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1 **Experimental evidence that polystyrene nanoplastics cross the intestinal barrier of**
2 **European seabass**

3

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18

19 **Highlights**

- 20 ● Plastic particles crossing the intestinal barrier is debated
- 21 ● PS-NP particles were tested in ex-vivo time series experiments using Ussing chambers
- 22 ● Two PS-NP concentrations were tested on two locations of adult Seabass guts
- 23 ● PS-NP directly crossed the intestinal barrier of adult Seabass within few minutes
- 24 ● PS-NP translocation was confirmed by 3 complementary techniques

25

26 **Abstract**

27 Plastic pollution in marine ecosystems constitutes an important threat to marine life. For
28 vertebrates, macro/microplastics can obstruct and/or transit into the airways and digestive tract
29 whereas nanoplastics (NPs; < 1000 nm) have been observed in non-digestive tissues such as
30 the liver and brain. Whether NPs cross the intestinal epithelium to gain access to the blood and
31 internal organs remains controversial, however. Here, we show directly NP translocation across
32 the intestinal barrier of a fish, the European seabass, *Dicentrarchus labrax*, *ex vivo*. The luminal
33 side of median and distal segments of intestine were exposed to fluorescent polystyrene NPs
34 (PS-NPs) of 50 nm diameter. PS-NPs that translocated to the serosal side were then detected
35 quantitatively by fluorimetry, and qualitatively by scanning electron microscopy (SEM) and
36 pyrolysis coupled to gas chromatography and high-resolution mass spectrometry (Py-GC-
37 HRMS). Fluorescence intensity on the serosal side increased 15–90 min after PS-NP addition
38 into the luminal side, suggesting that PS-NPs crossed the intestinal barrier; this was confirmed
39 by both SEM and Py-GC-HRMS. This study thus evidenced conclusively that NPs beads
40 translocate across the intestinal epithelium in this marine vertebrate.

41

42 **Key words:** polystyrene nanoplastic beads, translocation, intestinal epithelium, *Dicentrarchus*
43 *labrax*, Ussing chambers

44

45 **1. Introduction**

46 Whether plastic particles delivered through the food chain enter the circulation and reach organs
47 by crossing the intestinal barrier, i.e. by translocation, remains a question of discussion
48 (Catarino et al., 2019; Paul-Pont et al., 2018; Schür et al., 2019; Triebkorn et al., 2019). Almost
49 50 years ago, Volkheimer used histology and staining methods to show that during normal
50 digestion in mammals, particles of about 1 µm passed between adjacent epithelial cells of the

51 intestine and entered the subepithelial compartment, from where they entered the bloodstream
52 (Volkheimer, 1974). That study also found that particles up to 50 μm in diameter passed through
53 enterocytes by a pinocytosis-like process. Later, Volkheimer showed that 5–110 μm particles
54 of polyvinyl chloride appeared in the blood 2 h after ingestion by dogs, and smaller particles
55 were translocated more rapidly than larger ones (Volkheimer, 1975). In fish, the translocation
56 of plastic particles across the intestine has been implied by the discovery of particles in various
57 tissues of several species (Collard et al., 2017; Elizalde-Velázquez et al., 2020; Jovanović et
58 al., 2018; Kim et al., 2020; Mattsson et al., 2017; Zeytin et al., 2020), although the evidence is
59 not indisputable. For example, the presence of two microplastic (MP) particles of 39 and 90 μm
60 was reported in the livers of wild anchovy, *Engraulis encrasicolus* (Collard et al., 2017), but
61 the precise location of the particles in or near blood cells could not be accurately inferred due
62 to inadequate tissue preparation. Experimental studies have also suggested transfer of 200–
63 600 μm MPs from the digestive tract to the liver in grey mullet, *Mugil cephalus*, and the
64 presence of polystyrene (PS) particles (ranging from 214 to 288 μm) was observed in the livers
65 of a few individual seabream, *Sparus aurata*, 45 days after their addition to the diet (Jovanović
66 et al., 2018). In Crucian carp, *Carassius carassius*, fed food containing 53 nm and 180 nm
67 polystyrene nanoplastic (PS-NPs) particles, the particles were detected in the brain by
68 hyperspectral imaging (Mattsson et al., 2017). Similarly, in fathead minnow, *Pimephales*
69 *promelas*, 60–70 nm PS-NPs were found in liver and kidney 48 h after their addition to the diet
70 (Elizalde-Velázquez et al., 2020).

71 All these studies suggest that translocation explains the presence of plastic particles in the
72 various digestive and non-digestive organs. Before reaching internal organs, plastic particles
73 need to cross the intestinal barrier, i.e. the tight junction sealed-epithelial cell monolayer that
74 faces the luminal side as well as the lamina propria housing numerous immune cells. Besides
75 translocation across the intestine, other mechanisms, such the way some seaweeds accumulate

76 plastics by adherence (Gutow et al., 2016), have been suggested by some authors (Abbasi et al.,
77 2018). Recent studies tried to demonstrate the passage of NPs from the digestive tract to organs
78 by using fluorescently labelled plastic particles (Ding et al., 2018; Pitt et al., 2018a, 2018b; van
79 Pomeroy et al., 2017). Those studies are now considered inconclusive, however, because the
80 authors did not control for possible leaching of the fluorescent dye from the NPs (Catarino et
81 al., 2019; Schür et al., 2019). Indeed, commercial fluorescently labelled NPs do leach their
82 fluorophores, and the fluorophore alone can accumulate within internal tissues, as seen in
83 studies of zebrafish larvae (Catarino et al., 2019; Schür et al., 2019). Thus, in uptake and
84 translocation studies simple measurements of fluorescence in the target tissue may be
85 misleading. Very recently, accumulation of palladium-doped polystyrene was evidenced in the
86 mucosa and muscularis layers of the whole intestine of salmon and potential passage in the
87 blood was only found in the anterior intestine (Clark et al., 2022). These results based on an *ex-*
88 *vivo* gut sac exposure system are a tremendous step forward but, at the same time, raise some
89 questions about a passage only through the pyloric caeca of the anterior intestine, which would
90 necessarily limit the scope of this observation only to species that have them (Barrington, 1957;
91 Kapoor et al., 1976). Complementary and innovative methods are still needed to ascertain
92 whether NPs cross the intestinal barrier (Paul-Pont et al., 2018).

93 Here, the passage of PS-NPs across the intestine of the European seabass, *Dicentrarchus*
94 *labrax*, a temperate carnivorous fish of ecological and economic interest has been evaluated.
95 We use an *ex vivo* approach, based on Ussing chambers (Ussing and Zerahn, 1951) recognized
96 since a long time as an optimal method for studying transport/absorption processes in intestine
97 of several species including fish (Ašmonaitė et al., 2018; Clarke, 2009; de Jonge et al., 2004;
98 Grosell and Genz, 2006; Guffey et al., 2011; Haslam et al., 2011; Thomson et al., 2019;
99 Weinrauch et al., 2021). Median and distal sections of intestine (mid- and hindgut) were
100 exposed to two concentrations of 50 nm fluorescent PS-NPs on their luminal sides. To identify

101 particles that crossed the intestinal barrier, the serosal side was analyzed by using three
102 complementary techniques: fluorescence measurement, visual inspection by scanning electron
103 microscopy (SEM), and chemical analysis by high-resolution mass spectrometry (Py-GC-
104 HRMS). Thus, we show directly the translocation of PS-NPs across the intestinal epithelium.

