Supplementary Material

2. Methods

Composition of PS NP stocks

According to the manufacturer, unlabelled PS-NH_2 stock (PA02N, lot: 12839) was supplied at 10% solids (w:v) in deionized water with no stabilizers. The unlabelled PS-COOH stock suspension (PC02N, lot: 11652) contained 10.1% solids (w:v), 0.1% sodium dodecyl sulfate (SDS) and 0.05% sodium azide (NaN_3) as stabilizers. Fluorescently labelled PS-COOH stock suspension (FC02F, lot: 11587, Dragon green labeling ex/em 480/520), used for uptake and translocation experiments, contained 1% solids (w:v), 0.1% Tween 20 and 2 mM NaN_3. At the concentrations used in our study (5 and 25 µg mL^{-1}), unlabelled and fluorescently labelled PS-COOH suspensions contained a very low residue of stabilizers, as reported in Table S1.

Table S1. Calculated concentration of SDS, Tween 20 and NaN_3 as stabilizers in the unlabelled and fluorescently labelled PS-COOH suspensions at 5 and 25 µg mL^{-1}.

	Unlabelled PS-COOH (PC02N, lot: 11652)		**Labelled PS-COOH** (FC02F, lot: 11587, Dragon green labeling ex/em 480/520)	
	SDS	NaN_3	Tween 20	NaN_3
5 µg mL^{-1}	0.05 µg mL^{-1}	0.025 µg mL^{-1}	0.5 µg mL^{-1}	0.001 mM
25 µg mL^{-1}	0.25 µg mL^{-1}	0.125 µg mL^{-1}	2.5 µg mL^{-1}	0.005 mM

By comparing the amount of SDS and NaN_3 in the concentrations tested of PS-COOH (5 and 25 µg mL^{-1}) with E(L)C_{50} values in marine model organisms (Table S2), the effect of the stabilizers can be considered negligible. E(L)C_{50} values are in fact from 1 to 3 orders of magnitude higher than the concentrations of SDS and NaN_3 in PS-COOH working suspensions. We already clarified the negligible effects of stabilizers from different batches of PS-COOH at these final concentrations (Bellingeri et al., 2019; Bergami et al., 2020).

Therefore, the toxicological effects documented for either PS-COOH and PS-NH_2 are fully ascribable to the nanoscale dimension of the particles and their surface charges and not to the presence/absence of stabilizers (i.e., considering also SDS- and NaN_3-free PS-COOH used in Bergami et al., 2016, 2017, 2019; Manfra et al., 2017; Della Torre et al., 2014).

We agree to the need to assess the potential adverse effects of stabilizers present in commercially available NP stocks, however, it is important to take into account also the experimental conditions used

to assess the toxicity (e.g., time of exposure, culturing media, temperature, pH, dark/light). So far the majority of the studies to evaluate the role of stabilizers as confounding variables in PS NP testing has mainly been conducted on freshwater species (Pikuda et al., 2019; Heinlaan et al., 2020). With this regards, our previous studies on marine organisms (Bergami et al., 2016, 2017; Pinsino et al., 2017; Della Torre et al., 2014) have wrongly been cited by Pikuda et al. (2019) (i.e., in a study on the freshwater crustacean *Daphnia magna*) as an example of PS NP suspensions containing stabilizers, although they were all stabilizers-free.

Table S2. SDS and NaN_3 E(L)C_{50} values and exposure time (h) in different marine taxa. E(L)C_{50} values are reported as mean ± SD or (95% CI).

