



NOTE

# Examining effects of surfactants on particle clearance rate and capture efficiency of the blue mussel *Mytilus edulis*

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**ABSTRACT:** Suspension-feeding bivalve molluscs perform important ecological roles by coupling pelagic and benthic systems during their feeding activities. Particle capture, and thus feeding, is dependent on particle encounter and retention on the gill filaments, with several factors influencing this process. Over the past 30 yr, different types of synthetic microspheres have been used to examine aspects of particle capture and ingestion by bivalves. Critics of this work have posited that manufactured particles may contain surfactants, chemicals commonly used in manufacturing to reduce surface tension, that could produce spurious capture and ingestion rates. The goal of this work was to experimentally assess whether the presence of different types of surfactants on manufactured polystyrene particles can result in instantaneous effects on particle capture by the blue mussel *Mytilus edulis*. The effects of 3 different types of common surfactants (sodium dodecyl sulfate, benzalkonium chloride, Triton-X) on clearance rates (CR) and capture efficiencies (CE) were tested. Results indicated that none of the surfactant treatments had an effect on CR. Treatment with one of the surfactants (Triton-X) significantly lowered CE for 3 µm sized spheres compared to the control spheres (Milli-Q treated). None of the other tested surfactants significantly affected CE when compared to the control treatment. These data add to an understanding of particle handling by bivalves, and suggest that concentrations of surfactants found on commercially available microspheres used for experiments or found in the environment have little immediate effect on feeding processes.

**KEY WORDS:** Bivalves · Microplastics · Polystyrene · Suspension feeding

## 1. INTRODUCTION

Suspension-feeding bivalve molluscs are a critical component of benthic food webs, mediating the distribution of organic materials, cycling nutrients, and contributing significantly to biodeposition of organic and inorganic matter (Smaal et al. 2019). Bivalves,

such as mussels, use their ctenidia (=gills) in both oxygen exchange and to capture particles from the surrounding water. The hydrosol filtration system of bivalves utilizes mucociliary processes to efficiently capture a wide range of particle sizes (Møhlenberg & Riisgård 1978, Ward 1996, Strøhmeier et al. 2009, Yahel et al. 2009, Rosa et al. 2015, 2017).

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Much of the basic research on mechanisms of particle capture and selection by suspension-feeding bivalves has relied on the use of synthetic polystyrene microspheres. These particles are uniform in size and can be formulated with fluorescent or colored dyes and reactive functional groups for covalently binding different compounds (e.g. neoglycoproteins). These features, and the ease at which they can be enumerated, are ideal for studying effects of size, concentration, and surface properties on particle feeding by bivalve larvae and adults (e.g. Solow & Gallagher 1990, Silverman et al. 1995, Ward 1996, Yahel et al. 2009, Rosa et al. 2015, 2017). Critics of these studies, however, have posited that manufactured particles may contain surfactants, chemicals commonly used in the manufacturing of plastics to reduce surface tension, that could produce spurious capture and ingestion rates (Ostroumov 2003, Cranford et al. 2016). Dissolved surfactants (sodium dodecyl sulfate [SDS], Triton-X) have been reported to inhibit clearance of particles in *Mytilus edulis* (Ostroumov & Widdows 2006), but only at high, environmentally unrealistic, concentrations (1–5 mg l<sup>-1</sup>). In contrast, numerous studies have examined the capture and ingestion of various types of plastic particles by bivalves without observed effects on feeding (see Ward et al. 2019). Although microspheres have been used to examine clearance rates (CR) of several suspension-feeding taxa with no reported toxic effects (Sanders et al. 1989), to our knowledge no research has experimentally assessed whether an adsorbed surfactant can affect instantaneous CR or capture efficiencies (CE) of a suspension-feeding bivalve.

In this study, we tested the instantaneous effects of 3 different types of common surfactants on particle capture by the blue mussel *M. edulis*.

## 2. MATERIALS AND METHODS

### 2.1. Animal maintenance

Blue mussels *Mytilus edulis* sized 50 ± 5 mm were collected from local populations or purchased from aquaculture facilities (Prince Edward Island, Canada). Their shells were cleaned of fouling material, and the animals transferred to recirculating aquaria at 20°C for no more than 2 wk. During that period, they were fed a mixed ration of live microalgae and Shellfish Diet (Reed Mariculture®). This diet delivered a near continuous concentration of ca. 10<sup>4</sup> cells ml<sup>-1</sup> to the mussels, a level appropriate for maintaining bivalves in the laboratory (Rosa et al. 2018).

