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Effect of Microplastic on the Gills of the Shore Crab *Carcinus maenas*

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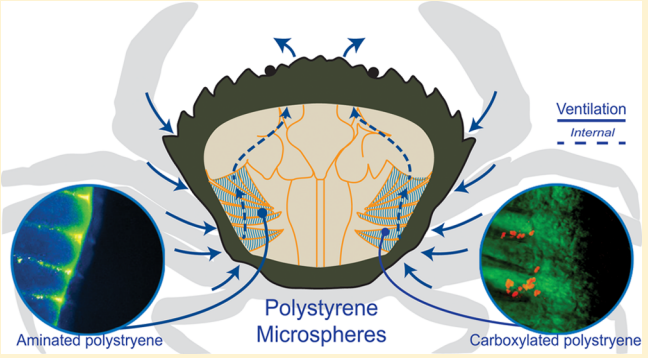
Supporting Information

ABSTRACT: Microscopic plastic debris (microplastics, <5 mm in diameter) is ubiquitous in the marine environment. Previous work has shown that microplastics may be ingested and inhaled by the shore crab *Carcinus maenas*, although the biological consequences are unknown. Here, we show that acute aqueous exposure to polystyrene microspheres (8 μm) with different surface coatings had significant but transient effects on branchial function. Microspheres inhaled into the gill chamber had a small but significant dose-dependent effect on oxygen consumption after 1 h of exposure, returning to normal levels after 16 h. Ion exchange was also affected, with a small but significant decrease in hemolymph sodium ions and an increase in calcium ions after 24 h post-exposure. To further asses the effects on osmoregulation, we challenged crabs with reduced salinity after microplastic exposure. Neither microspheres nor natural sediments altered the crab's response to osmotic stress regardless of plastic concentration added. Carboxylated (COOH) and aminated (NH_2) polystyrene microspheres were distributed differently across the gill surface, although neither had a significant adverse impact on gill function. These results illustrate the extent of the physiological effects of microplastics compared to the physiological resilience of shore crabs in maintaining osmoregulatory and respiratory function after acute exposure to both anthropogenic plastics and natural particles.



INTRODUCTION

Microplastics (plastic particles <5 mm)[1] are an emerging environmental problem and have been accumulating in coastal habitats for at least four decades.[2] Microplastics come from sewage releases of microbeads added to cosmetic products to give exfoliation properties, paints, coatings, and industrial pellets and from the breakdown of larger plastics.[3] This second source is enhanced by abiotic processes such as wave action, UV degradation, and general heat stress and by biological transformation. For example, the shore crab *Carcinus maenas* is able to break down any microscopic rope fibers that it has ingested through the gastric mill digestive processes.[4] Ingestion of microplastics has been documented in over 200 marine and aquatic species.[5] Polystyrene microspheres (0.4−30.6 μm) have been found to be consumed by numerous organisms such as zooplankton,[6,7] filter-feeding molluscs,[8] and scavenging decapod crustaceans.[9,10] Most of these studies have concentrated on the uptake of microplastic by ingestion and the potential for feeding activity to then be disrupted. For example, Wright et al.[11] showed depletion of energy reserves of up to 50% in lugworms (*Arenicola marina*) cultured for up to a month in sediment spiked with polyvinyl chloride (PVC), an effect attributed to reduced feeding activity. Similarly, a decrease has been reported in the energy available for growth in *C. maenas* when they consumed plastic-contaminated food.[4]

Shore crabs are omnivores and frequently feed on bivalves such as the common blue mussel *Mytilus edulis*. Trophic-transfer experiments[9,10] have shown that crabs can ingest microplastics from contaminated mussels, leading to a reduced allocation of energy for growth.[4] Crabs can also take up microplastics by ventilation into the gill chambers,[10] where they may remain for up to 22 days. Most microplastics in *C. maenas* are found adhered to the posterior gills, which are a known major site for ion regulation.[12] The emerging paradigm is that ingestion of microplastic can reduce fitness in marine species by altering their food consumption and energy allocation.[13] The purpose of this paper is to assess whether inspiration of microplastic through the ventilatory mechanism can also reduce fitness.

