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Examining the physiological plasticity of particle capture by the blue mussel, *Mytilus edulis* (L.): Confounding factors and potential artifacts with studies utilizing natural seston

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ABSTRACT

Historically, particle capture efficiency (CE) in suspension-feeding bivalve molluscs has been shown to be strongly dependent on particle size, increasing asymptotically to a maximum of about 100% for particles ca. ≥4 μm in diameter. Recent advances in the analysis of the particulate matter of seston have allowed for more precise studies of bivalve feeding under natural conditions. Some studies have reported that the mechanisms associated with particle capture exhibit physiological plasticity, and under certain conditions smaller cells and particles are captured in preference to larger ones. For bivalves, however, there is no mechanistic explanation that would account for such fine-scale control of CE based on size. The current study experimentally assessed the seasonal control of CE by the blue mussel, *Mytilus edulis*, employing a flow-through system to examine particle capture of natural seston. The natural particle field was analyzed using two different types of particle analyzers, the LISST-100X and the Coulter Multisizer IIe. Mussels were simultaneously delivered synthetic microspheres of defined diameter (2–45 μm) to control for the effects of seasonal differences in the size and shape of natural particles. The capture of microspheres was quantified by means of flow cytometry (FCM), and results cross-checked with the Multisizer. Additionally, gene expression of a mucosal lectin (MeML) associated with the feeding organs of mussels was examined as a biomarker for physiological response to seasonal changes in the particle-food supply. Results demonstrated that for microspheres ≥4 μm CE of mussels was always near 100%, and did not change seasonally. In contrast, there was an apparent seasonal shift in CE of natural particles, with particles 17-to-35 μm in equivalent spherical diameter (ESD) occasionally being captured at lower efficiencies than particles 4-to-15 μm in ESD (e.g., during September and December). No relationship between MeML expression and seasonal CE was found. These findings call into question the physiological plasticity of CE in mussels and alternative hypotheses are presented. We suggest that the purported changes in CE are not a consequence of behavioral or physiological responses of mussels, but rather a result of one or more of the following confounding factors; 1) instrument artifacts that can arise as a result of the way in which laser and electronic particle counters calculate ESD to estimate particle size; 2) disaggregation of flocculent material collected from control chambers; 3) post-capture escape of highly motile phytoplankton cells from the infrabranchial camber; 4) qualitative factors of the particles that could affect capture; or 5) mathematical happenstance of calculating CE on particle-size classes that contain widely different numbers of particles.

1. Introduction

Suspension-feeding bivalve molluscs are one of the most important groups of animals in coastal ecosystems, often dominating the macrobenthos (Dame, 1996). Bivalves are exposed to large amounts of suspended matter that include both nutritious and non-nutritious particles (Newell, 1965; Owen, 1966; Newell et al., 1989). As a way to process efficiently the complex mixture of material that they encounter, bivalves have evolved capabilities for selective feeding that allow them to reject some of the captured material in pseudofeces (Loosanoff, 1949; Foster-Smith, 1975; Shumway et al., 1985; Bacon et al., 1998). Selective feeding can be based on physical factors such as particle size and shape (Bayne et al., 1977; Shumway et al., 1985; Cognie et al., 2003; Mafra et al., 2009), as well as physicochemical interactions between the particles and the feeding organs (see reviews by Jørgensen, 1996; Ward and Shumway, 2004). Rosa et al. (2013) demonstrated that the

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eastern oyster, *Crassostrea virginica*, and the blue mussel, *Mytilus edulis*, can discriminate between particles of the same size based upon the surface charge and wettability of particles. More specific chemical interaction between lectins in the mucus of pallial organs and carbohydrates present on the surfaces of microalgal cells has also been demonstrated. In several studies, workers confirmed that carbohydrate-lectin interactions are involved in mediating particle sorting in both *C. virginica* and *M. edulis* (Pales Espinosa et al., 2009, 2010a). A mucosal C-type lectin (dubbed MeML) in the blue mussel, *M. edulis*, whose expression could be involved in particle sorting was also identified (Pales Espinosa et al., 2010b). Most recently, Pales Espinosa and Allam (2013) demonstrated that the expression of MeML is regulated in response to the quality and quantity of food offered, further suggesting a physiological basis for qualitative particle selection.

Although post-capture selection has been well studied, much less is known about selective retention during particle capture. Particle capture is the first step in suspension feeding and is a consequence of two interrelated processes: particle encounter and particle retention (Ward et al., 1998). Encounter efficiency relates to the proportion of particles that come into contact with the capture unit, in this case the gill filaments, whereas retention efficiency is the proportion of encountered particles that are actually retained (see Shimeta and Jumars, 1991). Although previous workers have used the term "retention efficiency" to describe particle capture efficiency in bivalves (Riisgård, 1988; MacDonald and Ward, 1994; Cranford and Hill, 1999; Strøhmeier et al., 2012), unless in vivo techniques are employed to differentiate the number of particles that are encounter from those that are actually retained (Ward et al., 1998), retention efficiency cannot be determined. Therefore, in this study we use the term capture efficiency (CE) to accurately describe the process being measured. Future studies using in situ techniques such as those described herein should use CE, which is the more exact term, in place of retention efficiency.

In general, capture efficiency has been reported to increase non-linearly with increasing particle diameter to a maximum of about 100%, with some species of bivalves being more efficient at capturing small particles than other species (see Ward and Shumway, 2004). If particles were differentially captured at the larger size threshold, this would be a form of particle selection. Preferential retention of particles could thus be an important discriminatory mechanism that alters the composition of material subjected to post-capture selection and ingestion. Although previous studies have demonstrated that many feeding processes of bivalves are under physiological control and respond to changing environmental conditions (= physiological plasticity; see Bayne, 2004 for review), uncertainty exists regarding the physiological plasticity of CE. Some studies suggest that different bivalve species can shift their maximum capture efficiency as a response to changes in the particle size distribution of the seston (reviewed in Ward and Shumway, 2004). This includes shifts in CE to coincide with larger particles containing a higher organic content by the rock-tide bivalve *Venerupis corrugatus* (Stenton-Dozey and Brown, 1992), and lower capture of smaller particles as the concentration of inorganic particles and overall particle loads increase by the scallop *Placopecten magellanicus*, and the oyster *Crassostrea gigas* (e.g., clay, Cranford and Gordon, 1992; Barillé et al., 1993, respectively). Recently, Strøhmeier et al. (2012) reported a seasonal variation in capture efficiency in the blue mussel *M. edulis*. These workers used a flow-through system to calculate CE and clearance rate, and compared these to the size distribution and concentration of particles in the natural seston. They reported that CE increased and reached a maximum for larger particles (30 to 35 μm) in early summer. Later in the season, when smaller particles dominated the seston, 7-to-15 μm particles were retained at higher efficiencies over the larger particles (30 to 35 μm), indicating a seasonal, size-dependent shift in particle capture. Overall, these studies suggest shifts in CE as a result of changing environmental conditions (e.g., tide, season).

In a few cases, qualitative factors have also been shown to affect CE. The European oyster, *Ostrea edulis*, for example, was found to capture the dinoflagellate *Prorocentrum minimum* preferentially over two other algal species of the same size (Shumway et al., 1985). Other studies have reported similar results for mussels and scallops (Newell et al., 1989; Shumway et al., 1997), and the authors suggested that capture was based on cell properties other than size. Hernroth et al. (2000) examined the effects of surface properties on particle uptake, and found that changing the electrostatic charge of bacteria (~1 μm) affected their capture by *M. edulis*. Similarly, Yahel et al. (2009) reported size-independent capture of particles in the tropical bivalve *Lithophaga simplex*. Using flow cytometry to examine the types of particles entering the inhalant siphon and comparing these with particles exiting the exhalent siphon, the authors determined that some algal species were captured at higher proportions than others, despite the overlap in mean cell size distribution.