Reagent	Model organism	E(L)C_{50} (µg/mL)	Time (h)	Reference
SDS	*Vibrio fischeri*	2.62 ± 0.90	0.25	(Mariani et al., 2006)
	Artemia spp.	23.20 ± 6.50	24	(Libralato et al., 2016)
NaN_3	*Vibrio fischeri*	> 100	0.5	(Heinlaan et al., 2020)
	Artemia spp.	84 (76-92)	24	(Sleet and Brendel, 1985)

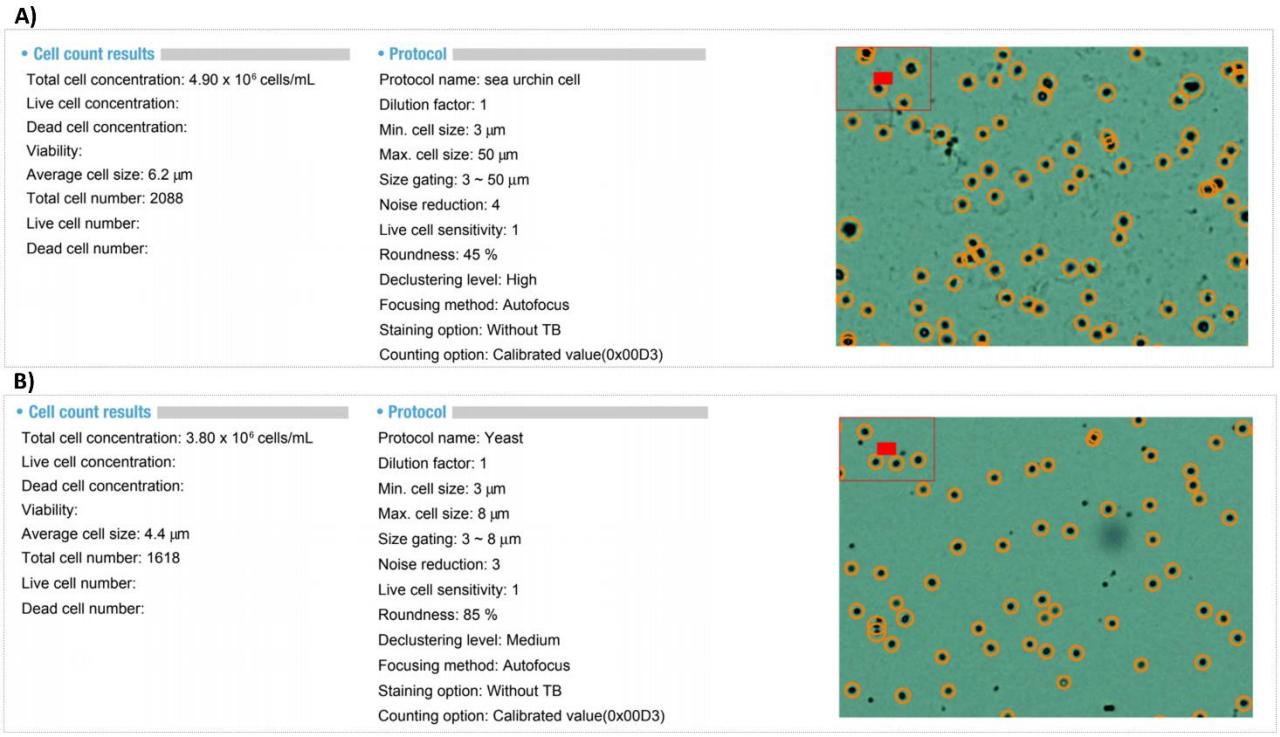

Figure S1. Luna II Automated cell counter (Logos Biosystems Inc) instrument settings used for cell counting. Settings for (A) heterogeneous coelomocytes populations (phagocytes, vibratile cells, white and red amebocytes) suspended in CF and (B) yeast cells of *Saccharomyces cerevisiae* suspended in 0.22-µm filtered NSW.

