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1 **Environmental concentrations of antifouling paint particles are toxic to sediment-dwelling**
2 **invertebrates**

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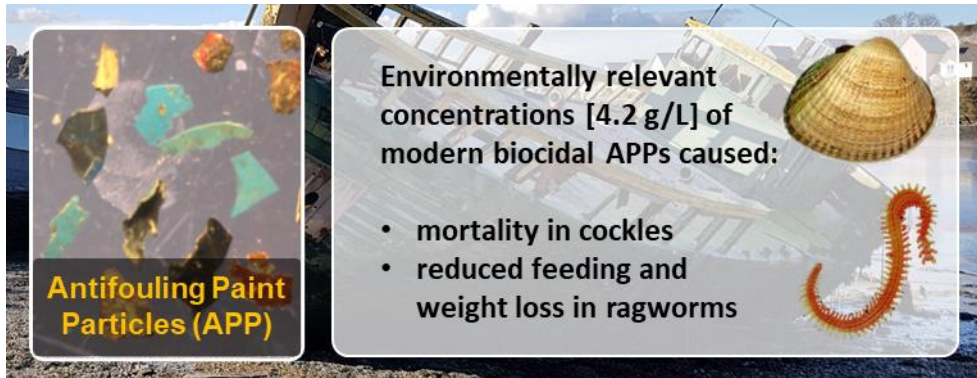
15 Accepted September 2020

16 **Abstract**

17 Antifouling paint particles (APPs) and associated metals have been identified in sediments around
18 boatyards and marinas globally, but the effects of APPs on benthic organisms are largely unknown. Sub-
19 lethal endpoints were measured following laboratory exposures of the harbour ragworm (*Hediste*
20 *diversicolor*) and the common cockle (*Cerastoderma edule*) to environmentally relevant concentrations of
21 biocidal ('modern' and 'historic') and biocide-free ('silicone') APPs added to clean estuarine sediment.
22 Further, the 5-day median lethal concentrations (LC₅₀) and effects concentrations (EC₅₀) for modern
23 biocidal APPs were calculated. For ragworms, significant decreases in weight (15.7%; $p < 0.01$) and feeding
24 rate (10.2%; $p < 0.05$) were observed in the modern biocidal treatment; burrowing behaviour was also
25 reduced by 29% in this treatment, but was not significant. For cockles, the modern biocidal treatment led
26 to 100% mortality of all replicates before endpoints were measured. In cockles, there was elevated levels
27 of metallothionein-like protein (MTLP) in response to both modern and historic biocidal treatments.
28 Ragworms had a higher tolerance to modern APPs (5-day LC₅₀: 19.9 APP g L⁻¹; EC₅₀: 14.6 g L⁻¹) compared to
29 cockles (5-day LC₅₀: 2.3 g L⁻¹ and EC₅₀: 1.4 g L⁻¹). The results of this study indicate that modern biocidal
30 APPs, containing high Cu concentrations, have the potential to adversely affect the health of benthic
31 organisms at environmentally relevant concentrations. The findings highlight the need for stricter
32 regulations on the disposal of APP waste originating from boatyards, marinas and abandoned boats.

33

34 **Graphical abstract**



35

36

37 **Keywords:** Microplastic, Fouling, Biocidal, Toxicity, Copper

38

39 **Highlights**

40

- Antifouling paint particles (APPs) are a type of microplastic debris

41

- Biocidal APPs containing copper proved toxic to sediment-dwelling biota

42

- Biocidal APPs were toxic at environmentally relevant concentrations

43

- Non-biocidal silicone-based APPs showed little toxicity

44

- Cockles were far more sensitive to APPs than ragworms

45

46 **Capsule**

47

In contrast to non-biocidal silicone formulations, copper-based biocidal antifouling paint particles (APPs) proved toxic to cockles and ragworms at environmentally relevant concentrations.

49

50 **1. Introduction**

51

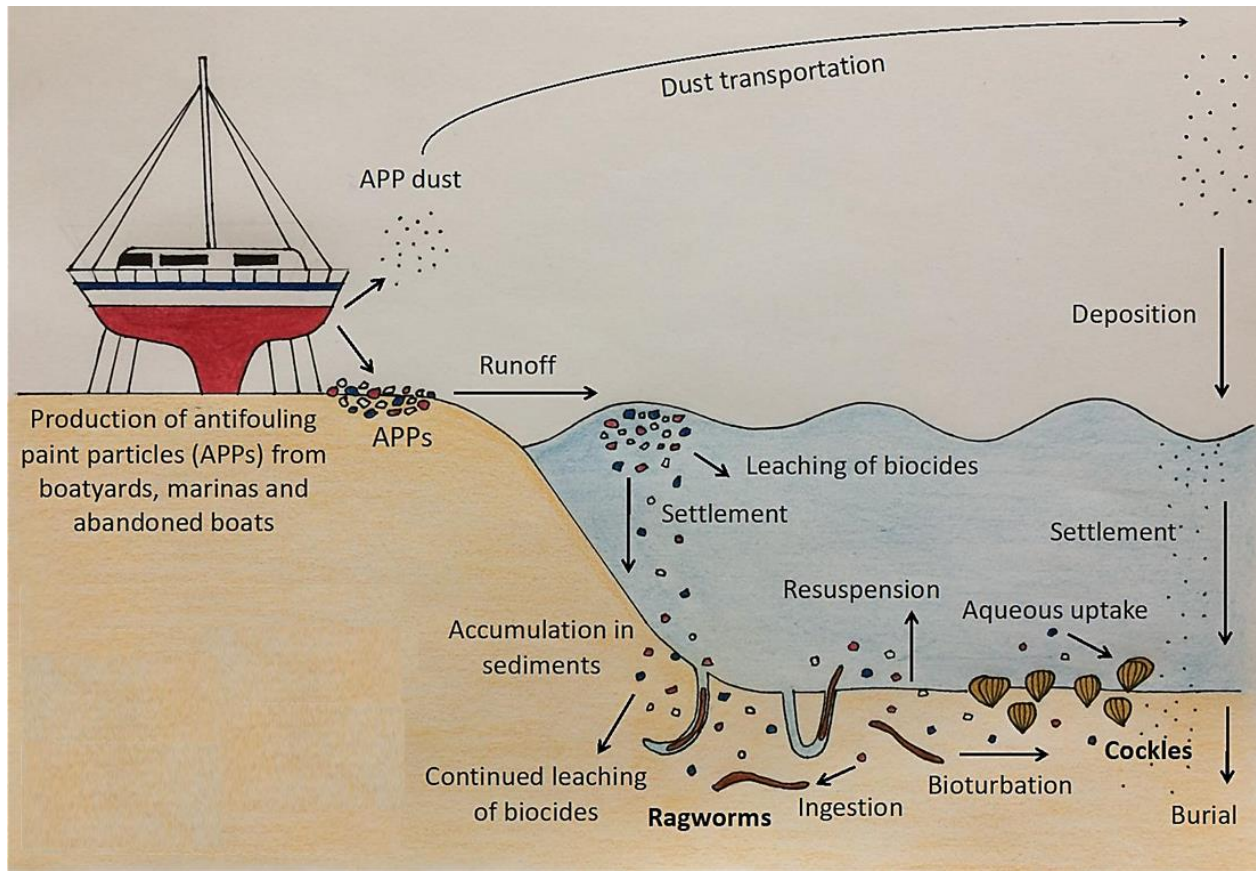
Antifouling paint is widely applied to marine structures to reduce biofouling, with the antifouling paints and coatings market estimated to be worth US\$9.22 billion by 2021 ([Markets, 2016](#)). Biofouling of submerged marine structures can lead to increased frictional drag, reduced manoeuvrability 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