Some studies have also reported that bivalves can capture smaller particles at a higher efficiency than larger particles (e.g., Strøhmeier et al., 2012). Lesser et al. (1991), for example, reported the clearance of the toxic dinoflagellate *Alexandrium tamarensis* (30–45 μm) by scallops at significantly lower rates than smaller (~16 μm diameter) phytoplankton species. Using a Coulter counter to examine particle selection in *M. edulis*, Bayne et al. (1977) reported that cells of *Phaeodactylum tricornotum* (~6 μm) were captured in higher quantities than larger inorganic particles, a shift the authors suggested may be a result of the coulter counter using spherical equivalents to calculate diameter. Using flow cytometry, Pile and Young (1999) reported that the cold-seep mussel *Bathymodiolus childressi* captured bacteria at significantly higher proportions than larger protozoans. Although the above reports suggest that particle capture in bivalves may be physiologically plastic and responsive to changes in the particle food supply, controls for the effects of seasonal changes in the relative abundance of different shaped particles, such as using microspheres of defined geometries, were not usually included in these studies. Apparent changes in CE could result from changes in the proportion of particles with different geometries, or other effects unrelated to physiological plasticity. To date, no mechanism has been proposed for bivalves that would account for the capture of smaller particles in preference to larger sized particles. Resolving results from field studies of CE with current models of particle capture mechanisms is important not only for a deeper knowledge of particle capture and selection processes, but also for a better understanding of how selective grazing by bivalves could affect phytoplankton species composition and impact food web dynamics in near-shore waters (see Dame, 1996).

This project was designed to assess experimentally the seasonal shifts in particle CE of *M. edulis*, and to compare CE of natural particles to that of microspheres with defined size and shape in order to specifically examine the physiological plasticity of particle capture. Changes in gene expression of a mucosal lectin (MeML), previously shown to be involved in particle feeding in mussels (Pales Espinosa et al., 2010b), were also investigated. A correlation between seasonal changes in CE and expression levels of MeML would further demonstrate a physiological response to changing particle fields.

2. Materials and methods

2.1. Study animals

Blue mussels, *M. edulis*, were collected locally from wild populations at the Avery Point Campus of the University of Connecticut in Groton, CT, USA. Animals were maintained in lantern nets hung from a dock adjacent to the intake line of the flow-through experimental system (see below). Mussels were acclimated to these conditions for at least 2 weeks prior to start of the feeding experiments.

2.2. Feeding experiments

Studies were carried out seasonally on two separate days for a period of 1 year (March, May, July, September, December 2013 and March 2014). A flow-through experimental system (Galimany et al., 2011) was employed to simulate environmental conditions experienced by bivalves in situ. The system was set up on a floating dock, and water was pumped directly from Long Island Sound into a common aerated head tank, which minimized particle settling (data not shown). Water was distributed to individual rectangular chambers, measuring 45 × 180 × 60 mm (width × length × height). Mussels (mean shell length 48 ± 5 mm [SD]) were secured to each chamber and the flow rate was set to 100 ml min^{-1} to prevent water recirculation. Animals were acclimated for 1 h prior to collection of water samples. The concentration of particles in the water exiting the control chambers (n = 4, no mussels) and the experimental chambers (n = 16, one mussel each) was measured using two different types of particle analyzers commonly used in bivalve mollusc feeding studies. First, a multi-parameter laser in situ scattering and transmissometry instrument (LISST-100X, Sequoia Science) was used to quantify particle size distribution and volume. The system was baffled against stray light that would interfere with the optics during measurements. Particle size distribution and their concentrations in the water column were determined by applying an inversion algorithm to the scatter data (Agrawal and Pottsmith, 2000). Within an hour of the LISST-100X analysis, a 250 mL water sample was collected from each chamber. These samples were gently inverted (10×), passed through a 70 μm mesh screen and analyzed by means of an electronic particle counter (Coulter Multisizer IIe) fitted with a 100 μm aperture. Both of these instruments measure particle volume, using light scattering or electrical impendence, and assume a spherical shape to calculate particle size. These sizes are reported as equivalent spherical diameters (ESD, μm).

To control for the effects of differences in particle shapes and presence of easily disrupted aggregates of natural seston on the calculated efficiencies, mussels also were delivered a mixture of equal concentrations of 2-, 4-, 6-, 10-, 25-, and 45-μm spherical fluorescent polystyrene particles (Polysciences, Inc.). These synthetic particles were delivered directly to the inhalant aperture using a pipette, and water leaving the exhalent aperture was collected using a sampling tube (2-mm diameter) attached to a peristaltic pump and positioned by means of a micromanipulator (similar to the InEx system, see Yahel et al., 2009). Special care was taken not to touch the inhalant and exhalant regions during sampling. Samples were analyzed by means of a flow cytometer (Accuri C6) and cross-checked on the Multisizer.

2.3. Environmental parameters

Seston samples were collected from each of the control chambers to calculate total particulate matter (TPM), particulate organic matter (POM), chlorophyll *a* concentration, and the distribution of phytoplankton species. To determine TPM and POM, replicate (n = 4) 1-L water samples were vacuum filtered through pre-ashed and pre-weighed 47-mm GF/C filters. Filtered samples were washed with 10 mL of ammonium formate (30 ppt) to remove residual salts, dried to a constant mass in an oven at 70 °C, and weighed to determine TPM. The filters were then placed in a muffle furnace overnight (450 °C), cooled in a desiccator, and re-weighed to determine the fraction of POM as weight loss on ignition (MacDonald and Ward, 2009). The concentration of chlorophyll *a* pigments was determined using the acidification method outlined by the Environmental Protection Agency (Arar and Collins, 1997). Briefly, replicate water samples (120–200 mL depending on seston concentration) were syringe filtered through GF/F filters, rinsed with 1 mL of magnesium carbonate solution to buffer the cells, and the chlorophyll extracted by placing it in 7 mL of 90% acetone overnight. The fluorescence intensity of the supernatant was measured by means of a TD 700 laboratory fluorometer (Turner Systems). After the initial measurement, 5% HCl was added to acidify the sample. Concentration of chlorophyll *a* was calculated using the formula:

$$\frac{\mu\text{g-chlorophyll-}a}{\text{L}} = \frac{\text{CF} \times \frac{\text{AF}}{(\text{AF}-1)} \times (\text{F}_1 - \text{F}_2) \times 7\ \text{mL}}{\text{V}}$$

where CF is the calibration factor of the instrument, AF is the acidification factor, 7 mL is the volume of acetone used to extract the chlorophyll, V is the total volume of sample filtered (in L), F_1 is the fluorescence reading before acidification, and F_2 is the fluorescence reading after acidification. Water temperature and salinity, measured by means of a refractometer and reported as parts per thousand, were recorded during each field season.

2.4. Phytoplankton distribution

A 1-L sample of water was taken from each of the head tanks in the flow through system, and from the overflow hose attached to the system intake pump. Samples were preserved in 2% Lugol's Iodine, and maintained on ice and in the dark until they were processed. First, water samples were allowed to settle for 1 week in the dark at 4 °C. After settling, a peristaltic pump was used to remove 750 mL of supernatant of the sample. The remaining 250 mL, including the settled material, was transferred to a clean cylindrical container and settled for another 2 weeks (dark, 4 °C). After this settling period, 225 mL of the supernatant was removed and the remaining 25 mL was transferred to clean scintillation vials for identification and counts. A gridded Rafter cell was used to count triplicate sub-samples of each concentrated sample. Phytoplankton were identified to genus (using the flora classification per Hoppenrath et al., 2009) and photographed. The longest dimension of representative cells in each genera was measured. More extensive sizing measurements were not collected due to time and resource limitations. The concentration of cells (cells mL^{-1}) belonging to major genera was determined, and percent abundance of all major genera calculated for each sampling date. Calculated abundances of identified genera for each of the samples (head tanks and overflow) were within 5% of each other, and thus averaged for clarity of presentation.

