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Environmental concentrations of antifouling paint particles are toxic to sediment-dwelling invertebrates☆

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ABSTRACT

Antifouling paint particles (APPs) and associated metals have been identified in sediments around boatyards and marinas globally, but the effects of APPs on benthic organisms are largely unknown. Sub-lethal endpoints were measured following laboratory exposures of the harbour ragworm (*Hediste diversicolor*) and the common cockle (*Cerastoderma edule*) to environmentally relevant concentrations of biocidal ('modern' and 'historic') and biocide-free ('silicone') APPs added to clean estuarine sediment. Further, the 5-day median lethal concentrations (LC_{50}) and effects concentrations (EC_{50}) for modern biocidal APPs were calculated. For ragworms, significant decreases in weight (15.7%; $p < 0.01$) and feeding rate (10.2%; $p < 0.05$) were observed in the modern biocidal treatment; burrowing behaviour was also reduced by 29% in this treatment, but was not significant. For cockles, the modern biocidal treatment led to 100% mortality of all replicates before endpoints were measured. In cockles, there was elevated levels of metallothionein-like protein (MTLP) in response to both modern and historic biocidal treatments. Ragworms had a higher tolerance to modern APPs (5-day LC_{50}:19.9 APP g L^{-1}; EC_{50}: 14.6 g L^{-1}) compared to cockles (5-day LC_{50}: 2.3 g L^{-1} and EC_{50}: 1.4 g L^{-1}). The results of this study indicate that modern biocidal APPs, containing high Cu concentrations, have the potential to adversely affect the health of benthic organisms at environmentally relevant concentrations. The findings highlight the need for stricter regulations on the disposal of APP waste originating from boatyards, marinas and abandoned boats.

1. Introduction

Antifouling paint is widely applied to marine structures to reduce biofouling, with the antifouling paints and coatings market estimated to be worth US$9.22 billion by 2021 (Markets, 2016). Biofouling of submerged marine structures can lead to increased frictional drag, reduced manoeuvrability of marine vessels, higher fuel consumption and increased cleaning and maintenance costs (Chambers et al., 2006; Yebra et al., 2004). Antifouling coatings typically work by leaching biocides into the surrounding seawater and forming a protective microlayer (Nurioglu et al., 2015; Singh and Turner, 2009a). Following the worldwide ban on organotin-based antifouling paints (e.g. tributyltin) in 2008, owing to toxic effects on non-target organisms (Evans et al., 1995; IMO, 2018), new tin-free antifouling paints were developed (Yebra et al., 2004). Most contemporary biocidal antifouling paints contain Cu (I) as the main biocide, in the form of cuprous oxide (Cu_2O) or copper thiocyanate (CuSCN), in combination with Zn-based compounds such as zinc oxide (ZnO) (Turner, 2010); booster biocides such as zinc pyrithione (ZnPT), Irgarol 1051 and diuron are also added to antifouling formulations to increase their effectiveness (Turner, 2010). Owing to their toxicity to marine life, a range of biocide-free antifouling formulations are also currently being developed. For example, silicone coatings work by reducing the adhesion of organisms to the surface of boats (Almeida et al., 2007) and have been found to be less toxic to marine organisms (Karlsson and Eklund, 2004).

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Antifouling paint particles (APPs) are waste products, generated in boatyards and marinas during maintenance and cleaning of boat hulls and grounded ships and boats (Turner, 2010). The disposal of APP waste is largely unregulated in the recreational boating industry and APPs can be readily transported from hard-standings and slipways into the marine environment via run-off (Connelly et al., 2001; Thomas et al., 2003; Turner, 2010) (Fig. 1). APPs also originate from weathering of old abandoned boats, which are often coated in numerous layers of historic antifouling paint and may contain banned or restricted compounds such as TBT and metals like Pb, historically used in antifouling and non-antifouling marine paints (Rees et al., 2014; Turner, 2010). Fine APP particulates might also be aerially dispersed, as observed with microfibres (Liu et al., 2019). APPs are highly heterogeneous in their chemical make-up, owing to the variety of antifouling formulations used over the past fifty years (Sandberg et al., 2007; Turner, 2010). Once in the marine environment, APPs can accumulate in benthic sediments around marinas, boatyards and abandoned boats; in the Plym estuary (UK), sampling revealed APP concentrations of 430 particles L^{-1} (0.2 g L^{-1}) next to a boat maintenance facility and 400 particles L^{-1} (4.2 g L^{-1}) in an area containing abandoned boats (Muller-Karanassos et al., 2019). APPs continue to leach biocides into the surrounding environment, with several studies finding high metal concentrations, often exceeding environmental standards, in sediments contaminated with APPs (Eklund et al., 2014; Muller-Karanassos et al., 2019; Rees et al., 2014; Sapozhnikova et al., 2013; Singh and Turner, 2009b; Soroldoni et al., 2018a). Biological activity (e.g. bioturbation, bioirrigation) has the capacity to redistribute and resuspend particles and metals present within intertidal habitats (He et al., 2017; Näkki et al., 2017). Modern antifouling coatings are polymeric with an alkyd resin base (Toben, 2017) and as such, APPs can be considered as a type of microplastic (plastic debris, 1 μm - 1 mm in size) (Boucher and Friot, 2017; Hartmann et al., 2019). Studies have shown that microplastic ingestion by marine organisms may lead to a reduction in feeding, reproduction (Cole et al., 2015) and energy reserves (Wright et al., 2013). Owing to their high metal concentrations, including Cu, Zn, Sn and Pb, APPs pose an additional toxic threat to sediment-dwelling biota. Benthic organisms are essential for the functioning of marine coastal ecosystems and play an important role in energy transfer between pelagic and benthic ecosystems. Laboratory studies have shown that exposure to APPs can lead to an accumulation of biocidal metals (Cu and Zn) in the tissues of benthic marine organisms including the common mussel *Mytilus edulis* (Turner et al., 2009), the common periwinkle *Littorina littorea* (Gammon et al., 2009) and the lugworm *Arenicola marina* (Turner et al., 2008). Uptake of metals is thought to occur through both aqueous exposure to APP leachate and via direct ingestion of APPs, with a recent study finding evidence of APP ingestion by the harbour ragworm *Hediste diversicolor* collected from contaminated sediments in the Plym estuary (UK) (Muller-Karanassos et al., 2019). Exposure to APP leachate has been found to have sub-lethal effects on marine organisms including a reduction in growth, larval development and bioluminescence (Ytreberg et al., 2010). Only three publications — all focussed upon modern biocidal antifouling materials — have considered the direct toxicity of APPs these studies revealed exposure to increasing concentrations of APPs can cause: a decrease in fecundity and survival in the epibenthic copepod *Nitokra* sp. (Soroldoni et al., 2017); decreased survival rates in pelagic copepods (Molino et al., 2019); and decreased survival in the benthic microcrustaceans *Monokalliapseudes schubarti* (a tanaid) and *Hyalella azteca* (an amphipod) (Soroldoni et al., 2020).