57

58 non-target organisms ([Evans et al., 1995](#); [IMO, 2018](#)), new tin-free antifouling paints were developed
59 ([Yebera et al., 2004](#)). Most contemporary biocidal antifouling paints contain Cu (I) as the main biocide, in
60 the form of cuprous oxide (Cu₂O) or copper thiocyanate (CuSCN), in combination with Zn-based
61 compounds such as zinc oxide (ZnO) ([Turner, 2010](#)); booster biocides such as zinc pyrithione (ZnPT), Irgarol
62 1051 and diuron are also added to antifouling formulations to increase their effectiveness ([Turner, 2010](#)).
63 Owing to their toxicity to marine life, a range of biocide-free antifouling formulations are also currently
64 being developed. For example, silicone coatings work by reducing the adhesion of organisms to the
65 surface of boats ([Almeida et al., 2007](#)) and have been found to be less toxic to marine organisms ([Karlsson
66 and Eklund, 2004](#)).

67 Antifouling paint particles (APPs) are waste products, generated in boatyards and marinas during
68 maintenance and cleaning of boat hulls and grounded ships and boats ([Turner, 2010](#)). The disposal of APP
69 waste is largely unregulated in the recreational boating industry and APPs can be readily transported from
70 hard-standings and slipways into the marine environment via run-off ([Connelly et al., 2001](#); [Thomas et al.,
71 2003](#); [Turner, 2010](#)) (Figure 1). APPs also originate from weathering of old abandoned boats, which are
72 often coated in numerous layers of historic antifouling paint and may contain banned or restricted
73 compounds such as TBT and metals like Pb, historically used in antifouling and non-antifouling marine
74 paints ([Rees et al., 2014](#); [Turner, 2010](#)). Fine APP particulates might also be aerially dispersed, as observed
75 with microfibrils ([Liu et al., 2019](#)). APPs are highly heterogeneous in their chemical make-up, owing to the
76 variety of antifouling formulations used over the past fifty years ([Sandberg et al., 2007](#); [Turner, 2010](#)).
77 Once in the marine environment, APPs can accumulate in benthic sediments around marinas, boatyards
78 and abandoned boats; in the Plym estuary (UK), sampling revealed APP concentrations of 430 particles L⁻¹
79 (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
80 abandoned boats ([Muller-Karanassos et al., 2019](#)). APPs continue to leach biocides into the surrounding
81 environment, with several studies finding high metal concentrations, often exceeding environmental
82 standards, in sediments contaminated with APPs ([Eklund et al., 2014](#); [Muller-Karanassos et al., 2019](#); [Rees
83 et al., 2014](#); [Sapozhnikova et al., 2013](#); [Singh and Turner, 2009b](#); [Soroldoni et al., 2018a](#)). Biological activity
84 (e.g. bioturbation, bioirrigation) has the capacity to redistribute and resuspend particles and metals
85 present within intertidal habitats ([He et al., 2017](#); [Näkki et al., 2017](#)). Modern antifouling coatings are
86 polymeric with an alkyd resin base ([Toben, 2017](#)) and as such, APPs can be considered as a type of
87 microplastic (plastic debris, 1 µm - 1 mm in size) ([Boucher and Friot, 2017](#); [Hartmann et al., 2019](#)). Studies
88 have shown that microplastic ingestion by marine organisms may lead to a reduction in feeding,
89 reproduction ([Cole et al., 2015](#)) and energy reserves ([Wright et al., 2013](#)). Owing to their high metal

90 concentrations, including Cu, Zn, Sn and Pb, APPs pose an additional toxic threat to sediment-dwelling
91 biota. Benthic organisms are essential for the functioning of marine coastal ecosystems and play an
92 important role in energy transfer between pelagic and benthic ecosystems. Laboratory studies have
93 shown that exposure to APPs can lead to an accumulation of biocidal metals (Cu and Zn) in the tissues of
94 benthic marine organisms including the common mussel *Mytilus edulis* ([Turner et al., 2009](#)), the common
95 periwinkle *Littorina littorea* ([Gammon et al., 2009](#)) and the lugworm *Arenicola marina* ([Turner et al., 2008](#)).
96 Uptake of metals is thought to occur through both aqueous exposure to APP leachate and via direct
97 ingestion of APPs, with a recent study finding evidence of APP ingestion by the harbour ragworm *Hediste*
98 *diversicolor* collected from contaminated sediments in the Plym estuary (UK) ([Muller-Karanassos et al.,](#)
99 [2019](#)). Exposure to APP leachate has been found to have sub-lethal effects on marine organisms including
100 a reduction in growth, larval development and bioluminescence ([Ytreberg et al., 2010](#)). Only three
101 publications – all focussed upon modern biocidal antifouling materials – have considered the direct
102 toxicity of APPs these studies revealed exposure to increasing concentrations of APPs can cause: a
103 decrease in fecundity and survival in the epibenthic copepod *Nitokra* sp. ([Soroldoni et al., 2017](#));
104 decreased survival rates in pelagic copepods ([Molino et al., 2019](#)); and decreased survival in the benthic
105 microcrustaceans *Monokalliapseudes schubarti* (a tanaid) and *Hyalella azteca* (an amphipod) ([Soroldoni](#)
106 [et al., 2020](#)).
107



108
 109 **Figure 1.** Potential abiotic and biotic transport pathways for antifouling paint particles (APPs) in intertidal
 110 habitats.

