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Juvenile fish caging as a tool for assessing microplastics contamination in estuarine fish nursery grounds

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30 **Abstract**

31
32 Estuaries serve as nursery grounds for many marine fish species. However, increasing human activities
33 within estuaries and surrounding areas lead to significant habitat quality degradation for the juveniles. In
34 recent years, plastic pollution has become a global environmental issue as plastic debris are found in all
35 aquatic environments with potential adverse impacts on marine biota. Given the important ecological role
36 of estuaries and implications of microplastics (MP) in ecosystems, here we assess the occurrence,
37 number, size and polymer types of MP ingested by wild and caged juveniles European flounder
38 (*Platichthys flesus*). We deployed caged fish for one month at five sites in three estuaries in the Eastern
39 English Channel. The Seine estuary, heavily impacted by manmade modifications and one of the most
40 contaminated estuaries in Europe, was compared to two smaller estuaries (Canche and Liane) less
41 impacted by industrial activities. We found that juvenile flounders (7- 9 cm) were vulnerable to plastic
42 ingestion. 75% of caged fish and 58% of wild caught fish had the presence of MP items in their digestive
43 tract. Fibers (69%) dominated in the fish's digestive tract at all sites. An average of 2.04 ± 1.93 MP items
44 were ingested by feral juveniles flounder and 1.67 ± 1.43 by caged juveniles flounder. For the caged fish,
45 the three sites impacted by wastewater treatment plant (Liane, Le Havre Harbor and Rouen) were those
46 with the highest percentage of individuals that have ingested MP items. Most of the isolated items were
47 fibers and blue in color. Polymers identified by micro Raman spectroscopy were Polycaprolactam,
48 Polyethylene Terephthalate and Polyurethane. Although other environmental factors may have affected
49 caged fish condition and mortality, we found no significant correlation with the number of ingested MP.
50 However, the high occurrence of MP ingested by juvenile fish on nursery grounds raises concerns on their
51 potential negative effects for fish recruitment success and population renewal. Finally, this study
52 describes, for the first time, the feasibility of using caged juvenile fish as an assessing tool of MP
53 contamination in estuarine nursery grounds.

54
55 **Keywords:** Microplastics; caging; juvenile flounder; estuaries; Raman spectroscopy.

1. Introduction:

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57
58 The occurrence of microplastics (defined as particles <5 mm in their longest size) in aquatic
59 ecosystems (marine and freshwater) is well documented (for review: Wright et al. 2013; Cole et
60 al. 2014; Van Cauwenberghe et al. 2015). Due to their different densities ranging from 0.9 g/cm³
61 (Polystyrene and Polypropylene) to 1.39 g/cm³ (Polyethylene terephthalate and Polyvinyl
62 chloride), they are either found at the water surface layer (Ivar do Sul and Costa 2014) or sunk to
63 the bottom (Woodall et al. 2014). Therefore, both pelagic (Collard et al. 2015) and benthic
64 species (McGoran et al. 2017) may be affected by these plastic pieces. Many aquatic species
65 have now been reported to ingest plastics from the environment. Microplastics (MP) can enter
66 the food web of aquatic environments via direct or indirect pathways, including inhalation,
67 entanglement, ingestion from incidental capture, being mistaken for food, or by the ingestion of a
68 prey species already containing microplastics (Au et al. 2017; Setälä et al. 2018). Because of
69 their ubiquitous presence, their small size, and the chemical pollutants existing in plastics (such
70 as additives or adsorbed contaminants from the surrounding environment), MP could threaten the
71 health of various organisms (Auta et al. 2017). Indeed, the ingestion of MP may cause both
72 direct physical and toxicological effects. Physical effects include internal abrasions and gut
73 blockages, which may lead to starvation (Wright et al. 2013; Gall and Thompson 2015). Among
74 other potential effects, the ingestion of MP instead of food may lead to a delay in growth (e.g.
75 due to starvation), a decrease in the individual fitness, and even causing death, with likely
76 negative effects on population dynamics (Rochman et al. 2013; Luis et al. 2015; Lönnstedt and
77 Eklöv 2016; Critchell and Hoogenboom 2018). In recent years, an increasing number of studies
78 have been carried out to assess the occurrence and effects of MP in marine fish species (e.g.,
79 Lusher et al. 2013, 2017; Nadal et al. 2016; Neves et al. 2015). However, few studies have
80 concerned estuarine fish (but see McGoran et al. 2017; Vendel et al. 2017; Bessa et al. 2018;
81 Ferreira et al. 2018). These studies focused mainly on tropical estuaries and on wild-caught adult
82 fish. Estuaries are known as essential fish habitats because they act as nursery grounds for
83 juveniles of various marine fish species, providing refuge, food, and habitat (Beck et al. 2001;
84 Selleslagh and Amara 2008). Despite their ecological importance, estuaries are amongst the most
85 modified and threatened aquatic environments (Halpern et al. 2008). These areas are exposed to
86 a growing anthropogenic pressure, particularly through acute and chronic pollutions such as

87 industrial and wastewater effluents discharge. Estuarine ecosystems have been identified as
88 microplastics hotspots (Browne et al. 2011, Wright et al. 2013).

89 In order to compare different sites or estuaries, it is necessary to investigate the same species of
90 the same age range at each site. However, it is almost impossible to find a species that is present
91 in all sites of interest. To cope with this problem, transplant experiments can be conducted
92 (Oikari 2006; Kerambrun et al. 2011). Caging experiments present many advantages (Oikari
93 2006) including the selection of well-characterized homogenous organisms (number, age, size,
94 weight, and sex) and the control of their exposure (location, time, and season). In addition, this
95 technique offers advantages over simple field collection of organisms since it is possible to study
96 an impacted zone surrounding a relatively precise outlet discharging pollutants. Such approach
97 was successfully used to monitor microplastics contamination in mussels (Catarino et al. 2018;
98 Railo et al. 2018). To the best of our knowledge, juvenile fish caging, as a tool for assessing
99 estuarine microplastics contamination, has not been investigated before.

100 The aims of this research were to estimate the occurrence, number, size and polymer types of MP
101 ingested by wild and caged juveniles European flounder (*Platichthys flesus*) and to test the
102 caging method as a tool to quantify and assess MP contamination of juvenile fish. We also tested
103 the hypothesis that ingested plastic adversely affects the condition and survival of caged fish.
104 The European flounder, was selected for the study because it is one of the most important
105 components of the juvenile demersal fish assemblage in European estuarine waters (Selleslagh et
106 al. 2009). This benthic species is commonly used for environmental monitoring studies in
107 northern European waters (e.g. Marchand et al. 2003; Amara et al. 2009).

108

109 **2. Materials and methods:**

110

111 **2.1. Study sites**

112

113 The study area was located along the French coast of the Eastern English Channel. Three
114 estuaries were investigated: the Liane, Canche and Seine estuaries (Fig 1). Liane and Canche are
115 small estuaries with small freshwater input: 3 and 7 m³.s⁻¹, respectively. The Liane estuary is
116 mainly affected by a municipal wastewater treatment plant (WWTP) that treats the wastewater of
117 ca. 200,000 inhabitants. The Canche estuary is not impacted by any important human activity

118 and is considered as clean estuary (Amara et al. 2009). The Seine estuary, the largest one in the
119 English Channel (150 km² at high tide), displays a strongly urbanized and industrialized basin
120 since it concentrates approximately 40% of the economic activity of France. In spite of
121 significant efforts to restore environmental quality during the past few decades, it remains one of
122 the most chemically polluted estuaries in Western Northern Europe (Dauvin et al. 2007).

123

124 **2.2. Sampling and caging experiment**

125

126 In September 2017, 150 0-group juveniles' flounder (7- 9 cm total length, TL) were collected in
127 the Canche estuary using a small beam trawl. After capture and before deployment in cages, the
128 fish were acclimatized for one week in a 500 liter aquarium supplied with an open seawater
129 circuit and were daily fed on frozen Mysidacea and brine shrimps (*Artemia* sp.).