The aim of this study was to determine if exposure to both biocidal and non-biocidal APPs, at environmentally relevant concentrations, negatively affects the health of benthic organisms with different feeding modes. Two sediment-dwelling estuarine species were chosen for this study, the harbour ragworm, *H. diversicolor*, and the edible cockle, *Cerastoderma edule*. *H. diversicolor* is a polychaete that is widely distributed in estuaries within Northwest Europe (Budd, 2008), having an important role in estuarine ecosystems as a bioturbator and as a food source for numerous species of wading birds (Goss-Custard et al., 1989) and flatfish (Budd, 2008). *C. edule*, is a commercially important species eaten widely throughout Europe and is also an important food source for wading birds and pelagic species in intertidal mudflats, where it can make up a significant proportion of biomass (Romano et al., 2011). Two

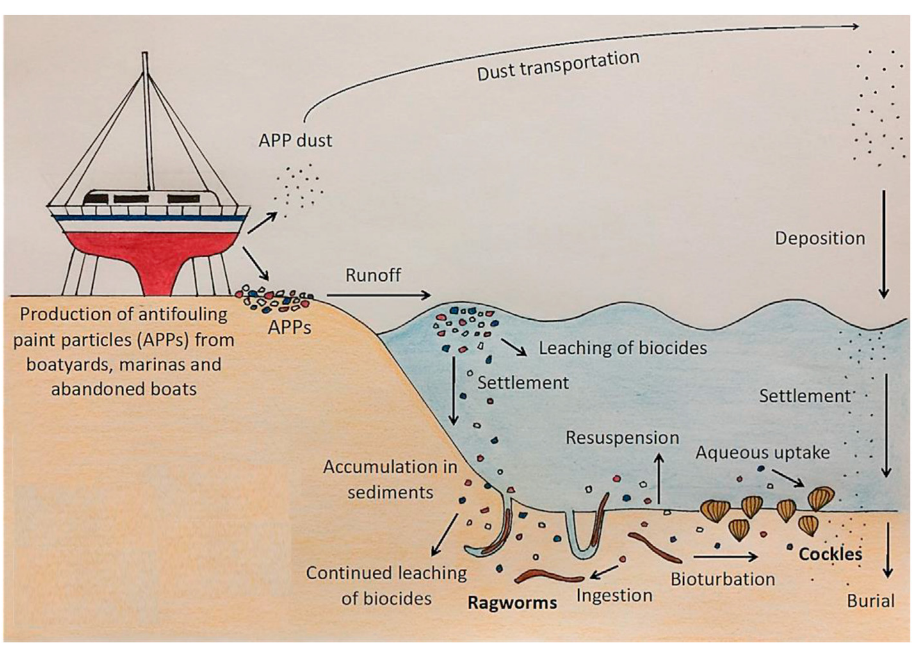

Fig. 1. Potential abiotic and biotic transport pathways for antifouling paint particles (APPs) in intertidal habitats.

laboratory studies were conducted: (1) an 18-day exposure to investigate sub-lethal health effects; and, (2) a 5-day assay to calculate how APP concentrations affected mortality (i.e. LC50s). The findings of this study have implications for the health of bio-diverse intertidal ecosystems and the management of antifouling waste.

2. Methods

2.1. Specimen collection and husbandry

Adult *H. diversicolor* (ragworms; 0.29–1.01 g wet weight) and *C. edule* (cockles; 26–36 mm shell length, 10.5–25 g wet weight) were collected by hand from an intertidal mudflat at Saltram Park (N 50° 22′ 43.284″ W 4° 6′ 0.755″) located within the Plym Estuary, UK (Fig. 1) in June and July 2018 and transported to PML within 1 h of sampling. There is no boating activity at Saltram Park and a recent field study showed minimal APP and metal contamination at this site (Muller-Karanassos et al., 2019). A salinity of 31.1 was measured at high water using an Oakton SALT 6+ handheld probe. On return to the laboratory, ragworms and cockles were allowed to acclimate for 4–7 days in polypropylene tanks (49 × 35 × 15 cm) containing approximately 5 cm depth of sieved estuarine sediment (<1 mm) collected at Saltram Park and 5 cm depth of aerated filtered seawater (FSW, 0.2 μm Millipore filter diluted to 31.1 salinity with Milli-Q water).

2.2. APP generation and characterisation

Three types of APPs were generated for use in the exposures, including two biocidal ('historic' and 'modern') and one biocide-free ('silicone'). 'Historic' biocidal antifouling paint flakes were collected by hand from abandoned boats at Hooe Lake, in the Plym Estuary (N 50° 21′ 22.464″ W 4° 6′ 28.943"; Fig. 2), and cleaned from any visible dirt and algae (Singh and Turner, 2009a). 'Modern' biocidal antifouling paint was scraped off rolled mild steel panels that had been painted with three commercially available biocidal paints in 2012, including an anti-corrosive layer, tie coat and top coat, and submerged in natural seawater off the coast of Orkney (Scotland) for 8 weeks in 2014. Non-biocidal 'silicone' antifouling paint was scraped off rolled mild steel panels painted with commercially available silicone paint and submerged in natural seawater for 5–10 days at station L4 (off the coast of Plymouth, UK; www.westernchannelobservatory.co.uk) in 2018. APPs were prepared by grinding down paint flakes using a pestle and mortar with the aid of liquid nitrogen; paint particles were passed through stainless steel sieves to collect the 100 μm–1 mm fraction, a size range considered bioavailable to the target species and for which APP particles have been identified in estuarine sediments (Muller-Karanassos et al., 2019). Particle size distribution was evaluated by measuring a representative sub-sample of 100 APPs under a microscope. Metal analysis of the three APP types was carried out non-destructively using an energy-dispersive portable x-ray