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In aquatic organisms, gills are the main site for gaseous and ionic exchanges and acid−base balance.[14] Therefore, any factor such as microbial growth[15] or contaminants[16] impairing gill function might have detrimental consequences for the organism. Exposure to marine contaminants such as dichlorodiphenyltrichloroethane (DDT), arsenite, cadmium, silver, copper, and mercury have, in fact, been reported to have detrimental effects for osmoregulation and ion exchange.[17] Although the uptake and retention of microplastics across the gill surface have been documented,[10] the potential effects on the crab's gaseous exchanges and ability to ion and osmoregulate have not been evaluated to date.

Because microplastics are retained on the gills of *C. maenas*, we hypothesized that acute exposure to waterborne plastic microspheres could significantly impact oxygen uptake, ion exchange, and osmoregulatory capacity of crabs. We tested this hypothesis by determining the impact on crabs of an acute 24 h exposure to polystyrene microspheres of diameter 8 μm. We chose polystyrene because it is a frequent feature of marine debris, and previous experiments have confirmed this size range to be retained within the outer surface of the gill lamellae. We also tested two further types of polystyrene with different surface coatings (carboxylated (COOH) and aminated (NH_2)) to compare the influence of surface composition on biological accumulation and effects. We measured the crab's ion regulation and respiratory processes in the presence and absence of a low-salinity challenge.

MATERIALS AND METHODS

Aquarium Procedure. Non-ovigorous (without eggs) and intermoult female shore crabs (*C. maenas*) were collected from the Exe estuary, Devon, UK (50°35.2′N, 3°23.59′W) and kept for 2 weeks in full-strength (33 ppt) artificial sea water (ASW) to acclimatize to aquarium conditions (14.5 °C, 12:12 h light/dark cycle). Crabs were fed for 12 days every other day with frozen mussels (*Mytilus edulis*) and then starved for 2 days prior to the experiments. Crabs were transferred to individual 5 L tanks filled with 2 L of ASW with an air stone used to keep the partial pressure of oxygen (PO_2) close to 100% saturation. Crabs were left to acclimate to this experimental setup overnight. The next morning, microplastic (8 μm polystyrene microspheres, Spherotec; neutral, carboxylated, or aminated) or natural sediment (ca. 28 μm, concentration 10^6 L^{-1}) was added. Plastic was added at two experimental concentrations (10^6 and 10^7 microspheres per L), with a set of crabs held in identical conditions without plastic to act as the controls. These concentrations were chosen to emulate the acute exposures in Watts et al.[10] Oxygen consumption was determined at 1, 16, and 24 h after the addition of plastics. At the end of the last oxygen-consumption determination, a 500 μL hemolymph sample was taken. Samples were taken from the base of the third walking leg using an ice-cooled 1 mL syringe.[18] A subsample of hemolymph was immediately transferred to a clean ice-cold 1.5 mL tube, from which 10 μL was taken and diluted in 4 mL of milli-Q water (<18 MΩ; Millipore Advantage 10 UV; Thermo Fisher Scientific), vortexed, and stored at −20 °C for later ion analysis. The remaining hemolymph was then centrifuged at 8000*g* for 2 min. Subsequently, a second subsample of 5 μL was diluted in 200 μL of ultrapure water (1:40 dilution) for later hemocyanin concentration. The remainder was mixed with anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA-Na_2, pH 7.3, 850 mOsm kg^{-1}) at 3:1 ratio, vortexed, and stored at −20 °C for later analysis. Hemolymph osmolality was determined in a vapor pressure osmometer (Wescor 5520; Wescor Inc., South Logan, UT).

For salinity challenge experiments, crabs were treated as above, but a further group was subsequently transferred into clean tanks containing ASW of reduced salinity (10 ppt). Oxygen consumption and water samples for ammonia were taken at 1, 6 and 24 h post-salinity challenge. At 24 h post-treatment, 500 μL of hemolymph was sampled and treated as above.