According to the manufacturer's instructions, the fluorescently labelled PS-COOH (60 nm) used in our study have the fluorescent Dragon green dye embedded in the polymer structure (Bangs Laboratories Inc. Product Data Sheet 731). Therefore, a quality control protocol was set up to assess the leaching of the dye and run at the same conditions used for short-term cultures of sea urchin coelomocytes (i.e., sterile vials, 18°C, in the dark). After 4 h of suspension in CF, aliquots of fluorescently labelled PS-COOH (25 µg mL^{-1}) were centrifuged in centrifugal filter units (Microcon®- 10 kDa) at 7000 x g for 30 min at 18°C. CF only was used as a reference. The fluorescence intensity of the resulting filtered solution ($radius_{min}$=1.42 nm of a smooth sphere according to Erickson, 2009) was recorded through the Tecan spectrophofluorometer (Infinite M1000 Pro). Before transferring the aliquots of CF only and CF + NPs to the centrifugal filter units, the fluorescence intensity was determined (CF: 234 ± 10 a.u.; CF + NPs: 42746 ± 120 a.u.). After centrifugation and filtration, no significant differences were observed in the fluorescence intensity of the resulting solution between CF and CF + NPs (CF: 227.3 ± 17 a.u.; CF + NPs: 218 ± 2.5 a.u.) (Fig S2). These results confirm that under the experimental conditions used no loss of the fluorophore from the labelled particles was detected.

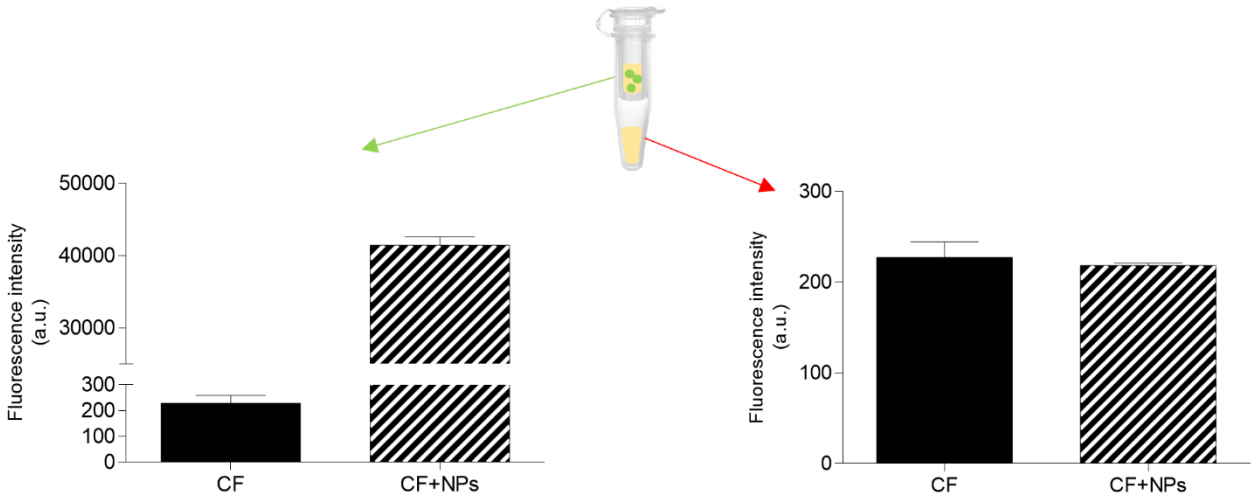

Figure S2. PS NP fluorophore leaching test. Fluorescence intensity of the resulting solution before and after centrifugation of CF (only) and PS-COOH suspended in CF at 25 µg mL^{-1} (CF+NPs) in centrifugal sample clarification units of 10 kDa ($radius_{min}$=1.42 nm of a smooth sphere in according to Erickson, 2009).

A) Coelomocytes viability

	Cell viability (%)
CTRL	80.3±1.6
PS-COOH 5	78.1±1.9
PS-COOH 25	70.9±7.5
PS-NH_2	62.4±3.7

B) Neutral red retention time

Destabilized lysosomes(%)

	30 min	45 min	60 min
CTRL	6.5±1.9	9.6±4.7	15.2±5.9
PS-COOH 5	8.6±2.9	15±4.3	18.2±6.7
PS-COOH 25	16.5±3.4	21.7±5.6	23.75±4.9
PS-NH_2	23±3.7	25.7±4.9	30.8±2.2

C) Phagocytosis activity

	PC (%)	PI (%)
CTRL	73.6±6.5	167.4±12.1
PS-COOH 5	52.9±4	163±14.5
PS-COOH 25	34.1±6.5	146±12.5
PS-NH_2	32±1.8	124.3±14.3

D) PS-COOH uptake

Fluorescence intensity (a.u.)