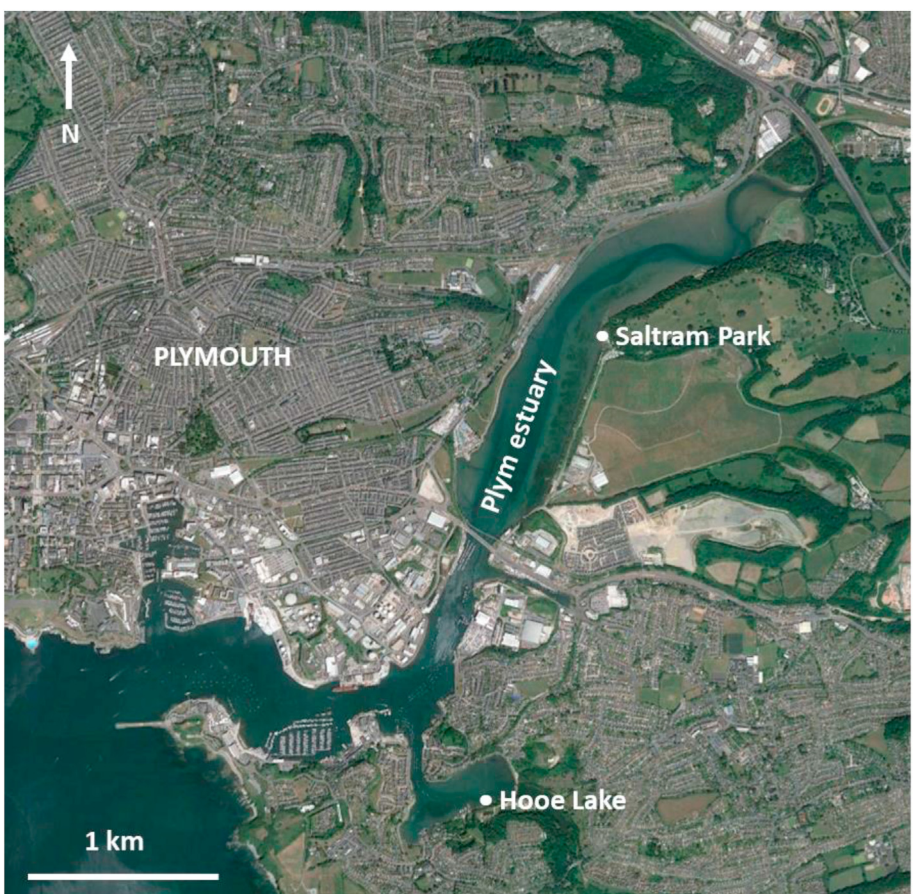

Fig. 2. Animals and sediment were collected from Saltram Park, Plym Estuary (UK). Historic APPs were sampled from abandoned boats at Hooe Lake. Map data: Google Earth, Landsat/Copernicus.

fluorescence (XRF) spectrometer (Niton XL3t He GOLDD+), with the focus on metals that are or have been commonly employed as biocides in antifouling paints (Cu, Hg, Pb, Sn and Zn). The instrument was operated in a low-density plastics mode with small-spot 3-mm collimation and thickness correction (between 0.5 and 3 mm) and performance was verified by analysis of Niton plastic reference discs containing known concentrations of various metals. APP fragments were characterised in clear polyethylene zip-bags for 60 s each at five different locations.

2.3. Experimental set-ups

Two laboratory-based exposure experiments, an 18-day and 5-day exposure, were carried out for each species in a temperature-controlled facility (13 ± 1 °C) under a 12:12 h light:dark cycle. Estuarine sediment was collected from Saltram Park, where our samples revealed no evidence of APPs (Muller-Karanassos et al., 2019); sediment was collected to a depth of approximately 20 cm at the same time as the biota, and sieved through a 1 mm stainless steel sieve with the aid of seawater to remove macro-debris. Sediment was stored in a plastic tub covered with aluminium foil until experiments were carried out. Prior to exposures, pre-weighed APPs were mixed into sediments in individual containers using a stainless-steel spatula and then allowed to settle for 24 h prior to the introduction of animals. Owing to the high-density of the APPs, we observed that particles remained within sediments during water changes. For the 18-day exposure, APP concentrations were based on environmental concentrations identified at Hooe Lake (0–18.8 g L^{-1}; mean 4.2 g L^{-1}) (Muller-Karanassos et al., 2019) adjusting for the density differences of the different APPs (SI, Table S1). Trial experiments carried out using the maximum environmental APP concentration (18.8 g L^{-1}) led to mortality of all *H. diversicolor* and *C. edule* in the modern treatment within 6 days and, therefore, the mean environmental APP concentration was used in order to assess sub-lethal effects. Density-corrected APP concentrations were: 4.2 g L^{-1} for the historic biocidal treatment; 3.0 g L^{-1} for the modern biocidal treatment; and 2.1 g L^{-1} for the non-biocidal silicone treatment. For the 5-day LC_{50} exposure, modern biocidal APPs (selected owing to their comparatively higher toxicity) were used at concentrations ranging from 0 to 30 g L^{-1} (ragworms) and 0–6 g L^{-1} (cockles).

After acclimation, individual organisms were weighed to ascertain pre-exposure wet weight. Ragworms were transferred into individual 100 mL polyethylene containers (1 ragworm per container) containing 50 mL of sieved sediment pre-mixed with APPs (silicone: 0.105 g, historic: 0.209 g, modern: 0.152 g) or control sediment, and 50 mL of aerated FSW. Cockles were transferred into 1 L food-grade containers (1 cockle per vessel) containing 250 mL of control sediment or sediment pre-mixed with APPs (historic: 1.047 g; modern: 0.759 g; silicone: 0.525 g) and 700 mL of well-aerated, natural seawater filtered through a 0.2 μm glass fibre filter and diluted with ultrapure water to a salinity of 31.1 (matching estuarine conditions). Water was changed every 2–3 days and water quality parameters including temperature, pH, salinity, conductivity and dissolved oxygen were measured using YSI Pro 1030 and Pro 20 m at every water change (SI, Table S2). For the 18-day exposure, ragworms ($n = 10$ per treatment) were each fed 8 g of hatched *Artemia salina* nauplii (Instant Baby Brine Shrimp, Ocean Nutrition) every other day (Moreira et al., 2005), and cockles ($n = 10$ per treatment) were fed every other day with an alternating diet of live *Thalassiosira rotula* (4000 cells mL^{-1}) and freeze-dried multi-cell algal culture (5 Species Phytoplankton, Reefphyto). For the 5-day exposure, ragworms ($n = 5$ per APP concentration) and cockles ($n = 5$ per APP concentration) were not fed.