105

106 **2. Materials and methods**

107

108 Ethic statement: All fish manipulations were performed according to the Animal Care
109 Committee of France (ACCF), and the authors complied with the ARRIVE guidelines.

110

111 *2.1. Polystyrene nanoplastics (PS-NP)*

112 Commercially available polystyrene nanoplastics (PS-NP) 50-nm beads at 5000 mg L⁻¹ with a
113 blue (excitation 358 nm and emission 410 nm) fluorescent label L0780-1 mL were purchased
114 from Sigma-Aldrich (Saint Louis, USA), and stored at 4°C prior to experiments. Previous
115 Raman microspectroscopy analysis confirmed the PS nature of the polymer (Tallec et al., 2018).
116 PS-NP commercial suspensions were in ultrapure water (UW) with Tween-20© surfactant
117 (<0.1%) to limit aggregation; Tween-20© had previously been demonstrated to be innocuous
118 for marine invertebrates at this dose (Ostroumov, 2003). Based on previous Dynamic Light
119 Scattering analyses (DLS), we decided to use beads with amine group functionalization (NH₂)
120 because they remain at the nanometric scale: the 50-NH₂ suspension exhibited a nanometer-
121 scale size (mean ± SD; 125.6 ± 0.2 nm) and a positive surface charge (mean ± SD; 13.2 ± 1.0
122 mV) in natural filtered seawater. Before each use, particles were vortexed to prevent particle
123 aggregation and ensure good suspension homogenization. The Z-average (size; nm) and the ζ-
124 potential (mean surface charge; mV) were determined by DLS (nano-Zetasizer ZS Malvern
125 Instruments) (Tallec et al., 2019) in the four media used in this study (see the next material and

126 methods sections): (i) Ultrapure water, (ii) 10 mM Krebs-Glucose, (iii) 10 mM Krebs-Mannitol,
127 (iv) trichloroacetic acid (TCA). The PS-NP suspensions were added in disposable folded
128 capillary cells (DTS 1060C, Malvern Instruments; Final volume = 1 mL). All analyses were
129 conducted in triplicates (13 runs and 10 sec measure⁻¹ for Z-average; 40 runs and 10 sec
130 measure⁻¹ ζ -potential; concentration: 100 g m⁻³). DLS measurements were performed as
131 described by [Tallec et al., 2019](#) with a nano-Zetasizer ZS (Malvern Instruments, UK) using an
132 angle of 173° Backscatter, a temperature of 20 °C and an equilibration of 120 s. We used the
133 implemented data analysis software to measure the mean size of particles/aggregates (Z-
134 average; nm), the aggregation state and the mean surface charge (ζ -potential; mV) of NP. The
135 accuracy of all measures was verified with the report quality from the implemented software
136 and a counting rate being always higher than 100 kcps.

137

138 2.2. *Biological material and rearing conditions*

139 Fish used in this study were 23 healthy adult *Dicentrarchus labrax* individuals (about 1.5 kg, 2
140 males and 21 females) reared in an open circulating system at Ifremer (French Institute for Sea
141 Exploration) facilities in Plouzané, France. Running seawater passed successively through a
142 tungsten heater and a degassing column packed with plastic rings and the water parameters
143 followed that of the environment during the experimental period (from October 2018 to
144 November 2019). Water temperature (15°C ± 3°C), oxygen (99% ± 1%) and pH (8 ± 0.1) were
145 controlled once a week using multiparameter WTW probes (OXI 340, and pH3310,
146 respectively). Fish were fed *ad libitum* industrial food (Le Gouessant®, Lamballe, France)
147 twice a week.

148 Portions of the median and distal intestine tested in the present study can be collected from the
149 same animal.

150

151 2.3. *Ussing experiment*

152

153 2.3.1. *Experimental set-up*

154 The experimental set-up for Ussing experiment is presented in Fig. 1. It is composed of two
155 glass half chambers: one luminal and one serosal between which the intestine is mounted as a
156 flat sheet. Each half chamber is connected by a tubing system to a U-shaped reservoir filled
157 with Krebs that was supplemented with 10 mM glucose on the serosal side to ensure energy
158 provision to the intestinal cells and 10mM mannitol on the luminal side to counterbalance
159 osmolarity. Ussing chambers compartments were carefully washed with filtered distilled water
160 to avoid any potential plastic contamination. The 10 mM Krebs-Glucose was made with 1/11
161 vol. of 10X Krebs (NaCl: 1.16 M, KCl: 0.05 M, KH₂PO₄: 0.01 M, MgSO₄-7H₂O: 0.023 M,
162 NaHCO₃: 0.25 M) at pH 7.2, 1/100 vol. 10X CaCl₂ at 0.0075 M, and 10 mM D-glucose. The
163 10 mM Krebs-Mannitol solution was made similarly, replacing the D-glucose by D-Mannitol.
164 All chemicals were supplied by Sigma-Aldrich (Saint-Quentin Fallavier, France).
165 The area of exposure of the intestinal epithelium was 2.35 cm². Oxygenation and stirring of
166 solutions were ensured by an air-lift on both sides of the intestinal segments.

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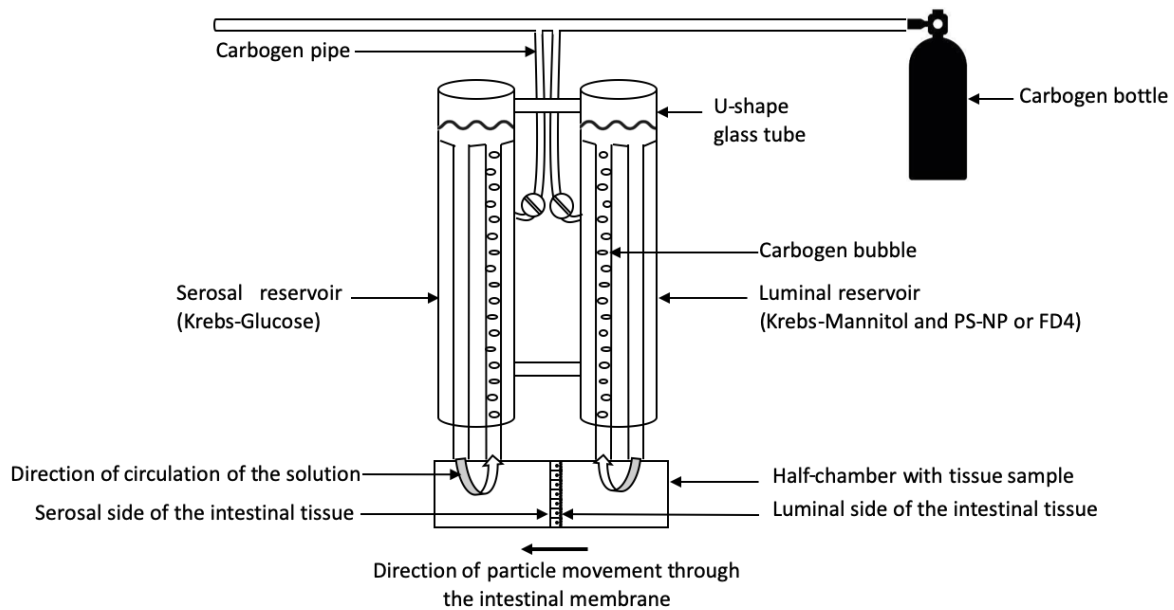
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176 **Fig. 1**

177



178

179 **Fig. 1:** Experimental set-up of a circulating Ussing device. It is composed of glass
180 compartments. It contains two half chambers: one luminal and one serosal between which the
181 intestine is mounted as a flat sheet. Two U-shaped reservoirs, filled with 8 mL oxygenated
182 Krebs-Glucose (on the serosal side) or 8 mL Krebs-Mannitol (on the luminal side) are
183 connected to each half chamber by a tubing system. Carbogen bottle contains 95% O₂ and 5%
184 CO₂. The CO₂ aimed to buffer the Krebs solution that contains HCO₃⁻ and then helped to
185 maintain a pH of 7.3 in each chamber. Polystyrene nanoplastic beads (PS-NP) or fluorescein
186 isothiocyanate dextran 4000 molecule (FD4) were added to Krebs-Mannitol in the luminal part.
187 The direction of circulation of the solutions is indicated for the two half-chambers. An arrow
188 indicates the direction of potential particle movement from the luminal side to the serosal side
189 of the intestinal membrane.