	1h	2h	3h	4h
PS-COOH 5	704.3±51.5	806.7±163.9	815±57.7	854.7±145.4
PS-COOH 25	3220±361.45	3861.3±135.6	3975±179	4443±484

E) PS-COOH clearance

Fluorescence intensity (a.u.)

	1h	3h depuration
PS-COOH 5	704.3±51.5	430±40.4
PS-COOH 25	3220±361.4	1930±37

Figure S3. Summary of the results obtained in this study. Results of (A) coelomocytes viability (mean % ± SD); (B) neutral red retention time (mean % ± SD); (C) phagocytic activity (mean % ± SD); (D) PS-COOH uptake (mean a.u. ± SD) and (E) PS-COOH clearance (mean a.u. ± SD).

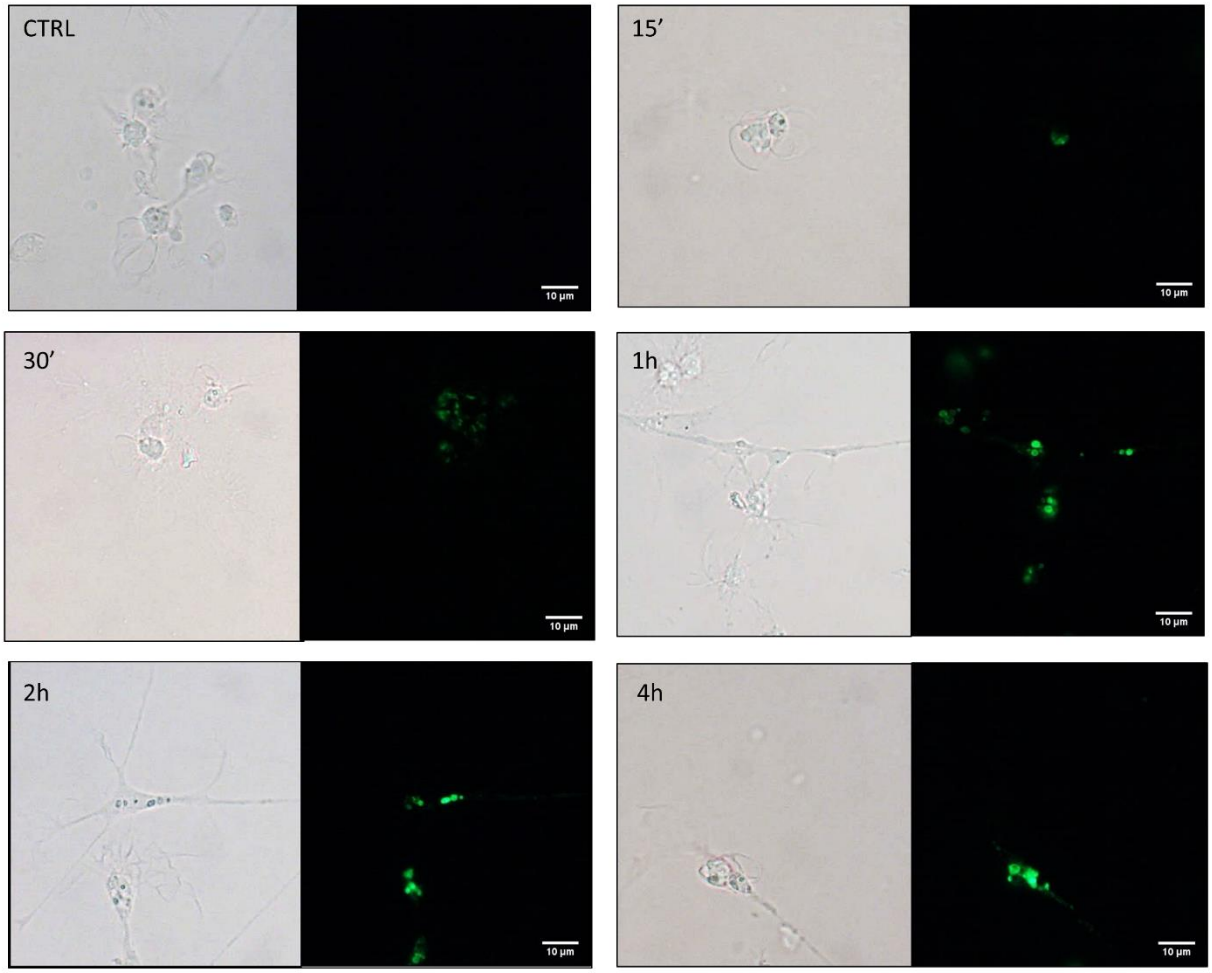

Figure S4. **Time-resolved disposition of PS-COOH in sea urchin phagocytes.** Fluorescently labelled PS-COOH (25 µg mL^{-1}) internalization at different times of exposure in phagocytes of sea urchin *Paracentrotus lividus*. Images were recorded at the same gain using an optical microscope (Olympus U-25ND6) equipped with fluorescence GFP-filter (488 λ_{ex}- 510 λ_{em}). Scale bar: 5 µm.

