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Environmental Toxicology and Chemistry

Short Communication

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THE FATE OF MICROPLASTICS DURING UPTAKE AND DEPURATION PHASES IN A BLUE MUSSEL EXPOSURE SYSTEM

Running title: Microplastic fate in a blue mussel exposure system

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Abstract

We present a blue mussel exposure system where the fate of microplastics (polystyrene beads) is tracked during exposure and depuration phases. This enabled the establishment of a complete mass balance. Quantification of beads in mussels was done with a novel enzymatic digestion protocol. We found a similar relative distribution of beads for two environmentally realistic concentrations (5 and 100 beads L^{-1}) and no substantial egestion of particles within 2h depuration. This article is protected by copyright. All rights reserved

Graphical abstract

A complete mass balance of microplastic beads in a blue mussel exposure system was established by quantifying beads in four compartments: the exposure water, the mussel after exposure, the depuration water and the mussel after depuration.

Keywords: aquatic invertebrates; emerging pollutants; microplastics; fate and transport; enzymatic digestion; quantification

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INTRODUCTION

In recent years research has shown that aquatic environments globally are polluted with microplastics (Eerkes-Medrano et al. 2015; W.C. Li et al. 2016). The abundance of microplastics in the oceans is heterogeneous and reported concentrations range between $2.2 \cdot 10^{-5}$ and 9.2 particles L^{-1} in water columns (Hidalgo-Ruz et al. 2012; Desforges et al. 2014; Qu et al. 2018). However, concentrations can be considerably higher in coastal hotspots like in the vicinity of plastic production plants where up to 102 particles L^{-1} have been documented (Noren 2007). Particle sizes between 1 µm and 5 mm overlap with the size of plankton (Enders et al. 2015) and the presence of plastics in water columns and sediments makes them available to a variety of plankton and sediment feeding organisms. Accordingly, a large number of species have been found to ingest microplastics (Avio et al. 2016). Numerous studies have focused on the suspension feeding blue mussel (*Mytilus edulis*) (Browne et al. 2008; Wegner et al. 2012; Van Cauwenberghe et al. 2015) as this is a keystone species in many coastal habitats (Ragnarsson and Raffaelli 1999). The species faces a strong exposure to microplastics due to its high filtering activity (Riisgård et al. 2014) and is thus considered a promising bio-indicator for microplastic pollution (Lusher et al. 2017). Although *M. edulis* is a selective feeder and able to reject particles with low or no nutritional value (Riisgård et al. 2011), ingestion of different plastic particle sizes (30 nm to several mm) and shapes (fragments, beads, fibers, films) have been demonstrated (Wegner et al. 2012; J. Li et al. 2016). Laboratory studies have also found adverse effects of ingested microplastics on a cellular and physiological level (Wegner et al. 2012; Paul-Pont et al. 2016). However, the tested concentrations are frequently several orders of magnitude higher than what has been documented in the environment (Lenz et al. 2016). Furthermore, particle fate in

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the used exposure systems is seldom investigated thoroughly. In some exposure studies of marine invertebrates particle ingestion has been quantified, most commonly by counting particles in animal tissues or faeces (e.g. Watts et al. 2014; Setälä et al. 2016), however the fate of the remaining particles is rarely determined. This hampers the understanding of how the particles behave and where they end up in the system. Sussarellu et al. (2016) exposed oysters continuously to polystyrene (PS) particles and quantified the particles in the inlet and outlet of the exposure tank. However, as particle ingestion was only calculated and not actually measured it remains unclear how the oysters interacted with them. To the best of our knowledge, no study has aimed at making a complete mass balance of all microplastic particles in such exposure systems.

The aim of the present study was to analyze microplastic ingestion and egestion by *M. edulis* at environmentally realistic concentrations in a completely controlled exposure system to trace the fate of all particles. In this way, a mass balance of microplastics in the exposure system can be established, giving new insights into the interactions of the test organism and the tested particles.

