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## Cryogrinding and sieving techniques as challenges towards producing controlled size range microplastics for relevant ecotoxicological tests

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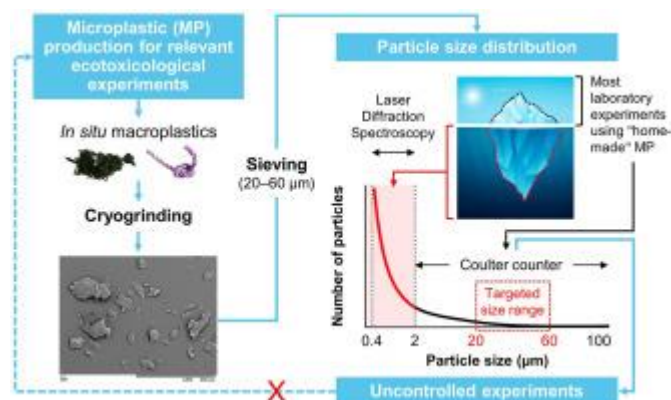
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### Abstract :

The impact of microplastics (MP) has attracted much attention from the scientific community and many laboratory assessments have been made of their effects on aquatic organisms. To produce MP from real environmental plastic waste, which would enable more realistic experiments, we used plastic pearl farming equipment from French Polynesian lagoons. Here, the pearl oyster *Pinctada margaritifera* could encounter MP coming from their breakdown in its surrounding environment. We tested an established method based on mechanical cryogenic grinding and liquid sieving. Our desired size range was 20–60 µm, corresponding to the optimal particle size ingested by *P. margaritifera*. The protocol was effective, generating MP particles of 20–60 µm (~17,000–28,000 MP µg<sup>-1</sup>), but also produced too many smaller particles. The peak in the desired size range was thus flattened by the many small particles <3 µm (~82,000–333,000 MP µg<sup>-1</sup>; 53–70% of total analysed particles), visible at the limit of Coulter counter analysis (cut-off point: 2 µm). Laser diffraction analysis (cut-off point: 0.4 µm) provided greater detail, showing that ~80–90% of the total analysed particles were <1 µm. Diverging particle size distributions between those expected based on sieving range and those really observed, highlight the need to perform fine-scaled particle size distribution analyses to avoid underestimating the number of small micro- and nanoplastics (MNP) and to obtain an exact estimation of the fractions produced. Size and microstructure characterization by scanning electron microscopy suggested spontaneous particle self-assembly into crystal superstructures, which is the supposed cause of the divergence we observed. Overall, our results emphasize that particle self-assembly is a technical hurdle requiring further work and highlight the specific need to finely characterize the size distribution of MNP used in ecotoxicological experiments to avoid overestimating effects.

## Graphical abstract



## Highlights

► “Homemade” microplastics may underestimate exposure level and overestimate impact. ► Particle sieving range was inconsistent with size distribution. ► Particle self-assembly may cause biased sieving and size distribution. ► Particle size distribution needs to be systematically characterized very finely. ► For standardization, it is vital to improve microplastic manufacture and sizing.

**Keywords:** Laboratory experiment, Microplastic production, Sieving, Particle self-assembly, Uncontrolled size distribution, Underestimated concentration

## 60 1. Introduction

61 Microplastic particles (MP, <5 mm) are the most numerically abundant form of solid waste on  
62 Earth (Eriksen et al., 2014). Over the past fifteen years, the scientific community has studied  
63 this global concern by evaluating environmental concentrations in different marine  
64 compartments (Eriksen et al., 2014; Cózar et al., 2014; Woodall et al., 2014; van Sebille et al.,  
65 2015), as well as the adverse effects on aquatic organisms in laboratory conditions (reviewed  
66 in Thornton Hampton et al., 2022). Historically, the vast majority of laboratory experiments  
67 used a single polymer type, in pieces of spherical shape (*i.e.* microbeads), and particle  
68 concentrations mostly higher (in number of particles per volume) than those found in the field  
69 (Phuong et al., 2016; Lenz et al., 2016). These biases were mainly caused by a lack of more  
70 appropriate polymer types and shapes, and uncertainties regarding *in situ* concentrations of  
71 particles below 300 µm. Although an increasing number of papers now aim to consider the  
72 heterogeneity of microplastics (*i.e.* by including a variety of particle shapes, sizes, polymer  
73 types, surface properties; Rochman et al., 2019), the gap between potential and actual risks  
74 remain concrete considering the lack of relevance in ecological terms (Connors et al., 2017; de  
75 Sá et al., 2018).

76 A rigorous understanding of the environmental impacts of MP on marine biota requires fine  
77 evaluation of concentrations and characteristics of the smallest MP sizes in order to conduct  
78 relevant environmental experiments in laboratory conditions. The first step to using more  
79 realistic MP scenarios is to conduct an evidence-based approach, which means targeting the  
80 most relevant particles in terms of shape (*i.e.* fragments rather than spheres), size (*i.e.*  
81 polydisperse rather than monodisperse), polymers (*i.e.* those mostly found locally), and surface  
82 properties (*i.e.* aging and biofilm). For instance, recent studies demonstrating physical and  
83 chemical differences between weathered plastics and pristine ones or post-production consumer  
84 products have highlighted the need to use materials for ecotoxicology tests that are as similar

85 as possible to those found *in situ* (Jahnke et al., 2017). Using collected weathered plastics, such  
86 as beached macroplastic litter, to produce MP therefore offers a way to obtain relevant test  
87 materials for impact assessment studies (Kühn et al., 2018).

88 In the lagoons of French Polynesia, pearl farming has been identified as a specific source of  
89 plastic pollution through the accumulation of operational and abandoned plastic equipment (*e.g.*  
90 ropes, collectors, buoys) (Andréfouët et al., 2014). A recent monitoring study in pearl farming  
91 lagoons revealed a widespread contamination by MP in both seawater and cultured pearl oyster  
92 (Gardon et al., 2021). Among the possible ecological disturbances discussed in Andréfouët et  
93 al. (2014), one comes from a preliminary experiment conducted by Gardon et al. (2018)  
94 highlighting a dose effect of polystyrene microbeads on energy balance (Gardon et al., 2018)  
95 and dose-specific transcriptomic disruption of gene expression (Gardon et al., 2020b) in the  
96 pearl oyster (*Pinctada margaritifera*). As *P. margaritifera* is a species of commercial interest  
97 and the second most important economic resource in French Polynesia, risk assessment using  
98 realistic MP (*i.e.* sourced from local pearl-farming gear debris) is very important. It will form  
99 the crucial next step to improving both the simulation of the pearl oyster environment and the  
100 realism of results that can support decision making.