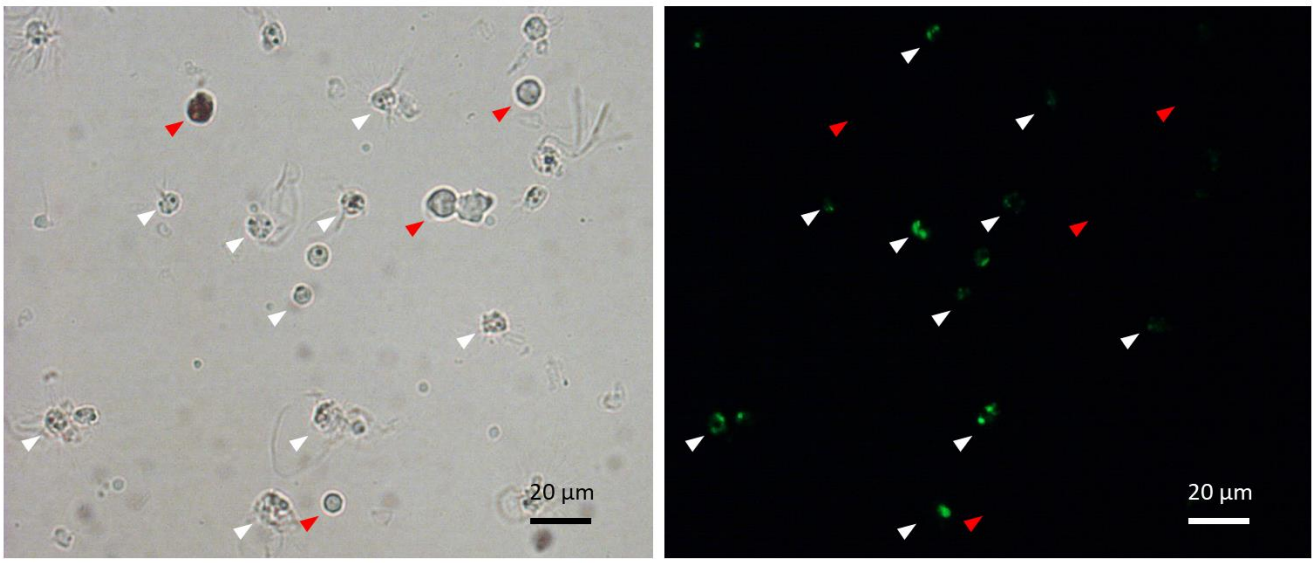

Figure S5. **Disposition of PS-COOH in different *P. lividus* coelomocytes sub-populations.** Optical and fluorescence images (on the left and right panel, respectively) of coelomocytes after 4 h of exposure to fluorescently labelled PS-COOH (25 µg mL^{-1}). PS-COOH agglomerates were localized on phagocytes (white arrowheads), while they were absent on other cell sub-populations (red arrow heads), such as vibratile cells and red and white amoebocytes. Scale bar: 20 µm.

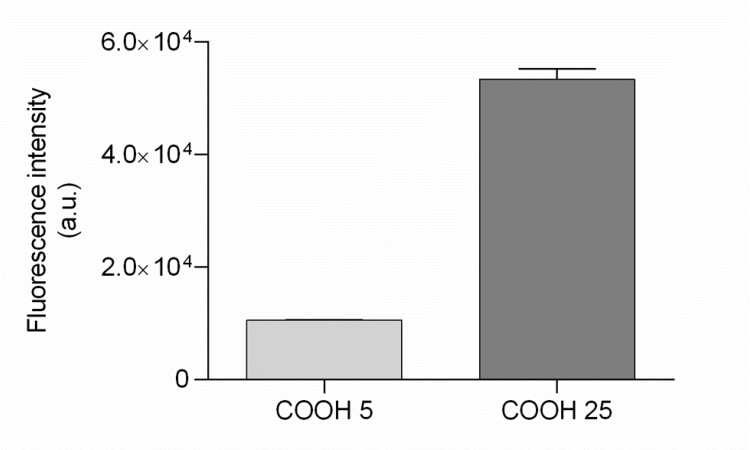