MATERIALS AND METHODS

Model microbeads

The microplastic particles used in the present study were black Thermo Scientific™ ChromoSphere-T™ PS microspheres (49.1 ± 1.3 μm, density: 1.05 gcm^{-3}), purchased as a dry powder from Distrilab (Leusden, Netherlands). Polystyrene is a commonly used model particle in exposure and uptake studies (e.g. Wegner et al. 2012; Van Cauwenberghe et al. 2015; Cole and Galloway 2015) and due to its density close to that of water readily available to filter feeding organisms. A stock solution was prepared by adding the product in 1000 mL distilled water. As

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no particle aggregation was observed by visual inspection a homogeneous suspension was simply achieved by turning the bottle several times by hand. Particle concentration in the stock solution was determined by counting particles in three sub-samples of the stock solution (161.0 ± 6.4 particles 100 μL^{-1}) and the designated particle counts in the exposure systems were administered with the help of a micropipette.

Enzymatic digestion protocol

An enzymatic digestion method was developed to digest mussel tissue and in this way retrieve the ingested plastic particles. Proteases have been shown to efficiently digest mussel tissues in several different digestion methods using Proteinase K (Karlsson et al. 2017), trypsin (Courtene-Jones et al. 2017) and industrial proteases (Catarino et al. 2016; Railo et al. 2018). Especially the use of industrial enzymes for digesting blue mussel tissues has been recommended by Catarino et al. (2016) as they are relatively inexpensive, mostly supplied in liquid form and therefore easy to handle with a low hazard. The specific enzyme, Corolase 7089 (bacterial protease, AB Enzymes), used by Catarino et al. (2016), was however not available for purchase. To be used as a standard method the enzymes should be easily available and work without any use of additional chemicals as in the method by Railo et al. (2018) using sodium dodecyl sulphate (SDS) alongside industrial detergents. We compared six industrial proteases supplied by Novozymes® (Alcalase, Neutrase, NovoBate, Peltec, Ronozyme ProAct and Savinase). In a preliminary test (see SI) Alcalase showed the most promising performance and was selected for a more thorough testing of digestion efficiency. Enzyme solutions of 5, 2.5, 1.25, 0.5 and 0.25% (v/v) were prepared and 20 mL of one solution were added to the soft tissues of single blue mussels (n=5), similar to the method described by Courtene-Jones et al. (2017). The addition of

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pure MilliQ water served as a control. After agitation on a shaking table overnight at 37°C the digest was filtered onto a 10 µm steel filter. For the two lowest concentrations an additional pre-filtering step with a 100 µm mesh was necessary to avoid extensive clogging of the 10 µm filter. The filters were subsequently dried at 105°C for 24h to obtain the dry weight (dw) of the remaining tissue. To evaluate the digestion efficiency weights of the initial mussel tissue (wet weight, ww) were noted as well as the remaining tissue after digestion (ww and dw). In addition, 10 extra mussels were used to measure the ratio of ww and dw before digestion in order to calculate digestion efficiencies based on dw ($dw_{initial} = ww_{initial} \cdot 0.177$). It was also tested whether the PS beads were affected by the enzymes by adding the beads to the respective solutions under the same treatment as before. They were then visually compared to untreated beads using a stereomicroscope (Leica MZ6, 40x).

Blue mussel exposure system

Mussel sampling and preparation. Blue mussels (*Mytilus edulis*) were collected from long lines on a mussel farm in Limfjorden near Sallingsund (Denmark) one week prior to the experiment and kept in a flow-through system with natural plankton at 17 to 18°C and a salinity of 26 to 28 PSU. For the exposure experiment, 250 mussels with a shell length of 5 to 5.5 cm were selected and distributed in three 50 L tanks. The floor of the tanks was covered with individual loose ceramic tiles (21 cm^2) for the mussels to attach to. During the experiment, mussels were moved between containers by moving the tiles, thereby avoiding touching the animals to reduce stress. Only tiles with one attached mussel were used.