190

191

192

193 2.3.2. *Kinetic protocol*

194 Each day of experiment, one or two 72h-starved fish were euthanized by an anesthetic overdose
195 (Eugenol 1 mL L⁻¹; Fili@vet Reseau Cristal, France), and their intestine was entirely removed.

196 Then, the median or the distal segment were isolated.

197 Each portion of the intestine was then opened in order to obtain the luminal side on one face
198 and the serosal face on the other. The intestinal segments were mounted in Ussing chambers
199 (Fig.1), and 8 mL of Krebs-Mannitol and 8 mL of Krebs-Glucose solutions were added to the
200 luminal and serosal sides of the epithelium, respectively. The whole experiment was performed
201 at room temperature (approx. 20°C).

202 In order to study the transfer rate of an experimental substance across the epithelium, one
203 volume of Krebs-Mannitol was replaced by one volume of the experimental substance. Two
204 experimental substances were tested: i) the fluorescein isothiocyanate dextran 4000 molecule
205 (Sigma-Aldrich), which is an inert marker of gut permeability and integrity (Thomson et al.,
206 2019; molecular weight 3,000–5,000), hereafter called FD4, and ii) the PS-NP dispersions. A
207 volume of 200 µL of serosal Krebs-Glucose solution was sampled at T0 (just before adding the
208 experimental substance), and then at 15, 30, 45, 60, 90, 120, and 150 min after addition of the
209 experimental substance.

210

211 2.3.3. *Test for the integrity of the intestinal epithelium using FD4*

212 Because NPs themselves or the Tween added to the NP particles in order to prevent aggregation
213 could affect epithelial cells integrity, the integrity and the impermeability of the intestinal
214 epithelium to soluble macromolecules was determined by incubating the luminal side of median
215 or distal sections of fish intestine in the Ussing chamber (Fig. 1) with FD4, and with FD4
216 combined with two concentrations of 50-nm PS-NP fluorescent beads (20 mg L⁻¹, i.e. NP[20]
217 and 200 mg L⁻¹, i.e. NP[200], that are further used in the experiment; see 2.3.4). This test was

218 performed on 14 median (including 13 females and 1 male) and 15 distal intestinal segments
219 (14 females and 1 male). One volume of Krebs-Mannitol was replaced by either the same
220 volume of a FD4 solution (FD4 molecule diluted at 10 mg mL^{-1} in Krebs) in the luminal side
221 (initial FD4 concentration in the luminal side: $125 \mu\text{g mL}^{-1}$), or by one volume of FD4 + PS-
222 NP. Then kinetic responses were measured, through the appearance of fluorescence on the
223 serosal side at intervals up to 150 min (Fig. 2, and S5 for statistical results), and compared to
224 T0.

225 A potential adsorption of FD4 was analyzed on the NPs to test if that might have modified the
226 fluorescence of FD4, and thus have led to underestimate the presence of FD4 in the serosal side.
227 The fluorescence of a calibration curve of FD4 ($0, 62.5, 125, 500 \text{ ng mL}^{-1}$) was assayed in the
228 absence or presence of PS-NPs added at each FD4 concentration tested at 20 mg L^{-1} , *i.e.* the
229 largest PS-NP concentration we measured on the serosal side in our assays. FD4 fluorescence
230 was read at 485/528 nm (excitation/emission), as described in the following 2.4.1. section. The
231 regressions obtained from the calibration curves are not significantly different between FD4
232 alone or with NP addition (ANCOVA, $p\text{-value} = 0.1814$; see Supplementary Material S1). This
233 result indicates that if there is any FD4 adsorption on NP, it does not result in any change in
234 FD4 fluorescence.

235

236 2.3.4. *Test for the PS-NP transfer across the intestine using PS-NP beads*

237 To test for the passage of PS-NP across the intestinal epithelium, the luminal part of both
238 median and distal segments of sea bass intestine was exposed to the two concentrations of 50
239 nm PS-NP fluorescent particles tested above (NP[20] and NP[200]). PS-NP transfer through
240 the intestinal epithelium was tested by replacing one volume of Krebs-Mannitol in the luminal
241 side by the same volume of the PS-NP dispersion stock (5000 mg L^{-1} diluted in 4 mL Krebs-
242 Glucose). The PS-NP concentrations tested were based on the range of values reviewed for

243 exposure experiments of cationic amino polystyrene (PS-NH₂) on different phylum (from
244 micro-algae to molluscs (Gonçalves and Bebianno, 2021):

245 - 20 mg L⁻¹, obtained by replacing 32 µL of Krebs-Mannitol by the same volume of PS-
246 NP dispersion (5000 mg L⁻¹ diluted in 4 mL Krebs-Glucose) to the luminal side
247 (NP[20]: n=6 median and n=6 distal, including 6 females). This corresponds to
248 2.88x10⁸ particles mL⁻¹ (Tallec et al., 2021).

249 - 200 mg L⁻¹, obtained by replacing 320 µL of Krebs-Mannitol by the same volume of
250 PS-NP dispersion (5000 mg L⁻¹ diluted in 4 mL Krebs-Glucose) to the luminal side
251 (NP[200]: n=6 distal and n=6 median, including 2 females and 4 males). This
252 corresponds to 2.88x10⁹ particles mL⁻¹ (Tallec et al., 2021).

253 The choice of a model nanoparticle with spherical shape and surface functionalization available
254 commercially was only driven by the fact that it stays at the nanometric scale, a necessity for
255 testing translocation. The use of these non-environmental concentrations can be viewed as proof
256 of concept producing ground-breaking data for testing translocation. At this stage, it does not
257 suggest an environmental risk. The concentration used in our study were high compared to the
258 highest concentration of microplastics reported at sea (e.g. 8 particles mL⁻¹; (Brandon et al.,
259 2020) even if the nanoplastic concentrations in the ocean are expected to be higher than
260 microplastics (Wagner and Reemtsma, 2019).

261 The leakage of dye was controlled using the dialysis technique described in Çatıno et al.
262 (2019). Briefly, 20 µl (at 5000 mg L⁻¹) of PS-NP was added in 5 Pur-A-Lyzer dialysis tubes
263 (200 µL of Krebs-Mannitol) provided by the Pur-A-Lyzer™ Mini Dialysis Kit (Sigma-Aldrich,
264 USA). After 1:30 of incubation, fluorescence was read in the total volume of the 5 replicates
265 dialysis tubes at the PS-NP wavelength 360/460 nm (excitation/emission) as described in the
266 following 2.4.1.