130 One day before the caging experiment, flounders were anaesthetized (Eugenol 35 mg/L),
131 weighed (to the nearest 1 mg), measured for total length (within 0.1 mm), and individually
132 marked (Visual Implant Tag, 1.2 mm×2.7 mm, Northwest Marine Technology).

133 Cage placement was carried out the 12th and 13th of September 2017 at five sites. Three sites
134 were chosen in the Seine estuary: Rouen (49°22.995' N; 01°00.676' E), Le Havre harbor (49° 28.
135 853' N; 00° 05.590' E) which are both affected by a wastewater treatment plant (Emeraude and
136 Edelweisse, respectively), and Fosse Nord (49°27.328' N; 00°07. 493' E) in the main channel of
137 the estuary. Two other cages were put in the Canche (50°30.982' N; 01°37.852' E) and the Liane
138 estuaries (50°42.08' N; 01°36.59' E). The number of fish placed inside the cages was between 15
139 and 20 fish per cage. The cages were made of Stainless steel without any plastic material to
140 avoid contamination. Their length was of 1 m, whereas their width and height were of 0.6 m.
141 Their mesh size was 15 mm allowing water circulation and enough space for fish to feed. The
142 cages were fixed to the bottom with a screw anchor secured by scuba-divers at depths varying
143 between 4 to 8 m. Following the one month caging exposure, all fish were rapidly transferred to
144 the laboratory, identified (tag), weighed, and measured. In order to evaluate the potential effect
145 of microplastics contamination on juvenile fish, we calculated the Fulton's K condition index as
146 an indicator of the fish general well-being.

147 $K = 100 W/L^3$; where (W) is the body mass (mg) and (L) is the total length (mm).

148 Along with the caging experiment, feral juvenile flounders of the same age (G0) and size (7- 9
149 cm TL) were collected in September 2017 at two sites near the caging sites: the Canche and the

150 Seine (Fosse Nord) estuaries in order to compare microplastics contamination between feral and
151 caged fish. Although we sampled in all the sites used for the caging experiment, we did not
152 capture flounder at the other three sites.

153

154 **2.3. Microplastics analysis**

155

156 Flounders were dissected under a laminar flow hood and their digestive tract (stomach and gut)
157 were weighted, preserved in aluminum foil, and conserved at -20°C until analysis. Cotton
158 laboratory coats were worn at all times during samples analysis, dissecting instruments were
159 cleaned with MilliQ water after every dissection, and the usage of plastic material was avoided.
160 Prior to digestion, digestive tracts were taken out of the freezer and left to thaw. All the
161 following procedures were done under a laminar flow hood. Solutions used (besides MilliQ
162 water) were filtered three times using glass fiber filters GF/A with a pore size of 1.6 µm
163 (Whatman, France). All materials were cleaned with MilliQ water, filtered ethanol 70%, and
164 MilliQ water, respectively. The digestive tract of each individual was taken and emplaced in an
165 Erlenmeyer with a volume of 100 mL of filtered KOH 10% (m/v, ChimiePlus, France) (Dehaut
166 et al. 2016; Hermsen et al. 2017). With every digested lot constituted of 9 erlenmeyers each one
167 containing one digestive tube; one control was made containing only 100 mL of KOH and has
168 undergone the same digestion conditions as the samples. These Erlenmeyers were put on a
169 heating magnetic stirrer for 24 hours at 60°C. Then, each solution was vacuum filtered on a 47
170 mm GF/A filter (Whatman, France). Each filter was put inside a clean glass Petri dish and
171 surrounded with parafilm. Filters remained covered until Raman analysis to avoid prolonged
172 exposure to atmospheric contamination from dust.

173

174

175 **2.4. Stereomicroscope observation and micro-Raman spectroscopy analysis**

176

177 After digestion, filters were observed under 120x magnification using Leica M165 C
178 Stereomicroscope and images of plastic items recovered were taken with a Leica M170 HD
179 camera and LAS (Leica application suite) software. The filters were methodically examined
180 from left to right along the first row, right to left along the second row and so on, to prevent

181 double-counting of MP. Microplastics were classified according to their physical characteristics
182 into fibers, fragments, and films. They were counted, measured at their longest dimension, and
183 their color was noted. During stereomicroscope inspection, samples remained closed inside the
184 Petri dish. Whereas for Raman analysis, filters were placed inside and the machine was directly
185 closed to avoid airborne contamination.

186
187 Five filters containing potential MPs, were randomly selected per site and analyzed using a
188 Micro-Raman Xplora Plus (HORIBA Scientific® France). Each filter corresponds to the
189 digestive tract of an individual fish. Two lasers were used with a wavelength of 532 nm and 785
190 nm with a range of 200-3500 cm^{-1} . Two objectives (Olympus, Rungis, France) were used: x10
191 and x100. Filters were either analyzed manually or using ParticleFinder module for LabSpec
192 (Frère et al. 2016). This latter is an automated application that locates particles and performs
193 Raman analysis on these particles by moving each particle beneath the laser spot. Each particle
194 spectrum is compared to Database polymer identification software (KnowItAll, BioRad®) and a
195 personal library made with specific polymers obtained from Goodfellow (France). The
196 identification is considered correct when the HQI (Hit Quality Index) was above 80 (ranging
197 from 0 to 100).

198 **2.5. Statistical analysis**

199
200 Data were analysed using XLSTAT software. The conditions for applying parametric tests, i.e.
201 homogeneity of variance and normality, were verified using Fisher and Shapiro-Wilk tests
202 respectively. As result of these tests, non-parametric tests (Kruskal-Wallis (KW) and Mann-
203 Whitney U-test) were used in order to highlight significant differences of MP contamination in
204 flounder caged at different locations and with feral individuals collected at the same site.
205 Differences between groups were considered as significant when $p < 0.05$. The KW test was
206 followed by a post hoc test Multiple Comparisons of p-value (MCP) when it was significant at p
207 < 0.05 . Data are expressed in mean \pm standard deviation (SD).

208 **3. Results**

209

210

211 **3.1. Caging experiment**

212
213 After one-month of exposure, all the cages were recovered. The number of fish in each cage was
214 counted and survival percentage was calculated. The mean percentage of survival was 70.59%
215 and all sites had a high survival rate (> 70%) except for the Canche estuary where the cage was
216 partially silted and, therefore, having the lowest survival percentage of 37% (Fig. 2). The
217 Fulton's K condition factor of each individual flounder analyzed varied between 0.55 and 1.39
218 $\text{mg}\cdot\text{mm}^{-3}$ (mean value $0.79 \pm 0.11 \text{ mg}\cdot\text{mm}^{-3}$). Individuals from Le Havre Harbor and Fosse Nord
219 had a significantly lower K compared to the Canche and Rouen (Fig. 2).

220
221

222 **3.2. Microplastics occurrence in fish**

223
224 A total of 86 fish (feral and caged) were analyzed. In all the examined fish, 149 items were
225 identified on the filters using the stereomicroscope as potential MP consisting of 103 fibers, 43
226 fragments, and 3 films (Fig. 3). Fibers (69%) dominated in fish's digestive tract whereas films
227 were only observed in feral fish in Fosse Nord and caged fish in Le Havre Harbor with an
228 average number of 0.2 ± 0.42 and 0.083 ± 0.28 , respectively. An average of 75% of caged fish
229 had at least one MP items (fragments, fibers, and films) in their digestive tract (64 caged fish
230 analyzed) with Le Havre harbor having the highest percentage of 91.7% (Fig. 4). For the feral
231 fish, Fosse Nord had a higher percentage of contaminated fish (80%) than that of the Canche
232 estuary (36.4%) (Fig. 4). An average of 2.04 ± 1.93 items were ingested by feral juvenile
233 flounder and 1.67 ± 1.43 in caged juvenile flounder (Fig. 5). Although not significantly different
234 (Mann-Whitney U-test, $p=0.097$), the number of MP items in feral fish was higher in the Seine
235 estuary (Fosse Nord) compared to the Canche estuary. For the same site, where both feral and
236 caged fish were analyzed, the number of MP items ingested by feral fish was higher but only
237 significant for Fosse Nord (Mann-Whitney U-test: Fosse Nord $p=0.011$; Canche $p=0.970$) than in
238 caged fish (Fig. 5). For caged fish, the number of ingested items was highest in the Liane ($2.47 \pm$
239 1.51) and lowest in the Canche (0.90 ± 0.99) and Fosse Nord (0.93 ± 0.70). A significant
240 difference was only observed between the Liane and Fosse Nord (KW test, $p=0.004$). There was
241 no significant correlation between juvenile fish condition and the number of MP ingested at each
242 site ($p=0.336$). In addition, the mortality rate observed in caged fish at each site is not correlated

243 to the mean number of MP ingested ($p= 0.09$). On the contrary, the sites with the lowest
244 mortality (Liane, Le Havre Harbor and Rouen) corresponded to those with the highest number of
245 MP ingested.