Mussel filtration prior to the exposure experiment. Right before the actual experiment was started, mussel filtration rates were measured to ensure the active and constant filtration of the

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animals at the start of exposure to the PS beads. This as well as the exposure experiment was run at 15°C in a temperature controlled climate room. We used 10 L white plastic buckets with aeration stones to ensure constant mixing of the water. Two individuals were placed in each bucket (n=4, containing only filtered seawater) and allowed to acclimatize for 30 min. Then, *Rhodomonas salina* was added at a concentration of 10,000 cells mL^{-1} and water samples of 1 mL each were taken every 8 min for 24 min in total to determine the filtration rate. The algae concentration (c) was measured as fluorescence (f) with a handheld fluorometer (AquaFluor, Turner Design) and calculated from a calibration curve ($c=350f–4039$, $R^2=0.99$). To ensure that mussel filtration was stable this procedure was repeated immediately after the first measurement was completed.

Exposure and depuration experiment. The exposure experiment was initiated immediately after the second cycle of filtration rate measurements. Mussels were exposed to either 5 beads L^{-1} (low concentration) or 100 beads L^{-1} (high concentration) for 10, 20 or 40 min. Simultaneously with the PS beads, *R. salina* was again added to reach a level of 10,000 cells mL^{-1}. After the respective exposure time (10, 20 or 40 min) both mussels were removed from the exposure bucket and thoroughly rinsed with filtered seawater. One mussel was immediately frozen, while the other one was moved to a 10 L bucket with clean seawater (filtered through 1 µm and UV treated) for 2h of depuration (fig. 1). At the beginning and after half the depuration time (1h) 10,000 cells mL^{-1} of *R. salina* were added, and after 2h the mussel was removed, rinsed as before and frozen. In parallel to each treatment one control group was treated in the same way, except for the addition of plastic beads.

Quantification of PS beads in the experimental compartments. The water of the exposure and depuration bucket, respectively, was filtered through a 20 µm mesh, together with additional

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water that was used for rinsing the bucket walls and the aeration stone thoroughly. All material was then rinsed off the 20 µm mesh into a glass beaker.The solution in the beaker was filtered onto a 10 µm plankton mesh, using a vacuum pump. The number of plastic beads per filter was counted using a stereomicroscope (Olympus SZX12).

The number of PS beads in the mussels was quantified in the frozen mussels after thawing for 1h. The shells were opened and the whole soft tissues were scraped into glass bottles using scalpel and forceps. Digestion was done with a 2.5% Alcalase solution using the procedure described above. The digested tissue was filtered onto a 10 µm plankton mesh. The plastic beads on each filter were counted with a stereomicroscope as described above.

To establish the mass balance of beads, the experimental system was divided into 4 compartments for which the distribution of beads was quantified individually: the seawater from the exposure bucket, the mussel that was frozen after exposure, the seawater from the depuration bucket and the mussel that was frozen after depuration.

Statistical analysis

All statistical analyses and graphs were made in R (version 3.2.5). Differences between groups were analyzed using ANOVA (analysis of variance). The homogeneity of variances was checked with the Fligner-Killeen-Test and normality of residuals with the Shapiro-Wilks-W-Test. In case of a significant finding with the ANOVA a post hoc test (Tukey's HSD) was conducted. The enzyme data was non-normal, therefore a Kruskal-Wallis test was used with the Dunn's test for post hoc testing.

