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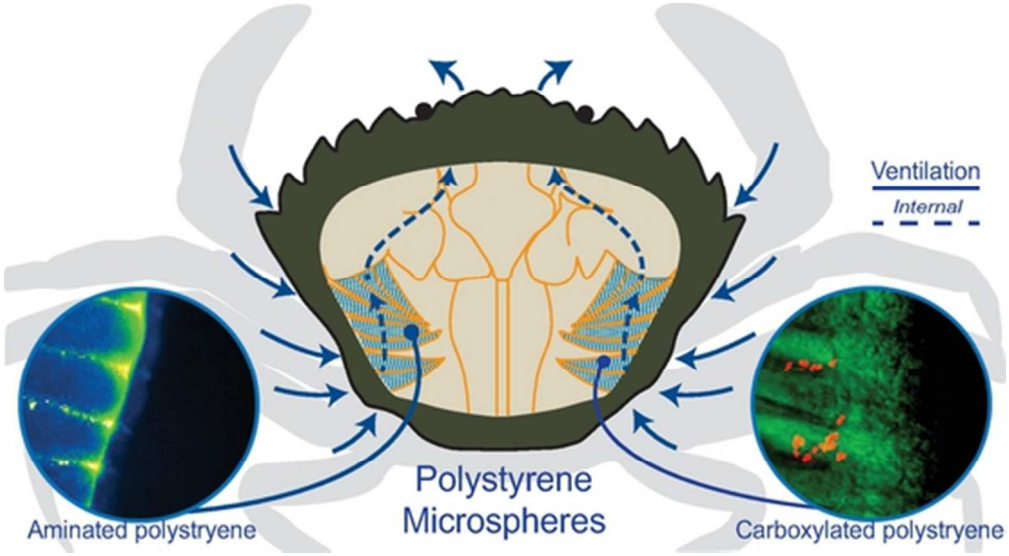

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Effect of microplastic on the gills of the Shore Crab *Carcinus maenas*

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Abstract

Microscopic plastic debris (microplastics, <5mm 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 (8μm) 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 hour 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, crabs were challenged 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.

1. 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 product 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 fibres 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 polyvinylchloride (PVC), an effect attributed to reduced feeding activity. Similarly, a decrease has been reported in the energy available for growth in *C. maenas* when consuming 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.

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.

Since 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.

Methods

Aquarium procedure

Non-ovigorous (without eggs) and intermoult female shore crabs (*Carcinus 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 acclimatise to aquarium conditions (14.5 ^{o}C, 12h:12h 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 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 set up 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 L^{-1}) 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 h, 16 h and 24 h after the addition of plastics. At the end of the last oxygen consumption determination, a 500 μL heamolymph sample was taken. Samples were taken from the base of the 3rd walking leg using an ice cooled 1 mL syringe.[18] A subsample of heamolymph was immediately transferred to a clean ice cold 1.5 mL tube, from where 10 μL were taken and diluted in 4 mL of milli-Q water (<18 MΩ; Millipore Advantage 10 UV; Thermo Fisher Scientific), vortexed and stored at -20 ^{o}C for later ion analysis. The remaining heamolymph was then centrifuged at 8000 g for 2 minutes. Subsequently, a second subsample of 5 μL was diluted in 200 μL of ultrapure water (1:40 dilution) for later haemocyanin 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 ^{o}C for later analysis. Hemolymph osmolality was determined in a vapour pressure osmometer (Wescor 5520; Wescor Inc., South Logan, UT, USA).

For salinity challenge experiments, crabs were treated as above, but a further group was subsequently transferred into clean tanks containing ASW of reduced salinity (10ppt). Oxygen consumption and water samples for ammonia were taken at 1, 6 and 24h post salinity challenge. At 24 h post treatment, 500 μL heamolymph 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 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 where the frequency of the first laser is constant, while 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 off resonance, providing a vibrational contrast in a CRS image. Here six crabs were exposed to non-labelled polystyrene spheres with different surface characteristics (three with carboxyl groups; three with amino groups) at a concentration of 1 x 10^5 spheres L^{-1} for 19h. Posterior gills were dissected fresh and analysed with CRS microscopy.

Oxygen consumption

Oxygen consumption was assessed by closed respirometry. Briefly, air was switched off and initial dissolved oxygen was determined and re-measured after 1 h. The air was then switched back on. Six supplementary tanks (not containing crabs) with 2 L 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, irrigated with water at the same temperature as the crabs (14.5 ^{o}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, the chamber PO_2 values were always 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}.

Haemocyanin and protein in the heamolymph

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

Ions

Haemolymph 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, a General Linear Model (GLM) was performed 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 behaviour were noted in any of the plastic treatments.