2.5. MeML transcript expression

The relative expression of the mucosal lectin (MeML) transcripts associated with the pallial organs were determined by means of qPCR on samples taken from the labial palps of mussels used in the flow through experiments. Labial palps were used as a proxy for gills because previous studies have shown that MeML expression in mussel labial palp and gill tissue is correlated (Pales Espinosa and Allam, 2013). Secondly, the labial palps have been found to have a higher signal-to-noise ratio than the gill tissue. Following each experiment, mussels were placed on ice and taken to the laboratory where labial palps were separately collected using sterile techniques. Tissue samples were then placed in liquid nitrogen and maintained at −80 °C until further processing. Total RNA of each sample was extracted using TRI-Reagent® RT (Molecular Research Center Inc., Cincinnati, Ohio) and reverse transcribed into cDNA using M-MLV RT (Promega, Madison, Wisconsin). MeML transcript abundances were measured in duplicate with a set of specific primers designed by Pales Espinosa et al. (2010b) (forward 5′-ATGCTCAATTGGCTGGCATCATGG-3′ and reverse 5′-ATGC TCAATTGGCTGGCATCATGG-3′) and using 18S ribosomal RNA as a housekeeping gene targeted with the primer set forward 5′-CTGGTT AATTCCGATAACGAACGAGACTCTA-3′ and reverse 5′-TGCTCAATCTCG TGTGGCTAAACGCCACTTG-3′. Real-time PCR monitoring was performed using an Eppendorf RealPlex cycler with 96 well plates. Relative

quantification was carried out in 10-μL reactions, including 5 μL of 2 × Brilliant II SYBR® Green qPCR Master Mix (Agilent), 1 μL of each primer at 10 μM concentration and 3 μL of cDNA template to obtain a final concentration of 5 ng/μL cDNA in the reaction volume. The thermal profile for real-time PCR assay was an initial denaturation step at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s, and annealing and extension at 60 °C for 1 min. A melting curve was set up and analyzed after each run to guarantee a specific amplification. The relative MeML expression level of each sample was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), using the 18S data for the first normalization and data from March 2014 as a calibration for the second normalization. March 2014 was selected as a calibration month because it had the lowest mean fold average ΔC_T, reducing the noise-to-signal ratio of the $\Delta\Delta C_T$ values. Data are presented as a fold change, where the value indicates the change in the expression level of MeML for each season relative to March 2014.

2.6. Particle analysis and feeding calculations

Particle size classes recorded by the LISST-100X ranged from 1 μm to 200 μm, and those recorded by the Multisizer ranged from 1 μm to 64 μm. Particle size bins were established based on counts from water exiting the control chambers and then used for all comparisons. Data for particle sizes were binned in 1-μm increments, unless total counts were <100 particles mL^{-1}, in which case data for the next measured size class were added until this threshold concentration was reached. This procedure ensured sufficient particle numbers for robust comparisons. Because few large particles were present in the seston, the maximum size bin that met the 100-particles mL^{-1} threshold was 61 μm for the LISST-100X and 25 μm for the Multisizer. The fact that these two truncation points are not the same demonstrates the variation in data that can be generated when the same particle field is analyzed by two different instruments. Capture efficiency (CE) was calculated for each size class of particles using the formula:

$$CE = 1 - \frac{PC_f}{PC_c}$$

where PC_f is the particle concentration exiting each individual animal chamber, and PC_c is the particle concentration exiting the control chamber (Cranford and Grant, 1990). Values of CE were then standardized by setting the highest CE of each mussel to 1 and increasing the values for the other size classes proportionately (Cranford and Gordon, 1992; MacDonald and Ward, 1994). This procedure reduces variation due to slight differences in the size-frequency distribution of seston, the flow rate through the holding chambers, and clearance rates of the individual mussels. Particle clearance rates (CR) for several defined size classes (<4 μm, 4 to 10 μm, 11 to 25 μm), and for the entire size range were calculated using the formula:

$$CR = f \times \left(\frac{PC_f - PC_c}{PC_c}\right)$$

where f is the set flow rate through each chamber (Bacon et al., 1998). The concentrations of polystyrene microspheres in the collected samples were determined by means of flow cytometry (Accuri C6), and cross-checked with the Multisizer. Capture efficiencies for these microspheres were also calculated using the above equation.

2.7. Statistical analysis

Seasonal differences in environmental parameters (TPM, POM) were tested using analysis of variance. To examine the effects of season and particle size on capture efficiencies, a mixed-model analysis of variance for repeated measures was used (ANOVAR; Systat 13), with particle size class as the within subject effect (repeated) and season as the between subject effect. Data were tested for normality and homogeneity prior to analysis. Following ANOVAR analyses, a Tukey's HSD post-hoc test was applied to examine differences between levels of the independent variables. Differences in expression of MeML were tested on ΔC_T values (C_Ts normalized with the housekeeping 18S gene) by means of a one-way analysis of variance by ranks, followed by a Dunn's post hoc, due to the heteroscedastic nature of the data (Kruskal–Wallis; SigmaStat). For all statistical tests, an alpha level of 0.05 was used.

3. Results

3.1. Environmental and seston parameters

As expected, environmental conditions varied seasonally during the one-year study (Table 1). Water temperature ranged from 3.5 to 24.5 °C, and salinity ranged from 30 to 35. The total particulate matter (TPM) ranged from 4.04 mg/L in December to 7.50 mg/L in March 2014. There were no significant differences in TPM between each of the seasons sampled. The particulate organic matter (POM) fraction was significantly higher during March 2013 (3.09 mg/L) and significantly lower in December 2013 (0.55 mg/L) compared to values for other sampling months. The lowest concentration of chlorophyll *a* was recorded in December (0.06 μg/L), and was significantly different than values for all other months. The highest concentration of chlorophyll *a* was recorded in May (5.13 μg/L), but this value was not significantly different than values for other months (except December). The seston was dominated by small particles (1–3 μm) in all months sampled (data not shown), with the number of particles analyzed by the LISST on the order of 10^8 particles mL^{-1} and by the Multisizer on the order of 10^6 particles mL^{-1}. Particle concentrations rapidly dropped to 10^3 particles mL^{-1} for particles ranging from 4 to 10 μm, and to ca. 10^2 particles mL^{-1} for larger particles. This overall particle-size distribution (PSD) did not change seasonally. Additionally, the PSD of the seston did not change significantly over the sampling period, or between the two sampling days. Therefore, data obtained for each of the two days were pooled.

3.2. Capture efficiencies

For the polystyrene microspheres, data analyses revealed no significant effect of season on CE for spheres ≥4-μm (ANOVAR, $P > 0.05$; Fig. 1A). For the 2-μm spheres, a significant seasonal effect was found, with CE significantly higher in December than in all other months (Tukey's HSD, $P < 0.01$). There was also a significant effect of microsphere size on CE (ANOVAR, $P < 0.01$). The 2-μm spheres were captured with lower efficiencies than all other size spheres (Tukey's HSD, $P < 0.01$). No significant differences in CE were found between any of the remaining size classes (4-, 6-, 10-, 25-, and 45-μm; Tukey's HSD, $P > 0.05$; Fig. 1A).

For natural seston analyzed by means of the LISST-100X, data analysis demonstrated significant effects of both season and particle size on CE (ANOVAR, $P < 0.01$; Table 2, Fig. 1B). There were also significant interaction effects between these two independent variables. For example, in May, July, and September particles ca. 3-μm in size were captured at significantly higher efficiencies than in March (2013 & 2014, Tukey's HSD, $P < 0.01$). In May, particles in the 22–31 μm range were captured at significantly higher efficiencies than in September and December (Tukey's HSD, $P < 0.01$; Table 2, Fig. 1B). Other seasonal differences in CE of particles in the same size class were also evident (Table 2). Overall, CE were lower for particles smaller than 3 μm in size. In March and May, CE generally increased or stayed constant as particle size increased above 5 μm. In September, however, CE were significantly lower for particle size classes ranging from 31 μm to 44 μm, compared to size classes 6 μm to 13 μm. In December, a similar trend was observed, with significantly lower CE for particle size classes ranging from 22 μm to 31 μm (Tukey's HSD, $P < 0.05$), compared to smaller and larger size classes

Table 1
Environmental parameters measured during the seasonal study. For chlorophyll *a*, total particulate matter (TPM), and particulate organic matter (POM) measurements, significant differences between sampling dates are denoted by asterisks (*, Tukey HSD, $P < 0.05$). Where appropriate, data are presented as means ± SD (in parentheses; n = 6–8). Seasonal designations are: Mar 13 = March 2013; May 13 = May 2013; Jul 13 = July 2013, Sept 13 = September 2013, Dec 13 = December 2013, and Mar 14 = March 2014.