246 **3.3. Characterization of microplastics**

247
248 Color distribution of ingested items was mostly uniform across all analyzed sites, blue MP being
249 the most common (54%), followed by red (21%) and black (13%), while other colors such as
250 pink, white and green were less frequent (Fig. 6a). The size of fibers ranged from 70 μm to 4510
251 μm and for the fragments between 5 μm and 66 μm . Fragments, films, and fibers were divided
252 into several size classes: 0-200 μm , 200-400 μm , 400-600 μm , 600-800 μm , 800-1000 μm , and >
253 1 mm. Most of the isolated MP belonged to the smallest (< 200 μm) and largest (> 1mm) size
254 class with respectively 35.6% and 24.2% while the other size classes had a similar distribution
255 (Fig. 6b). There was no inter-sites difference in ingested item size except for the Canche (for
256 feral and caged fish) where the largest size class dominates.

257 Five filters were randomly selected from each site and analyzed using μ -Raman to confirm if the
258 particles extracted were plastics by identifying their chemical composition. In the Raman
259 spectrum of fibers, only fluorescence could be observed, although an optimization including the
260 reduction of laser power and bleaching was attempted. In addition, for the colored items, the
261 spectrum was hidden by the additives (dyes) existing on particles. Only 37 fragments were
262 successfully analyzed with the Raman. Among these fragments, eleven were identified as
263 polymers: Polycaprolactam (PA-6), Polyethylene Terephthalate (PET) and Polyurethane (PUR).
264 For colored particles (blue and green), the observed spectrum was that of the dye. Two
265 corresponding spectra were observed: Copper Phthalocyanine (specific of blue items and the
266 most frequent obtained spectrum) and Hostasol Green G.K (which is characteristic of green
267 items). Fibers were not identified with the Raman due to its delicate procedure when identifying
268 thin and small fibers; suggesting that microplastics ingestion might have happened in lower
269 proportion than mentioned above.

270 The spectral range of PA-6, PET, PUR and Copper phthalocyanine are presented in the
271 Supplementary Material: The PA-6 having its characteristic peaks between 900 cm^{-1} and 1500
272 cm^{-1} , and 2500 cm^{-1} and 3000 cm^{-1} . Whereas for PET, characteristics peaks were between 600
273 cm^{-1} and 1700 cm^{-1} and 3000 cm^{-1} and 3400 cm^{-1} (decreased trend).

274 When excluding the colored items, we observed that in wild caught fish from the Canche and
275 Fosse Nord, the MP items were made of Copper Phthalocyanine and PA, respectively. In caged
276 fish, the MP items were made of PA in the Liane and in the Canche. Whereas for the three Seine
277 estuary sites, PET was the predominant polymer (61%) followed by PA and PUR (Fig. 7).

278

279 **4. Discussion**

280

281 This research identified and quantified, for the first time, the presence of ingested microplastics
282 in feral and caged juvenile fish (≤ 9 cm TL) from the Eastern English Channel estuaries
283 highlighting their potential negative effects. In this region, estuaries provide nursery areas for a
284 wide variety of marine fish species including commercially important fish such as seabass, sole,
285 plaice, and flounder (Selleslagh et al. 2009). Estuaries are also used by adults as reproduction,
286 migration, and feeding grounds (McLusky and Elliott 2004). These ecosystems play an important
287 role in maintaining biodiversity and constitute an essential fish habitat supporting future
288 recruitment to adult fish stocks (Beck et al. 2001). However, increasing human activities within
289 estuaries and surrounding areas, lead to a significant habitat loss for the juveniles and a decrease
290 in the quality of the remaining habitats as was reported for the Seine estuary (Gilliers et al. 2006;
291 Courrat et al. 2009).

292 Several studies have identified the presence of microplastics in the digestive tracts of wild-
293 caught fish. However, the level of fish contamination in transitional systems such as estuaries is
294 less known. Most of the studies were conducted in tropical estuaries (Dantas et al. 2012; Ramos
295 et al. 2012; Possatto et al. 2011; Vendel et al. 2017; Bessa et al. 2018; Ferreira et al. 2018). Only
296 two studies have been conducted in temperate estuaries: McGoran et al. (2017) in River Thames,
297 UK and Bessa et al. (2018) in the Mondego estuary (Portugal). We found that estuarine juvenile
298 flounders are vulnerable to plastic ingestion: 75% of caged fish and 58% of wild caught fish had
299 the presence of MP items in their digestive tract. In a recent study, McGoran et al. (2017) found
300 that over 70% of River Thames adults European flounder had ingested plastics. These results are
301 high compared to previously published estimates of plastic ingestion by marine fish (both pelagic
302 and demersal species) which ranged from 2.6 % in the North Sea (Foekema et al. 2013), 18% in
303 the Central Mediterranean (Romeo et al. 2015), 28% in the Adriatic Sea (Avio et al. 2015), and
304 41% in the Eastern Mediterranean (Guvén et al. 2017). In comparison with our study area,

305 Lusher et al. (2013) reported that 37% of fish in the English Channel had ingested MP, whereas
306 this ingestion was only 5.4% in the southern North Sea (Foekema et al. 2013). The high
307 occurrence of MP in estuarine fish suggests that MP are more common within estuarine water
308 column and sediments than in the marine environment (Anderson et al. 2018). These transitory
309 waters are important transport routes of MP into the marine environment since about 80% of
310 marine plastics are derived from land-based anthropogenic sources (Andrady 2011; Schmidt et
311 al. 2017). Mean concentration in rivers is roughly 40–50 times higher than the maximum
312 concentration observed in the open ocean (Schmidt et al. 2017). Estuaries are also dominated by
313 fine sediments in the subtidal and intertidal mudflats which can act as important short-term and
314 longer-term sinks for MP (Browne et al. 2010; Horton et al. 2017; Leslie et al. 2017) as often
315 occurs with other contaminants such as metals, hydrocarbons, and pesticides. For example, in
316 two South Carolina Estuaries, intertidal sediments contained a greater amount of microplastics
317 than the sea surface microlayer (Gray et al. 2018). Estuaries are considered as hotspots of MP
318 contamination (Browne et al. 2011; Wright et al. 2013). This means that estuarine fish are
319 exposed to a higher concentration of MP and, thus, have a higher probability of MP ingestion
320 than marine species.