RESULTS AND DISCUSSION

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Enzymatic digestion

The novel enzymatic digestion method did not lead to any visual alterations of the used PS beads and proved to be very efficient for the blue mussel tissue. It resulted in visually homogeneous solutions at all tested concentrations and digestion efficiencies of 98.45 to 99.44% (ww) and 98.3 to 99.35% (dw) (Table 1) which are comparable to or even higher than reported efficiencies of Proteinase K (> 97%) and trypsin (88%) (Cole et al. 2014; Courtene-Jones et al. 2017). The two lowest concentrations required however a pre-filtering step through a larger mesh to prevent clogging, making them less practical to work with. Some weight reduction was observed in the control samples, incubated only in MilliQ water without addition of enzyme. However, the soft tissue was still intact after shaking and incubation and the sample could only be filtered on the 100 µm pre-filter as the 10 µm filters clogged. Due to this loss the calculated weight reductions of the control are overestimated. Even though there was no significant difference between the three highest concentrations we chose 2.5% for the experiment as it showed the highest efficiency (dw). In general, Alcalase is a very promising enzyme for digesting soft tissues, also at lower concentrations, and it is easy to handle in only one treatment step without adding other chemicals as required in the methods using Proteinase K (Karlsson et al. 2017) or Biozym (Railo et al. 2018). The enzyme has also been used successfully to digest oysters for isolating peptides, which was however in combination with bromelin (Liu et al. 2008). In comparison to other digestion methods using chemicals, enzymes have been found to have minimal effects on the plastics while showing high tissue digestion efficiencies. This is supported by our results and demonstrated in several other studies (Cole et al. 2014; Karlsson et al. 2017; Railo et al. 2018). Therefore enzymes should be used when working with microplastics in mussel tissue. The digestion method of the present study furthermore has several advantages in comparison to other

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methods that have been used so far. Alcalase showed high digestion efficiencies (>98%) also at low concentrations and does not require the addition of any other chemicals, reducing handling time, costs and hazard. Therefore, it has the potential of becoming a standard method for digesting mussel tissue.

Filtration rates prior to exposure

With the exception of one treatment group (high, 20 min), the filtration rates of the mussels prior to the exposure were all in a similar range, between 6.2 and 10.6 Lh^{-1} (mean: 9.3 ± 1.7 Lh^{-1}) (fig. 2). Using the average dry weight (1.04 ± 0.21 g) per mussel of this population which was measured on a subsample, these filtration rates correspond to 6 to 10.2 $Lh^{-1}g^{-1}$(dw) (mean: 8.9 ± 1.6 Lh^{-1}). In comparison to the literature our filtration values are at the high end but filtration rates between 7 and 8 $Lh^{-1}g^{-1}$(dw) have been reported (Riisgård 2001).

Recovery and distribution of beads in the exposure system

The overall number of plastic beads that were recovered, including the water and mussels, were on average 964, 1078 and 1027 for the treatment groups with high microplastic concentration, which correspond to 96, 108 and 103% of the theoretically applied particle number of 1000 beads (100 L^{-1}). In case of the low microplastic concentration the numbers were 55, 58 and 62, which are equal to 110, 117 and 125% of the theoretical number of 50 beads (5 L^{-1}) (fig. 3). The slight deviations from the nominal concentrations likely result from pipetting uncertainties when adding a certain volume of the particles stock suspension. Still, the proximity of nominal and measured particle numbers illustrates the successful handling of this system, which allows the tracking of all plastic beads.

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The distribution of beads in the 4 compartments of the experimental system changed over time (fig. 4). At all time points (10, 20 and 40 min exposure) the largest fraction was found in the exposure water, although this decreased with time. Simultaneously the number of beads in the mussels increased. This illustrates that a larger water volume was filtered and thereby cleared of the beads over time. There was however not enough time for the mussels to filter the entire volume, explaining the fraction remaining in the exposure water. The smallest fraction was found in the depuration water and increased only slightly with time. This finding as well as the fact that we found no significant difference between the number of beads in the exposure and depuration mussels (figs. S1 and S2 in SI) indicates that very little or no egestion took place during the 2h. As we also did not observe particles incorporated in mussel faeces in the depuration water, it is possible that the few particles that were found in the depuration water itself had not been ingested but adhered to the surface structures of the mussel and in this way released despite thorough rinsing. To clarify this, it is recommended for future studies to examine mussels with a stereomicroscope and look for particles on the surface after exposure and rinsing. The depuration time of 2h was not sufficient for egestion to take place despite active filtering of the mussels which was ensured by the addition of algae during depuration. This is in line with observations by Bayne et al. who found gut passage times for *M. edulis* of 1.81 to 3.23h, depending on the diet and temperature (Bayne et al. 1989). Egestion also depends on the particle size as observed by Ward and Kach (2009) who found different egestion times for PS nanoparticles (highest egestion after 72h) and 10 µm beads (highest egestion after 6h). As our beads were bigger it can be expected that they are egested even faster. It is therefore recommended to prolong the depuration phase in future experiments to 4h or more. This will

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increase the ecological relevance and enhance the mechanistic understanding of microplastic egestion.