Season	Temperature (°C)	Salinity	TPM (mg/L)	POM (mg/L)	Chlorophyll *a* (μg/L)
Mar 13	6.5	30	7.35 (1.42)	3.09 (1.82)*	2.30 (1.38)
May 13	16	30	7.50 (1.04)	1.89 (0.14)	5.13 (0.71)
Jul 13	24.5	30	6.66 (1.51)	1.70 (1.12)	3.90 (1.27)
Sept 13	17.5	31.5	5.75 (0.46)	1.69 (0.25)	2.33 (1.31)
Dec 13	9	35	4.04 (1.15)	0.55 (0.16)*	0.06 (0.05)*
Mar 14	3.5	35	7.47 (0.59)	0.87 (0.28)	1.85 (0.68)

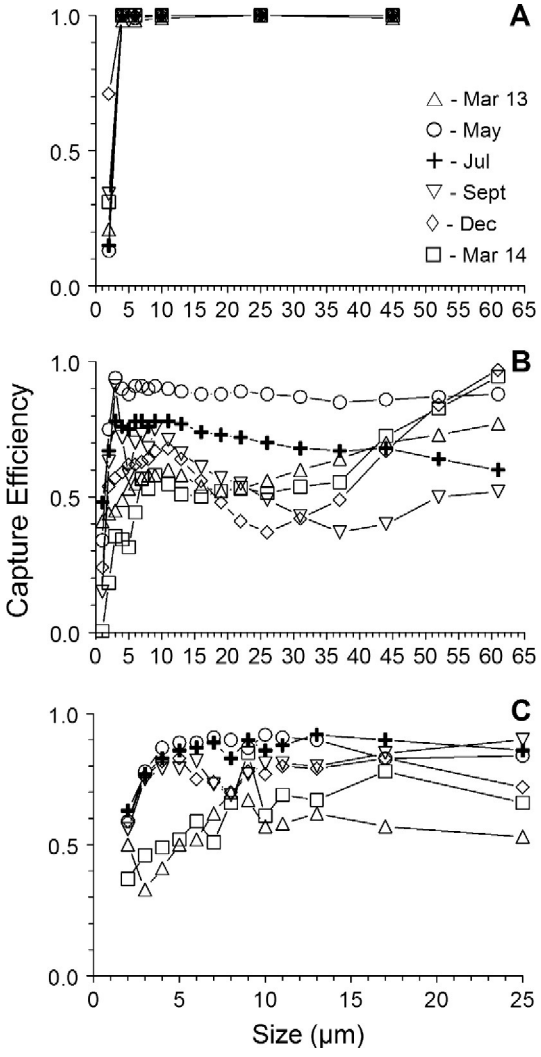

Fig. 1. Normalized capture efficiencies (CE) of mussels for all sampling dates calculated for (A) spherical synthetic particles analyzed by mean of flow-cytometry; (B) natural seston analyzed by means of LISST-100X; and (C) natural seston analyzed by means of the Coulter Multisizer IIe. For clarity, standard deviations and designations of statistical differences are not presented and can be found in Table 2. No significant differences in CE were found for synthetic spherical particles ranging from 4-μm to 25-μm (A), although CE of 2-μm spheres were significantly lower than CE for other sizes in all months (Tukey's HSD, $P < 0.05$). For natural seston analyzed by means of the LISST-100X (B), CE were often significantly lower for particles less than 5-μm compared to other sizes (e.g. Mar 2013, May, July; Tukey's HSD, $P < 0.05$). In September and December, particles 4-to-6-μm were captured at significantly higher rates than larger particles (31-to-44-μm in Sept and 26 in December; Tukey's HSD, $P < 0.05$). For natural seston analyzed by means of the Coulter Multisizer IIe (C), CE of particles less than 5-μm were significantly lower than for other sizes only in the March sampling dates (Tukey's HSD, $P < 0.05$). In March 2014, CE for 10-μm particles were lower than for 9-μm particles (Tukey's HSD, $P < 0.05$). Data are presented as means, SD not included for clarity.

(Table 2). These findings indicate an apparent seasonal shift in the size spectrum of natural particles captured by mussels, including the capture of smaller particles in preference to larger particles (Table 3).

For natural seston analyzed by means of the Multisizer, data analysis demonstrated significant effects of season and particle size on CE (ANOVAR, $P < 0.01$; Fig. 1C). As with data analyzed with the LISST-100X, there were significant interaction effects between these two independent variables, which were examined by means of post-hoc tests. For example, CE of mussels in March 2013 and 2014 were significantly different than CE in all other months (Tukey's HSD, $P < 0.01$), but not significantly different from each other ($P > 0.05$). In December, CE was significantly different from July and May (Tukey's HSD, $P < 0.05$). Within months, there were few significant differences in CE between size classes ≥4 μm. In March 2013, for example, CE were significantly higher for 8 μm particles than for particles 25 μm in size. In March 2014, CE were significantly higher for 9 μm particles than the CE of the 10 μm particles (Tukey's HSD, $P < 0.05$; Table 3). Generally, larger particles were captured at higher efficiencies than smaller particles.

3.3. Clearance rates

Clearance rates of mussels were generally lowest during the March sampling months, and highest during May (Fig. 3). Mean rates (L h^{-1}) for the defined particle-size classes ranged from 0.3 ± 0.56 (SD) to 2.8 ± 1.94 (SD) for particles <4 μm, 0.5 ± 0.53 (SD) to 3.7 ± 1.38 (SD) for particles 4 to 10 μm, and 0.7 ± 0.64 (SD) to 3.7 ± 1.33 (SD) for particles 11 to 25 μm. In most cases, particles <4 μm were cleared by mussels at significantly lower rates than particles between 4 and 25 μm. In several months, CR calculated by the two particle analyzers (LISST-100X vs. Multisizer) differed significantly for the <4 μm (September, December, March 2014) and the 4 to 10 μm (December) size classes (Tukey's HSD, $P < 0.05$). Significant differences between the calculated rates for the full range of particle sizes (LISST-100X = 1 to 61 μm, Multisizer = 2 to 25 μm) were also found in September, December, and March 2014 (Tukey's HSD, $P < 0.05$; Fig. 2).

3.4. Phytoplankton diversity and abundance

A total of 30 genera of phytoplankton were identified during the seasonal study (Table 4). The plankton species, all common to Long Island Sound (Hoppenrath et al., 2009), ranged in size from 6 to 110 μm. Total phytoplankton abundance was highest during March (70 cells mL^{-1}), which corresponded to the spring bloom (data not shown) and lowest during December (20 cells mL^{-1}). Overall, diatoms dominated the seston, with only seven of the most common genera being dinoflagellates. The dinoflagellate genera were the numerically dominant phytoplankton in July (*Prorocentrum* and *Peridinium*). During September and December, phytoplankton abundance was dominated by chain forming diatoms (*Cerataulina*, 15 μm diameter and ~50 μm length; *Skeletonema*, 11.5 μm diameter

Table 2
Capture efficiencies of mussels feeding on natural seston for each size-class bin, calculated by data from the two different particle-analysis instruments. Lower case letters (a, b, c) denote significant differences in CE of particle sizes between seasons (between subjects effects, columns; $P < 0.05$, Tukey HSD). Data are presented as means ± SD (in parentheses; n = 20–36). See Table 1 for description of measured environmental parameters for each season.