321 Several studies showed higher frequencies of fibers compared with other forms of microplastic
322 in a variety of marine environments (see Cole et al. 2013 and Wright et al. 2013). In most
323 studies, fibers were the dominant type of microplastics ingested by estuarine fish (> 90%)
324 (Ferreira et al. 2018; Bessa et al. 2018). In our study, fibers (69%) constituted the majority of
325 items found in the digestive tract of the juvenile flounders. This percentage was similar to the
326 one observed in flounders (70%) from the River Thames (McGoran et al. 2017). The dominance
327 of fibers seems to be a typical pattern for many other demersal fish in other locations (e.g.
328 Lusher et al. 2017; Bessa et al. 2018). As suggested by Ferreira et al (2018), filaments may
329 resemble as natural food items for juvenile flounders (such as nematodes, amphipods, and
330 polychaetes) resulting in mistaking them as preys. The high contamination of fibers in estuarine
331 organisms supports Jabeen et al. (2017) suggestion that freshwater systems and estuaries
332 (transitional systems) are more likely to be contaminated by fibers. For example, in the Solent
333 estuary (UK) more than 80% of particles collected in the water column were fibers (Gallagher et
334 al. 2016). In the Seine River water, Dris et al. (2015) found that fibers were dominant with an
335 average of 45 fibers/m³ and 0.54 fragments/m³ in the water column. Even though the main

336 sources of fibers in these systems are not fully determined, they could be related with
337 Wastewater Treatment Plants (WWTPs) (Browne et al. 2011; Klein et al. 2015). While they are
338 able to retain a high proportion, e.g., from 83% to 95%, WWTPs effluents still constitute an
339 important source of fibers (Dris et al. 2015; Leslie et al. 2017). Fibers of all colors were found in
340 the gut of juvenile flounders, but blue fibers were predominant. This is also a typical observation,
341 reported worldwide, for estuarine fish species (Possatto et al. 2011; Vendel et al. 2017, Bessa et
342 al. 2018; Ferreira et al. 2018) and also for marine and freshwater species (Lusher et al. 2017). A
343 recent study investigating the removal of microplastics by WWTPs determined that blue
344 microplastic fibers were most often released from WWTPs (Conley 2017). During the caging
345 experiment, the three sites (Liane, Le Havre Harbor, and Rouen) that are affected by wastewater
346 treatment plant effluent presented the highest ingested number of fibers per fish, suggesting the
347 role of WWTPs as an important source of fibers in estuaries. However, abandoned ropes, fishing
348 gears (Browne et al. 2011) and atmospheric fallout of fibers (Dris et al. 2017) could be as
349 potential sources of fiber contamination in the aquatic systems.

350
351 The characterization of the extracted particles involved an identification of the plastic component
352 using micro-Raman spectroscopy. In the Raman spectrum of fibers, only fluorescence could be
353 observed, although an optimization including the reduction of laser power and bleaching was
354 attempted (see Kappler et al. 2016). Yet, when the sample is thin, Raman tends to detect the
355 underlying substrate instead of the sample (Kappler et al. 2015) which explains the problem we
356 had when identifying fibers' nature. Raman is able to achieve a better spatial resolution (down to
357 1 μm) than FT-IR (10 μm) (Lenz et al. 2015) but the identification of fibers relies mainly on FT-
358 IR as Raman analyses did not prove to be efficient so far for this type of microplastics (Kappler
359 et al. 2016). For the colored items, the spectrum was hidden by the additives (dyes) existing on
360 particles. Even if these spectra were subtracted, polymers could not be identified due to the
361 intense additives' spectra (Van Cauwenberghe et al. 2013; Van Cauwenberghe and Janssen
362 2014). This problem was discussed by many authors (see Collard et al. 2015; Lenz et al. 2015;
363 Frère et al. 2016) and, therefore, preventing polymer identification. As the analysis of fibers was
364 not conclusive with the Raman, most of the analyzed MP items were fragments. As previously
365 mentioned, the majority of MP items identified were fibers so we only have a partial
366 representation of the type of polymers ingested by flounders. A combination of identification

367 techniques is necessary for a complete and reliable characterization of the chemical composition
368 of plastics (Kappler et al. 2016; Hermabessiere et al. 2018). The types of polymers identified
369 were Polycaprolactam (PA), Polyethylene Terephthalate (PET), and Polyurethane (PUR). Less
370 dense MP such as polyethylene (PE) and PUR can be found on the surface or in the water
371 column while denser plastics like PA and PET sink and reside primarily in sediments
372 (Chubarenko et al. 2016). The presence of Polyurethane in fish caged in Rouen may be explained
373 by the presence of numerous petrochemical industries in and near this site. Another explanation
374 could be that this low dense polymer (PUR) may have sunk to the bottom since the site of Rouen
375 is characterized by a low water density (salinity = 0.4) compared to the other sites which are
376 characterized by higher water salinity ranging from 17.5 at Fosse Nord and 30.5 at Le Havre
377 Harbor. However, the buoyancy of microplastics can also be affected by chemical contaminants
378 and biofouling.

379
380 To the best of our knowledge, all the studies that have investigated the ingestion of microplastics
381 by fish have been conducted from wild caught species or in laboratory experiments. However,
382 the migration of many fish species for feeding and breeding creates uncertainty about how well
383 the analysis made on an individual truly reflects the environmental contamination by MP in or
384 around the site of capture (Oikari 2006). In this study, we tested for the first time the feasibility
385 of using caged juveniles to quantify and assess MP contamination of in estuarine nursery
386 grounds. Such approach was successfully used to monitor microplastics contamination in
387 mussels (Catarino et al. 2018; Railo et al. 2018). Our results demonstrated that the fish caging
388 approach is suitable to assess MP contamination in estuaries and to a lesser extent their effects on
389 fish condition. An average of 2.04 ± 1.93 MP items was ingested by feral juveniles flounder and
390 1.67 ± 1.43 by caged juveniles flounder. Similar levels (1.9 ± 0.1 items/individual) were
391 previously reported for different adult fish species by Lusher et al. (2013) in the English Channel
392 or in others estuaries: 1.67 items/individual (Bessa et al. 2018), 3.03 (Ferreira et al. 2018) and
393 1.06 (Vendel et al. 2017).

394 The higher number of fragments and fibers in wild fish when compared with the caged ones
395 suggest that the latter are probably limited in their feeding zone and, therefore, will have a lower
396 number of ingested items. During the caging experiment, most of the fish have lost weight and it
397 is likely that food availability in the cages was rather low due to the limited cage dimension. The

398 more frequent occurrence of MP in benthic species compared to pelagic fish (e.g. Neves et al.
399 2015; McGoran et al. 2017; Jabeen et al. 2017) suggests that plastic occurrence may be high near
400 the sea floor and/or in sediments, or that benthic fish are less selective feeders. In the Thames
401 estuary, McGoran et al. (2017) found that 70% of sampled European flounder had plastic fibers
402 in their gut compared with only 20% of European smelt, *Osmerus eperlanus* (a pelagic species).
403 The generalist feeding behavior of juvenile flounders which feed on benthic preys and ingest
404 large quantities of sediment (Selleslagh and Amara 2015) suggest that everything is a potential
405 prey to feed on, including microplastics being mistaken as food source.

406 In this study, we compared exposed juvenile fish from 5 sites in 3 different estuaries. Except the
407 Liane and Fosse Nord, we did not observe significant differences in the number of MP ingested
408 by caged fish. However, the three sites impacted by WWTP (Liane, Le Havre Harbor and
409 Rouen) are those with the highest percentage of individuals that have ingested fibers. This
410 suggest the possible contribution of WWTPs as a source of MP in estuaries.

411 While microplastic ingestion by fish has been confirmed in laboratory and wild caught
412 specimens, we know little about the impact of MP consumption by fish. However, the quantities
413 observed in fish guts are generally very low, usually less 1 to 2 particles per individual (Lusher et
414 al. 2017). Although other environmental factors may have affected caged fish condition and
415 mortality, we found no significant correlation between the condition factor and the mortality rate
416 with the MP number ingested by fish. Other studies also found that the condition factor of wild
417 captured fish was similar for those with or without MP ingestion (Ramos et al. 2012, Foekema et
418 al. 2013). However, these results did not exclude the possibility of physiological and
419 toxicological consequences. Risks associated with the ingested MP come from the material itself
420 and from the chemical pollutants included in plastic such as additives or contaminants adsorbed
421 from the surrounding water. Hazards associated with the complex mixture of plastic and
422 accumulated pollutants are largely unknown (Browne et al. 2013; Lusher et al. 2017). Metabolic
423 and physiological negative responses have been only observed under laboratory conditions,
424 where in most cases; very high levels of microplastics were tested under exposure scenarios that
425 were not representative of natural environmental conditions (e.g. Rochman et al. 2013; Peda et
426 al. 2016; Critchell and Hoogenboom 2018 and review in Lusher et al. 2017). Recently
427 microplastics were isolated in the gills, liver, and digestive tract of the Zebra danio (*Danio*

428 *rerio*); which caused inflammation, oxidative stress, and disrupted energy metabolism (Lu et al.
429 2016). Rochman et al (2013) showed that Japanese medaka (*Oryzias latipes*), exposed to a
430 mixture of polyethylene with chemical pollutants sorbed from the marine environment, can
431 bioaccumulate these chemical pollutants leading to liver toxicity and pathology. Fish behavior
432 may also be affected by microplastic exposure: the common goby (*Pomatoschistus microps*)
433 displayed reduced predatory performance, abnormal swimming behavior, and lethargy (De Sa et
434 al. 2015).

