for scoring. Swimming nauplii were counted as live, while nauplii observed to be immobile for more than one minute was considered dead. The nauplii from each treatment were collected, pooled, washed with aged seawater using a 50 μm sieve to remove excess polymer nanoparticles and fixed with 1% glutaraldehyde for further imaging.

To determine statistical significance and compare the mortality response from the polymer nanoparticle exposure, a two-factor ANOVA was used to test the effects of polymer types (three levels) and concentrations (four levels) on the survival of barnacle larvae. Post-hoc pairwise comparisons were carried out where Bonferroni's corrections applied to adjust for multiple comparisons. Data was transformed using $\log(X + 1)$ for homogeneity of variances, evaluated using Kolmogorov-Smirnov Test of Normality, to fulfil the assumptions of the tests.

2.5. PVC uptake and adhesion

During our study, PVC nanoparticles were found to adhere strongly to naupliar carapace, likely causing inhibition of locomotion and high

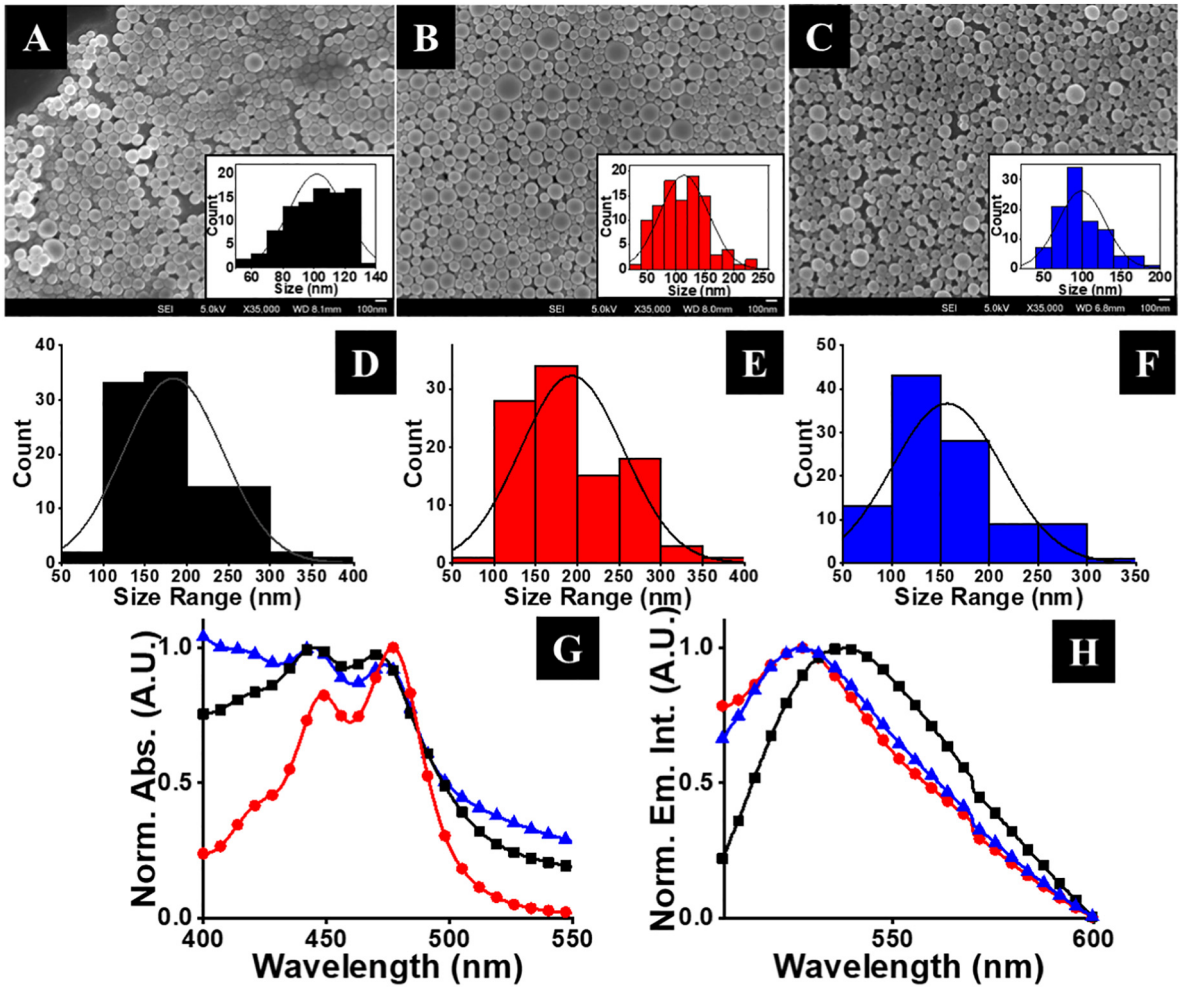

Fig. 1. SEM micrographs of PMMA (A, 101.7 nm), PS (B, 113.8 nm), and PVC (C, 98.6 nm) show polymer particles of spherical morphology, and smaller average particle size compared to their hydrated diameters as measured by DLS. The particle size distribution obtained via DLS for PMMA (D, 172.0 nm), PS (E 175.5 nm), and PVC (F, 141.0 nm). DLS curves, for all polymer nanoparticles dispersed in ultrapure water. Absorbance (G) and emission (H) spectra of PTE encapsulated in PMMA (—■—, λ_{max} = 444 nm, 470 nm/λ_{emi} = 527 nm), PS (—●—, λ_{max} = 444 nm, 470 nm/ λ_{emi} = 527 nm), and PVC (—▲—, λ_{max} = 449 nm, 477 nm/ λ_{emi} = 537 nm). The values obtained in filtered seawater (27 PSU) are provided in the Supporting Information Fig. S1.