Size (μm)	Mar 13	May 13	Jul 13	Sept 13	Dec 13	Mar 14
LISST-100X						
1	$0.41^{a,b}$	$0.34^{a,b}$	0.48^{a}	$0.15^{b,c}$	$0.24^{a,c}$	0.01^{c}
	(0.41)	(0.44)	(0.36)	(0.30)	(0.22)	(0.02)
2	$0.44^{a,c}$	0.75^{b}	$0.67^{a,b}$	$0.63^{a,b}$	$0.54^{a,b}$	0.18^{c}
	(0.24)	(0.23)	(0.21)	(0.14)	(0.19)	(0.23)
3	0.45^{a}	$0.94^{b,c}$	0.78^{b}	$0.91^{b,c}$	$0.57^{a,b}$	0.36^{a}
	(0.15)	(0.07)	(0.19)	(0.14)	(0.15)	(0.40)
4	0.49^{a}	0.90^{b}	$0.76^{a,b,c}$	$0.72^{a,b}$	$0.59^{a,c}$	0.34^{c}
	(0.18)	(0.08)	(0.13)	(0.13)	(0.14)	(0.22)
5	$0.53^{a,c}$	0.88^{b}	$0.75^{a,b}$	$0.58^{a,c}$	$0.62^{a,b}$	0.32^{c}
	(0.19)	(0.09)	(0.14)	(0.25)	(0.15)	(0.20)
6	0.55^{a}	0.91^{b}	$0.78^{a,b}$	$0.70^{a,b}$	0.62^{a}	0.44^{a}
	(0.20)	(0.09)	(0.13)	(0.17)	(0.14)	(0.24)
7	0.57^{a}	0.91^{b}	$0.78^{a,b}$	$0.73^{a,b}$	0.63^{a}	0.57^{a}
	(0.19)	(0.09)	(0.14)	(0.17)	(0.15)	(0.21)
8	0.58^{a}	0.90^{b}	$0.76^{a,b}$	$0.68^{a,b}$	$0.64^{a,b}$	0.53^{a}
	(0.20)	(0.09)	(0.15)	(0.20)	(0.15)	(0.23)
9	0.58^{a}	0.91^{b}	$0.78^{a,b}$	$0.75^{a,b}$	$0.67^{a,b}$	0.58^{a}
	(0.19)	(0.10)	(0.16)	(0.17)	(0.16)	(0.23)
11	0.60^{a}	0.90^{b}	$0.78^{a,b}$	$0.71^{a,b}$	$0.68^{a,b}$	0.55^{a}
	(0.18)	(0.11)	(0.17)	(0.20)	(0.17)	(0.21)
13	0.58^{a}	0.89^{b}	$0.77^{a,b}$	$0.66^{a,b}$	$0.64^{a,b}$	0.51^{a}
	(0.17)	(0.11)	(0.17)	(0.21)	(0.16)	(0.21)
16	0.54^{a}	0.88^{b}	$0.74^{a,b}$	$0.61^{a,b}$	0.56^{a}	0.50^{a}
	(0.17)	(0.10)	(0.17)	(0.21)	(0.15)	(0.21)
19	0.52^{a}	0.88^{b}	$0.73^{a,b}$	0.57^{a}	0.48^{a}	0.52^{a}
	(0.17)	(0.10)	(0.18)	(0.23)	(0.14)	(0.20)
22	$0.53^{a,c}$	0.89^{b}	$0.72^{b,c}$	$0.55^{a,c}$	0.41^{a}	$0.53^{a,c}$
	(0.19)	(0.11)	(0.19)	(0.27)	(0.15)	(0.19)
26	$0.56^{a,c}$	0.88^{b}	$0.70^{b,c}$	$0.49^{a,c}$	0.37^{a}	$0.52^{a,c}$
	(0.19)	(0.12)	(0.19)	(0.30)	(0.19)	(0.20)
31	$0.60^{a,b}$	0.87^{b}	$0.68^{a,b}$	0.43^{a}	0.42^{a}	0.54^{a}
	(0.20)	(0.14)	(0.20)	(0.31)	(0.22)	(0.15)
37	$0.64^{a,b,c}$	0.85^{b}	$0.67^{a,b}$	0.37^{c}	$0.49^{a,c}$	$0.55^{a,b,c}$
	(0.17)	(0.16)	(0.20)	(0.32)	(0.22)	(0.19)
44	0.70^{a}	0.86^{a}	0.68^{a}	0.40^{b}	$0.67^{a,b}$	0.73^{a}
	(0.18)	(0.17)	(0.23)	(0.33)	(0.18)	(0.11)
52	$0.73^{a,b}$	0.87^{a}	$0.64^{a,b}$	0.50^{b}	0.84^{a}	0.83^{a}
	(0.27)	(0.18)	(0.35)	(0.35)	(0.11)	(0.08)
61	$0.77^{a,b}$	0.88^{a}	$0.60^{a,b}$	0.52^{b}	0.97^{a}	0.94^{a}
	(0.35)	(0.22)	(0.44)	(0.41)	(0.10)	(0.08)
Coulter Multisizer IIe						
2	0.50^{a}	0.59^{a}	0.63^{a}	0.56^{a}	0.59^{a}	0.37^{a}
	(0.35)	(0.28)	(0.22)	(0.19)	(0.38)	(0.22)
3	0.33^{a}	0.78^{b}	0.77^{b}	0.75^{b}	0.76^{b}	0.46^{a}
	(0.29)	(0.10)	(0.17)	(0.16)	(0.26)	(0.17)
4	0.41^{a}	0.87^{b}	0.83^{b}	0.79^{b}	0.82^{b}	0.49^{a}
	(0.29)	(0.09)	(0.14)	(0.13)	(0.18)	(0.19)
5	0.50^{a}	0.89^{b}	0.86^{b}	0.79^{b}	0.83^{b}	0.52^{a}
	(0.34)	(0.11)	(0.15)	(0.14)	(0.11)	(0.25)
6	0.52^{a}	$0.89^{b,c}$	$0.87^{b,c}$	0.82^{c}	$0.75^{a,b,c}$	$0.59^{a,c}$
	(0.34)	(0.13)	(0.14)	(0.15)	(0.21)	(0.27)
7	0.62^{a}	0.91^{b}	0.89^{b}	$0.73^{a,b}$	$0.74^{a,b}$	0.51^{a}
	(0.28)	(0.15)	(0.08)	(0.16)	(0.22)	(0.33)
8	0.70^{a}	0.90^{a}	0.83^{a}	0.69^{a}	0.70^{a}	0.66^{a}
	(0.27)	(0.18)	(0.14)	(0.26)	(0.24)	(0.19)
9	0.67^{a}	0.87^{a}	0.90^{a}	0.77^{a}	0.78^{a}	0.85^{a}
	(0.30)	(0.16)	(0.13)	(0.21)	(0.24)	(0.26)
10	0.57^{a}	0.92^{b}	0.86^{b}	$0.81^{a,b}$	$0.77^{a,b}$	0.61^{a}
	(0.26)	(0.11)	(0.12)	(0.14)	(0.19)	(0.28)
11	0.58^{a}	0.91^{b}	0.88^{b}	$0.81^{a,b}$	$0.80^{a,b}$	$0.69^{a,b}$
	(0.25)	(0.10)	(0.12)	(0.18)	(0.18)	(0.25)
13	0.62^{a}	$0.90^{b,c}$	0.92^{c}	$0.80^{a,b,c}$	$0.79^{a,b,c}$	$0.67^{a,b}$
	(0.27)	(0.12)	(0.08)	(0.18)	(0.18)	(0.24)
17	0.57^{a}	0.83^{b}	0.90^{b}	$0.85^{a,b}$	0.83^{b}	$0.78^{a,b}$
	(0.29)	(0.19)	(0.13)	(0.16)	(0.16)	(0.17)
25	0.53^{a}	0.84^{b}	0.86^{b}	0.90^{b}	$0.72^{a,b}$	$0.66^{a,b}$
	(0.31)	(0.20)	(0.20)	(0.21)	(0.19)	(0.14)

and 45 μm length) and pennate diatoms (*Navicula*, 14 μm width and ~70 μm length; *Nitzschia*, 6 μm width and ca. 50 μm length). Centric diatoms (e.g., *Thalassiosira*, 27 μm) were mainly abundant during the spring bloom (March). In none of the samples, however, were centric genera the numerically dominant phytoplankton group.

Table 3
Selected comparisons of mean capture efficiencies of mussels feeding on natural seston of different sizes as calculated by the two instruments. In many cases the instruments yielded data that resulted in different conclusions. Also, in all examples (except Mar 13 for the Multisizer), larger particles were captured at lower efficiencies than smaller particles within the given months. Significance of these comparisons often depended on the data generated by each of the two instruments (LISST-100X vs Multisizer). * denotes significant differences (Tukey HSD, $P < 0.05$; n = 20–36), N/A denotes particle size comparisons not available (due to low counts).