435

436 **Conclusion**

437

438 Regarding the present study, we can conclude that caged fish are suitable to assess microplastic
439 contamination in aquatic environment. Both caged and wild caught European flounder from
440 three estuaries of the Eastern English Channel ingested MP, mainly fibers, in an amount higher
441 to that generally observed in other marine fish species. This would confirm previous studies that
442 have indicated that MP are more common within estuaries than in the marine environment
443 (Schmidt et al. 2017; Horton et al. 2017). European flounder is an opportunistic species that
444 tolerates a wide range of salinity (0 to 35) and can be an ideal indicator to study MP
445 contamination along a salinity gradient. Since microplastic contamination may vary in space and
446 time, particularly in estuarine systems affected by tide and river flow, the caging approach may
447 be useful for assessing the spatial and temporal variability in MP and the many factors that
448 influence this.

449 The high occurrence of MP ingested by juvenile fish in nursery grounds raises concerns on their
450 potential negative effects for fish recruitment success and population renewal. No negative
451 effects on juvenile fish condition was observed. However, further researches are required to fully
452 understand the ecological impact of MP within these essential fish habitats. The caging approach
453 may be useful to study the potential effect of MP ingestion on physiological and toxicological
454 responses fish by measuring different biomarkers.

455

456

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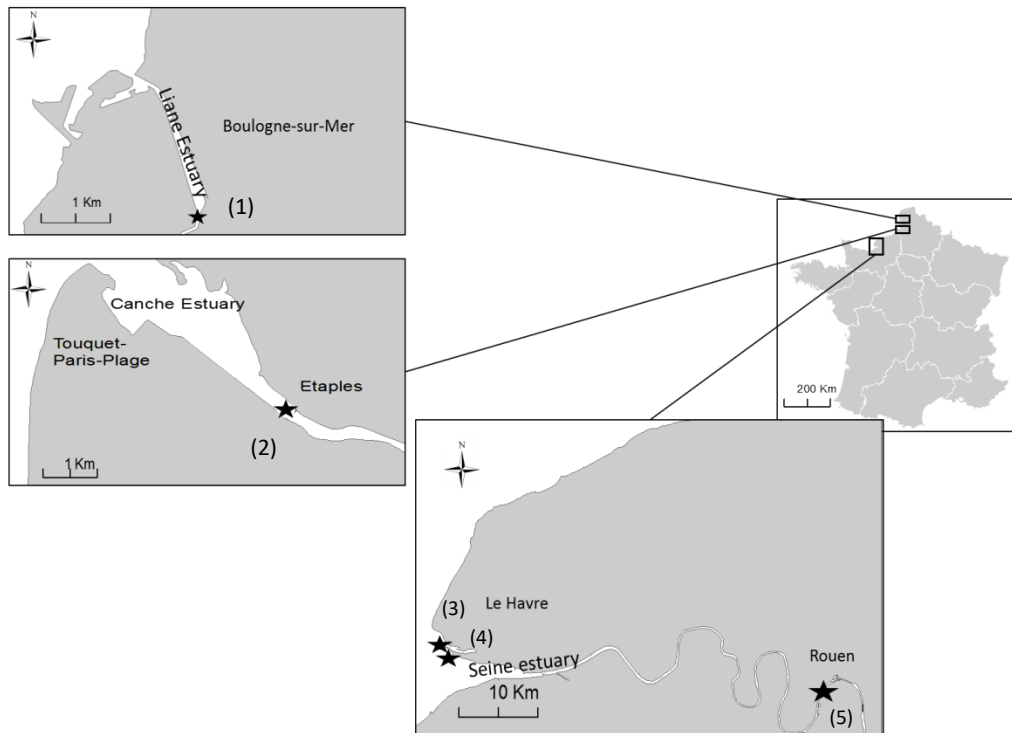


Fig. 1 Sampling and caging sites of juveniles flounder in (1) the Liane, (2) the Canche, and the Seine estuary: (3) Le Havre Harbor, (4) Fosse Nord and (5) Rouen

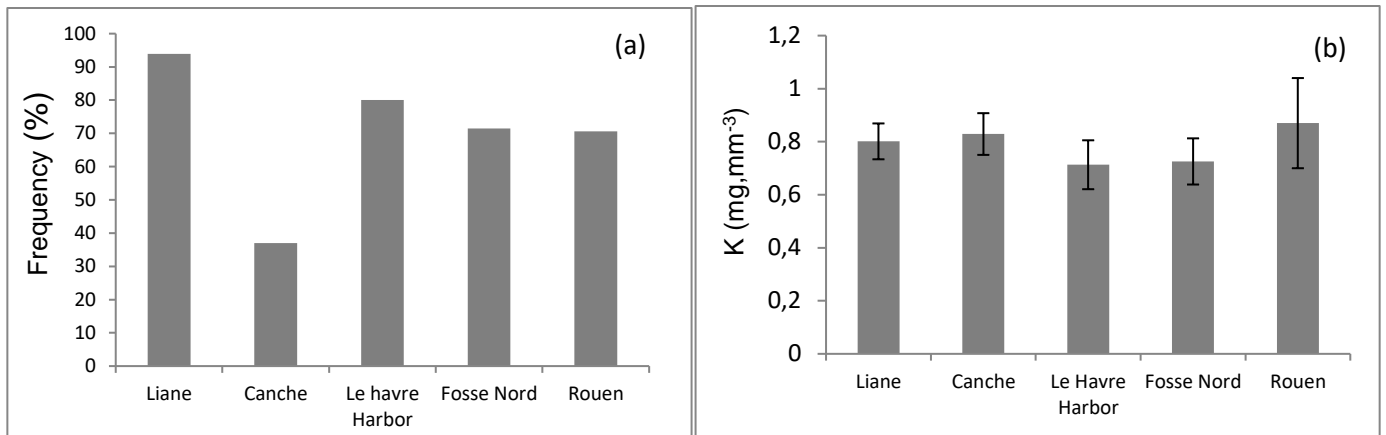


Fig. 2 Percentage of survival of juvenile flounder following one month caging experiment at the different sites (a) and, (b) Fulton K condition factor (mean \pm SD)