mortality at concentrations of 15 and 25 mg/L. As such, the ingestion and adhesion rate of PVC over time was investigated by exposure of freshly released nauplii to PVC nanoparticles at 25 mg/L. Four 20 mL glass vials were loaded with 100 nauplii each and aged seawater (9 mL). PVC nanoparticle stock solution (1 mL) at a concentration of 250 mg/L was added to the glass vial to get a final PVC nanoparticle concentration of 25 mg/L. After 30, 75, 105, and 165 min of exposure, nauplii from the individual vial were removed using a filter (50 μm mesh) and washed with aged seawater to remove any un-adhered polymer particles. The nauplii collected were imaged using a confocal microscope.

2.6. Analysis of commercial polymer powder for impurities

The impurities and small molecular weight fractions in all laboratory grade polymer powder used in nanoparticle preparation (PMMA, PS, and PVC) were tested using mass spectrometry. Polymer powder (20 mg) was dissolved in dichloromethane (5 mL) and insoluble solids were removed via filtration using a 0.22 μm syringe filter. The filtered dichloromethane solution with all soluble contaminants or oligomers was poured into methanol (10 mL) to precipitate high molecular weight polymer chains. The solution was filtered using a 0.22 μm syringe filter to remove all solid particles and the clear filtrate was analysed using Electro-Spray Ionisation Mass Spectrometry (ESI-MS) to identify the presence of low molecular weight components and other additives in the commercial polymer powder. Gel permeation chromatography (GPC) of the polymers were performed using tetrahydrofuran (THF) as the eluent.

2.7. Imaging methods and analysis

Barnacle nauplii fixed with glutaraldehyde solution (1%) were placed directly on a glass slide and covered with a glass cover slip. Microscopic imaging was performed on a Zeiss LSM 900 upright confocal microscope with a 488 nm laser for the excitation of the PTE dye. Scan speed, laser power, and signal amplification were set to minimise background noise. *Z*-stack images of a minimum of 10 unique nauplii were collected from each concentration of the polymer nanoparticle treatment. Z-stack slices were recorded simultaneously using differential interference contrast (DIC) mode and in fluorescence (FITC) mode. Fluorescence intensities of the images on the fluorographs were quantified using ImageJ analysis and the average quantities of polymer nanoparticles assimilated after each treatment were determined. One-tailed *t*-tests were conducted on the fluorograph emission intensities of the individual nauplii to determine the statistical differences in the amount of polymer nanoparticles assimilated by each treatment group versus the control.

3. Results and discussion

3.1. Preparation and characterisation of polymer nanoparticles

The morphological characterisations of the polymer nanoparticles prepared in this study were performed using a scanning electron microscope and all polymer particles showed monodispersed spherical morphologies (Fig. 1A–C). The size distribution of the particles is given in inset and the average dry sizes of the nanoparticle were calculated as PMMA (101.7 nm, Fig. 1A), PS (113.8 nm, Fig. 1B), and PVC (98.6 nm, Fig. 1C). From the DLS measurements, the hydrodynamic diameters of the PMMA (172 nm, Fig. S1A), PS (175.5 nm, Fig. S1B) and PVC (141 nm, Fig. S1C) nanoparticles in ultrapure water were obtained. Similarly, the zeta potentials of the nanoparticles were measured as PMMA (−28.4 mV, Fig. S2A), PS (−40.3 mV, Fig. S2B) and PVC (−32.4 mV, Fig. S2C). The particles of all three polymers showed medium to large polydispersed aggregates in filtered sea water with aggregated particle sizes for PMMA (2.03 μm, Fig. S1D), PS (1.55 μm, Fig. S1E) and for PVC (1.68 μm, Fig. S1F). During this study, such aggregation did not affect ingestion by the nauplii, as the nanoparticle aggregates were smaller than the typical size range (8–10 μm) of their microalgal feed (Arora et al., 2013; Piazza et al., 2012).

As PTE was used as the fluorescent dye in the preparation of all polymer nanoparticles, the optical absorbance and emission characteristics were similar for all particles. Aside from the polymer type, there were no major differences among the structural characteristics of the nanoparticles of all three polymers as the zeta potentials (Fig. S2) and morphology (Fig. 1A, B, C) and size (Fig. 1D, E, F) of all three polymer nanoparticles were found to be similar in nature. All polymers, PS, PMMA, and PVC have alkyl (i.e. -CH_2-CHX-) backbones, differing only in the functional groups (i.e. X) attached to the polymer chain. Any difference observed in toxicity among the three polymer nanoparticles were attributable to the different functional groups of the polymer.

Both PMMA and PS nanoparticles showed absorbance maxima at 444 nm and 470 nm, respectively (Fig. 1G), and emission maximum at 527 nm (Fig. 1H), which corresponds to the optical properties of encapsulated PTE dye. However, PVC particles showed a red shift with the absorbance maxima at 449, and 477 nm (Fig. 1G), and the emission maximum at 537 nm in water. This is expected due to the differences in interactions between the PTE molecules and the polymer chains, which modulate the optical properties. However, such differences in absorption and emission maxima did not affect the visualisation or imaging of the polymer nanoparticles under confocal microscopy as the 488 nm laser was able to excite the PTE fluorophore in all three polymer nanoparticles and the signal detection cut-off at 590 nm encompassed the emission range of the PTE dye. All polymer nanoparticles were observed to aggregate when dispersed in seawater (27 PSU, Fig. 1G, H, I). However, as the hydrophobic PTE fluorophore was encapsulated inside the polymer particle, the optical absorbance and emission characteristics of the particles were not altered by the media used for the investigations (Fig. S1G) (Bhargava et al., 2018a, 2018b).