Figure S6. **Labelled PS-COOH suspended in CF.** Fluorescent intensity (arbitrary units, a.u.) of labelled PS-COOH (5 and 25 µg mL^{-1}) suspended in CF and incubated for 4 h in sterile vials in the dark at 18°C. Bars represent mean ± SD (n = 3). Fluorescence intensity values are normalized subtracting the auto-fluorescence of the control group (CF only).

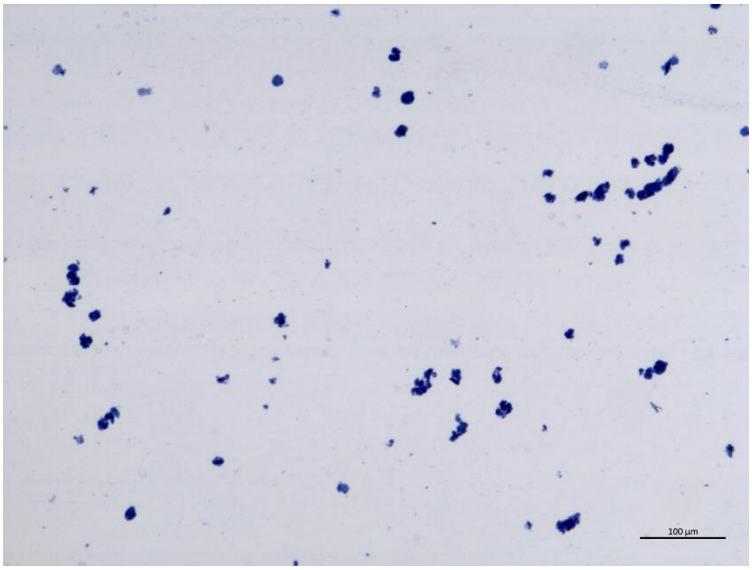

Figure S7. Trypan blue exclusion test. Trypan blue (0.4%) does not fully dissolve in *P. lividus* CF (CF: trypan blue 1:1), with the formation of dye clots. Images were recorded using an optical microscope (Zeiss Apotome.2). Scale bar: 100 µm.

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