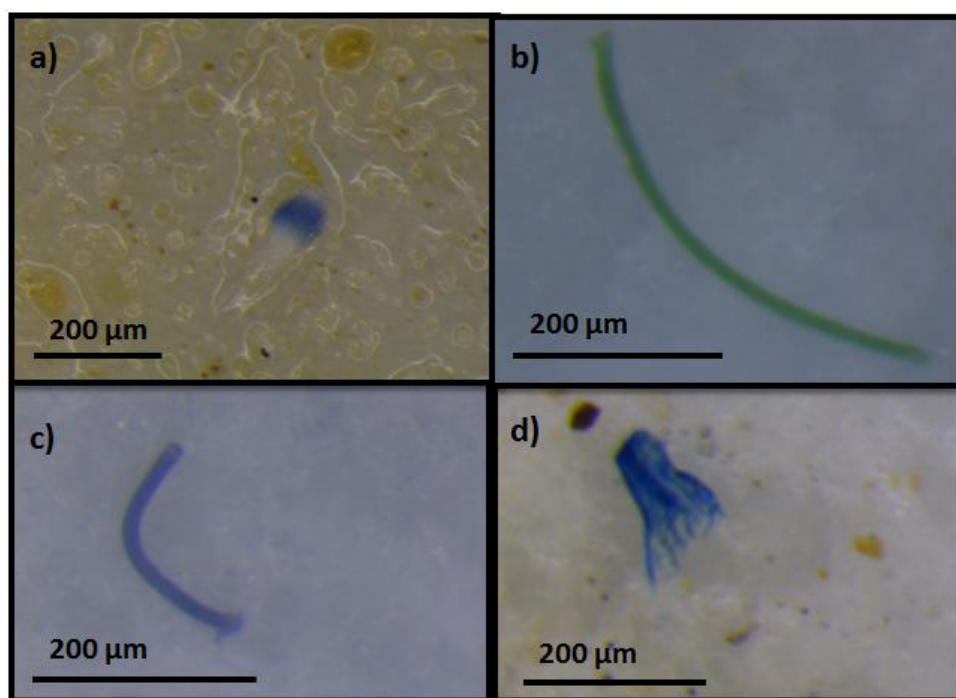


Fig. 3 Examples of microplastics found in the digestive tract of juveniles flounder: a) represents a fragment; b) and c) filaments; and d) films

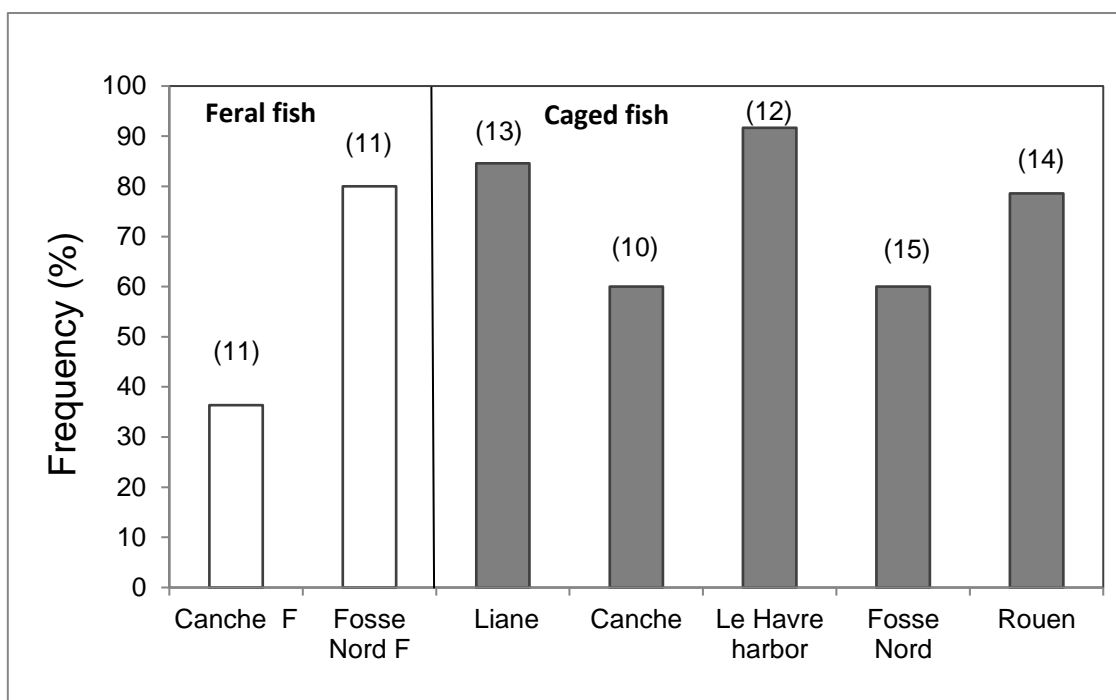


Fig. 4 Percentage of juvenile flounder that have ingested items. White bars: feral fish and grey bars: caged fish. Between brackets are presented the total number of analyzed individuals

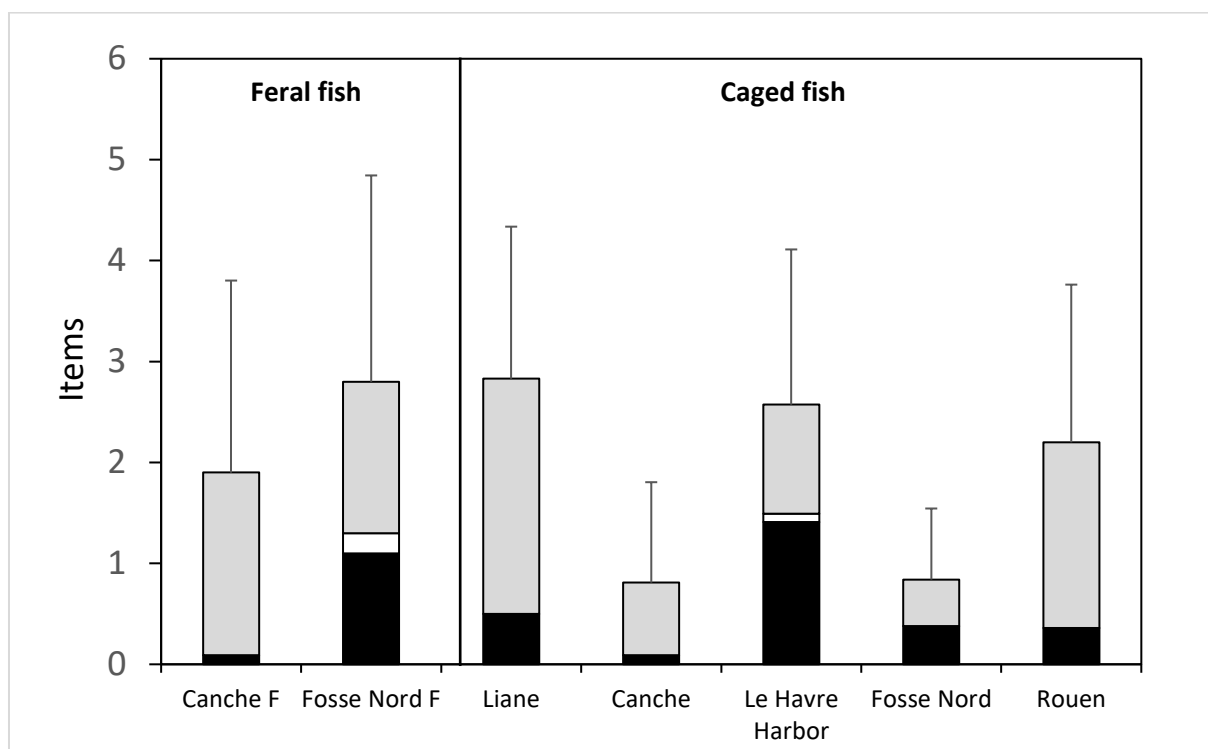


Fig. 5 Average (+ SD) number of items (fragments, fibers, and films) ingested by feral and caged juveniles flounder at the different estuarine sites. Grey: fibers; black: fragments; white: films