101 A few recent studies provide protocols for preparing model micro- and nanoplastics (*e.g.* Cole,  
102 2016; Eitzen et al., 2019), some of which present cryomilling as an effective way to obtain  
103 reference particles (Kühn et al., 2018). However, the particles thus-obtained from aged  
104 polymers were mostly in the range of several hundred  $\mu\text{m}$  (Kühn et al., 2018) or nano sized (El  
105 Hadri et al., 2020). The production of standardized size particles targeting a range of around 1  
106 to 100  $\mu\text{m}$ , which is a typical size range used in most of laboratory studies (de Sá et al., 2018;  
107 Paul-Pont et al., 2018), remains under-investigated. The present analytical investigation thus  
108 proposes to test a cryogrinding and sieving protocol for laboratory MP production from  
109 weathered plastic pearl farming equipment as this is a potential source of MP in pearl farming

110 lagoons (Gardon et al., 2021). The objective was to obtain a specific size range of particles  
111 included in the *P. margaritifera* particle size retention range (2 to 200  $\mu\text{m}$ ; Pouvreau et al.,  
112 1999). Thus, in view of our environmental data showing the prevalence of MP 20–200  $\mu\text{m}$  in  
113 the cultured pearl oysters (Gardon et al., 2021) and to keep close to the most MP size used in  
114 laboratory studies (<50  $\mu\text{m}$ ; de Sá et al., 2018), we focused on the obtention of polydisperse  
115 particles ranging from 20 to 60  $\mu\text{m}$ . With the aim of questioning difference between the  
116 expected and the real particle sizes, we hypothesized that cryogrinding and sieving techniques  
117 do not make it possible to obtain a specific particle size range regardless of the grinding time.  
118 Yet, this approach has already been used in some recent (eco)toxicological studies (*e.g.* Revel  
119 et al., 2019; Schür et al., 2020; Carrasco-Navarro et al., 2021). However, the overall particle  
120 size distribution of the obtained plastic powder was not always considered, depending on the  
121 method used to characterize these "lab MP". A commonly used approach is a Coulter counter  
122 equipped with a 100  $\mu\text{m}$  aperture tube, which allows the detection of particles ranging from 2  
123 to 100  $\mu\text{m}$  (Revel et al., 2019; Schür et al., 2020; Zimmermann et al., 2020), while the number  
124 of particles <2  $\mu\text{m}$  remains unknown if no further analysis is performed. The lack of  
125 consideration of the smaller size range is therefore an omission that deserves attention. In our  
126 study, we examine the potential scale of the error caused by ignoring the nano fraction. This  
127 would bias ecotoxicological results interpretation, especially considering that the toxicity of  
128 particles increases as their size decreases (Jeong et al., 2016; Ma et al., 2016). We demonstrate  
129 the need to improve the standardization of particle concentrations used in exposure experiments  
130 and highlight the real challenges of producing microplastics with a controlled size range.

## 131 2. Materials and methods

### 132 2.1. Plastic selection

133 Several types of plastic pearl farming gear were collected from a pearl farm in Manihi atoll  
134 (14°24'10.4"S, 145°57'29.2"W), among which synthetic ropes and spat collectors were the most  
135 abundant (Andréfouët et al., 2014). Both ropes and collectors are suspected to be potential  
136 sources of polyethylene (PE) and polypropylene (PP) microplastics in the environment of  
137 cultivated pearl oysters (Gardon et al., 2021). The polymers of the gear were identified by  
138 Fourier-transform infrared spectroscopy (FTIR) on both plastic pieces and MP particles  
139 produced, confirming that the spat collector (shade-mesh) was made of PP (**Figure 1A–B**) and  
140 the rope was made of PE (**Figure 1D–E**).

### 141 2.2. Microplastic production

142 The sampled spat collectors and ropes were first washed in saline water (100 g of NaCl L<sup>-1</sup>)  
143 overnight (o/n) to remove as many potentially present microorganisms as possible, then rinsed  
144 thoroughly and dried at 60°C for 3 days in a proofer. They were then cut into ≤1 cm pieces  
145 using clean stainless-steel scissors (**Figure 1C** and **F**; **Figure 2**, step 1). A second washing in  
146 saline water was performed o/n on the plastic pieces, followed by rinsing with filtered (1.2 µm)  
147 distilled water and drying in a proofer at 60°C for 3 days. Two-gram aliquots of dried plastic  
148 pieces were packaged in aluminium and immersed in liquid nitrogen for mechanical cryogenic  
149 grinding. Steel grinding jars (50 ml) with a steel ball 2 cm in diameter were cooled in liquid  
150 nitrogen and filled with an aliquot (2 g of plastic pieces per jar) once all materials were cooled  
151 down (**Figure 2**, step 2). The grinding jars were then placed in a Mixer Mill MM 400 (Retsch™)  
152 for grinding (**Figure 2**, step 3). Three running times were tested for each MP type: 1-, 2- and

153 3-minutes run of grinding. After grinding, the MP from the spat collector (MP<sub>coll</sub>) and rope  
154 (MP<sub>rope</sub>) were dried at 60°C o/n before sieving (**Figure 2**, step 4).

155 A multi-step sieving phase was carried out to collect the MP 20–60 µm fraction. First, the MP  
156 powder was sieved through the 60 µm sieve and rinsed with filtered 70% ethanol from a wash  
157 bottle, aiming to avoid MP aggregates as much as possible and facilitate particle passage  
158 through the sieve (**Figure 2**, step 5). The remaining >60 µm fraction was then suspended in 500  
159 ml of filtered distilled water (*i.e.* ~1 g L<sup>-1</sup>) and the resulting solution sonicated at 30 kHz for 5  
160 min to dissociate potential aggregates (**Figure 2**, step 6) in order to improve the subsequent  
161 second sieving through the 60 µm sieve with filtered 70% ethanol (**Figure 2**, steps 7–8). The  
162 60 µm sieve was then dried at 60°C o/n and the dried >60 µm MP fraction was weighed with a  
163 digital precision weighing instrument (RADWAG® PS 600.R2; read accuracy = 0.0001 g) by  
164 putting the fraction in a tared cup (**Figure 2**, step 8). The MP 20–60 µm fraction was put through  
165 the same liquid sieving described above (**Figure 2**, steps 9–12) with the addition of Tween-20®  
166 (0.1% of the total volume) to the suspended solution before sonication to improve the final  
167 sieving phase. The MP 20–60 µm fraction was then rinsed a last time with filtered 70% ethanol  
168 and dried at 60°C o/n before recovery and weighing (**Figure 2**, step 12). The obtained MP 20–  
169 60 µm fraction was conserved in stock solution resuspended in filtered ethanol at 1.5 g L<sup>-1</sup> for  
170 particle size distribution and microstructure characterization (**Figure 2**, steps 13–14).