Coherent Raman Scattering Microscopy. Coherent Raman scattering microscopy (CRS) is a multiphoton microscopy technique that provides label-free contrast of both the target sample and the surrounding biological matrix, based on vibrational spectroscopy. The applications of CRS range from medical research[19] to more recent usage in ecotoxicology.[20] Plastics have previously been successfully imaged using the CRS technique in zooplankton[6] and in crab gills.[10] For a more detailed explanation of the theory behind CRS imaging of biological samples, see Goodhead et al.[20] Briefly, Raman scattering provides a great deal of chemical information by examining the light that is scattered by molecular vibrations. Raman scattered light is emitted at a slightly shifted wavelength with respect to the incident light, the shift in energy corresponding to the vibrational frequency of a molecular bond within the sample. The CRS process involves two lasers in which the frequency of the first laser is constant, and the frequency of the second one can be tuned in a way that the frequency difference between the two lasers equals the frequency of the Raman active or vibrational mode of interest. The molecules in resonance produce a larger signal than those of resonance, providing a vibrational contrast in a CRS image. Here, six crabs were exposed to nonlabeled polystyrene spheres with different surface characteristics (three with carboxyl groups and three with amino groups) at a concentration of 1×10^5 spheres per L for 19 h. Posterior gills were dissected fresh and analyzed with CRS microscopy.

Oxygen Consumption. Oxygen consumption was assessed by closed respirometry. Briefly, air was switched off, the initial dissolved oxygen was determined immediately and remeasured after 1 h. The air was then switched back on. A total of six supplementary tanks (not containing crabs) with 2 L of ASW (either 10 or 33 ppt) were used as controls to measure oxygen diffusion from the air−water interface or bacterial-oxygen consumption. The exact time the air was switched off, oxygen measured and air switched back on was recorded for each tank. For full-salinity experiments (33 ppt), oxygen was assessed in ∼0.5 mL via a Strathkelvin oxygen electrode connected to a 781 oxygen meter. The oxygen electrode was housed in a water jacket that was irrigated with water at the same temperature as the crabs (14.5 °C). For the salinity challenge experiments, dissolved oxygen was assessed using a needle-type fiber-optic sensor (Firesting OXR 230) connected to a FSO2-4 optical oxygen meter. Oxygen electrodes were calibrated daily with fully aerated water (100% oxygen saturation) and a saturated sodium sulphite solution (0% oxygen saturation). To avoid compensatory responses associated with depleted dissolved oxygen concentrations, we ensured that the chamber P_{O_2} values were in excess of ∼120 mmHg (∼15.5 kPa). Oxygen consumption was calculated as the difference in water oxygen content over time and displayed in ml O_2 g^{-1} h^{-1}.

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Hemocyanin and Protein in the Hemolymph. A 5 μL subsample of hemolymph was added, per triplicate, to a 96 well plate followed by addition of 200 μL of Milli-Q water and mixed for 45 s. The absorbance at 335 nm was measured with path-length correction on a plate reader (Tecan, NanoQuant Infinite M200 Pro). Oxy-hemocyanin concentration was determined using an extinction coefficient (ε) of 17.26 calculated on the basis of a functional subunit of 74 000 Da for crabs.[21] Protein in the hemolymph was quantified via Bradford[22] using a bovine serum albumin standard curve.

Ions. Hemolymph Na^+, K^+, and Ca^{2+} were quantified in the diluted samples (10 μL in 4 mL) via flame photometry (Sherwood Instruments). Standard curves were constructed using 1 mM solutions of NaCl, KCl, and CaCl.

Statistics. To test if the microplastic treatment or the salinity challenge explained the variation observed in the physiological parameters, we performed a general linear model (GLM) followed by a Tukey post hoc test when the GLM was significant. Parametric assumptions of normality of residuals and homogeneity of variances was met. The GLM and post hoc analysis was performed in MINITAB. A repeated measures ANOVA (Sigma plot) was used to determine differences in oxygen consumption over time. Differences were considered significant at a $p \leq 0.05$.