### 2.2. Microsphere and surfactant preparation

Fluorescent polystyrene microspheres (fluorescent 3, 6, 10 µm; plain 10 µm; YG, Polysciences) were treated with 3 different types of commercially available surfactants commonly used in microsphere manufacturing: SDS (anionic), benzalkonium chloride (BKc; cationic) and Triton-X (nonionic; Keppler et al. 1977, Polysciences Inc. 2013). Working solutions of 0.2 mg ml<sup>-1</sup> of each surfactant were prepared. This concentration was selected because previous work on bivalve feeding physiology demonstrated a significant response to neoglycoproteins and adsorbed carbohydrates (methyl cellulose) at this concentration (e.g. Rosa et al. 2013, 2017).

Stock microsphere suspensions were prepared following standard methods to remove residual surfactants that could persist with the purchased spheres (Cole & Galloway 2015, Rosa et al. 2015, 2017, Gray et al. 2017). Briefly, particles were dispersed by sonicating for 30 s, then transferred to ultrapure Millipore water (MQ) at a concentration of 9 × 10<sup>7</sup> particles ml<sup>-1</sup>. The suspension was then centrifuged at 2000 × *g* for 10 min, the supernatant discarded, and the pellet re-suspended in MQ water. This washing procedure was then repeated. After the final wash, microspheres were separately treated with the different surfactants by adding a 0.2 mg ml<sup>-1</sup> solution of surfactant and incubating for 30 min at room temperature on a shaker table. Control microspheres were prepared by incubating particles in MQ water. Treated microspheres were centrifuged at 2000 × *g* for 10 min, and the pellet stored in the refrigerator for use within 24 h.

### 2.3. Measuring CR

Treatment suspensions were made up in 0.2 µm (nominal) filtered seawater (FSW) containing a 50:50 mixture of microspheres (10 µm) treated with one of the 3 aforementioned surfactants or MQ, and a similarly sized microalga (*Tetraselmis chui*). Total concentration of spheres and algae in each beaker was ca. 2 × 10<sup>4</sup> particles ml<sup>-1</sup>. Suspensions with 100% *T. chui* at the same concentration were also prepared as a control. A total of 8 replicate mixtures per treatment were distributed in 1 l beakers and aerated to maintain particles in suspension. The microalga-only control was duplicated for a total replication of 16 beakers. A second control, consisting of the experimental suspensions with no added mussels, was used to monitor particle loss due to

settling. On the day of the experiment, individual mussels were transferred from the aquarium in which they were held to a beaker containing one of the aforementioned treatments. After showing signs of feeding (shell open, mantle extended, exhalant siphon formed), a 1 ml aliquot of water was taken from each beaker to determine the concentration at time zero. Samples were then taken every 10 min over a 30 min period. Concentration of particles in each water sample was analyzed by means of an electronic particle counter (Coulter Counter<sup>®</sup> Multi-sizer IIe) and particle counts used to calculate CR. The CR was calculated as:

$$CR = \frac{v}{t} \times \left[ \ln \left( \frac{C_o}{C_t} \right) \right] \quad (1)$$

where  $v$  is the volume of the container (l),  $t$  is time between samples (h),  $C_o$  is the particle concentration at the beginning of the sampling period, and  $C_t$  is the particle concentration at the end of the sampling period (i.e. every 10 min; Coughlan 1969, Cranford et al. 2011). Rates were corrected for the small changes in particle concentration measured in the settling-control beakers (no mussels).

#### 2.4. Measuring CE

Microspheres were prepared separately as above, but 3 size classes of microspheres were used (3, 6, and 10  $\mu\text{m}$ ). In total, 10–12 animals were used, and all mussel were exposed to each treatment and control suspension. On the day of the experiment, all 3 sizes of microspheres, treated with one of the 3 aforementioned surfactants or MQ, were suspended in 0.2  $\mu\text{m}$  (nominal) FSW at a density of  $3 \times 10^4$  particles  $\text{ml}^{-1}$ . The spherical microalgae *Cricosphaera carterae* ( $x = 8 \mu\text{m}$ , range = 5.6–14.9  $\mu\text{m}$ ) was also used as a reference to determine if a living particle was handled similarly to the polystyrene microspheres, or if any residual surfactants on the microspheres affected CE. On the day of the experiment, mussels were transferred from the aquarium in which they were held to individual beakers with FSW. After showing signs of feeding (shell open, mantle extended, exhalant siphon formed), microspheres were directly delivered to individual animals using previously described methods (Rosa et al. 2015, 2017). Briefly, a volumetric pipette was used to deliver each suspension to the inhalant aperture of the mussel, while the exhalant siphon was simultaneously sampled using a tube attached to a peristaltic pump, and posi-