3.2. Toxicity of the polymer nanoparticles

The polymer nanoparticles were ingested by the filter-feeding nauplii, as evidenced by the highly fluorescent polymer nanoparticles observed inside the body under the confocal microscope (Fig. 2A).

The exposure of *A. amphitrite* nauplii to all three polymer particles (PMMA, PS, PVC) revealed strong fluorescence from nanoparticles accumulated in the digestive tract and gut (Fig. 2A). The relative fluorescence emission intensities of a minimum of 10 individual nauplii were measured using the software ImageJ to quantify the amount of polymer nanoparticles ingested by the exposed nauplii (Fig. 2B). The control nauplii showed minimum autofluorescence during the imaging, arising from Schiff's base structure formation from glutaraldehyde fixing of the nauplii (Cai et al., 2016; Clancy and Cauller, 1998; Schelkle et al., 2017). The PTE dye encapsulated polymer nanoparticles showed intense fluorescence emission, which is significant considering that background fluorescence may cause errors in imaging of such biological samples. The average fluorescence intensity generally increased (i.e., five times for PMMA, and four times for PS nanoparticles, and four times for PVC) between the lowest nanoparticle concentration exposure at 1 mg/L, to the highest concentration exposure at 25 mg/L (Fig. 2B).

The fluorescent nanoparticles were detected in the digestive tract of nauplii exposed to all concentrations, demonstrating that nauplii ingested all polymer nanoparticles, without showing significant mortalities, except in the case of high concentrations of PVC nanoparticles (Fig. 3 (■), Table S1). Overall results from the two-factor ANOVA revealed that the mortality of nauplii was significantly influenced by polymer types ($p < 0.0001$) and concentrations ($p < 0.01$) (Table S1). At 1 mg/L, there were no significant differences in mortality across the PMMA, PS and PVC exposed nauplii. The mortality of exposed nauplii to PMMA and PS was also not significantly different across all other exposure concentrations ($p > 0.05$), with reported values of only ≤15% of nauplii mortality. Mortalities arising from PMMA, and PS exposure are still relatively low compared to nauplii exposed to PVC at 15 and 25 mg/L. Results suggest that PMMA and PS are not detrimentally toxic to nauplii with no LC_{50} values calculated (Fig. 3, Table S1). This agrees well with our previous observation that PMMA nanoparticles at a concentration of 25 mg/L induced low acute toxic effects on *A. amphitrite* nauplii (Bhargava et al., 2018a).

Our results are consistent with previous studies reporting non-significant acute toxicity from PS nanoparticles (Cole et al., 2015; Gambardella et al., 2017). A notable exception was the exposure of *Artemia franciscana* to cationic amino-modified PS nanoparticles, which showed significant mortality and increased moulting due to an upregulation of *clap* and *cstb* genes to cope with the exposure to cationic PS nanoparticles (Bergami et al., 2017). Exposure to PVC nanoparticles at 1 and 5 mg/L produced average mortalities around 4% for both concentrations, which was not significantly different when compared to the control. The exposure to PVC nanoparticles at 15 and 25 mg/L caused significant mortality in nauplii compared to all other treatments (Table S1). LC_{50} value of the PVC nanoparticles was calculated as 7.66 ± 0.03 mg/L, 95% CI (Finney, 1952). Determining the actual amount of nanoparticles consumed by the nauplii is complicated due to many reasons, which include periodic loss of the consumed nanoparticles from the body via defecation and moulting (Bhargava et al., 2018a), and observed adhesion of the particles on the nauplii carapace (Fig. 4A, B). Thus it is difficult to provide absolute quantification of how much nanoparticles are taken up by the individual nauplius.

All three nanoparticles prepared in this study showed negative zeta potential, with PMMA nanoparticles showing the lowest value at −28.4 mV (Fig. S2A), PS nanoparticles with highest absolute zeta potential at −40.3 mV (Fig. S2B), and PVC particles showed a zeta potential at −32.4 mV (Fig. S2C). The polymer nanoparticles used in the present study were prepared in water in presence of the stabilizer, sodium dodecylsulfate (Bhargava et al., 2018b), resulting in its negative zeta potential (Selvamani, 2018). Exposure to both PMMA and PS nanoparticles did not induce significant mortality at all concentrations tested, it can be concluded that the negative zeta potential of the particles was not the cause of the elevated naupliar mortality seen in the case of PVC nanoparticle exposure at concentrations of 15, and 25 mg/L (Fig. 3). In addition, all our nanoparticles showed spherical morphologies under the same preparation methods (Fig. 1A, B, C). Occasionally, nanoparticles of different polymers showed different surface morphologies and adhesion forces when probed by atomic force microscopy (Zimmermann et al., 2020).