The fractions of beads in the different compartments were very similar for both concentrations, which means that filtration rates were not adjusted to the different particle amounts but stayed constant. This could be due to the rather small difference between 5 and 100 beads L^{-1}, which may not be large enough for the mussels to sense quantitatively. When comparing the total volume of plastic beads in the system to the volume of algae, the beads are equivalent to 0.025% (for 5 beads L^{-1}) and 0.5% (for 100 beads L^{-1}) of the algae volume. Plastic particles only make up a small fraction in comparison to food in most aquatic habitats. Therefore, it may be expected that mussels do not adjust their filtering activity to this, also considering that they are additionally exposed to natural suspended solids.

As we used environmentally realistic particle concentrations and provided food alongside it can be expected that a similar uptake would take place in the environment. Since blue mussels are an important food source for many marine species and the trophic transfer of microplastics from blue mussels to crabs has been observed in a laboratory study (Farrell and Nelson 2013), this microplastic ingestion could also affect other species in the bentho-pelagic food web.

CONCLUSION

Our experiment shows that a high recovery of plastic beads is achievable in controlled exposure systems with low, environmentally realistic microplastic concentrations, enabling the establishment of a mass balance of particles. For quantifying the number of ingested beads per mussel an easy and efficient enzymatic digestion protocol using Alcalase was developed. We found a similar distribution of 50 µm PS beads between the mussels and the water for a low (5

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beads L^{-1}) and high (100 beads L^{-1}) concentration, which indicates that no adaptation of filtration to the number of particles in the water took place. The depuration period of 2h was not sufficient for the mussels to egest the particles and should be prolonged to 4h or more.

We recommend that future studies incorporate an analysis of particle fate in exposure systems. This enhances our understanding of the interactions between test organisms and microplastics and can elucidate why effects are observed in certain exposure systems while being absent in others.

Supplemental data

The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx

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Data availability

Data, associated metadata, and calculation tools are available from the corresponding author (siri@env.dtu.dk).

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Figure captions

Fig. 1: Overview of the exposure system and subsequent analysis steps.

Fig. 2: Filtrations rates (Lh^{-1}, means ± SD) prior to the experiment in the treatment groups (grey) that were afterwards exposed to a low (5 beads L^{-1}) or high (100 beads L^{-1}) microplastic concentration for 10, 20 or 40 min, and in the respective control groups (white).

Fig. 3: Total number of polystyrene beads (means ± SD) that were recovered from the exposure system of different treatment groups that had been exposed to a low (5 beads L^{-1}) or high (100 beads L^{-1}) microplastic concentration for 10, 20 or 40 min, respectively. Dashed lines show the theoretical applied numbers of 50 (low) and 1000 (high) particles in the systems.

Fig. 4: Relative distribution of polystyrene (PS) beads in the four components of the exposure system: the water in the exposure bucket, the mussel sampled after exposure, the water in the depuration bucket and the mussel sampled after depuration. Percentages refer to the total number of beads that were recovered from the system. A) shows the treatment groups at a low (5 beads L^{-1}) and B) at a high (100 beads L^{-1}) microplastic concentration, with exposure lengths being 10, 20 or 40 min, respectively.

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Table 1: Alcalase digestion efficiency (defined as the weight reduction of mussel tissue in percent) of different enzyme concentrations, given for wet and dry weights. Initial dry weights were calculated using a measured ratio of dry to wet weight of mussel tissue ($dw_{initial} = ww_{initial} \cdot 0.177$).