Uptake of carboxylated and aminated polystyrene microparticles

All (6) 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 1h, 16h and 24h post treatment with neutrally charged polystyrene is shown in Figure 2A. After 1h post treatment crabs with the highest concentration of plastic (10^7 microspheres L^{-1}) 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 16h and 24h 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.

Heamolymph constituents

There was a slight but significant drop in the concentration of Na^+ ions (Figure 2B) within the heamolymph 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 L^{-1} presented a heamolymph Na^+ concentrations of 546 ± 5.62 mmol L^{-1} which was not significantly different from the controls. Crabs, however, treated with 10^7 microspheres L^{-1} had significantly lower concentration of Na^+ (522 ± 14.31 mmol L^{-1}) than control crabs (Tukey $p<0.05$). There was a significant increase in the concentration of Ca^{2+} ions (Figure 2C) within the heamolymph 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 L^{-1} had 62.9 ± 0.92 mmol L^{-1} Ca^{2+}, and crabs treated with 10^7 microspheres L^{-1} 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 significant difference in the concentration of K^+ ion ($F_{2,29} = 1.05$, $p = 0.363$).

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

There was no significant effect on haemocyanin ($F_{5,33}$ = 0.17, p = 0.974), heamolymph 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, carboxyl or amino coated plastics.

As there was a dose dependant 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 1h, 6h and 24h post salinity challenge is shown in Figure 3A. There wereno significant differences between any treatments or control after 1h ($F_{4,44}$ = 0.40, P = 0.808) or 6h ($F_{4,44}$ = 2.05, p = 0.106). However, after 24h there was a significant increase in oxygen consumption ($F_{4,44}$ = 5.25, p = 0.002) with control crabs kept at 33ppt having a significantly lower oxygen consumption than crabs at reduced salinity with 0, 10^5 and 10^6 microspheres per L^{-1} (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.

Heamolymph 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 heamolymph osmolality ($F_{4,40}$ = 35.83, p < 0.001) (osmolality and K+ seen in SI.2). Whilst 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 haemocyanin concentration ($F_{4,43}$ = 1.57, p = 0.201) or heamolymph protein concentration ($F_{4,42}$ = 0.62, p = 0.649) between salinity or plastic treatments were also found.

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, but that the physiological consequences to the crabs, under the short term exposure conditions of our experiments, were minimal.Transient, dose dependent

changes in oxygen consumption and ion regulation were found, which 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, 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 lamellae 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 *Carcinus maenas* was exposed to 10 mg L^{-1} of copper, Na^+ decreased from 347 ± 14 mmol L-1 to 269 ± 54 mmol L^{-1} a drop of 22 %.[23] In this study a change of salinity from 33 ppt to 10 ppt in crabs not dosed with microplastic resulted in a 39.8 % drop in Na^+ plasma concentration (from 508 ± 4.35 mmol L^{-1} 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 low salinity challenge, suggesting that no additive effect or interaction occurs between the mechanisms by which plastics affects ion balance and crab ion regulation.

We were able to show using bio-imaging that polystyrene microspheres with different surface coatings carboxylated (COOH) and aminated (NH_2) were taken up into the gill chambers. We categorised the potential charge of these plastics (Supplemental Information SI.3) showing that the small positive or negative charges would be masked in the external medium by the large buffering capacity of sea water. 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* behaviour of micro and nano polymers varies depending on numerous physico-chemical 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 negatively charged ones, This has been attributed to the interaction of cations with the negatively charged cell membrane. [27]

Acrylic ester nano- and micro-polymers 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 lung 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 acute inhalation of polystyrene microsphere into the gill chambers of crabs lead 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 plastic concentration added. Carboxylated (COOH) and aminated (NH_2) polystyrene microspheres were distributed differently across the gill surface; 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.

Supporting information

This information is available free of charge via the Internet at http://pubs.acs.org/

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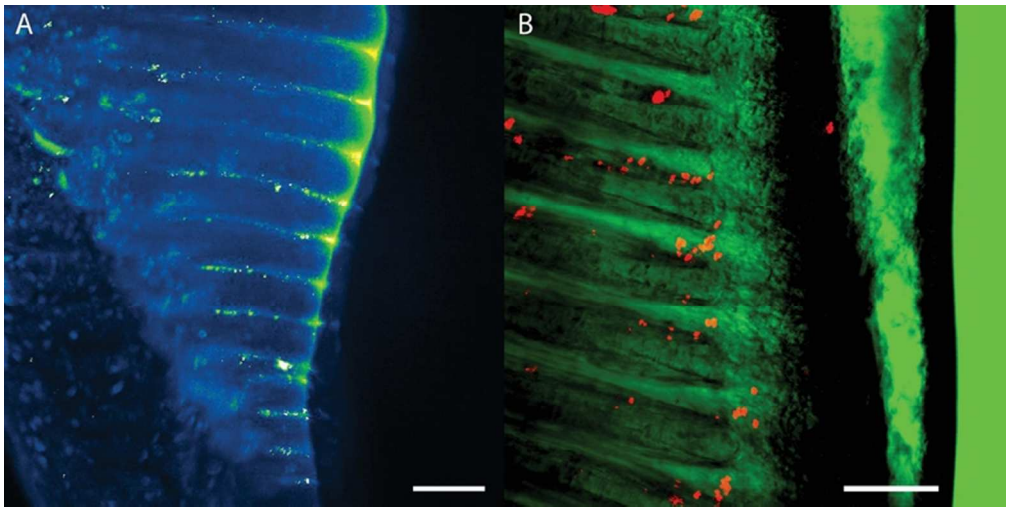