tioned with a micromanipulator. The number of particles in each sample was counted using a flow cytometer (Accuri C6 Plus) to more accurately distinguish and enumerate the 3  $\mu\text{m}$  spheres from the background particles, and values were used to calculate CE as follows:

$$CE = 1 - \frac{C_{\text{out}}}{C_{\text{in}}} \quad (2)$$

where  $C_{\text{out}}$  is the number of particles exiting the exhalant aperture (i.e. particles not captured) and  $C_{\text{in}}$  is the number of particles delivered to the inhalant aperture. A CE of zero indicates that none of the particles were captured, and conversely a value of 1 indicates all of the particles were captured by the mussels.

#### 2.5. Statistical analyses

All data were tested for normality and homoscedasticity prior to statistical analyses. Only CE data violated these assumptions, and data were arcsine transformed prior to analysis. Calculated CR of mussels in each treatment were analyzed using an analysis of variance procedure (ANOVA, General Linear Model). To examine effects of treatment on mussel CE across the 3 size classes of microspheres, data were analyzed in SYSTAT<sup>®</sup>, using a 2-way mixed model ANOVA for repeated measures. If significant differences were found, then a multiple comparison test (Tukey's HSD) was used to determine differences between means. A significance level of  $\alpha = 0.05$  was used in all analyses, and data are presented as means  $\pm$  SD.

### 3. RESULTS

#### 3.1. CR

Mussel CR ranged from  $58.7 \pm 23.6 \text{ ml min}^{-1}$  for mussels feeding on *Tetraselmis chui* to  $74.6 \pm 18.0 \text{ ml min}^{-1}$  for mussels feeding on *T. chui* and microspheres treated with a saturating solution of BKc. No significant differences in mussel CR were found between the 5 treatments (ANOVA,  $F = 1.23$ ,  $p = 0.312$ ; Fig. 1). During the experiment, one mussel in the treatment with 100% microalgae (no microspheres) closed after 10 min, and one in the SDS treatment spawned and did not feed. As outliers, these data points were removed from the final analysis.

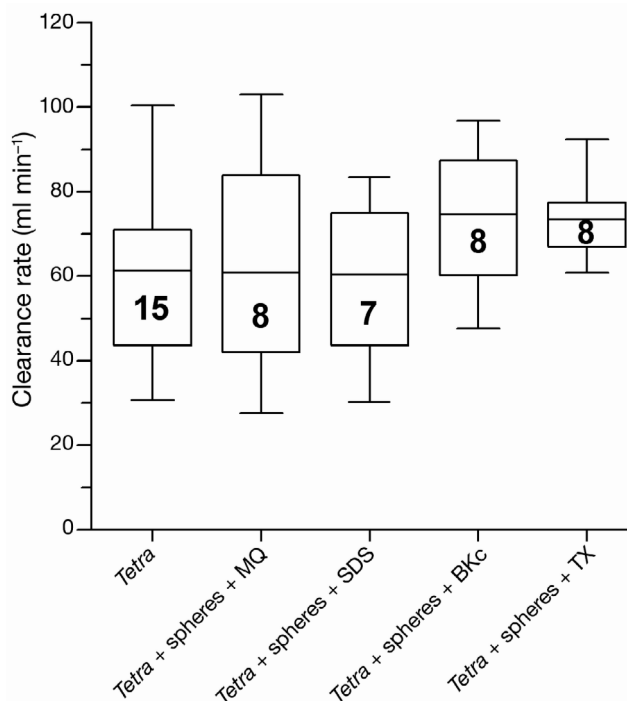


Fig. 1. Clearance rates (CR) of blue mussels feeding on the microalga *Tetraselmis chui* (*Tetra*) alone or in combination with MQ-treated (control) or surfactant-treated microspheres. No significant differences in CR were found between the 5 treatments (ANOVA,  $F = 1.23$ ,  $p = 0.312$ ). Data presented as means (horizontal line), with the 25<sup>th</sup> to 75<sup>th</sup> percentile (box) and 5<sup>th</sup> and 95<sup>th</sup> percentile of the data (whiskers) shown. Numbers on each bar: replicates per treatment. See Table 1 for explanation of surfactant abbreviations