	Digestion efficiency (%)[a]		
% Alcalase	ww	dw	Used filters
0	39.71 ± 10.12	56.77 ± 8.40	100 µm
0.25	98.45 ± 0.49	98.30 ± 0.33	100 µm and 10 µm
0.5	99.25 ± 0.43	98.50 ± 0.45	100 µm and 10 µm
1.25	99.17 ± 0.49 *	98.50 ± 1.23 *	10 µm
2.5	99.42 ± 0.31	99.35 ± 0.33	10 µm
5	99.44 ± 0.43	99.21 ± 0.38	10 µm

[a] Data given as means ± standard deviation (n=5), asterisks (*) indicate significant differences between treatments ($p<0.05$)
Abbreviations: ww = wet weight, dw = dry weight

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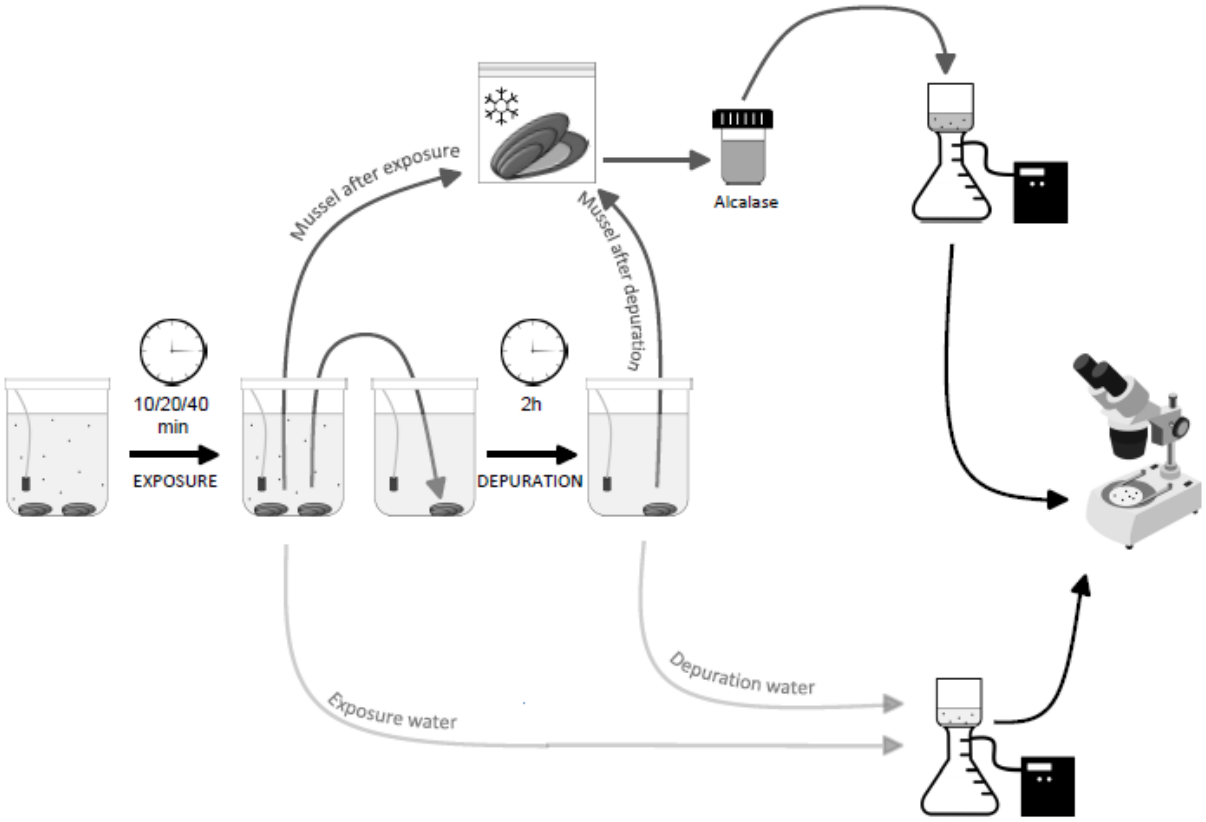