111
 112 The aim of this study was to determine if exposure to both biocidal and non-biocidal APPs, at
 113 environmentally relevant concentrations, negatively affects the health of benthic organisms with different
 114 feeding modes. Two sediment-dwelling estuarine species were chosen for this study, the harbour
 115 ragworm, *H. diversicolor*, and the edible cockle, *Cerastoderma edule*. *H. diversicolor* is a polychaete that
 116 is widely distributed in estuaries within Northwest Europe (Budd, 2008), having an important role in
 117 estuarine ecosystems as a bioturbator and as a food source for numerous species of wading birds (Goss-
 118 Custard et al., 1989) and flatfish (Budd, 2008). *C. edule*, is a commercially important species eaten widely
 119 throughout Europe and is also an important food source for wading birds and pelagic species in intertidal
 120 mudflats, where it can make up a significant proportion of biomass (Romano et al., 2011). Two laboratory
 121 studies were conducted: (1) an 18-day exposure to investigate sub-lethal health effects; and, (2) a 5-day
 122 assay to calculate how APP concentrations affected mortality (i.e. LC50s). The findings of this study have
 123 implications for the health of biodiverse intertidal ecosystems and the management of antifouling waste.

125 **2. Methods**

126

127 **2.1 Specimen collection and husbandry**

128 Adult *H. diversicolor* (ragworms; 0.29-1.01 g wet weight) and *C. edule* (cockles; 26-36 mm shell
129 length, 10.5-25 g wet weight) were collected by hand from an intertidal mudflat at Saltram Park (N 50°
130 22' 43.284" W 4° 6' 0.755") located within the Plym Estuary, UK (Figure 1) in June and July 2018 and
131 transported to PML within 1 hour of sampling. There is no boating activity at Saltram Park and a recent
132 field study showed minimal APP and metal contamination at this site ([Muller-Karanassos et al., 2019](#)). A
133 salinity of 31.1 was measured at high water using an Oakton SALT 6+ handheld probe. On return to the
134 laboratory, ragworms and cockles were allowed to acclimate for 4-7 days in polypropylene tanks (49 x 35
135 x 15 cm) containing approximately 5 cm depth of sieved estuarine sediment (<1 mm) collected at Saltram
136 Park and 5 cm depth of aerated filtered seawater (FSW, 0.2 µm Millipore filter diluted to 31.1 salinity with
137 Milli-Q water).

138



139
140 **Figure 2.** Animals and sediment were collected from Saltram Park, Plym Estuary (UK). Historic APPs were
141 sampled from abandoned boats at Hooe Lake. Map data: Google Earth, Landsat / Copernicus.

142
143 **2.2 APP generation and characterisation**

144 Three types of APPs were generated for use in the exposures, including two biocidal ('historic' and
145 'modern') and one biocide-free ('silicone'). 'Historic' biocidal antifouling paint flakes were collected by
146 hand from abandoned boats at Hooe Lake, in the Plym Estuary (N 50° 21' 22.464" W 4° 6' 28.943"; Figure
147 2), and cleaned from any visible dirt and algae ([Singh and Turner, 2009a](#)). 'Modern' biocidal antifouling
148 paint was scraped off rolled mild steel panels that had been painted with three commercially available
149 biocidal paints in 2012, including an anti-corrosive layer, tie coat and top coat, and submerged in natural
150 seawater off the coast of Orkney (Scotland) for 8 weeks in 2014. Non-biocidal 'silicone' antifouling paint

151 was scraped off rolled mild steel panels painted with commercially available silicone paint and submerged
152 in natural seawater for 5-10 days at station L4 (off the coast of Plymouth, UK;
153 www.westernchannelobservatory.co.uk) in 2018. APPs were prepared by grinding down paint flakes using
154 a pestle and mortar with the aid of liquid nitrogen; paint particles were passed through stainless steel
155 sieves to collect the 100 μm –1 mm fraction, a size range considered bioavailable to the target species and
156 for which APP particles have been identified in estuarine sediments ([Muller-Karanassos et al., 2019](#)).
157 Particle size distribution was evaluated by measuring a representative sub-sample of 100 APPs under a
158 microscope. Metal analysis of the three APP types was carried out non-destructively using an energy-
159 dispersive portable x-ray fluorescence (XRF) spectrometer (Niton XL3t He GOLDD+), with the focus on
160 metals that are or have been commonly employed as biocides in antifouling paints (Cu, Hg, Pb, Sn and
161 Zn). The instrument was operated in a low-density plastics mode with small-spot 3-mm collimation and
162 thickness correction (between 0.5 and 3 mm) and performance was verified by analysis of Niton plastic
163 reference discs containing known concentrations of various metals. APP fragments were characterised in
164 clear polyethylene zip-bags for 60 s each at five different locations.

165

166 **2.3 Experimental set-ups**

167 Two laboratory-based exposure experiments, an 18-day and 5-day exposure, were carried out for
168 each species in a temperature-controlled facility ($13\pm 1^\circ\text{C}$) under a 12:12 hour light:dark cycle. Estuarine
169 sediment was collected from Saltram Park, where our samples revealed no evidence of APPs ([Muller-
170 Karanassos et al., 2019](#)); sediment was collected to a depth of approximately 20 cm at the same time as
171 the biota, and sieved through a 1 mm stainless steel sieve with the aid of seawater to remove macrodebris.
172 Sediment was stored in a plastic tub covered with aluminium foil until experiments were carried out. Prior
173 to exposures, pre-weighed APPs were mixed into sediments in individual containers using a stainless-steel
174 spatula and then allowed to settle for 24 h prior to the introduction of animals. Owing to the high-density
175 of the APPs, we observed that particles remained within sediments during water changes. For the 18-day
176 exposure, APP concentrations were based on environmental concentrations identified at Hooe Lake (0–
177 18.8 g L^{-1} ; mean 4.2 g L^{-1}) ([Muller-Karanassos et al., 2019](#)) adjusting for the density differences of the
178 different APPs (SI, Table S1). Trial experiments carried out using the maximum environmental APP
179 concentration (18.8 g L^{-1}) led to mortality of all *H. diversicolor* and *C. edule* in the modern treatment within
180 6 days and, therefore, the mean environmental APP concentration was used in order to assess sub-lethal
181 effects. Density-corrected APP concentrations were: 4.2 g L^{-1} for the historic biocidal treatment; 3.0 g L^{-1}
182 for the modern biocidal treatment; and 2.1 g L^{-1} for the non-biocidal silicone treatment. For the 5-day LC₅₀

183 exposure, modern biocidal APPs (selected owing to their comparatively higher toxicity) were used at
184 concentrations ranging from 0-30 g L⁻¹ (ragworms) and 0-6 g L⁻¹ (cockles).