Season	Particle size range (μm)	LISST-100X *P*-value	Coulter Multisizer IIe *P*-value
Mar 13	3 vs 11–13	1.000	0.017*
Sept 13	4 vs 31–44	0.000*	N/A
	6 vs 31–44	0.001*	N/A
Dec 13	4 vs 26	0.041*	1.000
	6 vs 26	0.006*	1.000
Mar 14	9 vs 10	1.000	0.022*

3.5. MeML expression

Analysis of MeML-transcript data showed significant differences in expression between seasons (ANOVA, $P < 0.01$; Fig. 3). For the majority of the months, there was a high degree of variability with one or two samples displaying low C_T, resulting in high folds. Results suggest a variation of the expression of MeML gene in the labial palps with sampling season. Transcript levels of MeML increased from March to July 2013 before displaying a sharp decrease in September followed by a rebound in December. Overall, MeML transcript levels were significantly higher in March 2013, May, and December as compared to March 2014 (K–W statistic, $P < 0.05$). In contrast, MeML transcript levels in July and September were not significantly different from those measured in March 2014.

4. Discussion

Results reported here demonstrate several important points regarding the capture of particles by mussels and point out potential errors associated with use of different instruments. First, the capture of the spherical particles ≥4 μm in diameter was consistently high across all sampling months, with only the 2-μm particles being captured at a lower efficiency than particles of greater size. These data are consistent with current understanding of hydrosol filtration mechanisms employed by bivalves (Shimeta and Jumars, 1991; Ward et al., 1998; Riisgård and Larsen, 2010). Small particles encounter gill filaments at a lower rate, and thus are captured at a lower efficiency than larger particles. As particle size increases, CE increases and approaches 100%. The particle size above which CE is maximum is species specific, and for *M. edulis* it has been reported as ca. 4 μm (Møhlenberg and

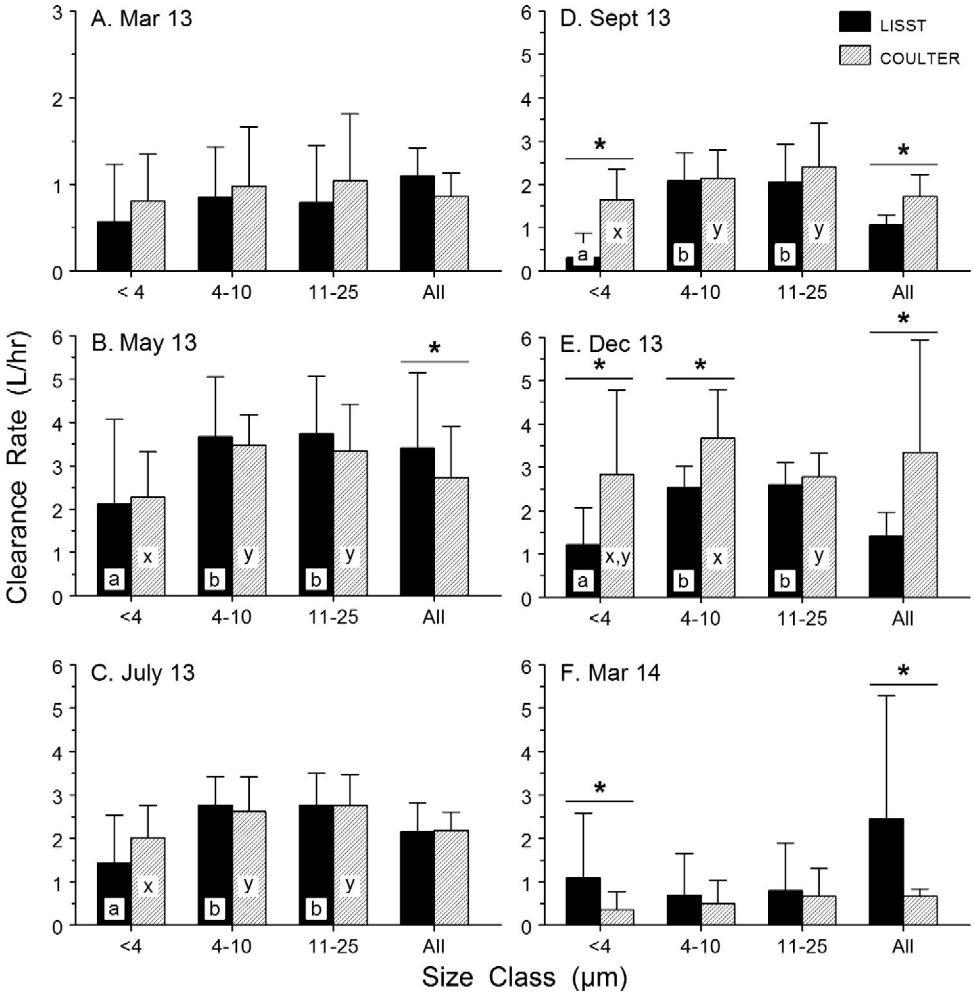

Fig. 2. Comparison of clearance rates ($L\,h^{-1}$) of mussels calculated by means of the LISST-100X and Multisizer particle analyzers. Clearance rates (CR) were calculated for 3 different size classes (particles <4 μm, 4 to 10 μm, and 11 to 25 μm) and for the full range of sizes (LISST-100X = 1 to 61 μm, Multisizer = 2 to 25 μm). Within a month, and for each instrument, significant differences in CR between each size class are denoted by lower case letters (a, b for LISST-100X; x, y for Multisizer; Tukey's HSD, $P < 0.05$). Within a month and within each size class, significant differences in CR between the two instruments are indicated by an asterisk (*, Tukey's HSD, $P < 0.05$). Data presented as means ± SD (n = 10–30). See Table 1 for description of seasonal designations.

Table 4
Phytoplankton distribution for each month during the one-year study. Data presented as percent (%) abundance of each genus in samples for a given date. There were 32 major genera of phytoplankton represented in the samples, all of which are common to the study site. The seston was dominated by diatoms, with less than a third of the identified genera being dinoflagellates. The identified phytoplankton genera consisted largely of non-centric forms, including pennates and chain forming diatoms.

Genus	Size (μ)	Mar 13	May 13	Jul 13	Sept 13	Dec 13	Mar 14
Nitzschia	6	9.7	5.3	18.3	19.2	30.8	16.6
Leptocylindrus	6.5	0.0	0.0	0.7	0.0	0.0	0.0
Pyramimonas	7.5	0.0	0.0	0.3	0.0	0.0	0.0
Gyrosigma	11	1.6	1.2	0.6	7.6	13.5	1.7
Skeletonema	11.5	7.0	7.4	7.2	23.1	6.7	7.9
Euglena	13	0.8	0.2	0.0	0.0	0.1	2.2
Navicula	14	8.7	7.4	6.9	12.8	12.4	17.3
Cerataulina	15	0.0	43.1	0.0	0.0	0.0	<0.1
Cocconeis	15	0.7	0.6	0.0	0.0	0.0	0.0
Dinophysis	16.5	0.0	0.0	0.2	0.0	0.0	0.0
Diploneis	20	<0.1	0.0	0.0	0.0	0.0	0.0
Gyrodinium	25	0.9	0.1	0.0	0.0	0.0	0.0
Chaetoceros	27.5	0.4	10.7	2.5	1.2	1.5	3.2
Thalassiosira	27.5	34.0	10.3	7.8	12.2	9.7	1.3
Prorocentrum	35	0.0	0.0	7.6	0.0	0.0	0.0
Rhizosolenia	38.5	6.4	0.1	2.2	8.5	0.7	5.9
Ceratium	40	<0.1	0.0	0.1	0.0	0.1	0.7
Ditylum	42.5	0.2	0.0	2.4	0.5	0.7	0.1
Melosira	43	2.2	1.0	1.5	1.8	2.6	1.0
Disteplanus	45	0.1	<0.1	0.1	0.2	0.3	0.1
Thalassiothrix	45	16.6	6.2	9.2	2.8	6.6	12.7
Eucampia	49	<0.1	0.1	0.8	2.7	1.3	<0.1
Peridinium	50	0.0	0.0	21.6	0.0	0.0	0.0
Biddulphia	55	0.7	0.3	0.9	0.4	0.6	0.3
Licmophora	62.5	1.3	2.1	0.1	1.2	3.0	4.7
Asterionella	67.5	6.0	0.6	6.0	<0.1	0.2	14.9
Grammatophora	72.5	0.1	0.6	0.3	1.1	1.8	0.7
Coscinodiscus	75	1.6	0.6	1.2	0.3	0.4	7.0
Amphiprora	84	0.2	1.1	0.8	3.2	4.9	1.3
Rhabdonema	110	0.9	1.1	0.6	1.2	2.2	0.5