2.4. 18-Day exposure

2.4.1. Feeding rate

For ragworms, a feeding rate experiment was carried out following methods in Moreira et al. (2005). In summary, ragworms were transferred into individual Petri dishes containing 100 *A. salina* nauplii and 20 mL of diluted seawater and allowed to feed in darkness for 1 h. Remaining *A. salina* nauplii were counted and feeding rate was determined as the number of nauplii consumed per hour.

For cockles, clearance rate was assessed using an algal feeding assay. A known concentration of *T. rotula* cells were introduced to experimental vessels and aliquots of water removed at the start of the experiment and after 1 h. Five blank vessels were used to account for algal growth and settling. Algal concentration was assessed using the Sedgwick-Rafter counting method. Clearance rate (*CR*) was calculated as described in Romano et al. (2011), as follows:

$$CR = V \times \frac{logC_1 - logC_2}{t} \times n$$

where V is the volume of water in the experimental vessel, C_1 is the initial algal concentration, C_2 is the final algal concentration, $t$is experimental duration, and n is the number of cockles per vessel.

2.4.2. Weight change

Animals were re-weighed (post-exposure wet weight) and weight change was determined as the difference between pre- and post-exposure wet weight.

2.4.3. Burrowing

For ragworms, burrowing behaviour was analysed following methods by Buffet et al. (2011). Ragworms were placed in 100 mL containers with 50 mL clean estuarine sediment from Saltram Park and 50 mL of aerated filtered seawater. Burial state was recorded every 2 min for a total of 30 min.

For cockles, burrowing was analysed as in Byrne and O'halloran (2000) and Møhlenberg and Kiørboe (1983). Cockles were placed in individual containers set up in experimental chambers containing clean estuarine sediments, and burrowing state was recorded after 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 120 and 180 min. Cockles were judged to be burrowed when approximately 50% of the shell was covered by sediment.

2.4.4. Metallothionein-like protein assay

Metallothionein-like protein (MTLP) concentration in whole tissue was quantified for both species using the method described by Viarengo et al. (1997) and UNEP (1999) with a few alterations. Using frozen specimens, soft tissues were pooled in Petri dishes for each treatment (control, silicone, historic, modern) and defrosted in an oven for 10 min at 50 °C. Samples were weighed and volume of tissue measured before homogenisation of tissue in 2 vol of homogenising buffer containing β-mercaptoenthanol, phenylmethylsuphonylfuride and leupeptin. Homogenisation was performed using a 600 W Morphy Richards 402058 hand-blender in beakers until tissue was smooth enough to be removed using a 1 mL Gilson pipette. Two mL of sample was pipetted into 2 mL centrifuge tubes (6 replicates per treatment) and centrifuged at 2 °C, 14,000×*g* for 45 min. One mL of supernatant was carefully removed by pipette and added to 1.05 mL of cold (−20 °C) absolute ethanol and 80 μL of chloroform in 2 mL centrifuge tubes stored on ice. Tubes were inverted and vortexed before centrifugation at 0 °C and 6000×*g* for 10 min. Supernatant was carefully pipetted off into 15 mL tubes and volume recorded for each sample. Ten μL of a

100 mg mL^{-1} stock of RNA, 40 μL of 37% HCl and 3 vol of cold absolute ethanol were added before vortexing and storing at −20 °C for 1 h. Samples were then centrifuged at 0 °C and 4000×*g* for 15 min to form a pellet. Supernatant was carefully removed and the pellet washed with 5 mL of a *cleaner* solution (87:1:12 ethanol/chloroform/homogenising buffer, stored at −20 °C). Samples were re-centrifuged at 0 °C and 4000×*g* for 5 min before removal of supernatant and drying. 150 μL of 0.25 M NaCl solution and 150 μL of 1N HCl containing 4 mM EDTA were added and then vortexed. Standards were made up using a 1 mg mL^{-1} solution of glutathione in 0.25 M NaCl, and blanks were made up with 150 μL of 0.25 M NaCl solution and 150 μL of 1N HCl containing 4 mM EDTA. Just before analysis, 0.43 mM (7.14 mg/42 mL) DTNB (Ellman's reagent) was dissolved in 0.2 M phosphate buffer pH 8 containing 2 M NaCl and 4.2 mL of this solution was added to all samples, standards and blanks. All tubes were then centrifuged at 3000×*g* for 5 min at room temperature. Absorbance was measured at 412 nm in a VWR UV-3100 PC spectrophotometer.

2.5. 5-Day exposure

2.5.1. LC_{50} and EC_{50}

Daily checks were carried out for mortality of both species. Healthy ragworms will naturally burrow to avoid predation (Kalman et al., 2009) and have a fast reaction time (personal observations). Ragworms were recorded as dead when they appeared to be motionless at the surface of the sediment and there was no response to touch, and adversely affected when they were found on the sediment surface and only reacted slightly to touch. Cockles were also deemed to be dead when no response was observed on gentle touching of valves or foot, and adversely affected when exhibiting gaping behaviour with minimal response to touch (Thompson and Richardson, 1993).

2.6. Statistical analysis

All ragworm and cockle data were checked for normality using Shapiro-Wilk's test. For the non-parametric ragworm data a Kruskal-Wallis test followed by Wilcoxon Rank Sum test was used, while a one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare differences in parametric cockle data. A generalised linear model (GLM) with binomial distribution was used to compare the number of animals burrowed after 30 min across treatments. The 5-day LC_{50} and EC_{50} values were calculated by probit analysis in SPSS. All other analyses were carried out using R statistical software v3.5.1 (R, 2019). Biological data is presented as mean values ± standard error, while metal chemistry data is presented as mean values ± standard deviation; significant difference is attributed where $p < 0.05$.

3. Results

3.1. APP characterisation

APPs ranged from 0.0625 to 1 mm in size, with particle size varying between the three APP types (Fig. 3). Overall, historic biocidal APPs had the highest proportion of particles in the size range 0.125–0.25 mm (37.6%), modern biocidal APPs had the smallest particle size, with 43.6% of particles in the size range 0.0625–0.125 mm. Non-biocidal silicone antifouling paint had a "rubber-like" consistency and was much harder to breakdown into smaller particles via cryogenic grinding, and as a result these APPs had the largest particle size, with 39.7% of particles in the size range 0.5–1 mm.