### 3.2. CE

Mussel CE ranged from  $0.69 \pm 0.30$  for mussels delivered 3  $\mu\text{m}$  spheres treated with a saturating solution of Triton-X, to  $0.94 \pm 0.04$  for mussels delivered 6  $\mu\text{m}$  spheres treated with BKc. There was no significant effect of microsphere size on mussel CE (ANOVA,  $F = 2.40$ ,  $p > 0.09$ ; Table 1). There was a significant effect of surfactant treatment on mussel CE (ANOVA,  $F = 14.92$ ,  $p < 0.001$ ; Table 1). This effect was a result of the significantly lower CE for the 3 and 10  $\mu\text{m}$  spheres treated with Triton-X. The 3  $\mu\text{m}$  spheres treated with Triton-X were captured with lower efficiency ( $0.69 \pm 0.30$ ) than all other treated and control 3  $\mu\text{m}$  spheres (Tukey's HSD,  $p < 0.05$ ). The 10  $\mu\text{m}$  spheres treated with Triton-X were captured at significantly lower efficiency than the microspheres treated with BKc (Tukey's HSD,  $p < 0.05$ ), but at the same efficiency as the MQ- and SDS-treated microspheres (Tukey's HSD,  $p > 0.05$ ; Fig. 2). Within a particle size class, no other significant treat-

Table 1. Results of the 2-way mixed-model ANOVA for repeated measures, and Tukey's tests examining the fixed effects of microsphere treatment (surfactant) and size on capture efficiency in blue mussels. Multiple comparison tests between treatments within each microsphere size are presented in Fig. 2. Control: Milli-Q (MQ); SDS: sodium dodecyl sulfate; BKc: benzalkonium chloride; TX: Triton-X. **Bold** font indicates significance ( $p \leq 0.05$ )

ANOVA	df	F	p
Treatment (surfactant)	3	14.92	<b>&lt;0.001</b>
Size	2	2.40	0.096
Interaction	6	0.94	0.471
Treatment	t	p	
Control (MQ) vs. SDS	-0.77	0.868	
Control (MQ) vs. BKc	1.95	0.215	
Control (MQ) vs. TX	4.12	<b>&lt;0.001</b>	
TX vs. BKc	6.29	<b>&lt;0.001</b>	
TX vs. SDS	5.06	<b>&lt;0.001</b>	
Microsphere size (TX)	t	p	
3 vs. 6	-2.11	0.093	
3 vs. 10	0.55	0.848	
6 vs. 10	-1.56	0.266	

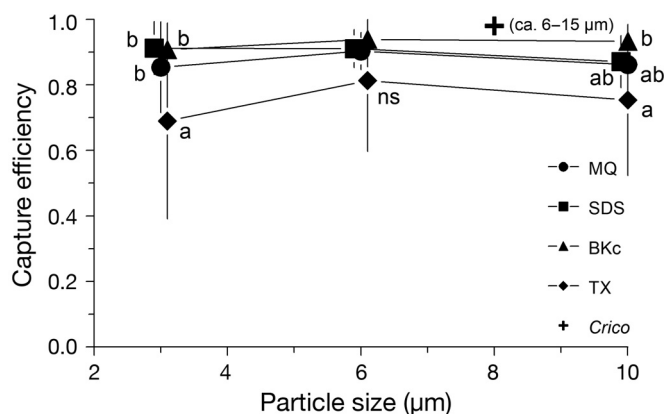


Fig. 2. Capture efficiencies of blue mussels feeding on microspheres treated with MQ (control) or surfactants, or on the microalga *Cricosphaera carterae* (*Crico*). There was a significant effect of surfactant treatment on the efficiency at which mussels captured 3 and 10  $\mu\text{m}$  spheres (mixed model ANOVA,  $F = 14.92$ ,  $p < 0.001$ ). However, only 3  $\mu\text{m}$  spheres treated with TX were captured at a significantly lower efficiency than the same sized control microspheres (Tukey's HSD,  $p < 0.05$ ). Mean cell size for *C. carterae* is plotted, but cell size ranged from ca. 6.0–15.0  $\mu\text{m}$ . Data presented as means  $\pm$  SD,  $n = 10$ . Within a particle size, different letters indicate significant differences between means (Tukey's,  $p < 0.05$ ); ns: not significant. See Table 1 for explanation of surfactant abbreviations

ment effects were detected. There was no significant interaction effect of microsphere treatment and size on CE (ANOVA,  $F = 0.94$ ,  $p > 0.47$ ). The efficiency at which mussels captured *Cricosphaera carterae* ( $0.98 \pm 0.03$ ) was slightly higher than the CE for 6 and 10  $\mu\text{m}$  spheres, likely resulting from the broad size range of the cells (ca. 6.0–15.0  $\mu\text{m}$ ; Fig. 2).