It is noted that the PVC nanoparticles form large (1.7 μm) sticky aggregates at a concentration of 25 mg/L in seawater, but remain adequately small and suspended in the water column with 27 PSU (Fig. S1). Similar results were reported from anionic and carboxylate functionalised PS nanoparticles, which showed strong adhesion and

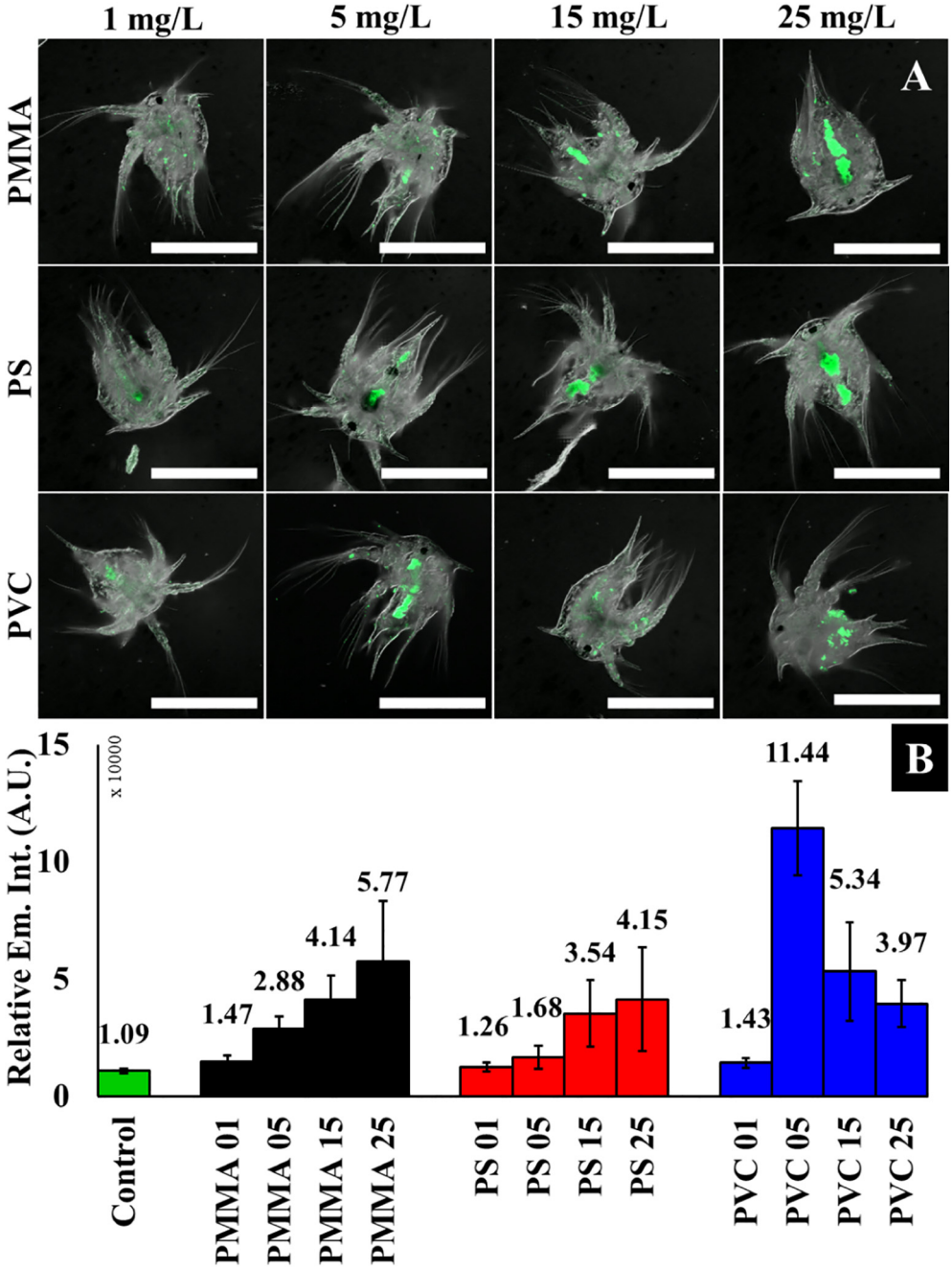

Fig. 2. Confocal micrographs of barnacle nauplii exposed to 1, 5, 15, and 25 mg/L PTE tagged polymer nanoparticles (A), and the average fluorescence intensities from confocal fluorographs of the ten unique nauplii exposed to the same concentration were measured using ImageJ (B) for easy comparison. All micrographs in (A) are recorded at the same magnification, the scale bars are at 200 μm. The glutaraldehyde fixed nauplii showed low levels of autofluorescence in the unexposed control nauplii (B, ■). The nauplii exposed to PMMA (■), PS (■) and PVC (■) nanoparticles showed generally higher fluorescence emission with increasing concentrations of the polymer nanoparticles under confocal microscopy. The separate DIC and fluorescence confocal micrographs of all polymer nanoparticle exposed nauplii are available in the Supporting Information Figs. S4–S6. The separate DIC and fluorescence confocal micrographs of a nauplius unexposed to polymer nanoparticles is available in the Supporting Information (Fig. S7).

retardation of the swimming speed of *Crassostrea gigas* spermatozoa at a concentration of 25 μg/mL (Tallec et al., 2020). The comparatively weaker adhesion was observed on *C. gigas* spermatozoa for the anionic PS particles at lower concentrations (i.e. 1 and 5 mg/L) (Tallec et al., 2020). Tallec et al. proposed that favourable interaction between cationic amino-functionalised PS nanoparticles and spermatozoa cell membrane enhanced adhesion induced toxic effects such as reduced spermatozoa motility and reduced embryogenesis (Tallec et al., 2020). Positively charged particles strongly interact with negatively charged lipid membrane and cause adverse impacts on the organism (Cho et al., 2009; Gaikwad et al., 2019; Nangia and Sureshkumar, 2012).