Metal concentrations varied considerably between APP types

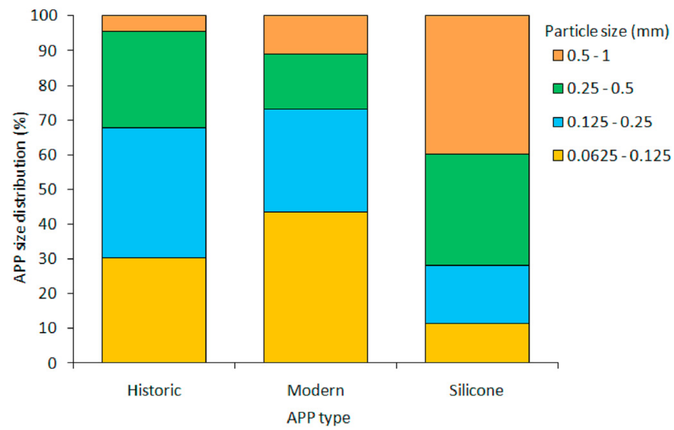

Fig. 3. Particle size distribution of the different types of APPs used in exposure experiments.

(Table 1). Historic biocidal APPs had the highest concentrations of Zn, Pb and Hg, and relatively high Cu concentrations, whereas modern biocidal APPs had the highest Cu concentrations with much lower concentrations of Zn. Copper was detected in one reading taken from the non-biocidal silicone APPs but Zn was not detected; however, there were detectable concentrations of Sn and Hg in the these APPs.

3.2. 18-Day assay

Individual ragworms (*H. diversicolor*) were used for testing sub-lethal end-points across four treatments (control n = 9, non-biocidal silicone n = 8, historic biocidal n = 9 and modern biocidal n = 10). Three ragworms, from the control, non-biocidal silicone and historic biocidal treatments, spawned during the exposure and were removed from further analysis to avoid bias. One ragworm from the non-biocidal silicone treatment was lost during processing before end-point experiments were carried out. For cockles (*C. edule*), 100% mortality was observed for the modern biocidal treatment after 10 days, so sub-lethal endpoints could not be assessed; all cockles survived in control, non-biocidal silicone and historic biocidal treatments (n = 10).

3.2.1. Feeding rate

The highest feeding rate occurred in the control treatment (17 ± 4 nauplii h^{-1} ind.$^{-1}$) and the lowest was observed in the modern biocidal treatment (6 ± 2 nauplii h^{-1} ind.$^{-1}$). The feeding rate of ragworms differed significantly between treatments (One-way ANOVA: $F_{3,32} = 3.131$, $p < 0.05$; Fig. 4A), and the post-hoc test showed a significant difference between the modern biocidal treatment and control (Tukey: $p < 0.05$).

No significant difference in clearance rates of cockles between treatments was observed (control: 0.60 L h^{-1} ind.$^{-1}$; non-biocidal silicone: 0.63 L h^{-1} ind.$^{-1}$; historic biocidal: 0.82 L h^{-1} ind.$^{-1}$; ANOVA: $F_{2,27} = 2.797$, $p = 0.08$; Fig. 4B).

3.2.2. Weight change

Ragworms showed a significant difference in mean weight change between treatments (Kruskal-Wallis: $p < 0.01$; Fig. 4C). Exposure to biocidal APPs resulted in a marked decrease in weight, however when compared with controls, significant differences were only observed in ragworms exposed to modern biocidal APPs (18.5 ± 4% weight loss; Wilcoxon: $p < 0.01$).

Weight change in cockles was not significantly affected by treatment (One-way ANOVA: $F_{2,27} = 3.30$, $p = 0.052$; Fig. 4D).

Table 1
Metal concentrations (mg kg^{-1}) in APPs used in 18-day and 5-day assays. The mean of 5 measurements is shown for each APP type ± standard error. Metal concentrations not detected were replaced with measurement detection limits (values where < is shown) for the calculation of means.

APP type	Cu	Zn	Sn	Pb	Hg
Historic	148000	69000	412	1030	1620
	142000	64500	408	2010	1350
	176000	73200	326	565	1930
	181000	87100	576	503	2570
	168000	78200	510	1210	1590
Mean	**163000** ± 769	**74400** ± 3901	**446** ± 44	**1060** ± 272	**1810** ± 211
Modern	440000	4900	977	401	399
	427000	9180	853	<237	394
	428000	18000	941	<225	459
	420000	17200	782	<246	436
	421000	13500	750	<254	411
Mean	**427000** ± 357	**12600** ± 247	**861** ± 44	**272** ± 32	**420** ± 12
Silicone	<126	<81	838	<24	69
	<140	<89	913	<30	77
	150	<99	863	<42	69
	<130	<86	705	<38	74
	<145	<94	702	<34	78
Mean	**138** ± 4	**<90** ± 3	**804** ± 43	**34** ± 3	**73** ± 2

However, compared with controls, cockles exposed to non-biocidal silicone APPs did show a significantly greater weight loss (Tukey: $p < 0.05$).

3.2.3. Burrowing

An average of 60 ± 16% of ragworms exposed to modern biocidal APPs had buried after 30 min, while an average of 88–100% of individuals in the other treatments had successfully buried in this time period. However, there were no significant differences between treatments (GLM: $p > 0.05$; Fig. 4E).

Approximately two-thirds of cockles had successfully buried after 180 min across all treatments, with no significant difference between treatments (GLM: $p > 0.05$; Fig. 4F).

3.2.4. Metallothionein-like protein

MTLP concentrations in ragworms averaged 59 μg g^{-1} in controls, with no significant difference between treatments (Kruskal-Wallis: $p = 0.07$); however, ragworms exposed to the historic biocidal APPs showed significantly reduced MTLP concentrations compared with the controls (38.3 μg g^{-1}; Wilcoxon: $p < 0.05$; Fig. 4G).

Whole-tissue MTLP concentrations in cockles averaged 20.6 μg g^{-1} in controls, which is comparable with other studies (Aly et al., 2014). MTLP concentrations were significantly affected by treatment (One-way ANOVA: $F_{3,18} = 13.49$; $p < 0.01$; Fig. 4H), with MTLP levels significantly elevated in historic biocidal (26.2 μg g^{-1}; Tukey: $p < 0.05$) and modern biocidal (31.1 μg g^{-1}; Tukey: $p < 0.01$) treatments, as compared with controls.