Figure 1 Coherent Raman scattering visualisation of gill lamellae, compromised of a backwards detected coherent anti-Stokes Raman image, a forwards detected stimulated Raman scattering image and a transmitted light image, merged in false colour. (A) 8 µm amino coated polystyrene (green dots) indicate amino coated polystyrene trapped between the gill lamellae (B) 8 µm carboxylated polystyrene (red dots) indicates 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.
70x35mm (300 x 300 DPI)

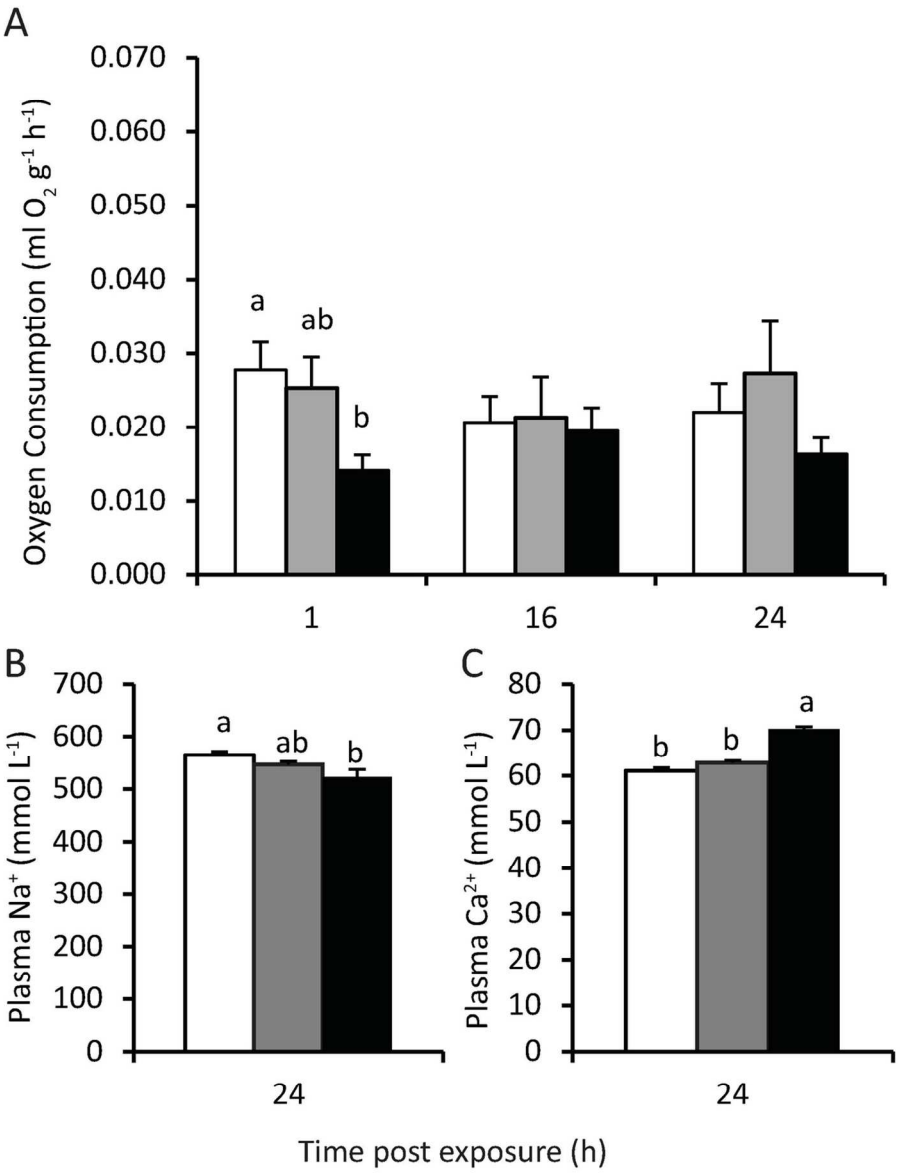

Figure 2 Results from the full salinity experiments. a) Oxygen consumption at 1, 16 and 24 h post addition of plastic, b) Plasma Na^+ ion concentration, c) Plasma Ca^{2+} ion concentration at 24 h post addition of plastic in the shore crab *Carcinus maenas* subjected to three treatments of 8 µm microplastic. White bars crabs with no plastic added to the tank (n=10), grey bars crabs with 10^6 microspheres L^{-1} within 2 L of water. Black bars represent crabs with 10^7 microspheres L^{-1} 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 each other time points.
114x146mm (300 x 300 DPI)