Although anionic particles are not favourable, electrostatic interaction is still possible from the presence of positively charged sites on the cell membrane (Becucci et al., 2019), and negatively charged particles. The polymer nanoparticles prepared and used in the present study are stabilised by sodium dodecylsulfate and imparting a negative surface charge to the particles, as evidenced by the negative zeta potentials measured in all three polymer nanoparticle dispersions (Bhargava et al., 2018b). The strong adhesion of our PVC nanoparticles at 15 and 25 mg/L to naupliar carapace and limbs may have resulted in locomotion inhibition, which negatively affect zooplankton survival (Frydkjær et al., 2017; Gambardella et al., 2017; Ziajahromi et al., 2017).

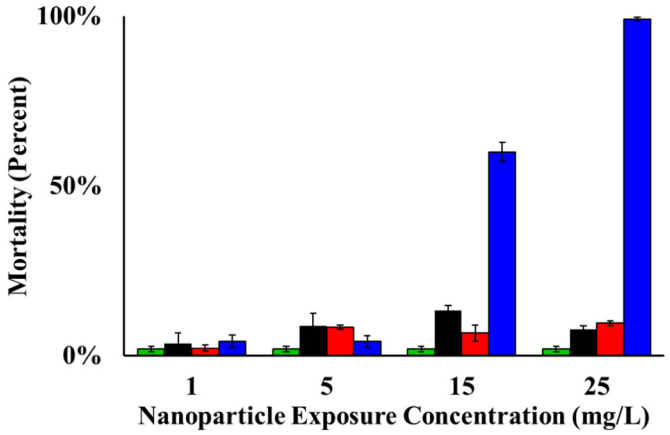

Fig. 3. Barnacle nauplii mortality after 24-hour exposure to 1, 5, 15, and 25 mg/L of PMMA (■), PS (■), and PVC (■). Control samples of nauplii (■) were not exposed to polymer nanoparticles but maintained in seawater with the same condition as the other experimental samples. The statistical treatment of values is given in the supporting information, Table S1.

All exposed nauplii were rinsed with aged seawater to remove excess polymer nanoparticles prior to fixing with glutaraldehyde. In the case of nauplii exposed to high concentrations at 15 mg/L (Fig. 4A) and 25 mg/L showed fluorescent PVC nanoparticles adhering to the carapace and limbs of the nauplii (Fig. 4B). Nauplii exposed to PS (Fig. 4C), and PMMA (Fig. 4D) nanoparticles at a concentration of 25 mg/L were also observed to have small quantities of PS or PMMA nanoparticles adhering to the carapace (white arrows in Fig. 4) even after washing. Most of the observed PS and PMMA nanoparticles were located inside the nauplii (red arrows in Fig. 4). The nauplii exposed to fluorescent polymer nanoparticles over the 24-hour exposure period are expected to feed continuously, which showed the presence of fluorescent polymer nanoparticles in large amounts in the fore and hind gut region of exposed nauplii (Bhargava et al., 2018a; Yu and Chan, 2020). Generally, the simple washing with seawater did not completely remove nanoparticles adhered onto the surfaces of nauplii, especially PVC (Fig. 5A, Fig. 5B), which implies stronger surface interaction between PVC nanoparticles and naupliar carapace compared to the other polymers.

3.3. PVC uptake and adhesion

In the present study, a potential correlation between the observed toxicity and adhesion of PVC particles on the barnacle nauplii was explored further to understand the mechanism. A few studies have reported on exposure of polymer nanoparticle resulting in adhesion, for example, adhesion of PS microparticles to the carapace of the copepod *Temora longicornis* (Cole et al., 2013), and to *C. gigas* spermatozoa showed a negative impact on embryogenesis (Tallec et al., 2020). Exposure of *Protopalythoa* coral to PVC microparticles resulted in adhesion and subsequent assimilation of the PVC particles inside the coral, which ultimately caused reduction in population of symbiotic zooxanthellae by 71% as the PVC microparticles caused coral bleaching (Jiang et al., 2021).