3.3. 5-Day assay: LC50s

The 5-day LC_{50} was 19.9 APP g L^{-1} for ragworms (Fig. 5A) and 2.3 g L^{-1} for cockles (Fig. 5B). In the control treatment, there were no mortalities for either species. The 5-day EC_{50} values were calculated as 14.6 g L^{-1} for ragworms and 1.4 g L^{-1} for cockles (data not shown).

4. Discussion

4.1. 18-Day exposure

Results from the 18-day study demonstrate that exposure to modern biocidal APPs at environmentally relevant concentrations leads to adverse health effects in the ragworm *H. diversicolor* and mortality in the cockle *C. edule*. As compared with controls, significant net decreases in weight (15.7 ± 5%) and feeding rate (10.2 ± 4%) were observed in ragworms exposed to modern biocidal APPs; furthermore modern biocidal APPs were associated with the highest percentage of un-burrowed ragworms, with 29 ± 20% fewer ragworms burrowed after 30 min compared to the average control, although this difference was not statistically significant. Modern biocidal APPs were acutely toxic to cockles, resulting in mortality of all replicates in the first 10 days of exposure. Both historic and modern biocidal APPs caused significant increases in MTLPs in cockles. Largely, historic biocidal and non-biocidal silicone APPs did not cause substantial sub-lethal health effects in ragworms or cockles, although it was observed that exposure to historic biocidal APPs led to substantial weight loss in the ragworms.

A reduction in weight, feeding and burrowing activity of ragworms could lead to a range of consequences in the natural environment. Impairment of feeding activity in marine organisms can directly affect population parameters such as growth, reproduction (Maltby et al., 2001) and energy intake (Kalman et al., 2009). Previous studies have demonstrated that Cu exposure can have lethal and sub-lethal effects on ragworms, including reduced burrowing activity (Thit et al., 2015) and feeding (Moreira et al., 2005). Reduced burrowing efficiency in ragworms and cockles increases the likelihood of predation (Kalman et al., 2009), and reduces bioturbation activity – a vital process for sediment processing and deposition of organic matter in estuarine ecosystems (Moreira et al., 2006). Increased mortality of cockles caused by exposure to APPs could lead to a decrease in natural populations, with implications for ecosystem functionality (e.g. bioturbation and food webs). Soroldini et al. (Soroldoni et al., 2017; Soroldoni et al., 2020) similarly found an increase in mortality in copepods and benthic invertebrates with increasing concentrations of APPs originating from a commonly-used contemporary commercial antifouling paint containing high concentrations of Cu and Zn (Cu:

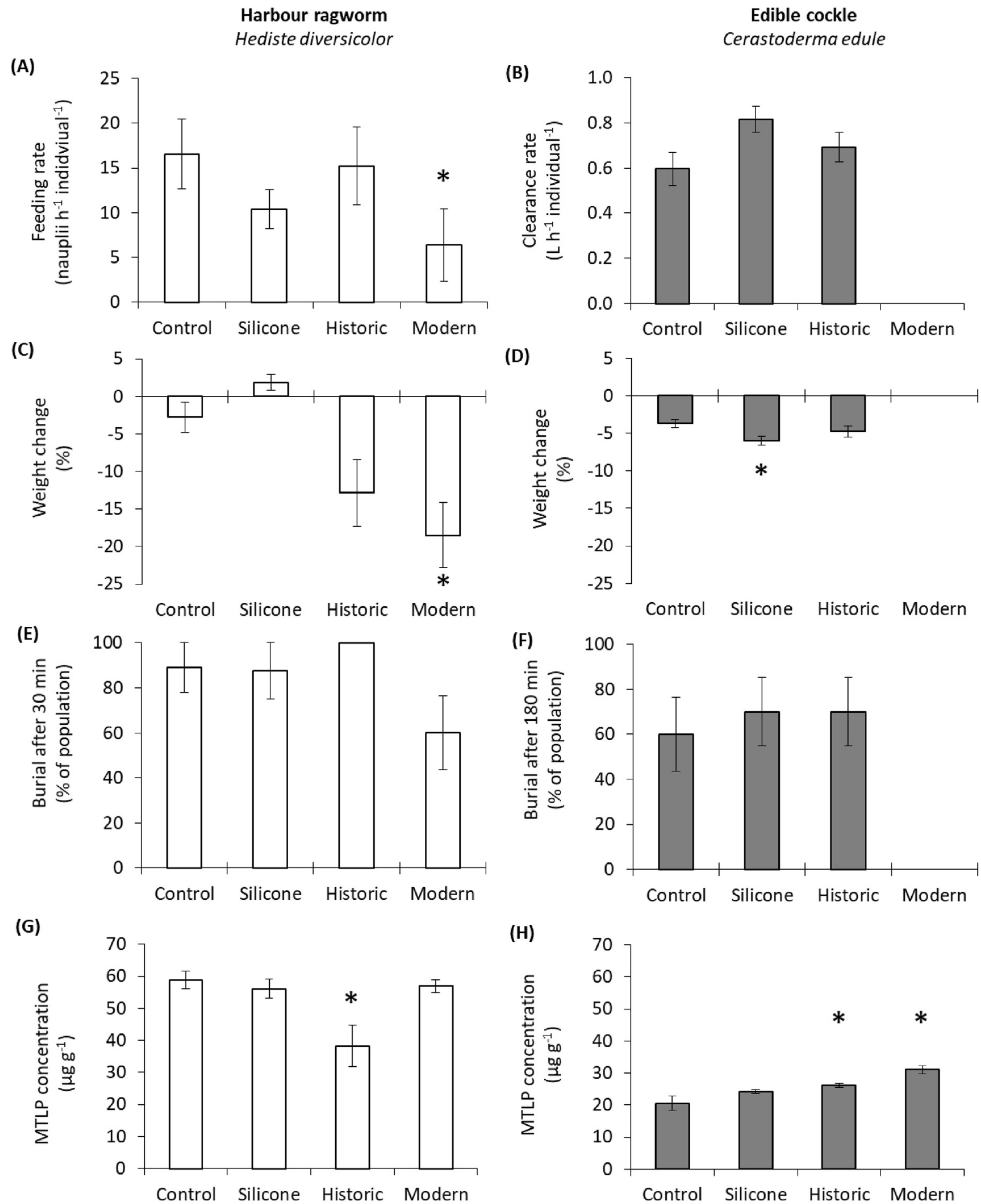

Fig. 4. Sub-lethal health responses in ragworms (white bars) and cockles (grey bars) following an 18-day exposure to controls and silicone, historic or modern APP treatments: (A) Feeding rates (nauplii individual^{-1} hour^{-1}); (B) Clearance rates (L h^{-1} individual^{-1}); (C-D) Weight change (%); (E-F) Percentage of individuals burrowed after 30 min (ragworm) and 180 min (cockles); (G-H) Metallothionein-like protein (MTLP) concentration, noting that cockles in modern treatment died before the end of the 18-day exposure period. * denotes significant difference from control treatment. Error bars indicate standard error.