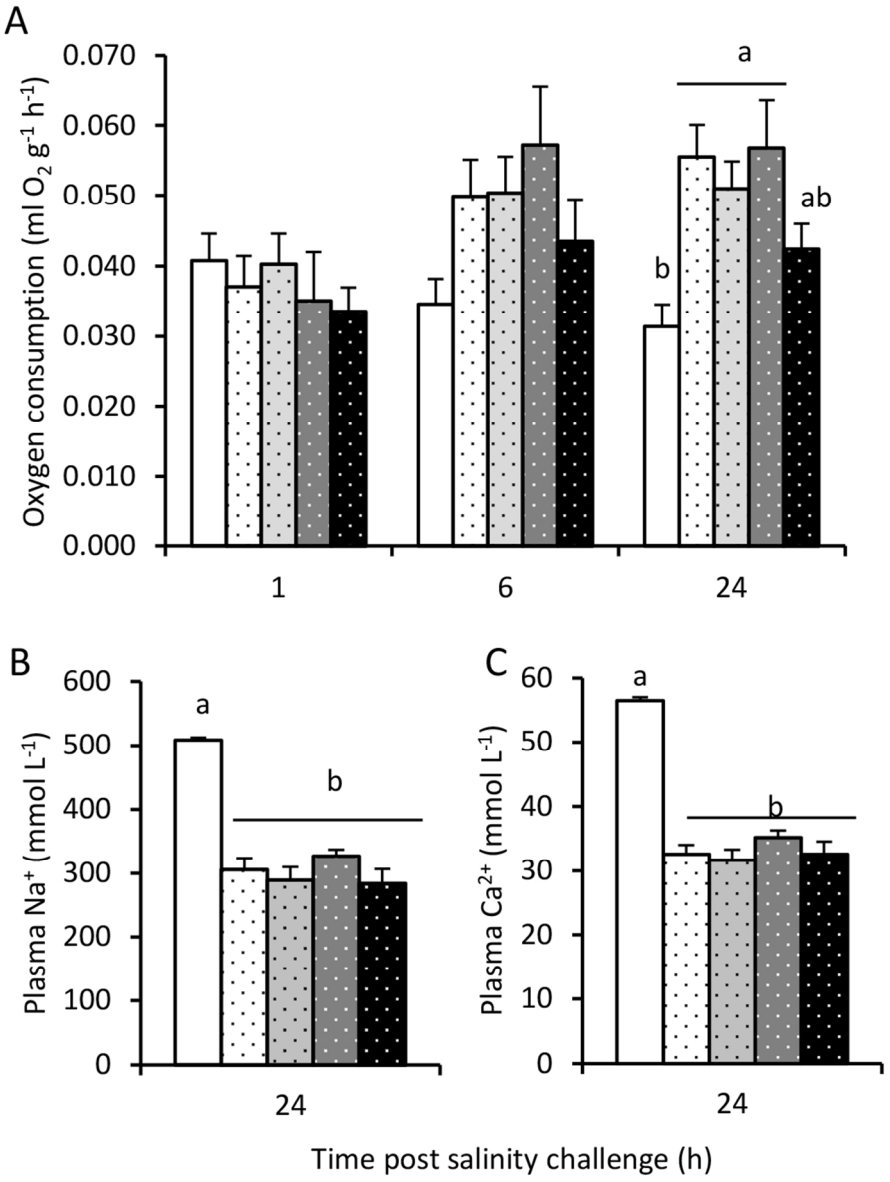

Figure 3 Results from the reduced salinity experiments. a) Oxygen consumption at 1, 6 and 24 h post salinity change, b) Plasma Na^+ ion concentration, c) Plasma Ca^{2+} ion concentration at 24 h post salinity change in the shore crab *Carcinus maenas* subjected to four treatments of 8 µm microplastic. White bars crabs with no plastic added to the tank (n=10), light grey bars crabs with 10^5 microspheres L^{-1} grey bars crabs with 10^6 microspheres L-1 within 2 L of water. Black bars represent crabs with 107 microspheres L^{-1} with 2 L water added. Dots within bars represent crabs that have been added to 10 ppt artificial sea water 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 each other time points.
91x119mm (300 x 300 DPI)