185 After acclimation, individual organisms were weighed to ascertain pre-exposure wet weight.
186 Ragworms were transferred into individual 100 mL polyethylene containers (1 ragworm per container)
187 containing 50 mL of sieved sediment pre-mixed with APPs (silicone: 0.105 g, historic: 0.209 g, modern:
188 0.152 g) or control sediment, and 50 mL of aerated FSW. Cockles were transferred into 1 L food-grade
189 containers (1 cockle per vessel) containing 250 mL of control sediment or sediment pre-mixed with APPs
190 (historic: 1.047 g; modern: 0.759 g; silicone: 0.525 g) and 700 mL of well-aerated, natural seawater filtered
191 through a 0.2 µm glass fibre filter and diluted with ultrapure water to a salinity of 31.1 (matching estuarine
192 conditions). Water was changed every 2-3 days and water quality parameters including temperature, pH,
193 salinity, conductivity and dissolved oxygen were measured using YSI Pro 1030 and Pro 20 meters at every
194 water change (SI, Table S2). For the 18-day exposure, ragworms (n=10 per treatment) were each fed 8 g
195 of hatched *Artemia salina* nauplii (Instant Baby Brine Shrimp, Ocean Nutrition) every other day ([Moreira](#)
196 [et al., 2005](#)), and cockles (n=10 per treatment) were fed every other day with an alternating diet of live
197 *Thalassiosira rotula* (4000 cells mL⁻¹) and freeze-dried multi-cell algal culture (5 Species Phytoplankton,
198 Reefphyto). For the 5-day exposure, ragworms (n=5 per APP concentration) and cockles (n=5 per APP
199 concentration) were not fed.

200

201 **2.4 18-day exposure**

202 **2.4.1 Feeding rate**

203 For ragworms, a feeding rate experiment was carried out following methods in [Moreira et al.](#)
204 [\(2005\)](#). In summary, ragworms were transferred into individual Petri dishes containing 100 *A. salina*
205 nauplii and 20 mL of diluted seawater and allowed to feed in darkness for 1 hour. Remaining *A. salina*
206 nauplii were counted and feeding rate was determined as the number of nauplii consumed per hour.

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

$$212 \quad CR = V \times \frac{\log C_1 - \log C_2}{t} \times n$$

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

215 **2.4.2 Weight change**

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

218 **2.4.3 Burrowing**

219 For ragworms, burrowing behaviour was analysed following methods by [Buffet et al. \(2011\)](#).
220 Ragworms were placed in 100 mL containers with 50 mL clean estuarine sediment from Saltram Park and
221 50 mL of aerated filtered seawater. Burial state was recorded every 2 minutes for a total of 30 minutes.

222 For cockles, burrowing was analysed as in [Byrne and O'halloran \(2000\)](#) and [Møhlenberg and](#)
223 [Kjørboe \(1983\)](#). Cockles were placed in individual containers set up in experimental chambers containing
224 clean estuarine sediments, and burrowing state was recorded after 5, 10, 15, 20, 30, 40, 50, 60, 80, 100,
225 120 and 180 minutes. Cockles were judged to be burrowed when approximately 50% of the shell was
226 covered by sediment.

227 **2.4.4 Metallothionein-like protein assay**

228 Metallothionein-like protein (MTLP) concentration in whole tissue was quantified for both species
229 using the method described by [Viarengo et al. \(1997\)](#) and [UNEP \(1999\)](#) with a few alterations. Using frozen
230 specimens, soft tissues were pooled in Petri dishes for each treatment (control, silicone, historic, modern)
231 and defrosted in an oven for 10 minutes at 50 °C. Samples were weighed and volume of tissue measured
232 before homogenisation of tissue in 2 volumes of homogenising buffer containing β -mercaptoethanol,
233 phenylmethylsulphonylfuride and leupeptin. Homogenisation was performed using a 600 W Morphy
234 Richards 402058 hand-blender in beakers until tissue was smooth enough to be removed using a 1 mL
235 Gilson pipette. Two mL of sample was pipetted into 2 mL centrifuge tubes (6 replicates per treatment)
236 and centrifuged at 2 °C, 14,000 x g for 45 minutes. One mL of supernatant was carefully removed by
237 pipette and added to 1.05 mL of cold (-20 °C) absolute ethanol and 80 μ L of chloroform in 2 mL centrifuge
238 tubes stored on ice. Tubes were inverted and vortexed before centrifugation at 0 °C and 6000 x g for 10
239 minutes. Supernatant was carefully pipetted off into 15 mL tubes and volume recorded for each sample.
240 Ten μ L of a 100 mg mL⁻¹ stock of RNA, 40 μ L of 37% HCl and 3 volumes of cold absolute ethanol were
241 added before vortexing and storing at -20 °C for 1 hour. Samples were then centrifuged at 0 °C and 4000
242 x g for 15 minutes to form a pellet. Supernatant was carefully removed and the pellet washed with 5 mL
243 of a *cleaner* solution (87:1:12 ethanol/chloroform/homogenising buffer, stored at -20 °C). Samples were
244 re-centrifuged at 0 °C and 4000 x g for 5 minutes before removal of supernatant and drying. 150 μ L of

245 0.25 M NaCl solution and 150 μL of 1N HCl containing 4 mM EDTA were added and then vortexed.
246 Standards were made up using a 1 mg mL⁻¹ solution of glutathione in 0.25 M NaCl, and blanks were made
247 up with 150 μL of 0.25 M NaCl solution and 150 μL of 1N HCl containing 4 mM EDTA. Just before analysis,
248 0.43 mM (7.14 mg/42 mL) DTNB (Ellman's reagent) was dissolved in 0.2 M phosphate buffer pH 8
249 containing 2 M NaCl and 4.2 mL of this solution was added to all samples, standards and blanks. All tubes
250 were then centrifuged at 3,000 xg for 5 minutes at room temperature. Absorbance was measured at 412
251 nm in a VWR UV-3100 PC spectrophotometer.