171 All the sieving steps were realised under an extractor hood and over a glass dish. All materials  
172 were cleaned and rinsed between each grinding treatment. All wastes, including liquids, were  
173 treated as chemical waste.



174 **2.3. Quantitative assessment**

175 Microplastic fractions were weighed to estimate the relative mass of the >60 µm and 20–60 µm  
176 fractions as well as the loss of plastic mass following production from an initial 4 g of plastic  
177 pieces (*i.e.* 2 g per jar) for each plastic type and grinding condition.

178 **2.4. Particle characterization**

179 Size distributions of the produced MP were assessed using a Multisizer™ 3 Coulter Counter  
180 (Beckman Coulter, Inc., Brea, CA) equipped with a 100 µm aperture tube (detection range of  
181 2–100 µm), an approach commonly used to analyse "homemade" microplastics (*e.g.* Schür et  
182 al., 2020). A 1.5 µl MP suspension was added to 15 ml electrolytic solution (0.9% NaCl  
183 solution) supplied by the manufacturer (Beckman Coulter, Inc., Brea, CA) and previously  
184 filtered on a 0.2 µm sterile filter. The solution was shaken before measurement and three  
185 repeated measures with the 100 µm capillary (100 µl analytical volume, 300 measured points  
186 from 2 to 100 µm size) were performed for each condition. Background measurements were  
187 also performed without any MP suspension to quantify contamination in the electrolytic  
188 solution. Concentrations and size distribution of microplastics were thus corrected by  
189 subtracting the number of particles measured in the electrolytic solution for each of the  
190 measured points.

191 Laser diffraction analyses were also done, using a Beckman Coulter LS 130 particle laser  
192 diffractometer (Beckman Coulter, Inc., Brea, CA) to measure the size distribution of the  
193 particles down to 0.4 µm. A few ml of stock solutions were injected until an obturation of 5%  
194 was reached.

195 The particle microstructure was observed using a Hitachi TM 3030 scanning electron  
196 microscope (SEM) at 15 kV (in charge-up reduction mode) to analyse particle shapes and

197 structures in dry conditions (on the remaining plastic powder) and following slow solvent  
198 (ethanol) evaporation (from resuspended MP stock solution).

## 199 **2.5. Data analyses**

200 The weights of the microplastic fractions gave the distribution between fractions  $>60\ \mu\text{m}$  and  
201  $20\text{--}60\ \mu\text{m}$ , as well as the plastic mass loss caused during the production method, which was  
202 calculated by subtracting the weighed MP fractions from the initial weight. Data on particle  
203 size distribution obtained by Coulter counter analysis of the  $20\text{--}60\ \mu\text{m}$  fraction were averaged  
204 and plotted on the 300 measured points (from 2 to  $100\ \mu\text{m}$ ). We determined the proportion of  
205 particles really included in the obtained  $20\text{--}60\ \mu\text{m}$  fraction as well as proportions of particles  
206 above and below 60 and  $20\ \mu\text{m}$ , respectively. Pairwise comparisons were done to determine  
207 significant differences between grinding times regarding frequency distributions of particle  
208 count across the size ranges of  $2\text{--}20\ \mu\text{m}$  and  $20\text{--}60\ \mu\text{m}$  using Fisher's exact test. Considering  
209 no difference, grinding times were then grouped by plastic type and used as replicates ( $n = 3$ )  
210 to compare the relative abundance of particle count between both size ranges. Data were  
211 therefore transformed by the arcsine square root function. Normality of data distribution and  
212 homogeneity of variance were tested with Shapiro-Wilk test and Bartlett test, respectively.  
213 Means ( $\pm$  standard deviation, SD) were then compared using a two-way ANOVA for plastic  
214 type and size range ( $\alpha = 0.05$ ). Tukey's post hoc test was used to determine the significant  
215 differences between the averages of each group. Results were considered significant at  $P < 0.05$ .  
216 Data on the differential volume (%) and number (%) of analysed particles obtained by laser  
217 diffraction analysis were plotted on 24 measured points (from  $0.375$  to  $200\ \mu\text{m}$ ). The average  
218 size (mean  $\pm$  SD) and relative abundance of total analysed particles in the sizes  $<1$ ,  $<10$ ,  $<20$ ,  
219  $<50$ ,  $<100$  and  $<200\ \mu\text{m}$  were obtained. All analyses were performed and graphics drawn in  
220 RStudio v4.0.5 statistics software.

## 221 **3. Results and discussion**

### 222 **3.1. A microplastic production method adaptable to plastic properties**

223 High mass proportions of MP >60  $\mu\text{m}$  were recovered for both types of plastic regardless of the  
224 grinding time (**Table 1**). The >60  $\mu\text{m}$  fraction made from the spat collector reached weights of  
225 3.96 g (99.0%), 3.94 g (98.4%) and 3.87 g (96.7%) after 1, 2 and 3 min of grinding, respectively;  
226 from the rope the weights were 3.64 g (91.0%), 3.10 g (77.6%) and 3.69 g (92.2%), respectively  
227 (**Table 1**). Accordingly, low mass proportions were recovered in the spat collector 20–60  $\mu\text{m}$   
228 fraction, reaching only 0.03 g (0.9%), 0.03 g (0.7%) and 0.03 g (0.8%) after 1, 2 and 3 min of  
229 grinding, respectively, while the weight of this fraction from rope reached 0.15 g (3.7%), 0.69  
230 g (17.2%) and 0.16 g (4.0%) for these grinding times (**Table 1**). Interestingly, a greater  
231 proportion of MP weight was obtained in PE rope following 2 min grinding before decreasing  
232 after 3 min grinding. Even though those results came from single replicate (and were therefore  
233 not tested statistically), it is likely that 2 min grinding reached an intermediate state of particle  
234 size improving the sieving phase. Indeed, smaller particle size might have favoured their  
235 passage through the 60  $\mu\text{m}$  sieve compared to 1 min grinding. Meanwhile, particle size  
236 produced might have decreased following 3 min grinding leading to larger and harder formation  
237 of aggregates > 60  $\mu\text{m}$  (Hotze et al., 2010; Ashraf et al., 2018). Despite a high variability  
238 according to grinding time in the weight of the 20–60  $\mu\text{m}$  fraction in MP made from rope, the  
239 mass of these particles was from 5 to 25 times greater than those made from spat collector. In  
240 addition, higher mass losses were observed from the rope than from the spat collector (**Table**  
241 **1**). This plastic mass loss may be explained in several ways, including the sticking of particles  
242 to lab materials, particle volatility or the production of small MP <20  $\mu\text{m}$ . In view of these  
243 results, grinding time seems to have a low influence on the production of 20–60  $\mu\text{m}$  particles  
244 and on the loss of plastic mass, and these effects are clearly smaller than the influence of plastic