#### 4. DISCUSSION

This study experimentally assessed the instantaneous effects of 3 common industrial surfactants on CR and CE of the blue mussel *Mytilus edulis*. The treatment of microspheres with surfactants at concentrations above those which are likely present on commercially available spheres had minimal apparent effects on feeding physiology of *M. edulis*. In particular, the presence of the 3 surfactants had no effect on CR, and little effect on CE. Treating microspheres with Triton-X resulted in significantly lower CE of the smallest microspheres tested (3  $\mu\text{m}$ ), but had no effect on the capture of the 6 and 10  $\mu\text{m}$  sized spheres compared to the control-treated microspheres. This is an important point, as most studies that utilize microspheres to examine feeding processes of bivalves use particles of at least 6  $\mu\text{m}$  in size. CE for the 3  $\mu\text{m}$  control spheres found in this study were comparable to that of similarly treated microspheres reported previously for *M. edulis* (Rosa et al. 2017). Although high concentrations of surfactants (0.2 mg ml<sup>-1</sup>) were used to treat microspheres, the low amounts that adsorbed onto the spheres precluded directly quantifying a surface concentration.

Use of synthetic microspheres as proxies for microalgal cells and other types of particles is prevalent in the literature. Microspheres have been used to examine the mechanisms of suspension- and deposit-feeding by invertebrates, including studies on particle capture (Solow & Gallager 1990, Silverman et al. 1995, Ward 1996, Conova 1999, Yahel et al. 2009, Rosa et al. 2015, 2017), selection (Taghon 1982, Ward & Targett 1989, Shimeta & Koehl 1997, Rosa et al. 2013), and digestive processes (Sanders et al. 1989, Cole & Galloway 2015, Gray et al. 2017). Critics have suggested that the presence of surfactants, added during the manufacturing process of the microspheres, could result in sub-lethal effects, disrupting CR and CE (Ostroumov & Widdows 2006, Cranford et al. 2016). The microspheres used in the present study arrived from the manufacturer suspended in distilled water, with no added surfactant (only residual amounts; Polysciences Inc. 2013).

The practice of washing microspheres (e.g. MQ  $\geq 3\times$ ) prior to use further ensures that residual surfactants are removed prior to use. As such, and given the results of the present study, it is suggested that microspheres have little if any instantaneous effect on particle capture in bivalves, and are good proxies for various natural organic and inorganic particles.

Concerns about the effects of surfactants on feeding rates are largely based on 2 published papers. In these studies, the effects of surfactants on CR of the Pacific oyster *Crassostrea gigas* (Ostroumov 2003) and the blue mussel *M. edulis* (Ostroumov & Widdows 2006) feeding on the microalgae *Isochrysis* sp. (Tahitian strain) were examined. Ostroumov & Widdows (2006) separately added 3 different dissolved surfactants to the experimental water in increasing amounts to reach targeted concentrations of 0–5 mg l<sup>-1</sup>, which were meant to represent near-shore areas with high levels of pollution. The surfactants SDS and Triton-X reduced CR of *M. edulis* at high concentrations (1–5 mg l<sup>-1</sup>), which were 3 orders of magnitude higher than that to which suspension-feeders are theoretically exposed during laboratory experiments (see Rosa et al. 2018). In contrast, the surfactant tetradecyltrimethylammonium bromide, a quaternary ammonium compound, substantially inhibited CR at a lower concentration (0.1 mg l<sup>-1</sup>). This surfactant, however, is not commonly used in the manufacturing of microspheres. Therefore, using the results of Ostroumov & Widdows (2006) to generalize about the potential effects of surfactants at much lower concentrations on particle feeding is misleading.

Interest in the effects of plastic particles on aquatic suspension-feeders has increased dramatically over the past several years. So too has the concern over surfactants that might accompany particles purchased from manufacturers or might be added by investigators in order to suspend microplastics in water. Results from this study suggest that residual surfactant concentrations found on commercially available microspheres have little instantaneous (short-term) effects on feeding processes. Similar to the recommendations put forward by Connors et al. (2017) in a critical review of the literature pertaining to environmental fate and effects of microplastics, future studies examining the effects of surfactants on aquatic animals should use concentrations relevant to physiological experiments and environmental conditions. Such methodological considerations will allow researchers to differentiate irrelevant from pertinent effects.

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