252

253 **2.5 5-day exposure**

254 **2.5.1 LC₅₀ and EC₅₀**

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

262

263 **2.6 Statistical analysis**

264 All ragworm and cockle data were checked for normality using Shapiro-Wilk's test. For the non-
265 parametric ragworm data a Kruskal-Wallis test followed by Wilcoxon Rank Sum test was used, while a
266 one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare differences in
267 parametric cockle data. A generalised linear model (GLM) with binomial distribution was used to compare
268 the number of animals burrowed after 30 minutes across treatments. The 5-day LC₅₀ and EC₅₀ values were
269 calculated by probit analysis in SPSS. All other analyses were carried out using R statistical software v3.5.1
270 ([R, 2019](#)). Biological data is presented as mean values \pm standard error, while metal chemistry data is
271 presented as mean values \pm standard deviation; significant difference is attributed where $p < 0.05$.

272

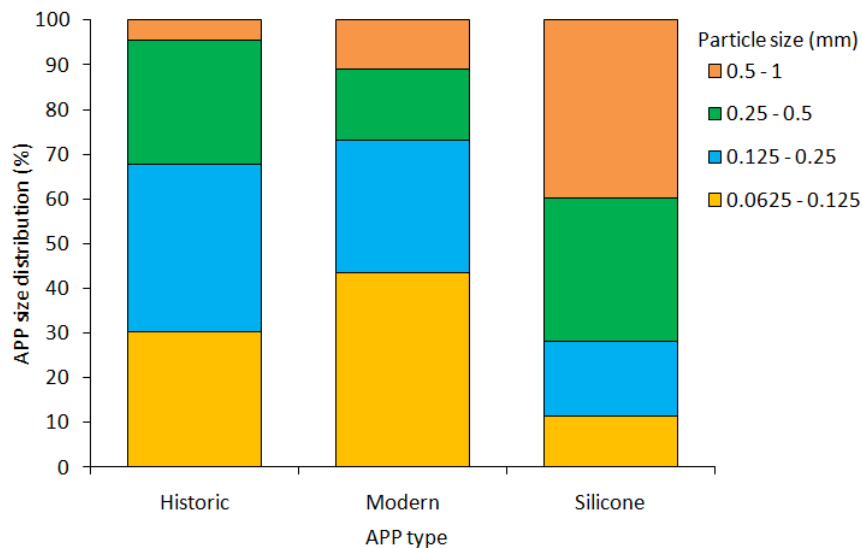
273 **3. Results**

274 **3.1 APP characterisation**

275 APPs ranged from 0.0625–1 mm in size, with particle size varying between the three APP types
 276 (Figure 3). Overall, historic biocidal APPs had the highest proportion of particles in the size range 0.125–
 277 0.25 mm (37.6%), modern biocidal APPs had the smallest particle size, with 43.6% of particles in the size
 278 range 0.0625–0.125 mm. Non-biocidal silicone antifouling paint had a “rubber-like” consistency and was
 279 much harder to breakdown into smaller particles via cryogenic grinding, and as a result these APPs had
 280 the largest particle size, with 39.7% of particles in the size range 0.5–1 mm.

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

286



287
 288 **Figure 3.** Particle size distribution of the different types of APPs used in exposure experiments.

289
 290 **Table 1.** Metal concentrations (mg kg⁻¹) in APPs used in 18-day and 5-day assays. The mean of 5
 291 measurements is shown for each APP type ± standard error. Metal concentrations not detected were
 292 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 |

293

294 3.2 18-day assay

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

303 3.2.1 Feeding rate

304 The highest feeding rate occurred in the control treatment (17 ± 4 nauplii h^{-1} ind. $^{-1}$) and the lowest
 305 was observed in the modern biocidal treatment (6 ± 2 nauplii h^{-1} ind. $^{-1}$). The feeding rate of ragworms
 306 differed significantly between treatments (One-way ANOVA: $F_{3,32}=3.131$, $p<0.05$; Figure 4A), and the post-
 307 hoc test showed a significant difference between the modern biocidal treatment and control (Tukey:
 308 $p<0.05$).

309 No significant difference in clearance rates of cockles between treatments was observed (control:
310 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: $F_{2,27}=2.797$,
311 $p=0.08$; Figure 4B).

312 **3.2.2 Weight change**

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

317 Weight change in cockles was not significantly affected by treatment (One-way ANOVA: $F_{2,27}=3.30$,
318 $p=0.052$; Figure 4D). However, compared with controls, cockles exposed to non-biocidal silicone APPs did
319 show a significantly greater weight loss (Tukey: $p<0.05$).