245 type. Differences in grinding fractions between spat collector and rope could be related to  
246 plastic type properties including density, crystallinity and additive content as well as shape and  
247 weathering. Indeed, even though liquid nitrogen makes plastic matter more brittle, grinding  
248 efficiency may be influenced by plastic density (*i.e.* by taking more or less space in the grinding  
249 jar and thus affecting mechanical grinding), which was higher for rope than spat collector (jar  
250 filling<sub>coll</sub> > jar filling<sub>rope</sub>). It may also be influenced by the shape of the plastic pieces before  
251 grinding, which was quite flat and thin for spat collector but cylindrical and thicker for rope  
252 (**Figure 1C and F**), favouring fragmentation in the latter (Oyinloye and Yoon, 2020). In  
253 addition, the grinding efficiency of plastic types is likely influenced by their intrinsic  
254 mechanical resistance, which is partly dependent on the additives incorporated during their  
255 manufacture (*e.g.* plasticisers, antioxidants), and their weathering at sea, characterized by the  
256 loss of structural integrity induced by biotic (*i.e.* biodegradation) and abiotic (*e.g.* photolysis,  
257 hydrolysis, thermal degradation) factors (Andrady, 2011), including the leaching of additives  
258 (Hahladakis et al., 2018). For example, phthalate esters, such as bis(2-ethylhexyl) phthalate  
259 (DEHP), are commonly used as plasticisers to improve the quality and durability of polymers  
260 by increasing their flexibility (Rowdhwal and Chen, 2018). A previous study showed DEHP to  
261 be one of the most important plastic additives in both plastic types, with a higher concentration  
262 quantified in aged rope (95.51  $\mu\text{g g}^{-1}$ ) than spat collector (70.26  $\mu\text{g g}^{-1}$ ) (Gardon et al., 2020a).  
263 It is likely that differences in concentrations of DEHP and/or other specific additives might  
264 have influenced the mechanical resistance of both plastic types to cryogrinding. Therefore, it  
265 seems that the mechanical properties of plastic materials, their fate and, probably, their ageing  
266 are important factors to consider in the choice of grinding time, both to limit the loss of materials  
267 and optimize the yield of the targeted size range fraction. In our study, ageing was probably not  
268 a driving variable as the infrared spectra did not show strong oxidation of the collected  
269 polymers. Indeed, the carbonyl absorption band in the 1780–1660  $\text{cm}^{-1}$  region, which can be

270 used as a probe to monitor surface oxidation of PE and PP (Rowenczyk et al., 2020), remained  
271 weak (**Figure 1A–B**).

### 272 **3.2. Inconsistency between particle sieving range and actual size distribution**

273 Size distribution analyses of the 20–60  $\mu\text{m}$  fractions revealed an inconsistency between the  
274 theoretical sieving range and the actual particle sizes, regardless of the plastic type or grinding  
275 time. The effectiveness of the protocol was shown by the increased number of MP in the range  
276 from 20  $\mu\text{m}$  ( $\sim 130\text{--}870 \text{ MP } \mu\text{g}^{-1}$ ) to 60  $\mu\text{m}$  ( $\sim 0\text{--}130 \text{ MP } \mu\text{g}^{-1}$ ) according to the Coulter counter  
277 analyses (**Figure 3A–B**, focusing on the particle size distribution in the range of 4–60  $\mu\text{m}$ ). The  
278 peak particle number appeared to be mostly centred around 20  $\mu\text{m}$ . However, there was a  
279 decrease in particle frequency around 8  $\mu\text{m}$ , and further down, a large increase in the number  
280 of particles of smaller size (around 5  $\mu\text{m}$  and below; **Figure 3A–B**). Coulter counter analyses  
281 (only considering particles  $>2 \mu\text{m}$ ) showed that the actual 20–60  $\mu\text{m}$  fractions only reached  
282 5.7% ( $\sim 26,000 \text{ MP } \mu\text{g}^{-1}$ ), 17.8% ( $\sim 28,000 \text{ MP } \mu\text{g}^{-1}$ ) and 16.1% ( $\sim 28,000 \text{ MP } \mu\text{g}^{-1}$ ) of the total  
283 analysed particles from PP spat collector after 1, 2 and 3 min of grinding, respectively; and  
284 3.6% ( $\sim 17,000 \text{ MP } \mu\text{g}^{-1}$ ), 6.1% ( $\sim 18,000 \text{ MP } \mu\text{g}^{-1}$ ) and 8.4% ( $\sim 18,000 \text{ MP } \mu\text{g}^{-1}$ ) from PE rope,  
285 respectively (**Table 2**). No difference occurred between grinding times in the frequency  
286 distributions of particles across size ranges of 2–20  $\mu\text{m}$  and 20–60  $\mu\text{m}$  ( $P > 0.05$ ). Using  
287 grinding times as replicates ( $n = 3$ ), the relative abundance of particle count in the 20–60  $\mu\text{m}$   
288 fraction reached  $13.2 \pm 6.6\%$  and  $6.0 \pm 2.4\%$  for PP spat collector and PE rope, respectively;  
289 while averages in the 2–20  $\mu\text{m}$  fraction reached  $86.8 \pm 6.6\%$  and  $94.0 \pm 2.4\%$ , respectively. No  
290 significant difference was observed between plastic types according to size range. However, a  
291 significant lower number of particles ( $P < 0.0001$ ) was obtained in the 20–60  $\mu\text{m}$  fraction  
292 compared to the 2–20  $\mu\text{m}$  fraction (**Figure 3C**). Particle size characterization therefore  
293 demonstrated that 53–70% ( $\sim 82,000\text{--}333,000 \text{ MP } \mu\text{g}^{-1}$ ) of the total analysed particles in the  
294 20–60  $\mu\text{m}$  fractions were in fact  $<3 \mu\text{m}$  (**Table 2**). Such a result indicates the potential