RESULTS

No mortalities were found during or after any of the experimental treatments. Furthermore, no evident changes in behavior were noted in any of the plastic treatments.

Uptake of Carboxylated and Aminated Polystyrene Microparticles. All six crabs sampled for coherent Raman scattering microscopy analysis had detectable microspheres on their gills. In Figure 1, gill tissue taken from a crab exposed to (A) aminated (NH_2) polystyrene and (B) carboxylated (COOH) polystyrene is shown.

Full-Strength Salinity (33 ppt). *Oxygen Consumption.* The oxygen consumption of crabs at 1, 16, and 24 h post treatment with neutrally charged polystyrene is shown in Figure 2A. After 1 h post-treatment, crabs with the highest concentration of plastic (10^7 microspheres per L) had a significantly lower oxygen consumption (0.014 ± 0.002 (mL O_2 g^{-1} h^{-1})) compared to the control (0.028 ± 0.004 (mL O_2 g^{-1} h^{-1})) ($F_{2,29} = 3.99$, $p = 0.030$). However, after 16 and 24 h post-treatment, there was no significant difference between either treatments and the control ($F_{2,29} = 0.05$, $p = 0.956$), ($F_{2,29} = 1.17$, $p = 0.325$). There was no significant difference in the oxygen consumption between treatment groups in the particle study with carboxyl and amino coated polystyrene or sediment at any time point.

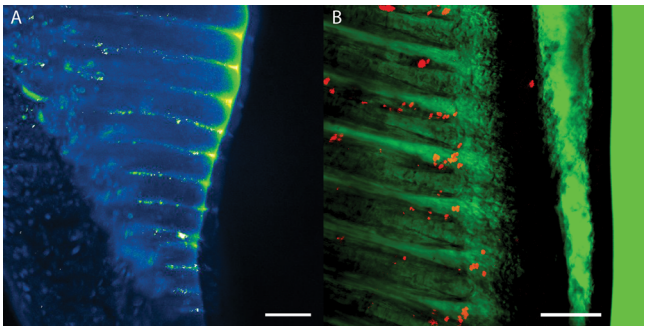

Figure 1. Coherent Raman scattering visualization of gill lamellae, composed of a backward-detected coherent anti-Stokes Raman image, a forward-detected stimulated Raman scattering image, and a transmitted light image, merged in false color. (A) 8 μm amino-coated polystyrene (green dots indicate amino-coated polystyrene trapped between the gill lamellae); (B) 8 μm carboxylated polystyrene (red dots indicate carboxylated polystyrene distributed across and around the gill lamellae). Both images were obtained at 3050 cm^{-1} and show particles adhering to the gill surface. Scale bars are 100 μm.