320 **3.2.3 Burrowing**

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

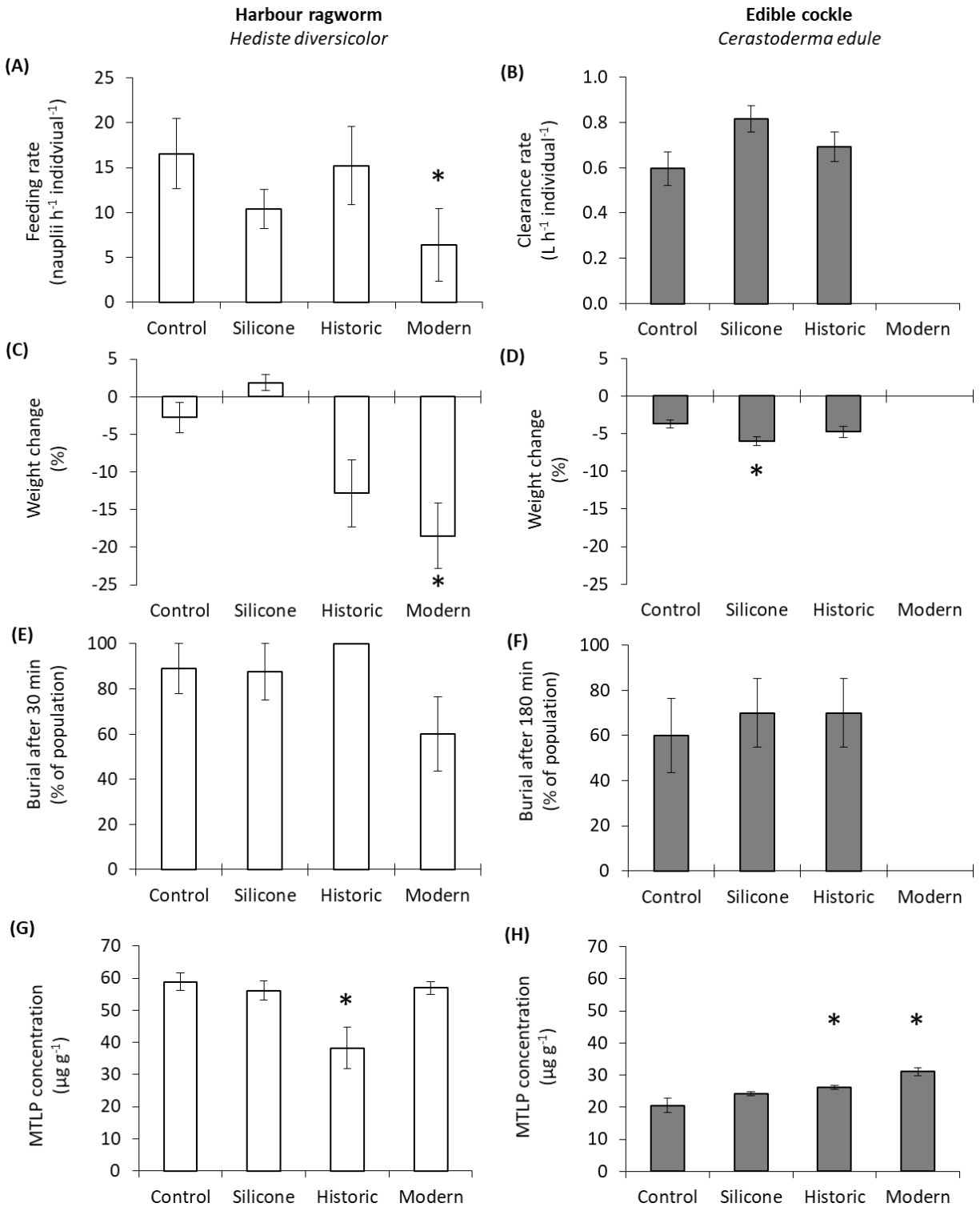
324 Approximately two-thirds of cockles had successfully buried after 180 minutes across all
325 treatments, with no significant difference between treatments (GLM: $p>0.05$; Figure 4F).

326 **3.2.4 Metallothionein-like protein**

327 MTLP concentrations in ragworms averaged 59 µg g⁻¹ in controls, with no significant difference
328 between treatments (Kruskal-Wallis: $p=0.07$); however, ragworms exposed to the historic biocidal APPs
329 showed significantly reduced MTLP concentrations compared with the controls (38.3 µg g⁻¹; Wilcoxon:
330 $p<0.05$; Figure 4G).

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

336



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Figure 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

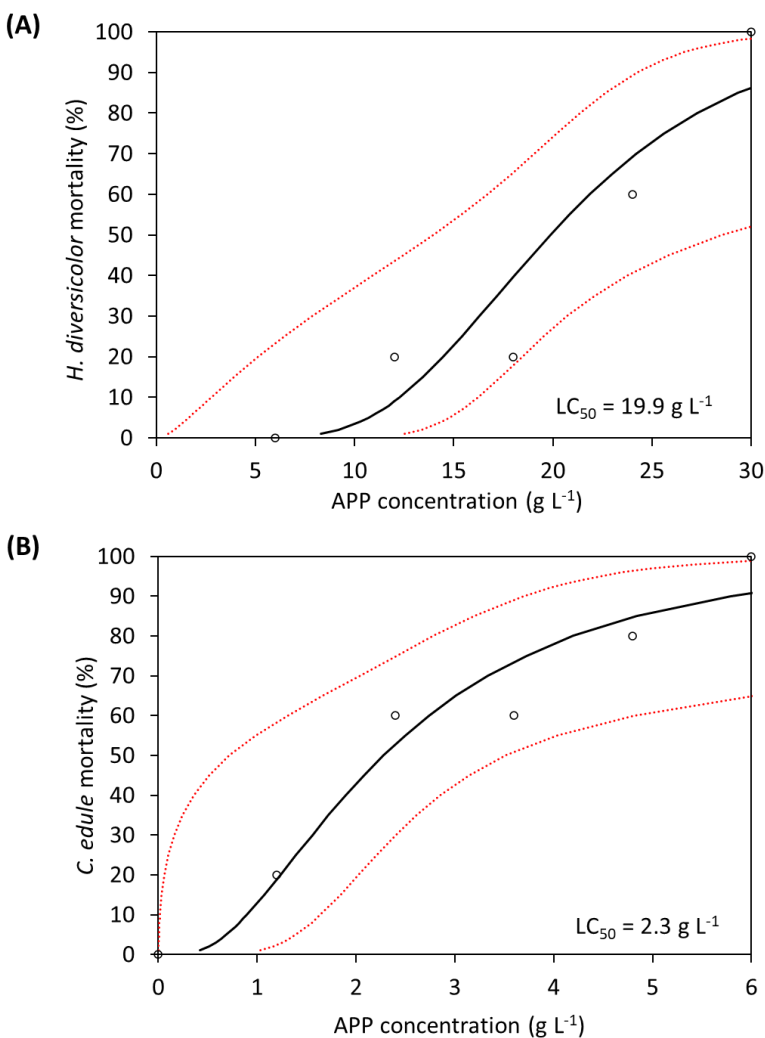
341 individuals burrowed after 30 minutes (ragworm) and 180 minutes (cockles); (G-H) Metallothionein-like
342 protein (MTLP) concentration, noting that cockles in modern treatment died before the end of the 18-day
343 exposure period. * denotes significant difference from control treatment. Error bars indicate standard
344 error.