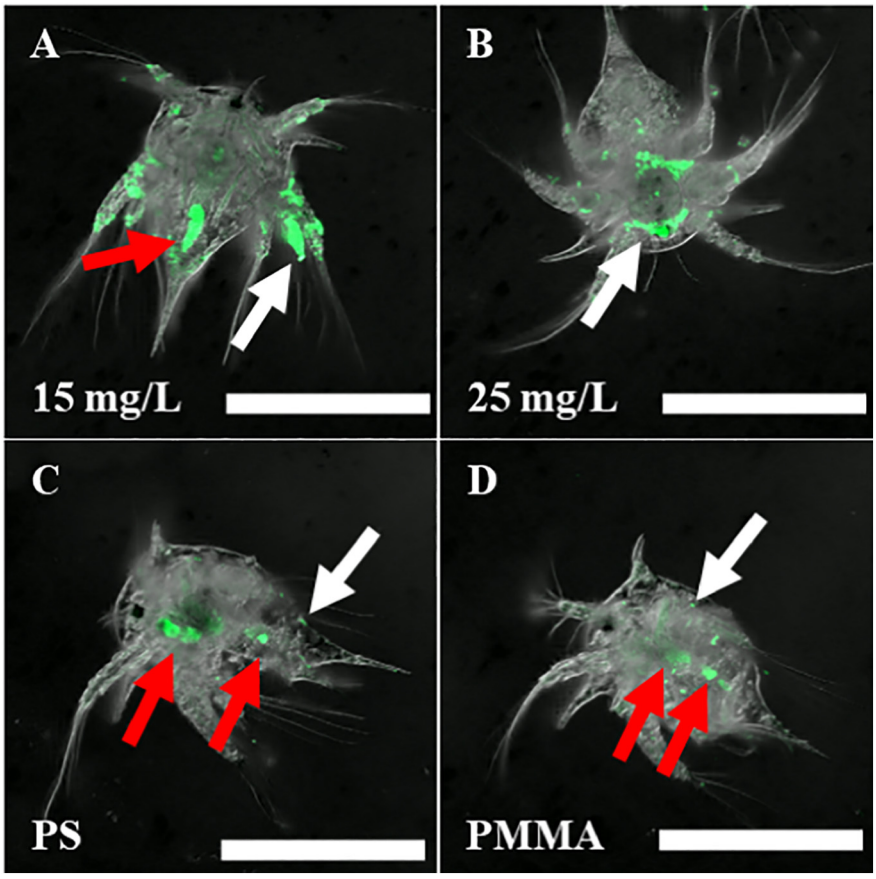

Fig. 4. Confocal micrographs of barnacle nauplii exposed to PVC nanoparticles at 15 mg/L (A), and 25 mg/L (B) showing strong adhesion of PVC nanoparticles to the nauplii after washing and fixing with glutaraldehyde (white arrows). PVC nanoparticle ingestion was also observed at high exposure concentration levels (red arrow). Nauplii exposed to 25 mg/L PS (C), and 25 PMMA showed fluorescent nanoparticles primarily inside the digestive tracts (C and D, red arrows). Some PS (C, white arrow), and PMMA (D, white arrow) particles were adhered to the carapace of exposed nauplii. All scale bars are at 200 μm. Three dimensional diagrams constructed from the confocal micrographs of PVC nanoparticles exposed nauplii are available in the Supporting Information (Fig. S3).

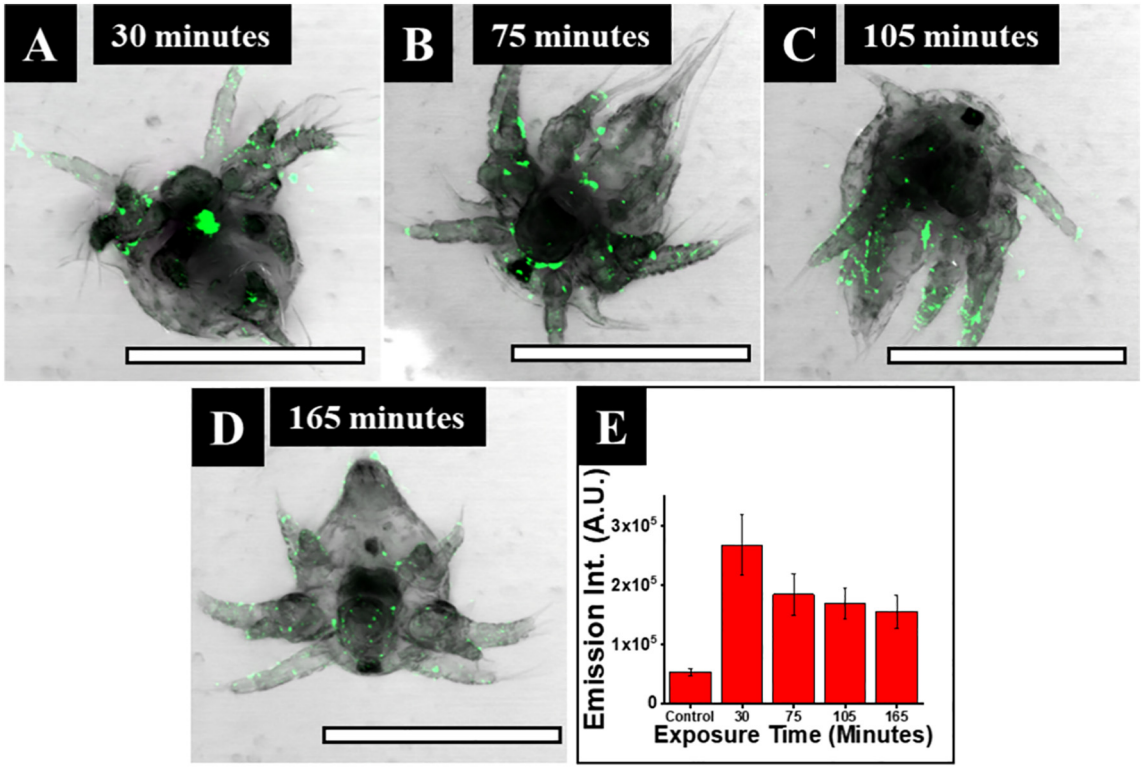

Fig. 5. Brightfield images overlaid with fluorescence confocal micrographs of barnacle nauplii exposed to PVC nanoparticles at 25 mg/L for 30 (A), 75 (B), 105 (C), and 165 min (D), showing PVC nanoparticle adhesion as early as 30 min of exposure. Micrographs in (A – D) were recorded at the same magnification, and all inset scale bars are 200 μm. Average fluorescence emission intensity of ten nauplii exposed to PVC nanoparticles for 30, 75, 105, and 165 min, as well as the same number of unexposed individuals (Control) were not statistically different from each other (E).

Since the hydrodynamic sizes (Fig. S1), zeta potentials (Fig. S2), identical protocols for the preparation and purification were employed, we attempted to understand the interaction between polymer nanoparticles and nauplii in the current investigation. The nauplii exposed to particles at a concentration of 25 mg/L were sub-sampled at 30, 75, 105, and 165 min. Adhesion of PVC nanoparticles occurred rapidly with particles appearing on the limbs and carapace of nauplii exposed within 30 min (Fig. 5A). A previous study reported that translocation of ingested fluorescent PMMA nanoparticles inside the nauplii occurred within 135 min (Bhargava et al., 2018a). It is unlikely that after 30 min exposure, the fluorescence derived from PVC nanoparticles on the limbs and carapace of nauplii are particles that are translocated from the digestive tract after ingestion. In addition, the nauplii exposed to particles for 75 (Fig. 5B), 105 (Fig. 5C), and 165 min (Fig. 5D) had similar amounts of PVC nanoparticles adhered on the body.