295 occurrence of the smallest MP and nanoplastics (MNP) in the 20  $\mu\text{m}$  sieved plastic powder  
296 despite all precautions taken to dispose of MP  $<20\ \mu\text{m}$  (*i.e.* liquid sieving, sonication steps and  
297 the use of Tween-20<sup>®</sup> dispersing agent). Considering the similar Coulter counter results among  
298 the different grinding times, we focused our laser diffraction analyses on the 1-min grinding  
299 treatment, which confirmed and fine-tuned particle size distribution (considering particles  $>0.4$   
300  $\mu\text{m}$ ; **Figure 4**). Results showed a high number of particles below the 2  $\mu\text{m}$  threshold, with  
301 89.1% ( $0.70 \pm 0.75\ \mu\text{m}$ ) and 81.6% ( $0.82 \pm 1.02\ \mu\text{m}$ ) of total particles  $<1\ \mu\text{m}$  from spat collector  
302 and rope, respectively (**Figure 4A** and C). It demonstrates the gap between a conventionally  
303 used method (*i.e.* a Coulter counter) to characterize particle size distribution up to the 2  $\mu\text{m}$   
304 threshold (*e.g.* Schür et al., 2020) and a finer analysis including the nano fraction capable of  
305 detecting particles up to the 0.4  $\mu\text{m}$  threshold (Balakrishnan et al., 2020). This gap emphasizes  
306 the need to be very cautious when assessing particle size distribution on a volume or number  
307 basis. Considering that small particles will count very little in terms of volume/weight, but a  
308 great deal in terms of numbers (**Figure 4**), it clearly appears that their presence should not be  
309 ignored, especially when conducting ecotoxicological studies. Overall, our results highlighted  
310 the discrepancy between what is expected from the sieving and what is really obtained in the  
311 relevant fractions. Such data confirm the need to thoroughly check the size distribution of  
312 "homemade" MP using appropriate analytical methods for both micro- and nanosized particles.

### 313 **3.3. Particle self-assembly induces biased sieving and size assessment**

314 A plausible explanation for the observed sieving inefficiency is the formation of anisotropic  
315 assemblies, in which physical properties of particles differ according to their orientation. Such  
316 phenomenon has already been demonstrated by spherical NP uniformly grafted with  
317 macroparticles that robustly self-assemble into a variety of anisotropic superstructures when  
318 they are dispersed in the corresponding polymer matrix (Akkora et al., 2009; Damasceno et al.,  
319 2012). Self-assembly is the process by which individual components arrange themselves into

320 an ordered structure. Self-assemblies and particle interactions are probably less frequent when  
321 a polymer matrix is diluted during size characterization (Jain et al., 2004), making it possible  
322 to observe these diverging results between quantitative and qualitative assessments. This  
323 physical phenomenon is likely illustrated by our results on size distribution obtained with the  
324 Coulter counter, which showed totals of 6,800, 2,300 and 2,600 analysed particles from spat  
325 collector samples after 1-, 2- and 3-min runs, respectively (**Figure 3A**), and 7,200, 4,500 and  
326 3,300 from the rope samples (**Figure 3B**). Even though those results came from pseudo-  
327 replicates, the number of analysed particles was higher after 1 min of grinding than after 2 and  
328 3 min with either plastic type, suggesting a large amount of particle self-assembly, possibly  
329 depending on the size reduction induced by the longer grinding time. The formation of  
330 aggregates in the MP 20–60  $\mu\text{m}$  fraction was confirmed by laser diffraction analyses showing  
331 4% and 14% of total volume particles  $>60 \mu\text{m}$  from spat collector (**Figure 4B**) and rope (**Figure**  
332 **4D**) samples, respectively. Characteristics such as shape, surface properties, charge,  
333 polarizability or mass could determine the degree of self-assembly, producing structures and  
334 patterns at all physical scales (Whitesides and Grzybowski, 2002). Molecular mechanisms  
335 involved in self-assembly include non-covalent or weak-covalent interactions such as van der  
336 Waals, electrostatic and hydrophobic interactions (Whitesides and Grzybowski, 2002; Deng et  
337 al., 2020). According to our results, these interactions may be plastic type-dependent (Yanar  
338 and Kwetkus, 1995; Park et al., 2008) as well as influenced by particle shape and size  
339 (Damasceno et al., 2012). Spontaneous MP self-assembly into crystal superstructures could  
340 explain why the sieving was not sufficiently effective (Lee et al., 2018; Deng et al., 2020), as  
341 illustrated in the SEM images (**Figure 5**). This physical phenomenon may have occurred  
342 between the drying phase (**Figure 5A–B**) and the liquid sieving phase over the 20  $\mu\text{m}$  sieve of  
343 which the ethanol efflux was slow. This condition could be compared to the solvent  
344 evaporation-driven assembly system developed by Lee et al. (2018) (**Figure 5C–I**). It seems

345 that the sonicating bath containing Tween-20<sup>®</sup> did not have the expected effect of preventing  
346 particle aggregation, as illustrated by the formation of 3D crystals of cuboid shape on all  
347 physical scales (**Figure 5C–H**). Further research and development are needed to reduce these  
348 particle interactions in polymer matrices to dispose of more of the MP <20 µm and get as close  
349 as possible to the desired size range. A promising investigation way is the introduction of  
350 stabiliser/protectant that plays an active part in preventing hard agglomerate formation during  
351 the drying process (Yeap, 2018). For example, De Jaeghere et al. (1999) examined the re-  
352 dispersibility of suspended polyethylene oxide-grafted nanoparticles previously frozen into ice  
353 crystals before subjecting to lyophilization with and without lyoprotectant (trehalose). Results  
354 showed that lyophilized nanoparticles without trehalose was found to form agglomerates even  
355 after sonication for re-dispersion procedure. Meanwhile, the ability of lyophilized nanoparticles  
356 with trehalose to be re-dispersed was significantly enhanced. In this study, we have therefore  
357 demonstrated the necessity of considering size distribution as well as the total number of  
358 particles and aggregates, since the aggregates are not dissociated during the measurement  
359 process.

360 Here, we emphasised the importance to better understand MNP fate and behaviour aiming to  
361 control particle size distribution in the corresponding polymer matrix. This constitutes a  
362 preliminary step towards the description of more complex aquatic system, such as experimental  
363 conditions involving more than two components interacting with one another, such as  
364 microalgae (Demir-Yilmaz et al., 2022), and/or by considering natural organic matter as small  
365 aggregates already present in the dispersing medium (Clavier et al., 2019). This can result in a  
366 complex combination of homo- and heteroaggregates (Praetorius et al., 2020), especially in  
367 marine water where all particle contacts are effective due to high ionic strength promoting  
368 various aggregation pathways and several possible attachment efficiencies (Clavier et al.,  
369 2019). These complex interactions make their theoretical and experimental determination



370 challenging (Praetorius et al., 2020), while their influence on exposure parameters remain  
371 concrete on bioavailability in ecotoxicological studies.