345

346 3.3 5-day assay: LC50s

347 The 5-day LC₅₀ was 19.9 APP g L⁻¹ for ragworms (Figure 5A) and 2.3 g L⁻¹ for cockles (Figure 5B). In
348 the control treatment, there were no mortalities for either species. The 5-day EC₅₀ values were calculated
349 as 14.6 g L⁻¹ for ragworms and 1.4 g L⁻¹ for cockles (data not shown).

350



351

352 **Figure 5.** 5-day median lethal concentration (LC₅₀) dose-response curves (black line) with 95% confidence

353 intervals (orange lines) for (A) ragworms and (B) cockles exposed to modern APPs.

354

355 4. Discussion

356

357 4.1 18-day exposure

358 Results from the 18-day study demonstrate that exposure to modern biocidal APPs at
359 environmentally relevant concentrations leads to adverse health effects in the ragworm *H. diversicolor*
360 and mortality in the cockle *C. edule*. As compared with controls, significant net decreases in weight
361 ($15.7 \pm 5\%$) and feeding rate ($10.2 \pm 4\%$) were observed in ragworms exposed to modern biocidal APPs;
362 furthermore modern biocidal APPs were associated with the highest percentage of un-burrowed
363 ragworms, with $29 \pm 20\%$ fewer ragworms burrowed after 30 minutes compared to the average control,
364 although this difference was not statistically significant. Modern biocidal APPs were acutely toxic to
365 cockles, resulting in mortality of all replicates in the first 10 days of exposure. Both historic and modern
366 biocidal APPs caused significant increases in MTLPs in cockles. Largely, historic biocidal and non-biocidal
367 silicone APPs did not cause substantial sub-lethal health effects in ragworms or cockles, although it was
368 observed that exposure to historic biocidal APPs led to substantial weight loss in the ragworms.

369 A reduction in weight, feeding and burrowing activity of ragworms could lead to a range of
370 consequences in the natural environment. Impairment of feeding activity in marine organisms can directly
371 affect population parameters such as growth, reproduction ([Maltby et al., 2001](#)) and energy intake
372 ([Kalman et al., 2009](#)). Previous studies have demonstrated that Cu exposure can have lethal and sub-lethal
373 effects on ragworms, including reduced burrowing activity ([Thit et al., 2015](#)) and feeding ([Moreira et al.,](#)
374 [2005](#)). Reduced burrowing efficiency in ragworms and cockles increases the likelihood of predation
375 ([Kalman et al., 2009](#)), and reduces bioturbation activity – a vital process for sediment processing and
376 deposition of organic matter in estuarine ecosystems ([Moreira et al., 2006](#)). Increased mortality of cockles
377 caused by exposure to APPs could lead to a decrease in natural populations, with implications for
378 ecosystem functionality (e.g. bioturbation and food webs). Soroldini et al. ([Soroldini et al., 2017](#);
379 [Soroldini et al., 2020](#)) similarly found an increase in mortality in copepods and benthic invertebrates with
380 increasing concentrations of APPs originating from a commonly-used contemporary commercial
381 antifouling paint containing high concentrations of Cu and Zn (Cu: $234 \pm 0.27 \text{ g kg}^{-1}$, Zn: $112 \pm 0.84 \text{ g kg}^{-1}$,
382 Pb: $0.51 \pm 0.01 \text{ g kg}^{-1}$). Another study found that leachate from sediment contaminated with APPs caused
383 a reduction in gram-negative bacteria (*Vibrio fischeri*) bioluminescence, decreased growth rate of the red
384 algae (*Ceramium tenuicorne*) and reduced larval development in the harpacticoid copepod *Nitocra*

385 *spinipes* ([Ytreberg et al., 2010](#)); while Cu was found to be more toxic to *V. fischeri* and *C. tenuicorne*, *N.*
386 *spinipes* was more sensitive to Zn ([Ytreberg et al., 2010](#)).

387 In comparing the metal profiles of the biocidal APPs, it was evident that the modern paints
388 contained far higher copper concentrations (Cu: modern: $427,000 \pm 7,000 \text{ mg kg}^{-1}$; historic: $163,000 \pm$
389 $15,600 \text{ mg kg}^{-1}$). The lower Cu concentrations in the historic biocidal APPs could be attributed to the
390 weathering of historic APP and a longer period of Cu leaching from aged historic paints ([Rees et al., 2014](#)),
391 leading to more inert APPs and therefore a reduced toxicity. However, historic APPs contained the highest
392 Zn concentrations ($74,400 \pm 7,800 \text{ mg kg}^{-1}$) and also contain other toxic metals such as Pb ($1,060 \pm 543 \text{ mg}$
393 kg^{-1}), Hg ($1,810 \pm 423 \text{ mg kg}^{-1}$) and Sn ($446 \pm 87 \text{ mg kg}^{-1}$), which may still pose an ongoing risk to non-target
394 organisms. Based on the heightened mortality and sub-lethal effects stemming from modern biocidal APP
395 exposure, we surmise Cu is the most toxic biocidal metal present. [Soroldoni et al. \(2017\)](#) similarly
396 identified that Cu was more toxic to copepods than Zn. Once metals have been taken up by an organism,
397 they can be detoxified by accumulation in metal-rich granules or by binding with metallothionein (MT)
398 and MTLP that regulate metal concentrations ([Rainbow, 2007](#)). The elevated MTLP in cockles from historic
399 and modern biocidal treatments compared to the control suggests an upregulation of MTLP in response
400 to metal exposure, particularly in the modern biocidal treatment (despite these cockles not surviving the
401 full 18-day exposure). MTLP concentrations in ragworms were not affected by treatment. Previous studies
402 also found no relationship between accumulated metal concentrations and MTLP levels in ragworms
403 ([Poirier et al., 2006](#); [Solé et al., 2009](#)), and several studies have shown that exposure to Cu leads to an
404 accumulation of this metal in *H. diversicolor* ([Berthet et al., 2003](#); [Geffard et al., 2005](#)). While ragworms
405 have an intolerance to Cu, they have shown the capacity to regulate Zn body concentrations, thought to
406 occur by reduced metal uptake rates, increased excretion rates and/or through storage of metals in
407 granules or MTLPs ([Berthet et al., 2003](#); [Geffard et al., 2005](#)).