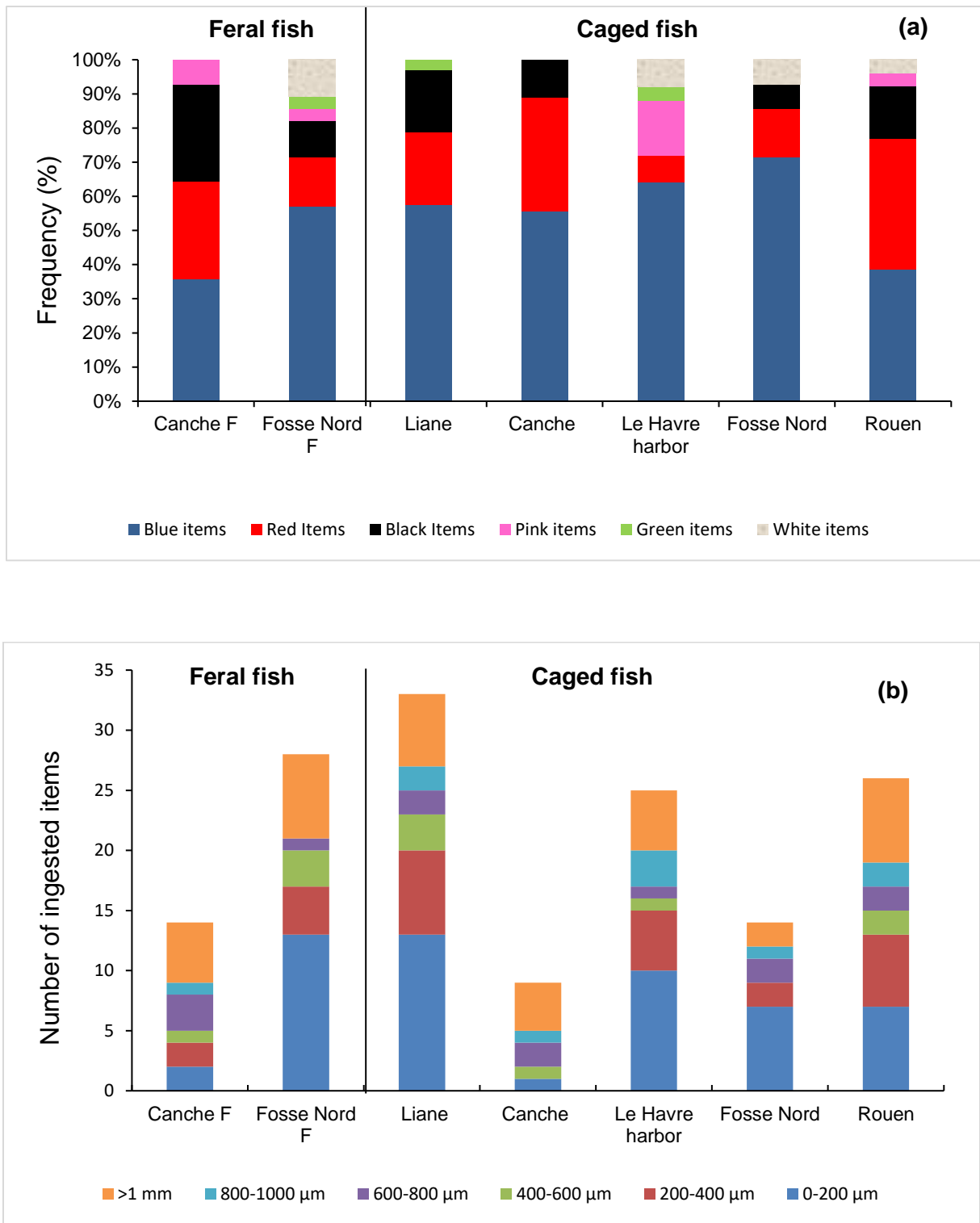


Fig. 6 Percentage of items ingested by feral and caged juvenile flounders at the different estuarine sites categorized by color (a) and the number of items ingested sorted by size class (b)

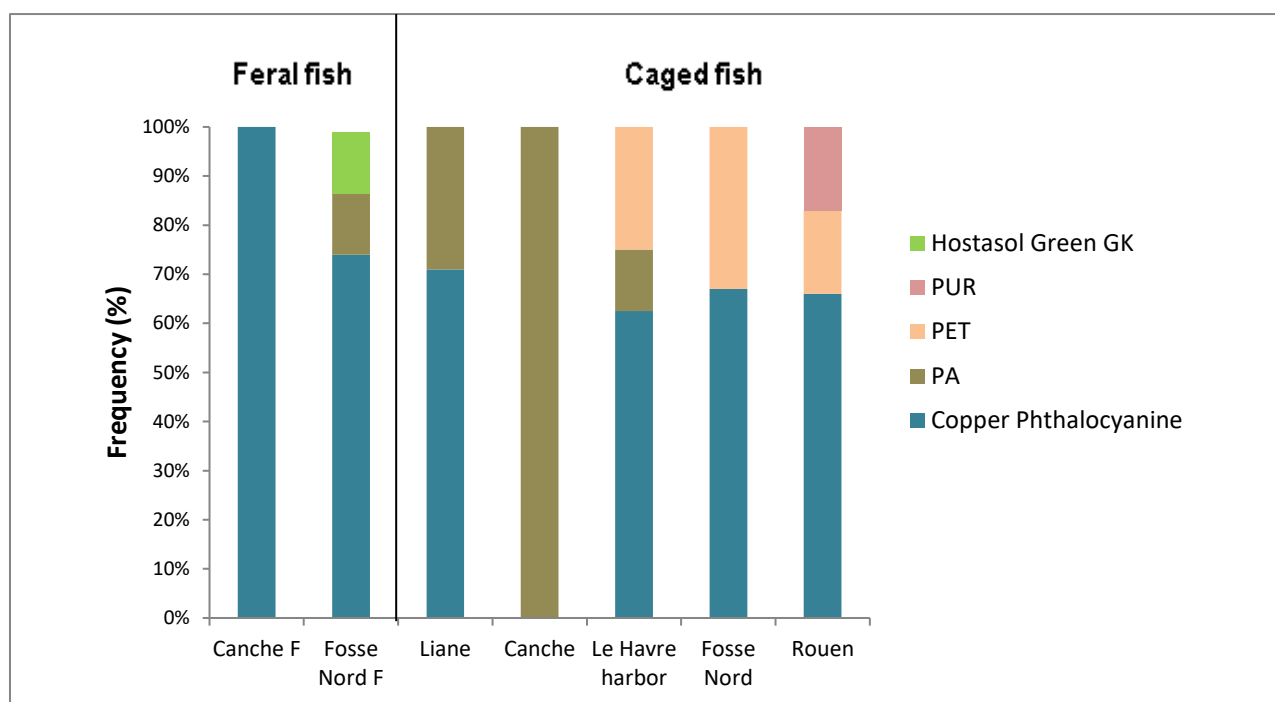


Fig. 7 Different polymers ingested by juvenile flounders (feral and caged) identified using micro-Raman spectroscopy