234 ± 0.27 g kg^{-1}, Zn: 112 ± 0.84 g kg^{-1}, Pb: 0.51 ± 0.01 g kg^{-1}). Another study found that leachate from sediment contaminated 1 with APPs caused a reduction in gram-negative bacteria (*Vibrio fischeri*) bioluminescence, decreased growth rate of the red algae (*Ceramium tenuicorne*) and reduced larval development in the harpacticoid copepod *Nitocra spinipes* (Ytreberg et al., 2010); while Cu was found to be more toxic to *V. fischeri* and *C. tenuicorne*, *N. spinipes* was more sensitive to Zn (Ytreberg et al., 2010).

In comparing the metal profiles of the biocidal APPs, it was evident that the modern paints contained far higher copper concentrations (Cu: modern: 427,000 ± 7000 mg kg^{-1}; historic: 163,000 ± 15,600 mg kg^{-1}). The lower Cu concentrations in the

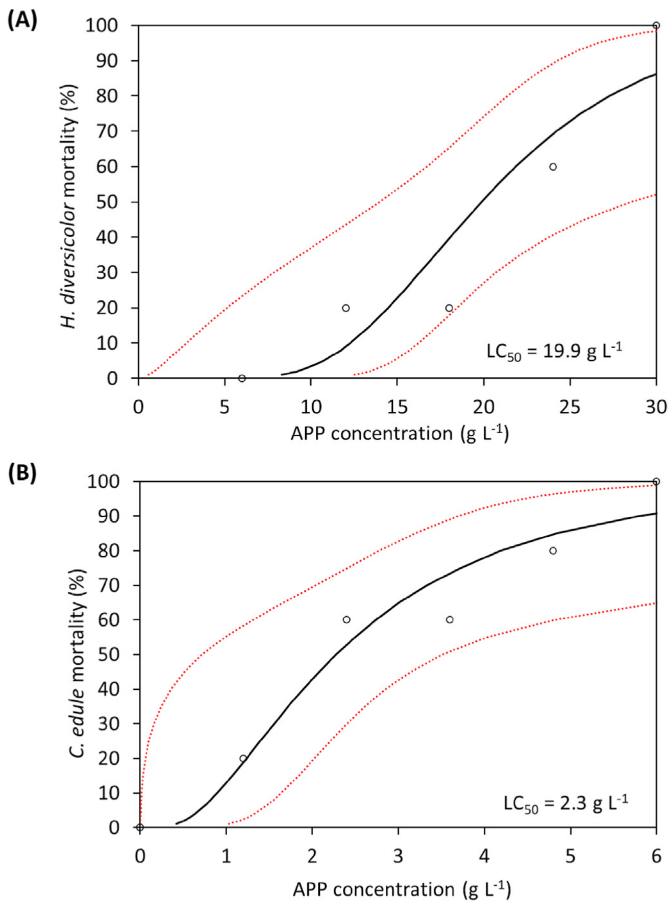

Fig. 5. 5-day median lethal concentration (LC_{50}) dose-response curves (black line) with 95% confidence intervals (orange lines) for (A) ragworms and B) cockles exposed to modern APPs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

historic biocidal APPs could be attributed to the weathering of historic APP and a longer period of Cu leaching from aged historic paints (Rees et al., 2014), leading to more inert APPs and therefore a reduced toxicity. However, historic APPs contained the highest Zn concentrations (74,400 ± 7800 mg kg^{-1}) and also contain other toxic metals such as Pb (1060 ± 543 mg kg^{-1}), Hg (1810 ± 423 mg kg^{-1}) and Sn (446 ± 87 mg kg^{-1}), which may still pose an ongoing risk to non-target organisms. Based on the heightened mortality and sub-lethal effects stemming from modern biocidal APP exposure, we surmise Cu is the most toxic biocidal metal present. Soroldoni et al. (2017) similarly identified that Cu was more toxic to copepods than Zn. Once metals have been taken up by an organism, they can be detoxified by accumulation in metal-rich granules or by binding with metallothionein (MT) and MTLP that regulate metal concentrations (Rainbow, 2007). The elevated MTLP in cockles from historic and modern biocidal treatments compared to the control suggests an upregulation of MTLP in response to metal exposure, particularly in the modern biocidal treatment (despite these cockles not surviving the full 18-day exposure). MTLP concentrations in ragworms were not affected by treatment. Previous studies also found no relationship between accumulated metal concentrations and MTLP levels in ragworms (Poirier et al., 2006; Solé et al., 2009), and several studies have shown that exposure to Cu leads to an accumulation of this metal in *H. diversicolor* (Berthet et al., 2003; Geffard et al., 2005). While ragworms have an intolerance to Cu, they have shown the capacity to regulate Zn body concentrations, thought to occur by reduced metal uptake rates, increased excretion rates and/or through storage of metals in granules or MTLPs (Berthet et al., 2003; Geffard et al., 2005).