267

268 2.4. *Sample analyses*

269

270 2.4.1. *Fluorescence data*

271 The transport kinetics was then measured based on fluorescence in the serosal part of the
272 intestine during 150 min, and the corresponding PS-NP concentration was calculated using
273 calibration curves. For that, 100 μ L of samples collected at each sampling time during the
274 kinetics were immediately read in duplicates on a high-binding flat bottom 96-well black plates
275 (Greiner bio-one, Les Ulis, France) with a fluorimeter (multi-detection microplate reader
276 Synergy HT, BioTek, Colmar, France) equipped with the Gen5 software (version 2.03.1). The
277 PS-NP fluorescence was read at 360/460 nm (excitation/emission), and FD4 fluorescence was
278 read at 485/528 nm (excitation/emission), with a gain of 50. FD4 and PS-NP standard curves
279 prepared with stock FD4 solution and PS-NP dispersion respectively, and Krebs-Glucose were
280 added on each plate and also read in duplicates. All standard curves had a $r^2 > 0.995$ for FD4,
281 and $r^2 > 0.978$ for the PS-NP (see Supplementary material S3).

282 At each wavelength, the PS-NP concentration was calculated for each technical replicate as
283 follows:

284
$$PS - NP \text{ Concentration}_{\text{sample } X} = \frac{(Fluo_{\text{sample } X} - Fluo_{\text{Mean } T_0})}{a}$$

285 Where $Fluo_{\text{sample } X}$ is the fluorescence read for the sample x (including sample at T0), $Fluo_{\text{Mean } T_0}$
286 is the mean fluorescence read for all the samples at T0, and a is the slope of the straight-line
287 $y=ax$ of the standard curve.

288 The natural autofluorescence of the tissue was measured on median and distal segment during
289 150 min without any additional substance and was not considered as significant (Table S4).

290

291 Statistics on fluorescence data were performed on the mean $Concentration_{\text{sample}}$ values obtained
292 from technical duplicates for each sample. Concentration data were analyzed with R (R Core

293 Team, 2020; version 3.6.1) using linear mixed-effects models (Package *nlme* version 3.1-140
294 (Pinheiro and Bates, 2000)) allowing for nested effects, using fish identification as a random
295 factor. The effect of gender was not tested because of the low number of males compared to
296 female samples. Pair-wise comparisons were performed using general linear hypothesis (glht)
297 in ‘multcomp’ package (Package *multcompView* version 0.1-8). A first model was used to test,
298 for each treatment (i.e. FD4, NP[20], NP[200]), if the two intestinal segments (median and
299 distal) differed significantly with regard to the substance concentration measured at each
300 sampling point compared to that measured at T0. This first model compared the kinetic
301 responses to a mean T0 calculated for both median and distal intestinal segments. To quantify
302 potential significant increase in concentration from T0 within each segment and treatment, a
303 second model was used for each segment independently, with a T0 calculated independently.
304 The models’ conditions of application have been verified using qqnorm plots. Results are
305 presented as mean \pm standard error.

306

307 2.4.2. *Sample preparation for further SEM and Py-GC-HRMS analyses*

308 To ensure that a potential fluorescence on the serosal side of the intestine sections is due to
309 translocation of NPs and not to leakage of the fluorophore, the solution from the serosal side of
310 the intestine (about 7 mL) was sampled at the end of the kinetic experiment (NP[200]
311 experiment, n=2 distal and n=2 median segments), and prepared to be analyzed by SEM and
312 Py-GC-HRMS. This serosal solution contained a lot of salts, present in the buffers used for the
313 experiment that can prevent further analyses because of their clumping potential (i.e.
314 agglutination potential), it was therefore necessary to remove them from the serosal solution.
315 For that, serosal samples were washed with trichloroacetic acid (TCA 10M) which separates
316 the beads from the salts by changing the polarity of the beads, and then facilitates their
317 precipitation by centrifugation commonly used for concentrating dilute protein/molecule

318 solutions and simultaneously removing undesirable substances (including salts and detergents,
319 Koontz, 2014). After a first centrifugation (20 min; 30,000 g), the pellet was dissolved in 1 mL
320 of filtered distilled water. TCA 99% was added (20% in volume, 10 M), and the solution was
321 centrifuged again (20 min; 30,000 g). The pellet was then resuspended in 1 mL of filtered
322 distilled water pending further analyses. The presence of fluorescence was controlled in the two
323 supernatant solutions and was about twice lower than in the pellet.

324

325 2.4.3. *Visual inspection by SEM*

326 After TCA precipitation, the solutions from the serosal sides of the distal and median segments
327 after injection of NP[200] [n = 2 for each segment]) were intended to be observed by SEM.
328 A volume of 6 mL was filtered through polycarbonate membranes (Millipore, 0.2 µm porosity).
329 Deposited samples were dried overnight in a laminar flow hood, fixed on aluminum stub with
330 a self-adhesive carbon pellet, and subsequently Au/Pd metallized under Ar flow using a sputter
331 coater Polaron SC7620. The SEM images were acquired on a SEM LEO 438VP.

332

333 2.4.4. *Py-GC-HRMS analyses*

334 In order to confirm the PS-NP composition of the beads observed, their chemical properties
335 were analyzed by Py-GC-HRMS. For that, a volume of 1 µL of pre-treated samples (from n=2
336 median; n=2 distal segments experiments, NP[200] experiment) was dropped off on quartz
337 discs into vials, previously cleaned at 1000°C and 2 quartz discs were added on top acting as a
338 cap. Samples were pyrolyzed at 600°C for 30 seconds with an autosampler of a CDS Pyroprobe
339 6150 (Oxford, US). For the transfer to gas chromatography GC, helium was used as carrier gas,
340 and temperatures of interface, valve oven and transfer line were set at 280°C, 300°C and 300°C,
341 respectively. The pyrolysis products were injected in a ThermoScientific Trace 1300 GC
342 (Villebon-sur-Yvette, France) with a temperature of 300°C and a split value of 5. Pyrolysis

343 products were separated using a RXi-5MS 15m x 0.25 mm x 0.25 μm column with a helium
344 flow of 1.0 mL min^{-1} . The programmed oven temperature was as follows: 2 min at 40°C, then
345 increased to 310°C at 9.0°C min^{-1} , hold for 10 min. Mass spectra were obtained with a
346 ThermoScientific Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer. All interfaces
347 between GC and ionization source were maintained at 300°C and pyrolysis products were
348 ionized at 70 eV and a mass range from 33.00000 to 500.00000 m/z with a resolution of 60,000.
349 Specific markers of PS were particularly targeted using their ion markers: styrene (78.046 and
350 104.061 m/z), styrene dimer (91.054, 117.069, 193.101 and 208.125 m/z) and styrene trimer
351 (91.054, 103.054, 195.112 and 312.187 m/z). A total of 10 blank samples were analyzed (3 pre-
352 measure blanks, 4 intermediate blanks after each median or distal sample analyzed, and 3 post-
353 measure blanks) to measure the basal signal of the laboratory, and thus check for the
354 contaminations. The sample values were statistically compared to the blank values using a
355 Wilcoxon rank sum test. The stock PS-NP dispersion was also analyzed. Results are presented
356 as average area \pm standard error.