### 372 **3.4. Implications for ecotoxicological studies**

373 In this study, we attempted to produce MP in a range of 20 to 60  $\mu\text{m}$  in order to match the  
374 retention size range of *P. margaritifera* (i.e. 2–200  $\mu\text{m}$ ) (Pouvreau et al., 1999), which would  
375 enable controlled exposition in future experiments. Particle characterization revealed that the  
376 MP produced by our method were  $\leq 60 \mu\text{m}$ , but results demonstrated an uncontrolled production  
377 of MP  $< 20 \mu\text{m}$ , mainly because of particles smaller than 1  $\mu\text{m}$  ( $> 80\%$  of the total particle  
378 number). This experimental artefact raises important questions for the interpretation of  
379 previously published result from (eco)toxicological studies that used grinding and sieving  
380 methods to produce MP without thoroughly characterizing the size range of the particles in the  
381 nanometric range (e.g. Schür et al., 2020). Some authors mention such bias to interpreting their  
382 toxicological data, as illustrated by Revel et al. (2019), who discussed a possible implication of  
383 NP in the observed toxicity with regards to their MP production method. Whenever possible,  
384 the proportion of small MP and NP must be characterized and considered in the exposition  
385 levels, especially with regard to the hypothesis that particle toxicity increases as size decreases  
386 (Jeong et al., 2016; Ma et al., 2016). This is a real problem for assessing the toxicity of MNP  
387 to obtain relevant data for decision support, but also because no data is available regarding *in*  
388 *situ* NP contamination (Schwaferts et al., 2019). In addition, the lack of consideration of the  
389 smallest particle sizes also raises an ethical issue associated to pollution with the possible  
390 release of NP in wastewater effluents from experiments. Indeed, NP may pass through most of  
391 the filtration devices, therefore entering the natural environment (Paul-Pont et al., 2018).

392 Overall, our study highlights that controlling the size distributions of small MP and NP  
393 following laboratory MP production poses some significant challenges since their hierarchical

394 self-assembly remains a physical problem. While waiting for better knowledge of *in situ*  
395 contamination, there is an important need to improve particle production methodology and  
396 associated characterization of particle populations.

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### 402 **Notes**

403 The authors declare no competing financial interest

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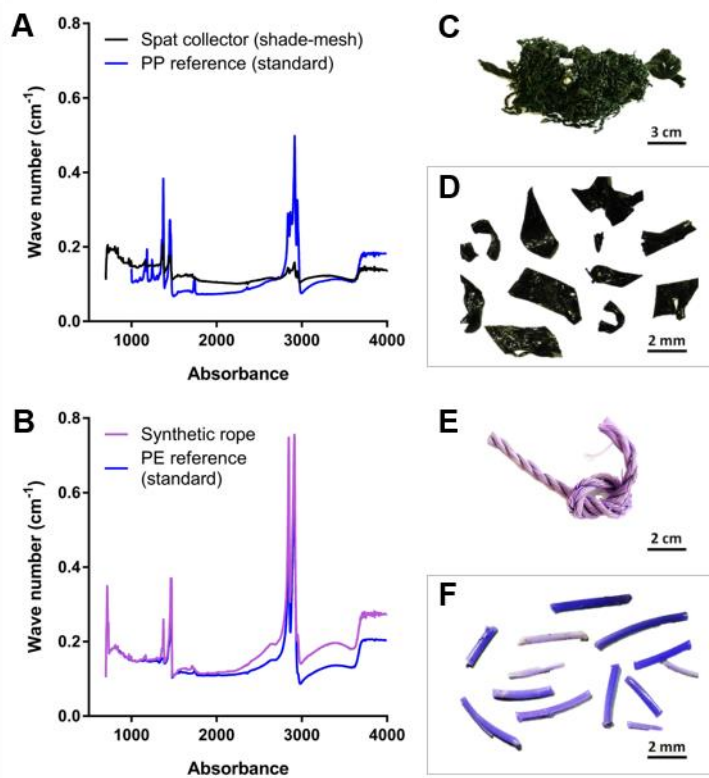
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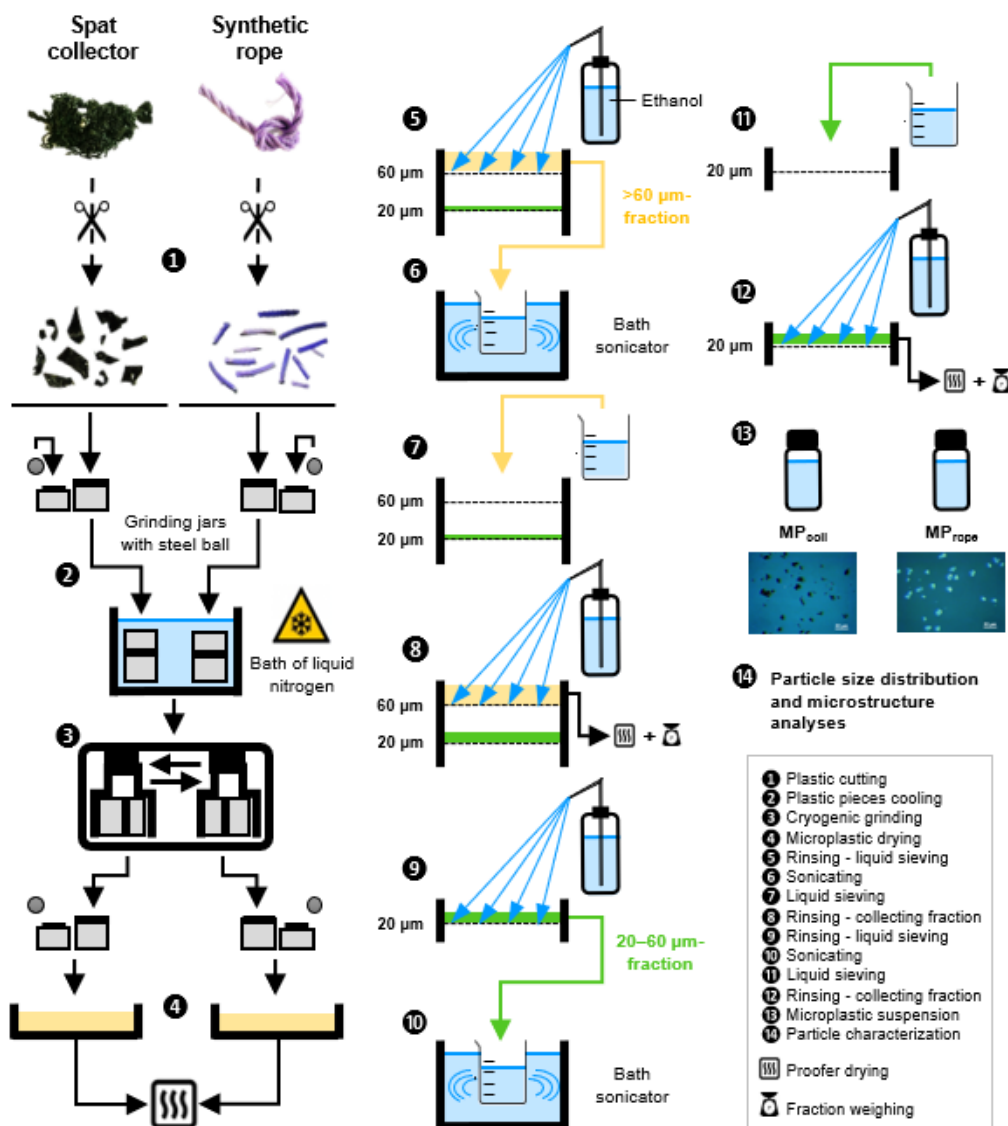