Figure 1

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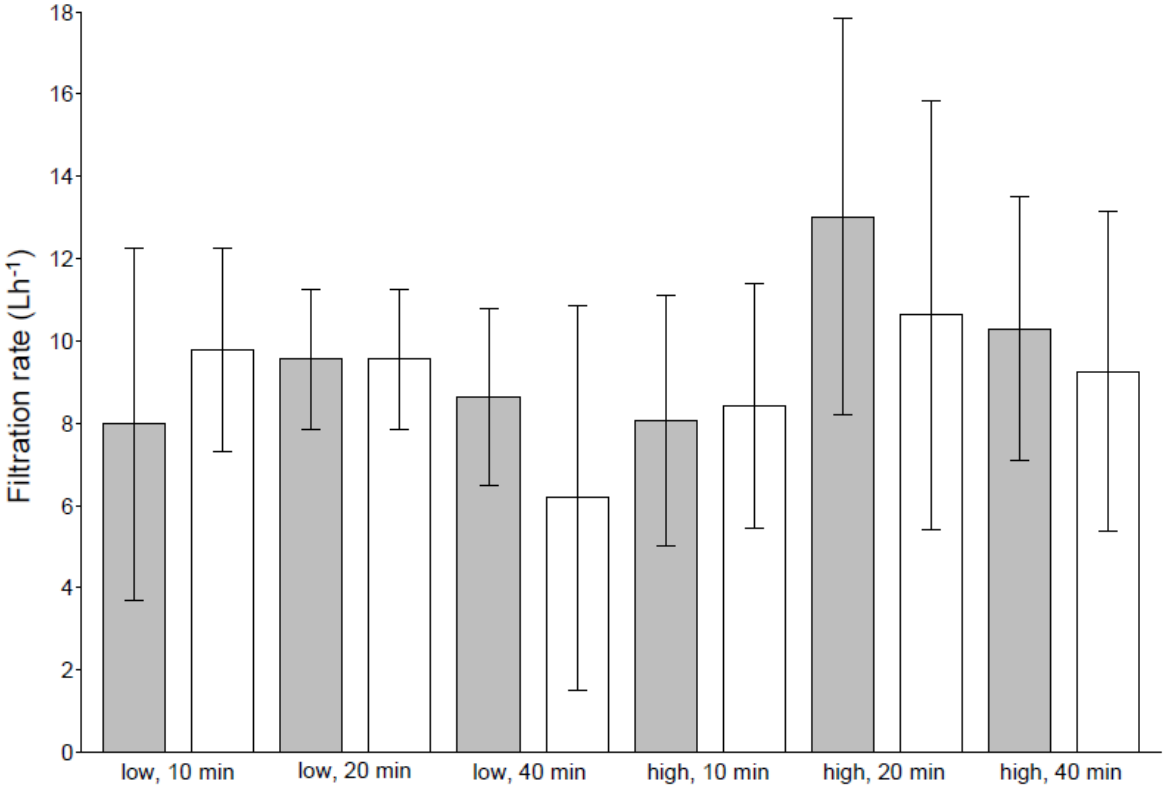