408 Other compounds found in antifouling paint such as booster biocides, solvents and binders can
409 also have toxic effects ([Karlsson et al., 2010](#)) and the mixture of metals and other compounds found in
410 APPs are likely to have synergistic effects ([Soroldoni et al., 2017](#)). It is therefore important to examine the
411 toxicity of APPs as a whole, in addition to that of individual compounds found within antifouling paints.
412 APPs were not tested for other compounds in this study, although they are likely to have contributed to
413 the high toxicity observed with modern biocidal APPs. Although uptake routes were not investigated in
414 the current study, exposure to biocides likely occurred via ingestion of APPs and uptake of dissolved
415 metals leached into the water column and sediment porewater. By design, biocidal antifouling paints will
416 constantly release metal ions into the surrounding water to prevent adherence and growth of fouling

417 organisms, so it is expected that biocidal APPs will continue to emit Cu and Zn throughout their lifespan,
418 although whether the rate of dissipation changes over time remains unclear. A number of studies have
419 exposed marine organisms to APP leachate solutions, which have been routinely demonstrated to be
420 readily taken up by biota and cause toxicity ([Gammon et al., 2009](#); [Katranitsas et al., 2003](#); [Soroldoni et](#)
421 [al., 2018b](#); [Tolhurst et al., 2007](#)). In the Plym estuary, the smallest APPs observed were ~0.5 mm in size
422 ([Muller-Karanassos et al., 2019](#)); detecting smaller particles was hindered by sampling and analytical
423 limitations, however abiotic and biotic processes can be expected to contribute to the proliferation of
424 even smaller APPs. In this study APPs were prepared to 0.0625-1 mm in size, with differences in particle
425 size profiles of biocidal and non-biocidal APPs observed: the majority of the “rubber-like” silicone APPs
426 being >0.5 mm, and the majority of biocidal APPs being <0.5 mm diameter. We cannot say for certain
427 whether the observed differences in particle size profiles might influence toxicity, however we consider
428 all particles to be in the normal prey size range of cockles and ragworms. Toxicity studies using plastic
429 particulates of distinct size (i.e. nano- vs micro) have revealed size dependent effects, with differences
430 stemming from different modes of biological action; for example, 50 nm polystyrene nanoplastics
431 triggered an immune response in mussels (likely owing to their capacity to readily translocate into
432 circulatory fluids) while exposure to 20 µm polystyrene microplastics caused no observable impact ([Cole](#)
433 [et al., 2020](#)). It is interesting to consider whether exposure to APPs of markedly different particle size (i.e.
434 nano vs micro) would significantly effect toxicity; for example, might smaller APPs with larger surface
435 areas increase metal leaching or penetrate deeper into tissues causing cellular toxicity ([Jeong et al., 2016](#))?
436 Further work is needed to clarify the mechanisms responsible for toxicity of APPs to benthic organisms
437 observed in the present study.

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

446 Owing to their alkyd-resin base, APPs can be considered as microplastics ([Hartmann et al., 2019](#)).
447 Microplastic debris can be ingested by a wide array of marine organisms, with evidence of sub-lethal harm
448 in a number of studies e.g. [Cole et al. \(2015\)](#), [Wright et al. \(2013\)](#). In this study, it is evident that non-

449 biocidal silicone APPs (absent of metal additives) have a far lower toxicity than APPs containing biocides,
450 highlighting the importance of considering additive and metal profiles when evaluating microplastic
451 toxicity. While the physical properties of a microplastic (e.g. size, shape) have been shown to interfere
452 with feeding and movement in marine biota (see [Galloway et al. \(2017\)](#) and [Setälä et al. \(2018\)](#)), their
453 chemical composition is often overlooked. However, microplastics more generally should not be
454 considered as a single polymeric compound, but a mixture of polymers, containing monomers and
455 additives including metals, emollients, phthalates and flame retardants ([Rochman et al., 2019](#)). These
456 additives can be highly toxic and have been associated with sub-lethal health effects and endocrine
457 disruption in marine invertebrates ([Browne et al., 2013](#); [Cole et al., 2019](#)).

458

459 **4.2 5-day assay**

460 Cockles were found to be much more sensitive to modern biocidal APPs when compared to
461 ragworms. The LC₅₀ and EC₅₀ for cockles (2.3 g L⁻¹ and 1.4 g L⁻¹ respectively) were almost an order of
462 magnitude lower than for ragworms (19.9 g L⁻¹ and 14.6 g L⁻¹ respectively). The maximum concentration
463 of APPs found within the Plym Estuary at Hooe Lake ([Muller-Karanassos et al., 2019](#)), where boating
464 activity is significant, was 18.8 g L⁻¹, which is well above the LC₅₀ and EC₅₀ for cockles and above the EC₅₀
465 for ragworms. This suggests that APP concentrations found in the natural environment have the potential
466 to cause mortality to cockles and adverse effects in ragworms.

467 Studies have shown that ragworms and cockles originating from metal-contaminated sites may
468 have a higher tolerance to Cu and Zn compared to uncontaminated sites ([Durou et al., 2005](#); [Mouneyrac
469 et al., 2003](#); [Naylor, 1987](#)). It is therefore not possible to generalise the findings of this study for all
470 ragworm and cockle populations since the LC₅₀ for APPs will likely differ between sites. Indeed, ragworm
471 and cockles could be identified in the vicinity of Hooe Lake, albeit in sparser numbers than elsewhere
472 (personal observations). Metal tolerance has also been found to differ between taxa and more sensitive
473 benthic organisms are expected to have lower APP LC₅₀ values. A study by [Buffet et al. \(2011\)](#) found that
474 the bivalve mollusc *Scrobicularia plana* was less tolerant to Cu nanoparticles compared to *H. diversicolor*,
475 supporting the findings of the current study. found a 4-day LC₅₀ value of 0.14 % of APPs by mass of dry
476 sediment for the epibenthic copepod *Nitokra* sp. The current study showed a similar 5-day LC₅₀ (0.15 % of
477 APPs by mass of wet sediment) for *C. edule* and a much higher 5-day LC₅₀ (1.42 % of APPs by mass of wet
478 sediment) for *H. diversicolor*. These values are not directly comparable since the exposure periods differ
479 and sediment wet weight was used instead of dry weight. However, it can be assumed that if water were
480 removed from sediment in the current study this would produce higher percentages of APPs by mass of

481 dry sediment. This suggests that cockles are more tolerant than copepod species to APP-contaminated
482 sediments, likely due to their larger body size and ability to accumulate metals.

483

484 **Conclusions**

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

492

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495

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