Other compounds found in antifouling paint such as booster biocides, solvents and binders can also have toxic effects (Karlsson et al., 2010) and the mixture of metals and other compounds found in APPs are likely to have synergistic effects (Soroldoni et al., 2017). It is therefore important to examine the toxicity of APPs as a whole, in addition to that of individual compounds found within antifouling paints. APPs were not tested for other compounds in this study, although they are likely to have contributed to the high toxicity observed with modern biocidal APPs. Although uptake routes were not investigated in the current study, exposure to biocides likely occurred via ingestion of APPs and uptake of dissolved metals leached into the water column and sediment porewater. By design, biocidal antifouling paints will constantly release metal ions into the surrounding water to prevent adherence and growth of fouling organisms, so it is expected that biocidal APPs will continue to emit Cu and Zn throughout their lifespan, although whether the rate of dissipation changes over time remains unclear. A number of studies have exposed marine organisms to APP leachate solutions, which have been routinely demonstrated to be readily taken up by biota and cause toxicity (Gammon et al., 2009; Katranitsas et al., 2003; Soroldoni et al., 2018b; Tolhurst et al., 2007). In the Plym estuary, the smallest APPs observed were ~0.5 mm in size (Muller-Karanassos et al., 2019); detecting smaller particles was hindered by sampling and analytical limitations, however abiotic and biotic processes can be expected to contribute to the proliferation of even smaller APPs. In this study APPs were prepared to 0.0625–1 mm in size, with differences in particle size profiles of biocidal and non-biocidal APPs observed: the majority of the "rubber-like" silicone APPs being >0.5 mm, and the majority of biocidal APPs being <0.5 mm diameter. We cannot say for certain whether the observed differences in particle size profiles might influence toxicity, however we consider all particles to be in the normal prey size range of cockles and ragworms. Toxicity studies using plastic particulates of distinct size (i.e. nano-vs micro) have revealed size dependent effects, with differences stemming from different modes of biological action; for example, 50 nm polystyrene nanoplastics triggered an immune response in mussels (likely owing to their capacity to readily translocate into circulatory fluids) while exposure to 20 μm polystyrene microplastics caused no observable impact (Cole et al., 2020). It is interesting to consider whether exposure to APPs of markedly different particle size (i.e. nano vs micro) would significantly effect toxicity; for example, might smaller APPs with larger surface areas increase metal leaching or penetrate deeper into tissues causing cellular toxicity (Jeong et al., 2016)? Further work is needed to clarify the mechanisms responsible for toxicity of APPs to benthic organisms observed in the present study.

Non-biocidal silicone paints, absent of Cu and Zn, caused no adverse health effects on cockles or ragworms, supporting our hypothesis that Cu, likely in combination with other compounds, is the most toxic component of APPs. This is further supported by other studies: for example, Watermann et al. (2005) found that silicone coatings did not negatively affect barnacle cypris larvae settlement and luminescence of the bacteria *V. fischeri*; and Karlsson and Eklund (2004) observed no changes to growth of two macroalgae (*C. tenuicorne* and *Ceramium strictum*) and no mortality of the copepod *N. spinipes* when exposed to silicone paint. Biocide-free silicone coatings may be a suitable alternative to modern biocidal antifouling paints for high speed vessels, but further studies are needed to assess long-term effects.

Owing to their alkyd-resin base, APPs can be considered as microplastics (Hartmann et al., 2019). Microplastic debris can be ingested by a wide array of marine organisms, with evidence of sub-lethal harm in a number of studies e.g. Cole et al. (2015), Wright et al. (2013). In this study, it is evident that non-biocidal silicone APPs (absent of metal additives) have a far lower toxicity than APPs containing biocides, highlighting the importance of considering additive and metal profiles when evaluating microplastic toxicity. While the physical properties of a microplastic (e.g. size, shape) have been shown to interfere with feeding and movement in marine biota (see Galloway et al. (2017) and Setälä et al. (2018))', their chemical composition is often overlooked. However, microplastics more generally should not be considered as a single polymeric compound, but a mixture of polymers, containing monomers and additives including metals, emollients, phthalates and flame retardants (Rochman et al., 2019). These additives can be highly toxic and have been associated with sub-lethal health effects and endocrine disruption in marine invertebrates (Browne et al., 2013; Cole et al., 2019).

4.2. 5-Day assay

Cockles were found to be much more sensitive to modern biocidal APPs when compared to ragworms. The LC_{50} and EC_{50} for cockles (2.3 g L^{-1} and 1.4 g L^{-1} respectively) were almost an order of magnitude lower than for ragworms (19.9 g L^{-1} and 14.6 g L^{-1} respectively). The maximum concentration of APPs found within the Plym Estuary at Hooe Lake (Muller-Karanassos et al., 2019), where boating activity is significant, was 18.8 g L^{-1}, which is well above the LC_{50} and EC_{50} for cockles and above the EC_{50} for ragworms. This suggests that APP concentrations found in the natural environment have the potential to cause mortality to cockles and adverse effects in ragworms.

Studies have shown that ragworms and cockles originating from metal-contaminated sites may have a higher tolerance to Cu and Zn compared to uncontaminated sites (Durou et al., 2005; Mouneyrac et al., 2003; Naylor, 1987). It is therefore not possible to generalise the findings of this study for all ragworm and cockle populations since the LC_{50} for APPs will likely differ between sites. Indeed, ragworm and cockles could be identified in the vicinity of Hooe Lake, albeit in sparser numbers than elsewhere (personal observations). Metal tolerance has also been found to differ between taxa and more sensitive benthic organisms are expected to have lower APP LC_{50} values. A study by Buffet et al. (2011) found that the bivalve mollusc *Scrobicularia plana* was less tolerant to Cu nanoparticles compared to *H. diversicolor*, supporting the findings of the current study found a 4-day LC_{50} value of 0.14% of APPs by mass of dry sediment for the epibenthic copepod *Nitokra* sp. The current study showed a similar 5-day LC_{50} (0.15% of APPs by mass of wet sediment) for *C. edule* and a much higher 5-day LC_{50} (1.42% of APPs by mass of wet sediment) for *H. diversicolor*. These values are not directly comparable since the exposure periods differ and sediment wet weight was used instead of dry weight. However, it can be assumed that if water were removed from sediment in the current study this would produce higher percentages of APPs by mass of dry sediment. This suggests that cockles are more tolerant than copepod species to APP-contaminated sediments, likely due to their larger body size and ability to accumulate metals.

5. Conclusions

Given the current evidence, it can be concluded that biocidal APPs present a source of contaminant metals to estuarine and coastal sediments. Exposure to these anthropogenic particles pose both a physical and toxic risk to benthic species and the wider food web, necessitating stricter regulations for antifouling waste in marinas and boatyards. APPs derived from copper-based biocidal paints proved most toxic to cockles and ragworms. While exposure to silicone-based non-biocidal APPs resulted in increased weight-loss in cockles, current evidence indicates non-biocidal antifouling paints to be less toxic to non-target organisms.

Credit author statement

Christina Muller-Karanassos: Conceptualization, methodology, analysis, investigation, writing, visualization; **William Arundel:** Conceptualization, methodology, analysis, investigation, writing, visualization; **Penelope Lindeque:** Investigation, resources, writing; **Thomas Vance:** Investigation, resources, writing; **Andrew Turner:** Conceptualization, methodology, investigation, writing, editing, supervision; **Matthew Cole:** Conceptualization, methodology, investigation, writing, editing, supervision, 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.

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Appendix A. Supplementary data

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