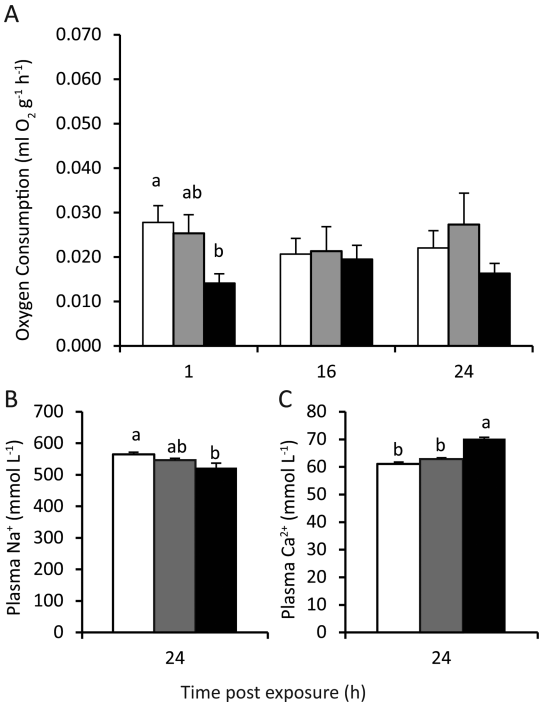

Figure 2. Results from the full salinity experiments. (a) Oxygen consumption at 1, 16, and 24 h post-addition of plastic, (b) plasma Na^+ ion concentration, and (c) plasma Ca^{2+} ion concentration at 24 h post-addition of plastic in the shore crab *C. maenas* subjected to three treatments of 8 μm microplastic. White bars indicate crabs with no plastic added to the tank, gray bars indicate crabs with 10^6 microspheres per L within 2 L of water ($n = 10$). Black bars represent crabs with 10^7 microspheres per L with 2 L water added. Error bars are one standard error. Means that do not share a letter are significantly different. Bars with no letters indicate no significant difference. Significant differences in oxygen consumption were tested at each time point independent of the other time points.

Hemolymph Constituents. There was a slight but significant drop in the concentration of Na^+ ions (Figure 2B) within the hemolymph with increasing neutral plastic dose ($F_{2,29} = 4.75$, $p = 0.017$). Crabs held in control conditions had an average of 564 ± 6.70 mmol L^{-1} Na^+, while crabs exposed to 10^6 microspheres per L presented a hemolymph Na^+ concentrations of 546 ± 5.62 mmol L^{-1}, which was not significantly different from the controls. Crabs, however, treated with 10^7 microspheres per L had a significantly lower concentration of Na^+ (522 ± 14.31 mmol L^{-1}) than did control crabs (Tukey $p < 0.05$). There was a significant increase in the concentration of Ca^{2+} ions (Figure 2C) within the hemolymph with increasing plastic dose ($F_{2,29} = 31.5$, $p < 0.001$). Crabs held in control conditions had an average of 61.1 ± 0.75 mmol L^{-1} Ca^{2+}, crabs treated with 10^6 microspheres per L had 62.9 ± 0.92 mmol L^{-1} Ca^{2+}, and crabs treated with 10^7 microspheres per L had 70.3 ± 0.93 mmol L^{-1} Ca^{2+}, significantly higher than the controls and lower plastic concentration (Tukey $P < 0.05$). There was no

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significant difference in the concentration of K^+ ion ($F_{2,29}$ = 1.05, p = 0.363).

There was no dose-dependent effect seen with hemolymph protein concentration. There was a slight but significantly higher concentration of hemocyanin within the hemolymph with increasing plastic dose ($F_{2,29}$ = 4.99, p = 0.014). Crabs held in control conditions had an average hemocyanin concentration of 0.48 ± 0.03 mmol L^{-1}, while crabs treated with 10^6 microspheres per L presented a significantly lower concentration (0.46 ± 0.01 mmol L^{-1}) (Tukey $p < 0.05$). Hemocyanin concentration were similar to controls at the highest concentration of plastic used (10^7 microspheres per L), with a value of 0.59 ± 0.04 mmol L^{-1}. Although protein concentration in the hemolymph followed the same pattern as hemocyanin, there was no significant differences found ($F_{2,29}$ = 2.72, p = 0.084) (see Figure SI.1).

There was no significant effect on hemocyanin ($F_{5,33}$ = 0.17, p = 0.974), hemolymph protein ($F_{5,33}$ = 0.72, p = 0.611), Na^+ ($F_{5,53}$= 1.98, p = 0.099), K^+ ($F_{5,33}$= 1.04, p = 0.405), or Ca^{2+} ($F_{5,53}$= 0.42, p = 0.832) ions when exposed to sediment or carboxyl- or amino-coated plastics.

Because there was a dose-dependent effect between neutral plastics and oxygen consumption and Na^+ and Ca^{2+} ions, a reduced-salinity experiment was performed to see whether these effects would exaggerate or disappear during an osmotic challenge.