598

599 **Figure 1.** Polymer identification in two plastic pearl farming gear types by FTIR.  
600 Polypropylene (PP) for the spat collector (A) and polyethylene (PE) for the synthetic rope (B).  
601 Photographs show the spat collector (C) and synthetic rope (E) and their associated plastic  
602 pieces (D and F, respectively) used for MP production. Spectra of particles produced were  
603 identical to those of plastic gears, hence not added here. Adapted from Gardon et al. (2020a).

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607 **Figure 2.** Synthetic diagram of the microplastic production method tested.

608



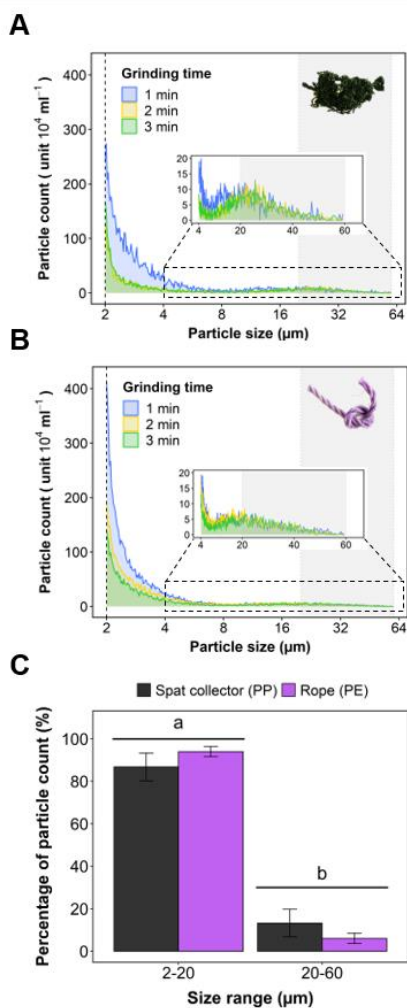
609 **Table 1.** Microplastic fractions produced from the two types of plastic pearl farming gear  
 610 according to grinding time.

Grinding time	MP fraction	Plastic type			
		PP spat collector		PE rope	
		Weight (g)	Proportion (%)	Weight (g)	Proportion (%)
<b>Initial</b>		4.0000	100.0	4.0000	100.0
<b>1 min</b>	<b>&gt;60 µm</b>	3.9588	99.0	3.6410	91.0
	<b>20–60 µm</b>	0.0344	0.9	0.1490	3.7
	<b>Loss*</b>	0.0068	0.2	0.2100	5.2
<b>2 min</b>	<b>&gt;60 µm</b>	3.9377	98.4	3.1050	77.6
	<b>20–60 µm</b>	0.0295	0.7	0.6900	17.2
	<b>Loss*</b>	0.0328	0.8	0.2050	5.1
<b>3 min</b>	<b>&gt;60 µm</b>	3.8676	96.7	3.6890	92.2
	<b>20–60 µm</b>	0.0305	0.8	0.1620	4.0
	<b>Loss*</b>	0.1019	2.5	0.1490	3.7

611

612 \* The loss of plastic mass was calculated by subtracting the weighed MP fractions from the  
 613 initial 4.00 g of plastic pieces.

614



616

617 **Figure 3.** Particle size distribution of microplastics obtained in the 20–60  $\mu\text{m}$  fraction as  
 618 assessed by Coulter counter analyses. Density plots of particle size distribution of MP 20–60  
 619  $\mu\text{m}$  fraction obtained from spat collector (A) and rope (B) samples according to grinding times  
 620 of 1, 2 and 3 minutes. Bar chart of the relative abundance of particle count across size ranges  
 621 of 2–20  $\mu\text{m}$  and 20–60  $\mu\text{m}$  according to plastic type (C). The dashed black line on the density  
 622 plots indicates the minimal 2  $\mu\text{m}$  detection thresholds from the Coulter counter (equipped with  
 623 a 100  $\mu\text{m}$  aperture tube) analysis. The grey area corresponds to the 20 to 60  $\mu\text{m}$  sieving range  
 624 used in the present microplastic production method. Bar chart illustrate mean  $\pm$  SD with  
 625 grinding times as replicates ( $n = 3$ ). Letters indicate statistically significant differences between  
 626 groups.

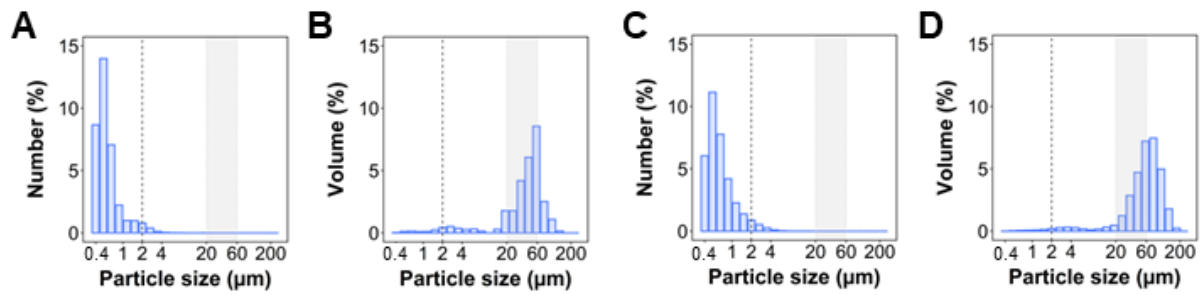
627 **Table 2.** Relative abundance of microplastics produced in the 20–60  $\mu\text{m}$  fraction according to  
628 their size distribution above the 2  $\mu\text{m}$  threshold.