357

358 **3. Results**

359 *3.1. Time series of fluorescence*

360 The higher the concentration of FD4 found in the serosal side, the greater its passage through
361 the membrane, and the greater the impairment of membrane integrity (Thomson et al., 2019).
362 The results obtained (Fig. 2) showed a FD4 concentration in the serosal part of both median
363 and distal segments was below the limit of detection (LOD; estimated at 23.4 ng mL^{-1} ; see
364 supplementary material to see the standard curves), so that it could not be detected. It did not
365 increase over time neither in the distal nor in the median segment all along the experiment (p-
366 values > 0.05 , see sections 1.2.2, 1.3.2 of the S5 for FD4), proving the integrity of the intestinal
367 tissue all along the 150 min experiment in both segments. Moreover this integrity was not

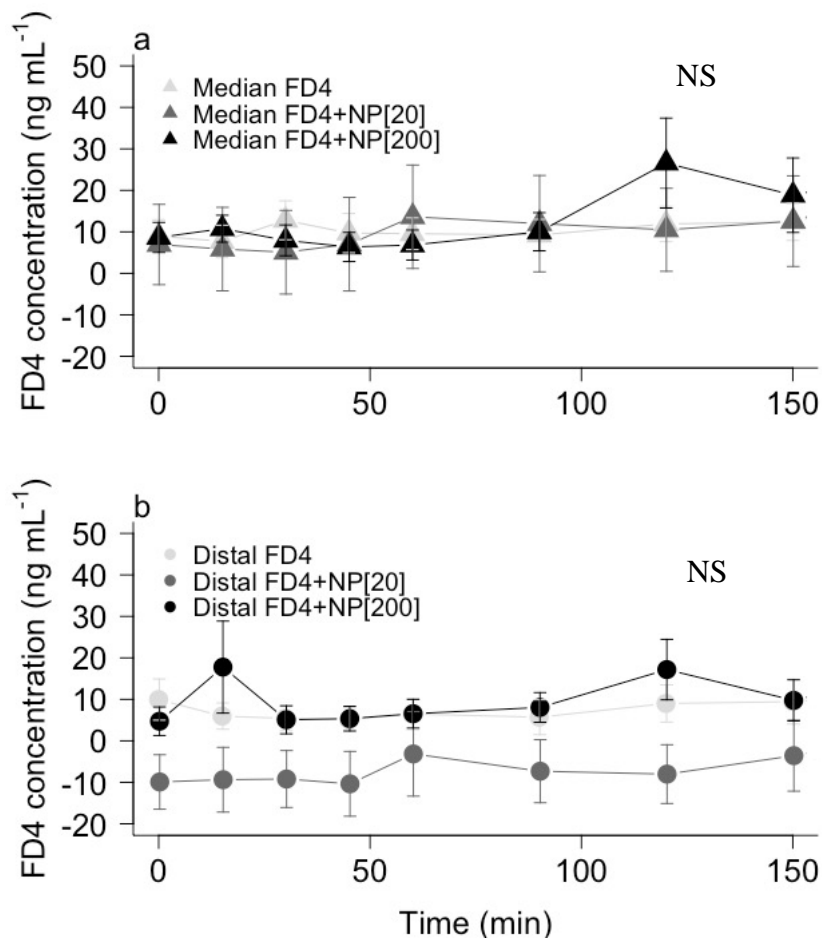
368 disturbed by addition of PS-NP (p-values > 0.05; see sections 2.2.2, 2.3.2 of S5 for FD4+NP20
369 and 3.2.2, 3.3.2 of S5 for FD4+NP200).

370 Results from fluorescence data show that PS-NP concentration in the serosal side of the
371 intestinal sections increased from T0 within 15 minutes after addition of PS-NP to the luminal
372 side in both the median and the distal sections (Fig. 3, and sections 4.3.2. and 5.2.2. of the S5).
373 In median sections, PS-NP concentration after NP[200] addition reached a plateau after about
374 50 minutes (section 4.2.2 of S5), whereas that after NP[20] addition, PS-NP concentration
375 continued to increase over 150 minutes and did not plateau (section 5.2.2 of S5). In distal
376 sections, PS-NP concentration after NP[20] and NP[200] addition reached a plateau after only
377 15 minutes and there was no further increase up to 150 minutes (see sections 4.3.2. and 5.3.2.
378 of S5). This difference between median and distal gut (p-value=0.0001; t-value=-4.457, see
379 section 4.1.3) cannot be explained at this time but could result on the specialization of each of
380 these intestinal segments in the assimilation process, considering that the median gut exhibits a
381 higher pinocytosis ability that can accommodate smaller and hydrophobic particles such as
382 polystyrene (Galloway et al., 2017; Urakami et al., 1994). For both median and distal segments,
383 the final PS-NP concentrations measured in the serosal side were higher in NP[20] treatment
384 than in NP[200] treatment compared to T0 (19-times and 17-times higher, respectively versus
385 about 7-times and 4-times higher, respectively; p-value = 0.09, z-value = 2.831 for NP200
386 compared to T0, and p-value<0.001, z-value = 8.517 for NP20, see sections 4.1.1 and 5.1.1.
387 respectively).