Reduced Salinity. *Oxygen Consumption.* The oxygen consumption of crabs at 1, 6, and 24 h post salinity challenge is shown in Figure 3A. There were no significant differences between any treatments or control after 1 h ($F_{4,44}$ = 0.40, p = 0.808) or 6 h ($F_{4,44}$ = 2.05, p = 0.106). However, after 24 h, there was a significant increase in oxygen consumption ($F_{4,44}$ = 5.25, p = 0.002) with control crabs kept at 33 ppt having a significantly lower oxygen consumption than crabs at reduced salinity with 0, 10^5, and 10^6 microspheres per L (Tukey p < 0.05). There were no significant changes in oxygen consumption within any of the treatments over time ($F_{4,46}$ = 1.605, p = 0.192, repeated measures ANOVA), nor a significant effect of plastic concentration.

Hemolymph Constituents. There was a significant effect of salinity on all ions (Na^+ ($F_{4,43}$ = 30.97, p < 0.001; Figure 3B), Ca^{2+} ($F_{4,43}$ = 56.11, p < 0.001; Figure 3C), and K^+ ($F_{4,43}$ = 50.17, p < 0.001)) and hemolymph osmolality ($F_{4,40}$ = 35.83, p < 0.001) (osmolality and K+ seen in Figure SI.2). Although all values were lower in the crabs challenged by low salinity, there was no significant effect of plastic concentration (Tukey, p > 0.05). There was no significant difference in the hemocyanin concentration ($F_{4,43}$ = 1.57, p = 0.201) or hemolymph protein concentration ($F_{4,42}$ = 0.62, p = 0.649) between salinity or plastic treatments were also found.

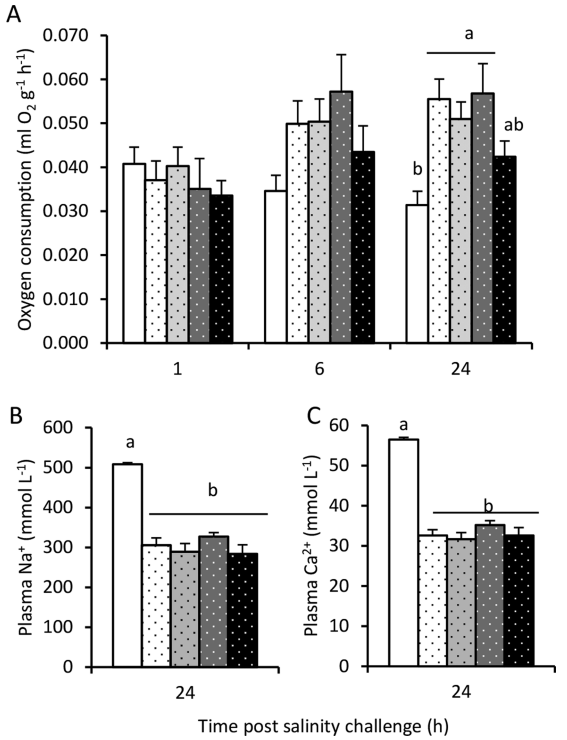

Figure 3. Results from the reduced salinity experiments. (a) Oxygen consumption at 1, 6, and 24 h post-salinity-change, (b) plasma Na^+ ion concentration, (c) plasma Ca^{2+} ion concentration at 24 h post-salinity-change in the shore crab *C. maenas* subjected to four treatments of 8 μm microplastic. White bars indicate crabs with no plastic added to the tank (n = 10), light gray bars indicate crabs with 10^5 microspheres per L, and gray bars indicate crabs with 10^6 microspheres per L within 2 L of water. Black bars represent crabs with 10^7 microspheres per L with 2 L of water added. Dots within bars represent crabs that have been added to 10 ppt artificial seawater after 16 h of plastic exposure. Clear bars represent crabs changed into clean 33 ppt ASW. Error bars are one standard error. Means that do not share a letter are significantly different. Bars with no letters indicate no significant difference. Significant differences in oxygen consumption were tested at each time point independent of the other time points.