Plastic type	Grinding time	Percentage of particle count (%)				
		2-3 $\mu\text{m}$	<5 $\mu\text{m}$	<10 $\mu\text{m}$	<20 $\mu\text{m}$	20–60 $\mu\text{m}$
PP spat collector	1 min	62	82	88	94	6
	2 min	53	66	71	82	18
	3 min	56	69	74	84	16
PE rope	1 min	70	88	92	96	4
	2 min	60	80	87	94	6
	3 min	60	79	84	92	8

629

630

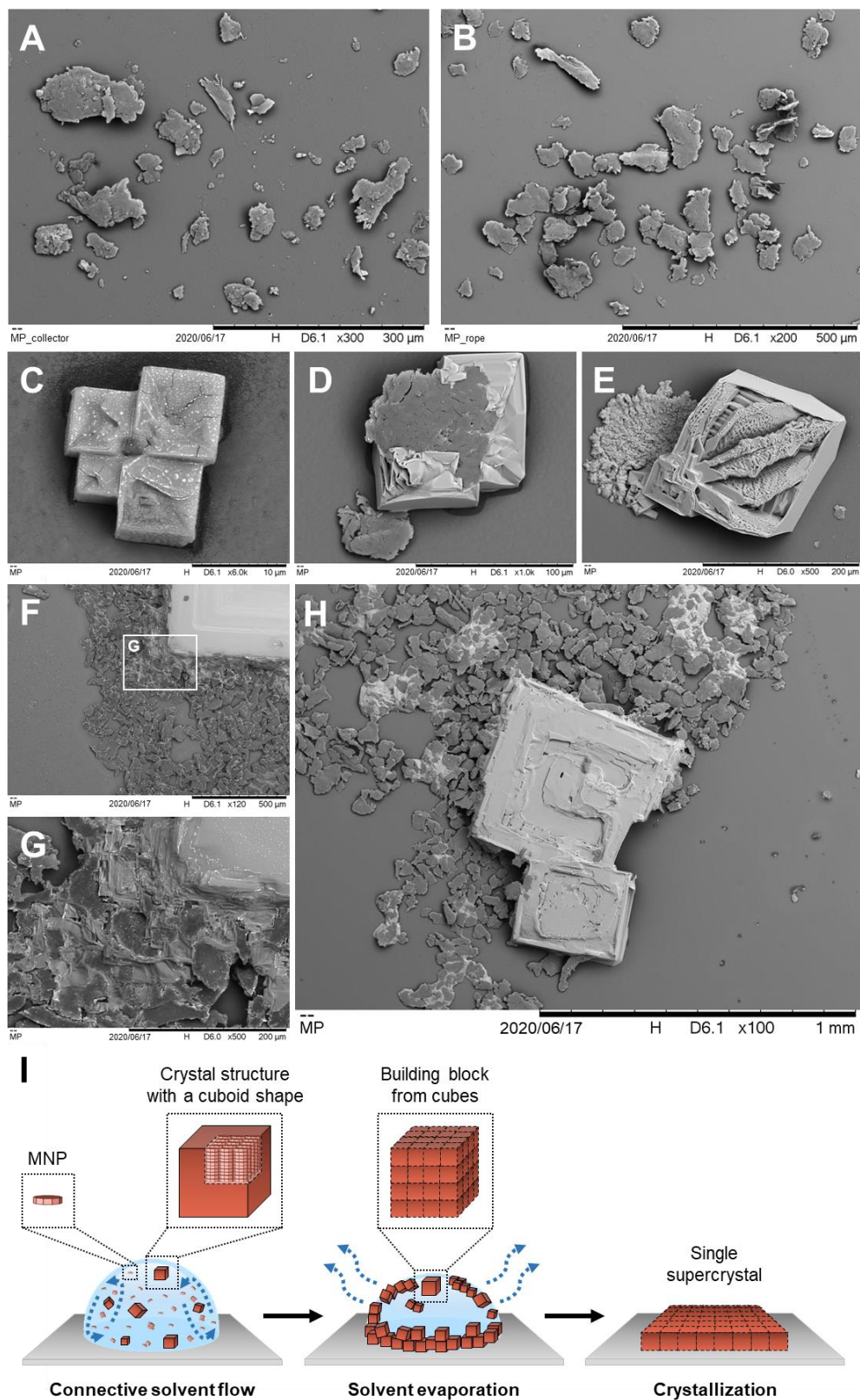
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632

633 **Figure 4.** Particle size distribution of microplastics obtained in the 20–60 μm fraction as  
634 assessed by laser diffraction analyses. Bar plots (A–D) show the differences in number and  
635 volume of particle sizes made from spat collector (A–B) and rope (C–D) by 1 min of grinding.  
636 The dashed black line indicates the minimal 2 μm detection thresholds from the Coulter counter  
637 (equipped with a 100 μm aperture tube). The grey area corresponds to the 20 to 60 μm sieving  
638 range used in the present microplastic production method.

639



641

642 **Figure 5.** Representative SEM images of microplastics in the 20–60  $\mu$ m fraction produced in  
 643 this study and their spontaneous self-assembly into crystal superstructures through slow solvent

644 evaporation. Pictures of MP made from spat collector (A) and rope (B) samples taken in dry  
645 conditions. Pictures of ordered assemblies from colloidal micro- and nanoplastics (MNP) of  
646 various shapes into crystalline structures with a cuboid shape at all physical scales (C–H). These  
647 spontaneous assemblies are associated with attractive forces among particles (*e.g.* van der  
648 Waals forces, electrostatic, hydrophobic interactions) and ligand–substrate interactions (*e.g.*  
649 chemisorption) responsible for forcing particles to self-arrange into ordered assemblies with  
650 multi-layered superstructures (F–G). Connective flow during solvent evaporation concentrates  
651 plastic particles and 3D crystals near the drying front, giving rise to building blocks that grow  
652 to larger dimensions until complete solvent evaporation occurs and supercrystals form at the  
653 millimetric scale (H). A synthetic scheme adapted and redrawn from Lee et al. (2018) and Deng  
654 et al. (2020) illustrates this process (I).

655