388

389 **Fig. 2**

390

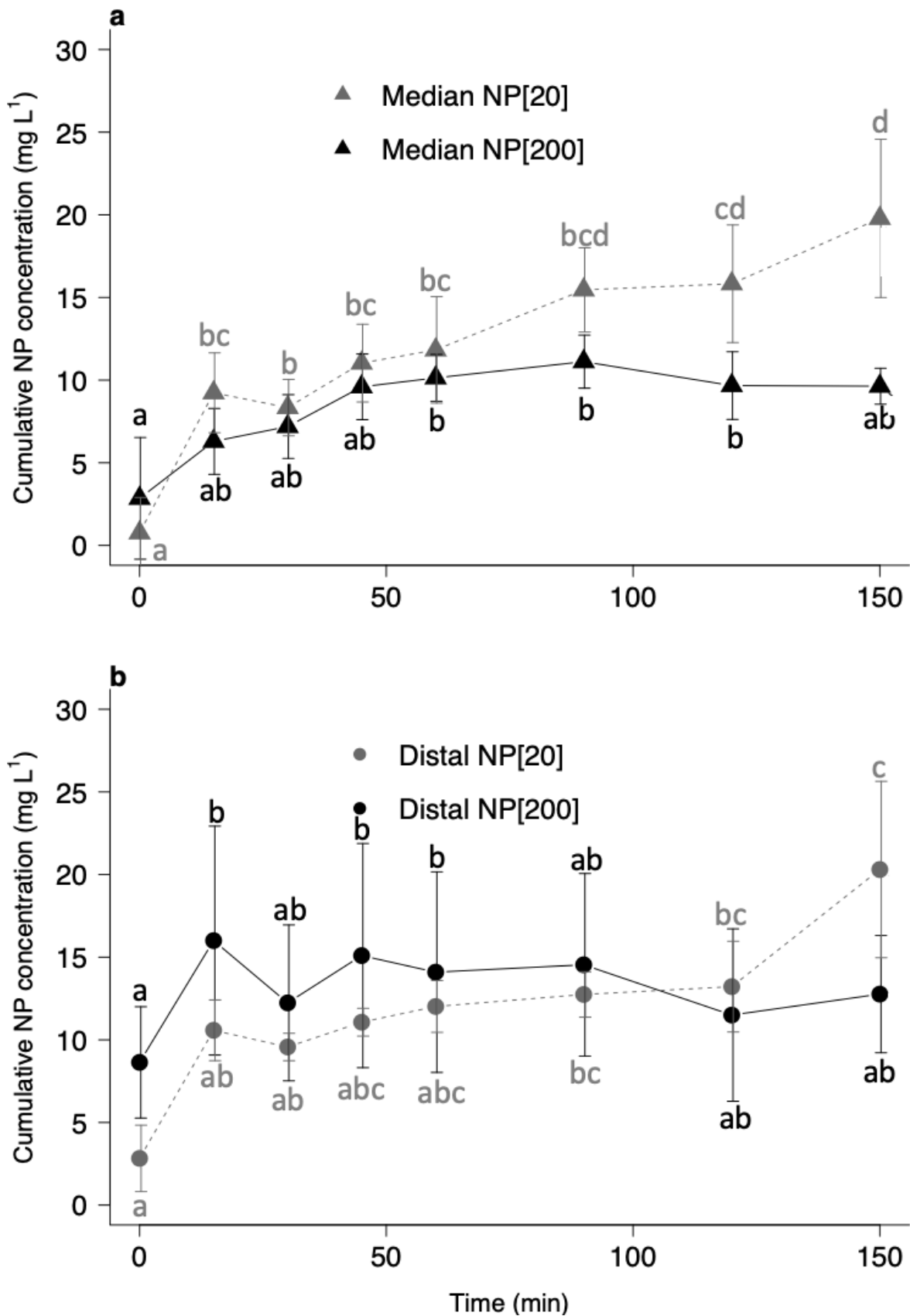


391
 392 **Fig. 2 :** Kinetics of FD4 passage in the serous compartment of Ussing chambers as an indicator
 393 of gut integrity and permeability. FD4 concentration (ng mL⁻¹; mean ± standard error) was
 394 measured over time in the serous side of the median (a) and distal (b) intestine of adult seabass
 395 (*Dicentrarchus labrax*) after addition of FD4 (initial concentration of 125 μg mL⁻¹; n=14
 396 median intestines and n=15 distal intestines) into the luminal side of the tissue, and after
 397 combined addition of FD4 with two PS-NP concentration further tested (20 mg L⁻¹, i.e. NP[20],
 398 and 200 mg L⁻¹, i.e. NP[200]). The limit of detection (LOD) for FD4 was estimated at 23.4 ng
 399 mL⁻¹ (see supplementary material S3 to see the standard curves). NS indicates no significant
 400 differences from T0, nor from each other sampling point whatever the treatments (linear mixed-
 401 effect model and general linear hypothesis with *glht()* in ‘mutlcomp’ package, see sections 1
 402 and 2, in supplementary material S5).

403

404 Fig. 3

405



406

407

408

409 **Fig. 3** : Kinetics of 50 nm polystyrene nanoplastic beads (NP) passage in the serosal part of
410 median and distal gut segments. NP concentration (mg L^{-1} ; mean \pm standard error) measured in
411 the serous side of the median (a) and distal (b) intestine of adult seabass (*Dicentrarchus labrax*)
412 after addition of pure NP at two concentrations (either 20 mg L^{-1} or 200 mg L^{-1} , $n=6$ per
413 intestinal segment) in the luminal side. For each NP concentration, means not sharing a
414 common letter are significantly different (linear mixed-effect model and general linear
415 hypothesis with *glht* in ‘mutlcomp’ package, see supplementary material section 4 and 5 of the
416 S5 for detailed statistical results). Fluorescence is not null at T0 because of the natural
417 fluorescence of the composed solutions used. This value T0 has been subtracted from the others
418 (see material and methods section). The limit of detection (LOD) for PS-NP was estimated at
419 9.2 mg L^{-1} (See supplementary material to see the standard curves).

420

421 3.2. SEM analyses on serosal samples

422 However, it is important to note that the increasing PS-NP concentrations calculated from
423 increasing fluorescence cannot be undoubtedly interpreted by a passage of PS-NP, as a leakage
424 of fluorophore from the beads may have occurred, all along the experiment (including in the
425 Krebs-Mannitol solution). The control of leakage by dialysis protocol revealed about 20% of
426 dye leakage (see supplementary material S2), supporting the need to use other techniques and
427 not only fluorescence to reveal the presence of PS-NP in the serous side.

428

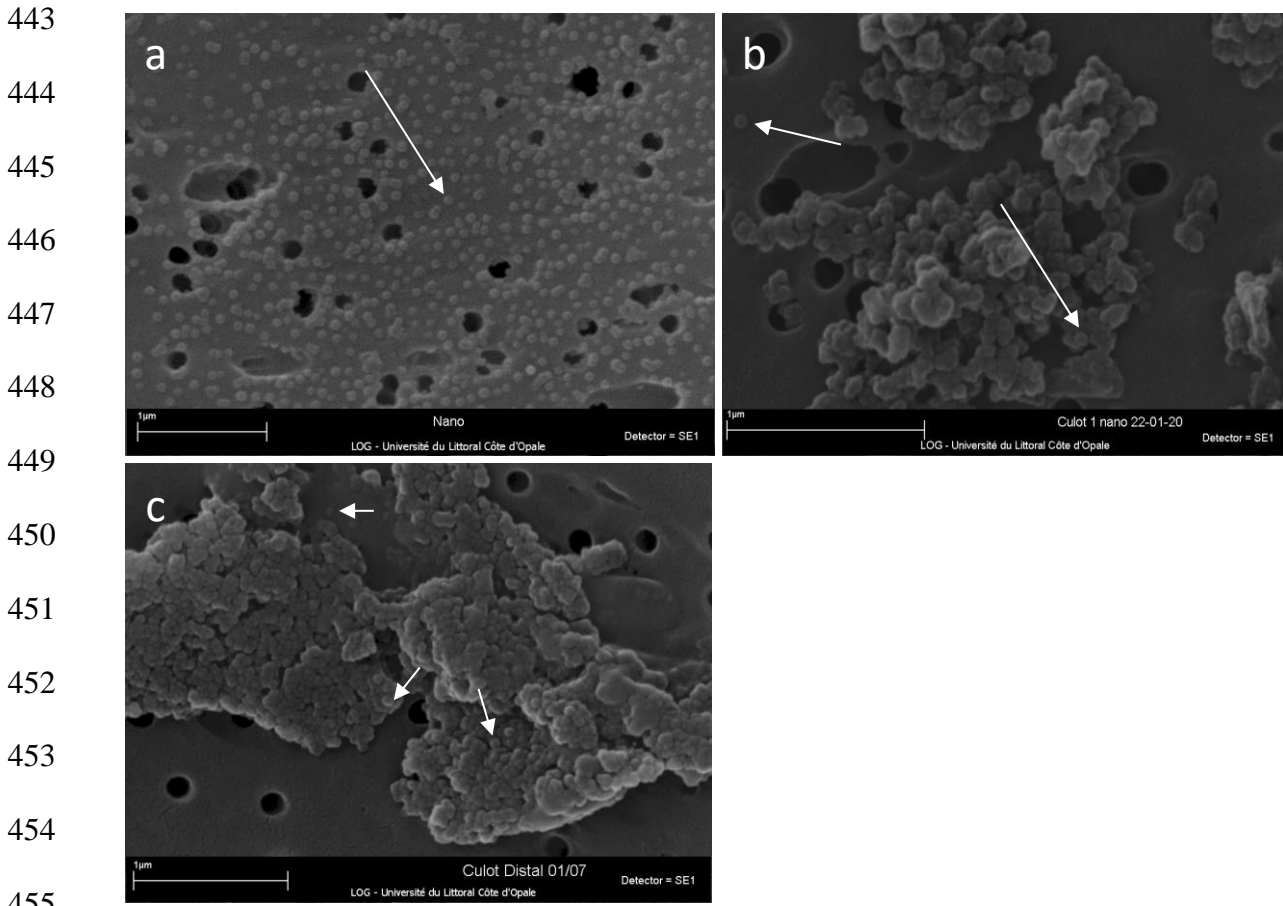
429 The SEM analyses revealed an agglutination of beads that was due to the TCA precipitation
430 protocol used, as the same was observed concerning the stock solution of fluorescent PS-NP
431 beads treated with TCA (Fig. 4). Also, a DLS analysis helped to confirm that after TCA washing