Riisgård, 1978). The results are also consistent with other studies that used uniform particles of known size (including phytoplankton cells) in which all particles above a threshold size were captured with high efficiency (see Ward and Shumway, 2004 for review). Second, because CE of microspheres ≥4 μm was not affected by season, there is no evidence that mussels adjusted either feeding behavior or physiology over time (e.g., changes in interfilamentar spaces, beat frequency of the laterofrontal cirri). If mussels were adjusting particle capture seasonally, and capturing smaller particles in preference to larger ones (e.g., as determined for natural seston in September and December), then CE of the 25-μm and 45-μm spheres should have declined. No decrease in CE of larger microspheres, however, was found in any season. The 2-μm spheres were captured at a significantly higher rate in December compared to the other months, though still not at a higher efficiency than larger particles. The reason for this finding is unclear. Temperature during December was at an intermediate level (9 °C), POM and chlorophyll *a* concentrations were at their lowest level, and TPM concentration was not significantly lower than during the other months (Table 1). Environmental factors, therefore, provide little insight as to why the 2-μm microspheres were captured at a higher efficiency in December compared to the other months. Work by Lucas et al. (1987), examining two populations of *M. edulis*, found that during times when bacterial numbers in the seston were high, animals were able to capture smaller-sized particles (0.5–1.58 μm) with up to 57% efficiency. Larger phytoplankton (>4 μm), however, were still captured with close to 100% efficiency. Such differences in the retention of particles at the lower threshold of capture warrants further research.

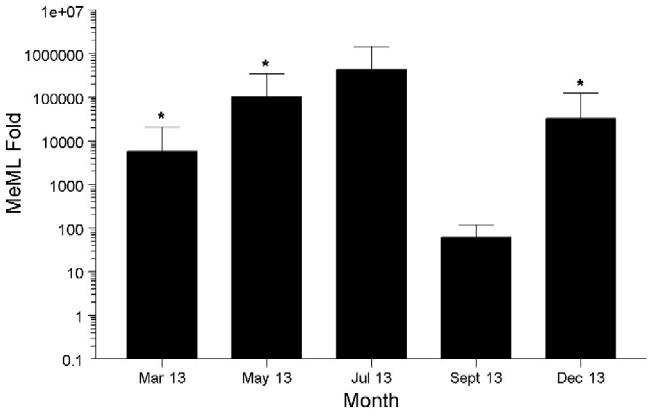

Fig. 3. Seasonal variation of MeML transcripts levels in labial palps of mussels. Transcripts levels were determined by quantitative real-time PCR and normalized to 18S RNA. Expression levels are presented relative to March 2014 (which had the lowest expression levels). Data presented as means ± SD (n = 5–9 mussels/sampling date). * denotes significant differences in expression level compared to March 2014 as determined by the non-parametric, pairwise multiple comparison Dunn's test ($P < 0.05$).

In contrast to the capture of microspheres, CE of mussels feeding on natural seston (measured on the same dates, with the same animals) appeared to change seasonally, and at times smaller particles were captured more efficiently than larger particles. These data are comparable to results obtained by previous workers using similar particle analyzers (e.g., Strøhmeier et al., 2012). For example, in March 2013 CE for particles ≤6 μm in size, calculated from data collected using the Multisizer, was significantly lower than CE for the same size particles in September. In September and December CE of particles between ca. 4 and 6 μm in size, calculated from data collected by the LISST-100X, were captured with a higher efficiency than particles ca. 22 to 37 μm in size. Other apparent differences in CE within and between months were found in the data sets collected by each of the two instruments. Additionally, in several months CE of mussels calculated from data generated by the LISST-100X and Multisizer were different. For example, in March CE calculated from Multisizer data increased from ~30% for 3-μm particles to ~70% for 8-μm particles, whereas CE calculated from LISST-100X data for particles in the same size range only changed from ~45% to ~60%. In September, the highest CE (91%) calculated from data collected by the LISST-100X was for particles 3 μm in size, with efficiency generally decreasing with increasing particle size to 50% for particles ca. 26 μm in size. Data collected at the same time using the Multisizer showed a different trend, with CE generally increasing with increasing particle size to a maximum of 90% for particles 25 μm in size.

The range of mean clearance rates measured in this study using both instruments (0.67 L h^{-1} to 3.34 L h^{-1}) was similar to rates that have been reported previously for *M. edulis* of similar size (MacDonald and Ward, 2009; Cranford et al., 2011). In all months except March (when water temperatures were lowest), particles <4 μm were cleared at a lower rate than larger particles. Our results also allow us to investigate whether the apparent shifts in CE could affect CR calculated from the collected data. In September and December, for example, data collected by the LISST indicated a significant decrease in CE of particles within the 11 to 25 μm size class compared to smaller sizes (between 4 and 10 μm), but no significant differences in CR between these two size classes. Data collected by the Multisizer in September indicated no significant difference in CE between particles in the 11 to 25 μm and 4 to 10 size classes, but a significantly lower CR for the smaller size particles. There also was no correlation in any month between clearance rates and CE of natural particles 25 μm in size (a particle size that showed some of the largest differences in CE) calculated from data collected by either of the two instruments. These findings demonstrate that an apparent shift in CE does not always translate to a change in CR. The most obvious result from this comparative analysis is that in many months (e.g., May, Sept, Dec, Mar 2014), CR calculated from data generated by the LISST was significantly different than that generated by the Multisizer. Differences were found within specific size classes and for the full range of particles. These results are striking, as they could affect the conclusions reached in studies using these different instruments.

Many aspects of feeding in bivalves have been shown to be under physiological control (e.g., clearance rates, digestive processes; Hawkins et al., 1985; Bayne et al., 1988; Iglesias et al., 1996; Smaal et al., 1997; MacDonald et al., 1998; Cranford et al., 2005; Bayne and Svensson, 2006). Results of this study, however, provide no evidence that CE is physiologically plastic. One indicator that mussels in the current study did not regulate CE came from results of the expression of the mucosal lectin MeML. Although MeML transcript levels displayed significant seasonal variations, these trends were decoupled from CE. Mussels exhibited the highest expression of MeML in July when CE, calculated from LISST-100X data, declined with increasing particle size. Similarly high transcript levels were measured in May when CE was constant over a wide range of particle sizes. In September, CE was higher for particles smaller and larger than 35 to 40 μm when mussels exhibited the lowest expression of MeML. In December, similar CE patterns were found, but MeML transcript levels were significantly higher than in September. Additionally, seasonal changes in MeML folds were not significantly correlated with either clearance rates of mussels (Pearson correlation, $P > 0.05$), or chlorophyll *a* content of the seston ($P > 0.05$). These findings suggest that seasonal changes in CE calculated for natural seston are not related to the expression of MeML, and that transcript levels in mussels are controlled by other exogenous or endogenous factors (i.e., reproduction, energy storage; Pales Espinosa and Allam, 2013).

Further, the striking differences between CE of microspheres and natural seston, suggest to us that factors other than shifts in feeding activity of mussels could be responsible for the data. We suggest that alternate explanations for the purported changes in CE measured in field studies need to be explored further, and propose that such data could be a result of one or more of the following: 1) artifacts associated with the way in which most particle analyzers calculate particle diameter; 2) disaggregation of flocculent material collected from control chambers that leads to the release of small particles; 3) post-capture loss from the gill and subsequent expulsion from the infrabranchial mantle chamber of strongly swimming phytoplankton cells (e.g., dinoflagellates) that increases the number of large particles exiting experimental chambers; 4) qualitative factors of the particles that could affect capture; or 5) mathematical happenstance of calculating CE on particle-size classes that contain widely different numbers of particles — i.e., larger size classes tend to be represented by fewer particle numbers.