Figure 2

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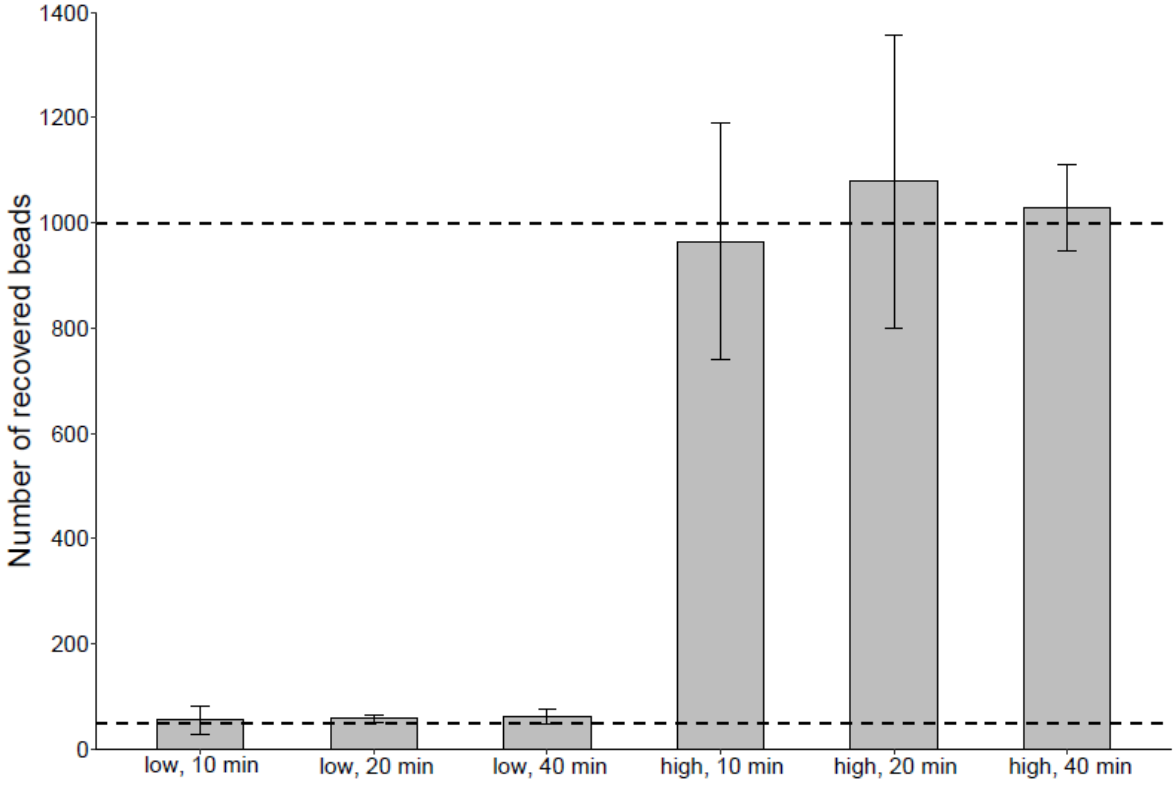


Figure 3

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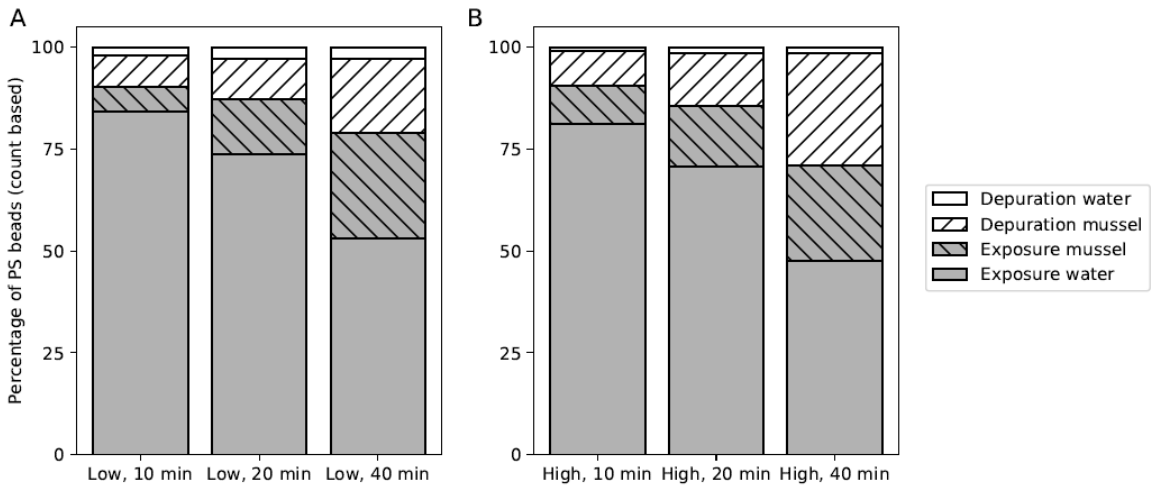


Figure 4

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