432 protocol, beads formed microscale aggregates (2351.3 ± 511.7 nm), displaying a negative
433 surface charge (-5.1 ± 0.6 mV), while they remained at a nanometric scale with a positive
434 surface charge in ultrapure water (66.1 ± 0.4 nm and 51.4 ± 2.1 mV), 10 mM Krebs-Glucose
435 (306.2 ± 61.1 nm and 19.4 ± 1.1 mV) and 10 mM Krebs-Mannitol (86.0 ± 0.4 nm and $18.5 \pm$
436 3.5 mV), used during the experiment. These results showed that most of the translocated
437 fluorescence observed was due to beads and not to leached fluorescence. Observing beads in
438 the median sample was technically challenging considering that the quality of the wash and
439 TCA precipitation was not optimal.

440

441

442 **Fig. 4**



456 **Fig. 4** : Observation of nanoplastics by scanning electron microscopy. (a) Sample of aqueous
457 suspension of polystyrene nanoplastic beads (PS-NP) at the concentration of 5000 mg L⁻¹ in
458 Krebs-Glucose (latex beads amine-modified polystyrene 0.05 μm mean particle size, Sigma-
459 Aldrich, France); (b) sample of the same aqueous PS-NP dispersion after the TCA precipitation
460 protocol (see details in material and methods section); (c) sample of Krebs-Glucose solution
461 collected from the serous side of the distal portion of the intestine, at the end of the 150-min
462 kinetic experiment during which the PS-NP dispersion diluted at 200 mg L⁻¹ was added to the
463 luminal side at T0, and washed according to the TCA precipitation protocol. The white arrows
464 show the nanoplastic beads observed.

465

466 3.3. Particles characterisation via Py-GC-HRMS analyses

467 At the end of this set of analysis, it remained to ascertain the polymeric composition of the
 468 beads by analyzing the pyrolysis products generated by Py-GC-HRMS (Table 1). The pyrolysis
 469 product of the PS-NP stock dispersion showed a strong signal of styrene and a weaker signal
 470 of styrene dimer (at such high pyrolysis temperature styrene trimer was nearly absent).
 471 Consequently, the styrene was conserved as the unique target marker for PS-NP. Substantial
 472 amounts of styrene were identified in the samples taken from the serosal side of both the median
 473 (surface area: 379,574,179 ± 198,468,159) and distal (surface area : 398,258,238 ±
 474 363,810,422) intestine sections after addition of NP[200]. No significant difference in these
 475 chemical properties was observed between median and distal intestinal segments.

476

477 **Table 1:** Styren signal intensities measured by Py-GC-HRMS in the serosal solutions (Krebs-
 478 Glucose) collected from the serous side of the distal (n=2) and median (n=2) portions of the
 479 intestine compared to the PS-NP stock dispersion (n=1). Data are expressed as a mean ±
 480 standard error.

481

| | Styrene surface area (arbitrary unit, mean area ± standard error) |
|---------------------------|--|
| PS-NP stock dispersion | 16 804 278 980 |
| Blank (n=10) | 4 501 980 ± 869 945 |
| Median gut (n = 2) | 379 574 179 ± 198 468 159 |
| Distal gut (n = 2) | 398 258 238 ± 363 810 422 |

482 Before analyses, serosal solutions were washed according to the TCA precipitation protocol, at
 483 the end of the kinetic experiment in Ussing chambers during which the 50 nm polystyrene
 484 nanoplastic (PS-NP) beads dispersion diluted at 200 mg L⁻¹ was tested. The PS-NP stock

485 dispersion was not treated with the TCA protocol before analyses. The 10 blank values in total
486 contain n=3 pre-measure blanks, n=4 intermediate blanks and n = 3 post-measure blanks. The
487 absence of standard error indicates that no second value was obtained. The bold values are
488 significantly different from the blank value (Wilcoxon test, $W = 40$, $p\text{-value} < 0.001$). Only the
489 styrene was statistically analyzed because it was the most important peak of the tested solutions,
490 the remaining peaks were represented by styrene dimer and trimer.

491

492 **4. Discussion**

493 *4.1. Strengths of combining techniques for monitoring nanometric particles in tissues.*

494 By coupling fluorescence, SEM and Py-GC/HRMS, completed by DLS analyses, the present
495 study evidences the translocation of 50 nm, -NH₂ functionalized PS-NP nanobeads, across the
496 intestine of adult European sea bass. This was presumably due to the fact that they remained in
497 the nanometre state in both Krebs-Mannitol and Krebs-Glucose as shown by the DLS analyses.
498 Indeed, they were only aggregated with TCA precipitation.

499 First, by measuring the increase in fluorescence on the serosal side of the intestine, we found a
500 regular increase of PS-NP concentration, even if less marked from 90 min, in the serosal side
501 of both median and distal segments of the intestine, after injection of 200 mg L⁻¹ and 20 mg L⁻¹
502 PS-NP (NP[20] and NP[200]) in the luminal side, suggesting a passage of PS-NP. Second,
503 SEM provided additional visual proof of the passage of PS-NP through the distal intestinal
504 segment at the PS-NP concentration of 200 mg L⁻¹ (NP[200]). And third, Py-GC/HRMS data
505 clearly showed that the amount of styrene in the samples was 4–90-times greater when
506 compared to the controls after intestinal luminal exposure to this concentration, confirming the
507 presence of PS in both median and distal samples, rather than leaching of fluorescence.

508 The aggregates of PS-NP observed by electron microscopy on the experimental samples can be
509 seen as an artefact of our methodologies because the TCA used to remove the salts to ensure

510 SEM good quality caused a shift in the ζ -potential leading to aggregation, while the PS-NP
511 remained at a nanometre state during exposures as confirmed by DLS. Although an
512 improvement of the methodology is certainly possible to avoid this aggregation, this does not
513 compromise the demonstration of the passage of PS-NPs through the intestinal epithelium.
514 This shows that using complementary methods is necessary to prove and to carefully interpret
515 the presence of PS-NP on the serosal part of the intestine.