One likely explanation for differences in the calculated CE of mussels feeding on natural particles versus microspheres relates to the way in which particle analyzers calculate an equivalent spherical diameter (ESD) to estimate particle size. Both the LISST-100X and Multisizer calculate ESD from measurements of particle volume. The LISST-100X uses near-forward scattering measurements to obtain information on the volume and concentration of suspended particles. The observed scattering measurements are then inverted and a spherical particle shape is assumed in order to estimate particle size from the measured particle volume (Agrawal and Pottsmith, 2000). Electronic particle analyzers, such as the Multisizer, use a similar approach, but measure the increase in the impedance of an electrical current that is applied across an aperture. The change in impedance is caused by particles passing through the current, which displace their own volume of the conducting liquid. The amplitude of the current fluctuation is directly proportional to the volume of the particle, which is then converted to ESD (User's Manual for Coulter® Multisizer IIe. England: Coulter Electronics Limited (1989)). Both of these techniques work well for particle shapes that approximate a sphere, but produce increasing error in ESD as particle shape deviates from spherical (e.g., chain-forming and pennate diatoms). The limitations of these instruments in accurately measuring particle size have been discussed previously (Jonasz, 1987; Karp-Boss et al., 2007; Reynolds et al., 2010). In particular, the analysis of a single species of phytoplankton with a complex shape by means of the LISST-100X can produce ESD data with peaks in several size ranges separated by tens or hundreds of micrometers, or broad size-frequency distributions that span tens or hundreds of micrometers (Karp-Boss et al., 2007). The error associated with calculating ESD and estimating actual particle size, changes with both the size of the particles (smaller particles tend to be more spherical) and season as the seston is dominated by different types of organic and inorganic particles, and different species of phytoplankton (Jonasz, 1987). Considering the complex assemblage of particles in the seston — with different sizes, shapes and concentrations — it is unlikely that a size-frequency distribution could be obtained that accurately reflects the true size range of natural particles in water before and after being processed by a suspension feeder. Additionally, phytoplankton with narrow widths (e.g., <3 μm) and length to width ratios >3 could be captured at a low efficiency, depending on their orientation as the approach the gill filaments, but would be recorded as particles with an ESD above the size that should be captured with ca. 100% efficiency. Bayne et al. (1977) previously reported that mussels apparently captured the non-spherical algae *Phaeodactylum tricornutum* at higher efficiencies than larger particles. They suggested that the data from their study, analyzed using a Coulter Counter, showed that the shape of diatom cells could result in higher CE than what would be expected for a spherical particle of similar volume. Data from the current study support these contentions. In the months of September and December, the dominant phytoplankton species were non-spherical diatoms (Table 4). During these months, CE calculated by means of the LISST-100X was significantly different than other months (e.g., May), and CE of large particles (ca. 20 and 40 μm) was significantly lower than small particles (ca. 6 μm). Particulate characteristics of the seston could, therefore, create considerable error in the size-frequency distributions generated by particle analyzers that lead to spurious calculations of CE and faulty conclusions regarding physiological plasticity of the capture process.

Another factor that could explain purported changes in CE is the disruption of marine aggregates (e.g., marine snow) during sampling handling and processing. Marine aggregates are a common component of the seston, with large proportions (>70%) of natural particulates being present in these aggregations during certain times of the year (Alldredge et al., 1993; Crocker and Passow, 1995; Syvitski et al., 1995). These loose agglomerations of organic and inorganic particles can be disrupted by shear (Kiørboe and Hansen, 1993; Manning and Dyer, 1999) such as that produced by the stirring or shaking of collected samples. Particle analyzers that rely on a vacuum to pull particles through an aperture, such as the Multisizer, can also form shear fields that disrupt aggregates and release constituent particles. Bivalves can capture aggregates of various sizes and ingest the constituent particles (Newell et al., 2005; Kach and Ward, 2008; Ward and Kach, 2009). Therefore, there would be more aggregates present in water samples taken before (i.e., control) than those taken after flowing over a bivalve. Disruption of aggregates and the release of small particles would bias the size distribution toward smaller sizes in the control samples. The calculated CE from such data would lead to the interpretation that the bivalve was able to capture more individual small particles than to which it was actually exposed. Because efficiency of capture is normalized to the particle size that produces the highest CE, this situation would result in higher CE for smaller particles and artificially lower CE for larger particles.

Another situation that could affect the calculated efficiencies is the presence of dinoflagellates or other highly motile cells in the seston. In the European oyster, *O. edulis*, cells of *Alexandrium tamarense* (a dinoflagellate with a mean length of 35 μm), can escape from the pallial organs after capture (Bricelj et al., 1998). The freely swimming cells congregate between the gill lamellae and are periodically expelled from the mantle cavity by the rapid adduction of the oyster shell valves. The consequence of such an expulsion of cells in a flow-through experiment would be the measurement of a high number of particles in the size range of those cells (e.g., 35 μm). Further, the calculated CE of particles in this size range would be low, with the interpretation that these

particles passed through the gills and were not retained when, in fact, they were retained but were expelled as unconsolidated pseudofeces. Although the specifics of the process of particle capture and transport by mussels differs from that of oysters, highly motile cells might be able to evade capture by the gills or swim from loosely bound material in the ventral grooves of mussels. In the current study, less than a third of the identified phytoplankton groups were dinoflagellates. Two of these identified genera, *Prorocentrum* and *Peridinium* (35 μm and 50 μm, respectively), constituted a large portion of the phytoplankton composition during the July sampling date. Assuming that the above scenario occurred, the presence of these dinoflagellates could have contributed to the lower CE measured for particles >35 μm compared to May.

Qualitative factors of particles, such as surface properties, could also contribute to the differential capture of natural seston; however, only a few papers have convincingly demonstrated such an effect. For example, work by Yahel et al. (2009) showed size-independent differences in CE of two different species of bacteria (0.4 μm) by the tropical mytilid *L. simplex*. Additionally, Hernroth et al. (2000) manipulated the surface charge of the bacterium *Salmonella typhimurium* (1 μm). These bacteria, which had a high electrostatic charge, were captured by *M. edulis* at higher rates than the non-manipulated bacteria of the same size. Additionally, the manipulated bacteria were also captured with the same efficiency as 15-μm polystyrene spheres also delivered to the mussels. These two reports suggest that differential capture of similar-sized particles can occur at the lower threshold of CE. Understanding the effects of particle surface properties on CE, especially of particles whose size is at the lower threshold of capture (e.g. <4 μm) is a fertile area for future research.

Finally, larger size classes tend to have orders of magnitude fewer particles than smaller size classes. Each individual particle in the larger size classes, therefore, contributes a greater proportion to the difference in counts between control and experimental samples, and thus the calculated efficiencies. For this reason, most studies pool data from size bins that do not meet some minimal particle concentration (e.g., 100 particles ml^{-1}) for the control samples. Little theoretical work has been done to examine the mathematical consequences of calculating CE on particle-size classes that contain widely different numbers of particles.

In conclusion, analyses of the same seston samples by two commonly used analytical instruments resulted in the calculation of different patterns of CE by mussels over a range of particle sizes and seasonal time frames, and often produced an "inverted" pattern in which smaller particles appeared to be captured more efficiently than larger particles. Such patterns of CE, which have also been generated by previous studies, are difficult to explain given the current knowledge of particle capture by the gill of bivalves (Ward et al., 1998; Riisgård and Larsen, 2010). Data for the capture of natural particles were also in conflict with data obtained for microspheres of defined size and shape that were delivered to mussels simultaneously. Results for CE of microspheres unequivocally showed that different size particles ≥4 μm were captured by *M. edulis* with close to 100% efficiency regardless of season. Finally, the lack of correlation between MeML expression and CE does not support the concept that mussel respond to seasonal exogenous factors by altering the efficiency of particle capture. The results of this study suggest that the purported changes in CE (e.g. Strøhmeier et al., 2012) may be a result of instrument artifacts or other factors associated with the seston, and not a result of behavioral or physiological responses of the mussel.

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