Both ingested and adhered fluorescent PVC nanoparticles were present on the exposed nauplii with only small differences in fluorescence intensities between samples collected at different exposure times. When compared to control (non-exposed) nauplii, those exposed to polymer nanoparticles for 30 min showed higher fluorescence intensity (5.04 times). Similarly, the observed enhancements in florescence intensities were 3.5, 3.2 and 3 times more than control nauplii after exposing to particles for 75, 105 and 165 min, respectively. This could be due to the saturation of PVC nanoparticles on nauplii carapace within 30 min of exposure with no continued adhesion after further exposure to particles. It is also noted that PVC nanoparticles adhered to the nauplii carapace were not removed during the multiple washing steps (Fig. 2B, Fig. 5E). The adhesions of nanoscale and micron-scale PS particles (Cole et al., 2013; Tallec et al., 2020), and micron-scale PE, PMMA, and PVC particles (Jiang et al., 2021), were also observed at high concentrations.

The fluorescence intensities from PVC nanoparticles exposed nauplii at a concentration of 25 mg/L for 30, 75, 105, and 165 min were compared to establish the relationship between exposure time and uptake of PVC nanoparticles (Fig. 5E). Unexposed nauplii were weakly emissive due to cellular autofluorescence arising from glutaraldehyde fixing (Mean fluorescence intensity $= 5.31 \times 10^4$) as compared to the nauplii exposed to polymer nanoparticles for 30 min that had significantly higher fluorescence emission intensity (Mean fluorescence intensity $= 2.68 \times 10^5$) (t-test; $t_{17} = -4.15$, $p = 0.0003$). It is understood that after initial intake of PVC nanoparticles, further ingestion by the nauplii is slowed or prevented due to the feeling of satiation.

The impacts of adhesion of plastic particles on the surface of organisms have been reported in the literature. For example, the exposure of *Ceriodaphnia dubia* to micron scale PE fibres caused carapace and antenna deformities due to adhesion and entanglement with the fibres (Ziajahromi et al., 2017). Furthermore, negatively charged PS nanoparticles have been reported to adhere to the surface of *D. tertiolecta*, but did not cause growth inhibition or mortality (Bergami et al., 2017). Hence, the strong adhesion of PVC nanoparticles to the limbs and carapace of exposed *A. amphitrite* nauplii may inhibit locomotion. In addition, the adhesion of polymer nanoparticles to the surface of organisms did not result in toxic response in all cases. The current investigation reveals that PVC is not toxic at lower exposure concentrations of 1 and 5 mg/L, but induced significant toxicity effects at the higher concentrations of 15 and 25 mg/L. This suggests that the acute toxicity observed after exposure is affected by the surface chemistry and surface charge of the nanoparticles. In addition, the factors such as surface charge or size of the nanoparticles may not be the sole cause of elevated toxicity in organisms, other factors such as chemical nature of the polymer, surface functional groups and adhesion of particles on the body parts could also induce significant adverse impacts on living organisms (Fig. 4). In order to probe the observed toxicity further, we investigated the chemical purity of the commercial polymer powders used to prepare the polymer particles for this investigation.

3.4. Analysis of polymer powders

In general, polyalkanes such as PMMA, PS and PVC are prepared through free radical or catalytic polymerization methods. Often traces of impurities such as initiators or unreacted monomers are present in the polymer and difficult to remove them. The radical polymerization method usually generates polymers with broad distribution of molecular weight. Gel permeation chromatographic (GPC) analysis of the commercial PMMA, PS and PVC powders were conducted with the solutions in tetrahydrofuran (THF). The PMMA powder showed an average molecular weight of 21,300 g/mol and had a polydispersity index of 1.77 (Fig. S7A). The PS had a bimodal distribution with a molecular weight 126,400, and 876 g/mol, with a polydispersity index of 1.87 and 1.27, respectively (Fig. S7B). The PVC had a molecular weight of 140,000 g/mol with polydispersity index of 2.28, and a peak at 1290 g/mol with polydispersity index of 1.14 (Fig. S8C). This indicates that most of the commercial polymers do contain small molecular weight polymer chains and oligomers.

We hypothesise that shorter polymer chains present in the polymer powder are expected to be more toxic than the high molecular weight polymer chains, due to easy translocation and interactions with cellular organelles. All monomers, including vinyl chloride is a known carcinogen and has been reported to cause angiosarcoma of the liver in humans over chronic exposure conditions ("HSDB: VINYL CHLORIDE,", 2013; Thompson et al., 2009). Although the volatility of vinyl chloride monomer reduces the risk of exposure and accumulation in marine organisms (De Rooij et al., 2004), the higher molecular weight vinyl chloride oligomers are able to persist in the aquatic environment. Short chain PVC molecules analogous to polychlorinated *n*-alkanes are known to be toxic to aquatic organisms (Feo et al., 2009). Both PMMA and PS nanoparticles prepared from the commercial powders did not cause elevated mortality over a 24-hour exposure period (Fig. 3). This could be due to many factors, which include differences in hydrophilicity, enhanced solubility in organic solvents and partial removal of small molecular weight contents during the nanoprecipitation, followed by the purification methods adopted for the polymer particles. In addition, the presence of monomers and oligomers in the PVC powder used for the nanoparticle preparation was ascertained using electrospray ionisation mass spectrometry (ESI-MS). The appropriate amount of PVC powder was dissolved in THF and precipitated from methanol and the low molecular weight components dissolved in methanol were analysed using mass spectrometry. In electrospray ionisation mass spectrometry using soft ionisation, high degree of molecular fragmentation was not usually observed. The mass spectra (ESI-MS) of the PVC powder in both positive (Fig. S9A, Table S2A), and negative (Fig. S9B, Table S2B) modes showed the presence of numerous small molecular species with a molecular weight below 1 kDa. Oligomer fragments such as tetramer, nonamer, tridecamer and pentadecamer were detected from the mass spectrometry data.