516 Surprisingly, for both median and distal segments, the final PS-NP concentrations measured in
517 the serosal side were higher in NP[20] treatment than in NP[200] treatment compared to T0.
518 This could be due to an overloading at the highest NP concentrations, possibly resulting in a
519 shutdown of the absorptive capacity as reported several years ago in grasscarp,
520 *Ctenopharyngodon idella*, when large amounts of food are suddenly available (Stroband and
521 van der Veen, 1981). It is not excluded that a concentration-dependent aggregation may have
522 also played a role inducing a bias for a non-constant exposure, but DLS data indicated that NP
523 remained in the nanometric scale after 15-30 min of incubation in the four media used in this
524 study. Additionally, we know that these NPs have a stable behavior over 48h in ultrapure water,
525 artificial seawater and 2-mm filtered natural (Tallec et al., 2019).

526 Our study makes it possible to resolve issues raised in a very recent study reporting the
527 translocation of PS-NP palladium labelled through the intestine of another fish species, the
528 Atlantic salmon *Salmo salar* (Clark et al., 2022). Clark's study and our study proposed different
529 ways to address the debated issue of PS-NP translocation. First, we used three complementary
530 methods to evidence the PS-NP translocation in a direct manner, whereas Clark et al. showed
531 the presence of PS-NP-palladium labelled measuring palladium by inductively coupled plasma
532 mass spectrometry (ICP-MS). Second, we confirmed the PS-NP passage through median and
533 distal intestinal segments, whereas Clark et al. found a passage in the anterior intestine,
534 containing pyloric caeca. This accumulation of PS-NP in anterior intestine would be a

535 consequence of the nature of pyloric caeca, which is a region of bacteria translocation through
536 endocytosis (Ringø et al., 2007), which is the same mechanism as nanoparticle uptake.
537 Pyloric caeca, by their structure, could thus represent a slightly different and very specific
538 portion of the intestine that could favor translocation. Therefore, it may not be representative
539 of the entire intestine of all vertebrates, which are generally devoid of such structures. Third,
540 our study shows the passage of PS-NP through an intact and functional intestine, as proven by
541 the FD4 experiment (Fig. 1). Our results showed the integrity of the membrane all along the
542 experiment, and that this integrity was not disturbed by addition of PS-NP. In Clark et al. the
543 ligations performed on both sides of the intestine could have favored local necrosis and
544 consequently the passage of PS-NP, though this should be confirmed. Finally, the Ussing
545 chamber we used has long been recognized as the most *ex-vivo* suitable method to study the
546 transport processes, while Clark et al. recognize “the limitations of the gut sac method” and call
547 for complementary innovative methods to their “proof-of-concept study”.

548

549 4.2. *Limitations of our study*

550 However, we are aware that our experimental design imposes some limitations on data
551 interpretation.

552 First, we used manufactured standardized polystyrene latex beads with a round regular shape,
553 whereas in natural marine habitats, nanoplastic debris vary in shape and composition (e.g.
554 polyethylene, polyester), and surface state (microorganisms and biomolecules attached to the
555 plastic surface, i.e. eco-corona), and these three factors may play a role in translocation
556 (Ramsperger et al., 2020; Rochman et al., 2019). For example, the styrene content of
557 polystyrene microplastics has been shown to strongly influence their ability to be phagocytized:
558 the greater the styrene content, the greater their ability to undergo phagocytosis (Urakami et al.,
559 1994). Also, the surface functionalization of the PS-NP (NH₂ in the present study) can affect

560 the physical behavior of the particles (e.g. aggregation, charge) in solution (Tallec et al., 2019),
561 and can consequently modify their bioavailability and thus their toxicity (Tallec et al., 2018).
562 The functionalization and surface charge are responsible for the interfacial dynamics, and thus
563 the interactions with living systems, especially biological membranes (Corsi et al., 2020).
564 Electrostatic interactions produced by the positive charges, such as NH₂, can reorient the
565 phospholipid headgroups of membranes, reducing lipid density and fluidizing the membrane
566 (Liu et al., 2020, 2011). This facilitates both passive diffusion and the membrane bending
567 associated with endo- and phagocytosis (Matthews et al., 2021), and can contribute to the fast
568 uptake of the PS-NH₂. Moreover, the environmental relevance of NH₂-functionalized NP is
569 questionable due to oxidation processes occurring in the environment leading to a carboxylic
570 surface and anionic charge (Fotopoulou and Karapanagioti, 2012).

571 Second, we showed a translocation process from 15 min after addition into the luminal side,
572 but this time does not consider the natural transit time of the particles along the gut. In mussels,
573 the translocation of plastic particles (particles of 3 and 9.6 μm) *in vivo* was estimated to start 3
574 days after ingestion, and was highest 12 days after ingestion. Smaller particles (3 μm) were
575 much more translocated than larger ones (9.6 μm) suggesting an interaction “translocation time
576 x particle size” that remains to be tested. Then, further experiments are required to validate our
577 findings *in vivo* in fish living in natural environment.

578 Finally, in order to avoid excessive manipulation of the intestinal segment that could have
579 impaired tissue integrity, the muscle layers were not dissected out. Therefore, the NP and FD4
580 passages measured in our set-up are probably underestimated since these molecules have more
581 cell layers to cross before reaching the serosal compartment, compared to the *in vivo* situation
582 where the molecules would reach the bloodstream more rapidly.

583
584 We are aware that the fluorescence measurement method has biases and we wanted to add
585 methods like SEM and Py-GC/HMRS, but which are not quantitative. Anyway, our idea was

586 to make the proof of concept that there is a passage but not to quantify this passage because
587 the Ussing method is too 'artificial' (e.g. no intestinal transit, nor blood flow, nor hormonal or
588 nervous influence in Ussing chambers).

589

590 **5. Conclusion**

591 This study reports direct evidence from an *ex vivo* system that translocation of 50 nm PS-NPs
592 across the intestine of the European sea bass occurs within a few minutes. Previous studies
593 showed the presence of NPs in various tissues, but either no clear or indirect evidence of
594 translocation was provided, or the data were compromised by inadequate tissue preparation or
595 methodology. Also, our study makes it possible to resolve issues raised in a very recent study
596 reporting the translocation in another fish species. The findings of our present study are
597 strengthened by the combination of fluorescence measurements, insufficient alone because of
598 possible fluorophore leaching, with two complementary analytical methods that proved the
599 physical presence of PS-NP on the serosal side of the intestine. Our data reveal that NPs beads
600 can be translocated across the intestinal barrier in the medial and distal intestine. This provides
601 a plausible mechanism to explain the appearance of NPs in internal organs by subsequent
602 transportation through the bloodstream.

603

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791 **CRedit authorship contribution statement**

792 All of the authors contributed to writing and editing the manuscript. M. Vagner performed the
793 experiment, analyzed the data; G. Boudry designed the analysis; L. Courcot performed the
794 scanning electron microscopy ; D.Vincent and A. Huvet advised on the methodology regarding
795 NP concentrations and detection strategies; K. Tallec performed the DLS analyses; G. Duflos

796 performed the management of Py-GC-HRMS; A. Dehaut. performed the Py-GC-HRMS data
797 treatments; J.-L. Zambonino. designed and performed the experiment and analyzed the data.

798

799 **Declaration of Competing Interest**

800 The authors declare no conflicts of interest.

801

802 **List of supplementary materials**

803 **Supplementary material S1 Testing the adsorption of FD4 to PS-NP**

804 **Supplementary material S2: Control for leakage of dye by dialysis**

805 **Supplementary material S3 : Calibration curves**

806 **Supplementary material S4: Determination of the autofluorescence of the tissue**

807 **Supplementary material S5 : Statistical analyses of the fluorescence dataset**

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