DISCUSSION

In the current study, we show that polystyrene microspheres with different surface coatings are readily taken up onto the gills of crabs following exposure through water. The physiological consequences to the crabs, under the short-term exposure conditions of our experiments, were however minimal. Transient, dose-dependent changes in oxygen consumption and ion regulation were found that returned to normal levels within the acute time frame of the exposures. This shows that the crabs are able to recover gas exchange, for example, by recruiting more lamellae or increasing perfusion or water flow in the branchial chamber. Na^+ and Ca^{2+} were both significantly altered by increasing concentrations of plastic with less Na^+ and more Ca^{2+} within the crab heamolymph at the highest concentrations of plastic. These are, however, minor differences; Na^+ dropped by 7.45%, and Ca^{2+} rose by an average 15.1% compared to the control. To put this into context, when *C. maenas* was exposed to 10 mg L^{-1} of copper, Na^+ decreased from 347 ± 14 to 269 ± 54 mmol L^{-1}, a drop of 22%.[23] In this study, a change of salinity from 33 to 10 ppt in crabs not dosed with microplastic resulted in a 39.8% drop in Na^+ plasma concentration (from 508 ± 4.35 to 306 ± 18.21 mmol L^{-1}). Evidently, crabs are able to overcome these minor effects on ion exchange induced by exposure to the polystyrene microspheres used here by minor physiological regulation.

When exposed to a low-salinity challenge, crabs also showed an increase in oxygen consumption.[24] This is thought to be associated with the increased cost of osmoregulation in the face of an osmoregulatory challenge (difference between internal and external mediums). No effects of either microplastics or sediments were found in the face of the low-salinity challenge, suggesting that no additive effect or interaction occurs between the mechanisms by which plastics affects ion balance and crab ion regulation.

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We were able to show using bioimaging that polystyrene microspheres with different surface coatings (carboxylated (COOH) and aminated (NH_2)) were taken up into the gill chambers. We categorized the potential charge of these plastics (Figure SI.3), showing that the small positive or negative charges would be masked in the external medium by the large buffering capacity of seawater. Once inside the gill chamber, we did not find any effect of these particles on oxygen consumption and ion exchange, although there were some qualitative variations in the pattern of distribution across the surface of the gills (Figure 1). In vertebrates, the in vivo behavior of micro- and nanopolymers varies depending on numerous physicochemical properties of the particles, including size, surface charge, aspect ratio, porosity, and surface corona.[25] The circulation time of particles within the body is significantly enhanced for hydrophilic and positively charged particles.[26] Positively charged particles generally show higher cytotoxicity across a range of model systems than do negatively charged ones. This has been attributed to the interaction of cations with the negatively charged cell membrane.[27]

Acrylic ester nano- and micropolymers showed low toxicity following inhalation in rats, which may have been due to their anionic surface charge.[28] Studies in which the surface charge of stearylamine−polylactic acid (PLA) polymer particles were modified from positive to negative confirmed that those with a positive charge showed higher toxicity in the lungs and were taken up more readily into cells.[29] The influence of surface characteristics of particles on the binding capacity within the gills of aquatic animals would be an intriguing avenue for future study.

In conclusion, we show here that the acute inhalation of polystyrene microspheres into the gill chambers of crabs leads to a small but transient change in oxygen consumption and ion regulation. Neither microspheres nor natural sediments altered the crab's response to osmotic stress, regardless of the plastic concentration added. Carboxylated (COOH) and aminated (NH_2) polystyrene microspheres were distributed differently across the gill surface; this is likely due to their interaction with the gill surface, although neither had a significant adverse impact on gill function. These results illustrate the physiological resilience of shore crabs in maintaining osmoregulatory and respiratory function after acute exposure to both anthropogenic plastics and natural particles.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01187.

Figures showing extra results from the normal- and reduced-salinity experiments and the change in pH to determine the buffering capacity. A table showing the general linear model output of experiments. (PDF)

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Author Contributions

‖First authorship is shared between A.J.R.W. and M.A.U. because they equally contributed to this manuscript.

Notes

The authors declare no competing financial interest.

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