In summary, both GPC and ESI-MS analyses of the stock PVC polymer powder showed the presence of significant amounts of low molecular weight components of the polymer. Although the proportion of low molecular weight species is relatively low, the chemical nature and quantities of material that leach out of the nanoparticle are harmful to living systems such as in the *Acorn Barnacle* nauplii, especially at high nanoparticle concentrations (Li et al., 2016). The increasing nauplii mortality with increasing nanoparticle concentration (Fig. 3) is similar to a dose-response behaviour observed for most toxins on living organisms (Calabrese, 2014).

4. Conclusion

The acute toxicity of luminescent PMMA, PS, and PVC nanoparticles was investigated and compared after exposing the freshly spawned *A. amphitrite* nauplii to polymer particles at concentrations of 1, 5, 15, and 25 mg/L for 24 h. All optically transparent nauplii exposed to PMMA, PS, and PVC nanoparticles were found to have ingested them, as observed under confocal microscope. The nauplii exposed to higher concentrations of PVC also had large quantities of PVC nanoparticles adhering to their carapace and limbs that persisted even washing. The strong adhesion of PVC nanoparticles to *A. amphitrite* nauplii may have inhibited naupliar locomotion and contribute greatly to mortality. Ingestion of PMMA and PS nanoparticles resulted in low, albeit significant, mortality rates, while exposure to PVC nanoparticles caused high mortality rates in nauplii at high concentrations of 15 and 25 mg/L, reaching an average of 60 and 99%, respectively. Both GPC and mass spectrometry analyses of the polymer powders indicated the presence of low molecular weight components.

Overall, the present comparative study examined a few hypotheses for the high toxicity of PVC nanoparticle exposure. Elevated mortality of nauplii is assigned to the chemical toxicity from the exposure to low molecular weight polychlorinated alkanes, after the particles are ingested or adhered to the nauplii. The strong adhesion of PVC nanoparticles at high concentrations inhibited locomotion and contributed indirectly to mortality. Elevated mortality in PVC exposed nauplii could also have been caused by a combination of both chemical toxicity of leachates and inhibition of locomotion through adhesion of particles. However, it should be noted that the PVC nanoparticles showed lethal effects only at elevated concentrations, much higher than the predicted environmental nanoplastics concentrations (Lenz et al., 2016).

The present study also demonstrates that the biological outcomes of nanoplastics investigations can vary significantly when different types of polymer particles are used. Unfortunately, the toxicity of a polymer nanoparticle is usually assessed in isolation with one or two animal models. However, environmental plastics pollution in an ecosystem comprises of many polymers in different amounts. Caution should be exercised when drawing general conclusions towards establishing the toxicity of nanoplastics using only one type of polymer particles. Future studies should involve combined effects of mixtures of polymer nanoparticles, which will provide data that is more representative of actual environmental conditions. Such studies will help in understanding the current and future consequences of environmental plastic pollution.

4.1. Justification

The present study compares acute toxicity from the exposure of nanoparticles prepared from three common polymers (PMMA, PS, PVC) on freshly spawned stage II Acorn Barnacle (*Amphibalanus amphitrite*) nauplii. To our knowledge, most nanoplastics studies draw their conclusions based on the effects of exposure of just one polymer particles to a single model system. Here we used three luminescent polymer particles and transparent nauplii to track the ingestion, translocation and fate of nanoparticles. Our study is also focused on the chemical makeup and toxicity of the multiple common plastics tested. Microscopic and chemical investigations revealed that different polymer particles with similar size and compositions induce different degree of toxicity in nauplii. PVC nanoparticles exposure resulted in high mortalities as compared to PMMA and PS particles at the same concentration. This study serves as an important data point and foundation for future investigations, demonstrating that general conclusions drawn from using a single polymer and single animal model is not sufficient to understand the adverse impact of polymer particles on living organisms.

CRediT authorship contribution statement

Yong Jie Yip: Methodology, Validation, Investigation, Data curation, Writing – original draft. **Serina Siew Chen Lee:** Methodology, Validation, Investigation, Writing – review & editing, Resources. **Mei Lin Neo:** Validation, Formal analysis, Writing – review & editing. **Serena Lay-Ming Teo:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Suresh Valiyaveettil:** Conceptualization,

Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgements

The authors acknowledge the funding support from the National Research Foundation, Singapore for research conducted under the Marine Science R&D Program (MSRDP-P07) at St. John's Island National Marine Laboratory, as well as St. John's Island National Marine Laboratory for providing the facility necessary for conducting the research. Part of this research is supported by the National Research Foundation, Singapore, under its MSRDP-P07/ R-143-000-676-281 and NRF-NERC-SEAP-2020-04/ R-143-000-B72-281. The authors would like to acknowledge Lynette Shu Min Ying and Gayathiri D/O Sivananthan for their contributions in shaping this study through earlier pilot experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150965.

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