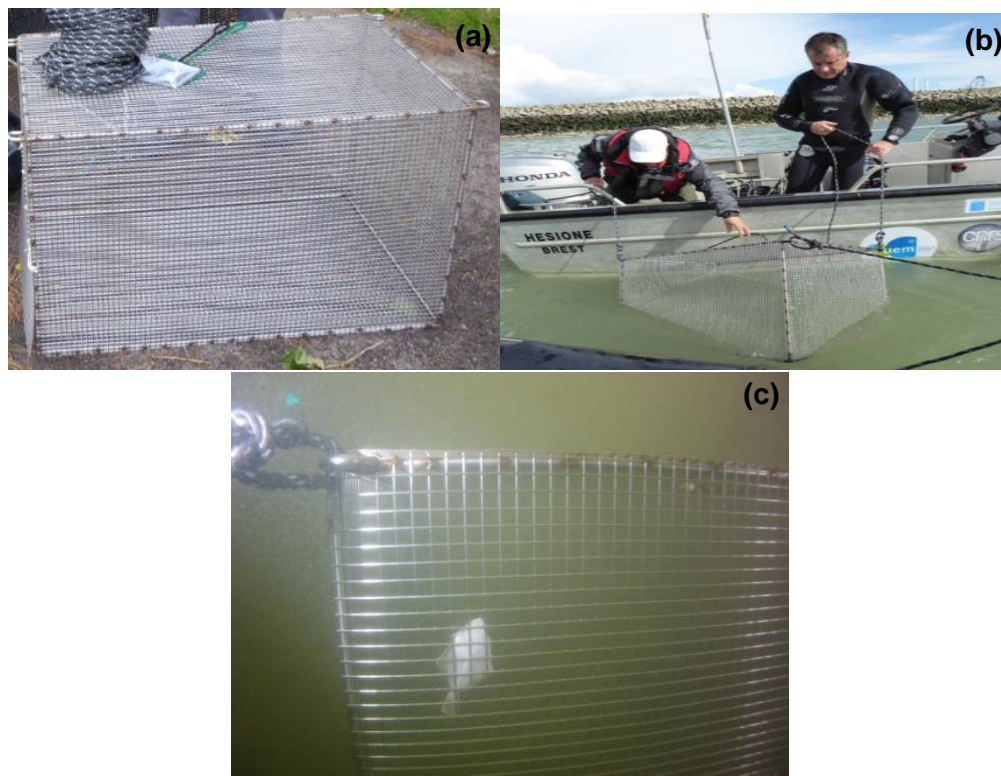
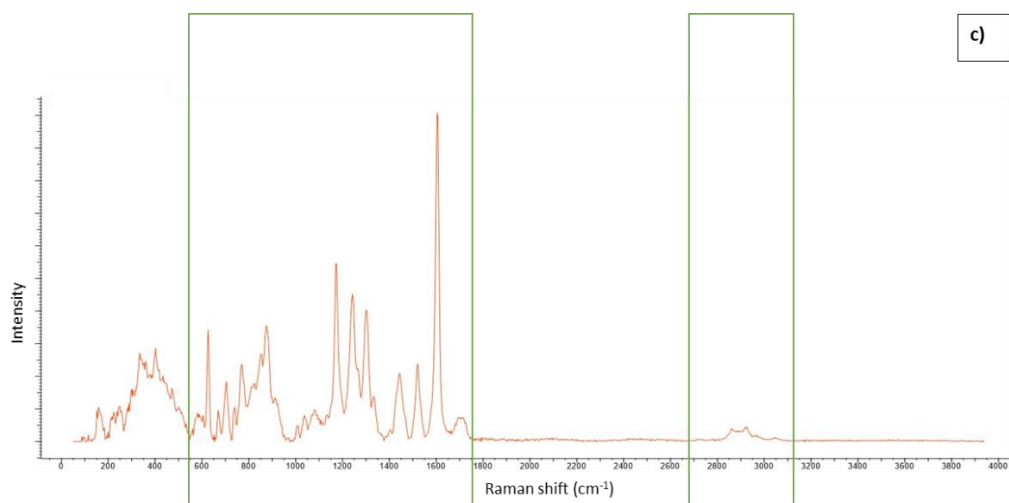
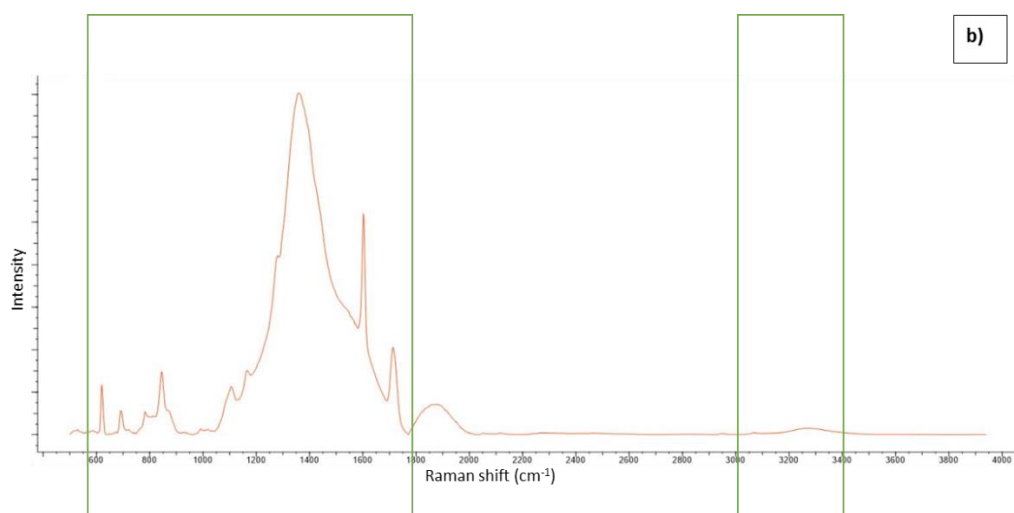
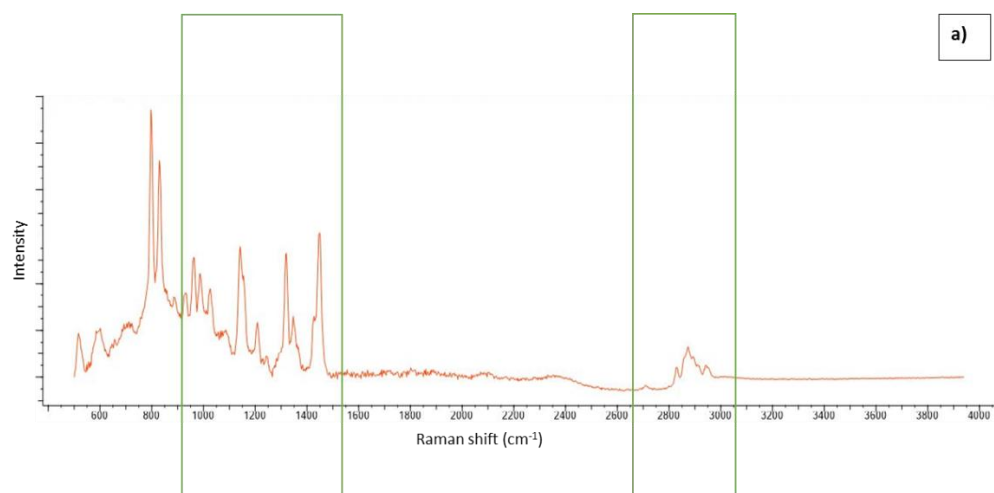


Fig. 1 The caging setup with (a) representing the stainless steel cage of 1 m length and a 0.6 m height and width. (b) representing the cage deployment and (c) representing *Platichthys flesus* swimming inside the cage after its attachment to the bottom



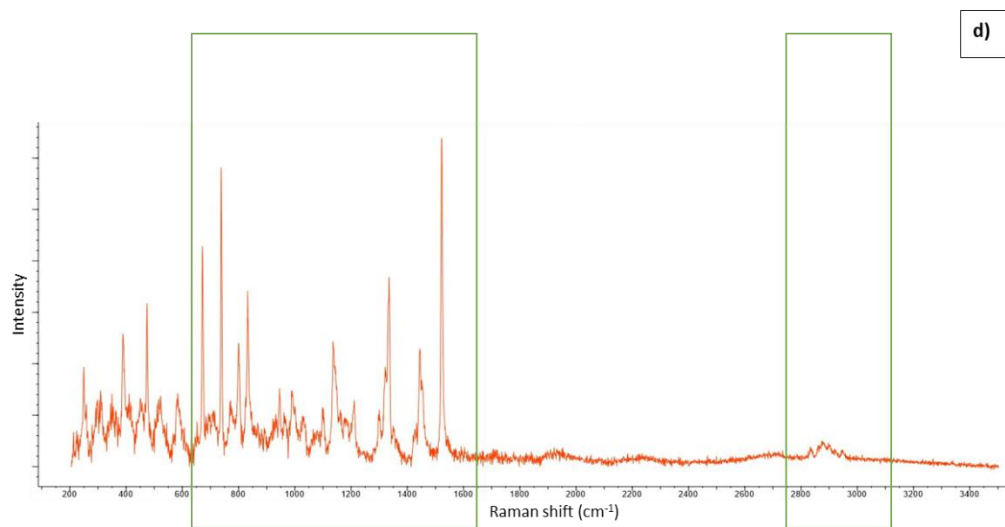


Fig. 2 Spectrum of Polycaprolactam (a), Polyethylene Terephthalate (b), Polyurethane (c), and Copper Phthalocyanine (d) obtained by micro-Raman spectroscopy